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A sequential recovery extraction and biological activity of water-soluble sulfated polysaccharides from the polar red macroalgae *Sarcopeltis skottsbergii*

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ABSTRACT

Algal biomass is comprised of a variety of biochemical components that make it a viable raw material for use in a variety of applications using a biorefining process. From the residual supernatant generated by high-pressure homogenization for the extraction of the R-phycoerythrin pigment, two soluble polysaccharides (neutral and acid fractions) were obtained. The composition of total carbon (C), hydrogen (H), nitrogen (N), and sulfur (S), gas chromatography–mass spectrometry (GC–MS), Fourier transform infrared (FTIR-ATR) spectroscopy, antioxidant capacity (ABTS) and antiproliferative *in vitro* and *in vivo* activities were evaluated. The two polysaccharides, which had a relatively high sulfate content, exhibited significant antioxidant activity in superoxide radical assay (ABTS) and high anti-proliferative effects on G-361, U-937, HCT-116 cancer cells. High selectivity index (>3) was observed for the acid extract on G-361 and U-937 cell lines, but a significant inhibitory effect on the growth of 1064sK healthy fibroblast cells was measured too. On the other hand, neutral fraction polysaccharides, with the lowest sulfate content and relatively lower radical scavenging rates, showed scarce antitumor activity, a high selectivity index on G-361 melanoma cancer cells and non-toxicity on healthy cells. This differential behavior was verified by testing toxicity and lethality of both polysaccharide extracts in and *in vivo* zebrafish embryo acute toxicity test. Following this method, the LC_{50} of the neutral fraction resulted much higher (1.477 mg mL^{-1}, $p \approx 0.0000$) than that of the acid fraction (0.080 mg mL^{-1}, $p \approx 0.0000$) at 72 h post-fertilization. During this period, a slight growth delay and/or rare teratogenic effects were also induced by these fractions at concentrations lower than their LC_{50}. Overall, this study indicates that the antitumor, antioxidant and toxicity effects of polysaccharides may be related to the combined effects of sulfate and galactose contents from the residual supernatant bioprocess. The nutraceutical potential of the acid fractions is thus finally discussed.

1. Introduction

An important part of the objectives of the blue bioeconomy is sustainable valorization of the aquatic biomass, marine, wild catch as well as aquaculture, to complement the terrestrial production of food, feed, non-food (biomaterials and chemicals) and bioenergy [1]. Seaweed is not only known as a rich source of nutrients but also as an important source of bioactive components. For centuries, seaweed has been used as both therapeutic and traditional medicines in many countries such as China and Korea [1,2]. In recent years, studies have shown that seaweed

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possesses polysaccharides with high antioxidant [3], anti-inflammatory, anticoagulant, antiangiogenic, and antitumor activities [4]. In general, the bioactive properties and their compounds vary from species to species. Most of the bioactive compounds in seaweed are typically from the aqueous extracts. This is probably due to the known traditional practice in several parts of the world where seaweed is boiled, infused with hot water, or prepared as a soup to extract their medicinal properties before being consumed by the patient [5,6]. Furthermore, a very important difference, essential for biorefinery processing, is that seaweed does not have the recalcitrant lignin such as lignocellulosic materials characteristic for terrestrial plants [7].

The new and upcoming use seaweed bioprocessing is facilitating the development of new types of higher-value products, which are not just tasty but also gut-health-promoting ingredients. The main challenge, which determines the industrial utilization of seaweed potential, in addition to biomass availability, is the development of efficient extraction methods while preserving most of the intrinsic qualities of the raw material, with its functionalities and bioactivities intact. Several procedures exist for extracting and separating biologically active compounds from the algal biomass. Traditional extraction techniques for seaweed generally consist of maceration of the material in solvents for several hours at temperatures ranging from ambient to 50–60 °C [8] Commonly used solvents include methanol, dichloromethane, or ethanol. Such conditions are quite severe and cause decomposition of most of the biologically active compounds and these solvents also have toxic effects. Thus, the primary challenge when extracting algal bioactive compounds will be to find a compromise between the cost of production of sufficient quantities and quality of compounds in the shortest timeframe, finding the optimum processing condition, and meeting the principles of green chemistry and green technology [9].

Recent trends to limit the use of chemicals have promoted the development of greener solvents, such as subcritical water extraction alone [10] or with ionic liquids as catalysts [11]. Moreover, methods assisted by microwaves [9], ultrasound [12] or high pressure [13], which reduces operation time and energy consumption compared with conventional extraction methods, are being used more frequently. However, depolymerization exhibits lower gel strength and viscosity [11].

High molecular weight polymers (up to 5000 kDa, average 200-800 kDa) are approved for food applications, whereas degraded polysaccharides (10-20KDa) are not authorized [14]. However, interesting activities of these degraded polysaccharides such as antitumor, antiviral, antibacterial, or immunostimulant have been reported [15–17].

A water-soluble polysaccharide has several biological activities. The sulfated polysaccharide extracted from algae show activities ranging from anticoagulant and antithrombotic, to immunomodulatory, antiviral, and antitumor effects [18]. A role of sulfated polysaccharides from algae as antineoplastic agents has also been suggested. Several investigations have reported that sulfated polysaccharides have antiproliferative activity in cancer cell lines *in vitro*, as well as inhibitive activity in tumors growing in mice [17–19].

Zebrafish embryo or rodent toxicity assays are also *in vivo* models of interest [20–23]. In zebrafish embryos, the anti-inflammatory [24,25], immunomodulatory [26–28], fungicidal [29], antioxidant [24,30], antitumor [31] or the toxic [32,33] activities of polysaccharides have been previously tested. Some of these studies, specifically analyze the effects of algal polysaccharides [34–37].

Sarcopeltis skottsbergii is an endemic species of Chile. The production of this macroalgae is around twenty thousand tons of dry alga to be commercialized mainly in Europe and Asia. The valuation of compounds in a biorefinery model is still in the early stages in algal biotechnology and thus have great prospects in the food, pharmaceutical, cosmeceutical, and nutraceutical industries [13,38,39].

Therefore, the modification of the process variables of technologies for the extraction and separation of sulfated polysaccharide from macroalgal biomass as well as the methodology followed are extremely important to keep extracts with high yield and the biotechnology properties. In previous studies in our group, we optimized water extraction by high-pressure homogenization (HPH-Assisted extraction) to recover R-phycoerythrin pigment from the red algae *S. skottsberggi*. The residue generated from the phycoerythrin aqueous extract was sequentially characterized funded high proportion of polysaccharides (neutral and acid) compounds. The aim of this study was to determine the chemical composition and biological activities of the residual water fraction by high-pressure homogenization. Both types of sulfated polysaccharides (neutral and acid) were characterized by chemical analyses (CHNS; FTIR; GC–MS). The antioxidant activity (ABTS), cytotoxic effects on different human tumor lines such as human colon cancer (HCT-116), human melanoma (G-361), human leukemia (U-937), human gingival fibroblasts cell line (1064SK) and toxic effects in the zebrafish embryo acute toxicity test (ZFET) were assessed.

2. Materials and methods

2.1. Algal biomass

In this study, the macroalgae was provided from the Magallanes Region by Gelymar Company S.A., Puerto Montt, Chile. The biomass was washed with filtered and distilled water to remove sand particles, epiphytes, and other undesirable materials. Biomass was lyophilized (Biobase BK-FD18PT) for 48 h and the substrate samples were carefully milled with a grinder machine (Sindelen Mol165IN, China) until the particle size was less than US sieve screen number 18 (1 mm openings). The samples were sieved using a Ro-Tap testing sieve shaker (model RX-29-10, W.S. Tyler, Mentor, OH) through a set of sieves (ASTM E11:95). The average particle diameter (dp = 0.70 mm) was determined using Eq. (1) of standard method S319.3 (American Society of Agricultural Engineers Standards, 2000).

$$d_p = log^{-1}\frac{\left[\sum_{i=1}^{n-} w_i \, log\overline{d}_i\right]}{\sum_{i=1}^{n} w_i} \quad (1)$$

$\overline{d}_i$ (mm) is the geometric mean diameter of particles on *i*th sieve, or $(d_i \, x \, d_{i+1})^{1/2}$ where di is the nominal sieve aperture size of the *i*th sieve, and d_{i+1} is the nominal sieve aperture size in the next one larger than *i*th sieve. w_i is the mass of particles with an average diameter of $\overline{d}_i$. The biomass was stored at −18 ± 2 °C and the desired quantity of biomass was taken out as and when required for the experimentation.

