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STRUCTURAL CHARACTERISTICS, ANTITUMOR AND ANTI-INFLAMMATORY PROPERTIES OF POLYSACCHARIDES ISOLATED FROM THE RED ALGAE *TRICLEACARPA FRAGILIS* GROWING ON THE LEBANESE COAST.

Zein[1] S., Haddad[1] M., Krivoruchko[2] E., El-Hajje[1] A., Hazimeh[1] G., Kassem[3] Z. and Kanaan H.[*1]

[1]Laboratory of Chemical Synthesis and extraction of Polysaccharides from seaweed, Faculty of Pharmacy, Lebanese University.

[2]Department of Pharmacognosy, National University of Pharmacy, Ukraine.

[3]PRASE, Platform of Research and Analysis in Environmental Sciences, Doctoral School of Sciences.

***Corresponding Author: Prof. Kanaan H.**

Laboratory of Chemical Synthesis and extraction of Polysaccharides from seaweed, Faculty of Pharmacy, Lebanese University.

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ABSTRACT

Objective: The purpose of this study was to extract, to determine the functional groups and to examine the anti-proliferative and the anti-inflammatory effect of the polysaccharides sulfated galactan and carrageenan extracted from the red algae *Tricleacarpa fragilis* growing on the Lebanese coast. **Materials and Methods:** Sulfated galactan and carrageenan were analyzed using ^{1}H NMR, and FTIR methods to reveal their structures. The polysaccharides were tested in-vitro for anti-proliferative activity against the colorectal cancer cell lines (HT 29 and HCT 116) for 24h, 48h and 72h respectively, by the MTT cell viability assay. The anti-inflammatory activity was tested on the RAW 264.7 cell line using the western blot analysis. **Results:** The extraction yield of sulfated galactan and carrageenan was 1.7% and 20% respectively. The structures of both polysaccharides were confirmed through the spectrum of ^{1}H NMR. For the anti-proliferative effect, sulfated galactan was able to inhibit the growth of HT 29 and HCT 116 more than carrageenan at the different time intervals. Sulfated galactan was found to have an anti-inflammatory effect, however carrageenan induced the inflammation. **Conclusion:** This study discusses the structure, the functional groups, the anti-proliferative and the anti-inflammatory effect of sulfated galactan and carrageenan. Both polysaccharides were able to inhibit the growth of HT 29 and HCT 116 with sulfated galactan the more potent. For the anti-inflammatory effect, sulfated galactan inhibited the inflammation while carrageenan induced it. These effects make them candidate in the field of medicine and food industry.

KEYWORDS: *Tricleacarpa fragilis*, sulfated galactan, carrageenan, anti-proliferative, anti-inflammatory.

I. INTRODUCTION

Medicinal plants have been widely developed in recent decades and they are considered as the richest natural resources of biologically compounds.[1] Among marine resources, algae are well known natural sources of polysaccharides.[2] Due to the secondary effects of synthetic chemicals, research was driven towards demand for natural therapeutics with bioactive compounds. In the last decades, nature became a relevant resource for the discovery of new drugs. Red algae, or Rhodophyta are one of the oldest groups of eukaryotic algae, and also one of the largest, with about 5000-6000 species of mostly multicellular, marine algae, including many notable seaweeds. It is composed from polysaccharides including: sulfated galactan and carrageenan along with agar. Carrageenan and agar are rarely present in the same species, which makes it possible to distinguish carrageenophytes from agarophytes, which are distributed among orders and families in a marked manner and are mainly used as thickening agents.[3] Medical and pharmaceutical industries are interested in marine plants since they have proven to be a rich source of structurally diverse bioactive compounds with valuable pharmaceutical and biomedical potential.[4] Indeed, the complex polysaccharides from red algae especially sulfated galactans and carrageenans defined as polymers of galactose possess broad spectrum of therapeutic properties. They are reported to exhibit anticoagulant[5] immunomodulating, antitumor.[6] and antioxidant activities.[7]

We have studied the structure and pharmacological activities of sulfated galactan and carrageenan in some species of red algae growing on the Lebanese coast, as *Pterocladia* and *Corallina*.[5, 8] We have followed the studies on the red algae, *Tricleacarpa fragilis.* The aim of this work was to extract, to determine the functional groups and to examine the anti-proliferative, and the anti-inflammatory effect of the polysaccharides sulfated galactan and carrageenan extracted from the red algae *Tricleacarpa fragilis* growing on the Lebanese coast.

