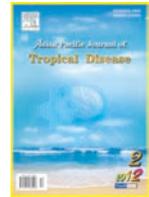




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In vitro antioxidant properties of sulfated polysaccharide from brown marine algae *Sargassum tenerrimum*

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ABSTRACT

Objective: In the present study the physico chemical characteristics, total antioxidant capacity (TAC), reducing power and the free radical scavenging potentials (DPPH radical, ABTS, H₂O₂ radical) of sulfated polysaccharide from marine brown algae *Sargassum tenerrimum* was investigated. **Methods:** The *Sargassum tenerrimum* seaweed, which have wide pharmaceutical application, were collected from the coastal region of Mandapam (Lat 09° 17'N, Long 79° 07'E), Tamil Nadu, India and evaluated for *In vitro* antioxidant properties. **Results:** The extract showed higher percentage of carbohydrate (8.20±1.23%) followed by sulphate (6.6±1.42%) and protein (0.86±0.42%). The free radical scavenging potential was found to be higher in ABTS (70.33±2.33%) followed by DPPH (64.66±2.08%) and H₂O₂ (61.56±2.05%). the TAC was found to be 62.55±1.40%. The characterization of sulfated polysaccharide by FT-IR spectrum showed the presence of carboxyl, hydroxyl and sulfate groups. The structure of mobility was assed by agarose gel electrophoresis which showed highest mobility at higher pH values especially in buffer carbonate–bicarbonate (pH 10). The molecular weight of the sulfated polysaccharide was determined by gradient polyacrylamide gel electrophoresis which was found to be 40 kDa. Finally, GC–MS analysis of sulfated polysaccharide from *S. tenerrimum* exhibited peaks corresponding to Benzenamine (31.67%) and Aminocarb (21.45%). The overall results have established that the sulfated polysaccharide from *S. tenerrimum* could be used as a promising antioxidant agent. **Conclusion:** Physico–chemical analysis and elemental analysis of crude seaweed polysaccharide from *Sargassum tenerrimum* a brown algae elicited the antioxidant activity.

1. Introduction

The Ocean, which is called the “Mother of origin of life”, is a source of structurally unique natural products that are mainly accumulated in living organisms. It is not truly known how many species inhabit the world’s oceans; however, it is becoming increasingly clear that the number of microbial species is many times larger than previously estimated, such that total marine species may approach 1 to 2 million. As a result of this intense competition, a high percentage of species have evolved chemical means by which to defend against predation, these chemical adaptations generally take the form of so called “Secondary metabolites” and involve

such well known chemical classes as terpenoids, alkaloids, polyketoides, peptides, sugars, steroids, polysaccharides and a multitude of mixed biogenesis metabolites. Several of the marine compounds show pharmacological activities and are helpful for the invention and discovery of bioactive compounds, primarily for deadly diseases like antioxidants, cancer and metastasis.

Free radicals are independent chemical species with one or more unpaired electrons. These include oxy–radicals, oxygen free radicals and various permutations and combinations of them. Together they are called as the reactive oxygen species (ROS). Due to the presence of one or more unpaired electrons, these species are paramagnetic which make them highly reactive[1]. Therefore it can accept electrons from other atoms or molecules, this process is known as oxidation and free radicals are known as oxidants.

Free radicals are capable of reacting with almost every known molecule at biological system in their vicinity. Free radicals

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damage proteins, cause breakdown of DNA strands and initiate the peroxidation of various compounds[2]. Almost all the vital components of cells are susceptible to damage by free radicals. Such damage accelerates the aging process and many diseases such as cancer, atherosclerosis, reperfusion injury and hepatic injury[3, 4].

Antioxidant substances which scavenge free radicals play an important role in the prevention of free radical induced diseases. The most commonly used antioxidants at present time are butylated hydroxyanisole (BHA), propyl gallate (PG), butylated hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ). However, these synthetic antioxidants have side effects such as liver damage and carcinogenesis[5]. There is a need for isolation and characterization of natural antioxidant having less or no side effects, for use in foods or medicinal materials to replace synthetic antioxidant.