2.2. Biochemical analyses total carbon, hydrogen, nitrogen and sulfur in biomass

Total carbon (C), hydrogen (H), nitrogen (N), and sulfur (S) were determined from dry biomass (20 mg) using the total combustion technique used in the LECO TruSppec Micro CHNSO-Elemental Analyzer according to the manual. This technique is based on the complete and instantaneous oxidation of the sample by pure combustion with controlled oxygen at a temperature of up to 1050 °C (C, H, N, S) and pyrolysis at 1300 °C (O) for decomposition of O as CO and oxidation to CO_2. The resulting combustion products, CO_2, H_2O, SO_2, and N_2, are subsequently quantified by a selective IR absorption detector (C, H, S) and TCD (N) differential thermo-conductivity sensor. The result of each element (C, H, N, S) is expressed in % with respect to the weight of the sample.

2.3. Polysaccharide extraction from the supernatant

The neutral and acid polysaccharides were extracted from the water residues from R-phycoerythrin extraction under high-pressure optimization according to the method reported by Castro-Varela et al. [40] (Fig. 1). For this, neutral polysaccharides were stirred at 90 °C and precipitated with the addition of ethanol (*v*/v) [41] and acid

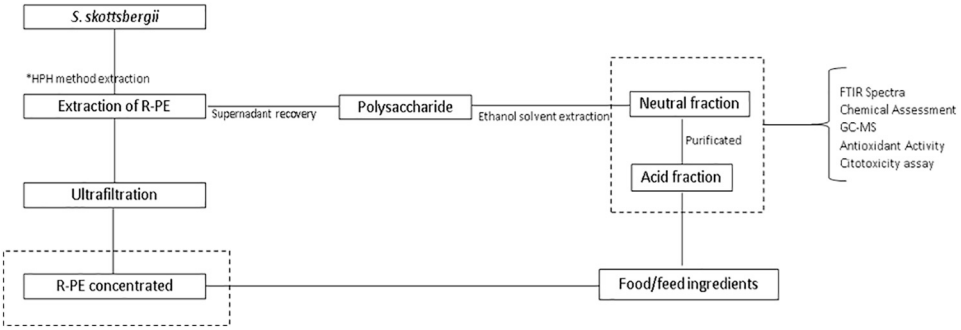

Fig. 1. Schematic representation of the biorefinery concept for the recovery of the neutral and acid polysaccharides. *Optimized extraction by Castro-Varela et al. [40].

polysaccharide with N-cetylpyridinium bromide (Cetavlon) (Sigma-Aldrich, St. Louis, MO, USA) 2 % (*w/v*) for 24 h [42]. Then, polysaccharides were centrifuged at 4500 rpm for 5 min, 4 °C. The supernatant was discarded, and 10 mL of 4-M NaCl (Sigma-Aldrich, St. Louis, MO, USA) were added. This mix was stirred until completely dissolved. Once cooled, the ethanol was placed in a ratio 1:1 (% *v*/v) and kept at 4 °C for 24 h. After centrifugation at 4500 rpm, 5 min, 4 °C, the pellet containing the polysaccharides and salts was placed on a dialysis membrane (Sigma-Aldrich, St. Louis, MO, USA) in a 0.5-M NaCl solution overnight at 4 °C. Then, the dialyzed EPS was centrifuged at 4500 rpm for 5 min, 4 °C, and washed with absolute ethanol. Finally, acid and neutral polysaccharides from supernatant extraction conditions (in triplicate, $n = 3$) were stored at −80 °C and subsequently lyophilized (Lyophilizer Cryodos, Telstar, Spain).

2.4. Total carbon, hydrogen, nitrogen, and sulfur in polysaccharides

Total carbon (C), nitrogen (N), and sulfur (S) were determined from the extracted polysaccharides using the total combustion technique used in the LECO TruSppec Micro CHNSO-Elemental Analyzer. This technique is based on the complete and instantaneous oxidation of the sample by pure combustion with controlled oxygen at a temperature of up to 1050 °C (C, H, N, S) and pyrolysis at 1300 °C (O) for decomposition of O as CO and oxidation to CO_2. The resulting combustion products, CO_2, H_2O, SO_2, and N_2, were subsequently quantified by a selective IR absorption detector (C, H, S) and TCD (N) differential thermoconductivity sensor. The result of each element (C, H, N, S) is expressed in % with respect to the weight of the sample.

2.5. Infrared analysis of polysaccharides

Fourier transform infrared (FTIR) spectra of the polysaccharides were obtained by using self-supporting pressed disks 16 mm in diameter from a mixture of polysaccharides and KBr (1 % *w*/w) with a hydrostatic press at a force of 15.0 tcm^{-2} for 3 min. The FTIR spectra were obtained with a Thermo Nicolet Avatar 360 IR spectrophotometer (Thermo Electron Inc., USA), having a resolution of 4 cm^{-1} with a DTGS detector and using an OMNIC 7.2 software (bandwidth 50 cm^{-1}, enhancement factor 2.6) in the 400–4000 cm^{-1} region. Baseline adjustment was performed using the Thermo Nicolet OMNIC software to flatten the baseline of each spectrum. The OMNIC correlation algorithm was used to compare sample spectra with those of the spectral library (Thermo Fisher Scientific, USA).

2.6. Antioxidant activity (ABTS) free-radical method

The ability of the polysaccharides to scavenge free radicals was evaluated using an ABTS assay according to Re et al. [43] with few modifications. Antioxidant capacity as 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) Assay Scavenging of Free Radical in Sulfated Polysaccharides and Biomass. The ABTS radical cation was produced through the reaction with ABTS. An aqueous solution containing was mixed 7 mM ABTS solution with 2.45 mM potassium persulfate for 16 h in the dark at room temperature. After incubation, the well-mixed solution was diluted to an absorbance of 0.7 at 413 nm with deionized water. The final concentrations of polysaccharide solution were: 25, 50, 75, 100, 150, 200, 300, 400, and 500 μg mL^{-1}. For biomass, 100 mg DW in 1.5 mL of phosphate buffer was made. A total of 50 μL of these samples were mixed with 940 μL of phosphate buffer and 10 μL of ABTS solution. The resulting mixture was measured with a spectrophotometer at 413 nm. ABTS radical scavenging capacity was calculated according to the following equation:

$$AA\% = [(A0 - A1)/A0]*100 \quad (2)$$

where, A0 is the absorbance of the ABTS radical in phosphate buffer at time 0 and A1 is the absorbance of the ABTS radical solution mixed with the sample after 8 min. All determinations were performed in triplicate ($n = 3$), [44].

2.7. Cell cultures

To perform the cytotoxicity analysis, the five following cell lines were chosen: human fibroblasts (1064sk, CIC-UGA, ES), human leukemia cell line (U-937, ATCC, USA), human malignant melanoma (G-361, ATCC, USA), and colon cancer cell line (HCT-116, ATCC, USA) stored in liquid nitrogen in the cell culture unit of the Central services for research support (SCAI) of Malaga University (UMA). To maintain the cells, 1064sk, G-361 and HCT-116 s were previously cultured using Dulbecco's modified Eagle's medium (DMEM) (Capricorn Scientific, ref. DMEM-HPSTA) supplemented with 10 % fetal bovine serum (Biowest, ref. S1810–500), 1 % penicillin–streptomycin solution 100× (Capricorn Scientific, ref. PS-B), and 0.5 % of amphotericin B (Biowest ref. L0009–100). U-937 were grown in RPMI-1640 medium (BioWhittaker, ref. BE12-167F) supplemented with 10 % fetal bovine serum (Biowest ref. S1810–500), 1 % penicillin–streptomycin solution 100×, and 0.5 % of amphotericin B. In the case of the cells in suspension (U-937), was harvested upon reaching 70–75 % confluence and centrifuged at 1500 rpm for 5 min at room temperature. Once centrifuged, the corresponding test or the subculture of the cells was carried out. The cells were kept under subconfluence in an atmosphere-controlled incubator with 5 % CO_2 at 37 °C.

