fermentation

MDPI

Article

Antioxidant and Anticancer Potential of Extracellular Polysaccharide from *Porphyridium aerugineum* (Rhodophyta)

Juliana G. Ivanova [1], Tanya S. Toshkova-Yotova [1], Reneta A. Toshkova [2], Veronika R. Deleva [1], Ani K. Georgieva [2] and Liliana G. Gigova [1,*]

[1] Institute of Plant Physiology and Genetics, Bulgarian Academy of Sciences, Acad. G. Bonchev Str. Bl. 21, 1113 Sofia, Bulgaria; juivanova@yahoo.com (J.G.I.); t_toshkova_yotova@abv.bg (T.S.T.-Y.); delevaveronika93@gmail.com (V.R.D.)

[2] Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Acad. G. Bonchev Str. Bl. 25, 1113 Sofia, Bulgaria; reneta_toshkova@yahoo.com (R.A.T.); georgieva_any@abv.bg (A.K.G.)

* Correspondence: gigova01@gmail.com

Abstract: *Porphyridium aerugineum* is a unicellular freshwater red microalga that synthesizes and secretes into the culture medium an extracellular polysaccharide (EPS). In this study, algal growth and polysaccharide production, as well as the antioxidant capacity and antitumor effect of *Porphyridium aerugineum* EPS (PaEPS), were investigated. Cultivation of the microalgae was carried out in a photobioreactor under controlled conditions. Algal growth and the amount of EPS were monitored daily. The accumulated polysaccharide was extracted and lyophilized. At the end of cultivation, the concentration of microalgal biomass and PaEPS reached 3.3 and 1.2 g L^{-1}, respectively. To examine the antioxidant capacity of PaEPS, FRAP and ABTS assays were performed. The cytotoxic activity of PaEPS was evaluated on the tumor cell lines MCF-7 (breast cancer) and HeLa (cervical adenocarcinoma) and on BJ (a non-tumor human skin fibroblast cell line), using MTT assay. The results obtained indicated that *P. aerugineum* polysaccharide exhibited a high ABTS radical-scavenging activity reaching up to 55%. The cytotoxic effect was best expressed in MCF-7 cells treated for 72 h with 1000 μg/mL PaEPS, where tumor cell proliferation was inhibited by more than 70%. Importantly, the PaEPS treatments did not significantly affect the viability of BJ cells. These findings promote the biotechnological production of *P. aerugineum* extracellular polysaccharide and reveal its potential as an anticancer and antioxidant agent for future applications.

Keywords: photobioreactor; *Porphyridium aerugineum*; extracellular polysaccharide; antioxidant activity; anticancer activity

Citation: Ivanova, J.G.; Toshkova-Yotova, T.S.; Toshkova, R.A.; Deleva, V.R.; Georgieva, A.K.; Gigova, L.G. Antioxidant and Anticancer Potential of Extracellular Polysaccharide from *Porphyridium aerugineum* (Rhodophyta). *Fermentation* **2024**, *10*, 259. https://doi.org/10.3390/fermentation10050259

Academic Editor: Diomi Mamma

Received: 5 April 2024
Revised: 10 May 2024
Accepted: 13 May 2024
Published: 15 May 2024

1. Introduction

Microalgae are unicellular eukaryotic microorganisms which thrive in both saltwater and freshwater environments. Despite the absence of a complex structure and organs when compared to plants, microalgae are able to perform photosynthesis using sunlight, carbon dioxide and water owing to the presence of photosynthetic pigments in their cells. These remarkable phototrophic microorganisms have the advantages of a high growth rate and high photosynthetic rate, and their capacity to fix carbon dioxide is 10 times that of terrestrial plants [1]. Microalgae can biosynthesize, metabolize, accumulate and secrete a wide variety of primary and secondary metabolites, many of which, such as lipids, proteins (amino acids), carbohydrates, carotenoids, phycobiliproteins, vitamins, etc., exhibit different biological activities and pharmacological properties, thus having current and future applications for the benefit of human health. The combination of high photosynthetic efficiency with the ability to produce a large amount of intracellular high-value biocompounds makes microalgae a suitable candidate to serve as an

industrial feedstock [2]. Moreover, growing in a variety of habitats and not requiring arable land or clean water, microalgae have advantages for mass culturing. Their cultivation can be carried out in closed, open or hybrid systems, with closed photobioreactors being considered the most efficient system [3]. The type of cultivation system, together with various physical and chemical factors, such as light, temperature, carbon dioxide, nutrients and pH, influence the yield of algal biomass and therefore the amount of compounds synthesized. After cultivation under suitable conditions, the accumulated biomass is harvested, processed and converted into the desired products. Cell-free culture medium can also be a source of bioproducts.

Among the numerous metabolites synthesized by microalgae that exhibit biological activities are sulphated polysaccharides (sPS). Rich and sustainable source of sPS are various species of red microalgae [4]. The cells of red microalgae are encased in a matrix of these polysaccharides. The thickness of the polysaccharide capsule varies with growth phase and culture conditions. During algal growth, polysaccharides from the outer part of the matrix are dissolved in the culture medium [5], thus facilitating their extraction. The ability to produce large amounts of sulphated extracellular polysaccharides (sEPS) under controlled culture conditions [6] and the possibility for efficient isolation of the accumulated sEPS [7] give advantages to red microalgae in the study and use of these valuable biological products. Indeed, sEPS produced by red microalgae have been the subject of a growing number of publications regarding their physicochemical properties as well as their biological activities, such as antioxidant, anti-inflammatory, antiviral, antifungal, antibacterial, anticancer, immunomodulatory, antiaging, etc. [4,8,9]. The great potential of sEPS from marine microalgae, including red microalgae, to be used as nutraceuticals, therapeutic agents or cosmetics, are approached in the review article by [10].

Since the characteristics of sulfated extracellular polysaccharides vary among different species of red microalgae and the available knowledge is the result of studies mainly on marine species, our investigation focused on the less-explored freshwater species *Porphyridium aerugineum*. In 1972, Ramus et al. found that *P. aerugineum* produced and secreted a high-molecular-weight sulfated heteropolysaccharide [11]. Recently, the chemical, structural and rheological properties of this EPS were characterized by Liberman et al. [8]. According to the authors, xylose was the predominant monosaccharide of the *P. aerugineum* EPS in concentrations of ~33 mol%, followed by glucose and galactose. The amounts of methylated sugars were 12.6–20.7 mol%, and the contents of glucuronic acid and sulfur were 4.4 mol% and 0.8%, respectively. Research on the biological activity of EPS from *P. aerugineum* is also scarce, although the antioxidant and anticancer potential of red microalgae polysaccharides has been investigated by a number of authors [12–17]. To date, only one publication has been identified regarding the antioxidant activity of *P. aerugineum* EPS [18]. The authors registered significantly decreased levels of malondialdehyde in the oxidant-damaged soybean-based infant formula in the presence of polysaccharide compared to the control (without polysaccharide).