II. MATERIALS AND METHODS

2.1. Sample collection

The marine red algae *Tricleacarpa fragilis* was collected from Sarafand beach 61km to the south of the capital Beirut, Lebanon, in May 2014 and separated from other species and sun dried.[9]

2.2. Extraction and isolation of sulfated galactans from *Tricleacarpa fragilis*

The extraction was carried out by the method of *Farias et al*. The dried sample (30 g) was cutted in small pieces, suspended in 250ml of 0.1M sodium acetate buffer (pH=6) containing 510mg of papain, 5M ethylendiaminetetraacetic acid (EDTA) and 5mM cysteine, and incubated at 60 °C for 24hr. The incubation mixture was then filtered and the supernatant saved. The residue was washed with 138ml of distilled water, filtered again, and the two supernatants were combined. Sulfated polysaccharides in solution were precipitated with 16ml of 10% cetylpyridinium chloride solution. After standing at room temperature for 24hrs, the mixture was centrifuged at 2560 × g, for 20 min, at 5 °C. The sulfated polysaccharides in the pellet were washed with 610ml of 0.05% cetylpyridinium chloride solution, dissolved with 172ml of 2M NaCl, ethanol (100:15 v/v) solution, and precipitated with 305ml of absolute ethanol. After 24hrs at 4 °C, the precipitate was collected by centrifugation (2560 × g, for 20 min, at 5 °C), washed twice with 305ml of 80% ethanol, and once with the same volume of absolute ethanol. The final precipitate was dried at room temperature overnight and the obtained product was weighed to calculate galactan yield.

Yield of sulfated galactan (%) = actual weight × 100/ theoretical weight.

2.3. Extraction and isolation of carrageenans from *Tricleacarpa fragilis*

The algae were washed with water to remove all possible impurities such as salt, sand, shell, and grinded to optimize the contact between samples and solvents at various subsequent operations. Then submitted to depigmentation: algae were treated with acetone overnight decanted and filtered to extract the hydrophobic pigments (chlorophylls and carotenoids), and the hydrophilic pigments were extracted with 80% ethanol by heating to reflux for 1hr, filtered, then treated them with absolute ethanol. Knowing that carrageenan compounds are very soluble in water, so this property is used for their extraction. 20g of algae pretreated were heated in 200ml of water at a slightly alkaline pH[8-9] (0.5M sodium hydrogen carbonate $NaHCO_3$ solution) in a water bath at 90 °C for 3hrs. This is the pH where the carrageenans are assumed to be stable. Then the mixture was filtered in order to remove the insoluble residues (cellulose), a viscous solution containing carrageenans 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 dried at room temperature for 24 hrs, then pulverized, reduced to powder in a mortar and finally sieved. The powdered product was weighed to calculate carrageenan yield.

Yield of carrageenan (%) = actual weight × 100/ theoretical weight.

2.4. Proton nuclear magnetic resonnance spectroscopy (^{1}H NMR)

In order to determine the position of proton in sulfated galactan and carrageenan, 3 mg of the samples were dissolved in 0.5 ml deutrium oxide (D_2O), and the NMR spectra of the samples were recorded using Ultrashield Brocker 300 spectrometer at room temperature, with a frequency of 300 MHZ, an acquisition time of 5.2 seconds and a pulse duration of 11 milliseconds. Tetramethylsilane was used as an internal standard.

2.5. Fourrier transforms infrared spectrometer (FTIR)

Sulfated galactan and carrageenan were mixed with potassium bromide (KBr), in a way that the % of sample/ KBr = 2%. The FTIR spectrum of the samples was recorded on a JASCO FT/IR 6300 spectrometer. The resolution was 4 cm^{-1} and data were collected in the range of 4000-400 cm^{-1}.

2.6. Cell culture

The cell lines used in this study were human colorectal cancer cell lines HCT 116 and HT 29 and murine macrophage cell line RAW 264.7. The cells were grown in plastic bottles (75 cm^2) containing Dulbecco's modified Eagle's medium complete media supplemented with 10% fetal bovine serum, 1% penicillin streptomycin (10000 IU/ml), and were maintained at 95% humidity in a CO_2 incubator at 37°C. During the experiments, cells were allowed to grow till 80-90% confluency, where all available space of the culture vessel is covered due to cellular expansion before passage. Cells were passaged 8 times in order to prolong life of the cells and allow them to proliferate.

