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Anticoagulant and antibacterial activities of polysaccharides of red algae *Corallina* collected from Lebanese coast

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ABSTRACT

In this work, we studied another species of red algae, *Corallina*, growing on the Lebanese coast of Batroun. The analysis of trace elements showed that *Corallina* was rich in K, Ca, Mg, Na, Si, Sr, P and Fe. *Corallina* was composed of 70.81% of saturated fatty acids, 25.54% of monounsaturated fatty acids and 3.65% of polyunsaturated fatty acids with palmitic acid as the main component. Moreover, the total yield of sulfated galactans and carrageenan was 2.5% and 10%, respectively. Both extracts exhibited anticoagulant effect but sulfated galactans were less potent than carrageenan. Sulfated galactans possessed inhibitory activity as well as bactericidal activity against all Gram-positive strains tested (*Enterococcus faecalis* ATCC 29212 and *Staphylococcus epidermidis* CIP 444). But, carrageenan was only able to inhibit the growth of *S. epidermidis* with a minimal inhibitory concentration (MIC) of 0.325 mg/mL. Furthermore, infrared spectroscopy (IR) revealed that the isolated carrageenan was of Lambda-type. *Corallina* could therefore be considered as a potential source of bioactive molecules that may be useful for the development of new pharmaceutical agents.

INTRODUCTION

Among the marine organisms, seaweeds are very attractive sources due to their huge biodiversity, high nutritional values, as well as the broad spectrum of biological activities of their extracts (Costa *et al.*, 2010; Wijesekara *et al.*, 2011). However, the Lebanese marine biodiversity remains partially unexplored. Recently, we have studied, for the first time, different species of brown algae *Sargassum sp, Padina pavonica* and *Dictyopteris polypodioides* growing in abundance on the Lebanese coast (Karaki *et al.*, 2013; Krivoruchko *et al.*, 2010; Men'shova *et al.*, 2012; Sokolova *et al.*, 2011; Yassine *et al.*, 2012). It has been reported that fucoidans isolated from *P. pavonica* exhibited more pronounced antitumor activity than those isolated from *D. polypodioides* and *Sargassumsp* (Men'shova *et al.*, 2012). In addition, Fucoidan, Laminaran and Mannuronan (FLM) isolated from *D. polypodioides* exerted significant antioxidant and anticoagulant activities (Karaki *et al.*, 2013).

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We continued experimenting on red algae growing on the Lebanese coast. Sulfated galactans and carrageenans were both extracted from the red algae, *Pterocladia* (Sebaaly *et al.*, 2012). It has been found that sulfated galactans had a greater anticoagulant and antioxidant effects than carrageenans (Sebaaly *et al.*, 2012). We also attempted to study another species of red algae, Corallina distributed widely in Batroun-Lebanon coast.

Previous studies have reported the biological properties of the extracts from the red algae of the genus *Corallina* collected from different countries. Indeed, the extract of *Corallina rubens* had an inhibitory effect on fibrinolysis with anticoagulant and anti-thrombin activities (Güven *et al.*, 1974). *Corallina elongata* collected from Moroccan coast exhibited an anti-inflammatory activity by inhibiting phospholipase A2 and elastase with an inhibition percentage above 70 and 95% respectively (Oumaskour *et al.*, 2013). Methanolic extracts of *C. elongate* showed antimicrobial activity by inhibiting *Staphylococcus aureus* (Oumaskour *et al.*, 2013). The methanol extract of the *Corallina pilulifera* collected from Korea was found to possess algicidal activity by strongly inhibiting the growth of the red tide microalga,

Cochlodinium polykrikoides, one of the most harmful dinoflagellates in Korea (Jeong *et al.,* 2000; Oh *et al.,* 2010). By inhibiting matrix metalloproteinase in human dermal fibroblast (HDF) cell, the *Corallina pilulifera* methanol extract may be considered as a potential source of natural anti-photoaging (Ryu *et al.,* 2009). Moreover, sulfated polysaccharides fractions rich in galactose and xylose extracted from *Corallina officinalis* possessed considerable antioxidant properties as reported by (Yang *et al.,* 2011). Moreover, the response of the antioxidant defense system of *C. officinalis* to different dosages of UV-B irradiation was also proved (Li *et al.,* 2010).

