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<u>Inclusion and Exclusion Criteria</u>
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<u>Attrition</u>
not required.
<u>Sex as a biological variable</u>
not required.
<u>Subject Demographics</u>
Age: not required.
Weight: not required.
<u>Randomization</u>
not detected.
<u>Blinding</u>
not detected.
<u>Power Analysis</u>
not detected.
<u>Replication</u>
Experiment was repeated in triplicate.
Number: Experiment was repeated in triplicate.

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Antibodies	Yes (indicate where provided: page no/section/legend)	n/a
For commercial reagents, provide supplier name, catalogue number and RRID, if available	No antibodies detected. Please add identifiers for all resources where possible	
Cell Materials	Yes (indicate where provided: page no/section/legend)	n/a
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By mohanad jasem

Characterization, and in vitro Antibacterial activity of sulfated polysaccharides from freshwater algae *Cladophora crispata*

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Keywords: *Cladophora crispata*, sulfated polysaccharides, function groups, Antibacterial activity.

35 **Abstract**

36 Barada River is characterized by an abundant growth of freshwater algae. In light of the increased
37 interest in algae to find new sources of bioactive compounds, this research, aimed to extract
38 sulfated Polysaccharides (SPs) from freshwater green algae *Cladophora crispata* using
39 ultrasonic-assisted extraction methods after cultivates it by the outdoor method. Also we
40 determined and selected its compounds, and we investigation effect SPs as antibacterial agent
41 After extraction, Sephadex gel G-100 was used for the Purification of SPs, where the purification
42 gave two fractions, namely F₁ and F₂. The chemical characterization of crude SPs and its fractions
43 were investigated. The highest carbohydrate and protein contents were found in crude SPs with
44 the ratios of 74.12%, and 4.02%, respectively, while the highest sulfate content 12.17% was
45 found in fraction 2, and the highest uronic content 18.46% was found in fraction 1. Fourier
46 transforms infrared Spectroscopy (FT-IR) used for confirmed the crude and fractions consist of
47 sugar, uronic acids, protein units, and sulfate groups. Both F₁, and F₂ consist of rhamnose,
48 galactose, xylose, and ribose based on separation on the High performance liquid
49 chromatography (HPLC). Each fraction has inhibitory effect on bacteria negative and positive
50 gram. F₂ have the lowest minimization inhibitory concentration (MIC) value against *Enterobacter*
51 *aerogenes* and *Staphylococcus aureus*; where MIC were 28 and 10 mg/ml respectively.
52 Polysaccharides had the most important interest because they have antibacterial properties,
53 making them interesting in pharmacy and food applications.

54 **Data summary**

55 no data was generated during this research.

56

57

58

59 1.Introduction

60 Green algae (Chlorophyta) are the primary producers in aquatic ecosystems, their count
61 from 6,000 to 8,000 species. A lot of them are macroalgae, others are microalgae,
62 *Cladophora* (Ulvophyceae, Cladophorales) usually found in freshwater and marine habitats,
63 maybe it is the most ubiquitous freshwater macroalga worldwide[1].

64 *Cladophora* is multinucleate filamentous algae with a parietal perforate or reticulate
65 chloroplast, ¹⁵predominantly benthic, is often found in the region of unidirectional flow or periodic
66 wave action, and is mid to late succession species. ²⁹The presence of pigment chlorophyll a- b,
67 xanthophylls and β -carotene in *Cladophora sp* gave it bright green color[2].

68 Algae can cultivate in open and closed ponds, photobioreactors for biomass
69 production[3]. Polysaccharide were extracted from fungi, bacterial and yeasts[4-6], and from
70 algae; Polysaccharides are the most important products of algae[7], many papers studied the
71 structure and biochemical functions of polysaccharides, it has antibacterial, antifungal, and
72 antiviral properties[9-10], and they effectively scavenge on superoxide radicals, hydroxyl,
73 DPPH[•], and ABTS^{•+}[11-12], and have commercial applications in food industries[13].

³⁷The sulfated Polysaccharides(SPs) from the green algae *Cladophora oligoclada* have
74 anticoagulant properties[14], properties of these polysaccharides differ from species to species
75 and within the same genus[15]. Monosaccharide units in green algae species are different; it is
76 heteropolysaccharides[9,16].