The aim of the present study was to investigate the growth and extracellular polysaccharide production of *Porphyridium aerugineum* cultivated in a photobioreactor under controlled conditions and to evaluate the activity of the extracted extracellular polysaccharide as an antioxidant agent as well as its cytotoxic effects on human tumor cells and healthy cells.

2. Materials and Methods

2.1. Microalgal Strain

In this study, the freshwater red microalga *Porphyridium aerugineum* Geitler strain HINDAK 1983/2 (Piestany, Slovakia) maintained in the algal collection of the Laboratory "Experimental and applied algology", IPPG, BAS, was used. The strain was grown on medium of Pekarkova [19].

2.2. Microalgal Cultivation

Porphyridium aerugineum was cultivated for 7 days in photobioreactor Applikon with a 2.5 L vessel and control module Applikon ADI 1020 (Applikon, Schiedam, The Netherlands). The reactor was equipped with additional graphical software—BioXpert V2. The inner dimensions of the photobioreactor were 13 cm in diameter and 25 cm in height. The temperature was maintained at 26 °C (previously selected as optimal for maximum EPS production). The cultures, standardized at an initial density of 0.5 mg mL^{-1} dry weight (DW), were continuously illuminated by an LED light panel at an irradiance level of 7500 lux. The pH was kept within the range of 7.1–7.6 using a solution of 0.2 M NaOH. Algal cultures were aerated by sterilized air enriched with up to 2% CO_2 and the concentration of the outlet air was analyzed with a Hartmann & Broun Advance Optima gas analyzer (Minden, Germany). Stirring was conducted with a mechanical stirrer at 150 rpm. The working volume of the bioreactor was 1300 mL.

2.3. Dry Weight Determination

The growth of the microalgal culture was estimated following the increase in biomass dry weight. For this purpose, 10 mL of the algal suspension were centrifuged at 6000× *g* for 20 min (Rotofix 32A, Hettich, Tuttlingen, Germany). The supernatant was removed and the cells were dried at 105 °C for 16 h. Prior to drying, salts were eliminated by rinsing the biomass thrice with distilled water.

2.4. Cell Count

To evaluate the growth and development of the investigated strain, cell counts were carried out using a Burker counting chamber (Fisher Scientific, Tonbridge, UK).

2.5. Carbohydrate Analysis

The content of total soluble carbohydrates was determined by the phenol–sulfuric acid method proposed by Dubois et al. [20]. In brief, 0.5 mL of culture supernatant was added to 0.5 mL of 5% phenol and 2.4 mL of sulfuric acid. After 30 min of incubation at room temperature, the extinction was measured at 490 nm on a T70 UV/VIS spectrometer (PG Instruments, Ltd., London, UK). The concentration of carbohydrates was determined by reference to a standard curve using glucose as a standard and expressed in g L^{-1}.

2.6. Extracellular Polysaccharide Viscosity, Extraction and Purification

Measurement of the viscosity (η) of the culture supernatant was accomplished to follow the dynamics of extracellular polysaccharide accumulation during *P. aerugineum* growth. The viscosity (η) was measured by viscometer KF10 (DIN 53015 (Rheotec, Drezden, Germany). This falling ball viscometer uses Höppler's simple but precise principle to measure the viscosity of Newtonian fluids by measuring the time required for a ball to fall under gravity through a sample-filled tube [21]. The viscosity was calculated after measuring the time of passage of the falling sphere between two marks by the following formula:

$$\eta = t \times (Q_1 - Q_2) \times K \quad (1)$$

where η is the dynamic viscosity of the liquid (mPa·sec); tis time for the ball to fall in the viscosimeter; $Q_1$is the ball density; $Q_2$is the density of the studied liquid; and Kis the ball constant (mPa·cm^3 g^{-1}). Data presented are the mean of five replicates.

The isolation of *P. aerugineum* EPS was carried out according to Simon et al. [22]. At the end of cultivation, cultures were centrifuged (10,000× *g*, 10 min) and the supernatant containing the soluble extracellular polysaccharide was mixed with 96% ethanol (1:2, *v*/*v*). The gel-like precipitate was collected, dissolved, and dialyzed (2.3 cm dialysis tubing, MW cutoff 12.4 kDa) against distilled water. Dialysis took place for 48 h at 4 °C,

after which the polysaccharide was lyophilized and powdered for testing in the present experiments.

2.7. Evaluation of Antioxidant Activity

The first method applied for the antioxidant activity evaluation of PaEPS was the FRAP assay (Ferric Reducing Antioxidant Power), carried out according to Benzie and Strain [23]. The FRAP reagent was freshly prepared by mixing equal volumes of 300 mM acetate buffer (pH 3.6), 10 mM TPTZ (2,4,6-tripyridyl-S-triazine) solution in 40 mM HCl and 20 mM $FeCl_3$ (Merck, Darmstadt, Germany) in a ratio 10:1:1. The 300 μL sample was mixed with 2.7 mL of FRAP reagent and the absorbance of the reaction mixture was measured at 593 nm after incubating it for 10 min at 37 °C. The FRAP value was calculated and expressed as μmol Trolox Equivalent per gram dry weight of the sample (μmol TE g^{-1} DW) based on a calibration curve plotted using Trolox as the standard.

The second method used, the ABTS radical cation decolorization assay, was performed as described by Re et al. [24]. Briefly, ABTS radical cation solution was prepared through a 12–16h reaction of ABTS (2,20-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid));(7.4 mM), with potassium persulfate (2.6 mM) at room temperature in the dark. The solution was diluted 28 times using dH_2O to obtain an absorbance of 0.700 at 734 nm. A total of 100 μL of extract was mixed with the diluted ABTS (2.9 mL). The mixture was shaken for 10 sec and left undisturbed for 15 min. The absorbance at 734 nm was measured using a T70 UV/VIS spectrophotometer (PG Instruments, Ltd., London, UK). ABTS scavenging activity was expressed by inhibition percentage calculated as follows:

$$\text{Inhibition (\%)} = [(A_{control} - A_{sample})/A_{control}] \times 100 \quad (2)$$

where A_{sample} is the absorbance of ABTS solution mixed with extract, and $A_{control}$ is the absorbance of ABTS solution mixed with 100 μL dH_2O instead of extract.

2.8. Anticancer Activity

Stock solution of PaEPS: prepared in Dulbecco's Modified Eagle's Medium (DMEM) at a concentration of 2.0 mg mL^{-1} with working concentrations of 2000 μg mL^{-1}, 1000 μg mL^{-1}, 500 μg mL^{-1}, 250 μg mL^{-1}, and 125 μg mL^{-1}.

