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Characterization and *in vitro* antibacterial activity of sulfated polysaccharides from freshwater alga *Cladophora crispata*

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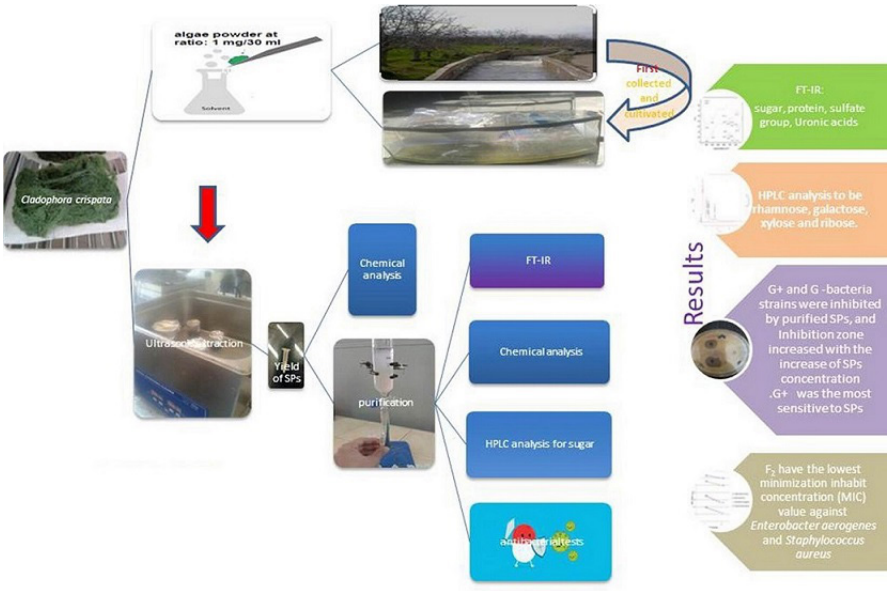

Graphical Abstract

Sulfated polysaccharides (SPs) from Cladophora crispata were extracted by ultrasonic bath: purify the crude SPs. Compounds of SPs were identified, and we investigated the effect of SPs as antibacterial agents.

Abstract

Barada River is characterized by an abundant growth of freshwater algae. *Cladophora* sp. algae have emerged as a new source of bioactive compounds. In this research *Cladophora crispata* was cultivated with the outdoor method, and algal sulfated polysaccharides (SPs) were extracted by an ultrasonic-assisted extraction method. After extraction, gel filtration was used to purify the crude SPs, SP compounds were determined and selected, and the effect of purified SPs as antibacterial agents was investigated. The purified extract gave two fractions (F1 and F2). The chemical components of both crude and purified SPs were then determined. The highest carbohydrate content (74.12%) and protein content (4.02%) was found in the crude extract, while the highest sulfate content (12.17%) was found in purified fraction F2, and the highest uronic acid content (18.46%) was found in purified fraction F1. Fourier transform infrared spectroscopy (FT-IR) was used to confirm that the crude extract and fractions consist of sugar, uronic acids, protein and sulfate groups. Both F1 and F2 consisted of rhamnose, galactose, xylose and ribose based on high performance liquid chromatography (HPLC) separation. Each fraction showed an inhibitory effect on Gram-positive and Gram-negative bacteria. F2 has the lowest minimum inhibitory concentration (MIC) value against *Staphylococcus aureus*, *Bacillus anthracis*, *Enterobacter aerogenes* and *Pseudomonas aeruginosa*, where its MIC values were 6, 13, 25 and 30 mg ml^{-1}, respectively. Algae polysaccharides are of key interest due to their antibacterial properties, which has led to them being included in pharmaceutics and food applications.

HIGHLIGHTS

- Sulfated polysaccharides (SPs) were extracted from *Cladophora crispata* using an ultrasonic bath.
- SPs consist of sugar, uronic acids, protein and sulfate groups.
- Gram-positive bacteria are more sensitive to SPs.

DATA SUMMARY

All supporting data, code and protocols have been provided within the article.

INTRODUCTION

Green algae (Chlorophyta) are the primary producers in aquatic ecosystems, with an estimated count of 6000 to 8000 species. Most of these are macroalgae, while others are microalgae such as *Cladophora* (Ulvophyceae, Cladophorales) [1]. These are multinucleate filamentous algae, and their cells contain a parietal perforate or reticulated chloroplast. The presence of pigments such as chlorophyll (a and b), xanthophylls and β-carotene give it its bright green colour. *Cladophora* is predominantly benthic [2], and it is usually found in the region of unidirectional flow or periodic wave action in fresh water and marine habitats. *Cladophora* is a mid to late successional species [1, 2].

Algae can be cultivated in open and closed ponds, and photobioreactors for biomass production [3]. Microbial polysaccharides are of great biotechnological and commercial interest, and have a wide application in the food, cosmetics and medical industries due to their emulsifying, thickening, flocculating, stabilizing, anti-oxidizing and antimicrobial properties. It is much easier and advantageous to use them because of the short life cycle of the microbes that allows quick production under controlled environmental conditions. Polysaccharides have been extracted from fungi, bacteria and yeasts [4–6], and they are the most important products of algae, representing 38 to 54 % of algal dry weight [7, 8]. They show important biochemical properties, which require further investigation to understand their structure and biochemical functions. Several studies have reported the antibacterial, antifungal and antiviral properties of sulfated polysaccharides (SPs) [9–11], as well as their scavenging activity against superoxide, hydroxyl, DPPH (1,2-diphenylpicrylhydrazyl) and ABTS radicals [2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonicacid) diammonium salt] [12, 13], and they have commercial applications in food industries [14]. Moreover, the SPs from *Cladophora oligoclada* have been reported to have anticoagulant properties [15]. Polysaccharides from green algae are heteropolysaccharides that are composed of different monosaccharides [10, 16].

Algae polysaccharides can be extracted by several green techniques such as microwave, enzyme and ultrasonic-assisted extraction (UAE). UAE reduces energy consumption and solvent use. In addition, the low temperatures and short times used in UAE processes can maintain the functionalities of the bioactive compounds [12, 17, 18]. The objectives for this study were (a) to isolate and cultivate *Cladophora crispata* to understand its growth dynamics, and (b) to study the chemical and functional properties of the crude and purified SPs produced by this species.

METHODS

Isolation and biomass production of algae

Algae samples were collected from the Barada River, Rabweh, North-West of Damascus city, Syria (Fig. 1), on 20 April 2021. Samples were kept in 5 l polyethylene bottles at 4 °C until they were shipped to the laboratory (Plant Biology Department, Faculty of Science, Damascus University, Syria), where they were washed with distilled water several times before identification; *Cladophora* cells were collected by grabber. Morphological diagnosis was performed as described by Prescott [19] by examination under a light microscope (Olympus CX41).

The identified isolates were purified by repeated cultivation in algae culture broth (Fluka medium; macronutrients $NaNO_3$, KH_2PO_4, alkaline EDTA, acidified iron solution, boron, trace metals solution) as indicated by West [20]. A single-batch system was used for this purpose, where 10 g algae was inoculated into 100 ml medium in a 250 ml conical flask, and incubated for 14 days at 23 °C and pH 8. Cultures were aerated by aerating pumps, and illuminated by fluorescent tubes with an intensity of 1800

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Keywords: antibacterial activity; *Cladophora crispata*; functional groups; minimum inhibitory concentration (MIC); quantity and quality determinations; sulfated polysaccharides.
Abbreviations: FT-IR, fourier transform infrared spectroscopy; HPLC, high performance liquid chromatography; MIC, minimum inhibitory concentration; PBS, phosphate-buffered saline; SP, sulfated polysaccharide; UAE, ultrasonic-assisted extraction.

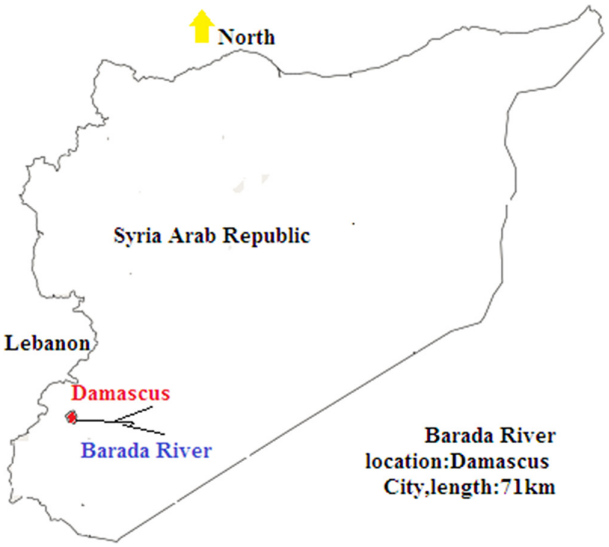

Fig. 1. Sampling site location.

lux, and a light/dark cycle of 16/8 h. Algae were transferred for outdoor cultivation for biomass production during the summer using open ponds with control of the nitrogen-to-phosphorus ratio [21], as shown in Table 1; a DR 2800 Lange spectrophotometer was used to estimate these elements in the water.