78 Algae polysaccharides extract by several extraction techniques, considered green
79 technology ⁹as microwave-assisted extraction, enzyme-assisted extract, and ⁹ultrasound-assisted
80 extraction (UAE). UAE can be extracted bioactive components at low temperature in very little

9
81 time, with lesser energy, and low solvent need, as a non-thermal extraction technology, better
82 equipped to keep the functionality of the bioactive compounds[11,17-18].

30 83 **2.Materials and Methods**

84 **2.1. Isolation and biomass production**

85 *Cladophora* sp was collected from the Barada River (Damascus – Rabweh, Spring 20, 2021). The
86 algae were put into 5-L polyethylene bottles at 4 °C, then brought them to the laboratory of the
87 Plant Biology Department, washed with distilled water several times. Morphological diagnosis
88 carries out according to Prescott [19], Then the isolates were purified by cultured in fluka (India)
89 medium as advice Andersen and Kawach [20]; 10 g of the algae were grown by the single batch
90 culture system and added to a conical flask containing 100 ml of medium, and incubated for 14
91 days, under conditions: aeration by aerating pumps, light sources" fluorescent tubes" with 16/8 h:
92 light/dark period, and intensity of illumination 1800 Lux, pH 8 and temperature 23 °C. The
93 biomass harvest in the stationary phase, then the algae cultivating by the outdoor method using
94 open ponds during summer for biomass production, control with the ratio of nitrogen to
95 phosphorous[21].

96 **2.2. Polysaccharide Extraction**

97 Sonication treatment: Polysaccharides were extracted by an ultrasonic bath as described
98 by Esmaeili et al[22]; followed by the sequential extracted assay as Song method with some
99 modified[11], Briefly; we mixed distill water and dried macroalgae powder, the mixtures were
100 put in the ultrasonic bath (Ultrasonic cleaner model:ps-60ar, 360 W,40 kHz frequencies, jeken,
101 China). The mixing ratio of distill water to dried algae powder (ml: g; 30:1 ml/ g), time of
102 extraction (120 min), and temperature of extraction (60 °C) 28, centrifuged for 10 min at 5000 rpm,
103 collected the supernatants, and the pure ethanol was added for precipitated polysaccharides

(incubated at 4°C for 48 h), Pellets re-solve using water, three times a mixture of absolute chloroform and absolute acetone (3:1, v/v) use for removed lipids content. Residual proteins were removed using Sevage reagent (1:4 v/v mixture of n-butanol and chloroform), the protein precipitate and separated by centrifugation (4000 rpm for 15 min) and discarded, the supernatants were collected and dialyzed by dialysis bags for 48 h. Polysaccharides precipitated by addition of pure ethanol three times at 4°C for 48 h, then collected using the cold centrifuge at 5000 rpm for 30 min at -20°C.

2.3. Purification of Crude sulfated Polysaccharides

Purification of crude Polysaccharides from *Cladophora crispata* was carried out according to Pier method[23] with some modifications; The crude SPs 0.5 g dissolved in 100 mL mobile phase; phosphate-buffered solution (PBS) consisting of: 6.8g Sodium chloride+ 0.43 g Potassium dihydrogen phosphate + 1.48 g Disodium hydrogen phosphate in 1-liter distill water (pH = 7.2). 3 ml of SPs and PBS solution was loaded into a chromatography column with Sephadex gel G-100 gel chromatography (2.6 × 20 cm), and eluted with PBS solution (flow rate: 1 ml/min). Each fraction about 700 µL of the eluted was collected, polysaccharide fractions were precipitated by the addition of pure ethanol three times at 4°C for 48 h, and object to the rotary evaporator at 60 °C, 100 rpm. Then shaking incubator (JSR model jssi-100) at 50 °C, 150 rpm for 12 h was used for crystallization the SPs, SPs was dried until water content 10 %.

2.4. The Fourier transforms infrared spectra (FT-IR)

Each fraction from polysaccharide (2 mg) was dried by oven for 12 h, at 40 °C. Then mixed with KBr powder. The FT-IR-4200 type A-C077661018 instrument was use within the range of frequency from 400 to 4000 cm⁻¹ to examine function groups in purified SPs[11].

2.5. Determination of sulfated Polysaccharides components

127 was using ⁶ the phenol-sulfuric acid method was used to determine The carbohydrate
128 content of each fraction of SPs[24], the sulfate content in SPs calculates by the barium chloride
129 gelatin assay[25], while Lowry's method was used to determine the protein contents[26].