Test system: HeLa (ATCC® No. CCL-2™) tumor cells—a permanent human cervical adenocarcinoma tumor cell line; MCF-7 (ATCC® No. HTB-22™) tumor cells—a permanent human mammary adenocarcinoma tumor cell line; BJ—a non-tumor permanent cell line of human skin-derived fibroblast cells (normal human skin fibroblasts).

2.8.1. Cell Cultivation

The three cell types (HeLa, MCF-7, and BJ) were grown as a monolayer culture in DMEM, containing 10% fetal calf serum (FBS) and antibiotics (100 IU mL^{-1} Penicillin, 0.1 mg mL^{-1} Streptomycin) in 50 cm^3 plastic tissue culture dishes. Cell cultures were incubated in a thermostat at 37 °C, 5% CO_2 and 95% humidity.

2.8.2. MTT Test (to Prove Cell Viability/Vitality/Proliferation)

The antitumor activity of PaEPS was evaluated by the viability (proliferation) of the examined cells after their treatment. The effect of PaEPS applied at different concentrations for 48 and 72 h on the viability of human tumor (HeLa and MCF-7) and normal (BJ) cells was assessed calorimetrically by the MTT assay, as reported by Mossmann [25]. Cells (2×10^4 $well^{-1}$) were seeded into a 96-well tissue culture plate and incubated to form monolayer. After achieving 60–70% confluency (about 24 h), the medium was removed and fresh medium supplemented with polysaccharide solution (each concentration at 100 μL final volume) was added in each well and cells were

incubated for 48 and 72 h. Each concentration of PaEPS was tested in five replicates. Cell cultures supplemented with medium alone (DMEM with 10% FBS) were used as a negative control for the experiments. After the treatment, the medium was aspirated and 100 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) working solution (5.0 mg mL^{-1} in PBS) was added to each well, followed by additional incubation at 37 °C, 5% CO_2 and 95% humidity for 3 h. The formazan crystals formed due to MTT reduction from living cells were dissolved in 100 µL of a solution containing DMSO and EtOH (1:1, *v*/*v*). Optical density (OD) was measured at 570 nm and 620 nm (as a reference wavelength) on an ELISA spectrophotometer (TECAN, SunriseTM, Grödig/Salzburg, Austria). Cell viability was calculated using the formula

$$\text{Cell viability (\%)} = OD_{570}\text{ (experiment)}/OD_{570}\text{ (negative control)} \times 100 \quad (3)$$

where OD_{570} (experiment) and OD_{570} (negative control) represent the absorbance value of treated and nontreated cells, respectively. The concentrations required for 50% inhibition (IC_{50}) of cell growth were calculated using nonlinear regression analysis (GraphPad Prism 4 Software).

2.9. Statistical Analysis

All the experiments were performed in triplicate unless otherwise stated and the quantitative data are presented as mean and standard deviations (SD). One-way analysis of variance (ANOVA) followed by Bonferroni's post hoc test was performed. Values of * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ were considered significant, using software package GraphPad Prism 5, USA.

3. Results and Discussion

3.1. Growth and Polysaccharide Production

The closed photobioreactor ensures high photosynthetic efficiency and very good control [26]. Some of the main advantages of a closed system are controlling the growth of microalgae, preventing contamination, limiting the transfer of gases and bacteria between the cultivation vessel and environment, manipulating the system and optimizing physiological parameters. During phototrophic growth, in order to multiplicate, microalgae consume dissolved carbon dioxide and produce oxygen (O_2).

The online measured CO_2 uptake rate and O_2 production rate can be used as inputs for the metabolic model and give insight into the biomass yield. However, high concentrations of dissolved oxygen (dO_2) can inhibit the growth of the photosynthetic microalgae [27]. Oxygen produced in the culture is a major issue in closed photobioreactors during microalgal growth as oxygen is a by-product of photosynthesis and its concentration can reach over four times the air saturation in the culture, which constrains the photosynthesis rate. Stress due to oxygen oversaturation has been so far less studied, despite its negative effect on growth. At a high concentration, O_2 can compete with CO_2 for RuBisCo, the key enzyme in the Calvin cycle [28]. There are different mathematical models describing the effect of the dissolved oxygen concentration on the photosynthetic rate and biomass production [29,30].

In our research, the cell number and dissolved oxygen concentration were continuously monitored to ascertain the productivity of *P. aerugineum* (Figure 1). A slight decrease in dissolved oxygen at 72 and 144 h was recorded, which is probably related to a protective response of the strain to prevent oxidative stress from the accumulation of large amounts of oxygen in the suspension with a switch from photosynthesis to photorespiration, although the culture was illuminated continuously. During cultivation, the number of cells increased 5-fold in 168 h, and the concentration of dissolved oxygen increased 1.68-fold.

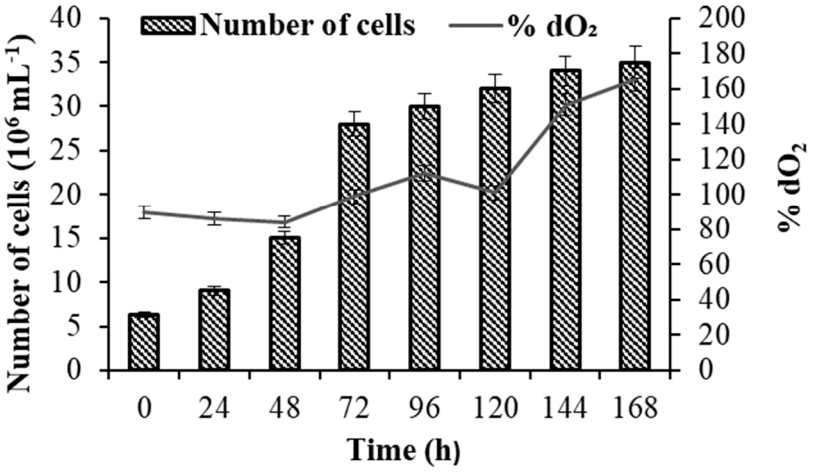

Figure 1. Biomass yield and dissolved O_2 in microalgal suspension.

The results obtained on the growth and polysaccharide production of *P. aerugineum* showed that this strain was easily cultivated in a suitable medium. The growth curves of the strain displayed a lag phase during the first two days and exponential growth in the next 3 days. Microalgal biomass increased to 3.3 g L^{-1} at the end of cultivation and the viscosity (η) of the culture medium (supernatant) increased to 4.1 mPa.sec due to the synthesis of large amounts of EPS (Figure 2).

