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Polysaccharides in CO_2 enriched *Arthrospira platensis*: Structure, physico-chemical properties, antioxidant and cytotoxicity activities and laser burn wound healing in rats

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ABSTRACT

This work was undertaken to determine the structural characteristics of polysaccharides extracted from CO_2-enriched *Arthrospiraplatensis* (Spirulina Water Soluble Polysaccharide: SWSP), as well as its antioxidant activities, cytotoxic effects and laser burn wound healing in rats. This SWSP was structurally characterized by Scanning Electron Microscopy (SEM), Fourier-transformed infrared (FT-IR), X-ray diffraction (XRD), high-performance liquid chromatography (HPLC), and thin layer chromatography (TLC). This novel polysaccharide was found to have an average molecular weight of 6.21 kDa. It is a hetero-polysaccharide composed of rhamnose, xylose, glucose and mannose. According to XRD and FT-IR spectra, the SWSP showed a semi-crystalline structure. It is composed of 100 to 500 µm geometric shaped units with flat surfaces and it was found to inhibit the proliferation of human colon (HCT-116) and breast (MCF-7) cancers. This polysaccharide display potential antioxidant activities determined through three different assays: scavenging activity against 2,2'-azino-bis-3-ethylbenzothiazoline-6-sulphonic acid (ABTS), 2,2-Diphenyl-1-Picrylhydrazyl (DPPH) scavenging assay and ferric reducing antioxidant power assay (FRAP). Results strongly support the beneficial effects of the SWSP to accelerate wound healing in rats. Indeed, its application significantly increased tissue re-epithelization and remodeling phases, after 8 days of the experiment. Findings herein demonstrated that SWSP could be a novel auspicious source of natural wound healing closure and/or cytotoxic remedy.

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Introduction

Carbone dioxide fixation using photosynthetic microalgae has become a very attractive potential approach to resolve global issues of energy (1). The production of algae is identified as one of the solutions of carbon sequestration to achieve the permanent removal of carbon from the atmosphere (2).

Arthrospira platensis also called *Spirulina plantensis*is one of the edible microalgae that has been used as health food and feed for a long time (3). Spirulina can produce numerous valuable compounds, such as lipids (6–13%; half of which are fatty acids), phycocyanin (20–28%), and carbohydrates (15–20%) and the major carbohydrates are polysaccharides (4). Nowadays, polysaccharides have emerged as an important class of biopolymers due to their potential for use in different fields of applied and industrial biotechnology. There is increasing evidence in the literature that the bioactivities and functional properties of polysaccharides mainly depend on several parameters, including their physicochemical properties, water solubility and even primary structures (5).

Studies are widely conducted on the isolation, purification and structural analysis of polysaccharides (6,7). In recent years, polysaccharides from plants, animals, and microorganisms have piqued the interest of many researchers, owing to their many biological activities (8, 9).

Although there are some studies on the extraction, isolation and purification of polysaccharides from microalgae such as *Chlorella*, *Dunaliella*, *Nostoc* and *Porphyridium* (10-12), polysaccharide extraction from Spirulina is less documented.

Besides, our recent research well documented that the use of CO_2 in the production of algae may have a positive environmental benefit to reduce the carbon pollution from the atmosphere and provide a better Spirulina production and quality (13).

Spirulinacontains polysaccharides, with anti-inflammatory, antioxidant and immuno-stimulating effects (14), however, none have handled with burn healing efficacy of this cyanobacteria.

Burn is defined as the destruction of the skin covering. It is characterized by its depth (the degree to which it has altered the skin), its surface area in relation to the total skin surface (SCT), as well as its etiology (thermal, chemical, electrical, radioactive).Itimplicates several steps, inclu-

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ding hemostasis, acute inflammation, granulation tissue formation, matrix formation, remodeling of connective tissue, collagenization and acquisition of wound strength (15). Therefore, the quality of wound regeneration depends mainly on the efficiency of wound care (15).

This study aimed to investigate the physico-chemical, and structural characteristics of water-soluble polysaccharide extracted from the CO_2-enriched blue algae, *Arthrospira platensis* (SWSP) in order to valorize the macromolecules synthesized in algae and to evaluate the relationship between the structure and the bioactivity of these molecules. It aimed also to elucidate the antioxidant activity and cytotoxicity effects of this SWSP against breast colon and ovary cancer strains and for the first time, the effects of SWSP on chronic wound healing in rat models.

Materials and Methods

Materials and reagents

Arthrospira platensis cultivation was performed in raceway ponds on the standard Zarrouk medium (described by Sassi-Aydiet al., 13). The cultures were illuminated under a low light intensity of 52–55 µmol m^{-2} s^{-1} and light/dark cycles of 16/8 h at 25°C and aerated continuously with 5% of CO_2 at a flow rate of 0.30 L min^{-1}. When the density is considered sufficient, biomass samples were harvested, dried using the solar panel hot air drying method and vacuum stored at room temperature, before extractions were carried out.

Extraction of SWSP

SWSP was recovered by the method of Liu et al. (16). Briefly, *A. platensis* powder was pre-extracted with 95% ethanol at room temperature (23±3°C), to remove pigments. The dry residue was extracted twice with 20 vols of deionized water at 90°C for 4 h, where the suspension was continuously mixed using a magnetic agitator (AREX Velp-Scientifica, Usmate, Italy). Extracts were combined and filtered, and filtrates were then evaporated using a rotary vacuum evaporator; BüchiRotavapor R-200 (BüchiLabortechnik, Flawil, Switzerland). The concentrated liquid was precipitated with 95% (v v^{-1}) ethanol at 4°C for 24 h and then centrifuged (4500 rpm) for 15 min using a HERMLE Z 513 K centrifuge (HERMLE Labortechnik, Wehingen, Germany). The final precipitate was redissolved in double distilled water. The water phase was dialyzed at 4°C against distilled water for 2 days. The dialysate was concentrated by the BüchiRotavapor under reduced pressure and freeze-dried using a freeze dryer (Bioblock Scientific Christ ALPHA 1–2, IllKrich-Cedex, France) to obtain SWSP. The latter were then stored at −20 °C for further use.

