

Induction of Sulfated Polysaccharides in *Spirulina platensis* as Response to Nitrogen Concentration and its Biological Evaluation

Abd El Baky H, Hanaa El Baz KF and EL-Latife SA

Plant Biochemistry Department, National Research Centre, Dokki, Cairo, Egypt

Abstract

Sulfated polysaccharides (SPS) extracted with hot water from *Spirulina* grown on nutrient medium containing 412 ppm (optimal N₂ level) and 45 ppm (limited N₂ level) ppm nitrogen were found to be rich in sulfate contents with values 5.02 % and 4.13%, respectively. Monosaccharides content of SPS in both algae cells were analyzed by HPLC and the results showed that: glucouronic acid and galactose were the predominating sugar in all extracts, followed by rhamnose, arabinose, glucose and ribose. The SPS analyzed by FT-IR spectra showed an intense band of absorption at 790-850 cm⁻¹ indicating the presence of sulfate group of polysaccharides. SPS of *S. platensis* exhibited anticoagulant activities as compared with heparin (standard anticoagulant drug). SPS showed a significant growth inhibit (%) against HepG2 and MCF7 cancer cell lines, with IC₅₀ value ranged between 4.0 and 0.54 g/ml. SPS showed antiviral activity against HSV-1 (standard strain) as a model of DNA virus (as assessed by a plaque reduction test and antioxidant activities toward DPPH. and ABTS⁺ radical.

Keywords: *Spirulina platensis*; Sulfated Polysaccharide; Blue Green Microalgae

Introduction

Spirulina has a high nutritional value due to its content of a wide range of essential nutrients, such as provitamin, minerals, proteins, polyunsaturated fatty acids such as gamma linolenic acid and sulfated polysaccharides [1,2]. In the last decades, high attention has focused on the biological properties of polysaccharides and their chemical derivatives, especially sulfated derivatives. Sulfated polysaccharides have a broad range of important bioactivities comprising antioxidant, anticoagulant and antithrombotic activities. They are also known to increase the resistance to some virus and inhibit some tumor development [3]. Sulfated polysaccharides comprise a complex group of macromolecules with a wide range of important biological properties. These anionic polymers are widespread in nature, occurring in a great variety of organisms [4].

Sulfated polysaccharides (SPS) are widespread in nature, especially occurring in marine algae. Many seaweed polysaccharides, such as agar and carrageenan, are used extensively in industry. Recently, there has been an increasing interest in systematic screening of biological activity of sulfated polysaccharides isolated from marine algae. The biological features of the SP reported till now are antioxidant, antitumor, immunomodulatory, inflammation, anticoagulant, antiviral, antiprotozoan, antibacterial, antilipemic.

Some of them have been developed as new drugs for antitumor, antiviral, anticoagulant and antihyperlipidemia treatment. These SPS have linear structures and possess many biological activities such as antiviral and antitumor activities. However, there is no report about the application of the SPS in the inhibition of urinary stones [5]. Calcium spirulan (Ca-SP) is a novel sulfated polysaccharide isolated from a hot water extract of a blue-green alga *Spirulina platensis* [6]. Hayakawa et al. [7] reported that calcium spirulan, showed an inhibitory effect on the replication of enveloped viruses. Recently Ca-SP is a potent inhibitor of thrombin through heparin cofactor II HC II, and that Ca-SP is a sulfated polysaccharide distinct from heparin or dermatan sulfate in an experiment of chondroitinase treatment.

The aim of the present work was to produce *Spirulina platensis* with

a high content of sulfated polysaccharides and evaluate their biological activities as anticoagulant, anticancer, antiviral, antimicrobial, and antioxidant.

Materials and Methods

Algal source

The blue green algae, *Spirulina platensis* was obtained from the Culture Collection of Texas University, Austin, Texas, USA.

Growth conditions

Algae were cultured in 4 L Erlenmeyer flasks containing autoclaved 3 L Zarrouk's medium [8]. The medium was enriched with nitrogen (as sodium nitrate) at concentrations of 45, 128, 293, 412 (optimum concentration usually use) and 622 ppm. The pH of all media was adjusted to 10.5 and the medium autoclaved. The cultures were gassed with 0.3% CO₂ in air and the algae were cultivated at 25 ± 3 °C. The cultivated flasks were illuminated 24 h with continuous cool white fluorescent lamps at 400 W (equal 477 lx) [9,10].

Growth measurements and harvesting

The growth rate of *Spirulina platensis* was monitoring every three days through cultivation period by determining the dry weight (d. w) and optical density at 670 nm methods Payer [11]. The cells were harvested at the stationary phase, by centrifugation at 10,000 g (4°C) for 15 min and the cells masses were stored at -20°C until analysis.

