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IN VITRO ANTIOXIDANT AND ANTITUMOR ACTIVITY OF POLYSACCHARIDE ISOLATED FROM *ULVA FASCIATA*

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ABSTRACT

A water soluble polysaccharide was extracted from the edible green algae *Ulva fasciata* and isolated by Gel filtration chromatography. The antioxidant activity of the purified polysaccharide fraction was evaluated *in vitro* by superoxide radical scavenging assay, lipid peroxidation inhibition assay, hydroxyl radical scavenging assay and nitric oxide scavenging assay. The trypan blue exclusion method was used to study the antitumor activity of the polysaccharide on Daltons Lymphoma Ascites (DLA) cells. The results indicated that the polysaccharide from *Ulva fasciata* has potent antioxidant and antitumor activity.

KEY WORDS : *Ulva fasciata*, Polysaccharide, anti oxidant and antitumor.

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INTRODUCTION

The green macroalgal genera *Ulva* and *Enteromorpha* are widely distributed from marine to fresh water all over the world [1,2]. The cosmopolitan genus *Ulva* Linnaeus, commonly known as the "sea lettuce", is represented by species distributed in all oceans and estuaries of the world [3]. *Ulva* sps is rich in cell-wall polysaccharides, including cellulose and water-soluble polysaccharides that contain sulphate groups. Sulphated polysaccharides from marine algae are known to exhibit many biological and physiological activities including anticoagulant, antihyperlipidemic, antiviral, antitumor and antioxidant activities [4,5,6,7.]

Antioxidant activity has become a subject of intensive investigations due to the ever increasing demand by the pharmaceutical industries to develop natural bioactive anti-aging and anticarcinogenic compounds that demonstrate measurable health benefits. Many synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, *t*-butyl-hydroquinone and propyl gallate may be used to retard lipid peroxidation in many fields [8]. However, the use of synthetic antioxidants is under strict regulation due to their potential health hazards [9,10]. Therefore, the search for natural antioxidants as alternatives to synthetic ones is of great interest. Marine seaweeds characteristically contain sulphated polysaccharides that are not found in land plants. However, there are very few studies in the literature on antioxidant activity associated to sulphated polysaccharides from seaweeds. In recent years, the antioxidant activities of several polysaccharides have been described. The polysaccharide from fungus *Keissleriella* sp. could scavenge superoxide radicals very effectively[11]. In this chapter the *in vitro* antioxidant and antitumor properties of polysaccharide isolated from *Ulva fasciata* was studied. The antitumor effect was confirmed by the trypan blue exclusion method.

MATERIALS AND METHODS

(i) Plant material and reagents

The study area was located along the 45 km long southwest coast of India (Indian Ocean), between latitude 08°54' N and longitude 76°38' E. Seaweed specimens were collected from the intertidal and subtidal habitat of Kollam prefecture (Thirumullavaram, Kerala, India) located on the southwest coast. The algal material was identified as *Ulva fasciata*. The seaweeds were washed thoroughly with tap water, dried in air and powdered. The powdered sample was defatted and depigmented by sequential extraction with petroleum ether, chloroform and acetone as solvents in a Soxhlet apparatus. DLA cells were originally obtained through courtesy of Amala Cancer Research Centre, Thrissur, Kerala, India. They were maintained by weekly intraperitoneal inoculation of 10^6 cells/mouse. All solvents and chemicals were of analytical grade and purchased from SRL chemical.

(ii) Extraction and purification of polysaccharide

Powdered algal sample (40 g) was stirred with 800 ml of distilled water for 3 h. The temperature of the extraction was adjusted as 90 °C. Separation of the residue from the aqueous extract was performed by centrifugation at 8000 ×g for 15 min. The pellet was re-extracted in a similar way. The supernatants were combined and dialysed extensively against water. The polysaccharides were precipitated with twice the volume of 95 % alcohol. The precipitate was collected by centrifugation at 10000 ×g for 20 min. The collected precipitate was washed with absolute alcohol and lyophilised. The precipitate obtained by the extraction procedure was re-dissolved in water and the protein content was removed by Sevag method[12]. The aqueous phase was recovered and dialysed against distilled water. The

polysaccharides were recovered by precipitation with absolute ethanol. The collected precipitate was washed and lyophilised. Partially purified polysaccharide was further purified by gel filtration chromatography on Sephacryl S-400. Distilled water was used as the mobile phase and the elution was done at 60 °C. The flow rate was adjusted to 1 ml/min. The fractions were collected and analysed by Phenol-sulphuric acid method [13]. The purified fractions were pooled and polysaccharides were reprecipitated with absolute ethanol and the fraction with high carbohydrate content (UPF 1) was lyophilised and used for further experiments.