Physico-chemical analysis

Color, pH (1% solution at 25°C) and viscosity at various concentrations in H_2O (0.5, 1, and 1.5 g L^{-1}) of SWSP were determined. The color was evaluated using a Color Flex spectrocolorimeter (Hunter Associates Laboratory Inc., Reston, VA, USA) and reported as L*, a* and b* values, referring to the measuring parameters of lightness, redness, and yellowness, respectively. The sample was filled in a 64 mm glass cup with three readings. The latter was determined in triplicate. The white tile and black glass were used to standardize the equipment. The pH was measured using a digital pH meter (Mettler-Toledo AG, Schwerzenbach, Switzerland) with complete immerging of the glass electrode into the solution. Viscosity measurements were determined at 25°C by using a digital viscometer (NDJ-1, Japon) at 30 rpm spindle rotation. The moisture and ash content were determined according to the AOAC standard methods 930.15 and 942.05, respectively (17). Crude protein content was evaluated after multiplying total nitrogen content by the factor of 6.25. Crude fat was determined gravimetrically after Soxhlet extraction of dried samples. Total sugars were determined by the phenol-sulfuric acid method (18).

Thin Layer Chromatography (TLC)

For the present study, different standards were used that polysaccharides may contain: galactose, arabinose, rhamnose, tagatose, glucose, xylose, mannose and fructose. The chromatographic plaques used are silica gel plates type Silica gel 60 F 254, the thickness of 0.25 mm. The mobile phase used was Butanol: Acetic acid: Water, in the proportions of 2:1:1 (v v^{1}). Fluorescent spots were located under a 254 nm UV lamp (UVP UVGL-58, Analytik Jena, USA).

Spectroscopic analysis

UV absorption peak detection

SWSP was dissolved in distilled water to a final concentration of 0.1%. The UV absorption spectrum of the sample was recorded in the wavelength range of 200-800 nm (19) using a UV-VIS Spectrophotometer (2005, JP SelectaS.A., Barcelona, Spain).

FT-IR spectrometric analysis

FT-IR spectrum of SWSP was determined on a Nicolet FTIR spectrometer equipped with a horizontal attenuated total reflection (ATR) accessory. The internal crystal reflection was made from zinc selenide and had a 45° angle of incidence to the IR beam. Spectrum was acquired at 4 cm^{-1}resolution, and the measurement range was 500-4000 cm^{-1} (mid-IR region) at room temperature. The spectral data were analyzed by the OPUS 3.0 data collection software program (Bruker, Ettlingen, Germany).

X-ray diffraction (XRD)

XRD pattern of SWSP was recorded at room temperature on an X-ray diffractometer (D8 advance, Bruker, Germany). The data were collected in the 2Θ ranges 5-80° with a step size of 0.05° and accounting time of 5s/step.

HPLC analysis

An aliquot of 2 mg of SWSP was hydrolyzed in 250 µL of 2 M sulfuric acid (H_2SO_4) at 100°C for 1 h. A 20 µL hydrolysate was added to 980 µL of deionized water and filtered through ahydrophobic PTFE 0.45 µm membrane filter (Sartorius GmbH, Goettingen, Germany). Monosaccharide composition was analyzed by HPLC using a sugar KS-800 column with a mobile phase of 0.001 M NaOH, a flow rate of 0.5 mL min^{-1}, and column temperature of 50°C. The monosaccharide composition assays were performed in two independent experiments. Glucose, fructose, sucrose, gluconic acid, mannose, arabinose, galactose, and xylose were used as standard monosaccharides.

Scanning Electron Microscopic (SEM)

Polysaccharides were examined by scanning electron

microscopy (SEM) model JEOL (JSM-IT100). Each dried polysaccharide was mounted on a metal stub and sputtered in carbon conductive adhesive tapes and the images were observed. The accelerating voltage was 1.0 kV.

Water-holding and oil-holding capacities (WHC and OHC)

WHC and OHC were assayed by the method of Nguyen et al. (20). Briefly, 0.5 g of SWSP was dissolved in 50 ml of distilled water or 10 ml of soybean oil. The mixed solution was kept at room temperature for 1 h and then centrifuged at 8000 rpm for 20 min. The supernatant was removed prudently and the centrifuged tube was kept on a filter paper for 30 min in order to drain, after being oriented to a 45° angle. The ratio between the weight of the tube content after draining and the weight of the lyophilized SWSP was determined and the capacity (%) was reported as grams of water or oil bound per gram of the SWSP on a dry basis.

Biological activities evaluation

In vitro antioxidant assays

DPPH radical scavenging assay

The stable free radical scavenging activity was evaluated using the DPPH assay (21). A methanol extract of *A. platensis* was mixed, at equal volume, with 2,2-diphenyl-1-picrylhydrazyl (DPPH, 100 mM), then incubated at room temperature, for 15 min. The absorbance was determined at 517 nm. Butylatedhydroxy-toluene (BHT) was used as a positive control. These measurements were done in triplicate and the percentage of inhibition of DPPH oxidation (Pi) was calculated using the following formula:

Pi = ((Ab-As)/Ab)*100

Where Ab is the absorbance of the control and As is the absorbance of the extract.

The 50% inhibitory concentration (IC50) was expressed as the quantity of the extracts to react with half of the DPPH radicals. The IC50 values were calculated using linear regression analysis and used to indicate antioxidant capacity.

Ferric-reducing antioxidant power assay (FRAP)

The FRAP assay was done according to Abreu et al. (22). The FRAP reagent was prepared from 0.3 M acetate buffer (pH 3.6), 10 mmol 2,4,6-tripyridyl-s-triazine (TPTZ) solution in 40 mmol hydrochloric acid and 20 mmol iron (III) chloride solution with the ratio 10:1:1 (v/v/v). Briefly, 50 µL of a sample of phytochemicals (three replicates) were added to 1.5 mL of the FRAP reagent. Four min after, the absorbance was determined at 593 nm. The standard curve was constructed using $FeSO_4$ solution (from 50 to 200 µg mL^{-1}), and the results were expressed as µg mL^{-1} Fe (II).