130 2.6. Monosaccharide analysis

131 Carbohydrate analysis requires prior hydrolysis by sulfuric acid, briefly;
132 30 mg from crude or F₁ and F₂ hydrolyzed with 300 μ L sulfuric acid 72% for 30 min at
133 30 °C, dilution by ⁶ 8.4 mL of distilled water and incubation at 121 °C for 1 h. The hydrolyze SPs
134 neutralized by CaCO₃ as described by Cui et al[27].

135 The monosaccharide selected ³¹ by the High performance liquid chromatography (HPLC)
136 according to Xu method[28], with some modify; ² the samples were analyzed on a NH₂ column
137 ² (250 $\times$ 4.40 mm). The column and RID temperatures were set at 30°C, The mobile phase was
138 composed of acetonitrile and deionized water (85:15, v / v), and the flow rate was 1.0 mL/min.
139 The injection volume was 20 μ L. Standard solution (from sigma) was injected to the
140 chromatographic equipment at concentration 0.5-50 mg in 1 mL deionized water. All samples
141 were diluted with deionized water and filtered through 0.45 μ m Nylon filters prior to HPLC-
142 analysis. Experiment ² was repeated in triplicate. The column was washed at the end of each
143 experiment period for more than 20 min with the mobile phase.

144 2.7. Antibacterial activity of SPs

145 The investigation of antimicrobial activity of crude SPs and its Fractions were carried out
146 against *Staphylococcus aureus* and *Bacillus anthracis* (Gram-positive), *Enterobacter aerogenes*,
147 and *Pseudomonas aeruginosa* (Gram-negative), which were obtained from microbiology and
148 algae laboratory, Damascus university, whom identified it using Bergey's manual[29]; The strains
149 grow on nutrient agar for 24 h at 37 °C, then by the loop, we take part of each bacterial inoculum

150 to sterile tube contains 5 mL nutrient broth and incubated for 4 h, shaking at 100 rpm at 37
151 °C[30].

152 2.7.1. Preparation of each inoculum

153 The bacterial suspension turbidity was adjusted to 0.5 McFarland scale using sterile
154 physiological saline solution; equal to 1×10^8 colony-forming units (cfu) /mL using the turbidity
155 method by McFarland assay, following by dilution until 10^5 cfu/mL concentration, after that; the
156 bacteria suspension inoculation into the Mueller-Hinton agar plates[31].

157 2.7.2. Antibacterial activity assay

158 Antimicrobial activity determined for each fraction. The bacterial suspension containing
159 10^5 cfu/ml (50 µl of standardized inoculum) inoculated into the Mueller-Hinton agar surface.
160 Wells were made in the medium (Mueller Hinton Agar) using a drill bit No 3, and removed agar
161 plugs with a sterile needle [32], a known concentration of each fraction from sulfated
162 polysaccharides ranges from 20 to 50 mg/ mL. Ceftriaxons ¹³ as a positive control (at a
163 ¹⁸ concentration of 0.45%), while negative control was ¹⁸ distilled water, then the agar plates were
164 ²³ incubated at 37 °C for 24 h. we recorded bacterial sensitivity. Inhibition Measure as ²³ the diameter
165 of the zone sizes to the nearest millimeter using a ruler [31].

166 2.7.3. In vitro ¹ minimum inhibitory concentration determination (MIC)

167 The minimum inhibitory concentration (MIC) of different SPs concentrations on the
168 bacterial is determined by dilution agar method, the SPs ¹ in Mueller-Hinton-agar (MHA). Freshly
169 a bacterial suspension of 10^5 cfu/mL was used, the SPs were ⁶ dissolved in distilled water, and the
170 serial dilution of SPs in the MHA is prepared, concentration of SPs range of 0.5 to 50 mg/mL.
171 SPs in MHA ¹ are placed in Petri dishes and exposed to drying, as well as Petri dishes without SPs.
172 Finally, 1 µl of each microbial strain was added to each Petri dish and placed in ¹ an incubator at
173 37 °C for 24 hours. For each concentration, three tests were carried out[33].