***Corresponding author:** Abd El Baky, Plant Biochemistry Department, National Research Centre, Dokki, Cairo, Egypt, Tel: 202 3377164; E-mail: abdelbakyh@hotmail.com

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Extraction of sulfated polysaccharides from *Spirulina platensis*

The sulfated polysaccharide was extracted from *S. platensis*, using two different extraction methods:

Extraction of sulfated polysaccharides with hot water: Fresh algae (5 g) were mixed with 20 ml of distilled water transfer to water bath at 100°C for 2 h, and the liquid was then filtered through Whatman filter paper No.54. Sulfated polysaccharides were then precipitated using ethanol absolute and centrifuged at 10,000 rpm to pool it and evaporated under vacuum at 60°C in rotary evaporator according to Asker et al. [12]. The precipitate was dried at 30°C, to yield brownish powder crude sulfated polysaccharides [13] and kept for analysis.

Extraction of sulfated polysaccharides with ethanol 85%: Sulfated polysaccharides of fresh algae (5 g) was extracted with ethanol 85%, transfer to water bath at 80°C for 2 h, the liquid was then evaporated under vacuum at 60°C, and kept for analysis according to Pugh et al. [14] and Yim et al. [15].

Determination of total carbohydrates, reducing sugars and Non- reducing sugars of algal sulfated polysaccharides

Total carbohydrates were estimated by phenol /sulfuric acid reagent using the method of Dubois et al. [16], while reducing sugars was determined by the dinitrosalicylic acid (DNS) using the method of Miller et al. [17]. Non- reducing sugars were calculated by difference between the total carbohydrates and the reducing sugars.

Determination of Sulfate content algal polysaccharides

Sulfate content in algal extracts was determined after hydrolysis with 1 N HCl at 100°C for 1h using sodium-rhodizo-nate method, described by [18] as followed: 0.5 ml of each samples and water were pipetted into test tubes and 2.0 ml ethanol absolute was added. If precipitation occurs the tubes were centrifuged until clear. 1.0 ml BaCl₂ buffer and 1.5 ml sodium rhodizo-nate solution were pipetted in and then the tubes were well shaken, and were allowed to stand for 10 min in the dark at room temperature. The intensity of the color was measured spectrophotometrically at 520 nm. The color remains unchanged for 30 min. A standard (Na₂SO₄) series were treated as the samples.

Determination of monosaccharides content of algal polysaccharides by HPLC

The monosaccharides in the extract of the higher sulfate content was quantified by HPLC on Shimadzu Shim-Pack SCR-101N column (7.9mm x 30 cm), using deionized water as the mobile phase (flow rate 0.5ml/min) and refractive index detector, as described by El-Sayed et al. [19].

Infrared spectroscopic measurements analysis of algal sulfated polysaccharides

The higher extracts in sulfate content were mixed with KBr, grounded and pressed into a 1 mm pellet. IR spectra of samples were recorded on JASCO FT/IR 6100A spectrometer [12].

Biological evaluation of *Spirulina* sulfated polysaccharide

Antimicrobial activity of sulfated polysaccharide: The following organisms were obtained from Northern Regional Research Laboratory (NRRL), USA: *Bacillus subtilis* NRRL B-543 (Gram (+), *Escherichia coli* NRRL B-210 (Gram (-), *Aspergillus niger* NRRL 599 and *Candida albicans* NRRL Y-477. The sterilized nutrient agar medium "50 ml" was

prepared by the method of [20]. An amount from the test solution (0.1 ml dissolved in ethanol) was poured inside the holes. Three holes were made for each sample to be assayed. The Petri dishes were incubated at 6°C for 3 h. to permit good diffusion and then transferred to an incubator of 28°C for 16 h. The diameter of the clear inhibition zone was measured for each sample. Due to the nature of the extracts under investigation serial dilutions of the sample in dimethylsulphoxide (DMSO) were prepared dilutions from 1: 10 to 1: 100 v/v.

After complete mixing, 0.1 ml of each diluted solution was separately tested for its activity against the test organisms. The Minimum Inhibitory Concentration (MIC) was recorded for SPS extracted with hot water from *Spirulina* grown on medium containing 350 mg/L sulfur as the minimum level preventing growth of *Candida albicans* and *Aspergillus niger* after 24 h [21].

Antiviral activity of *Spirulina* sulfated polysaccharides: Preparation of samples for antiviral bioassay: *Spirulina* sulfated polysaccharides were dissolved as 100mg each in 1ml of 0.1 M phosphate buffer (pH 7). The final concentration was 100 g/ml (Stock solution).

Viruses used: Herpes simplex virus type 1 (HSV-1) and Hepatitis A virus (cell culture adapted strain MBB). The virus was obtained from virology laboratory, NRC, Giza, Egypt. Viruses were propagated and titrated on Vero cell (HSV-1) strain.

Plaque infectivity reduction assay: The method described by Silva et al. [22] was used where, a 6-well plate was cultivated with Vero cell (HSV) and another plate containing HepG2 (HAV) culture (105cell/ml) and incubated for 2 days at 37°C. Virus was diluted to give 107 PFU/ml as final concentrations and mixed with the algal extract at the previous concentration and incubated overnight at 4°C. Growth medium was removed from the multiwell plate and virus-compound mixture was inoculated (100 µl/well). After contact time, inoculate were aspirated, agarose were overlaid, and plates were left to solidify then incubated until the development of virus plaques. Cell sheets were fixed in 10% formalin and stained with crystal violet stain.