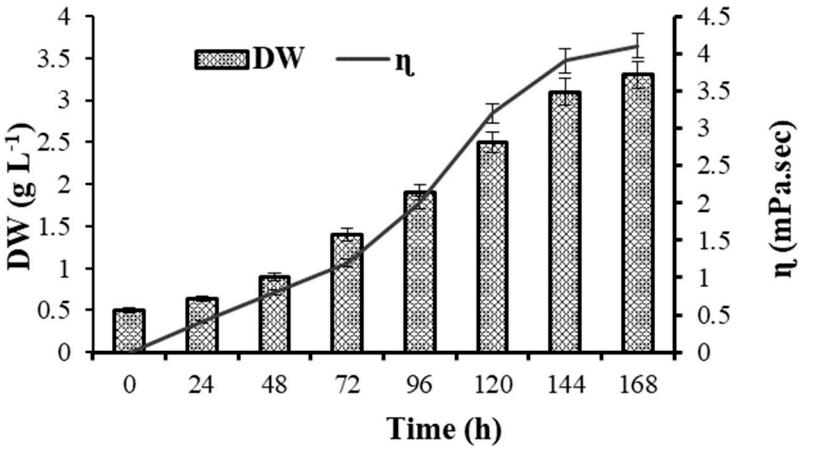

Figure 2. Growth of *Porphyridium aerugineum* and production of extracellular polysaccharide.

It should be noted that the viscosity of the culture medium directly correlates with the measured quantity of carbohydrates (extracellular polysaccharide) contained in it (Table 1). In fact, EPS production was observed throughout the growth process of *P. aerugineum*, with the highest amount accumulating at the entry into the stationary phase (168 h). Similar results were obtained in our study on the growth and polysaccharide production of other red microalgae, the marine species *Porphyridium cruentum* and *Porphyridium sordidum* [13, 31].

Table 1. Increase in *Porphyridium aerugineum* EPS amount during algal growth.

Time	48 h	72 h	96 h	120 h	144 h	168 h
Polysaccharide concentration (g L^{-1})	0.08 ± 0.04	0.2 ± 0.03	0.4 ± 0.02	0.68 ± 0.02	1.0 ± 0.01	1.2 ± 0.02

Our results are in agreement with the previously established negative correlation between cell division and total polysaccharide production in some microalgae; viz., increased polysaccharide production occurs during the stationary phase of growth [32,33].

3.2. Antioxidant Activity

The ferric-reducing antioxidant power (FRAP) and 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) colorimetric assays were used to assess the antioxidant potential of *P. aerugineum* EPS. The FRAP method has a single electron transfer mechanism (SET) and is based on the ability of antioxidant compounds to reduce the Fe^{3+} - tripyridyltriazine complex (Fe^{3+}- TPTZ) to the blue ferrous form (Fe^{2+} - TPTZ), while ABTS quantifies the decay of the colored stable free radical ($ABTS^{\bullet+}$) in the case of its interaction with electron or hydrogen donors (SET or hydrogen atom transfer; HAT mechanism).

There have been numerous reports evaluating the antioxidant activity of microalgae, but their polysaccharides, and especially extracellular polysaccharides, have only been the subject of research in recent years. Available information in the scientific literature indicates that the assessment of the antioxidant potential of microalgae polysaccharides may vary depending on the specifics of the assay used. Since ferric ions (Fe^{3+}) act as precursors to generate free radicals, the ability of the tested sample to reduce a ferric ion to a ferrous ion (Fe^{2+}) is an indicator of its antioxidant capacity. FRAP has been successfully applied to evaluate the antioxidant activity of intracellular extracts from microalgae. Ethanol/water extracts of *Porphyridium cruentum* and *Isochrysis* sp., for example, were reported to exhibit ferric ion reduction potentials of 10.89 μmol TE g^{-1} DW and 53.73 μmol TE g^{-1} DW, respectively [34]. Examining three types of green microalgae using this method, Ivanova et al. [35] found that the cell extract of *Chlorella vulgaris* showed the best antioxidant activity—32 μmol TE g^{-1} DW.

Our results from FRAP assay showed that the ferric ion-reducing potential of *P. aerugineum* extracellular polysaccharide is dose dependent (Table 2). At the highest concentration tested (1.5 mg mL^{-1}) the polysaccharide showed an FRAP relative to 25 ± 0.1 μmol Trolox Equivalent per gram dry weight of the sample.

Table 2. Antioxidant activity of *Porphyridium aerugineum* extracellular polysaccharide at different concentrations according to FRAP assay.

Polysaccharide	μmol TE g^{-1} DW
0.5 mg mL^{-1}	10 ± 0.15
1.0 mg mL^{-1}	18 ± 0.07
1.5 mg mL^{-1}	25 ± 0.10

In comparison, sulfated polysaccharides from the freshwater green microalga *Chlamydomonas reinhardtii* exhibited 28–68% ferric ion reduction potential at 0.5–5 mg mL^{-1}, respectively [36]. Crude and purified water-soluble polysaccharides from another green microalga *Tetraselmis* sp. KCTC 12432 BP had no detectable antioxidant activities according to the FRAP assay, whereas those from *Tetraselmis* sp. KCTC 12236 BP showed specific activities of 25.05 ± 4.03 and 8.77 ± 0.25 μmol g^{-1} min^{-1}, respectively [37]. In a study of Liberti et al. [38] the sulfated extracellular polysaccharides produced by the red marine microalga *Porphyridium cruentum* (CCALA415) were not able to scavenge the ABTS and DPPH radicals, whereas a significant activity (34% inhibition at a final concentration of 0.12 mg mL^{-1}) was observed for ferric ion reduction assay.

The ABTS radical scavenging activity of the EPS of *P. aerugineum* was evaluated at four different concentrations (0.1–1.5 mg mL^{-1}) (Table 3). The results showed 55 ± 0.08% inhibition of ABTS at a polysaccharide concentration of 1.5 mg mL^{-1}, and 47 ± 0.07% inhibition at 1 mg mL^{-1}.

Table 3. ABTS radical scavenging activity of *Porphyridium aerugineum* extracellular polysaccharide at different concentrations.