2.8. Analytic method (MTT assay)

For cancer and healthy cell viability assay, U-937, HTC-116, G-361 and 1064sk cells were incubated at polysaccharide concentrations of 5 to 4.76 × 10^{-6} mg mL^{-1} in serial dilutions (1:1) from the alga *S. skottsbergii*. The experiment was conducted individually with each cell line in a 96-well microplate for 72 h (37 °C, 5 % CO_2 in a humid atmosphere). The proliferation of these cell lines was estimated by the

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay [45]. Briefly, a volume of 10 μL of the MTT solution (5 mg mL^{-1} in phosphate buffered saline) was added to each well. The plates were incubated at 37 °C for 4 h. The yellow tetrazolium salt of MTT was reduced by mitochondrial dehydrogenases of metabolically active viable cells to form insoluble purple formazan crystals. Formazan was dissolved by the addition of acid–isopropanol (150 μL of 0.04 N HCl^{-2} propanol) and measured spectrophotometrically at 550 nm (Micro Plate Reader 2001, Whittaker Bioproducts, USA). The relative cell viability was expressed as the mean percentage of viable cells compared with the untreated cells. Four samples for each tested concentration were included in each experiment. Measurements were carried out in independent experiments in triplicate.

2.9. Calculation of the selectivity index (SI)

The selectivity index determines the cytotoxic selectivity of the compounds tested and is calculated by the ratio between the IC_{50} as indicated in the following formula:

$$SI = \frac{IC\ 50\ OF\ health\ cell}{IC\ 50\ of\ cancer\ cell.}$$

According to Indrayanto et al. [46], a selective compound presents a SI over 3.

2.10. Zebrafish embryo studies

2.10.1. Zebrafish embryo collection

Danio rerio embryos used are offspring of mating between wild type AB and/or transgenic *Tg(fli-1:EGFP)y1* adults from the breeding stock at the "Centro de Experimentación y Conducta Animal" at the University of Málaga (CECA-UMA). Lineages were established from adults purchased from Zebrafish International Resource Centre (ZIRC, Eugene, OR) and cultured by standard husbandry procedures [47,48] under National Laws (Royal Decree Laws 178/2004 and 367/2010, Spain). After fertilization, eggs were bleached and incubated at 28 ± 0.1 °C in Petri dish with E3 embryo medium [49].

2.10.2. Zebrafish embryo acute toxicity test

Four hours post-fertilization (hpf), eggs were placed in 300 μL embryo medium in 96-well plates (1 fish per well) [50]. Following the ZFET procedure [51], eight embryos were incubated for three days in different concentrations of each polysaccharide obtained by dilution of stock solutions in embryo medium. 1 and 4 mg mL^{-1} stock solutions were obtained dispersing lyophilized powder of each polysaccharide in E3 medium and storing them at 4 °C. Incubations in E3 embryo medium were used as negative controls. Experiments were run in triplicate. Experimental results were compared in parallel with effective concentrations of polysaccharides from *Ulva rigida* (Chlorophyta) reported by García-Márquez et al. [52]. Live embryos were over-anesthetized and eliminated as organic waste under UMA procedures.

2.10.3. Phenotypic evaluation

Larva were observed under the magnifying microscope (Nikon SMZ-445 model) or photographed in a Nikon Microphot-FX Fluorescence Microscope with a Nikon DS-L1 camera. During this visual inspection, viability, hatching, chorion lysis, and/or cardiac oedema of each specimen were daily annotated. From viability data, LC_{50} was estimated following a log-linear regression test [53]. Growth delay was estimated by measuring the standard length [47] from digital images of each larva [52] using ImageJ 1.50i settings (National Institutes of Health, USA). Descriptions and measuring were verified by single-blind evaluation. Stage estimation from these variables were not performed [52].

2.11. Gas chromatography–mass spectrometry (GC–MS)

2.11.1. Hydrolysis and derivatization of polysaccharides

Polysaccharide samples (2 mg) and monosaccharide standards were treated with the same procedure. First, 100 μL of the standard stock solution of 1 mg mL^{-1} of each monosaccharide was dried under nitrogen gas flow. Second, the samples of polysaccharides, and a mixture containing the standard monosaccharides included in the IS (Internal Standard), were methanolized in 2 mL methanol/3 M HCl at 80 °C for 24 h. The monosaccharides glucose, galactose, rhamnose, fructose, mannose, xylose, apiose and myo-inositol (Internal Standard, IS), as well as pyridine, hexane and methanol/3 M HCl solution, were purchased from Sigma-Aldrich. Then, the saccharides were washed with methanol and dried under nitrogen gas flow. Third, the trimethylsilyl reaction was accomplished with 200 μL of Tri-Sil HTP (Thermo Fisher Scientific, Franklin, MA, USA). Each vial with the sample was heated to 80 °C for 1 h. The derivatized sample was cooled at room temperature and dried under a steam of nitrogen. Fourth, the dry residue was extracted with hexane (2 mL) and centrifuged. Finally, the hexane solution containing silylated monosaccharides was concentrated and reconstituted in hexane (200 μL), filtered and transferred to a GC–MS autosampler vial. Sample preparation and analyses were performed in triplicate.

2.11.2. Gas chromatography-mass spectrometry (GC–MS) analysis

GC/MS analyses were performed using a Trace GC gas chromatograph (Thermo Scientifics), Tri Plus autosampler and DSQ mass spectrometer quadrupole (Thermo Scientifics). The column was ZB-5 Zebron, Phenomenex (5 % phenyl, 95 % dimethylpolysiloxane) with dimensions of 30 m × 0.25 mm i.d. × 0.25 μm. The column temperature program started at 80 °C (held 2 min) and underwent a gradient of 5 °C min^{-1} to reach a final temperature of 230 °C. The carrier gas was helium (flow 1.2 mL min^{-1}). The injection volume was 1 μL in splitless mode at 250 °C. The source and MS transfer line temperature were 230 °C. The mass spectrometer was set for a Select Ion Monitoring (SIM) program in electron ionization mode (EI) at 70 eV. The TMS-derivatives were identified by characteristic retention times and mass spectrum compared to those of the standards that were used for the identification of monosaccharides. The compounds were identified by comparing the mass spectra with those in the National Institute of Standards and Technology (NIST 2014) library.

2.12. Statistical analysis

The values obtained were expressed as means ± standard deviations (SD) or standard error of the mean (SEM) depending on the case. To determine the statistical differences between each treatment, a one-way analysis of *v*ariance (ANOVA) was performed followed by a Student Newman Keuls (SNK) test (Underwood, 1996). Homogeneity of variance was evaluated using the Cochran test and visual inspection of the residuals (Underwood, 1996). All *in vitro* and chemical analyses were performed using SPSS v.21 (IBM, USA). Differences between means of standard lengths of control and treated zebrafish embryos were statistically evaluated by *t*-test (Statgraphic, Statgraphic Technology, Inc).

3. Results

Biomass from the marine macroalgae *Sarcopeltis skottsbergii* (Rhodophyta) is distributed along the coast of Chile, from Puerto Montt to the Antarctic Peninsula (63° 23'S) [54]. Macroalgae biomass was obtained from Gelymar Company S.A. (Puerto Montt, Chile), washed *in situ*, and transported cold to the "Universidad de La Frontera" (Temuco, Chile). After lyophilization for 48 h, samples were milled to particle size smaller than 1 mm in diameter (see Materials and methods) and stored at −18 ± 2 °C. Neutral and acid polysaccharides were R-phycoerythrin extracted under high-pressure optimization [40]. Neutral polysaccharides were precipitated with ethanol 90 % (*v*/v) after stirring at 90 °C [41] and acid

polysaccharides were precipitated with N-cetylpyridinium bromide 2 % (*w/v*) for 24 h [42]. In general, precipitations were performed by centrifugation at 4500 rpm for 5 min, 4 °C. After discarding the supernatant, pellet was completely dissolved in 10 mL of 4-M NaCl, and ethanol kept at 4 °C for 24 h at a 1:1 (% v/v) ratio. Once centrifuged, the pellet was placed on a dialysis membrane in a 0.5-M NaCl solution overnight at 4 °C. After centrifuging the dialyzed EPS and washing it with absolute ethanol, acid and neutral polysaccharides were extracted from the supernatant, stored at −80 °C and lyophilized.