ABTS assay

The radical scavenging activity of SWSP was determined according to the procedure of Dissanayake et al. (23) with slight modifications. 2.2-azino-bis3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (7mM) and potassium persulfate (2.45 mM) solutions were mixed and stored in a dark room for 12-16 h before use. Before the analysis, the ABTS solution was diluted with ethanol to an absorbance of 0.700 ± 0.05 at 734 nm. Following the addition of 4.5 mL of the ABTS reaction mixture to the various concentrations (50-250 µg/mL) of the extracts (1 mg/mL), the reaction mixture was vortexed. After keeping it at room temperature for 15 min, the absorbance of the samples was read at 734 nm. The results were assessed as IC50 values.

Antibacterial activity

SWSP was tested individually against 9 human-pathogenic microbial strains: 6 Gram-positive bacteria (*Enterococcus faecalis*; *Listeria monocytogenes*; *Bacillus subtilis*; *Bacillus cereus*; *Bacillus thuringiensis*; *Micrococcus luteus*) and 3 Gram-negative ones (*Salmonella enterica*; *Escherichia coli*; *Klebsiellapneumoniae*). The bacteria used were selected for their involvement in the human skin, oral and intestinal tract. The antimicrobial activities of SWSP were assayed using the well diffusion method. An inoculum containing 10^6 colony-forming units (cfu) mL^{-1} of each bacterial culture to be tested was spread on Mueller-Hinton (MH) agar plates with a sterile swab moistened with the bacterial suspension. Subsequently, wells of 6 mm diameter were punched, using a sterile agar cutter, into the MH agar medium. The antimicrobial activity of the SWSP was checked by introducing 50 µL of 100 µg mL^{-1} concentrations into triplicate wells. An additional well in each plate was filled with the dimethyl sulfoxide (DMSO) solvent (2%), which served as the negative control while ampicillin (5 µg mL^{-1}) was used as a positive control. The culture plates were allowed to diffuse for 2 h at room temperature. The plates were then incubated, for 24 h, in the upright position at 37°C. After that, the antimicrobial activities of the algae extract and the standard antibiotic was determined. Zones of the inhibition around each of the extract and the antibiotic were measured to the nearest mm using a digital caliper (Shanghai Taihai-CongliangJu Co., Ltd., Shanghai, China). Three replicates were carried out for each test microorganism.

Cytotoxic activity

Cytotoxicity of SWSP against human breast (MCF-7), ovarian (OVCAR) and colon (HCT-116) cancer cell lines were estimated by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay of El Euchet al. (24). HCT-116 cell line was grown in Gibco RPMI 1640 medium (Thermo Fisher Scientific, Paisley, UK), while MCF-7 and OVCAR cell lines were grown in DMEM medium (Thermo Fisher Scientific, Inc.) supplemented with 10% fetal calf serum (Gibco, Langley, OK, USA), air and 5% CO_2. Briefly, 100 µL of cells were distributed in 96-well plates at a concentration of 10^4 cells per well and incubated at 37°C for 24 h. Then, 100 µL of cells in the exponential growth phase were incubated in a fully humidified atmosphere at 37°C for 48 h with the addition of 100 µL of the culture medium, supplemented with 2 mM L-glutamine and 50 µg mL^{-1} gentamycin, containing SWSP at a concentration of 50 mg L^{-1}. The medium was removed and cells were treated with MTT solution 50 µL, 1 mg. mL^{-1} in phosphate-buffered saline (PBS) and incubated at 37°C for 40 min. MTT solution was then discarded and DMSO (50 µL) was added to dissolve in soluble blue crystals. Optical density was determined at 605 nm. Tamoxifen was used as a positive control.

***In vivo* study of the effect of SWSP on laser wound healing**

In vivo assay

Adult Wistar rats (190-200 g) were obtained from the department of life sciences, faculty of sciences at the uni-

versity of Gabes, Tunisia. The animals were caged under controlled conditions of light (12 h light/dark cycles), room temperature (23 ± 1°C) and relative humidity (50% ± 10%) with free access to food and water ad libitum. The general guidelines on the use of living animals in scientific investigations (Council of European Communities) and the guidelines for the care and use of laboratory animals controlled by the Tunisian Research Ministry were followed.

Wound healing activity

Fractional CO_2 laser burn creation

Eighteen rats were anesthetized with ketamine 50 mg/kg, along with 5 mg/kg of midazolam. The back of each animal was shaved and exposed to partial-thickness skin burns (wound area = 2 cm^2) by a CO_2 Fractional Laser System (DSE, Korea) as follows: density: (level = 20, line = 29 x 29, dot = 0841), energy level = 25 MJ and depth level = 4.

Experiment protocol

After CO_2 laser burns, the animals were divided into 3 groups (n = 6) and treated respectively with glycerol solution (30%), "Cytolcentella" and SWSP hydrogel. Each animal was housed separately and treated every day until the first group was completely healed. "CytolCentella" cream was purchased from the local pharmaceutical industry. The hydrogel was prepared by dissolving the lyophilized SWSP in glycerol solution (30%), to give a final concentration of 15 mg/ml. The mixture was kept under agitation until a transparent hydrogel was formed.

Burn wound area measurement

During the wound healing period, the burn wound areas were traced manually using transparent paper. Wound areas were then measured on the day of the lesion (d1), three, seven and eight days afterward, using a computer software application for design and drafting (Auto CAD RL 14).

Hydroxyproline level measurement

Wound tissues were dried in a hot air oven at 60–70°C to a constant weight and then hydrolyzed in 6 N HCl for 4 h at 130°C in sealed glass tubes. The hydrolysates were neutralized to pH 7.0 and then subjected to Chloramine-T oxidation for 20 min. The reactions were terminated by the addition of 0.4 M perchloric acid and the color, developed with the help of Ehrlich reagent at 60°C, was measured at 650 nm using a spectrophotometer(JP Selecta). Hydroxyproline concentrations were calculated from the linear standard curve and presented as mg/100 mg of dry tissue weight (25).