174 2.9. Statistical analysis

175 All experiments were performed in triplicate, and experimental data was represented by
176 the average value $\pm$ SD of each sample. Statistical analyses were done by Spss version 20 using
177 one or three-way ANOVA at ($p \leq 0.01$).
178

179 3. Results and Discussion

180 3.1. Description of algae species and biomass production:

181 Freshwater green macroalgae *Cladophora crispata* are multicellular, filamentous, truly
182 branched, and cell patterns are cylindrical, the diameter about 50-70 micrometers, while the
183 lateral branches are small 20 -35 micrometers Figure 1, our result agrees with [34]. The structure
184 of algae and a large amount of biomass are related to habitat conditions. Depth of water, total
185 dissolved salts, orthophosphate, nitrate chloride, and Ch-a pigment content in water are key
186 parameters determining the composition of algae[35], in conditions such as water temperature at
187 26°C, weather temperature at 35 °C, illumination duration of 14.30 hours, the intensity of
188 illumination 790 lux. N: P ratio 3/1 (data not shown), so; our results indicate as the algae biomass
189 was exposed to a higher temperature to cause stress for algae, this led to an increase in the
190 percentage of carbohydrate content within their biomass; as carbohydrate synthesis requires less
191 energy, carbohydrates are synthesized before lipids in a rapid response to environmental
192 stress[36]. The synthesis of carbohydrates was as well affected by nitrogen concentration. The
193 highest carbohydrate content was in the culture with the highest nitrogen concentration at 27°C,
194 and these results were consistent with similar research [37], also; a culture that p-limited (low-p)
195 led to the accumulation of carbohydrate content, this agreement with [38].



Figure 1. *Cladophora crispata* under microscope40X.

3.2. Crude sulfated Polysaccharide from *Cladophora crispata*:

The sequential extraction method was used after Sonication, it can benefit to extract a maximum number of active compounds[39], The yield from SPs was 7.14%; the SPs content from 74.12 %carbohydrate and 9.08 % sulfate group.

3.3. Purification of Crude sulfated Polysaccharide from *Cladophora crispata*:

Purification of SPs from *Cladophora crispata* gave purified SPs with well-characterized, we purified the crude SPs extracted (60 °C,120 min, ratio 30:1 ml/g) by gel chromatography, 5 fractions were selected based on the total carbohydrate elution profile Figure 2. The crude of SPs extracted was composed of two fractions, which agrees with[11,23]; F₁ (fraction number 10-17) and F₂ (fraction number 22-27).

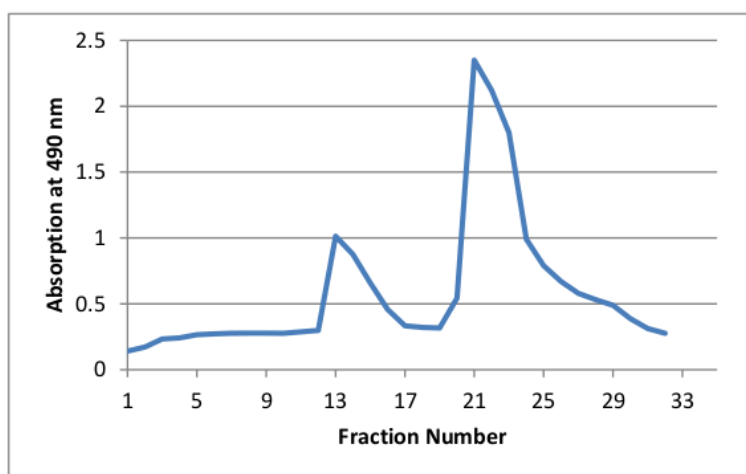


Figure 2. Sephadex gel G-100 chromatogram elution profile of crude sulfated polysaccharides of *Cladophora crispata*.

3.4. FT-IR Spectra:

Figure 3 expresses the infrared spectra of each of the two fractions obtained from the purification of crude polysaccharides, both F₁ and F₂ have the same spectra, With very slight differences in the wavenumbers. We noted The peak of the O–H stretching at 3397.21 and 3404.13 cm⁻¹, peaks of the C–H stretching vibration find at 2925.30 and 2926.44 cm⁻¹, this including CH, CH₂, and CH₃ stretching. The absorption peaks of amide I found at 1653.66 and 1663.30 cm⁻¹, and 1555.30 and 1556.27 cm⁻¹ indicate amide II. The C=O asymmetric stretching vibrations of the carboxylate groups (–COO–) observe at 1625.73 cm⁻¹, it indicates to present Uronic acids. The absorption peaks at around 1401.99 cm⁻¹ refers to C–H bending, and The peaks at 1250.61 and 1252.53 were due to the stretching vibration of S=O, in addition peaks at 1077.04 and 1074.15 were due to C–O. the C–O–S indicated that the majority of sulfate groups 847.56 cm⁻¹ and peaks at about 590–900 cm⁻¹ refer to pyranose[11,14,40].