2.7. Anti-proliferative activity: MTT cell viability assay

In order to determine the anti-proliferative effect of sulfated galactan and carrageenan on HT 29 and HCT 116, MTT assay was performed. This test is a good index of mitochondrial activity and thus of cell viability. It is based on the ability of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase enzymes in metabolically active cells to reduce the yellow tetrazolium dye MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) to its insoluble formazan, which has a purple color. The resulting intracellular purple formazan can be solubilized and

quantified by spectrophotometric means. HCT 116, and HT 29 cell lines were seeded in 96-well plates at a density of 15 000 cells/well and incubated in DMEM complete media for 24 hours. After incubation, media were aspirated and test compounds (galactan and carrageenan) were prepared with different concentrations (5mcg/ml, 25mcg/ml, 50mcg/ml, 100mcg/ml, and 200mcg/ml) and 100 µl were distributed into the wells in triplicate and incubated for 24 hours, 48 hours, and 72 hours. Control groups were treated with DMEM. After incubation is completed, 10 µl of MTT was added to each well. Three hours later, 100 µl of stop solution (HCl + Sodium Dodecyl Sulfate) was added and incubated for 1 hour to solubilize formazan. Then, the optical density (OD) of the samples was determined at 570 nm using enzyme-linked immunosorbent assay (ELISA) reader. Data were analyzed and percentages of cell viability were determined for the tested compounds.

$$\% \text{ of cell viability} = \frac{\text{Mean OD of treated Cells-Mean OD of blank} \times 100}{\text{Mean OD of Control-Mean OD of blank}}$$

Where the blank was the acidified isopropanol.

2.8. Anti-inflammatory activity: Western blot analysis

To determine the effect of galactan and carrageenan on the protein expression levels of COX-2 in LPS stimulated RAW 264.7 cells, western blot analysis was performed. RAW 264.7 cells were seeded in 96 well plates and incubated for 24 hours. After incubation, media were aspirated and the non-cytotoxic concentrations (>90% cell viability) of galactan (1 mcg/ml, 2 mcg/ml, 4 mcg/ml, 5 mcg/ml) and of carrageenan (5 mcg/ml, 25 mcg/ml, 50 mcg/ml, 100 mcg/ml) with 10 µl lipopolysaccharide (LPS) to stimulate inflammation in the cells were prepared and distributed to each well and incubated for 24 hours. Subsequently, supernatant was discarded and cells were washed with 500 µl phosphate buffered saline (PBS) and lysed with 60 µl radioimmunoprecipitation assay (RIPA) lysis buffer containing protease inhibitors and the adherent cells were dislodged by using a scrapper on ice. Then, extracts were centrifuged at 1000 rpm for 20 min at 4˚C and the supernatant was used for western blot analysis. Total protein (50 µg) was electrophoresed on 10% SDSpolyacrylamide gel (SDS-PAGE) and transferred to nitrocellulose membranes. Membranes were blocked with 5% non-fat dried milk in wash buffer (TrisPH = 8, 5 M sodium chloride, Tween 20, distilled water) for 2 hours with gentle shaking. Then, the membranes were washed with wash buffer six times for 5 min each time and incubated with primary antibody with gentle shaking at 4˚C overnight. Membranes were washed three times for 10 min each time, following which the membranes were incubated with corresponding horseradish peroxidaseconjugated (HRP) secondary antibody IgG for 2 h at room temperature. Following a final wash, protein bands were visualized by using enhanced chemiluminescence (ECL) reagents. GAPDH was used as a loading control. The basal levels of each protein were normalized by analyzing the level of GAPDH by using Image J program.