(iii) Superoxide radical scavenging assay

Assay is based on the ability of the polysaccharide to inhibit or scavenge the superoxide radical generated from the photo reduction of riboflavin[14].The reaction mixture contained, EDTA (6 mM) contained 3 µg NaCN; riboflavin (2 µM); NBT (50 µM); KH_2PO_4-Na_2HPO_4 buffer (67 mM, pH 7.8) and various concentrations of the polysaccharide in a final volume of 3 ml. The tubes were illuminated under the incandescent lamp for 15 min. The optical density (O.D) at 560 nm was measured before and after illumination against distilled water. The inhibition of the superoxide radical generation was determined by comparing the absorbance value of the controls and treated.

(iv) Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity was determined by studying the competition between deoxyribose and the polysaccharide for the hydroxyl radicals generated from Fe3+ ascorbate-EDTA-H_2O_2 system (Fenton's reaction). The hydroxyl radical attacked deoxiribose, which eventually result in a TBARS. The TBARS thus formed was measured [16]. The reaction mixture contained deoxiribose (2.8 mM); $FeCl_3$ (0.1 mM); K2HPO4-KOH buffer (20 mM, pH 7); EDTA(0.1 mM); H_2O_2 (1.0 mM); ascorbic acid (0.1 mM) and various concentrations of the polysaccharide in a final volumes of 1 ml. The reaction mixture was incubated at 30 °C for 60 min. The TBARS formed was estimated [16]. The hydroxyl radical scavenging activity was determined by comparing absorbance of control with that of treated. Catechin was used as standard.

(v) Nitric oxide scavenging assay

The nitric oxide scavenging activity of the polysaccharide was measured [17]. Immediately before the experiment, 10 mM stock solution of sodium nitroprusside was prepared in PBS (pH 7.4). Various concentrations of polysaccharide and sodium nitroprusside (1 mM) in a final volume of 3 ml were incubated at 25 °C for 150 min. After incubation, 2.5 ml of reaction solution was removed and mixed with 0.5 ml of Griess reagent (1 % sulphanilamide, 2 % orthophosphoric acid and 0.1 % naphthylethylenediamine dihydrochloride). The absorbance of the chromophore was read immediately at 546 nm against reagent blank. The nitric oxide scavenging activity was determined by comparing the absorbance of control with that of treated. Production of nitrite from solution of 1mM sodium nitroprusside solution incubated in the presence and absence of polysaccharide at various time intervals (50, 100 and 150 min) were also studied and compared to the absorbance of standard solutions.

(vi) Lipid peroxidation inhibition assay

Lipid peroxidation was induced by Fe^{2+} ascorbate system[15] in the rat liver homogenate in the presence and absence of polysaccharide to form TBARS and is measured[16]. The reaction mixture contained 0.1 ml of rat liver homogenate (25 % v/v) in Tris-HCl buffer 20 mM, pH 7); KCl (30 mM); $FeSO_4$ $(NH_4)2SO_4.6H_2O$ (0.16 mM); ascorbate (0.06 mM) and various concentrations of polysaccharide in a final volume of 0.5 ml. The reaction mixture was incubated for 1 h at 37 °C. After the incubation period, 0.4 ml of the reaction mixture was treated with 0.2 ml SDS; 1.5 ml thiobarbituric acid (0.8 %) and 1.5 ml acetic acid (20 %, pH 3.5). The total volume was made up to 4 ml by distilled water and then kept in a water bath at 95-100 °C for 1 h. After cooling 1 ml distilled water and 5 ml of n-butanol were added and centrifuged at

4000 rpm for 10 min. The organic layer was removed and its absorbance at 532 nm was measured against n-butanol-pyramidine mixture. Inhibition of lipid peroxidation was determined by comparing the optical densities by using Catechin as the standard.

(vii) Cytotoxicity assay

The *in vitro* short term cytotoxicity of the polysaccharide was assayed using DLA cell lines. Viable cells of each cell line were washed with phosphate buffered saline (pH 7.4) and centrifuged for 15 min at 1,500 rpm separately. The pellets were resuspended with PBS and the process was repeated three times. 1×10^6 viable cells of each cell lines were suspended in 0.1 ml of PBS, various concentrations of the polysaccharide(10-100 µl/ml) and phosphate buffer in a final volume of 1ml separately and incubated at 37 °C for 3 h after the incubation, the viability of the cells were determined by trypan blue exclusion method[18]. Percentage viability and the IC_{50} values were calculated.