Polysaccharide (mg mL^{-1})	Inhibition (%)
0.1	11 ± 0.20
0.5	27 ± 0.10
1.0	47 ± 0.07
1.5	55 ± 0.08

According to Casas-Arrojo et al. [39], the maximum antioxidant activity observed for *P. cruentum* (S.F.Gray) Nägeli sulfated extracellular polysaccharides using the ABTS assay was 6.92 ± 0.81% at a concentration of 0.2 mg mL^{-1}. The EPSs produced by *P. purpureum* and *P. cruentum* (CCALA 415) showed about 12% and 18% ABTS scavenging activities, respectively at a concentration of 1 mg mL^{-1} [40]. The ABTS radical inhibitory activity of polysaccharide from another red microalga *Rhodosorus* sp. was about 30% at a concentration of PS of 1 mg mL^{-1} and 64.42% at a concentration of 2 mg mL^{-1} [41]. Polysaccharides from the red macroalga *Phyllophora pseudoceranoides* also showed antioxidant activity, with a recorded ABTS scavenging effect of 30.3% at a concentration of 1.5 mg mL^{-1} [42]. Wang et al. [43] reported a similar inhibitory activity of the EPS produced by the green microalga *Botryococcus braunii* even at a concentration of 5 mg mL^{-1}, while at a concentration of 1 mg mL^{-1}, the inhibitory ability of the polysaccharide was slightly above 20%. The sulfated polysaccharides of the marine green microalgae *Chlorella sorokiniana* and two strains of *Chlorella* sp. showed significant antiradical activity, scavenging more than 90% of ABTS radicals when the polysaccharide concentration was 2 mg mL^{-1} [17].

3.3. Anticancer Activity

In our previous studies [13,16], it was found that treatment of cancer cells with the heteropolysaccharides from the marine red microalgae *Porphyridium sordidum* and *Porphyridium cruentum* caused a decrease in cancer cell viability. These results were an incentive for us to investigate the anticancer activity of the extracellular polysaccharide of freshwater *P. aerugineum*, for which there are no data yet. For this purpose, two different types of cancer cell lines, MCF-7 (breast cancer) and HeLa (cervical adenocarcinoma), were tested. The cell line BJ (normal human skin fibroblasts) was used as a control of healthy cells to assess the level of safety in potential use of the tested compound in human subjects. The cells were treated with PaEPS applied at concentrations of 2000 μg mL^{-1}, 1000 μg mL^{-1}, 500 μg mL^{-1}, 250 μg mL^{-1} and 125 μg mL^{-1} for 48 and 72 h. The results of the MTT assay performed at the 48th and 72nd hour of exposure of MCF-7, HeLa and BJ cells to the PaEPS action are presented in Figure 3, Figure 4 and Figure 5, respectively.

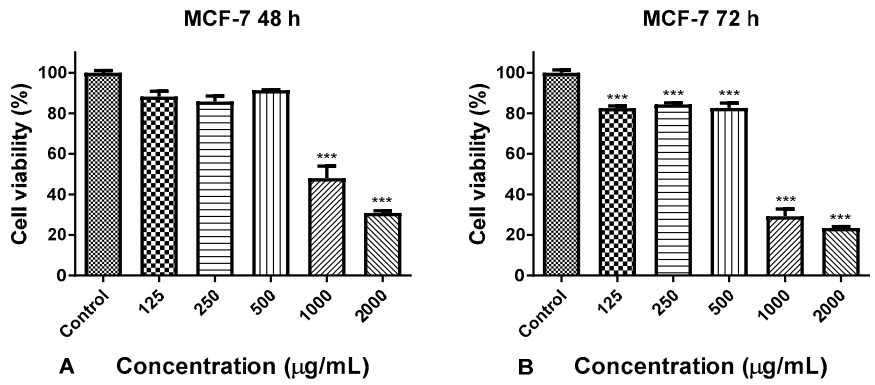

Figure 3. Effect of different concentrations of EPS from *P. aerugineum* on the proliferative activity/viability of MCF-7 tumor cells assessed at the 48th (**A**) and 72nd (**B**) hour of treatment. The

data are expressed as mean ± SD. *** $p < 0.001$ versus the control; no asterisk means no significant difference.

We found a significant inhibitory effect of EPS from *P. aerugineum* on the MCF-7 tumor cell line, which was dependent on both the EPS concentration and the duration of treatment. The proliferation/viability of MCF-7 tumor cells incubated in the presence of PaEPS for 48 h was significantly decreased ($p < 0.001$) compared to untreated cells (100% viability) only at a polysaccharide concentration of 1000 and 2000 μg mL^{-1} (48.02 ± 10.33% and 30.81 ± 1.99% viability, respectively) (Figure 3A). At 72 h, all tested concentrations of PaEPS inhibited the proliferation of MCF-7 cells to a statistically significant extent, with the most pronounced effect at concentrations of 1000 μg mL^{-1} and 2000 μg mL^{-1}, where the viability values were 29.21 ± 6.21% and 23.45 ± 1.18%, respectively (Figure 3B). However, no significant differences were observed between the concentrations 125–500 μg mL^{-1} and 1000–2000 μg mL^{-1}. The concentration of PaEPS capable of reducing MCF-7 cell viability by 50% compared to the untreated control (IC_{50}) was determined to be 1139 ± 74 μg mL^{-1} and 807 ± 76 μg mL^{-1} at 48 and 72 h treatment, respectively.

The PaEPS did not affect the viability of HeLa tumor cells as well as of BJ cells (normal human skin fibroblasts) (Figures 4A,B and 5A,B) even after 72 h of treatment. The reported viability values of the treated BJ cells and HeLa cells were not significantly different from those of the corresponding untreated control.

The results obtained with the MTT assay showed that unlike the BJ and HeLa cell lines, MCF-7 tumor cells were sensitive to *P. aerugineum* EPS. The growth inhibition of MCF-7 cells reached 76% when cells were treated with PaEPS at a concentration of 2000 μg mL^{-1} for 72 h. The selective effect exhibited by PaEPS can be explained by the different origins and expressions of different receptors in the three cell lines used.

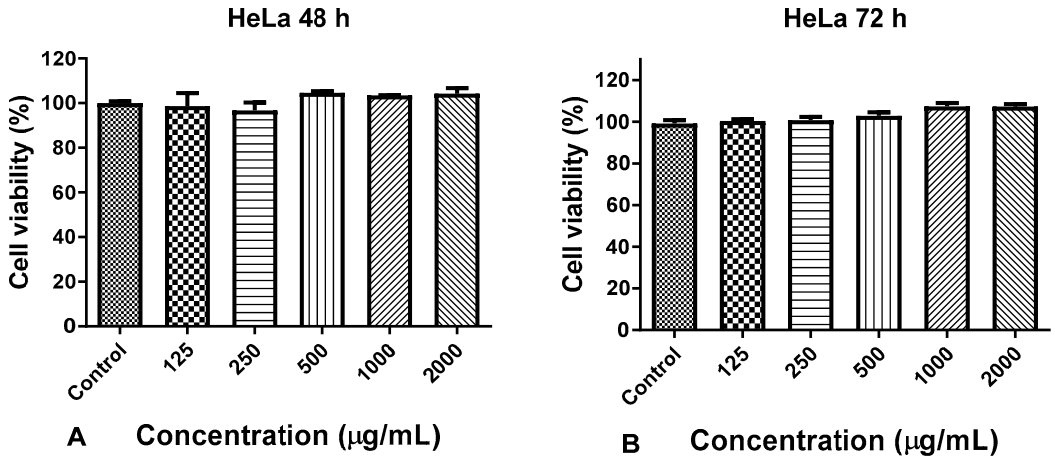

Figure 4. Effect of different concentrations of EPS from *P. aerugineum* on cell viability of HeLa tumor cells after 48 (**A**) and 72 (**B**) hours of treatment. The data are expressed as mean ± SD.