Algal growth rate was estimated by the method of Stein [22] based on measuring the optical density by spectrophotometer (UV/vis spectrophotometer model Optizen 2120 UV plus) at a wavelength of 650 nm. The algae biomass was isolated from the culture medium by centrifugation method at a speed of 3000 r.p.m. for 30 min, for the purpose of disassembling the strands into cells, sediment was discarded and the supernatant liquid was taken for measurement. Culture medium was used as the control solution, and the growth rate was calculated and expressed as the specific growth rate. Generation time was calculated according to the two equations developed by Huang and Wang [23]:

$$K = \frac{\log_{ODT} - \log_{OD0}}{T} X3.322$$

$$G = \frac{0.301}{K}$$

K is the specific growth rate (cells h^{-1}); ODT, optical density at the end of the experiment; OD0, optical density at the start of the experiment; T, time of the experiment; G, generation time (h).

Biomass was harvested at the stationary phase (where optical density was constant for a period of time then declined).

Table 1. Treatments of outdoor cultivation for biomass production

Treatment	KH_2PO_4 (mg l^{-1})	Yeast extract (mg l^{-1})
1	0.4	0.4
2	0.4	0.8
3	0.4	1.2
4	0.8	0.4
5	0.8	0.8
6	0.8	1.2
7	1.2	0.4
8	1.2	0.8
9	1.2	1.2
Control (distilled water)	0	0

Polysaccharide extraction

Polysaccharides were extracted using the UAE technique as described by Esmaeili *et al*. [24], followed by sequential extraction according to the method of Song *et al*. [12] with some modifications. Briefly, algae dried powder was mixed with distilled water in the ratio of 30:1 (ml:g), and the mixture was put in an ultrasonic bath (Ultrasonic cleaner model ps-60ar, 360 W, 40 kHz; JeKen) for 120 min at 60 °C. Algae biomass was separated from the extract by centrifugation at 5000 r.p.m. for 10 min. Polysaccharides in the supernatants were precipitated by adding pure ethanol (99.9%) at triple the volume of the extract volume. The mixture was incubated at 4 °C for 48 h, and the pellets formed were resolved using distilled water. Lipids were removed by adding chloroform and acetone mixture (3:1), at triple the volume. The residual proteins were removed by adding Sevage reagent (1:4, v/v, mixture of *n*-butanol and chloroform) and centrifugation at 4000 r.p.m. for 15 min; the precipitate was discarded and the supernatant was dialysed for 48 h using dialysis bags. Polysaccharides were precipitated by the addition of pure ethanol three times at 4 °C for 48 h, then collected using cold centrifugation at 5000 r.p.m. for 30 min at −20 °C.

SP purification

Purification of crude polysaccharides from *C. crispata* was carried out according to the method of Pier *et al*. [25] with some modifications; where 0.5 g crude SPs was dissolved in 100 ml phosphate-buffered saline (PBS; 6.8 g sodium chloride, 0.43 g potassium dihydrogen phosphate, 1.48 g disodium hydrogen phosphate dissolved in 1 l distilled water, pH 7.2). A total of 3 ml SPs and PBS was loaded into a gel filtration column [Sephadex G-100 gel (Sigma); column size 2.6×20 cm], and eluted with PBS at a flow rate of 1 ml min^{-1}. The eluted fractions were collected in a volume of 700 µl. Polysaccharides were precipitated by the addition of pure ethanol three times and incubation at 4°C for 48 h. SP solution was then concentrated via rotary evaporation at 60 °C and 100 r.p.m. The SPs were crystallized by incubation at 50 °C with shaking at 150 r.p.m. for 12 h by using a shaking incubator (JSR model JSSI-100) until reaching the water content of 10 %.

Fourier transform infrared spectroscopy (FT-IR)

FT-IR was used to recognize the functional groups in purified SPs. Each fraction (2 mg) from polysaccharide was dried in an oven at 40 °C for 12 h, and then mixed with potassium bromide (KBr) powder. FT-IR spectra were measured in the frequency range of 400 to 4000 cm^{-1} using a FT-IR-106 4200 type A-C077661018 instrument [12].

Determination of SP components

The phenol/sulfuric acid method was used to determine the carbohydrate content of each fraction of SPs [26]. The sulfate content in SPs was calculated by the barium chloride gelatine assay [27]. Protein content was determined according to the Lowry method [28].

Monosaccharide analysis

Carbohydrate analysis requires prior hydrolysis by sulfuric acid, briefly: 30 mg from each sample (crude SPs, F1 and F2) was hydrolysed with 300 µl of 72 % sulfuric acid solution for 30 min at 30 °C, and the mixture then was diluted by the addition of 8.4 ml distilled water and incubated at 121 °C for 1 h. The hydrolysed SPs were neutralized by calcium carbonate as described by Cui *et al*. [29].

The monosaccharides were selected by high performance liquid chromatography (HPLC) according to the assay of Xu *et al*. [30], with some modifications; the temperatures of the column and refractive index detector (RID) were set at 30 °C, the mobile phase was composed of acetonitrile and deionized water (85:15, v/v), flow rate was 1.0 ml min^{-1} and the injection volume was 20 µl. A standard solution (Sigma-Aldrich) was injected into the chromatography equipment at the concentration range of 0.5–50 mg in 1 ml deionized water. All samples were diluted with deionized water and filtered through 0.45 µm nylon filters prior to HPLC analysis. The experiments were repeated three times. The column was washed with the mobile phase at the end of each experiment period for more than 20 min.

Antibacterial activity of SPs

Analysis of the antimicrobial activity of crude SP and its fractions was carried out against both Gram-positive (*Staphylococcus aureus* and *Bacillus anthracis*) and Gram-negative bacteria (*Enterobacter aerogenes* and *Pseudomonas aeruginosa*), which were obtained from the Microbiology and Algae Laboratory, Damascus University, where they were identified according to Bergey's manual [31]. The strains were grown on nutrient agar for 24 h at 37 °C, a part of each bacterial inoculum was then taken into a sterile tube containing 5 ml nutrient broth and incubated for 4 h, with shaking at 100 r.p.m. at 37 °C [32].

Preparation of inoculum

The turbidity of bacterial suspension was adjusted to 0.5 McFarland scale (at OD_{600}) using sterile physiological saline solution, equal to 1×10^8 c.f.u. ml^{-1} using the turbidity method by McFarland assay; followed by dilution to a concentration of 10^5 c.f.u. ml^{-1} after that. The bacterial suspension was then inoculated onto Mueller–Hinton agar (MHA) plates [33].

Antibacterial activity assay

Antibacterial activity was determined for each fraction. Firstly, 50 µl standardized inoculum of the bacterial suspension (10^5 c.f.u. ml^{-1}) was inoculated onto the MHA surface. Then, wells were made in the MHA medium using a drill bit no. 3, where the agar plugs were removed with a sterile needle [34]. A known concentration of each fraction from SPs (20, 30, 40, 50 mg ml^{-1}) was inoculated into the wells. The positive control was ceftriaxone (at a concentration of 0.45%), while distilled water was the negative control. Then, the agar plates were incubated at 37 °C for 24 h, and the bacterial sensitivity was recorded. Inhibition measurement was carried out using a ruler, as the diameter of the zone sizes to the nearest millimetre [33].

In vitro minimum inhibitory concentration (MIC) determination

The MIC of SPs on the bacterial growth was determined by the agar dilution method. The SPs were dissolved in MHA medium, and serial dilution of SPs were prepared (concentration of SP solutions ranged from 0.5 to 50 mg ml^{-1}). SP solutions were placed in Petri dishes and exposed to drying; there were also Petri dishes without SP solutions. Fresh bacterial suspension (10^5 c.f.u. ml^{-1}) was used (1 µl each microbial strain was added to each Petri dish), and placed in an incubator at 37 °C for 24 h. The experiments were performed in triplicate [35].

Statistical analysis

All experiments were performed in triplicate, and experimental data are represented by the mean value ±SD of each sample. Statistical analyses were done by IBM SPSS version 20 using one- or three-way ANOVA at a level of significance of 0.01.