Anticoagulant activity of *S. platensis* sulfated polysaccharides: To 0.8 ml of the extract solution (1%), and 0.8 ml of standard heparin sodium solution (0.5 U.S.P. unit / 0.8 ml) was as positive control, or 0.8 ml saline solution as negative control was added these mixture in the test tube. Then, 1 ml rat plasma and 0.2 ml of 1% calcium chloride solution were added to each tube. Tubes were stopped immediately and the time was recorded, and inverting three times in such a way mixed the contents that the entire inner surface of the tube was wet. The time required for clotting was determined [23].

Antioxidant activity of *S. platensis* sulfated polysaccharides:

DPPH. scavenging radical assay: The antioxidant activity of sulfated polysaccharides extracts were determined based on DPPH. radical described by Ye et al. [24]. A 0.1 mM of ethanolic DPPH solution was prepared. The initial absorbance of the DPPH in ethanol was measured at 517 nm and did not change throughout the period of assay. An aliquot (0.1 ml) of each sample (with appropriate dilution if necessary) was added to 3.0 ml of ethanolic DPPH solution. Discolorations were measured at 517 nm after incubation for 30 min in the dark. Measurements were performed at least in triplicate. The percentage of DPPH which was scavenged was calculated using the following equation:

$$\text{Scavenging\%} = [1 - (\text{A sample} - \text{A blank} / \text{A control})] \times 100\%$$

Where, ethanol (3.0 ml) plus sample solution (0.1 ml) was used as a blank and 3 ml of DPPH-ethanol solution plus ethanol (0.1 ml) was also used as a negative control.

ABTS scavenging radical assay: TBAS radical scavenging activity of the SPS was determined according to the method of Urbani et al. [25] with some modifications as follows: ABTS^{•+} was produced by reacting 7 mM ABTS aqueous solution with 2.4 mM potassium persulfate in the dark for 12–16 h at room temperature. Prior to assay, this solution was diluted in ethanol (about 1:89 v/v) and equilibrated at 30°C to give an absorbance at 734 nm of 0.700 ± 0.02. After the addition of 1 ml of diluted ABTS solution to 10 µl of test sample in ethanol, absorbance was measured at 30°C exactly 30 min after the initial mixing. The inhibition percentage was calculated for the blank absorbance at 734 nm. Radical scavenging activity was expressed as the inhibition percentage of free radical by the sample and was calculated using the formula: % ABTS radical scavenging activity=(control OD-sample OD/control OD) ×100.

Anticancer activity of microalgal sulfated polysaccharides

Cytotoxicity activity Skehan et al. [26] of hot water and ethanol extracts which contain the highest sulfate content was tested on two cell lines, HepG2 (liver carcinoma cell line) and MCF7 (breast carcinoma cell line). The relation between surviving fraction and drug concentration is plotted to get the survival curve of each tumor cell line after the specified compound.

Statistical analysis

All experiments were performed in triplicate (n=3) and results were expressed as mean ± SD (standard deviation). Statistical analysis was carried out with (SPSS package version 10.0 MERANT, USA). Multiple comparisons of means were done by LSD (Least significance difference) at significance level p ≤ 0.05.

Results and Discussion

Effect of nitrogen concentrations on growth rate of *Spirulina platensis*

The effect of different nitrogen concentrations on growth of *S. Platensis* grown for 12 days cultures are shown in Figure 1. It's clear that the growth of *Spirulina* was increased gradually with culture age and reached the maximum (3.186 g/L) at 12 days of culture age and when the concentration of 412 ppm nitrogen was applied in the culture. Fabregas et al. [27]; Gordillo et al. [28]; Vermaas W.F.[29] and Colla et al. (2) mentioned that, in micro algal species, the concentration of

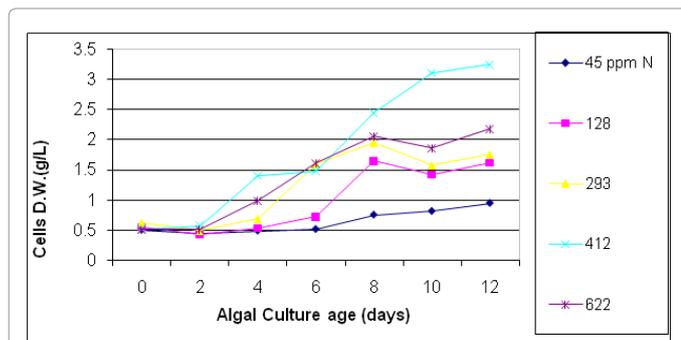


Figure 1: *Spirulina platensis* grown at different nitrogen concentrations growth rate.

photosynthetic pigments in the culture increases while nitrogen is the limiting factor of growth.