The sulfated polysaccharides from marine algae are known to exhibit many biological and physiological activities including anticoagulant, antihyperlipidemic, antiviral, antitumor and antioxidant activities[6]. The antioxidant activity of the sulfated polysaccharide protects the alga against reactive oxygen species produced under high solar irradiation, possibly by scavenging the free radicals produced in the cell under stress conditions and transporting them from the cell to the medium[7]. Considering the above fact the present study was undertaken to evaluate the antioxidant activity of sulfated polysaccharide from *Sargassum tenerrimum* with respect to their free radical scavenging properties.

2. Materials and Methods

2.1. Collection and processing of seaweed

The brown algae attached to the rocks and other materials were selected and carefully removed along the coastal region of Mandapam (Lat 09° 17'N, Long 79° 07'E). The collected brown seaweed *Sargassum tenerrimum* were initially, washed in seawater to remove the macroscopic epiphytes, sand particles and other extraneous matter and then rinsed in distilled water. This was then air dried in shady place and ground to fine powder which was used for further analysis.

2.2. Extraction of sulfated polysaccharide from seaweed

Sulfated polysaccharide from the brown seaweed *Sargassum tenerrimum* were extracted by the method followed by Subash *et al*[8]. 100 g of the dried seaweed powder was extracted with three volumes of water at 90–95 °C for 16 h. The brown coloured syrup was then filtered through Whatmann No. 3 filter paper, concentrated to 1/4th of the original volume, cooled and precipitated with three volumes of ethanol. The precipitate obtained was collected by centrifugation and dehydrated with diethyl ether to get a dried brown crude extract for further

analysis.

2.3. Physico – chemical analysis of crude polysaccharide

The total carbohydrate content of the crude extract was estimated by phenol sulphuric acid method proposed by Dubois *et al*[9]. Sulfate content was determined by the barium chloride gelatin method [10]. The amount of protein present in seaweed extract was estimated by the Lowry's method,[11].

2.4. Elemental analysis of seaweed polysaccharide

Carbon, hydrogen and nitrogen (CHN) contents were analyzed using elemental analyzer, Elementar vario EL III, Carlo Erba – 1180 at Sophisticated Analytical Instrument Facility, Central Drug Research Institute (CDRI), Lucknow, India.

Analysis of antioxidant activity in crude sulfated polysaccharide.

2.5. Determination of total antioxidant capacity (TAC)

Total antioxidant activity of seaweed extract was determined according to the method of Mitsuda *et al*[12]. 7.45 ml of sulphuric acid (0.6M), 0.99g of sodium sulfate (28mM) and 1.23g of ammonium molybdate (4mM) were mixed together in 250ml with distilled water and labeled as Total Antioxidant Capacity (TAC). 0.1 ml of the seaweed extract (50, 100, 250, 500 and 1000 µg) was dissolved in 1ml of TAC and the absorbance was read at 695 nm after 15 min. Ascorbic acid was used as standard.

2.6. Determination of reducing power

Reducing power of the extract was determined by the following method of Yamaguchi *et al*[13]. 0.75 ml of extract at various concentrations (200, 400, 600, 800 and 1000 µg) was mixed with 0.75 ml phosphate buffer (pH 6.6) and 0.75ml of 1% potassium ferricyanide was added. The mixture was incubated at 50°C for 20 min. 0.75 ml of 10% trichloroacetic acid was added to the mixture and centrifuged at 3000 g for 10 min. 1.5 ml of the supernatant was mixed with 1.5 ml of distilled water and 0.1ml of 0.1% ferric chloride. The absorbance was read at 700 nm after 10 min of incubation.

2.7. DPPH radical scavenging assay

The free radical scavenging activity of seaweed extract was measured by the 1–1–Diphenyl–2–picryl–hydrazyl (DPPH) following the method of Hong *et al*[14]. 0.1 mM solution of DPPH in methanol was prepared and 1ml of this solution was added to 3 ml of seaweed extracts at different concentrations (50, 100, 250, 500 and 1000 µg). After 10 min, absorbance was measured at 517 nm. The percentage scavenging activity values were calculated as below:

$$\% \text{ of scavenging} = ((A_0 - A_1) / A_0) \times 100$$

Where A_0 is absorbance of control and A_1 is absorbance of sample turbidity factor.