Histopathological examinations

The skin and sub-plantar muscle samples were fixed in 10% neutral buffered formalin solution, embedded in paraffin wax, and cut into 5 mm thick sections on a sliding microtome (Leica, Wetzlar, Germany). The specimens were deparaffinized with 80% xylene and ethyl alcohol, rinsed with phosphate buffer solution (PBS, pH 7.4), and stained with Mayer's haematoxylin solution and 1% eosin alcohol solution. Finally, a light microscope (Olympus, Tokyo, Japan) was used to examine the serial sections to identify the morphology of skin tissues.

Statistical analysis

All experiments were done in triplicate, and data were expressed as mean value ± standard deviation. The statistical analyzes were performed using the one-way analysis of variance (ANOVA) procedure with the Statistical Package for the Social Sciences (SPSS) version 20.0 software (IBM Corp. 2011, Armonk, NY). When $p<0.05$, differences were considered as statistically significant according to Fisher's LSD test.

Results and Discussion

Physico-chemical assessment analysis of SWSP

The extraction was achieved at 90°C then followed by incubation in 80% ethanol. The yield of *Spirulina* water-soluble polysaccharide (SWSP) was 5.8 g per 100 g (Table1). Indeed, several studies reported that extraction temperature has a major effect on the polysaccharide yield of *spirulina* species (26, 27). Hu et al. (28) extracted polysaccharides from *Chlorella pyrenoidosa* using the supercritical carbon dioxide method and obtained the highest yield of approximately 7.78%, while Suárez et al, (29) reported 15% of polysaccharide yield in *chlorella* green algae extracted with hot water. The SWSP levels of protein, fat and ash were 1.9, 0.02 and 1.92% respectively, and the moisture content attained 5.3%. The total sugar content reached 90.75% (Table 1). This showed that SWSP has a small amount of protein and fat, while sugars were the most abundant element as described in most extracted polysaccharides(30).

The molecular weight of SWSP was estimated by reference to a calibration curve constructed using the dextrans of known molecular weights. The estimated average molecular weight of SWSP was 6.21 KDa (Table 1). As suggested by many authors, polysaccharides have significantly different average molecular weights (31, 32). The molecular weight of SWSP in this study was different from that of the green algae polysaccharides, this may be due to the different sources, types and different determination

Table 1. Yield, chemical composition, physical properties and water holding, and oil holding capacities of SWSP.

Composition	(g/100 g wet weight)	Physical Parameters	Value	Properties	Capacities (%)
Yield	13.80±0.20	pH (solution 1%)	8.28±0.16	Water-holding capacity	11.31 ± 2.15
Protein	1.90±0.33	Color	4.90±0.33	Oil-holding capacity	1.46 ± 0.41
Fat	0.02±0.00	L*	31.98±0.14		
Moisture	2.30±0.23	a*	0.19±0.02		
Ash	1.92±0.12	b*	0.86±0.08		
Total sugars	90.75±0.26	Molecular weight (kDa)	6.21±0.25		

Values are given as mean ± SD (n = 5); SD: standard deviation.

methods of algae polysaccharides (26,33).

Table 1 showed the physical properties of SWSP: colour and pH. The changes in L*, a*, b* (lightness, redness, and yellowness) were analyzed. It can be noted that SWSP offered a light (L* = 31.98), a yellow color (b*= 0.86) and a slightly red color (a* = 0.19). Besides, 1% SWSP solution pH measured at 25°C showed an average of 8.28. A different pattern was reported in other plant polysaccharides (34).

Water-holding and oil-binding capacities are beneficial properties for the use of these preparations in food such as sausages and ham-burgers. The water-holding capacity of each preparation is important for the juiciness of a final product. In this paper, experiments were conducted to compare the influence of different drying methods on the swelling, water-holding and oil-binding capacities of b-glucan preparations.

UV–vis spectroscopy

The results of UV absorption by SWSP solution are reported in Figure 1. The SWSP sample exhibited a stronger absorption peak at 200–220 nm which may be due to the existence of unsaturated carbonyl, carboxyl, etc. Results herein showed no optical absorption peaks at 260–280 nm. This indicates that SWSP contains few proteins or nucleic acids since proteins and nucleic acids absorb light in 260 and 280 respectively. It can also be ascribed to other pigments released from the broken cells of *A. platensis*(35). Similar reports were described in other polysaccharides extracted from algae such as *chlorella*(11)or plants like *Allium*(30)and*Fungrec*(34).

High-performance thin-layer chromatography (HPTLC) was widely described as a suitable method for routine analysis of monosaccharides, or polymers (36) in pharmaceutical and herbal drug samples. Monosaccharides were determined by comparing them with standards arabinose, xylose, fructose, glucose, tagatose, mannose, rhamnose and galactose (Fig. 2).

The analysis showed that the SWSP was a heteropolysaccharide composed of rhamnose, xylose, and glucose. Al-Dhabi and Arasu(37) showed also that rhamnose was the major sugar in *Spirulina.*

Additional SWSP analysis was performed by HPLC in order to identify monosaccharides. Compared with the monosaccharide standards, SWSP was principally composed of glucose (61.70%), rhamnose (23.40%) and xylose (12.76%). These findings were similar to the HPTLC results already reported.

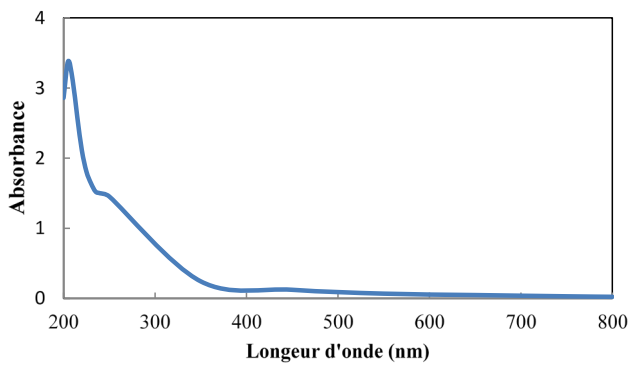

Figure 1. (A) Scan of SWSP within the wavelength range of 200–800 nm.