3.1. Chemical assessment in biomass and polysaccharide

Total carbon and sulfur contents were higher than the nitrogen content in biomass (Table 1). The polysaccharides from *S.skottsbergii* presented the highest percentage in carbon, hydrogen, and sulfur, the latter being the content of a higher order in acid polysaccharides compared to the neutral polysaccharides. In the case of nitrogen, the neutral polysaccharides had a higher percentage than those acid polysaccharides. The molar C/N ratio was 1.72 times higher in the case of acid polysaccharides (121.02 ± 0.4) *versus* neutral polysaccharides (70.0 ± 0.3) (Table 1).

3.2. Gas chromatography–mass spectrometry (GC–MS)

According to the monosaccharide TMS (trimethylsilyl) derivatives composition, the neutral fraction of polysaccharide, Glc (32.37 %) was the major component, followed by Gal (28.37 %) (Table 2). The other acid fraction of polysaccharides, five types of monosaccharide TMS derivatives, such as Rhamnose (Ram), Xylose (Xyl), Mannose (Mann), Galactose (Gal), Glucose (Glc) were detected through GC–MS analysis. The main monosaccharides were Gal, accounting for 67.21 % of the polysaccharide. From the different peaks identified, the high values of Ram, Xyl, Mann and Glc accounted for 0.1 %, 0.29 %, 0.12 %, 2.27 %, respectively (Table 2).

3.3. Fourier transform infrared spectroscopy

The FTIR spectroscopy of the polysaccharides obtained from *S. skottsbergii* revealed the presence of several bands (Fig. 2). In general, both polysaccharides show the characteristic peaks were very close. The strong and wide signal localized between bands 3500 and 2800 cm^{-1} was also observed at approximately 1500–1300 and 1000–800 cm^{-1}. However, peaks at 3000–2800 cm^{-1} and 1500–1400 cm^{-1} were only detected for acid polysaccharides. In fact, the peaks at 900–800 cm^{-1} and 1600–1400 cm^{-1} were caused by the bending vibrations of C—O—S of sulfate in an axial position and C—O of uronic acids respectively. The peaks between 1200 cm^{-1} and 1000 cm^{-1} were attributed to the sugar ring and glycosidic bond C—O stretching vibrations. Bands centered at 3500–3400 cm^{-1} and 2900–2800 cm^{-1} were assigned to hydrogen bonded O—H stretching vibrations and C—H stretching vibrations respectively.

Table 1
Total carbon (C), nitrogen (N), ratio C/N, hydrogen (H) and sulfur (S) (%) obtained in the biomass and polysaccharides (neutral and acid) from *Sarcopeltis skottsbergii*. The data represent the average ± standard deviation ($n = 3$).

	C	N	C/N	H	S
Total biomass	28.38 ± 0.5	1.19 ± 0.04	23.85 ± 0.49	4.83 ± 0.07	5.63 ± 0.05
Neutral polysaccharides	39.924 ± 0.2	0.57 ± 0.01	70.0 ± 0.3	7.219 ± 0.04	1.889 ± 0.02
Acid polysaccharides	25.968 ± 0.1	0.214 ± 0.3	121.02 ± 0.4	4.086 ± 0.08	3.478 ± 0.03

Table 2
Content of monosaccharides in neutral and acid polysaccharide analyzed by gas-liquid chromatography-mass spectrometry (GC–MS).

Fraction of polysaccharides	Monosaccharide	Retention time (min)	Peak area (%)
Neutral polysaccharides	Galactose (Gal)	28.21	1.30
	Galactose (Gal)	29.06	5.28
	Galactose (Gal)	29.29	28.37
	Glucose (Glc)	30.41	32.37
	Glucose (Glc)	29.71	21.60
	Glucose (Glc)	30.22	7.34
	Glucose (Glc)	32.38	3.74
Acid polysaccharides	Rhamnose (Ram)	19.39	0.10
	Rhamnose (Ram)	21.08	0.01
	Xylose (Xyl)	21.98	0.23
	Xylose (Xyl)	24.21	0.04
	Xylose (Xyl)	26.94	0.29
	Mannose (Mann)	27.81	0.12
	Galactose (Gal)	28.24	67.21
	Galactose (Gal)	29.08	25.31
	Galactose (Gal)	29.3	1.66
	Glucose (Glc)	29.73	2.27
	Glucose (Glc)	30.24	0.78
	Galactose (Gal)	30.44	1.92
	Glucose (Glc)	32.39	0.05

Xyl = Xylose; Mann = Mannose; Gal = Galactose; Glc = Glucose; Ram = Rhamnose.

3.4. Antioxidant capacity (ABTS assay) in biomass and sulfated polysaccharides

Antioxidant activity was evaluated in neutral biomass and fractions of polysaccharide.

In the total antioxidant capacity (expressed as percentage of the radical scavenging capacity), all samples showed activity (Fig. 3). The total biomass and neutral polysaccharide showed the lowest activity with 13 and 15 % of scavenging effects, respectively. Moreover, the acid polysaccharide had the greatest activities with approximately 20 % of scavenging effects. Significant differences ($p \leq 0.05$) were observed between the antioxidant activity of biomass, neutral and acid fractions of polysaccharide (Fig. 3).

3.5. Cell viability of lines HTC-116, G-361, U-937 and 1064SK

The cytotoxicity of neutral and acid polysaccharides was determined in the human cancer cells (HCT-116; G-361; U-937) and healthy cells (1064sk). After the treatment of the cells with increasing concentrations of the two samples, cell proliferation was evaluated using the MTT assay. As shown in Figs. 4 and 5, all the samples exhibited relatively high inhibition ratios at all concentration levels and demonstrated an inhibition on the growth of cancer cells. It was observed that the lowest IC_{50} value in G-361 with neutral polysaccharides was 243.2 ± 2.56 mg mL^{-1}, in U-937 IC_{50} cells was 561.6 ± 10.23 mg mL^{-1}, in HCT-116 IC_{50} cells was 963.9 ± 11.63 mg mL^{-1} (Fig. 5; Table 3). For acid polysaccharides the lowest IC_{50} value in G-361 cells IC_{50} was 0.6 ± 1.86 mg mL^{-1}, while in U-937 IC50 was 1.2 ± 0.4 mg mL^{-1} and in HCT-116 was 283.6 ± 12.3 mg mL^{-1} (Fig. 4; Table 3). On the other hand, in the healthy cells line in 1064sK the neutral and acid polysaccharides were 3459 ± 18.3 mg mL^{-1} and 53.6 ± 3.3 mg mL^{-1}, respectively. The highest SI values calculated for the acid fraction extract also belonged to the three above-mentioned cell lines including G-361 (>83.4), U-937(>45.4) and HCT-116 (>0.19) compared to the other cell lines (Table 3). The SI values calculated for the neutral fractions for all the cell lines were low, ranging between 3.59 and 14.22, depending on the cell line, indicating higher effectiveness of the acid fraction extract on the cancer cell lines compared to neutral fractions polysaccharides. These results show an acute cytotoxic effect on both cancer and healthy cells where the acid polysaccharide presented relatively higher antitumor activities against

