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Characterization of Sulphated Polysaccharide with Antiviral Activity from Marine Brown Alga *Cystoseira myrica* Collected from Jazan Coasts, KSA

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Abstract: The hot water extract of brown alga *Cystoseira myrica,* collected from Jazan coasts, was fractionated into a neutral fraction NP and a sulphated hetero-polysaccharaide SP. Based on the antiviral activity SP was fractionated by ion exchange chromatography on DEAE cellulose column into SP-1, SP-2 and SP-3 with molecular weight of 115 kDa, 90 kDa and 60 kDa on Sephacryl S-300 column, respectively. Fucose was the major sugar in SP-1. The fraction SP-1 showed a marked antiviral activity against both HSV and HAV. Its inhibition effect against HSV was 52.4 % where against HAV was 53.8 % at concentration of 10 µg/ml**,** where was 62.2 and 66.15 at concentration of 20 µg/ml respectively.
Key words: *Cystoseira myrica*, sulphated polysaccharides, antiviral, HSV, HAV.

Introduction

Significant amounts of seaweed derived polysaccharides are used in food, pharmaceuticals and other products for human consumption. Thus; the global seaweed polysaccharide industry operates in a highly regulated environment[1] . Over the last decade, bioactive sulfated polysaccharides isolated from brown seaweeds have attracted much attention in the fields of pharmacology and biochemistry. Functional polysaccharides such as fucans and alginic acid derivatives produced by brown seaweeds are known to exhibit different biological properties including anticoagulant, anti-inflammatory, antiviral and antitumor activities[2-4]. The ability of sulfated polysaccharides from seaweeds to inhibit the replication of enveloped viruses including herpes simplex virus (HSV) [5,6]. human immunodeficiency virus (HIV), human cytomegalovirus, dengue virus and respiratory syncytial virus is well established[7]. The original observations on antiviral activities of seaweed constituents go back more than 50 years to the observation that seaweed extracts protected chicken embryos against influenza B and mumps[8]. It was discovered a little later, somewhat serendipitously, that heparin inhibited HSV in leukocyte cultures[9]. An effect hypothesized to be due to electrostatic interference with viral attachment to the cell surfaces. This spurred research into the antiviral effects of various poly- anionic substances including sulfated polysaccharides from a number of seaweed species. In addition, polysaccharides were capable of inhibiting the *in vitro* replication of *Herpes simplex* virus type 1 (HSV-1) on Vero cells values of EC50 of 4.1 and 17.2 µg/mL, respectively[10]. The aim of our work is to study the antiviral activity of sulphated polysaccharide extracted from marine brown alga *Cystoseira myrica.*

Materials and Methods

Biomass collection and pretreatment

The brown alga, *Cystoseira myrica,* is widely spread on Jazan coasts and common in all seasons. It was collected during summer (2015) from red sea, Jazan, coasts KSA. It was cleaned from epiphytes then washed several times with running tap water then with distilled water, sun dried, grinded and kept in dry place till used[5]. Algal powder was depigmented using sequential extraction with petroleum ether and acetone. The residual material was air dried to yield depigmented algal powder (DAP) [11].

Extraction techniques and chemical analysis method.

Polysaccharides from seaweed *Cystoseira myrica* (DAP 100gm) were extracted in hot distilled water (1.5 L) at 80 °C for 8 h with magnetic stirring. Insoluble residues were eliminated by filtration and centrifugation (20 min, 30,000 g). The supernatant was poured into 2 volumes of absolute ethanol during one night at 4 °C. The precipitate were recovered and washed by absolute acetone, dried overnight at 50 °C, weighed and ground to a powder[12,13].

Fractionation of the crude extract

The crude extract was treated with 10% cetyl trimethyl ammonium bromide (CTAB) solution. The precipitate formed was centrifuged (30 min, 5000 rpm), washed with water and stirred with 20% ethanolic KI solution (3 x 50 ml). After washing with ethanol the precipitate was dissolved in water, dialyzed exhaustively and lyophilized to give SP. The supernatant was dialyzed, concentrated, and diluted with 4 volumes of ethanol. The precipitate formed was then dissolved in water and lyophilized to give a neutral polysaccharide (NP). The crude extract and the two fractions, SP and NP, were subjected to antiviral assay.