In this work, we performed the extraction of trace elements, fatty acids, sulfated galactans and carrageenans from *Corallina* red algae growing on the Lebanese coast. In addition, the anticoagulant and antibacterial effects of both sulfated galactans and carrageenans isolated were investigated. In order to elucidate their structures, ^{1}H NMR, ^{13}C NMR and IR spectroscopy were carried out.

MATERIALS AND METHODS

Composition of the red algae *Corallina*

Isolation of Sulfated Galactans

All The chemicals were purchased from Sigma Aldrich-Lebanon. The marine red algae, *Corallina* were collected by hand picking in the period of April-July 2012 from *Batroun-Lebanon* coast (Table 1). The algae were cleaned, washed in distilled water then sun-dried. The extraction was carried out according to the method of (Sebaaly *et al.,* 2012).

Table. 1: Nature of water and other local factors while picking red algae from Batroun coast.

Location	Batroun
Longitude	35°39.413’
Latitude	34°15.090’
Temperature (°C)	23
Oxygen (mL/L)	7.20
PO4 (µg/L)	1.60
NH4 (µg/L)	2.55
NO3 (µg/L)	0.40
Salinity (%)	40

The dried tissue (25 g) was cut into small pieces, suspended in 250 mL of 0.1 M sodium acetate buffer (pH 6.0) containing 510 mg of papain (Sigma-Aldrich, Switzerland), 5 mM EDTA and 5 mM cysteine, and incubated at 60°C for 24h. The incubation mixture was then filtered and the supernatant saved. The residue was washed with 138 mL of distilled water, filtered again, and the two supernatants were combined. Sulfated polysaccharides in solution were precipitated with 16 mL of 10% cetylpyridinium chloride solution. After standing at room temperature for 24h, the mixture was centrifuged at 2560 x g, for 20 min, at 5°C. The sulfated polysaccharides in the pellet were washed with 610 mL of 0.05% cetylpyridinium chloride solution, dissolved with 172 mL of a 2 M NaCl, ethanol (100:15, v/v) solution, and precipitated with 305 mL of absolute ethanol. After 24hr at 4°C, the precipitate was collected by centrifugation (2560 x g for 20 min at 5°C), washed twice with 305 mL of 80% ethanol, and once with the same volume of absolute ethanol. The final precipitate and dried at room temperature overnight and 500 mg (dry weight) of crude polysaccharide were obtained after these procedures.

Isolation and purification of carrageenan

After washing with water to remove all possible impurities such as salt, sand, shells... samples were grinded to optimize the contact between the samples and solvents at various subsequent operations. They were then submitted to depigmentation (algae were treated with acetone overnight stirring), decanted and filtered to extract the hydrophobic pigments (chlorophylls and carotenoids), and heated with 80% ethanol to reflux for 1 hour, filtered then treated them with absolute ethanol to extract the hydrophilic pigments. Knowing that carrageenan compounds are very soluble in water, this property is used for their extraction. 20g of pretreated algae were heated in 200 mL of water at a slightly alkaline pH (8-9) (0.5M NaHCO3 solution) in a water bath at 90°C for 3hr. This is the pH at which carrageenans are assumed to be stable.

Then the mixture was filtered in order to remove insoluble residues (cellulose), after which a viscous solution containing carrageenan was obtained and submitted to purification. This latter step is based on the ability of carrageenan to form a precipitate in the presence of excess alcohol or in a KCl solution. Therefore, a double volume of alcohol was added to the solution of carrageenan by stirring with a glass rod allowing the formation of a whitish filament carrageenan insoluble in alcohol. The carrageenan was washed with ethanol and was dried at room temperature for 24 h, then pulverized, reduced to powder in a mortar and finally sieved (Sebaaly *et al.,* 2012). This process allowed us to obtain 2g of carrageenan powder.

Determination of fatty acids

The following test was performed in the institute of Monocrystals, Kharkov, Ukraine. Approximately 1.2g of dry algae, *Corallina* previously grounded into particles of 0.5 mm was extracted three times with methanol-chloroform in portions of 10 mL for 3 hours. The mix was filtered through the paper filter into a 10 mL flask. 1g of anhydrous sodium sulfate was added to the extract obtained, which was evaporated at 60°C in the nitrogen stream until dryness (a residue of 40mg). 1 mL of diethyl ester, 5 mL of methanol and 0.2 mL of acetyl chloride were added to the residue and the flask was filled with nitrogen, and then it was boiled with the reflux condenser on the glycerin bath for 45 min at 70°C.