Effect of nitrogen concentrations on total carbohydrate, reducing and non- reducing sugars % of *S. platensis*

Total carbohydrate %, reducing and non- reducing sugars are different algal cells shown in (Table 1). The total carbohydrate, reducing and non-reducing sugars content reached the maximum (31.05, 2.28, 28.77 % respectively) when the concentration of nitrogen was 45 ppm in the culture. In the present study, the results mentioned above indicate that high concentration of nitrogen source supported the biomass concentration in contrast to the carbohydrate content. The decreased growth pattern in *S. platensis* under nitrogen deficient conditions. Fabregas et al. [27] observed that, the production of exopolysaccharide is inversely related to the amount of nitrogen available. According to Yeesang and Cheirsilp [30], under nitrogen deficient conditions, algal cells accumulate carbon metabolites as carbohydrate [30]. It has earlier been reported that under nitrogen starvation conditions, nitrogen containing macromolecules and carbon reserve compounds like carbohydrates and fats are accumulated.

Effect of nitrogen concentrations on accumulation sulfated polysaccharide extracted with hot water and ethanol 85%

As shown in Table 2, sulfated polysaccharide extracted with hot water from *S. Platensis* grown on medium containing 412 ppm nitrogen, showed the higher value of % total carbohydrate (17.8%). These result suggested that in the growth medium, algae cells start to store carbohydrate to use them on energy production. While, sulfated polysaccharide extracted with hot water from *S. Platensis* grown on medium containing 293 ppm nitrogen showed the maximum

Nitrogen Concentrations (ppm)	Total carbohydrate %	Reducing sugar %	Non-reducing sugar %
45	31.05* ±0.02	2.28* ±0.015	28.77* ±0.006
128	28.09* ±0.02	0.825* ±0.002	27.27* ±0.015
293	23.62* ±0.02	0.908 ±0.002	22.71* ±0.02
412(control)	25.26 ±0.02	0.905 ±0.002	24.36 ±0.015
622	26.68* ±0.025	1.42* ±0.02	25.26* ±0.045

Data are means of triplicate ± SD. *The mean difference is significant at P ≤ 0.05

Table 1: Effect of Nitrogen concentrations on total carbohydrates, reducing and non-reducing sugars contents of *Spirulina platensis* cells.

Treatments	Hot Water extracts Total		Ethanol extracts Total	
	carbohydrate%	Sulfate%	carbohydrate%	Sulfate%
<i>S. platensis</i> grown at 45 ppm N	7.60* ± 0.200	2.35* ± 0.020	8.06* ± 0.122	5.02* ± 0.015
<i>S. platensis</i> grown at 128 ppm N	8.60* ± 0.200	3.13* ± 0.120	9.30* ± 0.200	5.40* ± 0.200
<i>S. platensis</i> grown at 293 ppm N	10.30* ± 0.200	3.26* ± 0.020	10.80* ± 0.300	2.13* ± 0.162
<i>S. platensis</i> grown at 412 ppm N	17.83 ± 0.252	1.77 ± 0.153	12.77 ± 0.153	4.13 ± 0.153
<i>S. platensis</i> grown at 622 ppm N	9.40* ± 0.200	0.94* ± 0.064	13.16* ± 0.216	4.05 ± 0.02

Data are means of triplicate ±SD. *The mean difference is significant at P ≤ 0.05

Table 2: Effect of extraction methods of sulfated polysaccharides of *S. platensis* grown under different nitrogen concentrations on total carbohydrates.

concentration of sulfate (3.26%). However, in sulfated polysaccharide extracted with ethanol from *S. Platensis* grown on media containing 128 ppm nitrogen gave the highest concentrations of sulfate 5.4%. These results indicated that extraction with ethanol 85% gave the highest concentration of total carbohydrates and sulfate content.

Monosaccharides profile of *S. platensis* sulfated polysaccharides

The sulfated polysaccharides hydrolysates of *S. Platensis* grown at different concentration of nitrogen were analyzed by HPLC. The results showed that glucouronic acids (ranged from 2.075 -45.15 mg/g) was found to be predominant constituents in all extracts (Table 3), followed by Galactose (0.57 - 2.495 mg/g) and then glucose (1.88- 131.125 mg/g). While, rhamnose and arabinose was present as a minor constituents. However, the sulfated polysaccharide extracted with hot water from *S. Platensis* grown on media containing 128 ppm nitrogen had a highest value of glucouronic acid, and had new sugar appeared its ribose as combined with other treatments. It has been shown by previous workers that, the initial analyses indicated that calcium spirulan (Ca-SP) was composed of rhamnose, ribose, mannose, fructose, galactose, xylose, glucose, glucuronic acid, and galacturonic acid [31].

Infrared spectra of *S. platensis* sulfated polysaccharides

IR spectrum was recorded for all SPS extracted from *S. Platensis* (Figure 2). All the *S. Platensis* extracts showed similar IR spectra. They had a several peaks corresponding to sulfate ester: the intense band at 790-850 cm^{-1} derived from the bending vibration of C-O-S of sulfate in axial position and stretching vibration of S-O of sulfate, respectively [32]. In addition, a strong band at 740- 880 cm^{-1} was due to asymmetric stretch vibration of COO- of uronic acids; 1440 - 1460 cm^{-1} , symmetric stretch vibration of COO- and stretch vibration of C-O within COOH. The large absorption band centered on 3400-4450 cm^{-1} , which caused by a large amount of O-H stretching. These results are in agreement with that found by Yang et al. [33], Mao et al. [32] and Abd El Baky et al. [34] which describing a symmetrical C-O-S vibration associated to a C-O-SO₃ group at 819 cm^{-1} and a discernible shoulder at 857 cm^{-1} was also due to the symmetrical C-O-S vibration.