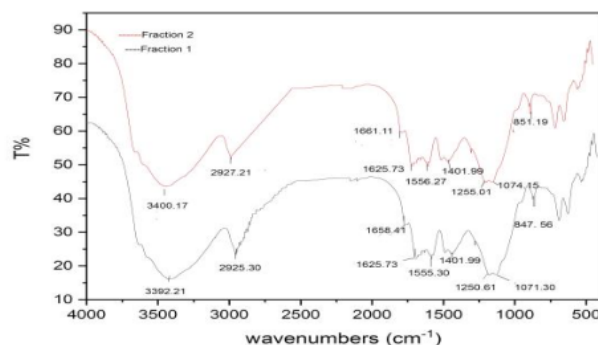


Figure 3: Fourier transform infrared (FT-IR) spectra of Fraction 1 and Fraction 2 of purified sulfated polysaccharides extracts from *Cladophora crispata*.

3.5. Chemical composition of purified SPs:

Properties of the two fractions (F₁ and F₂) shown in Table 1, we noted the carbohydrate content was associated with protein content, but not for the sulfate and uronic acids. Carbohydrate, protein, sulfate groups, uronic acids contents for crude and fractions are not the same. Proteins are contamination of cell wall SPs, because protein is part of the structure of cell walls, and were closely associated with polysaccharides. All SPs extracts were contaminated with protein because of the ester sulfate moieties, which can form strong anions and attract positively charged proteins [40], after purification, the high sulfate groups are tightly linked to the SPs chains [41].

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Table 1: Chemical compositions of crude and purified fractions of SPs

This obtained from the Sephadex gel G-100 Chromatography (Based on moisture content 10%)

Component	Total Carbohydrates %	Protein %	Sulfate content %	Uroinc acids %*
Crude SPs	74.12±0.003 A	4.02±0.0019 A	9.08±0.002 C	2.87±0.006 C
F₁	59.07±0.002 C	2.52±0.002 B	10.04±0.002 B	18.46±0.003 A
F₂	65.36±0.002 B	1.83±0.002 C	12.17±0.002 A	10.62±0.003 B

251

*%uronic acids=100- (carb¹²hydrate +sulfate+protein+ moisture content).

252

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

253

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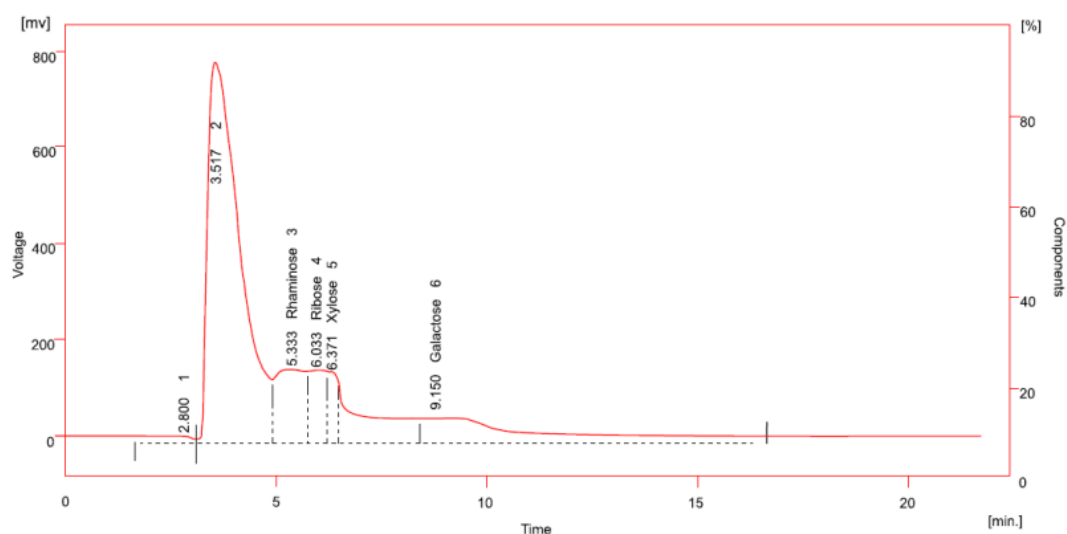
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259