The solution obtained was evaporated in the nitrogen stream to a volume of 0.3 mL. Then, 2 mL of cyclohexane were added and stirred for 1 min. After the complete stratification of the layers, the upper cyclohexane layer was used as a test sample which was filtered through a filter with 0.2 g of sodium sulfate. The resulting solution was subjected to analysis by gas chromatography Shimadzu GC-14B, FID chromatography under the following conditions: capillary column (60m x 0.32mm HP-23;

0.25µm), the column temperature was held at 175°C for 2 min, and then raised to 225°C with a rate of 3°C / min, injector and detector temperatures were 240°C and 250°C respectively, the carrier gas flow rate (nitrogen) was 1.0 mL/min, split ratio was 1:60. The content of each fatty acid was calculated by the internal regulation method (Kanaan *et al.*, 2005).

Determination of trace elements

The following tests were conducted in the Institute of Monocrystals, Kharkov, Ukraine. 2g of *Corallina* powder were placed in a capillary tube of fluorized polymers in order to disperse them under pressure and microwaves. After adding 5 mL of HNO3 (70%) the capillary was firmly closed and placed in a steam room for 20 min, under a pressure not exceeding 120 psi. After cooling and filtration, the substance was recovered in a 50 mL tube filled with water, thereby obtaining liquid number 1. 1 mL was taken from liquid 1 and put in a 100 mL tube filled with water up to 100 mL obtaining therefore liquid 2 (Kanaan *et al.*, 2005). In order to determine macro-elements percentages, a Thermo Jarrel Ash atomic absorption spectrometer was used. Liquid 1 was used to determine the percentage of Fe, Zn, Cu, Ni, Mn, Al and Se. And, liquid 2 was used to determine the percentage of Ca, Mg, K and Na.

Conditions: Liquid flow speed: 1.85 mL / 1mn 2 sec

The flow speed of added Argon (Ar): 1 l / min

The flow speed of initial Argon (Ar): 14 l / min

Structural analysis

^{1}H and ^{13}C NMR spectroscopy

About 3 mg of each sample (carrageenan or sulfated galactans) were dissolved in 0.5 mL of 99% D_2O. ^{1}H and ^{13}CNMR spectra were recorded at 27° C on an "Ultrashield 300 Bruker" spectrometer operating at a frequency of 300 MHz with an acquisition time of 5.29 s, and pulse duration of 11 µs. All chemical shifts were expressed in ppm and reported relative to an internal tetra-methyl silane reference.

Infrared spectroscopy

The infrared spectra were recorded on a "JASCO FT-IR 6300" spectrometer for a range of frequencies between 400 and 4000 cm^{-1}. The resolution was 4 cm^{-1}. All samples were analyzed as KBr pellet.

Biological activity assays

Anticoagulant activity assay

Activated partial thromboplastic time (APTT) assays were carried out by the method of (Andersson *et al.*, 1976). Normal human platelet-poor plasma (100 µl) was incubated with 10 µl of a solution of polysaccharide (0.05, 0.5, 2.5, 5.0 µg) at 37°C for 1 min. Then 100 µl of APTT reagent (Human Gesellschaft, Germany) were added and incubated at 37°C. After 2 min of incubation, 100 µl of 0.25 M CaCl2 were added to the mixtures and the clotting time was recorded on a coagulometer (Thrombotimer "Behnkelektronik").

Antibacterial activity assay

Bacteria strains

Three Gram-positive bacteria [*Staphylococcus epidermidis* CIP 444, *S. aureus* ATCC 25923 and *Enterococcus feacalis* ATCC 29212] and two Gram-negative strains [*Escherichia coli* ATCC35218 and *Pseudomonas aeruginosa* ATCC27853] were used in this study. CIP 444 is a clinical strain which was isolated from a patient with infected implanted device, hospitalized in the Mignot Hospital of Versailles, France (Chokr *et al.*, 2006).

This strain was identified and characterized for many features by Dr. Ali Chokr and deposited to be enclosed within the microorganisms of the collection of Institute Pasteur in 2007 (Chokr *et al.*, 2007; Chokr *et al.*, 2006; Kogan *et al.*, 2006; Sadovskaya *et al.*, 2006). The other strains are ATCC. The strains were stored at −80 ◦C in glycerol stocks and used as required. Brain heart infusion (BHI), Brain heart agar (BHA), and Mueller–Hinton broth (MHB) were purchased from HIMEDIA (Mumbai, India).