2.8. Hydrogen peroxide scavenging assay

The free radical scavenging activity of the extract was determined by Hydrogen peroxide assay^[15]. Hydrogen peroxide (10 mM) solution was prepared in phosphate buffered saline (0.1M, pH 7.4). 1ml of the extract (50, 100, 250, 500 and 1000 μ g) was rapidly mixed with 2 ml of hydrogen peroxide solution. The absorbance was measured at 230 nm in the UV spectrophotometer after 10 min of incubation at 37°C against a blank (without hydrogen peroxide). The percentage of scavenging of hydrogen peroxide was calculated using the formula:

$$\% \text{ of scavenging} = ((A_0 - A_1) / A_0) \times 100$$

Where A_0 is absorbance of control and A_1 is absorbance of sample.

2.9. ABTS inhibition assay

The free radical scavenging activity was also determined by ABTS (2,2'-azino bis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt) radical cation decolourization assay [16]. ABTS was generated by mixing 5 ml of 7 mM ABTS with 88 μ l of 140 mM potassium persulfate under darkness at room temperature for 16 h. The solution was diluted with 50% ethanol and the absorbance at 734 nm was measured. The ABTS radical cation scavenging activity was assessed by mixing 5 ml ABTS solution (absorbance of 0.7 ± 0.05) with 0.1 ml seaweed extract (50, 100, 250, 500 and 1000 μ g). The final absorbance was measured at 743 nm with spectrophotometer. The percentage of scavenging was calculated by the following formula:

$$\% \text{ of scavenging} = ((A_0 - A_1) / A_0) \times 100$$

Where A_0 is absorbance of control and A_1 is absorbance of sample.

2.10. Characterization of the seaweed sulfated polysaccharide

2.10.1. FT-IR spectrophotometer analysis

IR spectroscopies of seaweed extracts along with a standard, dextran sulfate were tested using Perkin-Elmer FT-IR instrument. One part of extract was mixed with ninety nine parts of dried potassium bromide (KBr) separately and then compressed to prepare a salt disc of 3mm diameter. These discs were subjected to IR- spectrophotometer. The absorption was read between 400 and 4000 cm^{-1} .

2.10.2. Agarose gel electrophoresis

The structure of mobility of the sulfated polysaccharide

using three different buffer systems at various pH such as acetate buffer (pH 5.2), citrate buffer (pH 3.6) and carbonate-bicarbonate buffer (pH 10) was performed by agarose gel electrophoresis (AGE) for one hour at 50V. After electrophoresis, the gel was fixed with 0.1% N-cetyl-N,N,N-trimethylammonium bromide for 12 h. The gel was then washed with distilled water, dried and stained with toluidine blue solution (0.1% toluidine in acetic acid, ethanol and water (0.1:5:5 v/v)). After staining, the gel was washed in destaining solution (acetic acid, ethanol and water in the ratio of 0.1:5:5 v/v) and were documented.

2.10.3. Gradient polyacrylamide gel electrophoresis (PAGE)

The molecular weight of the sulfated polysaccharide was examined by gradient polyacrylamide gel electrophoresis (PAGE) following the method of Vijayabaskar *et al*^[17]. The sulfated polysaccharides extracted from the seaweed *S. tenerrimum* were solubilized in sample loading buffer and loaded into 12–22% of gradient polyacrylamide gel and electrophoresed at a constant current (30 mA). After electrophoresis the gel was stained in staining solution (azure-A 0.08% w/v in PBS) and was destained in the destaining solution (methanol, glacial acetic acid and water in the ratio of 10:10:80).

2.10.4. GC-MS analysis

Polysaccharide was hydrolyzed to monomeric units and transformed in their alditol acetates. 0.1 g of crude sample were mixed with 1.25 ml of 72% sulfuric acid with a glass stick and incubated for 60 min at 30°C. The mixture were diluted with 13.5 ml of distilled water and incubated in boiling water bath for 4 h. After incubation, mixtures were cooled and 3.1 ml of 32% of NaOH (w/v) was added. At the end of hydrolysis, 0.2ml of sample was taken separately and 2 ml of 2% sodium borohydride in dimethyl sulfoxide was added. The mixtures were then shaken well at 40°C for 90 min after which 0.2 ml of glacial acetic acid was added to decompose excess of sodium borohydride. After cooling, 4 ml of acetic anhydride and 0.4 ml of 1-methylimidazole were added to the solution. The mixtures were then incubated for 10 min at room temperature and then 20 ml of distilled water was added to decompose excess of acetic anhydride. After cooling, 8 ml of dichloromethane was added and the mixture was shaken vigorously for total alditol acetate extraction. The upper layer was removed and the lower phase was washed three times with 20 ml of distilled water. The dichloromethane was evaporated at 40 °C under vacuum and final alditol acetate residues were dissolved in 1ml of dichloromethane and stored at -20 °C. Alditol acetates were separated on a 30 m x 0.25 mm ID x 0.25 μ m film thickness column DB 5 ms (agilent) attached to the GC- 2010 (GCM-QP 2010) SHIMADZU chromatography equipment with a flame-ionization detector and a split injector. High purity hydrogen was used as the carrier gas at a flow rate of 1.40 ml/min. the column temperature was maintained at 200 °C and 240 °C