Biological evaluation of *S. Platensis* sulfated polysaccharides

Antimicrobial activity of *S. Platensis* sulfated polysaccharides: Sulfated polysaccharide extracted with hot water from *S. Platensis*

grown on medium containing 128 ppm nitrogen recorded significant antimicrobial activity on yeast (8 mm) and fungi (10 mm), however there was no activity recorded on Gram-negative and Gram-positive

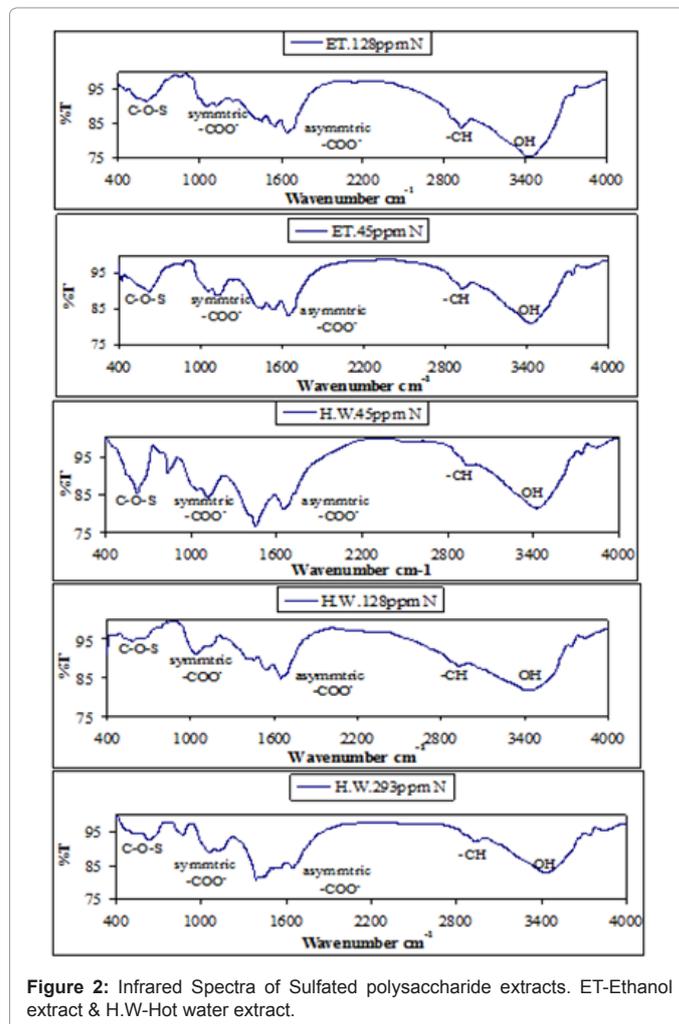


Figure 2: Infrared Spectra of Sulfated polysaccharide extracts. ET-Ethanol extract & H.W-Hot water extract.

EXTRACTS	Mono sugars content (mg/g)											
	Glucuronic acid(mg/g)	Molar Ratio	Galactose (mg/g)	Molar Ratio	Glucose (mg/g)	Molar Ratio	Rhamnose (mg/g)	Molar Ratio	Arabinose (mg/g)	Molar Ratio	Ribose (mg/g)	Molar Ratio
SPS Extracted with hot water from <i>Spirulina</i> grown on medium containing 45 ppm N	18.33	114.56	-		0.16	1	1.005	6.28	24.2	151.25	-	
SPS Extracted with hot water from <i>Spirulina</i> grown on medium containing 128 ppm N	45.15	1	-		-		-		-		387.22	8.576
SPS Extracted with hot water from <i>Spirulina</i> grown on medium containing 293 ppm N	3.28	41	2.495	31.18	0.08	1	-		-		-	
SPS Extracted with Ethanol from <i>Spirulina</i> grown on medium containing 45 ppm N	2.455	44.63	0.57	10.36	0.055	1	-		1.08	19.63	-	
SPS Extracted with Ethanol from <i>Spirulina</i> grown on medium containing 128 ppm N	2.075	24.41	0.575	6.76	0.085	1	0.635				-	

Molar ratio calculated as: By comparing the retention time of the standard peaks and the sample peak, the composition of the extracts

Table 3: HPLC Profile of *S.platensis* sulphated Polysaccharides.

bacterial species (Table 4). Anderson et al. [35] studied the interaction of heparan sulfate and other glycosaminoglycans (GAGs) with their respective receptors may also be involved in tissue penetration by spermatozoa and infectious microbes. GAGs have a key role in viral and bacterial infectivity, phagocytosis and endocytosis, intercellular communication, and cell-cell interaction. According to Mendiola et al. [36] four different microbial species, including a gram negative bacterium (*Escherichia coli*), a gram positive bacterium (*Staphylococcus aureus*), a yeast (*Candida albicans*) and a fungus (*Aspergillus niger*), were used to screen the possible antimicrobial activity of *Spirulina platensis* supercritical fluid extracts. The antimicrobial activity was quantitatively assessed by the determination of the minimum inhibitory concentration (MIC) and minimal bactericidal and fungicidal concentration (MBC). Results obtained showed that *C. albicans* was the most sensitive microorganism to all *Spirulina* fractions, with the lowest MBC values (15–10 mg/ml), whereas the least susceptible was the fungus *A. niger*. Sulfated polysaccharides serve a number of structural and protective roles in algal cell and plants. The extensive range of their physiological functions has only recently begun to be appreciated and explored [37].