RESULTS AND DISCUSSION

Algae species description and biomass production

Freshwater green macroalga *C. crispata* is multicellular, filamentous, truly branched, with cell patterns that are cylindrical, and the diameter of the head branches is about 50–70 µm, while ranging between 20 and 35 µm in the lateral branches, as seen in Fig. 2. The same microscopy observations have been reported by others [36]. Algae structure and a large amount of biomass production are related to habitat conditions. Depth of water, total dissolved salts, orthophosphate, nitrate chloride and Chlorophyll-a pigment content in water are key parameters to ubiquitous algae colonies [37]. In conditions such as a water temperature at 26 °C, weather temperature at 35 °C, an illumination duration of 14.30 h, the intensity of illumination 790 lux and N:P ratio 3:1, the specific growth rate was 0.3 cells h^{-1} and the generation time 0.97 h. So, our results indicate that the algae biomass was exposed to a higher temperature causing stress to the

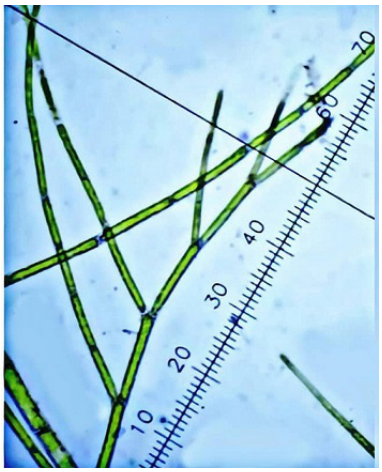

Fig. 2. Morphology of *C. crispata* under a microscope at ×40 magnification.

algae, this led to an increase in the carbohydrate content within their biomass; as carbohydrate synthesis requires less energy, carbohydrates were synthesized before lipids in a rapid response to environmental stress [38]. The synthesis of carbohydrates was affected by nitrogen concentration. The highest carbohydrate content (75.23%) was obtained in the culture supplemented with a higher nitrogen concentration at high temperatures, and these results were consistent with similar research [39]. A culture that was P limited (low P) led to the accumulation of carbohydrate content, this is in agreement with other work [40].

Purification of crude SP from *C. crispata*

A sequential extraction method was used after sonication to extract a maximum amount of SPs. The SPs yield was 7.14 %, which consisted of 74.12 % carbohydrate and 9.08 % sulfate group. Purification of SPs from *C. crispat*a gave purified SPs that were well characterized (high carbohydrate and low protein contents), crude SPs were purified by gel chromatography, and fractions of SPs were selected based on the total carbohydrate elution profile (Fig. 3). The crude SP extract gave two fractions, this result is in agreement with other studies [12, 25]: F_1 (fraction numbers 10–17) and F_2 (fraction numbers 22–27).

FT-IR spectra

The infrared spectra of each of the two fractions obtained from the purification of crude polysaccharides are shown in Fig. 4; both F_1 and F_2 have the same spectra, with little difference in the wave numbers. We noted the peak of the O–H stretches at 3397.21 and 3404.13 cm^{-1}, peaks of the C–H stretching vibration are found at 2925.30 and 2926.44 cm^{-1}, this is including CH, CH_2 and CH_3 stretching. The absorption peaks of amide I were found at 1653.66 and 1663.30 cm^{-1}, and 1555.30 and 1556.27 cm^{-1} peaks were indicated to be amide II. The C=O (carboxylate groups) asymmetric stretching vibrations were observed at 1625.73 cm^{-1}, it indicates the presence of uronic acids. The absorption peaks at around 1401.99 cm^{-1} refer to C–H bends, and the peaks at 1250.61 and 1252.53 cm^{-1} were due to S=O stretching vibration; also, peaks at 1077.04 and 1074.15 cm^{-1} were due to C-O. The C-O-S indicated the majority of sulfate groups, 847. 56 cm^{-1}, and peaks at about 590–900 cm^{-1} refer to pyranose [12, 15, 41, 42].

Chemical contents of purified SPs

The chemistry of the contents of crude extract and fractions F_1 and F_2 is shown in Table 2; we noted the carbohydrate content was associated with protein content (r=0.73; R^2=0.54) but not for the sulfate (r=−0.39; R^2=0.15) and uronic acids (r=−0.99; R^2=0.99). Carbohydrate, protein, sulfate groups and uronic acids contents for crude extract and fractions are not the same. A protein is contamination of cell wall SPs, because protein is a part of the structure of cell walls and was closely associated with polysaccharides. All SP extracts were contaminated with protein because of the ester sulfate moieties, which can form strong anions and attract positively charged proteins [43], after purification, the high sulfate groups are tightly linked to the SP chains [44].

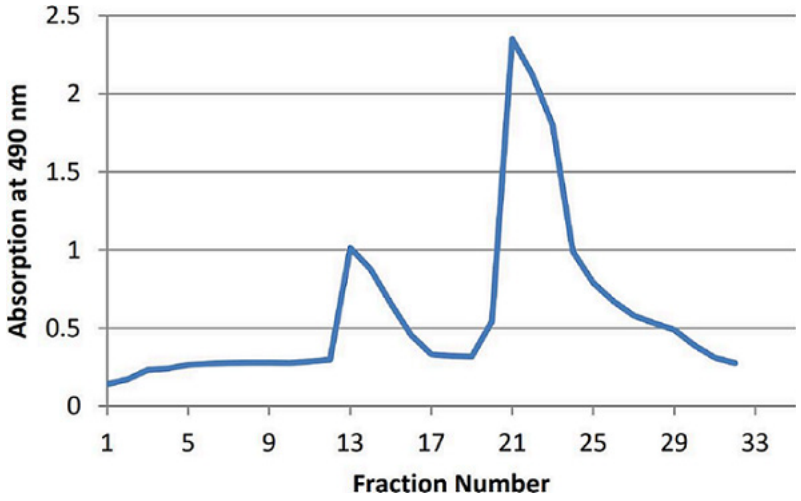

Fig. 3. Sephadex G-100 chromatogram elution profile of crude SPs of *C. crispata*.

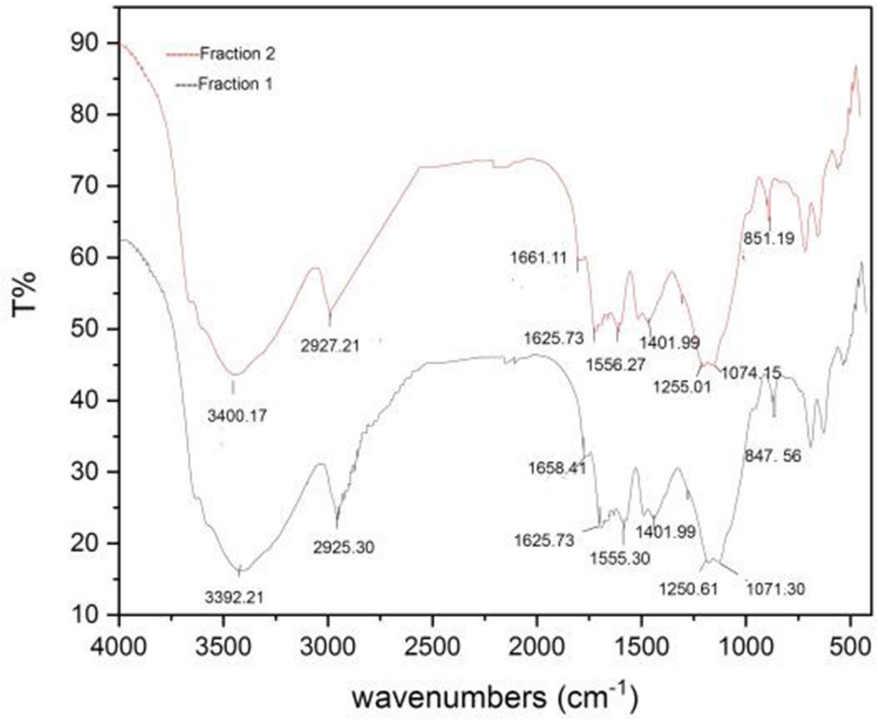

Fig. 4. FT-IR spectra of fraction 1 and fraction 2 of purified SPs extracts from *C. crispata*.

Table 2. Chemistry of the contents of the crude extract and purified fractions of SPs (based on moisture content 10%)

A, B, C, the same letters in the same column indicate no significant differences at 1%.

Component	Carbohydrate (%)	Protein (%)	Sulfate (%)	Uronic acids (%)*
Crude SPs	74.12±0.003 A	4.02±0.0019 A	9.08±0.002 C	2.87±0.006 C
F_1	59.07±0.002 C	2.52±0.002 B	10.04±0.002 B	18.46±0.003 A
F_2	65.36±0.002 B	1.83±0.002 C	12.17±0.002 A	10.62±0.003 B

*Percentage of uronic acids=100-(carbohydrate+sulfate+protein+moisture content).