260 3.6.Monosaccharide Composition:

261 The Monosaccharides in crude and fractions of SPs extracted from *Cladophora crispata*
 262 were: rhamnose, galactose, xylose, and ribose based on the HPLC analysis, Figure 4; This results
 263 agrees with[33]. The polysaccharides from green algae are ulvans, ulvan are
 264 heteropolysaccharides, and their compositions are rhamnose, xylose, sulfate group, and uronic
 265 acids such as iduronic acid or glucuronic acid. ²⁷ The composition of ulvan depends on the
 266 processing procedures of both biomass and ulvan, source species, and eco-physiology, ulvan has
 267 three types based on linked rhamnose to uronic acids; Nuclear magnetic resonance spectroscopy
 268 had used for this purpose, and other monosaccharides are reported (e.g., galactose, glucose,
 269 arabinose, and mannose), this structure is common in marine and freshwater algae[8, 42].



270

271 **Figure 4:** HPLC ⁵ analysis of the monosaccharide composition in crude
 272 and fractions of SPs extracted from *Cladophora crispata*.

273

274

3.7. Antibacterial activities:

Not only Gram-positive but also Gram-negative bacteria strains were inhibited by purified sulfated polysaccharides, and The antibacterial activity(Inhibition zone) of purified fractions of sulfated polysaccharides increased with the increase of sulfated polysaccharides concentration as shown in table 2. *Staphylococcus aureus* was the most sensitive to this sulfated polysaccharide; inhibit zone ranged from 7 to 27 mm, these results were similar to other reported[31]. The antibacterial activity of algae extracts against *Enterobacter sp* were classified as resistant or active. The purified ulvan from the green alga *Ulva reticulata* also had an antibacterial activity, the inhibition zone diameter was 20 mm[43], *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 the outer membrane such as lipopolysaccharide, and phospholipids[44]. According to other previous studies; ester sulfate of polysaccharides are related to their biological activity, such as antibacterial activities and preventing preformed biofilms[45]. *Staphylococcus aureus* had the lowest MIC 6 mg.L⁻¹ when we use F₂; while *Pseudomonas aeruginosa* had the highest MIC 32 mg.ml⁻¹ when we use F₁, figure 5. 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[31].

299
300
301

Table2: The antibacterial activity of the purified fractions of sulfated polysaccharides from *Cladophora crispata*.

Fractions	Concentration (mg/ml)	Inhabit zone(mm)			
		strains			
		<i>Staphcoccus aureus</i>	<i>Bacillus anthracis</i>	<i>Enterobacter aerogenes</i>	<i>Pseudomonas aeruginosa</i>
F₁	20	7.0±1 ^a F	6.6±1.1a G	0 ^b G	0b F
F₂	20	8.0±0.5 ^a F	7±1b G	0 ^c G	0c F
F₁	30	11.0±2 ^a E	9±2b F	7.3±0.5 ^c F	0d F
F₂	30	15.0±1 ^a D	11±1b E	9±1 ^c E	0d F
F₁	40	19.3±0.5 ^a C	17±1a D	11±1 ^c D	14±1c D
F₂	40	27.0±1 ^a A	25.3±0.5b B	14±1 ^d C	21±1c A
F₁	50	21.0±2a B	19±1b C	17±2 ^c B	16±1c C
F₂	50	27.0±1a A	27±1a A	19±1 ^b A	19.3±0.5b B
Positive control	0.05	22.0±1a B	18±1c CD	19±3 ^b A	11±1d E
Negative control	-	0a G	0a H	0a G	0a F