Antiviral activity of *S. Platensis* sulfated polysaccharides: As shown in Table 5 the extracts were evaluated for their antiviral activity against HSV-1 (standard strain) as a model of DNA virus by a plaque reduction assay. The sulfated polysaccharide extracted with hot water from *S. Platensis* grown on medium containing 45 ppm nitrogen showed the highest antiviral activity when the concentration of 20 µg was applied. An explanation of these results may be due to that this extract has a high molecular weight sulfated polysaccharides than the two other extracts. In general, the antiviral activities of sulfated polysaccharides increased with the degree of sulfation and molecular weight. This result is in agreement with that found by Asker et al. [12]. It is of interest to note that the influence of the distribution of sulfate groups along polymer chain and the conformational flexibility of this chain for adopting a definite shape which might be required during the formation of polysaccharide-virus complex. On the other hand, Chirasuwan et al. [38] studied the role of sulfate groups in the polysaccharides extracted from *S. Platensis* by eliminated sulfate groups

Extracts	Microorganism (Diameter of inhibition zone (mm))			
	<i>Bacillus subtilis</i> (Gr +ve bacteria)	<i>Escherichia coli</i> (Gr -ve bacteria)	<i>Candida albicans</i> (yeast)	<i>Aspergillus niger</i> (fungi)
SPS extracted with hot water from <i>Spirulina</i> grown on medium containing 45 ppm nitrogen	--	--	--	--
SPS extracted with hot water from <i>Spirulina</i> grown on medium containing 128 ppm nitrogen	--	--	--	--
SPS extracted with hot water from <i>Spirulina</i> grown on medium containing 293 ppm nitrogen	--	--	--	--
SPS extracted with ethanol from <i>Spirulina</i> grown on medium containing 45 ppm nitrogen	--	--	--	--
SPS extracted with ethanol from <i>Spirulina</i> grown on medium containing 128 ppm nitrogen	--	--	8	10

Table 4: Antimicrobial activity of sulfated polysaccharides of *Spirulina* grown under different nitrogen concentrations.

before testing for anti HSV-1, the results indicated that in the absence of sulfate groups in polysaccharide, no significant anti HSV-1 activity was detected.

Anticoagulant activity of *S. Platensis* sulfated polysaccharides:

Data concerning the anticoagulating activity of sulfated polysaccharide extracted with hot water and ethanol from *Spirulina platensis* are shown in Table 6. Heparin was used as standard anticoagulant. Generally, the anticoagulant activities of the extracts were less than heparin. The same results were obtained in sulfated polysaccharides extracted with hot water and ethanol from *S. Platensis* under different nitrogen concentrations. The effect of extracts, which possess anticoagulating activity, may be due to the presence of uronic acids. The polysaccharides containing uronic acids, carrying negative charge, have the ability for binding calcium ions and therefore prevent the formation of clot. In addition, variation in anticoagulating activity of the different extracts is probably due to the quantity of uronic acids in polysaccharides and some conformational differences in the molecules of these polysaccharides [34-39]. While, Mao et al. [32] mentioned that the anticoagulant activities of the sulfated polysaccharides from the green algae *Ulva conglobata* were weaker than heparin on the same concentration. In order to study the inhibitory mechanism of the sulfated polysaccharides, the effect on thrombin activity was investigated using chromogenic substrate in the presence and in the absence of antithrombin or heparin cofactor II. In general, heparin cofactor II is a serine protease inhibitor and selectively inhibits thrombin, and antithrombin inhibits all intrinsic pathway coagulation enzymes. These results suggest that the anticoagulant properties of the sulfated polysaccharides are attributed to the direct inhibition and heparin cofactor II media-ated inhibition of thrombin activity. Anti-coagulant activity is largely dependent on the sugar

Samples	%Anticoagulant activity* Time of clotting	
	Hot water	Ethanol
	extract	extract
Blank	--	--
Heparin	100%	100%
<i>S.platensis</i> grown at 45 ppm N	80%	60%
<i>S.platensis</i> grown at 128 ppm N	80%	60%
<i>S.platensis</i> grown at 293 ppm N	80%	60%
<i>S.platensis</i> grown at 412 ppm N	80%	60%
<i>S.platensis</i> grown at 622 ppm N	80%	60%

* Relative Percentage

Table 5: Anticoagulant activities of sulfated polysaccharide extracts of *Spirulina platensis*.