Monosaccharide composition

The monosaccharides in the crude extract and fractions of SPs extracted from *C. crispata* were: rhamnose, galactose, xylose and ribose, based on the HPLC analysis (Fig. 5); this result is in agreement with other research [36]. The polysaccharide from green algae is ulvan. Ulvan is a heteropolysaccharide; and the composition is rhamnose, xylose, sulfate groups and uronic acids, such as iduronic acid or glucuronic acid. The composition of ulvan depends on the processing procedures of the biomass, algae species and eco-physiology. Ulvan has three types based on linked rhamnose to uronic acids; nuclear magnetic resonance (NMR) spectroscopy has been used for analysis, and other monosaccharides were reported (e.g. galactose, glucose, arabinose and mannose). This structure is common in marine and freshwater algae [9, 43, 45].

Antibacterial activities

The block randomization method was used in this research, not only Gram-positive but also Gram-negative bacteria strains were inhibited by purified SPs, and the antibacterial activity (inhibition zone) of the purified fractions of SPs increased with their increasing concentration, as shown in Table 3. *S. aureus* was the most sensitive to this SP, with an inhibition zone ranging from 7 to 27 mm. These results were similar to those in other reports [33]. The positive control had an effect in all bacterial strains, but not the negative control, as shown in Fig. 6.

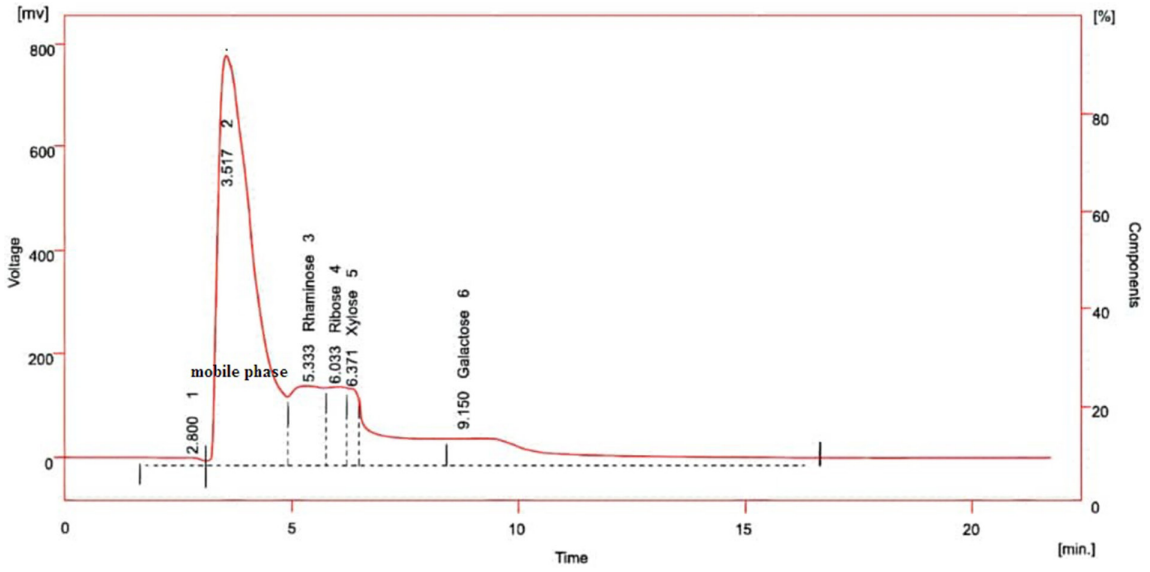

Fig. 5. HPLC analysis of the monosaccharide composition in crude extract and fractions of SPs extracted from *C. crispata*.

Table 3. Antibacterial activity of the purified fractions of SPs from *C. crispata*

A, B, C, D, E, F, G, H, the same alphabetical capital letters in the same column indicate no significant differences at 1%.

Fraction	Concn (mg ml^{-1})	Inhibition zone (mm)			
		Strains			
		Staphylococcus aureus	*Bacillus anthracis*	*Enterobacter aerogenes*	*Pseudomonas aeruginosa*
F_1	20	7.0 ± 1^{a} F	6.6 ± 1.1^{a} G	0^{b} G	0^{b} F
F_2	20	8.0 ± 0.5^{a} F	7 ± 1^{b} G	0^{c} G	0^{c} F
F_1	30	11.0 ± 2^{a} E	9 ± 2^{b} F	7.3 ± 0.5^{c} F	0^{d} F
F_2	30	15.0 ± 1^{a} D	11 ± 1^{b} E	9 ± 1^{c} E	0^{d} F
F_1	40	19.3 ± 0.5^{a} C	17 ± 1^{a} D	11 ± 1^{c} D	14 ± 1^{c} D
F_2	40	27.0 ± 1^{a} A	25.3 ± 0.5^{b} B	14 ± 1^{d} C	21 ± 1^{c} A
F_1	50	21.0 ± 2^{a} B	19 ± 1^{b} C	17 ± 2^{c} B	16 ± 1^{c} C
F_2	50	27.0 ± 1^{a} A	27 ± 1^{a} A	19 ± 1^{b} A	19.3 ± 0.5^{b} B
Positive control	0.05	22.0 ± 1^{a} B	18 ± 1^{c} CD	19 ± 3^{b} A	11 ± 1^{d} E
Negative control	–	0^{a} G	0^{a} H	0^{a} G	0^{a} F

The antibacterial activity of algae extracts against *Enterobacter* sp. was classified as resistant or active. The purified ulvan from the green alga *Ulva reticulata* also had an antibacterial activity with an inhibition zone diameter of about 20 mm [45]. *S. aureus* and *B. anthracis* bacteria are more sensitive to antibacterial agents than *E. aerogenes* and *P. aeruginosa*, as a result of having the additional protection afforded by an outer membrane, such as lipopolysaccharide and phospholipids [46]. According to other studies, the ester sulfate of polysaccharides is related to their biological activity, such as antibacterial activities and preventing preformed biofilms [47, 48]. *S. aureus* had the lowest MIC (6 mg l^{-1}) when we used F_2; while *P. aeruginosa* had the highest MIC (32 mg ml^{-1}) when we used F_1 (Table 4). Our results suggested that the SPs might be able to activate intestinal epithelial cells to produce cell-mediated immune response cytokines that initiate and amplify protective immune responses of the host [33].

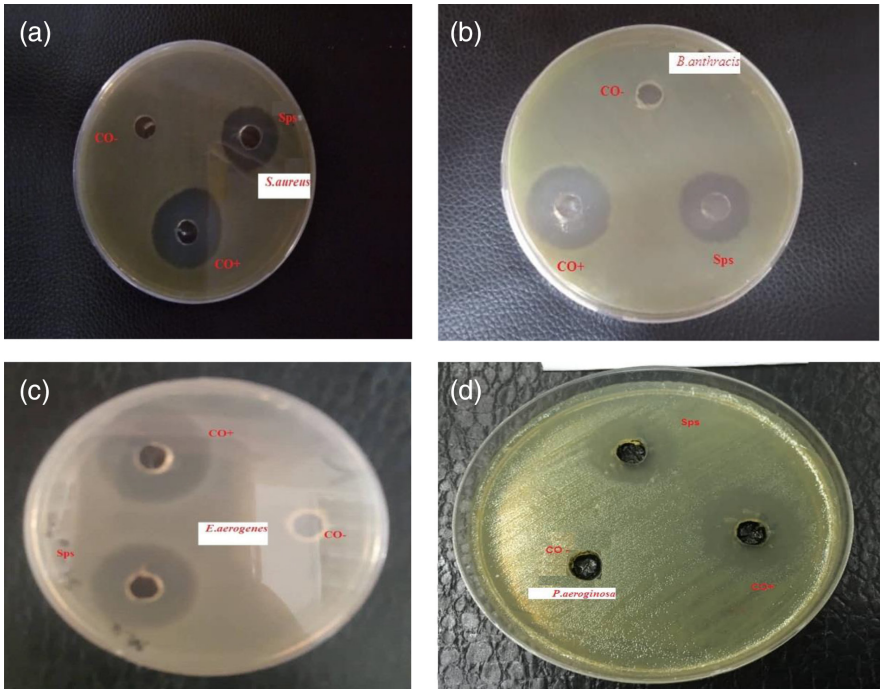

Fig. 6. Effect of SPs against bacteria. a) *S. aureus*, b) *B. anthracis*, c) *E. aerogenes*, d) *P. aeroginosa*

Table 4. MIC (mg ml^{-1}) for purified SPs against *S. aureus*, *B. anthracis*, *E. aerogenes* and *P. aeruginosa*

Bacterial strain	MIC (mg ml^{-1})	
	Fraction F1	Fraction F2
S. aureus	10	6
B. anthracis	18	13
E. aerogenes	28	25
P. aeruginosa	32	30

Conclusions

C. crispata is simple to classify because it has regular branches, and its growth in the outdoor ponds systems is an important feature for this species, because of the cheap equipment required for this purpose. *Cladophora* biomass is an unlimited, easily cultivated low-cost and valuable resource for different applications. Ultrasonic waves were considered a useful method for extracting SPs from *C. crispata*, with the following conditions: ratio of 30:1 ml g^{-1}, 60 °C, 120 min.