Ion exchange chromatography (IEC)

The crude extract SP (100mg in 5ml water) was subjected to a column ion exchange chromatography (2.0 x 40 cm) of DEAE cellulose, pre-equilibrated using phosphate buffer (0.1M) and eluted with continuous gradient of NaCl solution from 0.0 to 4.0M in the same buffer[14]. Fractions (5 ml) were collected and analyzed with phenol-H_2SO_4 reagent at 490nm using UV-VIS spectrophotometer, 2401PC Shimadzu[15]. The respective polysaccharide fractions were pooled and dialyzed overnight against deionized water and lyophilized. Each fraction was subjected to gel permeation**.**

Molecular weight determination of the fractions

The average molecular weight of the sulphated fractions were determined by a size exclusion chromatography (SEC). Standard dextrans (40, 500, 700 and 2000 KDa, Fluka Chemical Co., Bush, Switzerland) were passed through a (2.5 × 60 cm) Sephacryl S-300 column, and then the elution volumes were plotted against the logarithm of their respective molecular weights[16].

Desulphation of polysaccharide

The polysaccharide fractions (150 mg) were treated with 2% MeOH/HCl (20 ml) for 72 h at 25ºC and then dialyzed against deionized water followed by freeze drying[17].

Sulphate estimation

The polysaccharide fractions (100 mg) were hydrolyzed in a sealed tube with 1 ml HCl (1 M) at 85^{O}C for 24 h. Then it was neutralized to pH 7.0 with NaOH solution and diluted to 10 ml with distilled water. The sulphate content was determined by modified barium chloride method[18].

Sugar analysis of the fractions.

Total sugars were determined by the phenol-H_2SO_4 reagent[11], and *m*-hydroxydiphenyl[19], respectively. The polysaccharide fractions were hydrolyzed with 2 M trifluoroacetic acid in a sealed tube. Hydrolysis was carried out at 105 ºC for 2 h. After the hydrolysis, the acid was removed by flash evaporation on a water bath at

a temperature of 40 ºC and co-distilled with water (5 ml × 3) [20]. The purified hydrolyzates (20 µl) were analysis by HLPC as described before[21].

Infra red spectroscopy

The polysaccharide fractions were characterized using a Fourier transform infrared in Burcker Vector 22-spectrophotometer. The dried polysaccharides were ground with KBr powder and pressed into pellets for FT-IR spectra measurement in the frequency range of 100- 4000 cm^{-1} [18].

Antiviral activity by Plaque Reduction Assay.

The polysaccharide was dissolved as 10 mg in 1mL of 10% DMSO in water. The final concentration was 10µg/µL (Stock Solution). The dissolved stock solution was sterilized by the addition of 10 µg/m antibiotic antimycotic mixture (10 U penicillin G sodium, 10µg streptomycin sulfate and 250 µg amphotericin B) [22]. A 6-well plate was cultivated with Vero cell culture (105cell/mL) and incubated for 2 days at 37°C. HAV and HSV were diluted to give 104 (Plaques Forming Unit) PFU/mL final concentrations and were mixed with the tested polysaccharide at two concentrations and incubated 1 h at 40 °C. Growth medium was removed from the multi well plate and virus- polysaccharide mixture was inoculated (100 µL/well). After 1 h of contact time, the inocula were aspirated and 3 mL of DMEM with 1% agarose was overlaid on the cell sheets. The plates were left to solidify and incubated at 37 °C until the development of virus plaques occurred. Cell sheets were fixed in 10% formalin solution for 2 h, and stained with crystal violet stain. Control virus and cells were treated identically without polysaccharide. Virus plaques were counted and the percentage of reduction was calculated.

Results and Discussion

Extraction and fractionation of sulphated polysaccharide from *Cystoseira myrica*

Extraction of crude water soluble polysaccharides from *Cystoseira myrica* yielded approximately 5.3% of dried algal tissue collected at summer. The crude polysaccharide was used to evaluate the antiviral activity. The sulphated polysaccharide was fractionated by ion exchange column chromatography using DEAE-cellulose column and eluted with phosphate buffer containing NaCl (0.0-4.0 M) gradient. The sulphated polysaccharide was separated into three fractions, SP-1, SP-2 and SP-3. SP-1 was eluted with NaCl solution varied from 0.17 to 0.86 M whereas; SP-2 and SP-3 were eluted with NaCl varied from 1.25 to 2.05 M and 2.48 to 2.96 M, respectively (Fig.1). The three fractions were purified by gel chromatography on Sephacryl S-300 column. Moreover, the average molecular weights of SP-1, SP-2 and SP-3 were determined as 115 kDa, 90 kDa and 60 kDa respectively.