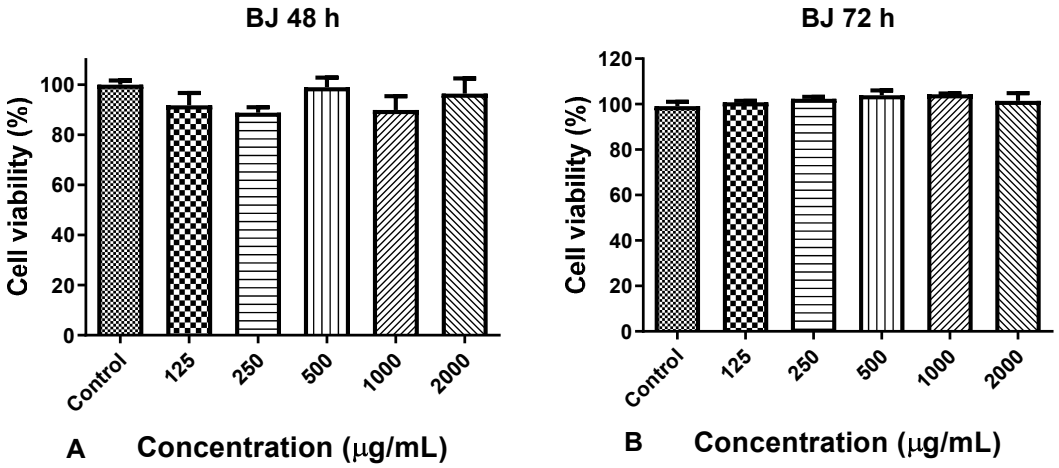

Figure 5. Effect of different concentrations of EPS from *P. aerugineum* on the proliferative activity/viability of BJ cells (normal human skin fibroblasts) after 48 (**A**) and 72 (**B**) hours of treatment. The data are expressed as mean ± SD.

The established dual antioxidant-anticancer activity of EPS derived from *P. aerugineum* is beneficial but not surprising, as such activity has also been reported for some polysaccharides from other algae. The extracellular polysaccharides isolated from the green microalgae *Chlorella pyrenoidosa* FACHB-9, *Scenedesmus* sp. and *Chlorococcum* sp. were shown to have the ability to scavenge hydroxyl radicals, with respective IC_{50} values of 0.75, 0.38 and 0.44 mg mL^{-1}. Their inhibitory effect on the viability of a human colon cancer cell line (HCT8) reached 22.9–38.6% at a polysaccharide concentration of 600 μg mL^{-1} [44]. Besides antioxidant activity (about 7% inhibition of ABTS at a concentration of 0.2 mg mL^{-1}), sEPS from *P. cruentum* exhibited cytotoxicity to five tumor cell lines, with IC_{50} ranging from 2311.20 μg mL^{-1} to 5498.14 μg mL^{-1} [39]. In a comparative study by Shao et al. [45], the authors showed that sulfated polysaccharides from the green alga *Ulva fasciata* (UFP), the red alga *Gloiopeltis furcata* and the brown alga *Sargassum henslouianum* (SHP) have in vitro antioxidant and antitumor activity. The two activities vary depending on the algae species and on the test system used, but mostly on the content of both sulfate and uronic acid in the polysaccharide. UFP (19.41% sulfate and 35.06% uronic acid) showed high antioxidant activity in the ABTS assay (36.34% inhibition at 0.1 mg mL^{-1} UFP), but demonstrated a weak inhibitory effect on the growth of MKN45 gastric cancer cells and DLD intestinal cancer cells. SHP with a lower sulfate and uronic acid content had relatively lower radical scavenging rates but showed significantly higher antitumor activity (47% and 28% inhibition effect on MKN45 and DLD cells, respectively, at 1000 μg mL^{-1} SHP).

Despite numerous studies demonstrating the biological activities of microalgal EPS, knowledge of the mechanisms by which they act is limited. The lack of complete characterization of the structures of these EPS makes it difficult to establish a clear relationship between structure and biological activity [33]. In a number of publications, however, it has been shown that a combination of some characteristics, such as monosaccharide components, sulfate content, sulfation pattern, uronic acid content, and molecular weight, are involved in the bioactivity of the polysaccharide, including antioxidant and/or antitumor activity [9,15–17,37, 45,46].

In the scientific literature, there are data on an increase in the antioxidant and anticancer activity of polysaccharides after using various techniques to reduce their molecular weight. Sun et al. [46], for example, found that high-molecular-weight polysaccharides from *P. cruentum* had no antioxidant activity, but after microwave degradation, low-molecular-weight fragments exerted an inhibitory effect on oxidative

damage. Gargouch et al. [15] showed increased efficacy against breast cancer cells of lower molar mass fractions of *Porphyridium marinum* polysaccharide. Microwave-derived polysaccharide fractions from *P. cruentum* EPS had a stronger inhibitory effect on the growth of HT-29 colon carcinoma cells (54.1% inhibition of cell viability) than the native polysaccharide (44% inhibition of cell viability), both administered at a concentration of 100 μg mL^{-1} [16]. Further studies on the primary structure of the polysaccharide from *P. aerugineum* Geitler strain HINDAK 1983/2 as well as on the biological activity of its polysaccharide fractions are in progress.

4. Conclusions

The poorly studied freshwater red microalga *Porphyridium aerugineum* was intensively cultivated in a photobioreactor. Basic physiological parameters of algal growth and extracellular polysaccharide accumulation dynamics were established. The extracted and purified polysaccharide showed high ABTS radical scavenging activity and ferric reducing antioxidant power (FRAP), and these in vitro antioxidant effects of the polysaccharide positively correlated with its concentration. Furthermore, *P. aerugineum* polysaccharide exhibited significant cell type-specific antiproliferative activity on the breast cancer cell line MCF-7, inhibiting cell growth by over 75%, but not on non-tumor, normal cells. These results suggest its potential pharmacological impact in cancer treatment without side effects. To the best of our knowledge, this study is the first to evaluate and report the antioxidant and anticancer activity of extracellular polysaccharide derived from *P. aerugineum*.