MIC and MBC assays

Minimum Inhibitory Concentrations (MIC) and Minimum Bactericidal Concentrations (MBC) of both sulfated galactans and carrageenans were determined using a microtiter broth dilution method as recommended by the Clinical and Laboratory Standards Institute (CLSI) (Wikler *et al.*, 2006). 0.1 mg/mL of each polysaccharide was prepared and autoclaved at 121°C for 15 minutes prior to inoculation.

Serial two-fold dilutions of the different extract sin MHBwere prepared in a 96-well plate (200µL Per Well) (Greiner Bio-One, Essen, Germany). Wells with no algal extract added were used as a positive growth control. a diluted bacterial suspension was added to each well to give a final concentration of 5×10^{5} Colony-Forming Units (Cfu)/ml, Confirmed by viable counts. Wells without bacterial inoculum were used as a negative growth control. The plates were incubated for 24 H At 37 °C.

The contents of the wells showing no visible growth were plated on brain heart agar (BHA) and the number of colonies was counted after overnight incubation at 37 °C to determine the MBC.

The MBC was defined as the lowest concentration reducing the initial inoculum by ≥99.9%. The MIC and MBC were determined for all strains. For each strain, at least three independent determinations were done and the modal value was taken.

RESULTS AND DISCUSSION

Polysaccharides content of red algae *Corallina*

The total yield of sulfated galactans and carrageenan extracted from red algae *Corallina* collected at the Lebanese coast was 2.5% and 10% respectively. Accordingly, using the same extraction method, the content of sulfated galactans and

carrageenans isolated from another species of red algae, *Pterocladia*, collected from Lebanese coast was 2.7% and 11.5 % respectively (Sebaaly *et al.*, 2012).

Fatty acids content of *Corallina* and *Pterocladia*

The fatty acids detected in the algal sample were shown in fig 1. *Corallina*, collected from Batroun-Lebanon coast, was composed of 70.81% saturated fatty acids, 25.54% monounsaturated fatty acids and 3.65% polyunsaturated fatty acids. The major fatty acid was saturated palmitic acid (52.13%) followed by the monounsaturated cis-vaccenic acid (18.2%), whereas the red algae *Pterocladia*, collected at Rawche beach-Lebanon, was composed of 69% of saturated fatty acids, 31% of monounsaturated fatty acids and did not contain polyunsaturated fatty acids (Sebaaly *et al.*, 2012). Its content in the main fatty acid, the palmitic acid (53.75 %) was slightly higher than that present in *Corallina* (52.13 %). This difference in fatty acids content between two different species of red algae harvested from different regions may indicate that algae's composition depends on both, species and environmental factors.

Trace elements content of *Corallina*

The elements detected in Corallina sample and their concentrations were shown in Fig. 2. *Corallina* was rich in K, Ca, Mg, Na, Si, Sr, P and Fe (Fig. 2). *Corallina* and *Pterocladia* also differ in their content of trace elements. Indeed, *Corallina* contains higher amounts of potassium K (3750 mg/100g), sodium Na (750 mg/100g), calcium Ca (2000 mg/100g) and magnesium Mg (750 mg/100g) compared to *Pterocladia* containing 2220 mg/100g of K, 150 mg/100g of Na, 740 mg/100g of Ca, 225 mg/100g of Mg (Sebaaly *et al.*, 2012). While *Pterocladia* is the richest in iron Fe (110 mg/100g), silicon Si (590 mg/100g), phosphorus P (125 mg/100g) and aluminum Al (59 mg/100g) (Sebaaly *et al.*, 2012). Both red algae contained negligible amounts of Pb and Hg thereby indicating that environment where they grow is unpolluted.