SPs from *C. crispata* are heteropolysaccharides, both F_1 and F_2 from purified SPs have the same monosaccharides, consisting of carbohydrate, protein, sulfate groups and uronic acids based on FT-IR analysis. It could be interesting to better purify the SP fractions by using ion-exchange resins to eliminate the presence of proteins. Purified SPs exhibit antibacterial activity because of their viscosity, water-solubility, hydroxyl and sulfate group. Mechanisms of antibacterial action for SPs are due to glycoprotein receptors present on the cell surface of polysaccharides that bind with compounds in the bacterial cell wall, cytoplasmic membrane and DNA, and cause increased permeability of the cytoplasmic membrane, protein leakage and binding of bacterial DNA. The antibacterial activity of SPs suggested possible use within the food industry, animal diets and pharmaceutical industries. We advise investigation of the antioxidant properties for SPs as further research, because the ester sulfate in SPs can donate an electron, which can give it antioxidant properties.

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Author contributions

M.K.J.: conceptualization, data curation, formal analysis, investigation, writing – review and editing, writing – original draft. A.-A.M: supervision, conceptualization, resources, methodology, writing – review and editing. A.A.N.: project administration, conceptualization, resources, methodology, writing – review and editing.

Conflicts of interest

The authors declare that there are no conflicts of interest.

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Peer review history

VERSION 4

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000537.v4.1

Girish Beedessee; University of Cambridge, UNITED KINGDOM

Date report received: 11 June 2023
Recommendation: Accept

Comments: The work presented is clear and the arguments well formed. This study would be a valuable contribution to the existing literature.

Author response to reviewers to Version 3

Letter for reviewers

Dear Professor

I did all the required modifications from reviewers,Editor and Editorial Office requirements briefly:

1. I have corrected the grammatical , typographical, and formatting errors were corrected.

2. Abbreviations were defined when they are first appearance.

3. I added graphical abstract with legend needed in my article.

4. I provide more detail in the Introduction and Methods Section.

1.In the Abstract Section:

I have corrected the grammatical , typographical, and formatting errors were corrected

had been emerged**(line 37)**=>have been emerged

2. In the Introduction Section:

I have corrected the grammatical errors**:**

İt is multinucleate filamentous algae. its cells contain a parietal perforate or reticulate chloroplast. The presence of pigments such as chlorophyll (a and b), xanthophylls, and β-carotene give it its bright green color**(line 67-70)**=>İt is multinucleate filamentous algae, its cells contain a parietal perforate or reticulated chloroplast. The presence of pigments such as chlorophyll (a and b), xanthophylls and β-carotene, give it its bright green color

quick production under controlled environmental conditions.polysaccharides were extracted from fungi, bacterial and yeasts [4-6], and from algae; polysaccharides are the most important products of algae**(line78-79)**=>quick production under controlled environmental conditions. Polysaccharides were extracted from fungi, bacteria and yeasts [4-6], and they are the most important products of algae, which represent 38 % to 54 % of algal dry weight [7-8],

3.In the Materials and Methods Section:

I addedFigure 1.sampling sites

I added more details in Table 1.treatments of outdoors cultivation for biomass production(line 116)=> Distill water without Yeast extracted and KH_2po_4

In section:Sulfated polysaccharides Purification

I have corrected the grammatical errors

In section : Antibacterial activityofSPs

I have corrected the grammatical errors

Antimicrobial activity determined for each fraction**(line 192)**=> Antimicrobial activity of crude SPs and its fractions was carried

where they were identified it's according to Bergey's manual**(line 196)**=> where they were identified according to Bergey's manual[

4. in the Results andDiscussionSection:

I have corrected the grammatical , typographical, and formatting errors

In section Algae species Description and biomass production:

the diameter about 50 - 70 micrometers, while the lateral branches are small diameter ranged**(line229-231)**=>the diameter of the head branches is about 50 - 70micrometers, while ranged between 20 -35 micrometers in the lateral branches, as seen in Figure 2, same microscopic observation were found with [36].

in section Purification of Crude Sulfated polysaccharide from *Cladophoracrispata*:

Purification of SPs from *Cladophoracrispat*a gave purified SPs with well-characterized (high carbohydrate and low protein contents), we purified the crude SPs by gel chromatography, fractions of SPs were selected based on the total carbohydrate elution profile (figure 3) **(line 251-252)**=> Purification of SPs from *Cladophoracrispat*a gave purified SPs with well-characterized (high carbohydrate and low protein contents), crude SPs were purified by gel chromatography, fractions of SPs were selected based on the total carbohydrate elution profile (figure 3).

in section **Monosaccharide Composition:**

nuclear magnetic resonance spectroscopy had used for this purpose, **(line)**=>

in section: Antibacterial activities:

Sulfated polysaccharide; inhibit zone**(line 308)**=> nuclear magnetic resonance (NMR) spectroscopy has used for this purpose,Abbreviations were defined **in Table 4**.The minimum inhibitory concentration (MIC mg/ml) forpurified Sulfated polysaccharides against *S. Aureus,B.anthracis*and *E. Aerogenes,P.aeroginosa*

4.Conclusions

I have corrected the grammatical

which caused increased**(line 370)**=> and causes increased

Thanks for you.

VERSION 3

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000537.v3.2

Girish Beedessee; University of Cambridge, UNITED KINGDOM

Date report received: 30 April 2023
Recommendation: Minor Amendment

Comments: The paper is poorly structured and written, which has prevented a proper assessment of the research done. The organisation of the files made it difficult to see what revisions were made where and in which file as the names are confusing.

It was very difficult to follow as no line numbering was used and the language made it hard and this affected the reviewing process as elaborated by Reviewer 1. Although the author made the required revision requested by the 2 reviewers, and to help guide that revision, I've added a few notes on the revised manuscript and highlighted points that seem to warrant special attention. I still think there is a need to improve the language for effective communication of your science (e.g. better figures) and throughout the manuscript, it is difficult to follow where a sentence starts and stops and where the next one starts. Throughout the manuscript, there is a need for better sentence construction such as proper tense namely at: Line 3: "had been emerged" Line 68:"or reticulatechloroplast. " Line 69:"carotene give it its " Line 78-79: It appears something is missing from this sentence., so please check "quick production under controlled environmental conditions polysaccharides were extracted from fungi, bacterial and yeasts [4-6], and from algae; polysaccharides are the most important Line 142:"modifications, Briefly; Dry powder " Line 158 (disolved -> dissolved). This also contained a hyperlink as it this was copy-pasted from a website. Line 194-105:"they were identified it's according to Bergey's manual" Line 205: "Antimicrobial activity determined for each fraction" Line 227: Spss Line 233: "branches are small the diameter ranged". Something is missing here. Line 238: "illumination duration of 14.30 hours." Is this correct? Line 256-258: Please rewrite this. Line 272: "it indicates to present uronic acids. " Please rewrite. Line 289: Please rewrite "proteins [41], after purification, the high sulfate groups are tightly linked to the SPs chains [43]." Line 321" Please write proper tense "spectroscopy had used for this " Line 324: Rewrite "polysaccharide; inhibit " Line 364: Rewrite "The ultrasonic waves consider a useful " Line 374:"which caused increased " Section 3.2 is irrelevant and can be merged with another section. The language is also confusing. Please clarify. Figure 1 needs to be of better quality with a proper legend. Table 1 needs to be designed better so as to make it easy understand what the control and distil water are for. Table 2 top legend: "Uroinc acids -> uronic" Table 3: Please redo this table as it is creating confusion between the small alphabets (abc) and capital (ABC) in the main table. Use different symbols to differentiate. Table 4: Please rewrite the bacteria ID (S. Aureus, B.anthracis and E. Aerogenes, P.aeroginosa) properly. (e.g. S. Aureus)

Reviewer 1 recommendation and comments

https://doi.org/10.1099/acmi.0.000537.v3.1

Anonymous.