It can be concluded that the biotechnologically obtained extracellular polysaccharide from *Porphyridium aerugineum* has potential as an anticancer and antioxidant agent for future applications in the pharmaceutical, food and cosmetic industries.

Author Contributions: Conceptualization: J.G.I.; methodology: J.G.I., R.A.T. and L.G.G.; investigation and data curation. V.R.D., J.G.I., A.K.G., R.A.T. and T.S.T.-Y.; writing—original draft preparation: J.G.I.; writing—review and editing: L.G.G., J.G.I. and R.A.T.; supervision: L.G.G. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Institutional Review Board Statement: Not applicable.

Informed Consent Statement: Not applicable.

Data Availability Statement: All data generated and analyzed during this study are included in the manuscript.

Conflicts of Interest: The authors declare no conflicts of interest.

References

1. Lalibertè, G.; Noüie, J.D.L. Auto-, hetero-, and mixotrophic growth of *Chlamydomonas humicola* (Cmloroimiyckak) on acetate. *J. Phycol.* **2010**, *29*, 612–620.
2. Tan, J.S.; Lee, S.Y.; Chew, K.W.; Lam, M.K.; Lim, J.W.; Ho, S.H.; Show, P.L. A review on microalgae cultivation and harvesting, and their biomass extraction processing using ionic liquids. *Bioengineered* **2020**, *11*, 116–129.
3. Mahmood, T.; Hussain, N.; Shahbaz, A. Sustainable production of biofuels from the algae-derived biomass. *Bioprocess Biosyst. Eng*. **2023**, *46*, 1077–1097.
4. Esqueda, B.A.; Gardarin, C.; Laroche, C. Exploring the diversity of red microalgae for exopolysaccharide production. *Mar. Drugs* **2022**, *20*, 246.
5. Arad, S.M.; Levy-Ontman, O. Red microalgal cell-wall polysaccharides: Biotechnological aspects. *Curr. Opin. Biotech.* **2010**, *21*, 358–364.
6. Khan, M.I.; Shin, J.H.; Kim, J.D. The promising future of microalgae: Current status, challenges, and optimization of a sustainable and renewable industry for biofuels, feed, and other products. *Microb. Cell Fact.* **2018**, *17*, 36–57.
7. Li, H.; Li, Z.; Xiong, S.; Zhang, H.; Li, N.; Zhou, S.; Liu, Y.; Huang, Z. Pilot-scale isolation of bioactive extracellular polymeric substances from cell-free media of mass microalgal cultures using tangential-flow ultrafiltration. *Process Biochem.* **2011**, *46*, 1104–1109.
8. Liberman, G.N.; Ochbaum, G.; Mejubovsky-Mikhelis, M.; Bitton, R.; Arad, S.M. Physico-chemical characteristics of the sulfated polysaccharides of the red microalgae *Dixoniella grisea* and *Porphyridium aerugineum*. *Int. J. Biol. Macromol.* **2020**, *145*, 1171–1179.

9. Prybylski, N.; Toucheteau, C.; El Alaoui, H.; Bridiau, N.; Maugard, T.; Abdelkafi, S.; Fendri, I.; Delattre, C.; Dubessay, P.; Pierre, G.; et al. Chapter 20—Bioactive polysaccharides from microalgae. In *Handbook of Microalgae-Based Processes and Products*; Jacob-Lopes, E., Maroneze, M., Queiroz, M., Zepka, L., Eds.; Academic Press: Cambridge, MA, USA; 2020; pp. 533–571.
10. Raposo, M.F.; de Morais, R.M.S.C.; de Morais, A.M.M.B. Bioactivity and applications of sulphated polysaccharides from marine microalgae. *Mar. Drugs* **2013**, *11*, 233–252.
11. Ramus, J. The production of extracellular polysaccharide by the unicellular red alga *Porphyridium aerugineum*. *J. Phycol.* **1972**, *8*, 97–111.
12. Gardeva, E.; Toshkova, R.; Minkova, K.; Gigova, L. Cancer protective action of polysaccharide, derived from red microalga *Porphyridium cruentum*—A biological background. *Biotechnol. Biotechnol. Equip.* **2009**, *23*, 783–787.
13. Nikolova, B.; Semkova, S.; Tsoneva, I.; Antov, G.; Ivanova, J.; Vasileva, I.; Kardaleva, P.; Stoineva, I.; Christova, N.; Nacheva, L.; et al. Characterization and potential antitumor effect of a heteropolysaccharide produced by the red alga *Porphyridium sordidum*. *Eng. Life Sci.* **2019**, *19*, 978–985.
14. Yang, S.; Wan, H.; Wang, R.; Hao, D. Sulfated polysaccharides from *Phaeodactylum tricornutum*: Isolation, structural characteristics, and inhibiting HepG2 growth activity in vitro. *PeerJ*. **2019**, *7*, e6409.
15. Gargouch, N.; Elleuch, F.; Karkouch, I.; Tabbene, O.; Pichon, C.; Gardarin, C.; Rihouey, C.; Picton Abdelkafi, L.S.; Fendri, I.; Laroche, C. Potential of exopolysaccharide from *Porphyridium marinum* to contend with bacterial proliferation, biofilm formation, and breast cancer. *Mar. Drugs* **2021**, *19*, 66.
16. Ivanova, J.; Konstantinidou, A.; Kabaivanova, L. Examination of exopolysaccharides from *Porphyridium cruentum* for estimation of their potential antitumour activity in vitro. *C. R. Acad. Bulg. Sci.* **2022**, *75*, 1146–1155.
17. Mousavian, Z.; Safavi, M.; Azizmohseni, F.; Hadizadeh, M.; Mirdamadi, S. Characterization, antioxidant and anticoagulant properties of exopolysaccharide from marine microalgae. *AMB Express* **2022**, *12*, 27.
18. Burg, A.; Oshrat, L.O. Salt effect on the antioxidant activity of red microalgal sulfated polysaccharides in soy-bean formula. *Mar. Drugs* **2015**, *13*, 6425–6439.
19. Pekarkova, B.; Hindak, F.; Smarda, J. Morphological characteristics and physiological properties of a coccoid rhodophycean alga *Rhodella grisea* from thermal springs at Piestany, Czechoslovakia. *Arch. Protistenkd.* **1988**, *135*, 69–83.
20. DuBois, M.; Gilles, K.A.; Hamilton, J.K.; Rebers, P.A.; Smith, F. Colorimetric method for determination of sugars and related substances. *Anal. Chem*. **1956**, *28*, 350–356.
21. Abbas, K.A.; Abdulkarim, S.M.; Saleh, A.M.; Ebrahimian, M.M. Suitability of viscosity measurement methods for liquid food variety and applicability in food industry—A review. *J. Food Agric. Environ*. **2010**, *8*, 100–107.
22. Simon, B.; Geresh, S.; Arad, S. Degradation products of the cell wall polysaccharide of the red microalga *Porphyridium* sp. (Rhodophyta) by means of enzymatic activity of its predator the dinoflagellate *Gymnodinium* sp. (Gymnodiales). *J. Phycol.* **1992**, *28*, 460–465.
23. Benzie, I.; Strain, J.J. The ferric reducing ability of plasma (FRAP) as a measure of "antioxidant power". The FRAP assay. *Anal. Biochem*. **1996**, *239*, 70–76.
24. Re, R.; Pellegrini, N.; Proteggente, A.; Pannala, A.; Yang, M.; Rice-Evans, C. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic. Biol. Med.* **1999**, *26*, 1231–1237.
25. Mossmann, T. Rapid colorimetric assay of cellular growth and survival: Application to proliferation and cytotoxicity assays. *J. Immunol. Methods* **1983**, *65*, 55–63.
26. Hossain, S.Z.; Alnoaimi, A.; Razzak, S.A.; Ezuber, H.; Al-Bastaki, N.; Safdar, M.; Hossain, M.M. Multiobjective optimization of microalgae (*Chlorella* sp.) growth in a photobioreactor using Box-Behnken design approach. *Can. J. Chem. Eng.* **2018**, *96*, 1903–1910.
27. Kazbar, A.; Cogne, G.; Urbain, B.; Marec, H.; Le-Gouic, B.; Tallec, J.; Takache, H.; Ismail, A.; Pruvost, J. Effect of dissolved oxygen concentration on microalgal culture in photobioreactors. *Algal Res.* **2019**, *39*, 101432.
28. Foyer, C.H.; Bloom, A.J.; Queval, G.; Noctor, G. Photorespiratory metabolism: Genes, mutants, energetics, and redox signaling. *Annu. Rev. Plant Biol.* **2009**, *60*, 455–484.
29. Darveheia, P.; Bahria, P.A.; Moheimanib, N.R. Model development for the growth of microalgae: A review. *Renew. Sustain. Energy Rev*. **2018**, *97*, 233–258.
30. López Muñoz, I.; Bernard, O. Modeling the influence of temperature, light intensity and oxygen concentration on microalgal growth rate. *Processes* **2021**, *9*, 496.
31. Vasileva, I.; Ivanova, J. Biochemical profile of green and red algae—A key for understanding their potential application as food additives. *Trakia J. Sci.* **2019**, *17*, 1–7.
32. Arad, S.; Friedman, O.; Rotem, A. Effect of nitrogen on polysaccharide production in a *Porphyridium* sp. *Appl. Environ. Microbiol*. **1988**, *54*, 2411–2414.
33. Laroche, C. Exopolysaccharides from microalgae and cyanobacteria: Diversity of strains, production strategies, and applications. *Mar. Drugs* **2022**, *20*, 336.
34. Goiris, K.; De Vreese, P.; De Cooman, L.; Muylaert, K. Rapid screening and guided extraction of antioxidants from microalgae using voltammetric methods. *J. Agric. Food Chem.* **2012**, *60*, 7359–7366.
35. Ivanova, J.; Kabaivanova, L.; Vasileva, I. Assessment of the production potential of valuable compounds with antioxidant properties of different green microalgae. *Oxid. Commun.* **2021**, *44*, 27.