Infrared analysis of *Corallina*

The sulfated galactans spectrum (fig. 3A) showed two bands located at 3554.16 and 3423.99 cm^{-1} corresponding to the stretching vibration of hydroxyl group (OH), a band at 2925.28 cm^{-1} due to the stretching vibration of C-H bonds and a band at 1616.06 cm^{-1} assigned to an asymmetric stretching vibration of O-C-O (Chopin *et al.*, 1999). The band of greater intensity located at 1420.32 cm^{-1} was assigned to the vibration of sulfate groups. The signals located at 1304.61 cm^{-1} and 1032.69 cm^{-1} were assigned to a stretching vibration of CO sulfate esters and hydroxyl groups respectively. The absorption band located at 889.987 cm^{-1} is characteristic of CH glycoside and the band at 822.491 cm^{-1} was assigned to stretching vibration of SO4-CO bonds and corresponds to the galactose-6-sulfate (Rochas *et al.*, 1986). Finally the band at 727.032 cm^{-1} may be due to C4 galactose sulfate (SO4-CO) (Fig. 3A). The IR spectrum of carrageenan showed the same bands as those observed with sulfated galactans (Fig. 3B). The spectrum didn't show the absorption band at 930 cm^{-1} characteristic of 3,6-anhydrobridge (Silva *et al.*, 2010) which is present in κ-and ι-carrageenan and absent in λ-carrageenan.

In addition, the absence of the absorption band located at 805 cm^{-1}characteristic of 3,6-anhydrogalactose-2-sulfate residue present in ι-carrageenan as well as the presence of 821.527 cm^{-1}characteristic of the galactose 6-sulfate residue in λ-carrageenan (Guibet *et al.*, 2007) indicated well that the carrageenan isolated from *Corallina* correspond to Lambda type.

^{1}H NMR analysis

The ^{1}H-NMR spectrum of carrageenan was characterized by a high viscosity as previously reported (Ciancia *et al.*, 1993; Stortz *et al.*, 1994), which explains the low spectrum resolution (data not shown).

On the other hand, both carrageenan and sulfated galactans spectrum showed a single signal at 1.03 ppm attributed to a proton methylene CH2. The presence of the signals in the area between 2.07-2.87 ppm can be attributed to proton acetyl group (Bubb, 2003). Doublet signal at 3.49 ppm may be due to O-CH3 group of the β bond (Farias *et al.*, 2000) (data not shown).

^{13} C NMR analyses

For the sulfated galactans spectrum, we noticed the presence of a signal at 215.24 ppm that can be attributed to the carbon acetate group (C = O) and a signal at 79.4 ppm which may be due to the chemical shift of carbon C3 or C5. The triplet signal 57.57-57.33-57.08 ppm is characteristic of C6 carbon of the glucosidic chain, the signal at 30.15 ppm corresponds to the carbon of the acetate moiety OAc- CH3 and triplet signal at 16.95-16.71-16,45 ppm is characteristic of the C6 carbon Levo configuration of the α-galactopyranose chain (L-Gα-C6 cp) (data not shown). The absence of the other signals may be related either to the high viscosity of lambda-carrageenan or to the temperature applied during the analysis.

Anticoagulant effect of Polysaccharides isolated from *Corallina*

Heparin has been identified and used for more than fifty years as a commercial anticoagulant and it is widely used for the prevention of venous thromboembolic disorders. However, several side effects of heparin have been reported such as development of avian influenza, bovine spongiform encephalopathy (Mendes *et al.*, 2009), thrombocytopenia, hemorrhagic effect, and incapacity to inhibit thrombin bound to fibrin (Pereira *et al.*, 2002). Therefore, there is an increasing interest in discovering new anticoagulation agents replacing heparin. Previous studies have shown that natural or chemically modified sulfated polysaccharides exhibit anticoagulant activities (Suwan *et al.*, 2009).

Therefore we studied the anticoagulant activity of the polysaccharides isolated from *Corallina* by APTT assay (table 2). After measuring the clotting time, the ratio was then calculated using the following formula: ratio= APTT/control = APTT/34 bearing in mind that the normal clotting time APTT is between 28-38s and depends on the laboratory and the reagents used. A

significant anticoagulant effect was obtained when the ratio is higher than 1.2. The results showed that an increase in the quantity of sulfated galactans form 0.05 to 5 µg induced a significant increase in the ratio from 1.21 to 3.07 which led to more pronounced anticoagulant effect (table 2). These findings were consistent with the recently published data by (Sebaaly *et al.*, 2012).