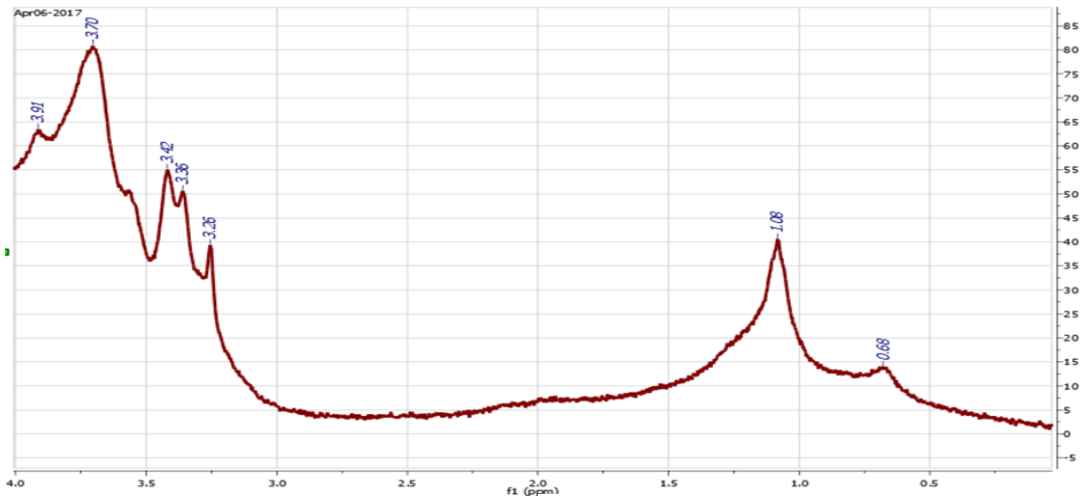
III. RESULTS

3.1. Percentage yield of polysaccharides

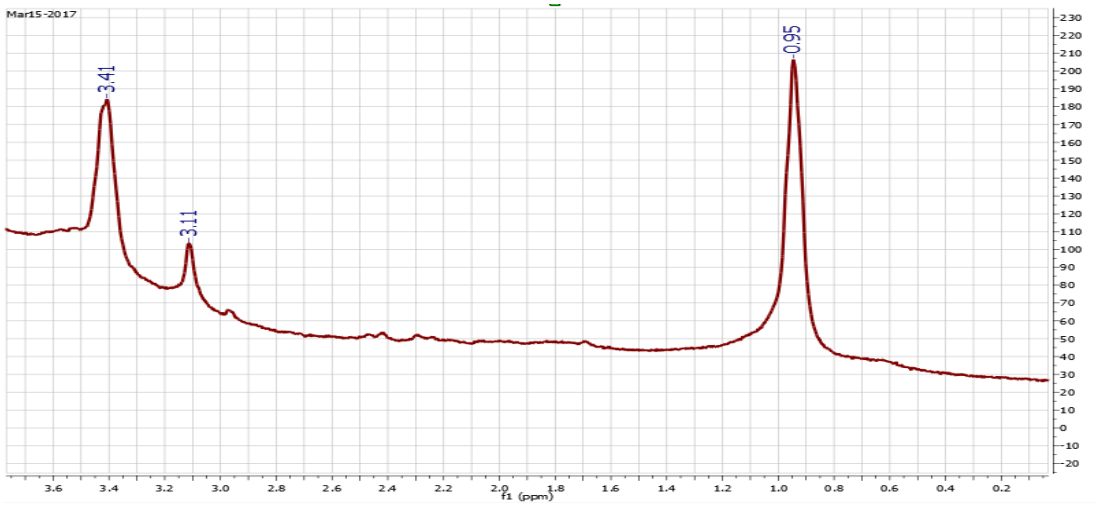
The quantity of sulfated galactan obtained from 30 g *Tricleacarpa fragilis* was: 0.51 g with a yield of 1.7% (0.51/30 × 100). While, the quantity of carrageenan was: 6 g with a yield of 20% (6/30 × 100).

3.2. Determination of the proton position: ^{1}H NMR analysis

The ^{1}H one dimensional spectra of sulfated galactan and carageenan from *Tricleacarpa fragilis* are shown in (Figure 1a and 1 b). These spectra reveal a single signal at 1.08 ppm and 0.95 ppm respectively attributed to a proton methylene CH2 and another single signal at 3.41 which may be due either to the presence of O-CH3 bond or is assigned to the β unit.[7]



a)



b)

Figure 1: ^{1}H NMR of a) sulfated galactan and b) carrageenan isolated from *Tricleacarpa fragilis.*

3.3. Determination of the functional groups: FTIR spectroscopic analysis of sulfated galactan

The FTIR spectrum of sulfated galactan showed one band located at 3422cm-1corresponding to the stretching vibration of hydroxyl group (OH), a band at 2920.66 cm-1due to the stretching vibration of C-H bonds and a band at 1636.3 cm-1 assigned to an asymmetric stretching vibration of O-C-O. The band of greater intensity located at 1414.53 cm-1 was assigned to the vibration of sulfate groups. The signals located at 1289.15 cm-1 and 1045.23 cm-1 were assigned to a stretching vibration of CO sulfate esters and hydroxyl groups respectively. The absorption band located at 934.342 cm-1 is characteristic of the 3, 6- anhydrogalactose residue. Finally, the band at 770.423 cm-1 may be due to C4 galactose sulfate (SO4-CO) (figure 2).