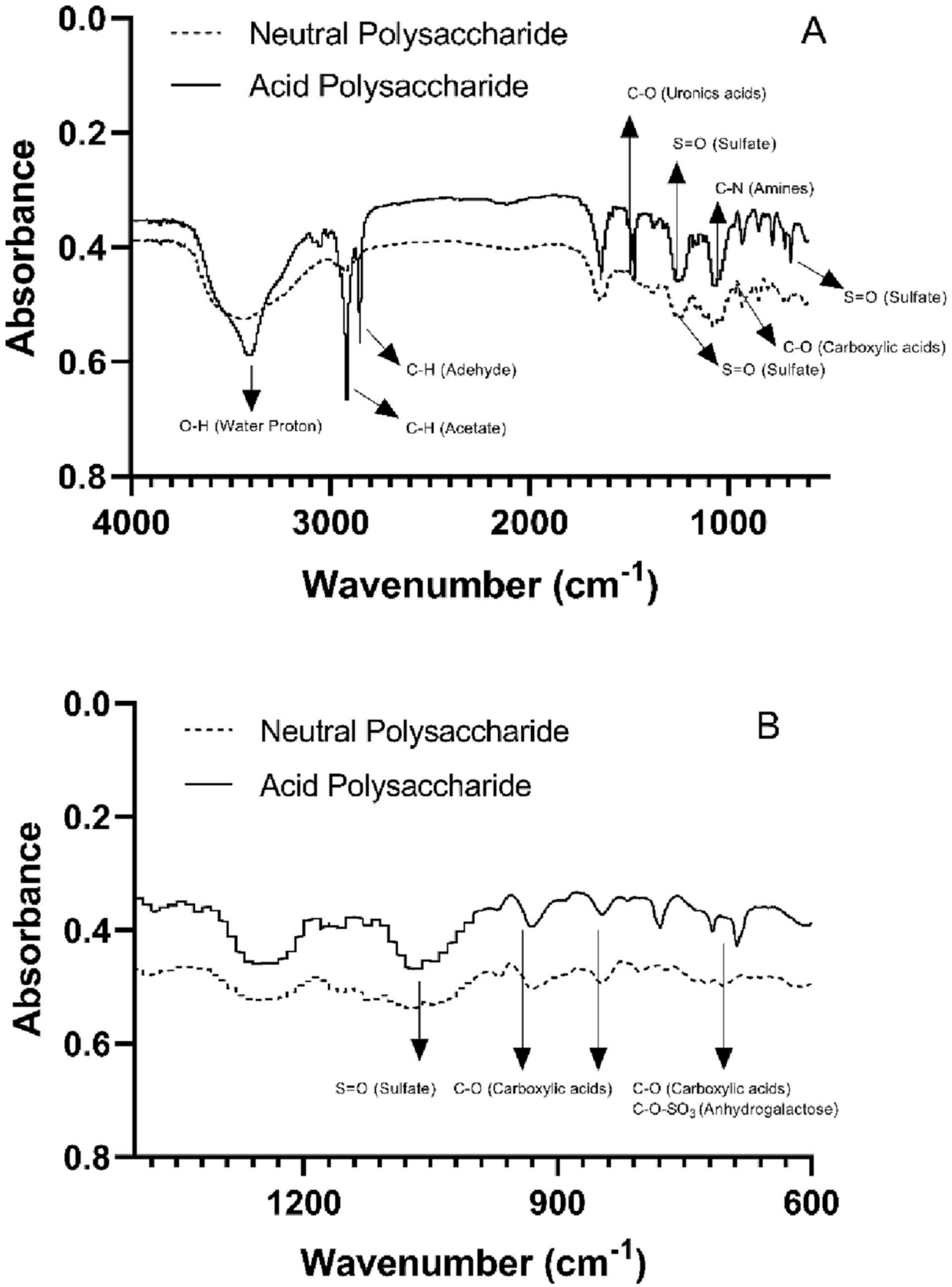

Fig. 2. FTIR spectra of the neutral and acid polysaccharides. A) total spectra from 600 to 4000 cm^{-1}. B) partial spectra from 600 to 1500 cm^{-1}.

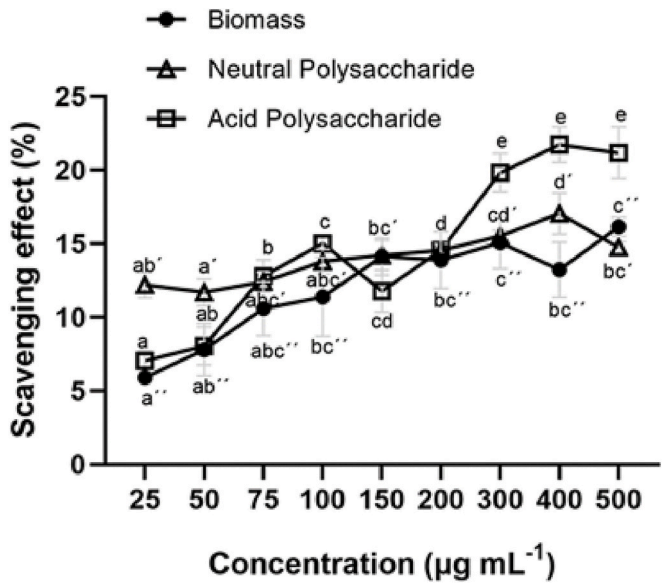

Fig. 3. Scavenging effects % of the sample of biomass, neutral and acid fractions in ABTS radical from *S. skottsbergii.* Different points with the same letter correspond to different concentrations with no significant differences between them (post-hoc Student-Newman-Keuls test).

G-361 cells.

3.6. Zebrafish embryo acute toxicity test of neutral and acid fractions

To study the effect of both fractions on zebrafish development, we have daily studied the viability and general anatomy of zebrafish embryos under each fraction in a variant of the zebrafish embryo acute toxicity test (ZFET), [51]. 4hpf embryos were each placed in a well of a 96-well plate containing 0.300 mL of a *S. skottsbergii* PS dispersion in embryo medium. Concentrations ranged between 0.060, 0.080, 0.100, 0.125, 0.25, 0.5, 1 and 2 mg mL^{-1} of each fraction. At high concentrations, a tenuous PS precipitate could be found over the well and the yolk sac. We have used 0.5 mg mL^{-1} Ulvan PS in E3 medium as a positive control [52] and performed three replica of each condition.

As in the MTT assay, the acid fraction induced higher mortality in zebrafish embryos than the neutral fraction. The increase of both neutral (Fig. 6A) and acid (Fig. 6B) fraction concentrations reduce 72 hpf embryo viability at specific ranges. Concentrations of the neutral fraction declining embryo viability to zero ranged between 0.5 and 2.5 mg mL^{-1} (Fig. 6A) whereas that of the acid fraction was between 0.060 and 0.125 mg mL^{-1} (Fig. 6B). Using a log-linear approach, the calculated LC_{50} of the neutral fraction was 1.477 mg mL^{-1} and that of the acid fraction was 0.080 mg mL^{-1}, much lower than the previous one.

Following the ZFET [51], a list of embryo characters was also observed at a daily basis during the three days. Several anatomical modifications [47] first suggested a reduction in the growing rate of embryos under the effect of increasing concentrations of the neutral fraction. To calculate this, we measured the standard length of neutral fraction-treated 72 hpf larvae from digital images. A slight reduction in embryo growth rate was observed (Fig. 7A-B). This effect is not observed when acid fraction-treated embryos were studied (Figs. 7C-D). When raw standard length was plotted against neutral fraction concentration, a linear reduction is observed ($R^2 = 0.3$; $p < 0.01$, Fig. 7E) with a slight slope of 0.179 mm mg^{-1} mL Only the SL of embryos treated with highest neutral fraction concentrations showed significant *t*-test difference with that of control embryos (Fig. 7E).

When the concentration of both fractions augmented, toxic, osmolarity and teratogenic effects were also observed as part of the ZFET assay (Fig. 8). In any case, no clear relationship between these