On the other hand, carrageenans exhibit more powerful anticoagulant activity than sulfated galactans. Indeed, at the lowest dose of polysaccharides (0.05 µg) used, the ratio was 2.31 for carrageenans being higher than that obtained with sulfated galactans (1.21) (table 2). A ratio higher than 3.53 was obtained with 0.5 µg of carrageenan while it reached 3.07 with the highest dose of sulfated galactans (5 µg) thereby indicating the highest effect of carrageenans. However, due to their high content of sulfate groups as well as their bulkiest structure, sulfated galactans from *Pterocladia* showed more pronounced anticoagulant effect than carrageenans(Sebaaly *et al.*, 2012). On the other hand, among the three types of carrageenan, the lambda-type showed the highest anticoagulant potential (Silva *et al.*, 2010). from that used in our study (lambda-type) explaining thereby the difference between their results and those obtained in our present study. In addition, it was previously reported that the anticoagulant activity of polysaccharides depends on their degree of substitution, molecular weight and the position of the sulfate group (Suwan *et al.*, 2009).

Antibacterial effect of sulfated galactans and carrageenans isolated from *Corallina*

Antibacterial activity of both sulfated galactans and carrageenan against Gram-positive and Gram-negative strains are presented in table 3. The MIC and MBC of Sulfated galactans were found to be 0.39 mg/mL and 3.125 mg/mL against *S. epidermidis* and 3.125 and 25 mg/mL against *E. faecalis* respectively (table 3). That suggests that sulfated galactans showed inhibitory activity as well as bactericidal activity against all Gram-positive strains tested. But, carrageenan was only able to inhibit the growth of *S. epidermidis* (MIC of 0.325 mg/mL). However, both polysaccharides isolated form *Corallina* did not exhibit either inhibitory or bactericidal activity against Bacterial Gram-negative strains since their MIC and MBC were of high value (Table 3).

Table. 2: Anticoagulant activity of polysaccharides isolated from *Corallina*.

Polysaccharides	Quantity (µg)	APTT (s)	Ratio
Carrageenans	0.05	78.4	2.31
	0.5	>120	>3.53
	2.5	>120	>3.53
	5	>120	>3.53
Sulfated Galactans	0.05	41.3	1.21
	0.5	55.9	1.64
	2.5	71.8	2.11
	5	104.3	3.07

APTT was measured in seconds by a coagulometer. Ratio was calculated by the formula: Ratio = APTT measured / APTT control = APTT measured / 34

Table 3: Antibacterial activity of polysaccharides isolated from *Corallina* on different bacterial strains

Bacterial type	Bacterial strain	Isolated Polysaccharides	MIC (mg/mL)	MBC (mg/mL)
Gram positive bacteria	*S. epidermidis* (CIP 444)	Sulfated Galactans	0.39	3.125
		Carrageenan	0.651	>83.3
	E. faecalis (ATCC29212)	Sulfated Galactans	3.125	25
		Carrageenan	>83.3	>83.3
	S. aureus (ATCC25923)	Sulfated Galactans	>100	>100
		Carrageenan	>83.3	>83.3
Gram negative bacteria	*E. coli* (ATCC35218)	Sulfated Galactans	>100	>100
		Carrageenan	>83.3	>83.3
	P. aeruginosa (ATCC27853)	Sulfated Galactans	>100	>100
		Carrageenan	>83.3	>83.3

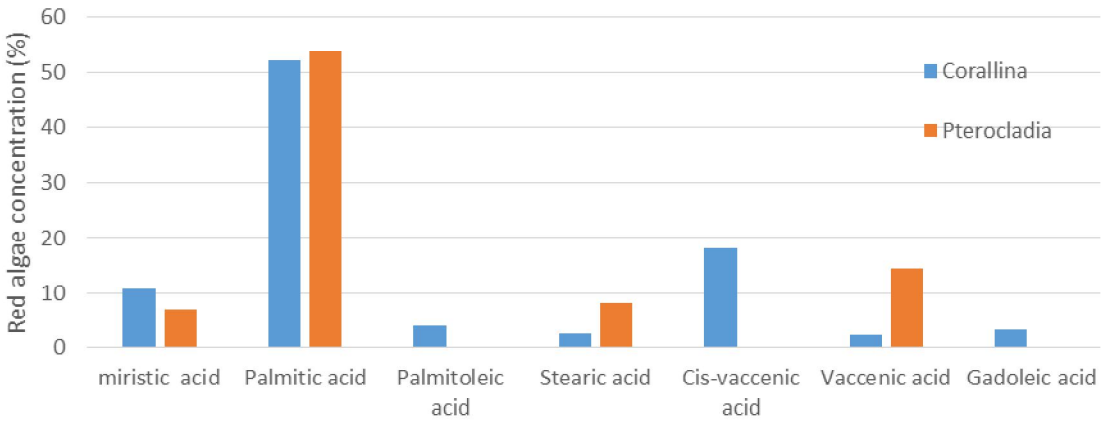


Fig. 1: Percent composition of fatty acids in *Corallina* and *Pterocladia* (red algae).