X-Ray Diffraction analysis

XRD analysis technique was applied on SWSP, and the crystalline degree of the polysaccharide was determined in Figure 3. Results were recorded from 0 to 100°C. Generally, SWSP exhibited low crystallinity, suggesting a semi-crystalline structure of the studied polymer. The main crystalline reflections were seen between 5 and 10° and at 13.25° (at the angle 2Θ). This may be due to the predominance of the amorphous components of SWSP as compared to crystalline ones (6). Our data is in agreement with those of the water-soluble polysaccharides of potato peels as reported by Ben Jeddou et al.(38).

FT-IR spectroscopy

For further characterization of SWSP and identification of its structure, FT-IR analysis was performed in the region of 4000–500 cm^{-1}. Results were exhibited in

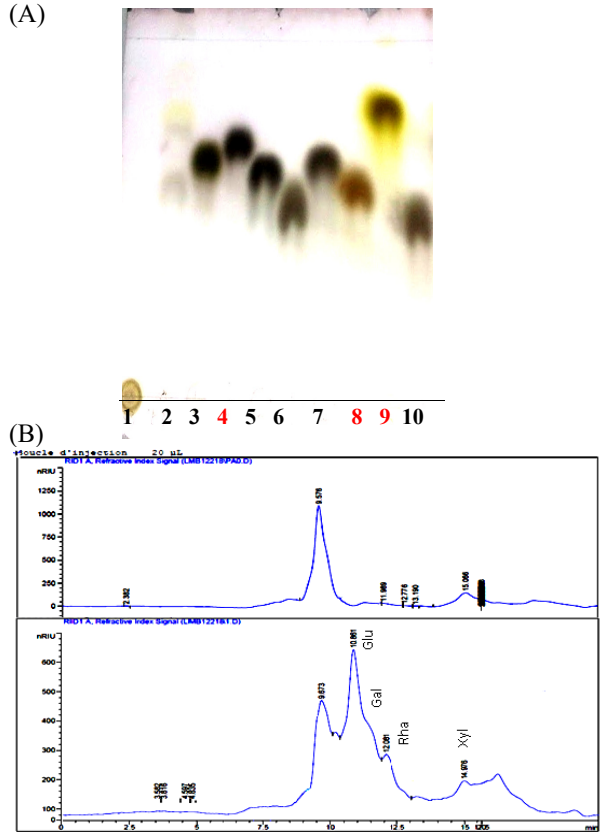

Figure 2. (A) TLC profile of the SWSP. 1: Non-hydrolyzed SWSP; 2: Hydrolyzed SWSP; 3: Arabinose; 4: Xylose; 5: Fructose; 6: Glucose; 7: Tagatose; 8: Mannose; 9: Rhamnose; 10: Galactose and (B)HPLC chromatogram profiles of non hydrolyzed SWSP (A), hydrolyzed SWSP (B). Each sample was applied to Sugar KS-800 column at a flow rate of 0.5 ml/min.

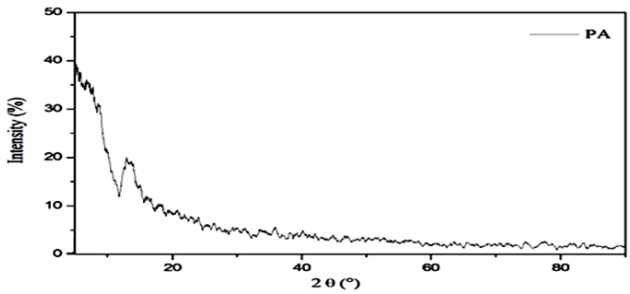

Figure 3. XRD patterns of AWSP.

Figure 4. The general form of the spectra collected for the SWSP showed different peak intensities at particular wave numbers. Actually, eight peaks dominate spectra for *spirulina* polysaccharide: 3280, 2940, 2080, 1600, 1520, 1400, 1240 and 1040. Each pick is assigned a functional group: According to Giordano et al.(39), the amide I and amide II bands represent proteins (around 1660 and around 1540 cm^{-1}) and the carbohydrate region (1200–900 cm^{-1}). Carbohydrate spectra were characterized by a strong feature at 1040 cm^{-1}.

The broad absorption peak at 3280 cm^{-1} represented the OH stretching vibration due to inter and intra-molecular hydrogen bands (39). Besides, in accordance with Wu et al.(40), the weak peak at 2940 $cm^{-1}$was attributed to the C-H stretching vibration of free sugars.

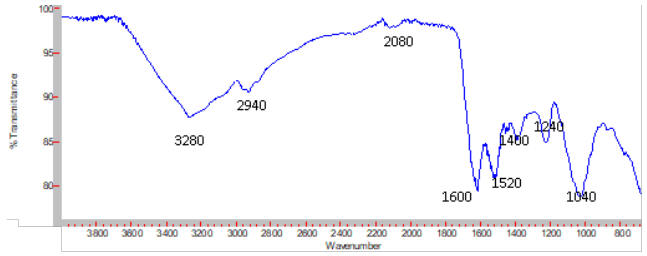

Figure 4. Fourier transformed the infrared spectrum of SWSP.

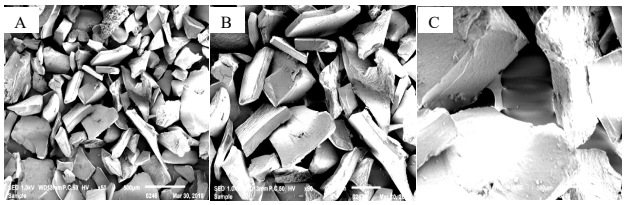

Figure 5. SEM images of SWSP. (A) Morphology at 50× (scale bar is 500 µm); (B) Morphology at 90× (scale bar is 200 µm); (C) Morphology at 250× (scale bar is 100 µm).

Scanning Electron Microscopic

With its high resolution, large field of view, and stereoscopic capabilities, SEM should be an excellent tool for studying polysaccharide surface structure (41). It is the prerequisite for gaining insight into structure-activity relationship (42). The SEM images of the SWSP are given in Figure 5. Our data showed that the SWSP is composed of 100 to 500 µm geometric-shaped units with flat surfaces. Different structures were given in the literature(42, 43)showing dissimilar polysaccharides structure. This could be due essentially to the sample preparation method before microscopy. Moreover, polysaccharides often have high molecular weights and tend to form aggregates in a solution that can mask the behavior of individual macromolecules (44).