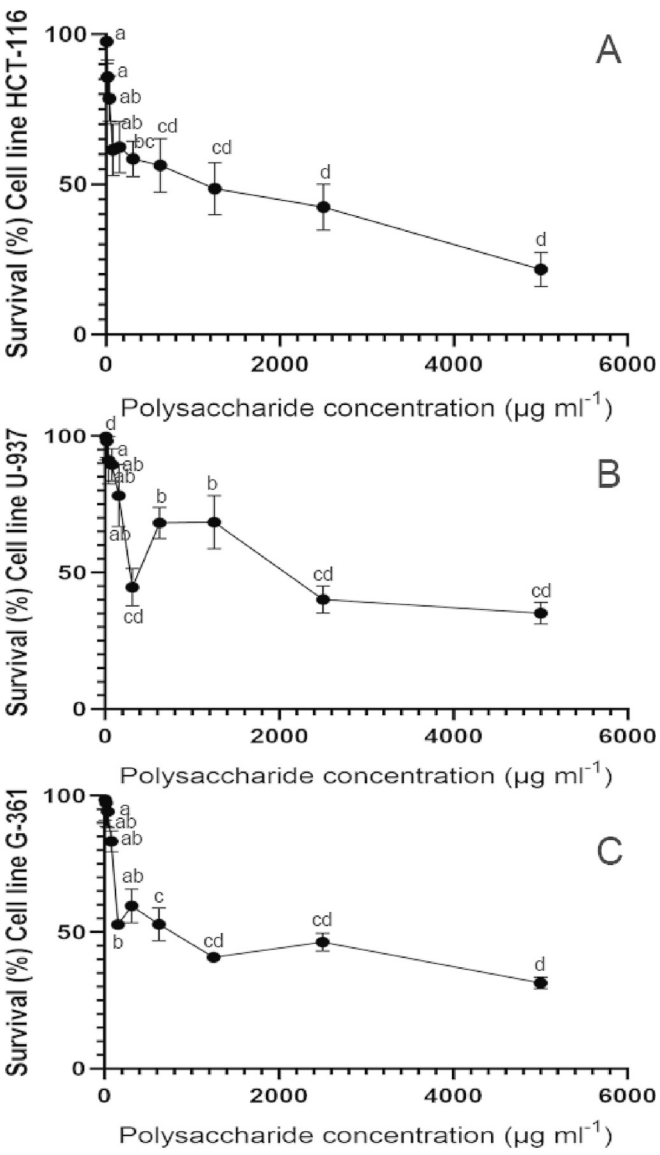

Fig. 4. Survival (%) of cell lines exposed to different concentrations of neutral polysaccharides. (A) Survival (%) of HTC-116 cell line; (B) Survival (%) of U-937 cell line; (C) Survival (%) of G-361 cell line. Different letters indicate significant differences ($p < 0.05$) (ANOVA).

characters and extract concentrations have been unambiguously established. Lordosis (Fig. 8A), scoliosis or chorion lysis (Fig. 8B) have been rarely found in acid fraction-treated embryos, but cardiac oedema have also been found in neutral fraction-treated embryos (Fig. 8C) with no clear relationship with fraction concentration.

4. Discussion

In this study, we first use polysaccharides from *S. skottsbergii* recovered from aqueous fractions derived from phycoerythrin extraction and study their potential bioactivity. Low temperature has been proposed to extract low-molecular-weight carrageenan and to maintain reducing, antiradical and anticoagulant activities, probably due to the higher sulfate content [55]. These activities would be lost after hot water, acid, and alkali treatments [55]. From our observations, high-pressure homogenization offers a process efficiency of depolymerization where desulfation was not observed. We been seen that the degree of sulfation is much higher in acid than total aqueous fraction as potential evidence of a greater bioactivity. In the same vein, Fenoradosoa, et al. [56]

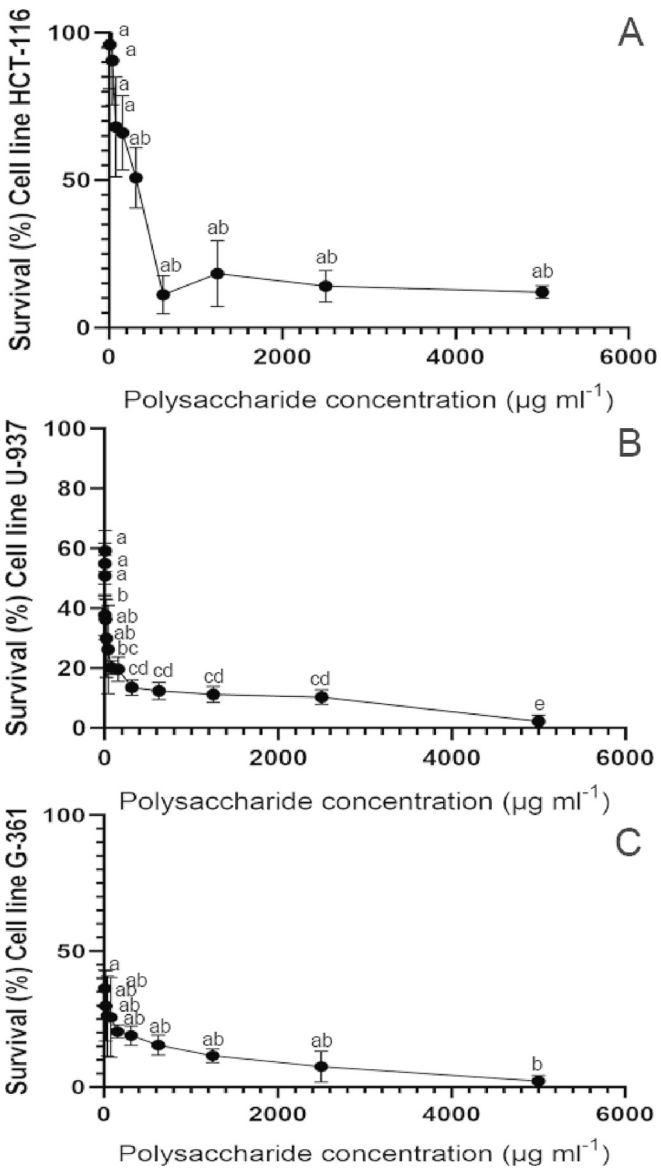

Fig. 5. Survival (%) of cell lines exposed to different concentrations of acid polysaccharides. (A) Survival (%) of HTC-116 cell line; (B) Survival (%) of U-937 cell line; (C) Survival (%) of G-361 cell line. Different letters indicate significant differences ($p < 0.05$) (ANOVA).

Table 3
Cytotoxicity index (IC_{50}) and selective index (SI) for different fractions of polysaccharides in the different cell lines: 1064 sK (human gingival fibroblasts cell line), HCT-116 (human colon cancer), G-361 (human melanoma cancer), U-937 (human leukemia cancer). Different letters indicate significant differences ($p < 0.05$) (ANOVA).

Fraction of polysaccharides	Healthy and carcinoma active cell lines	Cytotoxicity index IC_{50} (μg mL^{-1}).	Selectivity index (SI)
Neutral polysaccharides	1064sK	3459 ± 18.3^{d}	–
	HCT-116	963.9 ± 11.63^{c}	3.59
	U-937	561.6 ± 10.23^{b}	6.16
	G-361	243.2 ± 2.56^{a}	14.22
Acid polysaccharides	1064sK	53.6 ± 3.3^{b}	–
	HCT-116	283.6 ± 12.3^{c}	0.19
	U-937	1.2 ± 0.4^{a}	45.40
	G-361	0.642 ± 0.56^{a}	83.4

reported that high-pressure homogenization on the polysaccharide of *Halymenia durvillei* (red algae) provided feasibility and effectiveness and that high-pressure depolymerization led to ease and speed of preparation. del Río et al. [57] reported the use of microwave hydrothermal treatment for the sustainable valorization of macroalgae *Sargassum muticum* biomass. This procedure allowed the recovery of a liquid phase rich in fucoidan-derived compounds (up to 4.81 gL^{-1}), oligomers and phenols with an antioxidant capacity of interest in the feed/food sector. On the other hand, ultrasound-assisted processes, both alkaline and aqueous, shortened extraction times of polysaccharides from the red algae *Hypnea musciformis*. This avoids degradation of labile compounds, showing a slight variation in viscosity and sulfate, AG and Gal contents [58]. Youssouf et al. [12] reported that they doubled the yields attained in four to eight longer times with conventional extraction without affecting the chemical structure and molar mass distribution of carrageenans.