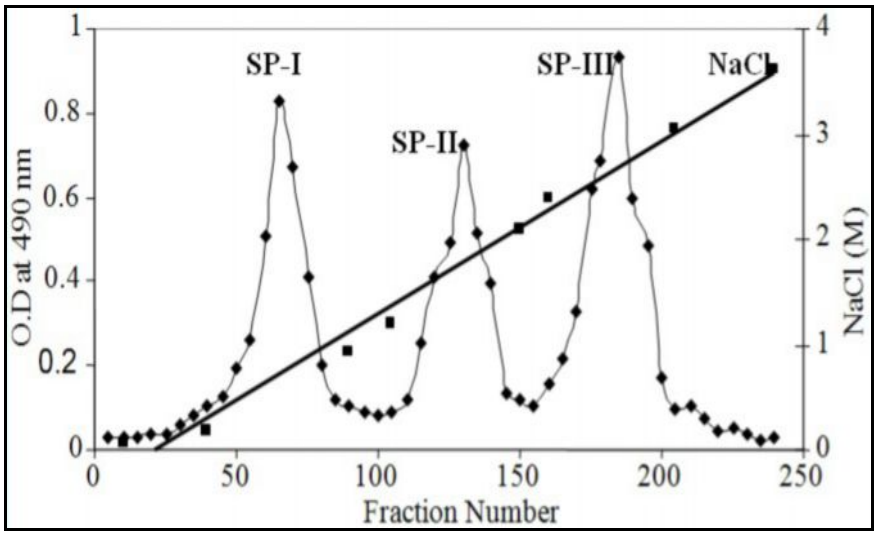

Fig. 1: A typical elution profile of the acidic polysaccharides on DEAE-cellulose column (2.0 x 50 cm, i.d.) previously equilibrated with 0.01M sodium phosphate buffer. A flow rate 0.6ml/min and 5.0 ml fractions were maintained.

Sulphate determination and compositional analysis of the crude sulphated polysaccharides SP and its fractions.

All polysaccharide fractions were hydrolyzed and subjected to HPLC analysis to determine the components and its molar rations as represented in Table 1. It was noticed that the faction SP-1 is the fraction with the highest sulphate content and containing the highest molar ration of fucose.

Table 1: Sulphate percentages, monosugar molar rations of sulphated polysaccharide fractions obtained from HPLC.

Fraction	SO_4^- %	Molar ratios			
		Fucose	Xylose	Glucose	Galactose
SP (crude extract)	22.3	11.2	1.9	6.3	2.8
SP-1	31.8	9.1	1.2	2.0	2.3
SP-2	12.6	6.1	1.32	4.8	1.5
SP-3	9.2	3.2	0.8	3.6	0.9

In vitro antiviral activity

The crude algal extracts as well as its two main fractions NP and SP were subjected to antiviral assay. The results showed that the crude extract showed a limited activity against both HSV and HAV where the neutral fraction showed minimum activity against HSV and no any activity against HAV, as represented in Table 2, while the fraction SP showed moderate activity against both HSV and HAV. Depending on this result the fraction SP was subjected to further fractionation for enrichment of the antiviral activity of the sulfated polysaccharide. The three fraction, SP-1, SP-2 and SP-3 showed variation in activity where the first fraction, SP-1 showed the highest antiviral activity than SP-2 and SP-3 where it showed inhibition percentage of 52.4 against HSV and 53.8 % against HAV at concentration of 10 µg/ml which elevated to 62.2 and 66.15% against HSV and HAV at concentration of 20 µg /ml respectively. An explanation of these results may be related to the higher sulphate ester groups and high molecular weightof SP-1 than the other fractions. In general, the antiviral activity of sulphated polysaccharides increases with the degree of sulphate and molecular weight[23].