Antibacterial activity

Results in Table 2 revealed that there was differential antibacterial activity possessed by the Spirulina polysaccharides: the latter had a selective and moderate antibacterial activity, where it does not inhibit all the tested bacteria strains. Antibacterial activity was observed against all strains of bacteria tested except for *E. faecalis* and *S. enterica*. It seems that the effect of SWSP was more harmful on Gram-positive bacteria strains than Gram-negative ones. However, the inhibition diameter remained lower than that against Ampicillin. This behavior can be explained by the properties and nature of the polysaccharides extract since the sugar content itself might give some effect on the bacteria growth instead of inhibiting it (45).

Cytotoxic activity

Anticancer activity of SWSP with different concentrations was determined by cell lines viability assay using human breast MCF-7, colon HCT-116 and ovarian OVCAR cancer cell lines. Table 3 summarizes the cytotoxic effect of the SWSP on the three studied cell lines viability and (IC_{50}) values. With the OVCAR line, no inhibition was detected, and the IC_{50} was more than 50 µg. mL^{-1}. The SWSP was found to inhibit the proliferation of human colon (HCT-116) and breast (MCF-7) cancers. Results showed that SWSP exhibit 50% inhibition (IC_{50}) for these two cell lines. The overall results indicated that SWSP exhibited the strongest anticancer activities towards cancer cells MCF-7 and the IC_{50} values were found 33.8 µgmL^{-1}.

In recent years, it has been found that polysaccharides with obvious antitumor effects can play a role in the tumorigenesis of various cell lines mainly by inhibiting tumor growth, inducing apoptosis, enhancing immune function and coordinating chemotherapy drugs. Polysaccharides extracted from the Solanaceae plant*Lyciumbarbarum* could inhibit MCF-7 tumor cell proliferation (46) and could induce cell apoptosis (47). The IC_{50} value of the red

Table 2. The average diameter of inhibition zone (mm) ± S.D for SWSP against 9 human-pathogenic microbial strains: 6 Gram-positive bacteria (*Enterococcus faecalis*; *Listeria monocytogenes*; *Bacillus subtilis*; *Bacillus cereus*; *Bacillus thuringiensis*; *Micrococcus luteus*) and 3 Gram-negative ones (*Salmonella enterica*; *Escherichia coli*; *Klebsiellapneumoniae*). Ampicillin was used as the positive control (25 µg).

Gram	Type of Bacteria	Diameter of inhibition	Ampicillin(25 µg)
G. positive	*E. faecalis*	N/A	18.1±1.2
	L. monocytogenes	9.1±0.3	15.3±1.2
	B. subtilis	8.3±0.1	16.4±0.5
	B. cereus	N/A	20.1±0.8
	B. thuringiensis	5.3±1.1	17.4±1.1
	M.s luteus	4.2±0.5	17.6±1.1
G.negative	*S. enterica*	N/A	19.5±1.5
	E. coli	1.4±0.2	18.4±0.8
	K. pneumoniae	1.1±0.2	15.9±0.5

Values are shown as mean±standard deviation (n=3).*N/A: No activity.

Table 3. Cytotoxic effect of SWSP on MCF-7; HCT-116 and OVCAR cell lines. IC_{50} value (µg mL^{-1}). Values are shown as mean±standard deviation (n=3).

Cell lines	SWSP	Tamoxifen
MCF-7	33.8±2.60	0.15±0.02
HCT-116	46.5±4.80	0.14±0.02
OVCAR	>50	0.19±0.03

alga *Janiarubens* polysaccharide extract was 0.312 mg. $mL^{-1}$for the breast MCF-7 cell lines and 20 mg. mL^{-1} for the colon CoCa2 cell lines(48).

The effect of SWSP on MCF-7 was more important than the plant and the marine red alga polysaccharides, this could be due to its small molecular weight. Many studies have reported that the activity of a polysaccharide is strongly associated with its molecular weight, and polysaccharides with a high molecular weight struggle to penetrate the cell membrane and exert a pharmacological effect (32; 49). Wang et al. (50) found that the entrance of a *Ganodermalucidum* polysaccharide (GLP) with 108 kDa into a cell was through the pathway of macropinocytosis.

Hence, new anticancer agents should be investigated from various resources. A great number of antitumor compounds are natural products or their derivatives, mainly produced by blue-green algae.

In vitro antioxidant activity

The results reported in Figure 6 show the scavenging capacities of the SWSP: DPPH, ABTSand FRAP compared with the free radical-scavenging activity of BHT. The DPPH and ABTS radical-scavenging tests offer a redox-functioned proton ion for unstable free radicals and play a critical role in stabilizing detrimental free radicals in the human body(22).SWSP showed better inhibitory activity against ABTS radicals than the DPPH radicals.It has been found that SWSPsignificantly quenched DPPH (IC_{50}= 1.7 mg. mL^{-1}) and ABTS (IC_{50}= 0.082 mg. mL^{-1}). Similar data were provided by Lee K.et al.(51). Indeed several studies reported that the antioxidant activities of polysaccharides extracted from numerous medicinal plants were significant and that those carbohydrates may be used as potential antioxidants (9, 34, 52).

In vivo burn healing study

Qualitative outcomes

The effect of SWSP on rats

Eight days after wounds induction, rats were sacrificed by cervical dislocation and weighed on a balance. The results revealed no significant body weight changes in all groups. Additionally, no death or other undesirable reaction happened throughout the experimental period (data not shown) which reveals that the doses of the SWSP used for wound healing were nontoxic.