36. Falcao, B.; Vishwakarma, J.; Jadav, H.; Vavilala, SL. In vitro evaluation of the antioxidant and anti-skin aging properties of green algal sulfated polysaccharides. *Arch. Microbiol. Immunol.* **2020**, *4*, 75–90.
37. Dogra, B.; Amna, S.; Park, Y.; Park, J.K. Biochemical properties of water soluble polysaccharides from photosynthetic marine microalgae *Tetraselmis S*pecies. *Macromol. Res.* **2017**, *25*, 172–179.
38. Liberti, D.; Imbimbo, P.; Giustino, E.; D'Elia, L.; Silva, M.; Barreira, L.; Monti, D.M. Shedding light on the hidden benefit of *Porphyridium cruentum* culture. *Antioxidants* **2023**, *12*, 337.
39. Casas-Arrojo, V.; Decara, J.; de los Ángeles Arrojo-Agudo, M.; Pérez-Manríquez, C.; Abdala-Díaz, R.T. Immunomodulatory, antioxidant activity and cytotoxic effect of sulfated polysaccharides from *Porphyridium cruentum* (S.F.Gray) Nägeli. *Biomolecules* **2021**, *11*, 488.
40. Wang, W.-N.; Li, Y.; Zhang, Y.; Xiang, W.-Z.; Li, A.-F.; Li, T. Comparison on characterization and antioxidant activity of exopolysaccharides from two *Porphyridium* strains. *J. Appl. Phycol.* **2021**, *33*, 2983–2994.
41. Wang, N.; Dai, L.; Chen, Z.; Li, T.; Wu, J.; Wu, H.; Wu, H.; Xiang, W. Extraction optimization, physicochemical characterization, and antioxidant activity of polysaccharides from *Rhodosorus* sp. SCSIO-45730. *J. Appl. Phycol*. **2022**, *34*, 285–299.
42. Trifan, A.; Sava, D.; Bucur, L.; Vasincu, A.; Vasincu, I.; Aprotosoaie, A.C.; Cioancă, O.; Miron, A. Isolation, characterization and antioxidant activity of the crude polysaccharide from *Phyllophora pseudoceranoides*. *Rev. Med. Chir. Soc. Med. Nat., Iaşi* **2015**, *119*, 603–609.
43. Wang, W.N.; Li, T.; Li, Y.; Zhang, Y.; Wu, H.L.; Xiang, W.Z.; Li, A.F. Exopolysaccharides from the energy microalga strain *Botryococcus braunii*: Purification, characterization, and antioxidant activity. *Foods* **2022**, *11*, 110.
44. Zhang, J.; Liu, L.; Ren, Y.; Chen, F. Characterization of exopolysaccharides produced by microalgae with antitumor activity on human colon cancer cells. *Int. J. Biol. Macromol*. **2019**, *128*, 761–767.
45. Shao, P.; Xiaoxiao, C.; Peilong, S. In vitro antioxidant and antitumor activities of different sulfated polysaccharides isolated from three algae. *Int. J. Biol. Macromol*. **2013**, *62*, 155–161.
46. Sun, L.; Wang, C.; Shi, Q.; Ma, C. Preparation of different molecular weight polysaccharides from *Porphyridium cruentum* and their antioxidant activities. *Int. J. Biol. Macromol*. **2009**, *45*, 42–47.