Date report received: 18 April 2023
Recommendation: Minor Amendment

Comments: The corresponding author responded to reviewer comments well. But I think that the resubmitted manuscript is not revised.

Please rate the manuscript for methodological rigour
Satisfactory

Please rate the quality of the presentation and structure of the manuscript
Satisfactory

To what extent are the conclusions supported by the data?
Partially support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

Author response to reviewers to Version 2

Letter for reviewers

Dear Professor

I did all the required modifications from reviewers,Editor and Editorial Office requirements briefly:

1. I have corrected the grammatical , typographical, and formatting errors were corrected.

2. Abbreviations were defined when they are first appearance.

3. I added graphical abstract with legend needed in my article.

4. I provide more detail in the Introduction and Methods Section.

1.In the Abstract Section:

Reviewer 1

I corrected thetypographical and formatting errors as:

crud" => "crude"

"sulfated Polysaccharides"

"infrared Spectroscopy"

"High performance"

I corrected theMIC values

Reviewer 2

Remove the algaehave …..*Cladophora*have been emerged as a new source for bioactive compounds.

Each fraction showed an inhibitory effect on the gram-positive and gram-negative bacteria.

I write: purified fraction instead of F2.

I added more Keywords

2. In the Introduction Section:

Reviewer 1

I correct thetypographical and formatting errors **as:**

amultinucleate" => "multinucleate"

I did theabbreviations were defined when they are first appearance as:

DPPH(1,2-diphenylpicrylhydrazyl), and ABTS radicals2,2′-azino-bis(3-ethylbenzothiazoline-6-sulphonicacid) diammonium salt.

I explain why polysaccharides are the most important products:

"Microbial polysaccharides are of great biotechnological and commercial interest and have wide application in the food, cosmetics, and medicine industries due to their emulsifying, thickening, flocculating, stabilizing, anti-oxidizing, and antimicrobial properties, and it is much easier and advantageous because of the short life cycle of microbes that allows quick production under controlled environmental conditions"

Reviewer 2

typographical, and formatting errors **as:**Syria

I addedreference for:

Green algae (Chlorophyta) are the primary producers in the aquatic ecosystems, with an estimated count from 6,000 to 8,000 species. Most of which are macroalgae, and others are microalgae such as *Cladaphora*., *Cladophora*(Ulvophyceae, Cladophorales) [1]. İt is multinucleate filamentous algae. its cells contain a parietal perforate or reticulatechloroplast. The presence of pigments such as chlorophyll (a and b) , xanthophylls, and β-carotene give it its bright green color.*Cladophora*is predominantly benthic[2], and it is usually found in the region of unidirectional flow or periodic wave action in freshwater and marine habitats.*Cladophora*is a mid to late successional species, [1,2].

I remove:

a.maybe it is the most ubiquitous freshwater macroalgae worldwide . Barada River is one of the most important aqueous habitat for algae in Syria.

b.In this study, UAE was used to extract SPs from Cladophora crispata that had been isolated from the Barada River and grown outdoors. We have further investigated the chemical composition, and in vitro antibacterial activity of crude and purified SPs.

I added

and show important biochemical properties which require thorough investigations to understand their structure and biochemical functions

I replace there is increasing concern in studying the structure and biochemical functions of polysaccharides' –by several studies have reported antimicrobial properties of sulfated polysaccharides

I added

The objectives for this study were a) to isolate and cultivate Cladophora crispata to understand its growth dynamics b)to study the chemical and functional properties of the crude and purified SPs produced by this species

GPS of sampling site was provided , the distance between river and lab is 10 km, so condition during storageisn't needed.

*Cladophora*cells was Collected by grabber

I added table 1.treatments of outdoors cultivation for biomass production, and we used we used DR 2800- lange to estimate elements (N,P)in the water

The pure ethanol (99.9%).

Sephadex gel G-100 gel(Sigma, Japan); the column distance 2.6 × 20 cm

I added more details of Xu's assay

I did not study the effect sulfated polysaccharides on Ecoli and Bacillus subtilis because it was studied previously

McFarland work at OD= 600 nm

3.In the Materials and Methods Section:

Reviewer 1

I corrected thetypographical, and formatting errors **as :**

Kawach" => "Kawachi"

Song" => "Song et al."

PBS saline" => "PBS"

"Cefteriaxons" => "Ceftriaxons"

2.9. Statistical analysis=>2.8

I added the abbreviations were defined when they are first appearance as:

KBr powder => Potassium bromide (KBr) powder

"RID"=> Refractive Index Detector (RID)

I corrected the fermentation medium toalgae culture broth, and added its composition

I provided the method about biomass determination

Reviewer 2

the Barada River, Rabwehnorthwest of Damascus city, Syria

Morphological diagnosis was performed according to Prescott by examination under the light microscope (Olympus-cx41).

I made a photopanel.

4. in the Results andDiscussionSection:

Reviewer 1

I correct the typographical, and formatting errorsas:

Carbohydrates => Carbohydrate in Table 1

"Inhabit zone" => "Inhibition zone in Table 2

I correct the formatting errors of the reference as:

The reference [34] is Athbi et al. (2012) instead of Zouaghi et al(2021)

Boulet et al (2007)and Kidgell (2021) which refer to the peak for pyranose.

Kidgellet al (2019) refer to the All SPs extracts were contaminated with protein

Epand et al (2016) refer to the additional protection afforded by the outer membrane such as lipopolysaccharide,and phospholipids

I added more information about the specific growth rate, Generation time

i delete the reference Ramadan et al

I added the carbohydrate content was associated with protein content,($r=0.73;R^2=0.54$)

I added an explanation for peak 1 (2.800) and peak 2 (3.517); the peak 1 (2.800) and peak 2 (3.517) are mobile phase.

Reviewer 2

well-characterized(high carbohydrate and low protein contents),

The crude SPs extract gave two fractions,this result agrees with otherstudies [11, 24].

the carbohydrate content was associated with protein content,($r=0.73;R^2=0.54$)

The purified ulvan from the green alga *Ulvareticulata*also had an antibacterial activity with inhibition zone diameter about 20 mm [44],

Ulvan is the sulfated polysaccharides from green algae , my article is the first study on *Cladophoracrispata*

I change the MIC graph to table.

I provide picture for effect Sulfated polysaccharides on the bacteria

Thanks for you.

VERSION 2

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000537.v2.5

Girish Beedessee; University of Cambridge, UNITED KINGDOM

Date report received: 22 February 2023
Recommendation: Major Revision

Comments: The reviewers have highlighted major concerns with the work presented. Please ensure that you address their comments. The language used is poor, which can cause ambiguity at times. Please carefully rewrite it. We offer a discounted translation service, Editage (https://www.editage.com/; see https://www.microbiologyresearch.org/prepare-an-article#13 for more information). Please provide more detail in the Methods section and ensure that software is consistently cited and its version and parameters included. The reviewers raise concerns regarding the scientific rigour and experimental design of the work.

Reviewer 2 recommendation and comments

https://doi.org/10.1099/acmi.0.000537.v2.4

Anwesha Ghosh; Indian Institute of Science Education and Research Kolkata, INDIA
https://orcid.org/0000-0003-1765-9832