Table 1.Percentage of total carbohydrates, sulphate, protein and elements CHN in *Sargassum tenerrimum*

Sample	Carbohydrate (%)	Sulphate (%)	Protein (%)	Carbon (%)	Hydrogen (%)	Nitrogen (%)
<i>Sargassum tenerrimum</i>	8.20 ± 1.23	6.60 ± 1.42	0.86 ± 0.42	22.44 ± 3.12	4.34 ± 1.02	1.83 ± 0.22

respectively, and 1 μ l sample in dichloromethane was injected through a glass-lined splitter, set at 1/90 ratio. The absorption was read between 40 m/z and 800 m/z.

3. Results

In recent years, a broad series of polysaccharides from edible seaweeds have emerged as an important class of bioactive natural products, possessing many important properties of pharmacological relevance [18]. Sulfated polysaccharides are widespread in nature, occurring in a great variety of marine organisms. Therefore an attempt was made to study the presence of chemical constituents, characterization and antioxidant activity of the sulfated polysaccharide from brown seaweed namely *Sargassum tenerrimum* extracted by following the method of Subash *et al* [8].

The yield of sulfated polysaccharide extracted from 100 gm of the seaweed powder were found to be 11.54 gm. Carbohydrate contents in sulfated polysaccharide was 8.20% ± 1.23%. The Sulphate content was found to be 6.6% ± 1.42%. Previous studies have indicated that the polysaccharide bioactivities depend on the degree and position of sulfation [19, 20]. Zhang *et al.* [21] have shown that difference in antioxidant activities between different polysaccharides may be due to differences in their sulfate content which corroborated with the finding of the present study. Protein content was 0.86% ± 0.42% which is typically very low. This is similar to the results of Tatiana *et al.* [22] which showed protein content of the polysaccharide from brown seaweed *Fucus evanescens* was less than 2%. Local variation in salinity has also been shown to be an important factor in the biological uptake of a number of elements [23, 24]. Hence elemental analysis was done in present study which showed high percentage of carbon (24.44% ± 3.12%) followed by hydrogen (4.34% ± 1.02%) and nitrogen (1.83% ± 0.22%) (Table. 1).

The total antioxidant assay was found to be 62.55% ± 1.40% (Fig. 1). The reducing properties are generally associated with the presence of reductions. Reductions were reported to be terminators of free radical chain reactions by donating a hydrogen atom. In most cases, irrespective of the stage in the oxidative chain, in which the antioxidant action is assessed, most non-enzymatic anti-oxidative activity is mediated by redox reactions [25]. In the present study the reducing power of the sulfated polysaccharide increases with increasing concentration (Fig. 2) as said by Rout and Banerjee, [26]. This result also indicated that compounds with strongest reducing power were concentrated in the brown seaweeds. This was similar to the result given by Costa *et al.* [27] which shows that the highest amount of reducing power was observed in sulfated polysaccharide from *Sargassum fillipendula*.

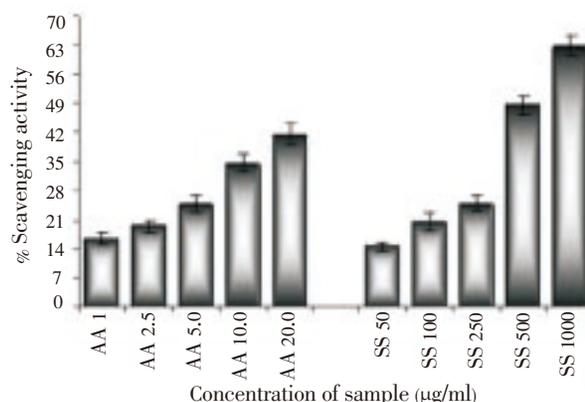


Figure 1. Total antioxidant activity of sulfated polysaccharide *S. tenerrimum* (ST) compared with standard Ascorbic acid (AA).

