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PURIFICATION AND *IN VITRO* ANTIOXIDANT ACTIVITY OF POLYSACCHARIDE ISOLATED FROM GREEN SEAWEED *CAULERPA RACEMOSA*

S. MAHENDRAN* AND S.SARAVANAN

Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai–608 502, Tamil Nadu, India.

ABSTRACT

The present investigation was to study the purification and *In vitro* antioxidant activities of the polysaccharide from green seaweed*Caulerpa racemosa*. The polysaccharide was isolated and purified from green seaweed *C. racemosa* by ethanol precipitation and DEAE-cellulose ion exchange chromatography. The chemical analysis of polysaccharide showed carbohydrate content (47.43%), sulfate content (12.86%) and uronic acid content (4.9%). The *In vitro* antioxidant activity was determined by free radical scavenging assays such as total antioxidant activity (80.24±0.56%), Reducing power assay [(0.243±0.04) - (1.624±0.07)],Hydrogen peroxide assays (77.22±0.67%),DPPH assays (71.42±0.74%), ABTS assays(73.32±1.27%), Hydroxyl assays (68.29±1.03), Superoxide anion assays (66.17±0.77 %) and Nitric oxide radical assay (40.64±1.82%). The antioxidant capacities of the *C. racemosa* polysaccharides showed potential rich sources of natural antioxidants.

KEYWORDS: ***Caulerpa racemosa*,polysaccharide, DEAE-cellulose, *In vitro* antioxidant activities.**

S. MAHENDRAN
Centre of Advanced Study in Marine Biology, Faculty of Marine Sciences, Annamalai University, Parangipettai–608 502, Tamil Nadu, India.

****Corresponding author***

INTRODUCTION

Seaweeds are considered as a source of bioactive compounds as they are able to produce a great variety of secondary metabolites characterized by a broad spectrum of biological activities[1] with antiviral, antibacterial and antifungal activities[2]which acts as potential bioactive compounds of interest for pharmaceutical applications[3]. Most of these bioactive substances isolated from marine algae are chemically classified as brominates, aromatics, nitrogen-heterocyclic, nitrosulphuric-heterocyclic, sterols, dibutanoids, proteins, peptides and sulphated polysaccharides[4]. The polysaccharides are an important component of algae. The immense interest in them is because of their broad spectrum biological activity. Polysaccharides exhibiting anticoagulant, antitumor and other activities have been isolated from green algae *Caulerpa racemosa*, *C. brachypus*, *C. okamurai*, *C. scapelliformis*, *Chaetomorpha crassa*, *C. spiralis*, *Codium adhaerens*, *C. fragille*, *C. latum*, *Enteromorpha compressa*, *Monostroma nitidum*, and *Ulva* sp[5].*Caulerpa racemosa* mainly grow in tropical regions, although some varieties may be found in subtropical regions. In South East Asian countries, it is usually served raw as a salad or eaten cooked. In addition, it is used as animal feed and in folk medicine to reduce blood pressure and to treat rheumatism[6]. The preliminary investigation on the crude methanol extract and phases from*Caulerpa racemosa* showed antinociceptive activity[7]. Sulfated polysaccharides (SP) from different sources have been studied in the light of theirimportant pharmacological activities, such as anticoagulant, antioxidant, antiproliferative, antitumoral, anticomplementary, anti-inflammatory and antiviral properties[8].Reactive oxygen species (ROS) such as superoxide radical, hydroxyl radical, peroxyl radical and nitric oxide radical attack biological molecules such as lipids, proteins, enzymes, DNA and RNA, leading to cell or tissue injury[9].The most commonly used antioxidants at present time are butyrate hydroxyanisole (BHA), propyl gallate (PG), butyrate hydroxytoluene (BHT) and tert-butylhydroquinone (TBHQ). However, these synthetic antioxidants have side effects such as liver damage and are suspected to be mutagenic and neurotoxic. Hence, most consumers prefer additive-free foods or a safer approach like the utilization of more effective antioxidants of natural origin[10,11,12].