Date report received: 18 February 2023
Recommendation: Major Revision

Comments: Dear authors, I have read through your manuscript very carefully. It is a good attempt but you need to include a lot of information in the manuscript. I suggest you to download Grammarly and run it on your word file. You will be able to fix the grammatical errors. 1. MIC graph is not needed 2. ZoI plate and MIC test tube images are mandatory 3. Sampling site map and sampling details need to be shared. See my comments in the file 4. Make a photoplate with the microscopic images that you have 5. Additional line by line comments found below: (Numbers refer to line numbers in manuscript) 21 & 24 – Syria S capital? 30 – Rethink keywords. These do not reflect the work. 36 – is this river in Syria? Please mention country here 37 – discontinuous sentence. Remove the algae have ....bioactive compounds. Start at, Cladaphora... I strongly suggest to download Grammarly and run it on your word file. It will help remove the formating errors 40 – which were further studied to understand their role as antibacterial agents 41 – line not needed. Sentence must start with capital letter 44 – F2 is the purified fraction. Please write purified fraction instead of F2. 45 – sentence is incomplete. Fix. 63 – reference? 64 – such as Cladaphora 69 – Last sentence, remove 71-72 – remove 73 – Keep 'Polysaccharides are the most important products of algae [7]' and add - and show important biochemical properties which require thorough investigations to understand their structure and biochemical functions 74 – 'there is increasing concern in studying the structure and biochemical functions of polysaccharides' - several studies have reported antimicrobial properties of sulfated polysaccharides 79 – for e.g. polysaccharides from green algae.... 85 – 'In this study'…. The objectives for this study were a) to isolate and cultivate Cladophora crispata to understand its growth dynamics b)to study the chemical and functional properties of the crude and purified SPs produced by this species 91 – which month? Saying 2021 spring is not enough 92 – GPS coordinates of sampling sites are needed. Please provide study site map as Fig. 1 or S1. white transparent bottles? What was the light condition during storage? 94 – these are microalgae. How do you wash them? If you collected benthic samples, you need to clarify samplng strategies. Did you use a corer? Grab? Provide detailed information 102 – give values and what did you use to add nitrogen and phosphate? Which salts? And what method was used to estimate these elements in the water? 105 – write in brief and put the reference in bracket. Otherwise its difficult for readers if you just give the reference 107 – what modifications? 111 – molecular grade absolute ethanol you mean? 119 – sulfated – capital letter 120 – polysaccharides – no capital letter 123 – company information missing. There is no hyphen. it should be 1L 133 – somewhere you have written full names and other places IUPAC names. Please stick to one format 147 – Xu's assay - brief details needed 157 – why did you use such dangerous pathogenic species? Why not Ecoli and Bacillus subtilis? 164 – Did you measure the OD? 172 – mention conc here. range is not enough 178 – write minimum inhibitory concentration (MIC) 188 – Which stat? You need to write. 194 – use micron sign. 195 – with a diameter of ~50-70 198-203 – you need to break these up into simple sentences 200 – this data is crucial You need to share it 205 – carbohydrate content - how much? High nitrogen - how much? 209 – which microscope was used?You need to make a photopanel. Something like these https://www.google.com/search?q=algae+image+photoplate&tbm=isch&ved=2ahUKEwi1g67voJ79AhWCg-YKHQyLBgQQ2-cCegQIABAA&oq=algae+image+photoplate&gs_lcp=CgNpbWcQAzoECCMQJzoICAAQgAQQsQM6CwgAEIAEELEDEIMBOgUIABCABDoECAAQHjoJCAAQgAQQChAYOgcIABCABBAYOgYIABAFEB46BggAEAgQHlDYBFinUGDBUmgJcAB4A4AB5gOIAYU6kgEKMS4xNC43LjQuNJgBAKABAaoBC2d3cy13aXotaW1nwAEB&sclient=img&ei=KFfwY7XPFYKHmgeMlpog&bih=685&biw=1517#imgrc=U4D35ZZXbLFfgM 216 – well-characterized … meaning? 219 – agrees with … what does this mean? 227 – little differences 236 – this image is resized. Please dont change the image size. 244 – how? positive correlation? 264 – agrees with … meaning? 282 – how did you measure this? 283 - 285 – what is the relevance of this information? 293 – you need to share the ZoI and MIC test images. Labeled petriplates and test tubes with date of test. 312 – i dont know what this graph means. There is no need to plot MIC on graphs

Please rate the manuscript for methodological rigour
Very poor

Please rate the quality of the presentation and structure of the manuscript
Very poor

To what extent are the conclusions supported by the data?
Partially support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

Reviewer 1 recommendation and comments

https://doi.org/10.1099/acmi.0.000537.v2.3

Eiichi Shoguchi; OIST: Okinawa Kagaku Gijustu Daigakuin Daigaku, JAPAN
https://orcid.org/0000-0003-3136-5558

Date report received: 09 February 2023
Recommendation: Minor Amendment

Comments: The manuscript has been revised well, including the corrections of grammatical errors. However, additional errors were found in revised sentences. My concerns are shown below. Specific comments: There are a lot of typos. Examples are below. Line 45: "crud" => "crude" Line 64: "amultinucleate" => "multinucleate" Line 97: "Kawach" => "Kawachi" Line 106: "Song" => "Song et al." Lines 123-125: "PBS saline" => "PBS" Line 173: "Cefteriaxons" => "Ceftriaxons" Line 186: "2.9." => "2.8." Table 2: "Inhabit zone" => "Inhibition zone". "samalphabetical" is a typo? Regarding format errors, incorrect use of capitalization is found in many places. Some examples are shown below. Line 38: "sulfated Polysaccharides" Line 45: "infrared Spectroscopy" Line 47: "High performance" Additionally, it would be nice to define what authors are omitting. Line 76: It would be better to spell out "DPPH" and "ABTS". Lines 132-133: KBr powder => Potassium bromide (KBr) powder Line 148: "RID" might be spelled out. Lines 48-51: In the abstract, MIC values do not correspond to results shown in Fig. 5. The data will need to be checked again. It would be good to indicate whether each bacterium is gram-positive or gram-negative. It makes the third sentence in the highlights section easier to understand. Lines 72-74: Yeasts also belong to fungi. It would be better to explain why polysaccharides are the most important products. "There are increasing interests" might be better than "there is increasing concern". Lines 97-99: It would be good to explain the composition of fermentation medium. Lines 102-103: How was the stationary phase of the biomass determined? I would like to see a brief explanation of that. Lines 193-195 and 459-462: "Our result agrees with [34]" is a vague explanation. Is it the result of microscopic observation? The reference [34] is Athbi et al. (2012). The format of the reference must be correct. Athbi AM, Ali DS, Abaas AN. The quantity determination of total carbohydrates and monosaccharides from some green algae (Chlorophyta). Marsch Bull. 2012. Available: https://www.iasj.net/iasj/download/b9815f379b8f731b Lines 198-207: It is difficult to discuss about biomass production because the result for biomass composition of Cladophora crispata is not shown. It would be good to show the data here. Lines 212-214: Why is Ramadan et al. [39] referred to here? Did you use the method for orange fruit peel? Authors should recheck the relationship between the text and the reference list. Lines 234-235: Kidgell et al. [40] is not described about the detail of pyranose. Did you refer to the peak for pyranose? It is better to explain using the values used as a reference. Lines 243-246: How did you note the association between carbohydrate and protein? I think that table 1 does not show it. It would be good to show a brief explanation here. Lines 247-250: Boulet et al. [41] does not explain high sulfate groups and SPs chains. Suitable references should be given. Lines 262-264: Zouaghi et al. [33] does not show monosaccharides, galactose, xylose, and ribose. The reference should be corrected, or it would be better to explain why it agrees. Lines 266-270: Kidgell et al. [40] that is the review paper for ulvan might be referred to here. Lines 283-285: Epand et al. [43] does not include the description of the ulvan. Should be confirmed references. Figure 4: It would be nice to add an explanation for peak 1 (2.800) and peak 2 (3.517). Table 1: "Total" for "Carbohydrates" is needed? Total Carbohydrates => Carbohydrate I do not understand the meaning of the same alphabetical letters. It would be good to discuss the results in the text.

Please rate the manuscript for methodological rigour
Satisfactory

Please rate the quality of the presentation and structure of the manuscript
Satisfactory

To what extent are the conclusions supported by the data?
Partially support

Do you have any concerns of possible image manipulation, plagiarism or any other unethical practices?
No

Is there a potential financial or other conflict of interest between yourself and the author(s)?
No

If this manuscript involves human and/or animal work, have the subjects been treated in an ethical manner and the authors complied with the appropriate guidelines?
Yes

SciScore report

https://doi.org/10.1099/acmi.0.000537.v2.1

iThenticate report

https://doi.org/10.1099/acmi.0.000537.v2.2

Author response to reviewers to Version 1

Letter for reviewers

Dear Professor

I did all the required modifications from reviewers, briefly:

1. I have corrected the grammatical errors(in red color) were corrected.

2.There are many sentences highlighted in yellow refers to typographical, and formatting errors were corrected.

3.Abbreviations were defined when they are first appearance.

1.In the Abstract Section:

√I Re –wrote the Abstract in the following style: Introduction; I added more sentence on the utilization/application of SPsin general.

Objective (why you did it), Methodology (how you did it), insert the highlighted important results, and added the conclusion at the end of the abstract inline numbers from 36-41:

Algae have been emerged as a new source for bioactive compounds, in this research *Cladophoracrispata*was cultivated in the outdoor method, and algae sulfated Polysaccharides (SPs) were extracted by ultrasonic-assisted extraction methods. After extraction, Gel filtration was used to purify the crude SPs; SPs compounds were determined and selected and we investigated the effect of SPs as antibacterial agents.

√İ provide a legend in the manuscript **as Highlights**line numbers from 53-57:

Highlights

- Sulfated polysaccharides (SPs) were extracted from *Cladophoracrispata*using ultrasonic bath.
- SPs consist of sugar, uronic acids, protein unite, and sulfate groups.
- Gram-positivebacteria are more sensitive to SPs.