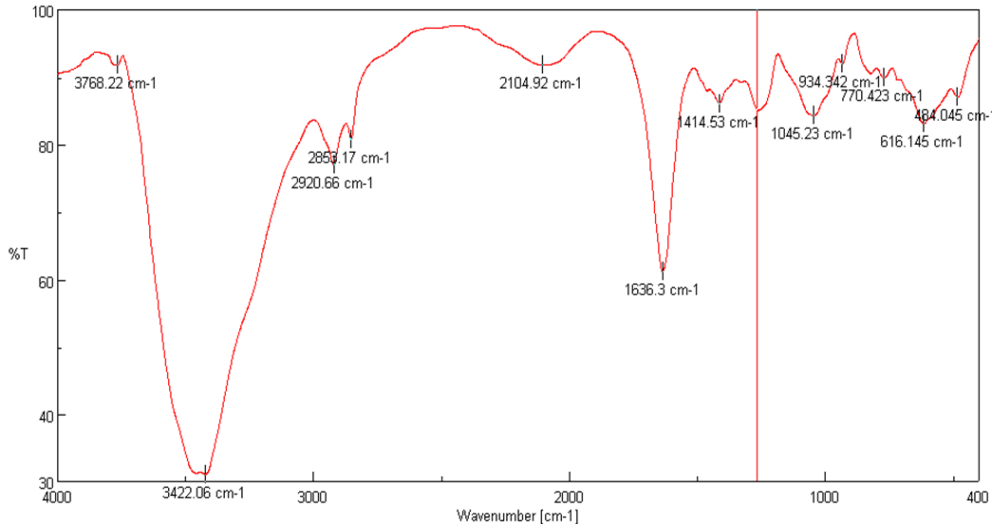


Figure 2: FTIR spectrum of sulfated galactan isolated from *Tricleacarpa fragilis.* %T: % Transmittance.

3.4. Determination of the functional groups: FTIR spectroscopic analysis of carrageenan

The FTIR spectrum of carrageenan showed the same bands as those observed with sulfated galactans (figure 3). But the absence of the absorption band at 805 cm-1characteristic of the 3, 6-anhydrogalactose-2-sulfate residue indicates that this type of carrageenan does not match iota-carrageenan. In addition, this spectrum does not show the two absorption bands characteristic of the λ carrageenan: a strong band at 830 cm-1 corresponding to the galactose-2-sulfate and a band at 820 cm-1 corresponding to the galactose 6-sulfate.[10] Thus the various peaks attributed above indicate that the studied carrageenan isolated from *Tricleacarpa fragilis* is probably of Kappa type.

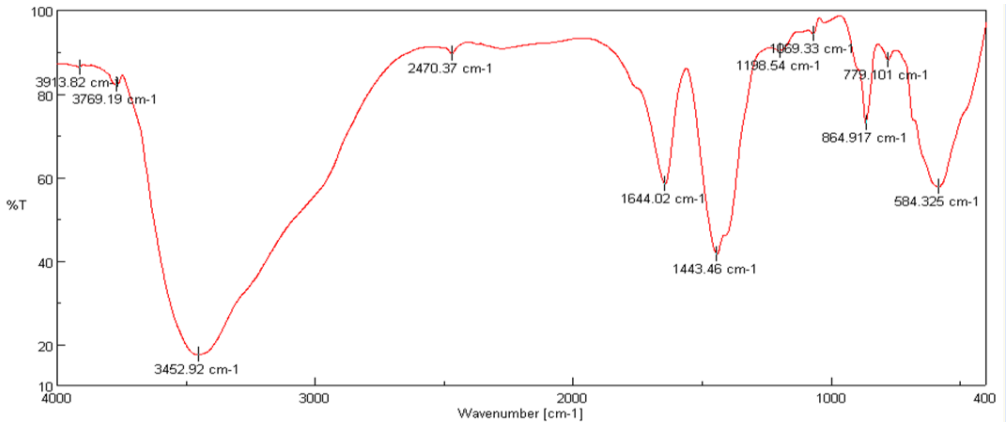


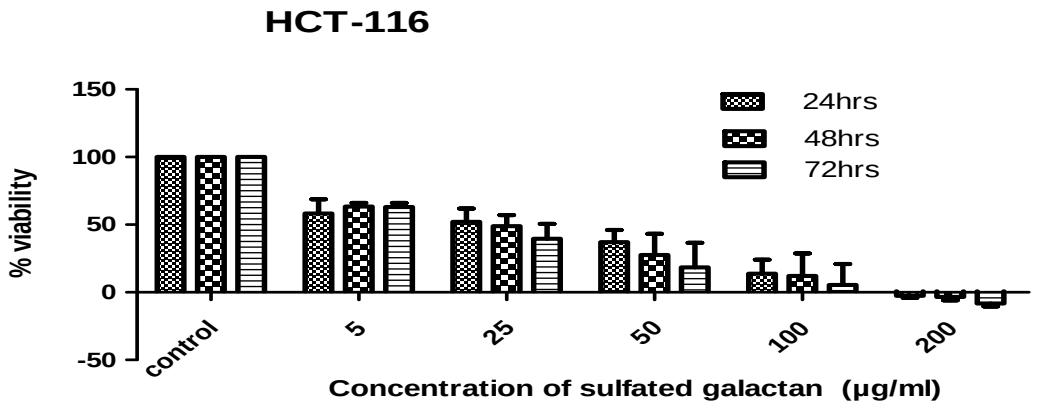
Figure 3: FTIR spectrum of carrageenan isolated from *Tricleacarpa fragilis.* %T: % Transmittance.