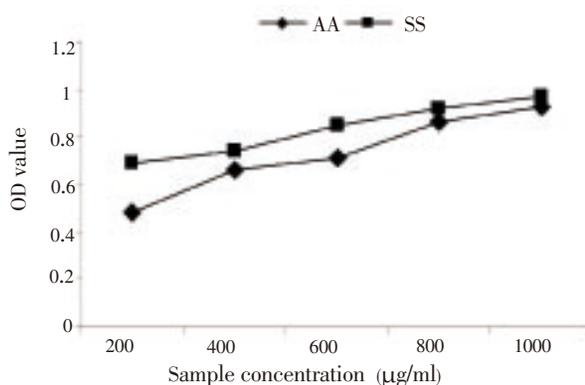


Figure 2. Reducing power of seaweed extract *S. tenerrimum* (ST) compared with standard Ascorbic acid (AA).

DPPH is a compound that possesses a nitrogen free radical and is readily destroyed by a free radical scavenger. This assay was used to test the ability of the anti-oxidative compounds functioning as proton radical scavengers or hydrogen donors [28]. The present study indicates DPPH scavenging activity for *Sargassum tenerrimum* extract as 64.66% ± 2.08% (Fig. 3). Sanaa, [29] have also reported the DPPH scavenging activity of *Sargassum dentifolium* to be around 82% which is much higher than that of extract used in the present study.

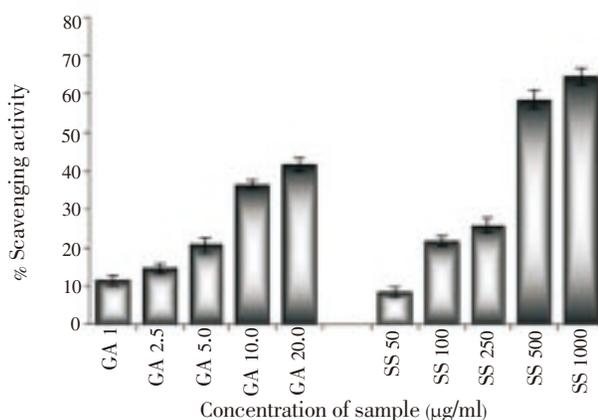


Figure 3. DPPH radical scavenging activities of seaweed extract *S. tenerrimum* (ST) compared with standard gallic acid (GA).

The measurement of H_2O_2 scavenging activity is one of the useful methods of determining the ability of antioxidants to decrease the level of pro-oxidants such as H_2O_2 [30]. It can cross membranes and may slowly oxidize a number of compounds. Hydrogen peroxide itself is not very reactive, but sometimes it can be toxic to cells because of rise in the hydroxyl radicals in the cells[31]. The inhibitive effect of seaweed extract was subjected to hydrogen peroxide scavenging assay and was found to be $61.56 \pm 2.05\%$ (Fig. 4).

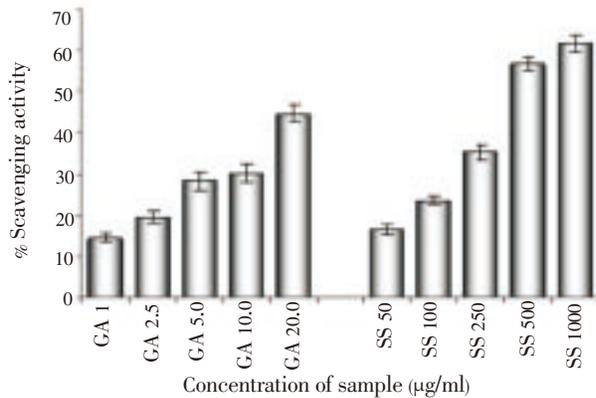


Figure 4. H_2O_2 radical scavenging activities of seaweed extract *S. tenerrimum* (ST) compared with standard gallic acid (GA).