Morphological evaluation

Skin integrity was reestablished and injured tissue was renewed after laser burning. Wound photographs could be a key parameter to estimate wound healing(53). The SWSP effect on wound healing was evaluated using the calorimetric assessment. Figure 7 exhibited the progress of treatment and the descriptive collections of wound photos of rats taken on 1, 3, 5, 7 and 8 days after burns from all groups during the wound-healing period. The control group (group I) treated with physiological serum, were compared with groups II, III and IV which were treated with glycerol, "Cytol Basic", and SWSPgel, respectively. In the beginning of the treatment, laser wounds exhibited an identical red coloration which evolved to dark brown on a postoperative day and persisted to the third day in all the group rats burns. By the fifth day, a novel scab was detected namely in the SWSP-treated wounds and started to disappear by the seventh day to allow the formation of a new pink-colored epithelium that completely covered the injury, eight days after laser burn induction. Nevertheless, no complete wound healing was noticed in the other rat groups by the end of the experiment (8 days). By decreasing downtime and accelerating re-epithelialization, SWSP gel seems to be the perfect wound healing. This could be namely allocated to its important antioxidant activity.

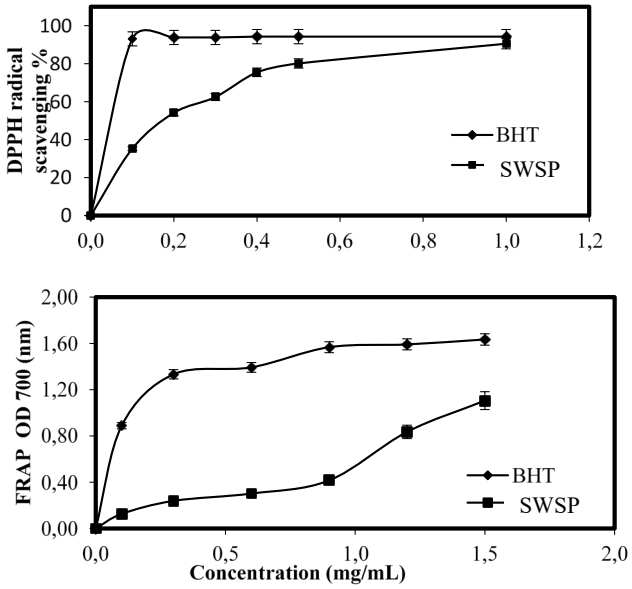

Figure 6. Antioxidant activity by 2,2-di-phenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity assay, Trolox equivalent antioxidant capacity (ABTS) assay, ferric reducing (FRAP) assay methods in SWSP. Values are the mean of three replicates ± standard deviation.

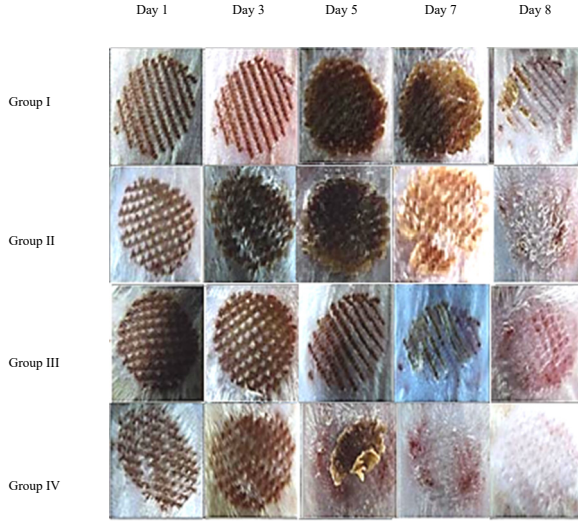

Figure 7. Laser Burn wounds chronicity was taken for the different groups on days 1, 3, 5, 7 and 8. Group I: was treated with physiological serum; Group II: was treated with glycerol; Group III was treated with "Cytol Basic" and Group IV: was treated with SWSP hydrogel.

Wound area assessment

Wound closure is a crucial method to check the evolution of burn wound healing. Wound area measurement of different groups of rats were gathered in Table 4. The healing progression was estimated over 8 days by checking regularly the dimension of the wound zone. Data herein showed that all treated groups revealed significant healing effects at the end of the experiment (8 days). Nevertheless, delayed wound healing processes were observed in group I compared to the fourth one, which presented significantly quicker closure times. Actually, the closure of the wounds seems to be totally accomplished in the SWSP-treated group reaching 0.05 cm against 0.25 cm in the "Cytol Basicthe " group, 0.36 cm in the glycerol group and 0.89 cm in the physiological serum group (table 4). This finding is in accordance with a previous work by Ktari et al. (34)and Zhang et al. (54) showing the benefic effect of polysaccharides for healing wounds.

Hydroxyproline and collagen turnover

The amino acid hydroxyproline is normally found in significant quantity in collagen and its measurement can be used as an indicator of collagen formation(55). Table 5 exhibited a significant increase in hydroxyproline content in "CytolCentella" treatment (842,82±5,44 mg. g^{-1})and namely in SWSP treated groups (1233,73±73,89 mg. g^{-1}) when compared to untreated (glycerol and control) ones, inferring more collagen synthesis, re-epithelialization and fibroblast proliferation. This denotes quicker wound healing progression in SWSP-treated groups during burn wound repair (56).

Histomorphometric study

Histological observations of wound tissue on the 8th post-laser day revealed a full re-epithelialization with a well-structured layer without cells inflammation in the SWSP-treated group (Fig. 8D). Similar results were observed in the positive group but with mild inflammatory cell infiltrations mainly in the perivascular site were seen (Fig. 8C). However, an invasive inflammatory cell infiltration without an epithelial layer and vacuolization of the dermal cells were seen in the untreated group (Fig. 8A). Further, a significant increase in hydroxyproline content (Fig. 8D) was equally shown in SWSP and CytolCentella groups ($P < 0.001$ and $P < 0.01$, respectively) when compared to the untreated group, which implies more collagen deposition and thus faster-wound healing process in treated groups.