3.5. Anti-proliferative activity: MTT Cell viability assay

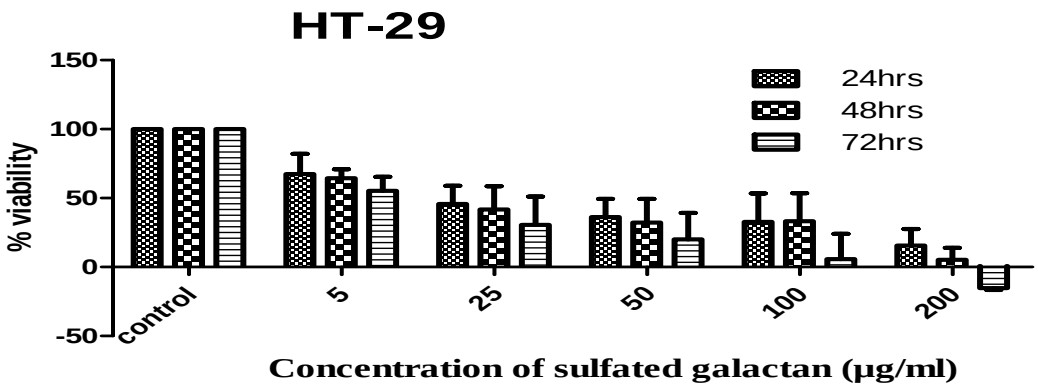
In order to examine the anti-proliferative effect of sulfated galactan and carrageenan, MTT cell viability assay was conducted on HCT 116 and HT 29 cells for 24, 48, and 72 hours. The results obtained are presented in figures 4, and 5.

3.5.1. Anti-proliferative effect of sulfated galactan on HCT 116 and HT 29

The results showed that there was a gradual decrease in the % of cell viability of HCT-116 and HT-29 cell lines with increasing the concentration from 5 to 200 mcg/ml. Also the effect was time-dependent, as the incubation time was increased from 24 to 72 hours, the decrease in the % of cell viability was better, with both cell lines (figure 4a, and 4b). For HCT 116 (figure 4a), at 200 mcg/ml the % of cell viability was decreased by 100 % at the three time intervals (figure 4a). However, with HT 29 the % of cell viability was decreased by 100 % at 200 mcg/ml at 72 hours only (figure 4b).



a)

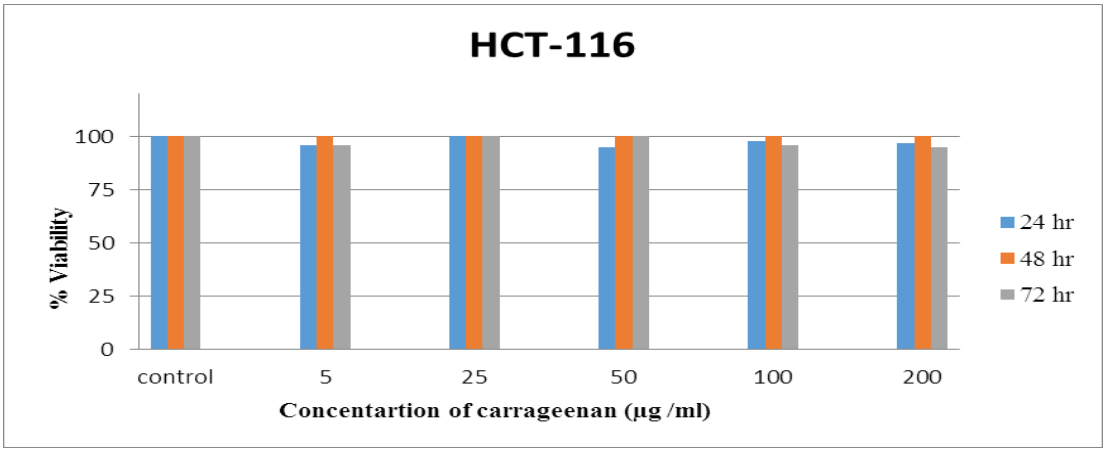


b)