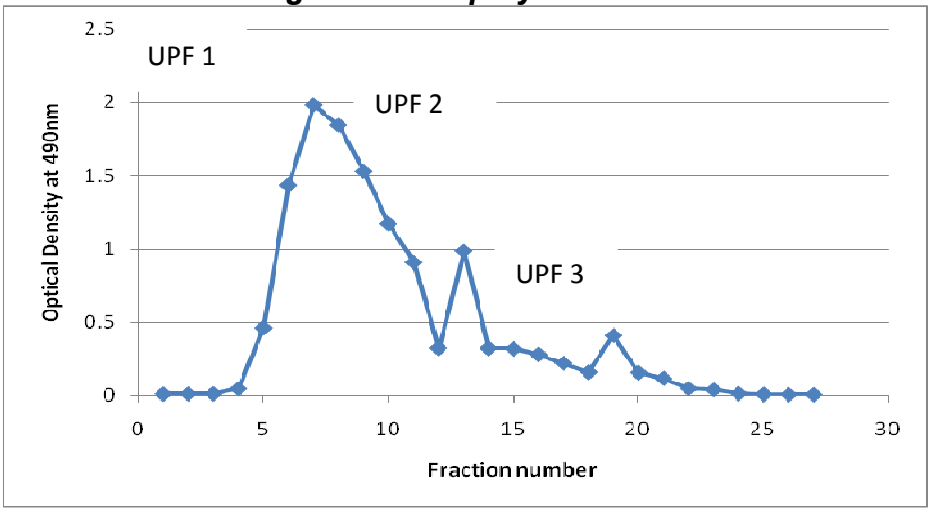
RESULTS

1. Polysaccharide extraction and purification :

The polysaccharide was extracted with hot water and the yield was found to be 23 % of algal dry weight. Partially purified polysaccharide was further purified by gel filtration chromatography with hot water as the mobile phase. The gel filtration chromatogram generated three peaks (Graph 1). Fractions obtained were pooled separately and carbohydrate content was analysed by Phenol-Sulphuric acid method. Fraction with high polysaccharide content (UPF1) was recovered.

Graph 1

Gel filtration chromatogram of the polysaccharide from Ulva fasciata



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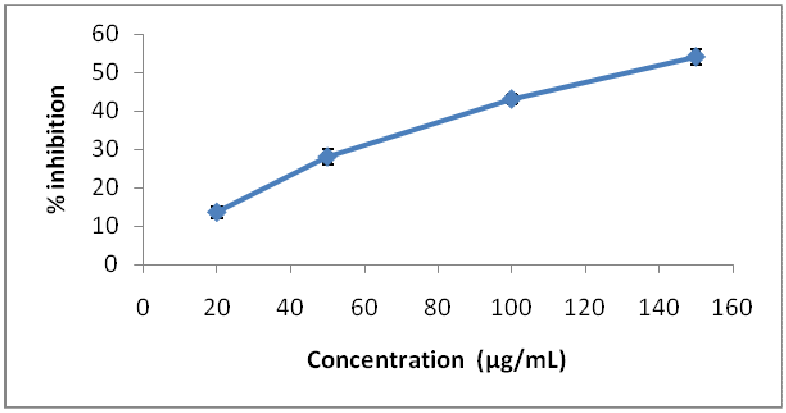
2. *Superoxide radical scavenging assay*

The Super oxide radical scavenging activity of the polysaccharide is given in Graph 2. The polysaccharide showed significant superoxide radical scavenging activity.