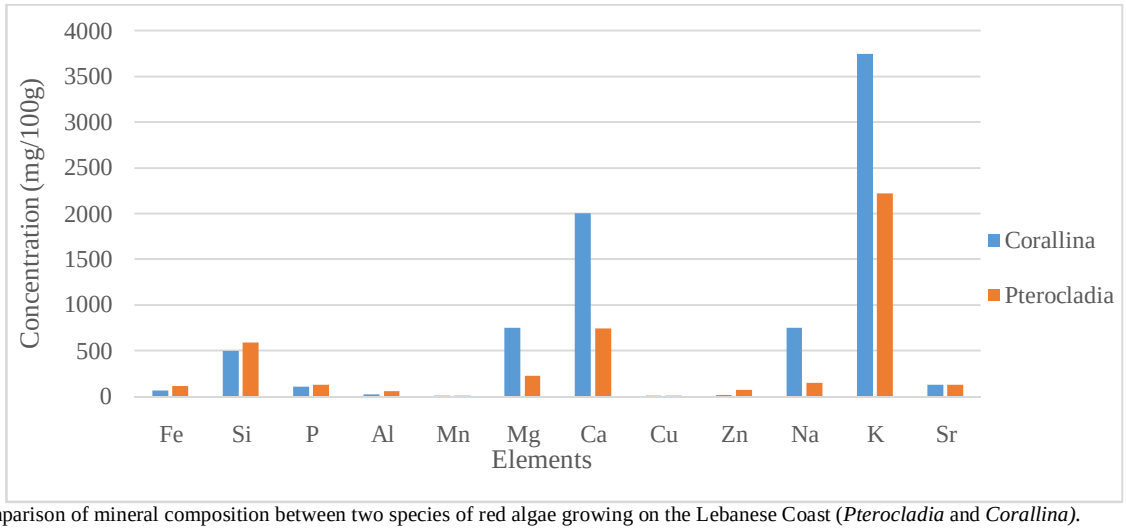
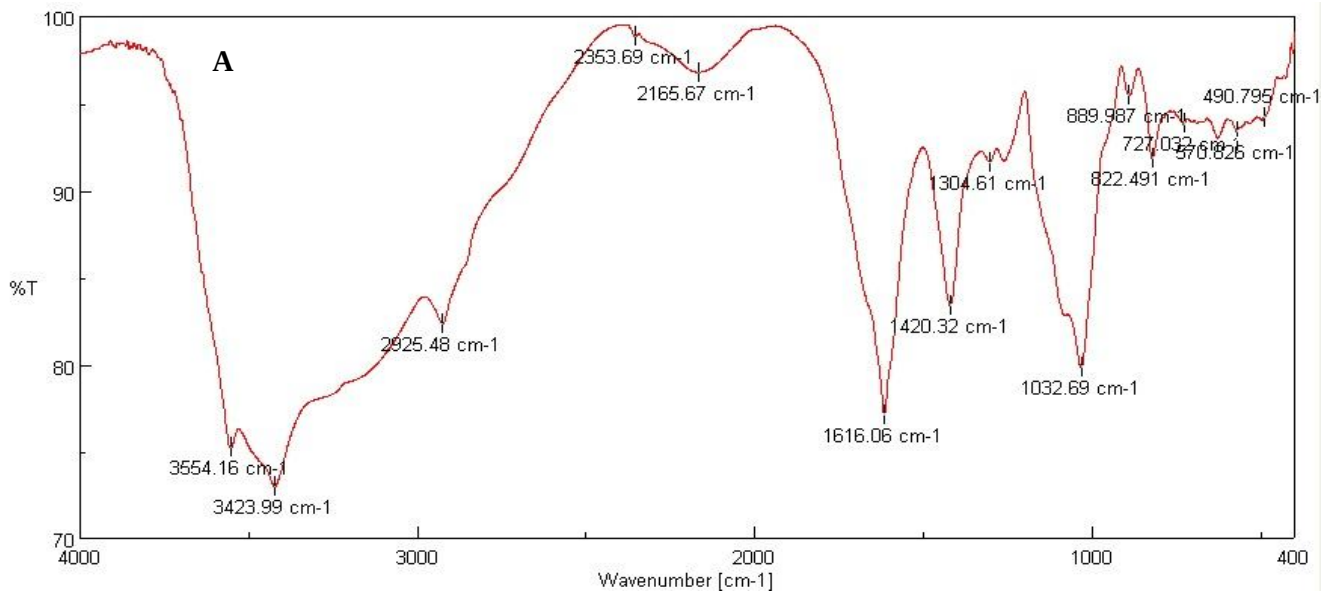