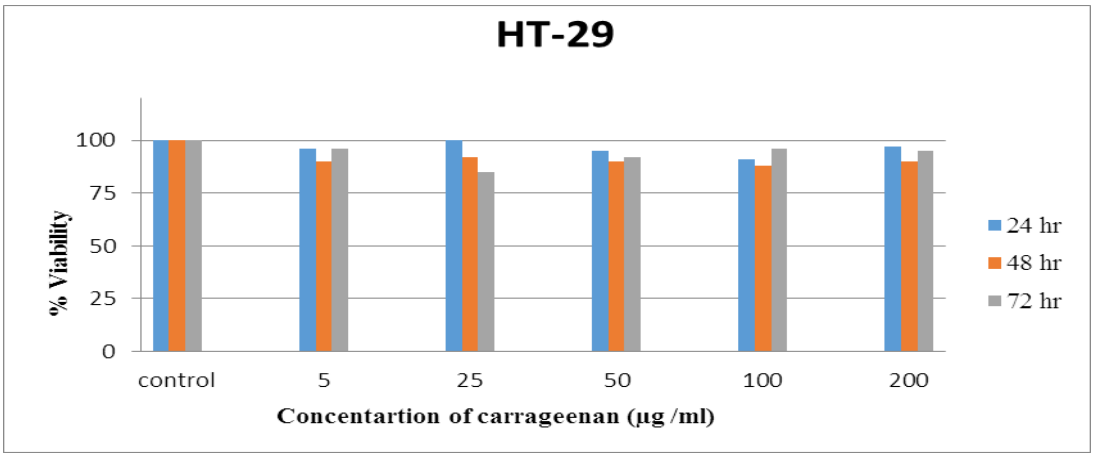
Figure 4: Viability of a) HCT 116 and b) HT 29 cells treated with different concentrations of sulfated galactan for 24, 48, and 72 hours. Control group is untreated cells.

3.5.2. Anti-proliferative effect of carrageenan on HCT 116 and HT 29

With HCT 116, at 24, 48, and 72 hours there was no effect and the % of cell viability was almost the same as the control (100%) (figure 5a). For HT 29, the results showed that there was a slight reduction in the % of cell viability (figure 5b).



a)



b)

Figure 5: Viability of a) HCT 116 and b) HT 29 cells treated with different concentrations of carrageenan for 24, 48, and 72 hours. Control group is untreated cells.

3.6. Anti-inflammatory activity: Western blot analysis

In order to examine the anti-inflammatory effect of sulfated galactan and carrageenan, western blot analysis was conducted on RAW 264.7 cell line. The results obtained are presented in figure 6.

3.6.1. Anti-inflammatory effect of sulfated galactan and carrageenan

According to our results, the ratio Cox2/GAPDH of sulfated galactan at the different concentrations (1 mcg/ml, 2 mcg/ml, 4 mcg/ml, and 5 mcg/ml) was lower than that of the LPS (approximately to the half of it) which indicated that sulfated galactan extracted from *Tricleacarpa fragilis* had an anti-inflammatory effect at any of the studied concentrations.

However, the ratio Cox2/GAPDH of carrageenan at the different concentrations (5 mcg/ml, 25 mcg/ml, 50 mcg/ml, and 100 mcg/ml) was higher than that of the LPS (some is doubled and other is tripled) which indicated that carrageenan was devoided from the anti-inflammatory effect and it may induce inflammation.

The quantification of the ratio Cox2/GAPDH is represented in table 1.

Table 1: Quantification of the ratio Cox2/GAPDH of sulfated galactan and carrageenan.

Name	GAPDH	Cox2	Cox2/GAPDH
Cntrl	15960.832	0	0
Lps	16695.439	18889.903	1.131440928
kg1	20921.551	13112.832	0.626761945
kg2	19525.317	10462.154	0.535825052
kg4	13301.016	10101.054	0.759419732
kg5	19817.388	12274.338	0.619372139
kc5	10884.296	20077.296	1.844611356
kc25	10346.439	24994.853	2.415792815
kc50	7445.004	17468.489	2.346337087
kc100	10346.439	30980.267	2.994292722

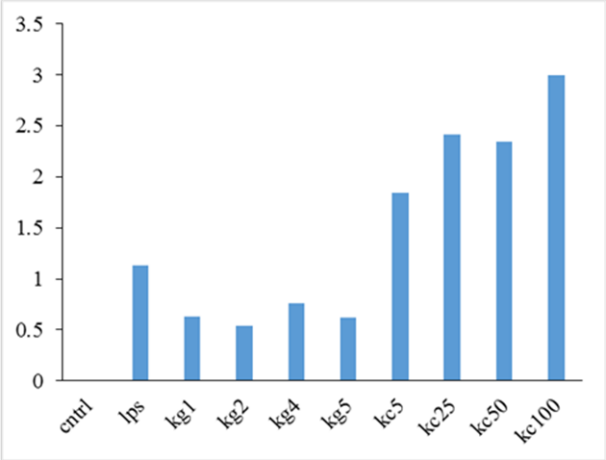


Figure 6: Western blot analysis results of sulfated galactan and carrageenan obtained upon calculating ratio Cox2/GAPDH. Note: kg is sulfated galactan and kc is carrageenan.

IV. DISCUSSION

In the present study the polysaccharides such as sulfated galactan, and carrageenan were extracted from red seaweed *Tricleacarpa fragilis* and characterized through FT-IR and NMR analysis and their anti-proliferative and anti-inflammatory effects were studied.