2. In the Introduction Section:

I have corrected the grammatical (in red color), typographical, and formatting errors (sentences highlighted in yellow).

Replaces thesentences, and both errors (red color andsentences highlighted in yellow). inline numbers from 62-69:

Green algae (Chlorophyta) are the primary producers in the aquaticecosystems, with an estimatedcount from 6,000 to 8,000 species. Most of whichare macroalgae, and others are microalgae, *Cladophora*(Ulvophyceae, Cladophorales) is amultinucleate

filamentous algae. It's cells contain a parietal perforate or reticulatechloroplast. The presence of pigments such as chlorophyll (a and b), xanthophylls,and β-carotene give it its bright green color.*Cladophora*is predominantly benthic, and is usually found in the region of unidirectional flow or periodic wave action in freshwater and marine habitats.*Cladophora*is a mid to late successional species, maybe it is the most ubiquitous freshwater macroalgae worldwide [1,2]

We add new sentences in line numbers no 70.

Barada River is one of the most important aqueous habitat for algae in Syria.

I have corrected the grammatical errors in line numbers no 71.

Algae can be cultivatedin open and closed ponds.

I have corrected the grammatical, typographical, and formatting errors

there is increasing concern in line numbers no73.

as well as their scavenging activity against in line numbers no76.

However, polysaccharides characteristics in line numbers no 79.

Polysaccharides from green algae are heteropolysaccharides which are composed of different monosaccharides in line numbers from 80-81.

can be extracted by several green in line numbers no 82.

UAE reduce energy consumption and solvent use. In addition, the low temperatures and short times used in UAE processes can keep the functionalities of the bioactive compounds in line numbers from 83-85.

We add new sentences in line numbers from 85-88.

In this study, UAE was used to extract SPs from *Cladophoracrispata*that had been isolated from the Barada River and grown outdoors. We have further investigated the chemical composition, and in vitro antibacterial activity of crude and purified SPs.

3.In the Materials and Methods Section:

I have corrected the grammatical(in red color), typographical, and formatting errors(sentences highlighted in yellow), and both errors (red color andsentences highlighted in yellow).

Isolation and biomass production of algae in Line numbers no 90.

Algae samples were in line numbers no 91.

Samples were kept in 5-L polyethylene bottles at 4 °C until they were shipped to the in Line numbers no 92.

where they were in line numbers no 94.

was performed in line numbers from 95.

The identified isolates were purified by repeated cultivation on algae culture broth (Fluka medium, India) as indicated by Andersen and Kawach [20]. The single-batch system was used for this purpose, where 10 g of the algae were inoculated into 100 ml of the fermentation medium in 250-mL conical flask, and incubated for 14 days at 23 °C and pH 8. Cultures were aerated by aerating pumps, and illuminated by fluorescent tubes with a light intensity of 1800 Lux, and a light/dark cycle of 16/8 h. Algae were transferred for outdoors cultivation for biomass production during summer using open ponds with controlling the nitrogen-to-phosphorus ratio [21]. The biomass was harvested at the stationary phase in line numbers from 95-103.

Polysaccharides were extracted using UAE technique as described by Esmaeili et al [22], followed by the sequential extraction according to the method of Song [11] with some modifications. Dry powder of the algae was mixed with distilled water with the ratio of30:1)ml: g), and the mixture was put in an ultrasonic bath (Ultrasonic cleaner model:ps-60ar, 360 W,40 kHz frequencies, jeken, China) for 120 min at 60 °C. Algae biomass was separated from the extract by centrifugation at 5000 rpm for 10 min. Polysaccharides in the supernatants was precipitated by the addition of pure ethanol which volume is three times that of extract in line numbers from 105-111.

Lipids were removed by the addition of three volumes of absolute chloroform and absolute acetone mixture (3:1). The residual proteins were removed by the addition of Sevage reagent (1:4 v/v mixtures of n-butanol and chloroform) and centrifugation at 4000 rpm for 15 min, and the precipitate was discarded in line numbers from 113-116.

where 0.5 g of the crude SPs was dissolved in line numbers no 121-122.

The eluted fractions were collected with the volume of 700 μL in line numbers from 125-126.

The SPs were then crystallized by incubation in line numbers no 128.

FT-IR was used to recognize the functional groups in purified SPs. Each fraction (2 mg) from polysaccharide was dried in an oven at 40 °C for 12 h, and then mixed with KBr powder in line numbers from 132-133.

Protein content was determined according to the method of Lowry in line numbers no 139.

30 mg from each sample (crude SPs, F1, and F2) was in line numbers no 142.

and the mixture then was diluted by the addition of 8.4 mL of distilled water and incubated at 121 °C for 1 h in line numbers from 143-144.

The monosaccharides were in line numbers no 146.

with some modifications; the temperatures of the column and RID were set at 30°C, The mobile phase was composed of acetonitrile and deionized water (85:15, v / v), and the flow rate was 1.0 mL/min, and the injection volume was 20 µL. A standard solution (Sigma-Aldrich) was injected into the chromatographic equipment at the concentration range of 0.5-50 mg in 1 mL of deionized water. All samples were diluted with deionized water and filtered through 0.45µm Nylon filters prior to HPLC-analysis. The experiments were repeated three times. Column was washed with the mobile phase at the end of each experiment period for more than 20 min in line numbers from 147-154.

against both gram-positive in line numbers no 157.

where they were identified it's in line numbers no 159.

The strains were grown in line numbers no 160.

with shaking in line numbers no 162.

The turbidity of bacterial suspension in line numbers no 164.

suspension was then inoculated in line numbers no 167.

50 µl of the standardized inoculum of the bacterial suspension (10^5cfu/ml) in line numbers from 169-170.

agar plugs were removed in line numbers no 171.

A known concentration of each fraction from SPs (20-50 mg/ mL) was inoculated into the wells in line numbers from 172-173.

bacterial growth was in Line numbers no 179.

concentration of SPs solutions range from 0.5 to 50 mg/mL (1 µl of each microbial strain was added to each wells in Petri dish) in line numbers from 181-182.

4. in the Results andDiscussionSection:

I have corrected the grammatical(in red color), typographical, and formatting errors(sentences highlighted in yellow), and both errors (red color andsentences highlighted in yellow).

Abbreviations were defined when they are first appearance(in blood).

Algae structure in line numbers no 196.

Morphology of *Cladophoracrispata*under microscope at 40X. in line numbers no 209.

(Figure 2) in line numbers no 218.

Figure 2.Sephadex gel G-100 chromatogram elution profile of crude

SPs of *Cladophoracrispata*.in line numbers from 221-222.

We noted thepeak inline numbers no 227.

present uronic inline numbers no 231.

1250.61 and 1252.53 cm^{-1}inline numbers from 233.

1077.04 and 1074.15 cm^{-1}inline numbers from 234.

Figure 3: FT-IR spectra of fraction1 and fraction2 of purified SPs extracts from *Cladophoracrispata*. in Line numbers no 239.

A protein is contamination in line numbers no 246.

was closely associated in line numbers no 247.

The content's chemistry of crude and purified fractions of SPs (Based on moisture content 10%) in line numbers from 254-255.

figure4; thisresultin line numbers no 263.

ulvan isheteropolysaccharide inline numbers no 264.

nuclear magnetic inline numbers no 268.

gram-positive in line numbers no 277.

gram-negative in line numbers no 278.

increase with in line numbers no 280.

*S. aureus*and *B. anthracis*bacteria in line numbers no 285.

*E.aerogenes*and *P. aeroginosa*;in line numbers no 286.

MIC (6 mg/L) in line numbers no 290.

(32 mg/ml) when in line numbers no 291.

Figure 5.The minimum inhibitory concentration (MIC) for sulfated polysaccharides against *S. Aureus,B.anthracis*and *E. Aerogenes*,*P.aeroginosa*

in line numbers from 311-312.

which caused in line numbers no 328.

which can in line numbers no 333.

Thanks for you.

VERSION 1

Editor recommendation and comments

https://doi.org/10.1099/acmi.0.000537.v1.3

Girish Beedessee; University of Cambridge, UNITED KINGDOM

Date report received: 14 December 2022
Recommendation: Major Revision

Comments: The language used is poor, which can cause ambiguity at times. Please carefully rewrite it. We offer a discounted translation service, Editage (https://www.editage.com/; see https://www.microbiologyresearch.org/prepare-an-article#13 for more information).

SciScore report

https://doi.org/10.1099/acmi.0.000537.v1.1

iThenticate report

https://doi.org/10.1099/acmi.0.000537.v1.2