Graph 2
Superoxide radical scavenging activity of the polysaccharide



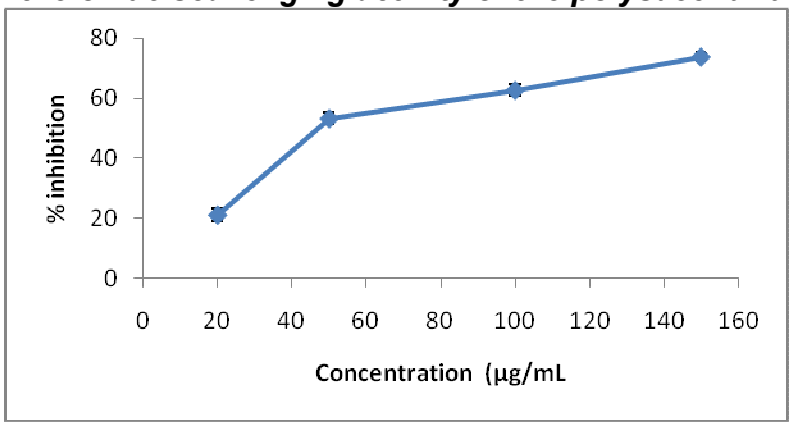
Data are mean±SD of three values

The concentration of the polysaccharide required to scavenge 50 % superoxide anion generated (IC_{50}) by the photo-reduction of riboflavin was found to be 132±.05 µg/ml and that of reference drug Quercetin was found to be 3.92±.01 µg/ml.

3. Nitric oxide scavenging assay:

The nitric oxide radical scavenging activity of the polysaccharide is given in Graph 3. Polysaccharide inhibited the nitric oxide released from the sodium nitroprusside.

Graph 3
Nitric oxide scavenging activity of the polysaccharide



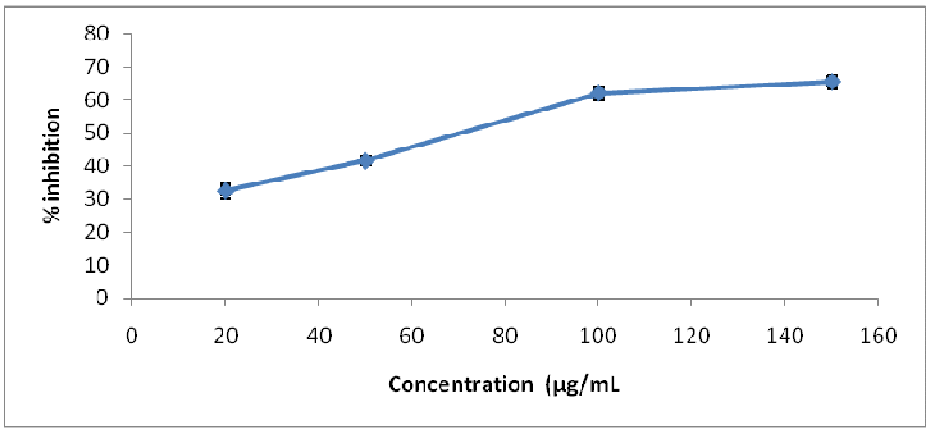
Data are mean±SD of three values

The IC_{50} for polysaccharide and reference drug Quercetin were 46±1.03 and 165.98±.652 µg/ml respectively.

4. Hydroxyl radical scavenging assay:

The hydroxyl radical scavenging activity of the polysaccharide is given in Graph 4. The degradation of deoxyribose to TBARS by hydroxyl radical generated from Fe^{3+}-ascorbate-EDTA-H_2O_2 system was markedly decreased by the polysaccharide.

Graph 4
Hydroxyl radical scavenging activity of the polysaccharide



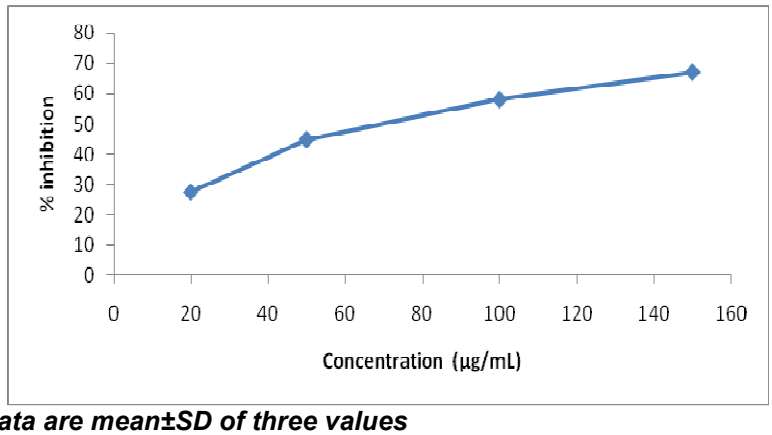
Data are mean±SD of three values

The IC_{50} was found to be 70±.9 for polysaccharide and 85.2±.9 µg/ml for reference drug Catechin.

5. Lipid peroxidation inhibition assay :

The inhibition of lipid peroxidation by the polysaccharide is given in Graph 5. Polysaccharide isolated from *Ulva* was effective in inhibiting the lipid peroxidation induced by Fe^{2+} ascorbate system in rat liver homogenate.