Table 2: Antiviral activity of polysaccharides extracted from *Cystoseira myrica* against HSV and HAV.

Extract	Conc µg/ml	HSV			HAV		
		Initial virus (PFU/ml)	Final virus (PFU/ml)	Inhibition %	Initial virus (PFU/ml)	Final virus (PFU/ml)	Inhibition %
Crude	10	$0.82X10^6$	$0.79X10^6$	3.7	$1.3X10^6$	$1.29X10^6$	0.76
	20	$0.82X10^6$	$0.76X10^6$	7.3	$1.3X10^6$	$1.25X10^6$	3.8
NP	10	$0.82X10^6$	$0.81X10^6$	1.2	$1.3X10^6$	$1.3X10^6$	--
	20	$0.82X10^6$	$0.81X10^6$	1.2	$1.3X10^6$	$1.3X10^6$	--
SP	10	$0.82X10^6$	$0.62X10^6$	20.73	$1.3X10^6$	$0.95X10^6$	26.9
	20	$0.82X10^6$	$0.61X10^6$	25.6	$1.3X10^6$	$0.8X10^6$	38.46
SP-1	10	$0.82X10^6$	$0.39X10^6$	52.4	$1.3X10^6$	$0.6X10^6$	53.8
	20	$0.82X10^6$	$0.31X10^6$	62.2	$1.3X10^6$	$0.44X10^6$	66.15
SP-2	10	$0.82X10^6$	$0.53X10^6$	35.4	$1.3X10^6$	$0.72X10^6$	44.6
	20	$0.82X10^6$	$0.5X10^6$	39.02	$1.3X10^6$	$0.63X10^6$	51.5
SP-3	10	$0.82X10^6$	$0.75X10^6$	8.5	$1.3X10^6$	$1.1X10^6$	15.3
	20	$0.82X10^6$	$0.73X10^6$	11	$1.3X10^6$	$1.1X10^6$	15.3

Fourier transforms infra red spectroscopy (FTIR)

FTIR spectra of SP-1, SP-2 and SP-3 were similar and showed an intense band of absorption at 1040-1250 Cm^{-1} indicating the presence of sulfate ester[24]. Two other bands 791 and 852 Cm^{-1} arising from the sulfate group of polysaccharides were also observed.

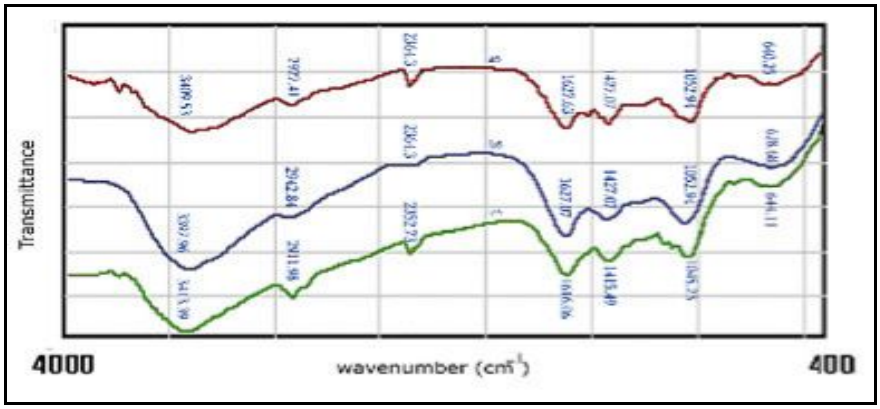

Figure 2: Infrared spectra of sulphated polysaccharides SP-1, the lower band SP-2, the middle band and SP-3 the higher band from *Cystosiera myrica*

Conclusion

Marine brown alga (cy*stosiera myrica)* showed an excellent source of natural antiviral polysaccharides. The antiviral activity of the polysaccharides increased after fractionation of the crude extract where the fraction SP-1 showed the highest activity on both type of virus HSV and HAV with activity of 52.4 and 53.8 % respectively at 10 µg/ml. Also this fraction showed the highest content of fucose sugar and sulphate content. The other two fractions, SP-2 and SP-3, showed lower activity. Our recommendation is that more research must be focused on the natural biologically active agents from marine algae.

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