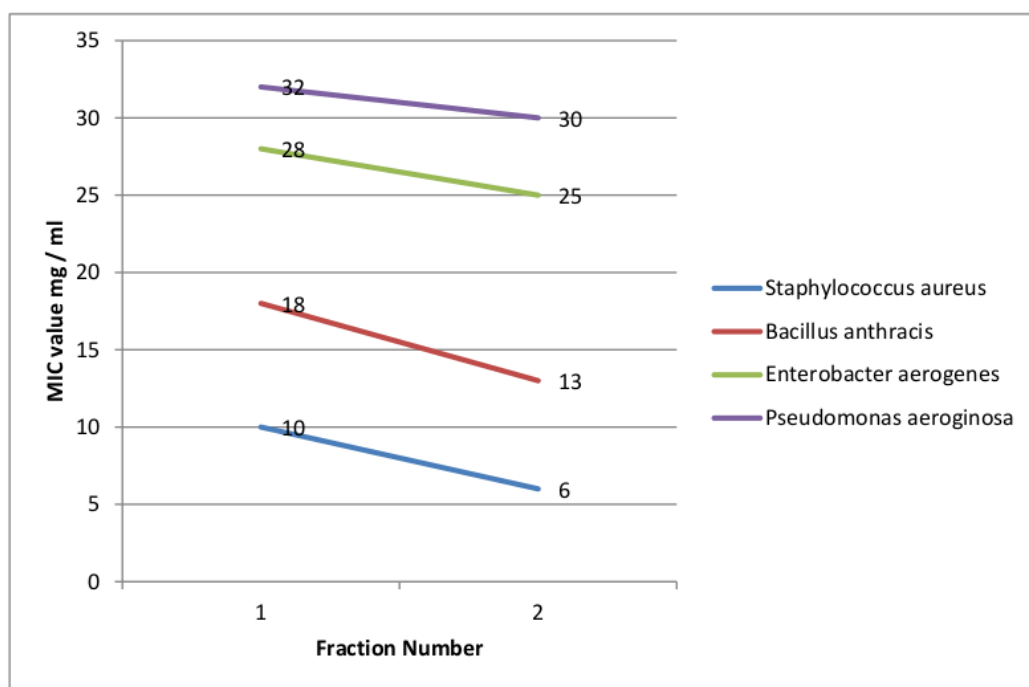
302

303 a,b,c,same alphabetical letters in the same row indicate no significant differences at 1%.
304 A,B,C,same alphabetical small letters in the same column indicate no significant differences at 1%.
305

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307

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309

310 **Figure 5.**The minimum inhibitory concentration (MIC) for sulfated polysaccharides against S.
 311 Aureus,B.anthraxis and E. Aerogenes,P.aeruginosa
 312

313 4.Conclusions

314 *Cladophora crispata* is simple to classify because it has regular branches, and its growth
 315 in the outdoor ponds systems is an important feature for this specie; because of the cheap
 316 equipment used for this purpose. ²⁰ *Cladophora* biomass is an unlimited, low-cost, and valuable
 317 resource for different applications. The ultrasonic waves consider a useful method for extracting
 318 sulfated polysaccharides from *Cladophora crispata* with conditions: ratio of 30:1 mL/g, 60
 319 °C,120 min.

320 Sulfated polysaccharides from *Cladophora crispata* are heteropolysaccharides, both F₁
 321 and F₂ from purified SPs have the same monosaccharide, and it consists of carbohydrate, protein,

sulfated group, and uronic acids based on FT-IR analysis. It could be interesting to better purify the SPs fractions by using ion-exchange resins to eliminate the presence of proteins. Purified Sulfated polysaccharides exhibit antibacterial activity because of their viscosity, water-solubility, hydroxyl and sulfate group, mechanisms of antibacterial action for SPs is due to glycoprotein-receptors present on the cell surface of polysaccharides which bind with compounds in the bacterial cell wall, cytoplasmic membrane, and DNA. These results in increased permeability of the cytoplasmic membrane, protein leakage, and binding of bacterial DNA; The act of sulfated polysaccharides as antibacterial suggested possible using it within the food industry, animal diets, and pharmaceutical industries.

We advise investigation of antioxidant properties for SPs for further research; because ester sulfate in sulfated polysaccharides can donate an electron which give it antioxidant properties.

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6. Disclosure and conflict of interest

The authors declare that they have no conflicts of interest.

7. Author contributions

Mohanad Khaled Jasem: Conceptualization, Data curation, Formal analysis, Investigation, Writing – review & editing, Writing – original draft; Dr. Meria. A.Al-W: Supervision, Conceptualization, Resources, Methodology, Writing – review & editing; Dr. Nizam. A.A: Project administration, Conceptualization, Resources, Methodology, Writing – review & editing.

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