4.1. Composition of polysaccharides and their antioxidant activity

The FTIR spectra suggest that aqueous supernatant from *S.skottsbergii* have different polysaccharides in their cell wall and intercellular space (matrix) mainly due to the different absorption in the range of 4000 to 500 cm^{-1} (Fig. 2). The broad stretching peak around 3500 cm^{-1} is characteristic of the hydroxyl group, while the weak stretching band at 3000 cm^{-1} suggests C—H bonds in neutral and acid polysaccharide. By contrast, the peak at 1600 cm^{-1} has been shown to indicate the presence of cyclic alkene C—C bonds [59] whereas the peaks between 1400 and 1000 cm^{-1} are attributed to C—O stretching vibration [60] and to a combination of N—H bending and C—H stretching vibration in amide complexes. On the other hand, the peak of strong intensity at 1250 cm^{-1} could be attributed to the S═O (ester sulfate); [61] stretching vibration, which is associated with the spatial distribution of sulfated groups where the acid polysaccharide has greater intensity. According to van de Velde [62], on the analysis of the FTIR-ATR spectrum, the composition of carrageenan's in *S.skottsbergii* (formerly *Gigartina skottsbergii*) showed more equal distribution between Kappa (59 % mol) and iota (about 41 % mol) hybrid carrageenans. Similar results were found on the FTIR-ATR spectrum described by Cotas et al. [63] in *Gigartina pistillata* where a strong percentage of kappa-carrageenan was supported by the 1066 cm^{-1} bands. Between 900 cm^{-1} and 970 cm^{-1} band is related to the presence of galactose units. Also, they found the different compositions of carrageenan extracts between life cycle phases, female gametophyte a kappa/iota hybrid carrageenan and tetrasporophyte a lambda/xi hybrid carrageenan. From a nutraceutical point of view, the kappa/iota composition and distribution are of increasing importance for their specific functionalities in biological systems [63]. In Fig. 2b, is possible to see a more permonorized spectrum between 500 and 1500 cm^{-1} wavenumber. Bands between 700 and 800 cm^{-1} region (characteristic of the lamba and kappa carrageenan spectra, and "mu" and "nu" biological precursors of kappa-carrageenan), indicate the presence of C—O—SO_3, which is corroborated by previous results in *Gigartina pistillata* [63]. Also, carrageenan spectra share strong absorption bands in the 1000–1200 cm^{-1} regions, corresponding to sulphated esters groups (S═O), which are always present in sulphated polysaccharide samples, according to similar vibrational bands by Pereira et al. [59].

The antioxidant capacity of sulfated polysaccharides was measured using an assay based on electron transfer that employed the $ABTS^{+}$ cation radical as an oxidant. ABTS radical scavenging activities of neutral and acid polysaccharide and concentration-dependent radical scavenging activity was thus observed. In general, the two polysaccharide samples exhibited lower scavenging activities (<50 %) at all tested concentrations. Unlike excellent performances in concentrations between 0.3 and 0.5 mg L^{-1}, the scavenging rates of acid polysaccharide was higher than 20 % with the high sulfate content of 3.47 %, while neutral polysaccharide achieved a radical scavenging average of 15 %. Different radical scavenging activities of acid polysaccharide might

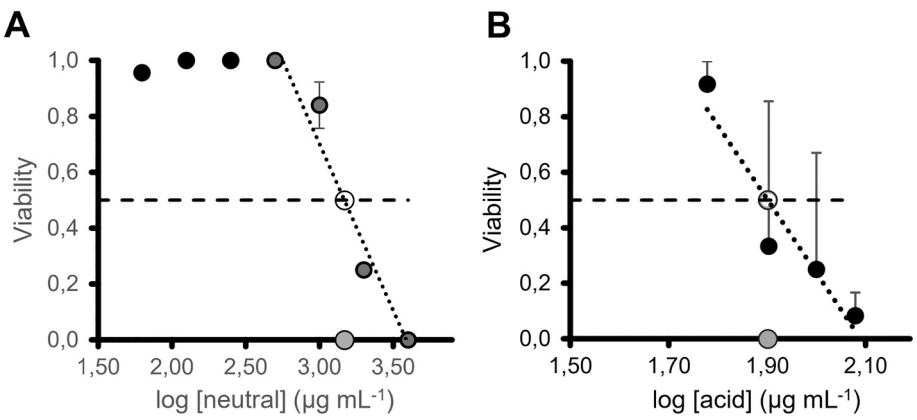

Fig. 6. Estimation of LC_{50} of neutral and acid *S. skottsbergii* polysaccharide-treatment over zebrafish 72 hpf embryos. Plots of viability frequency over logarithm of neutral (A) and acid (B) concentrations. Grey circles are used to estimate LC_{50}. Unused black circles are absent in B. Empty circles are intersections of regression line and 50 % viability. Light grey circles are log (LC_{50}) estimations. Vertical bars are standard deviations. Linear adjustments are Viability = −1.1926 log[neutral] + 4.2797 (R^2 = 0.9532, $p \approx$ 0.0000) (A) and Viability = −2.6566 log[acid] + 5.5499 (R^2 = 0.9049, $p \approx$ 0.0000) (B) (Excel, Microsoft Office). Each point is the average of data from the three replicas (about 24 larvae).

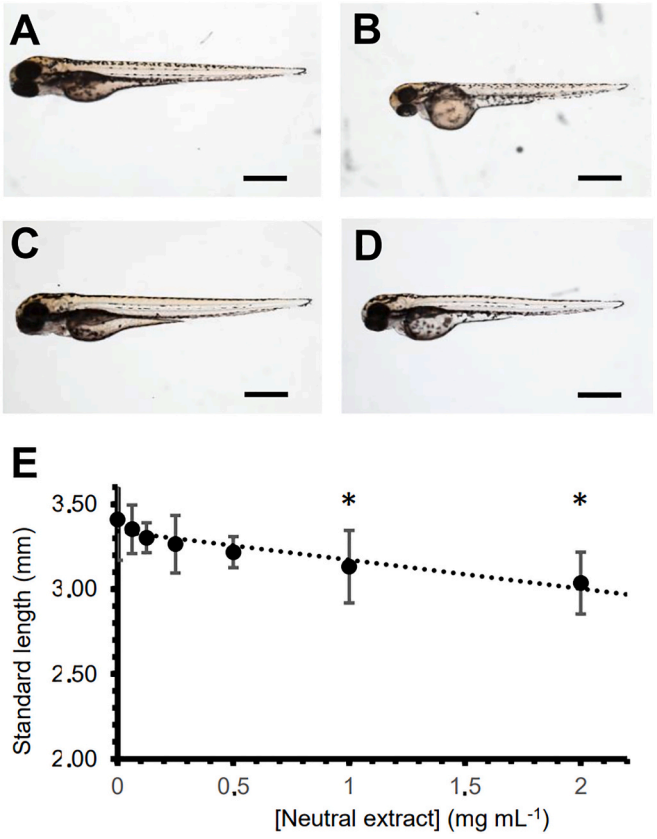

Fig. 7. Increasing concentrations of neutral *S. skottsbergii* polysaccharide fraction slightly reduces zebrafish growth rate. (A-B) Body size reduction after exposition to neutral fraction for three days. Embryos grown in E3 medium (A) and 2 mg mL^{-1} neutral extracts (B). (C-D) Acid fraction treatments do not show significant body size reduction. 72 hpf control (C) and 0.060 mg mL^{-1} acid fraction-treated (D) embryos. Bars represent 500 µm. (E) Linear regression between standard length and concentration of neutral fraction. Black circles are means and vertical bars standard deviation. Linear adjustment of raw data (not shown) is standard length = −0.179 [neutral extract] + 3.34 (R^2 = 0.3; $p \approx$ 0.000) (Excel, Microsoft Office). * is $p < 0.05$.

suggest that the chemical composition had a certain effect on antioxidant activity. Studies over the last several years have revealed that sulfated polysaccharides from several seaweed types have an appreciable antioxidant capacity. For example, sulfate polysaccharides from *Mastocarpus stellatus* (Red algae) had a significant positive correlation between sulfate and ABTS ($r = 0.83$) [55]. Similar results in acid polysaccharide from *Gelidium corneum* (Red algae) and *Porphyra umbilicalis* (Red algae) also showed significant antioxidant capabilities in hydroxyl radical scavenging assays [42]. Multifunctional antioxidant capacity of different polysaccharide fractions in seaweed can be directly correlated to increasing sulfate content [64] or to decreasing molecular weight of polysaccharides [65]. Our results suggested that sulfate content could also have a relevant effect on radical scavenging of acid fractions when compared to the neutral fractions. However, the high sulfate content in the total biomass showed lower ABTS activity. In this regard, other potential synergies should be tested by analyses such as DPPH, ORAC, among others.