Histopathological analyses showed a significant de-

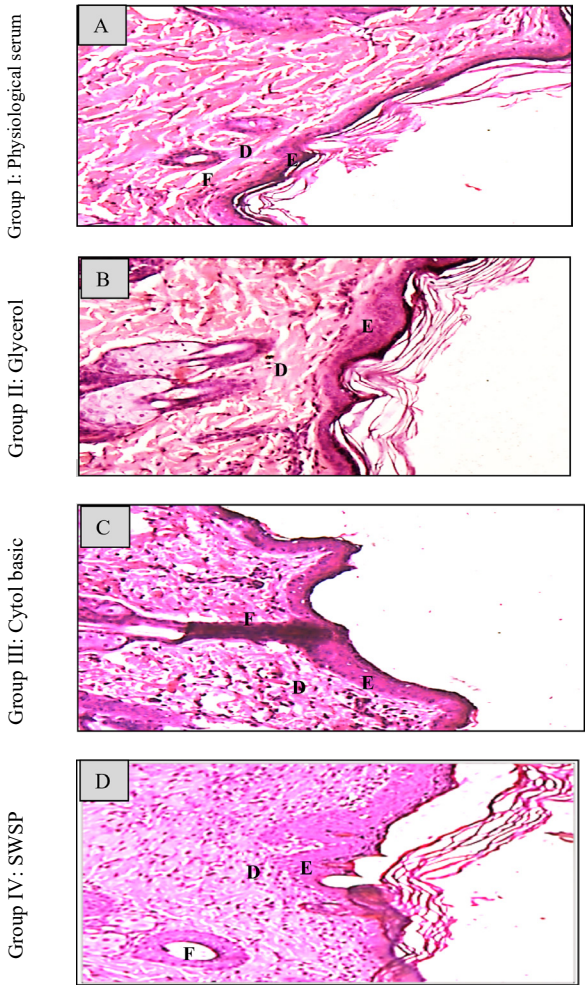

Figure 8. Representative photomicrographs of the epidermal and dermal architecture of wounds on the 8th day of treated rats with physiological serum (Group I); glycerol solution (Group II); CytolCentella (Group III) or SWSP hydrogel (Group IV). Tissues were stained with hematoxylin-eosin and visualized at 100 X magnifications. E: epidermis; D: dermis; F, hair follicle.

Table 5. Hydroxyproline content in the tissue of the different experimental animal groups.

Groups	Hydroxyproline (mg/g of tissue)
Group I	642.88±48.94^{d}
Group II	735.16±43.32^{c}
Group III	842.82±54.43^{b}
Group IV	933.67±73.89^{a}

Values are given as mean ± SD (n = 5 rats per group). a, b, c: different letters for each column represent significant differences at $P < 0.05$. Group I was treated with physiological serum; Group II was treated with glycerol; Group III was treated with "Cytol Basic" and Group IV was treated with SWSP.

Table 4. Evolution of the burns wound healing. Wound area measurement (cm) of different groups of rats.

Groups	Days				
	1	3	5	7	8
Group I	1.60±0.22^{a}	1.56±0.28^{a}	1.52±0.14^{ab}	1.22±0.11^{c}	0.89±0.17^{d}
Group II	1.60±0.13^{a}	1.51±0.11^{ab}	1.43±0.10^{b}	0.95±0.15^{d}	0.36±0.02^{e}
Group III	1.61±0.35^{a}	1.42±0.36^{b}	1.23±0.19^{c}	0.87±0.08^{d}	0.25±0.03^{e}
Group IV	1.61±0.17^{a}	1.41±0.21^{b}	1.19±0.23^{c}	0.79±0.09^{d}	0.05±0.03^{f}

Values are given as mean ± SD (n = 5 rats per group).

a, b, c: different letters for each column represent significant differences at $P < 0.05$.Group, I was treated with physiological serum; Group II was treated with glycerol; GroupIII was treated with "Cytol Basic" and Group IV was treated with SWSP.

crease in the number of fibroblast cells (Fig. 8) in the SWSP group when compared with control and glycerol. The group treated with SWSP also presented a significant decrease in inflammatory cells when compared with the controls, showing an anti-inflammatory activity (Fig. 8). In the same group was observed a higher presence of skin appendages, such as hair follicle and sebaceous glands, which was not achieved in the other groups, showing a tissular re-epithelialization capacity. The images show the formation of well-defined basal laminae in the SWSP group (Fig. 8D) when compared with the other groups. This result corroborates the complete re-epithelialization capacity of this extract in cutaneous wounds.

Conclusion

The healing effect of SWSP might be due to several mechanisms such as increasing rate of re-epithelialization and neo-vascularization, scavenging of destructive free radicals, inflammation reduction and control of infection, which might be due to antioxidant, anti-inflammatory and antimicrobial constituents of polysaccharide, as previously demonstrated by using other plant polysaccharides extract in wound healing activity.

Oxidative stress has a critical role in the progression of many disorders including wounds.

The balance between oxidants and antioxidants at the wound site interferes closely in the tissue regeneration process. Antioxidants are able to scavenge free-radical activity and inhibit its reaction propagation, favoring the healing process. The free radical scavenging properties of SWSP compounds could protect wound tissues from oxidative damage and reduce the risk of inflammation, contributing to improved wound healing. In summary, this work reported that the polysaccharide extracted from CO_2-enriched *Arthropsiraplatensis*seem to have the characteristics of polysaccharides but are different from polysaccharides extracted from other *Spirulina* grown in a CO_2-free medium. We reported also that CO_2 addition to the culture medium of *Spirulina*may improves its cytotoxic activity namely against human colon and breast cancer and its wound healing capacity.

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Declaration of competing interest

The authors confirm that they have no conflicts of interest with respect to the study described in this manuscript.

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Credit authorship contribution statement:

S. SASSI AYDI: Conceptualization, Formal analysis, Investigation, Writing- Original draft preparation, Project administration. ***S. AYDI***: Visualization, Writing-Original draft preparation, Funding acquisition, Supervision, Writing-Reviewing and Editing, Project administration. ***I. BKHAIRIA***: Methodology, Data Curation. ***R. RAHMANI***: Methodology, Software. ***N. KTARI***: Visualization, Validation. ***R. BEN SALAH***: Conceptualization, Resources, Writing - Review & Editing. ***J. BOUAJILA***: Funding acquisition, Resources, Supervision, Writing-Reviewing and Editing.

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