Graph 5
Inhibition of lipid peroxidation by polysaccharide



Data are mean±SD of three values

The IC_{50} of the polysaccharide and reference drug Catechin were 68±1.8 and 429.31±3.1 µg/ml respectively.

The *in vitro* antioxidant activity of the isolated polysaccharide was studied and the IC_{50} were calculated. The results are given in Table1.

Table 1
In vitro* antioxidant activity of polysaccharide isolated from *Ulva fasciata

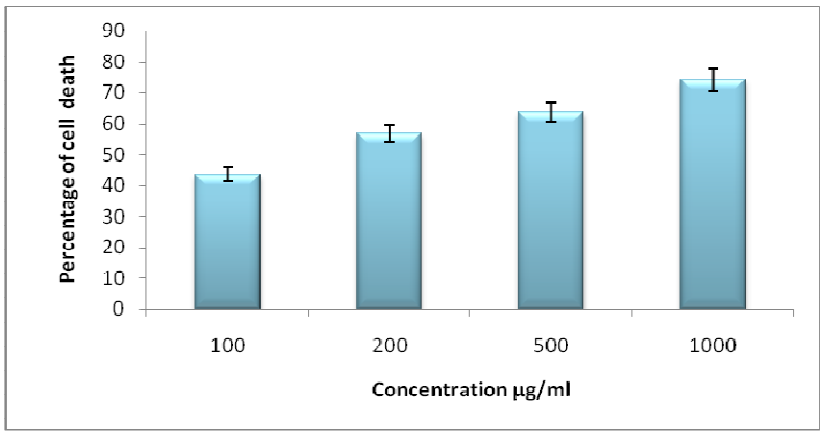
Assay	Polysaccharide IC_{50} (µg/ml)	Reference drug IC_{50} (µg/ml)	
		Catechin	Quercetin
Superoxide radical scavenging	132±.05	-	3.92±1.8
Nitric oxide scavenging	46±1.03	-	165.98±1.8
Hydroxyl radical scavenging	70±.9	85.2±4.56	-
Lipid peroxidation inhibiting	68±1.8	429.31±3.1	-

Values are expressed as mean±SD

6. Cytotoxicity screening by trypan blue exclusion method :

The result for trypan blue exclusion method for DLA cell lines is shown in Graph 6. Polysaccharide showed marked cytotoxicity for DLA cell lines. The concentration required for 50 % cell death IC_{50} was found to be 220 µg for DLA cell lines.

Graph 6
Effect of polysaccharide on Dalton's Lymphoma Ascites (DLA)



Data are mean ± SD of three values

DISCUSSIONS

The polysaccharide fraction UPF 1 was isolated by the gel filtration chromatography of the hot water extracted polysaccharide from *Ulva fasciata*. The antioxidant activity of polysaccharide isolated from *Ulva fasciata* indicated that the *in vitro* free radical scavenging activity was concentration dependent. The exact mechanism for radical scavenging activity is unknown and it may be due to the direct scavenging of superoxide anion generated from photo illumination of riboflavin.

In addition to the Reactive Oxygen Species, nitric oxide is also implicated in inflammation, cancer and other pathological conditions [17].The polysaccharide from *Ulva fasciata* showed a significant nitric oxide scavenging activity.

Recently, the antioxidant activity of polysaccharides from the chlorophyte *Ulva pertusa* was also investigated. All of the compounds analyzed showed that molecular weight (MW) had a significant effect on antioxidant activity [19]. The sulphated polysaccharides from *Laminaria japonica* and *Ecklonia kurome* showed free radical scavenging activities; fucans from *F. vesiculosus* exhibited considerable ferric reducing/antioxidant power and superoxide radical scavenging ability[20,21]. Superoxide radical scavenging activity correlated positively with the sulphate content of the polysaccharide fractions. Antioxidant properties of carrageenans and Ulvans also

related to sulphate content. Sulphated polysaccharides from *Ulva pertusa* were also demonstrated to have antioxidant activity including scavenging activity against superoxide, hydroxyl radicals, chelating ability, reducing power etc.

The results of present investigation revealed that polysaccharide with wide spectrum of bioactivities had cytotoxicity against DLA cell lines. The polysaccharide exhibited a concentration dependent cytotoxicity against mice cell lines. Cytotoxicity was one of the chemotherapeutic targets of antitumor activity [22].

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