The extraction yield of sulfated galactan was 1.7%. Those results are consistent with previous results. According to the literature, the content of sulfated galactan isolated from the red algae *Pterocladia* was 2.7%[5] and from *Corallina* was 2.5%.[8] For carrageenan, the extraction yield was 20%. Another studies showed the content of carrageenan extracted from other species of red algae collected from Lebanese coast, *Pterocladia and Corallina*, was 11.5% and 10% respectively.[8] This difference can be due to environmental factors and different species taking into consideration the significant content of carrageenan present in *Tricleacarpa fragilis.*

To determine the proton position in sulfated galactan and carrageenan, ^{1}H NMR analysis was performed. The spectrum of sulfated galactan and carrageenan showed a single signal at 1.08 ppm and 0.95 ppm respectively attributed to a proton methylene CH2 and another single signal at 3.41 which may be due either to the presence of O-CH3 bond or is assigned to the β unit. But these spectra do not show signals between 4.4 and 5.4 ppm relative to the region of anomeric sugars (11) nor the signal at 5.2 ppm corresponding to the unit α.[7] This seems to be related to the temperature applied during the analysis and which should be set at around 65˚C and even higher. In fact, temperature affects positively the viscosity reducing the height of the peaks and shifting the solvent resonance.[5]

For the anti-proliferative effect, the human colorectal cancer cell lines (HT 29 and HCT 116) were treated with our samples for 24, 48, and 72 hours. Sulfated galactan was found to have a more potent anti-proliferative effect compared to carrageenan with both cell lines and at the different time intervals. In addition, sulfated galactan was able to decrease the % of cell viability by 100%, and HT 29 was more resistant than HCT 116. Several investigations have reported that sulfated polysaccharides have antiproliferative activity in cancer cell lines in vitro, as well as inhibitive activity in tumors growing in mice. Moreover, *Gracilaria caudate*, among the Rodophyta division proved to have sulfated polysaccharides with antiproliferative properties.[7] Furthermore, three red algae *Sphaerococcus coronopifolius*, *Asparagopsis armata* and *Plocamium cartilagineum collected* from Portugal coast showed interesting results on human hepatocellular carcinoma (HepG-2 cells). These 3 red algae had the highest antiproliferative effects on HepG-2 cells compared to other studied algae.[12] It has been reported that the polysaccharide bioactivities of seaweeds are closely related to several structural parameters, such as the degree of sulfation, the molecular weight, the sulfation position, type of sugar and glycosidic branching.[13] So, the high anti-proliferative effect of the sulfated galactan may be explained by their unique structure including high level of sulfation as well as their distribution; however, these structural properties were absent in carrageenan.

For the anti-inflammatory effect, RAW 264.7 cell was used and inflammation was induced using LPS, and our samples were added. The results showed that sulfated galactan had a potent anti-inflammatory effect with the different concentrations used, while carrageenan did not show any anti-inflammatory effect and it was found to induce inflammation. A study showed that sulfated galactan extracted from the red marine algae *Gelidium crinale* showed anti-inflammatory as well as antinociceptive activities in a rodent model. Another red seaweed *Gracilaria cornea* was proved to have sulfated polysaccharides effective in reducing inflammation and nociception in mice models. Carrageenan from red marine algae is known to be a potent inflammatory agent in rodents; primes mice leucocytes to produce tumor necrosis factor-alpha (TNF-alpha) in response to bacterial lipopolysaccharide Moreover, some types of carrageenans induce potent macrophage activation, while some carrageenans and fucoidan appear to inhibit macrophage function.[14] Thus, carrageenans have two possible actions, either they can reduce inflammation or induce inflammation which was our case.

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V. CONCLUSION

In this context, *Tricleacarpa fragilis*, one of the red algae have been studied. In the present study, the yield of sulfated galactan was 1.7% and that of carrageenan 20%. The proton position and the functional groups of both

polysaccharides were determined. They have anti-proliferative effect against human colorectal cancer cells (HT 29, HCT 116); sulfated galactan was found to be more potent than carrageenan at different time intervals (24, 48, and 72 hours) and HT 29 was more resistant to treatment than HCT 116. For the anti-inflammatory effect, sulfated galactan was found to inhibit inflammation, while carrageenan induced it. This study once again allows the development of the exploitation of polysaccharides extracted from *Tricleacarpa fragilis* growing on the Lebanese coast in the medicinal field, in order to develop molecules with novel modes of action to face and fight emerging diseases, and substitute the synthetic drugs that are known to have multiple toxic effects. At the same time, it confirms that they can be used, after further analyses in the fields of pharmacy and food industry.

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