The acid fractions also show high amounts of galactose and small amounts of rhamnose, xylose, mannose, and glucose. In turns, the neutral fractions only contained glucose and galactose in its monosaccharide content. Moreover, the polymerization was higher in acid than the neutral fraction with high-pressure homogenization. Thus, high-pressure homogenization before hot water treatment was enough to allow further breakdown of the complex polysaccharide, very different to those of the neutral fractions. Cotas et al. [63] found in *Gigartina pistillata* (Red algae) lower depolymerization (only two monosaccharides) with sequential extraction when using a lower temperature (60 °C) with a water solvent. Furthermore, Mateos-Aparicio et al. [66] found high depolymerization with different concentration of alkaline solvent before applying the hot water treatment (85–90 °C) in the same species *Gigartina pistillata*. Although Torres et al. [17] did not analyze the biopolymer monosaccharides profile in the red algae *Mastocarpus stellatus*, ultrasound-assisted extraction was shown to be a practical method for polysaccharide degradation that provided low molecular weight oligosaccharides with new functionalities and bioactivities. The differences between the weight and content of monosaccharides are due to the physiological and nutritional stress of the macroalgae caused by environmental conditions, the different methods of extraction, derivatization, and analysis strategies [14,67,68]. Our results showed that shorter times of the pre-treatment of 46 °C under high-pressure homogenization before hot water extraction of polysaccharides enhances the extraction process performance.

4.2. Sulfated groups increase antitumor activity in cell culture and toxic activity in zebrafish embryos

It has been reported that the sulfated groups played a major role in the suppression of cancer cell growth by binding with cationic proteins on the cell surface, and the polysaccharides with higher sulfate contents exhibited stronger antitumor activities *in vitro* [19,60,65,69,70]. In this work, acid fractions of polysaccharides, which had higher sulfate contents that neutral ones, showed markedly higher antitumor activities against the G-361 cells, U-937 and HCT-116 cells. A previous study also showed that uronic acid in these types of extracts could affect their antitumor and immunomodulatory activities [71]. Although we have

Fig. 8. Teratogenic and toxic effects of polysaccharide extracts from *S. skottsbergii*. Slight lordosis (A) or scoliosis (B) and cardiac oedema (C) have been observed in zebrafish embryos treated with concentrations equal or over 0.060 mg mL^{-1} acid (A, B) or neutral (C) extracts. Chorion lysis (B) have also been observed at these conditions. Bars represent 500 μm.

not detected uronic acid in either polysaccharide, they showed significantly high inhibition ratios. Similar results were found by Sheng et al. [72] where different fractions of polysaccharides extracted from green microalgae *Auxenochlorella pyrenoidosa* (formerly *Chlorella pyrenoidosa*) exhibited high antitumor activity, exhibiting galactose and rhamnose as the mains monosaccharides.

Due to the absence of uronic acid, the antitumor and antioxidant activities observed *in vitro* may be related to comprehensive effects of their sulfate contents. On the other hand, the anticancer activity of carrageenan has been reported to be closely related to its molecular weight. When native carrageenan was hydrolyzed in boiling water with HCl acid for 4 h, a significant increase in anticancer activity was observed [73,74]. Moreover, carrageenan hydrolyzed in a microwave oven showed little improvement in anticancer activity [9]. This suggests that the anticancer activity of carrageenan could be significantly enhanced by lowering its molecular weight by depolymerization under mild conditions [67,75]. Other reasons could be associated to the concentrations and the effect on the osmotic equilibrium. In this sense, Willebrand et al. [76] found that concentrations over 40 mM of NaCl produce an osmotic shock and inhibit tumor growth in Human Melanoma cells (B16F10). Inclusive, Calvo et al. [77] suggest that the carrageenan-containing phagolysosomes eventually rupture due to osmotic swelling. The consequent release of hydrolytic enzymes into the cytosol causes irreversible damage and eventual lysis of macrophages. In this sense, the osmotic change generated by the depolymerization of carrageenan would be influencing the cell viability of tumor cells. Regarding the relation between sugar concentration and osmotic potential, we did not find any information about antitumoral activity of algae polysaccharides due to this mechanism of action. Ibrahim et al. [78] indicates an anticancer effect of an acidic polysaccharide extracted from *Bacillus sp.* NRC5 by regulating Na^+/K^+ ATPase, a transmembrane protein that plays an important role on cell ionic and osmotic equilibrium. In our case, further studies are necessary to elucidate the mechanism of action of the polysaccharide extracted with the osmotic potential. On the other hand, high-pressure homogenization may depolymerize our water-soluble polysaccharides leading to high levels of cytotoxicity index (IC_{50}) on G-361, U-937 and HCT-116. Zainal-Ariffin et al. [74] reported similar results, when effects of carrageenan from *Eucheuma denticulatum* (red algae) on human colon cancer Caco-2 with $IC_{50} = 1000$ (μg mL^{-1}) was analyzed. In our analyses on HCT-116, the acid polysaccharides from *S. skottsbergii* presented 18.89 times higher cytotoxicity than neutral polyssacharides in the same line of culture (HCT-116). In addition, Torres et al. [17] reported an activity enhancement of carrageenan extracts by ultrasound-assistance when compared with the conventional extraction method. The cytotoxicity observed was also 3.39 times more effective against the cancer cell line HT-29 than that induced by the conventional extracts obtained by Suganya et al. [79]. In general, the relation between the chemical characteristics and bioactivities of polysaccharides needs further investigation. The fractionation of polysaccharides by extractive green systems such as high pressure could be an important effect due to the high affinity with biology systems. Future studies in an *in vivo* system are necessary to relate antiproliferative and antioxidant activities as potential nutraceuticals.

In principle, the low IC_{50} values for G-361 (0.642 ± 0.56) and U-937 (1.2 ± 0.4) as well as the high SI values for the extract in these cells (>83.4 for G-361 and >45.4 for U-937) would suggest acid polysaccharide as a promising therapeutic candidate particularly on skin and blood cancers. Knowing that SI values over 3 indicate general toxicity and that the great SI value suggests more selectivity [46], we conclude that acid fraction extracts are good candidates for growth suppression of all the examined cell lines with SI values >10. Nevertheless, the significant activity over healthy cells of acid fractions would have a prejudicial narrowing effect of the potential therapeutic range of this fraction over patients. This interpretation is also supported by the study on our zebrafish embryo acute toxicity test. Acid fractions indeed showed low LC_{50} but small toxic, osmolarity, teratogenic or cardiac oedema effects [51] when compared with neutral fractions.

Finally, the two sulfated polysaccharides extracted from the red algae *S. skottsbergii* after previous extraction of phycoerythrin by high-pressure homogenization (HPH-assisted extraction) water extraction following a biorefinery approach exhibits antioxidant and antitumor activities. Specifically, acid polysaccharide with high sulfate and the galactose contents showed the highest activity in scavenging radical assays in the ABTS radical assay, *i.e.*, a radical scavenging rate of 20 % at

the concentration between 0.3 and 0.5 mg mL^{-1}. The antitumor activity of the acid fraction was significantly higher in all cancer cell lines tested than that of the neutral fraction. However, the neutral fraction with the lowest sulfate content and galactose content exhibited a middle level activity in the scavenging radical assay, but had higher antitumor activities against G-361 melanoma cancer cells with a high selective index of 14.22 and low toxicity on fibroblast healthy cells. To our certain knowledge, polysaccharide fractions recovered from a supernatant bioprocess as antitumor and antioxidant biocompounds could be applied in the pharmacology, cosmeceutical, and nutraceutical industry.

CRediT authorship contribution statement

Conceptualization, P.C-V., M.R., F.L.F. and R.A-D.; methodology, B. R. and P.C-V.; experiments, P.C-V and B.R.; zebrafish methodology: C.C-S and M.J.P.; zebrafish experiment supervision and writing, M.M.-B; data curation, P.C-V, B.R. and M.R.; validation, P.C-V and B.R.; writing original draft preparation, P.C-V.; writing-review and editing, P. C-V., M.R., F.L.F. M.M.-B. and R.A-D.; visualization, P.C-V; supervision, M.R., F.L.F. and R.A-D. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare that there are no conflicts of interest.

Data availability

The data that support the findings of this study are openly available from the corresponding author [P.C-V.] on request if the editor or reviewer needs it.

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Bioethical approvals

Experiments were conducted under notifications A/ES/12/I-22 and A/ES/12/24 to Ministryof Ecological Transition and Demographic Challenge and approval 100-2018-T of the UMA Bioethics commission.

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