Extracts	Conc. (µg)	% Viral Inhibition	IC ₅₀
SPS extracted with hot water from <i>Spirulina</i>	10µg	28.12	17.78
grown on medium containing 45 ppm nitrogen	20µg	28.12	
SPS extracted with hot water from <i>Spirulina</i>	10µg	9.37	53.36
grown on medium containing 128 ppm nitrogen	20µg	12.5	
SPS extracted with hot water from <i>Spirulina</i>	10µg	9.37	53.36
grown on medium containing 293 ppm nitrogen	20µg	25	
SPS extracted with ethanol from <i>Spirulina</i>	10µg	25	20
grown on medium containing 45 ppm nitrogen	20µg	34.37	
SPS extracted with ethanol from <i>Spirulina</i>	10µg	12.5	40
grown on medium containing 128 ppm nitrogen	20µg	15.6	

Table 6: In-Vitro antiviral activity of sulfated polysaccharides extracts assessed on HSV1.

composition, sulfate content, sulfate position and molecular weight of the compound. The correlation also suggests that suitable length and/or conformation and moderate extent of negative charge density of the polysaccharide molecule would be required for expression of its effective anticoagulant activity [40]. It has been shown that Ca-SP activates heparin cofactor II, a physiological inhibitor of thrombin, and exhibits antithrombin activity in vitro by a unique mechanism different from that of heparin [41].

Antioxidant activity of *S. Platensis* sulfated polysaccharides:

DPPH radical scavenging: DPPH. is used as a free radical to evaluate antioxidant activity of *S. Platensis* extracts. The degree of its discoloration might attribute to hydrogen donating ability of test extracts [42]. Table 7 showed % scavenging of extracts with different concentrations of all extracts, BHA and BHT Significant DPPH radical scavenging activity was evident in hot water extract of *S. Platensis* grown on medium containing 128 ppm nitrogen at concentration 300 µg. However, all extracts showed lower activities than BHA and BHT at the same concentration. Zhao et al. [43] mentioned that low molecular weight sulfated polysaccharide has the potential ability to stop free radical reactions from the start and to inhibit the damage induced by excess free radical. However, the relationship between the structure of algal polysaccharides and antioxidative mechanisms has not yet been elucidated [24].

ABTS⁺ radical scavenging activity: Table 8 showed % scavenging of extracts with different concentrations, and standard antioxidant Trolox. Significant ABTS⁺ radical scavenging activity was evident in hot water extract of *S. Platensis* grown in medium containing 293 ppm nitrogen at concentration 400 µg. However, all extracts showed lower activities than Trolox. Other results noted that there is a positive correlation between sulfate content and antioxidant activity [44]. These findings lend weight to the content of sulfated polysaccharides in different *S. Platensis* extracts, which might explain their high antioxidant activities, that a direct correlations between sulfated polysaccharides content of algal and its antioxidant activity is observed. Hence, the all tested sample with scavenging activity had the same structure feature in that all of them

Extracts	Scavenging%		
	Concentration(µg)		
	100	200	300
SPS extracted with hot water from <i>Spirulina</i>	30.55**	32.05**	35.36**
grown on medium containing 45 ppm nitrogen	±0.020	±0.025	±0.03
SPS extracted with hot water from <i>Spirulina</i>	39.73**	40.67**	68.29**
grown on medium containing 128 ppm nitrogen	±0.026	±0.02	±0.02
SPS extracted with hot water from <i>Spirulina</i>	28.23**	35.69**	44.66**
grown on medium containing 293 ppm nitrogen	±0.020	±0.025	±0.02
SPS extracted with ethanol from <i>Spirulina</i>	26.37**	28.05**	30.05**
grown on medium containing 45 ppm nitrogen	±0.020	±0.02	±0.006
SPS extracted with ethanol from <i>Spirulina</i>	25.75**	26.63**	30.16**
grown on medium containing 128 ppm nitrogen	±0.020	±0.025	±0.025
BHA	88.54	95.22	99.41
	±0.020	±0.02	±0.025
BHT	85.57*	94.76*	98.15*
	±0.025	±0.031	±0.046

Data are means of triplicate ± SD

(*)The mean difference is significant at P≤ 0.05 relative to BHA

(*)The mean difference is significant at P≤ 0.05 relative to BHT

Table 7: Antioxidant activity of sulfated polysaccharides extracts assessed by DPPH.

had many –OH and –OSO₃H groups in the molecules. Their groups had a significant effect on scavenging free radicals. Polysaccharides promote antioxidant activity, which exhibited the greater of abstraction of anomeric hydrogen from the internal monosaccharide units [34,45]. Also, polysaccharide like alginic acid can absorption of toxic chemicals and free radical [46]. Therefore, *S. Platensis* exert antioxidant action by breaking the free radical chain by donating a hydrogen atom [47].

Cytotoxic activity of *S. Platensis* sulfated polysaccharides: The cytotoxic tests (Table 9) revealed that, sulfated polysaccharide extracted with hot water from *S. Platensis* showed a significant inhibition % against HepG2 and MCF7 (86.99, 88.51 %, respectively) While IC₅₀ for sulfated polysaccharide extracted with hot water was 0.54 µg from *S. Platensis* grown on media containing 293 ppm nitrogen against HepG2. It was 0.40 µg for sulfated polysaccharide extracted with hot water from *S. Platensis* grown on media containing 293 ppm nitrogen against MCF7.