Figure 2: Comparison of mineral composition between two species of red algae growing on the Lebanese Coast (*Pterocladia* and *Corallina).*



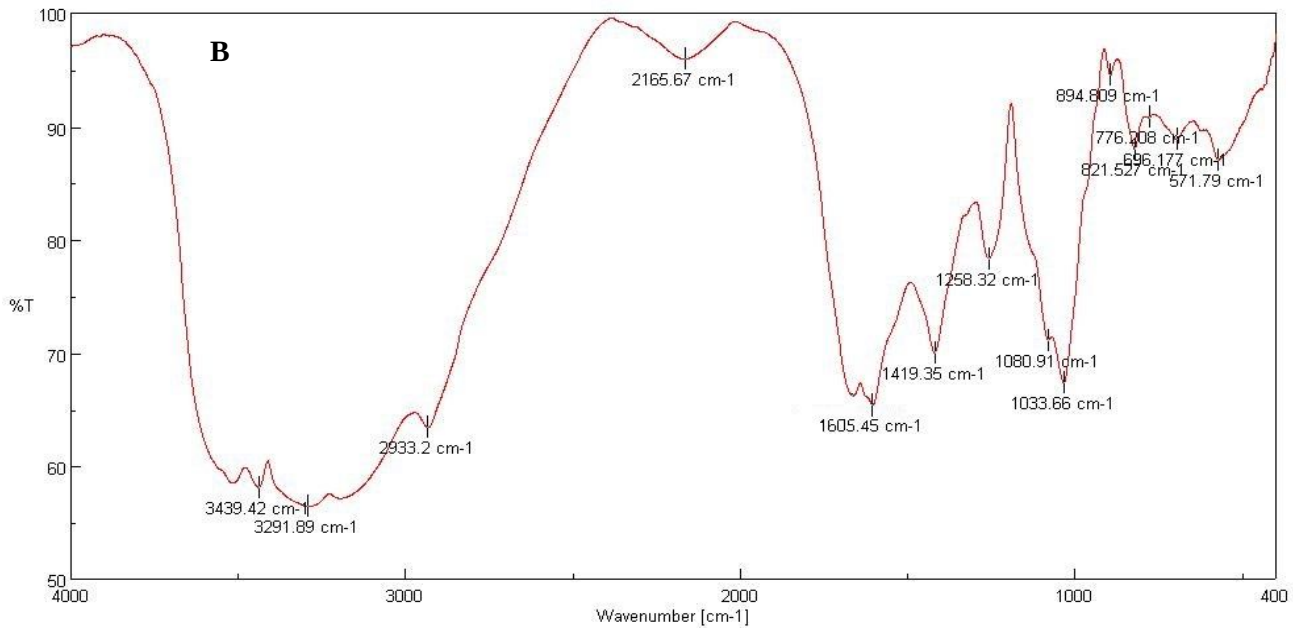


Fig. 3: Infrared spectrum of sulfated galactans (A) and carrageenan (B) isolated from *Corallina*

CONCLUSIONS

The red algae *Corallina* growing on the Lebanese coast were rich in minerals and saturated fatty acids. Red seaweeds *Corallina* could be considered as a potential source of bioactive molecules that may be useful for the development of new pharmaceutical agents. Indeed, both sulfated galactans and carrageenan isolated exhibited anticoagulant and antibacterial effects. Moreover, the anticoagulant effect of sulfated galactans was less potent than that of carrageenan. These polysaccharides isolated can be valuable tools for further studies of biological activities such as anti-inflammatory, antiviral and antioxidant effects.

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