The ABTS radical reactions involve electron transfer and the process take place faster rate when compared to DPPH radicals. In the present study the ABTS radical scavenging activity was more ($70.33 \pm 2.33\%$) (Fig. 5) when compared DPPH and Hydrogen peroxide. There are some reports in the literature on the antioxidant capacity of algae. Alcoholic and aqueous extracts of seaweeds have been evaluated for antioxidant activity by lipoxygenase inhibition, DPPH assay and deoxyribose assays[32, 33, 34]. Recently, several marine alginate derivatives, sulfated fucoidans from the brown seaweed *Laminaria japonica*, agar-like sulfated galactans from the red seaweed *Nori* and sulfated polysaccharides from *Fucus vesiculosus*, have been reported to have antioxidant activity[35, 36, 37].

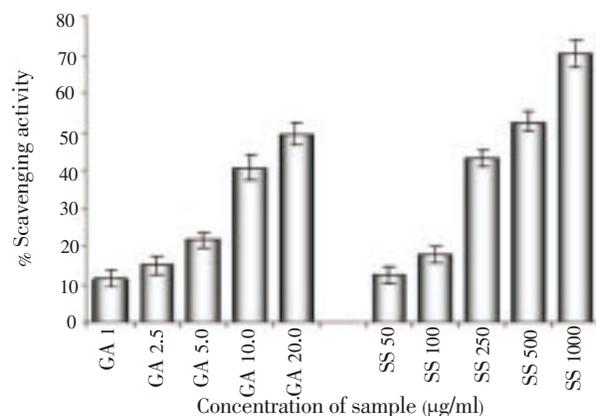


Figure 5. ABTS radical scavenging activities of seaweed extract *S. tenerrimum* (ST) compared with standard gallic acid (GA).

The presence of sulfated polysaccharide in the crude extract was further confirmed by FT-IR analysis. IR spectra of the extract revealed characteristic absorption bands for sulfated polysaccharides. It reveals the presence of carboxyl, hydroxyl

and sulfate groups. The peak at 3728.53 cm^{-1} indicates the O-H stretching. The signal at 1024.24 cm^{-1} indicate the presence of S=O (Sulfoxide stretching) (Fig. 6).

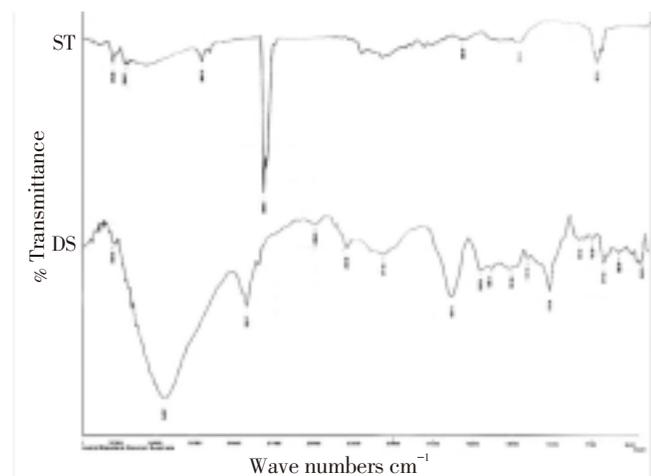


Figure 6. FT-IR analysis of sulfated polysaccharide of *S. tenerrimum* (ST) Compared to the Standard Dextran Sulphate (DS)

The electrophoretic migration of sulfated polysaccharides in agarose gel, using various buffer systems at various pH, depends on the structure of polysaccharide. The sulfated polysaccharide of the seaweed separates in the gel depending upon the size of the molecules. These molecules were compared with standard dextran sulfate. In the present study highest mobility was observed in higher pH 10 (Fig. 7). Dietrich *et al.*[38], Mariana and Barbara, [39] showed that the sulfated polysaccharides had different electrophoretic mobility for different buffer system depending on the structure of the polysaccharide.

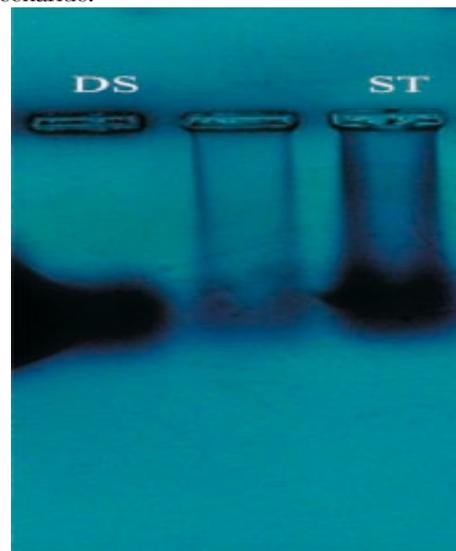


Figure 7. Structure of mobility analyses of sulfated polysaccharide in *S. tenerrimum* by highest carbonate – bicarbonate buffer (pH 10).