These results may be apparently related to their chemical constituents present in their algal polysaccharides, in particular, for sulfate contents in *S. Platensis* polysaccharide. The ability of algal polysaccharides to inhibit the proliferation of many cultured of cancer cells has been well documented [34,45]. Moreover, a marine algae

Extracts	Scavenging%			
	Concentration(µg)			
SPS extracted with hot water from <i>Spirulina</i>	40.04*	45.44*	46.37*	49.03*
grown on medium containing 45 ppm nitrogen	±0.025	±0.035	±0.025	±0.076
SPS extracted with hot water from <i>Spirulina</i>	39.41*	44.84	49.96*	51.81*
grown on medium containing 128 ppm nitrogen	±0.025	±0.020	±0.015	±0.020
SPS extracted with hot water from <i>Spirulina</i>	51.91*	57.74*	59.57*	71.12*
grown on medium containing 293 ppm nitrogen	±0.021	±0.035	±0.025	±0.020
SPS extracted with ethanol from <i>Spirulina</i>	34.17*	41.11*	46.69*	64.48*
grown on medium containing 45 ppm nitrogen	±0.020	±0.076	±0.776	±0.020
SPS extracted with ethanol from <i>Spirulina</i>	34.47*	38.77*	42.23*	56.09
grown on medium containing 128 ppm nitrogen	±0.020	±0.153	±0.025	±0.012
Trolox	92.35	95.66	98.23	99.45
	±0.020	±0.020	±0.038	±0.131

Data are means of triplicate ±SD. *. The mean difference is significant at P≤ 0.05

Table 8: Antioxidant activity of sulfated polysaccharides extracts assessed by ABTS⁺.

Extracts(10µg)	IC ₅₀ (µg)			
	% Inhibition			
	HepG2	MCF7	HepG2	MCF7
SPS extracted with hot water from <i>Spirulina</i>	80.59	81.85	3.09	0.74
grown on medium containing 45 ppm nitrogen				
SPS extracted with hot water from <i>Spirulina</i>	78.56	88.8	3.69	0.47
grown on medium containing 128 ppm nitrogen				
SPS extracted with hot water from <i>Spirulina</i>	83.67	88.09	0.54	0.4
grown on medium containing 293 ppm nitrogen				
SPS extracted with ethanol from <i>Spirulina</i>	85.1	88.51	1.68	0.74
grown on medium containing 45 ppm nitrogen				
SPS extracted with ethanol from <i>Spirulina</i>	86.99	82.88	2.82	0.47
grown on medium containing 128 ppm nitrogen				
Novatron (standard)	59.5	52.81	4	1.4

Table 9: Cytotoxic activity of sulfated polysaccharides extracts assessed on HepG2 and MCF7 cell lines.

contains large amounts of characterized polysaccharides such as fucoidan, carrageenan and calcium spirulan etc. exhibited anti-tumor, anti-cancer and anti-metastatic properties and they also reduce cell proliferation [48]. However, their activity may be correlated with the presence of sulfate and uronic groups in their compounds [45,49,50].

In the present work the effect of sodium nitrate concentration on the production of biomass was studied and the maximum growth was achieved in control medium (412 ppm nitrogen). However, cultivation of *S. Platensis* on media containing 412, 293 ppm nitrogen gave the highest values of sulfate and *S. Platensis* grown on media containing 45 and 128 ppm nitrogen gave the highest values of total carbohydrate, reducing and non-reducing sugars. In addition, the results showed that all extracts have anticoagulant activity but not as high as heparin. On the other hand, it acts as anticancer agent against liver carcinoma cell line (HepG2) and breast carcinoma cell line (MCF7), and has antimicrobial and antiviral activities. Further investigations on sulfated polysaccharides are needed to explore its mode of action and its related chemical composition and applications.

Polysaccharide can absorption of toxic chemical substances and it plays a major role as dietary fiber for the maintenance of animal and human health [41]. These dietary polysaccharides are not found in any land plants. They help protect against potential carcinogens *S. Platensis* grown on media containing 45 and 128 ppm nitrogen gave the highest values of sulfated polysaccharides and has a higher amount of sulfate ester groups and high molecular weight than the other fraction. In general, the bioactivity (as antiviral, anticancer and antimicrobial activity) of sulfated polysaccharides increases with the degree of salivation and molecular weight [51]. A next point of interest is the influence of the distribution of sulfate groups along polymer chain and the conformational flexibility of this chain for adopting a definite shape that might be required during the formation of polysaccharide-virus complex [52]. It is likely that post-infection involved inhibition of early post absorption steps such as virus internalization and inhibition of cell-to-cell transmission in successive cycles of replication.

Conclusion

Spirulina platensis accumulate a high amount of sulfated polysaccharides it had biological activities as anticoagulant, anticancer, antiviral, antimicrobial, and antioxidant.

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