In gradient PAGE analysis, the azure-A dye binds to polysaccharide because of the suitable arrangement of anionic sites for interaction with the dye dimer and does not bind to proteins or any other compounds[17]. The molecular weight of crude extracts of *Sargassum tenerrimum* was 40 kDa this was compared with the standard dextran sulfate (Fig. 8). This is

similar to the result of Hong *et al.*[40] who reported that the molecular weight of *Sargassum pallidum* polysaccharide was 50 kDa. Yu *et al.*[41] found that the effects of ulvan on lipid metabolism were modified when it was degraded to low molecular weights fractions without changing its chemical composition and structure, with the lower molecular weight sample more active. This result is in accordance with our results where the lowest molecular weight sample had the highest antioxidant activity.

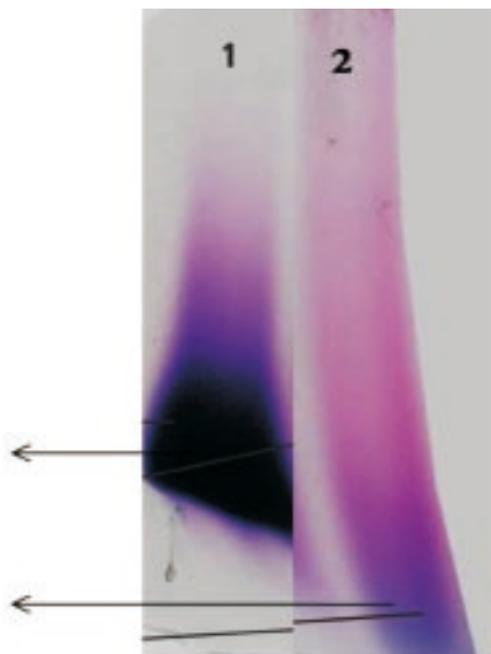


Figure 8. Gradient PAGE gel of sulfated polysaccharide of *S. tenerrimum*. Lane 1, standard dextran sulfate (50 kDa); lane 2, crude sulfated polysaccharide (40 kDa).

In case of complex carbohydrates, neutral sugars are identified by their derivatives, alditol acetates by GC–MS [42], which was described by Hoebler *et al.*[43]. Alditol acetates are often used only to determine the ratios of monosaccharides in polysaccharide hydrolysates [44]. Two major peaks are present corresponding to benzenamine (31.67%) and aminocarb (21.45%). A number of small peaks are also present, but these do not necessarily correspond to alditol acetate (Fig. 9).

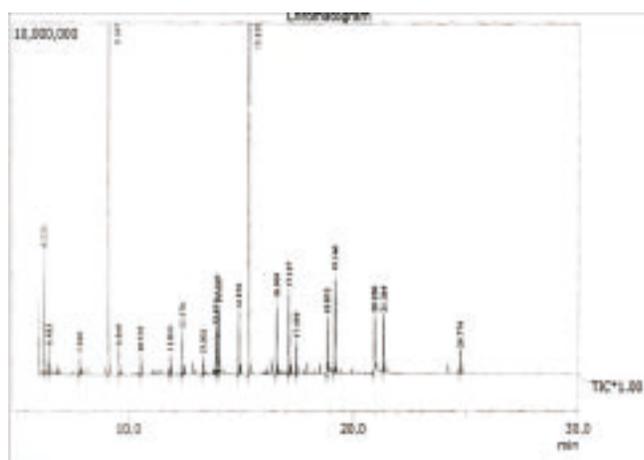


Figure 9. GC–MS Spectrum of sulfated polysaccharide of *Sargassum tenerrimum*.

The study on sulfated polysaccharide from *Sargassum tenerrimum* a brown algae elicited the antioxidant activity. It is in this context; in future the research establishment and scholars of our country should pay more attention for the development of drug systems from marine algae especially brown algae to save the life of man kind.

Conflict of interest statement

We declare that we have no conflict of interest.

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