The algal polysaccharides were reported to be useful candidates in the search for an effective non-toxic substance and have been demonstrated to play an important role as free radical scavengers *in vitro* and antioxidants for the prevention of oxidative damage in living organisms[13,14,15,16]. The antioxidant capacity of sulfated polysaccharides has been studied by different in vitro methods, including hydrogen peroxide, superoxide anion and hydroxyl radical scavenging assays. DPPH radical scavenging assay is frequently used for the analysis of food and substances obtained from natural sources[17]. The seaweeds possess wide application in food and in the pharmaceutical industry; the antioxidant activities of many types of seaweed in the South Indian coastal area are still unexplored. There is a need for isolation and characterization of natural antioxidant having less or no side effects, for using it in foods and medicines. The genus *Caulerpa* has attracted the attention of researchers due to its important secondary metabolite caulerpenyne (CYN) that is reported to exhibit the antineoplastic, antibacterial and antiproliferative activities[18,19]. The main objective of the present study is to evaluate the purification, chemical characterization and antioxidant activity of polysaccharidefrom *Caulerpa racemosa* green seaweed obtained from Tuticorin coastal area, Gulf of Mannar, India.

METHODS

Collection and processing of seaweeds

The green algae, *Caulerpa racemosa* (Chlorophyta) was collected from the intertidal region of Tuticorin coast, (Lat 08°45′; Long

78°12′E)Gulf of Mannar, South east coast of India. The study area is a marine biosphere which harbors unique biodiversity of global significance such as coral reef, seaweed and sea grass ecosystems.

Extraction of crude polysaccharide

The polysaccharide from the green seaweeds *Caulerpa racemosa* was extracted by the method followed by Subash*et al.*[20]. 100g of dried seaweed powder was extracted with three volumes of water at 90-95^{0}C for 16 hrs. The brown coloured syrup was then filtered through Whatman No. 3 filter paper and concentrated to 1/4th of the original volume; it was cooled and precipitated with three volumes of ethanol overnight at 4˚C. The obtained precipitate was collected by centrifugation and the pellet dehydrated with diethyl ether to yield 10% green crude polysaccharide (10% yield).

Purification of polysaccharide

The crude polysaccharide was further purified by column chromatography. Fifty milligrams of crude polysaccharide was dissolved in 10 ml of distilled water, it was applied to a DEAE-cellulose column(3×45 cm) pre-equilibrated with water and eluted in NaCl gradient(0-3 M) until no carbohydrate is detected. Each fraction was assayed for carbohydrates content by phenol-sulfuric acid method Dubois *et al.*[21]. The carbohydrate-positive fractions were pooled together and dialyzed (MWCO 14,000) for 24 hours against distilled water and then lyophilized.

Chemical analysis

The total carbohydrate content was estimated by phenol sulphuric acid method proposed by Dubois*et al.*[21]. Sulfate content was determined by barium chloride gelatin method according to the procedure of Lloyd *et al.*[22]. Uronic acid content was determined by the carbazole reaction[23] using D-glucuronic acid as a standard.

Free radical scavenging activity of polysaccharide

Determination of total antioxidant capacity (TAC)

Total antioxidant activity of seaweed polysaccharide was determined according to the method of Prieto *et al.*[24].Briefly, 0.3 ml of sample was mixed with 3.0 ml reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). The reaction mixture was incubated at 95º C for 90 minutes under water bath. Finally the antioxidant property of the sample was measured at 695 nm. Total antioxidant activity is expressed as the number of equivalents of ascorbic acid in milligram per gram of extract.

Determination of reducing power

Reducing power of the polysaccharide was determined by the following method of Yamaguchi *et al.*[25].Briefly, 4 ml of reaction mixture, containing samples of different concentration in phosphate buffer (0.2 M, pH 6.6), was incubated with potassium ferricyanide (1% w/v) at 50^{o}C for 20 min. The reaction was terminated by TCA solution (10% w/v). The solution was then mixed with distilled water and ferric chloride (0.1% w/v) solution and the absorbance was measured at 700 nm.

Hydrogen peroxide scavenging assay

The free radical scavenging activity of the polysaccharide was determined by hydrogen peroxide assay[26]. Hydrogenperoxide (10mM) solution was prepared in phosphate buffered saline (0.1M, pH 7.4). 1ml of the extract containing samples of different concentration (100, 250, 500, 750 and 1000µg) was rapidly mixed with 2ml of hydrogen peroxide solution. The absorbance was measured at 230 nm in the UV spectrophotometer after 10 minutes of incubation at 37^{o}C against a blank (without hydrogen peroxide). The percentage of scavenging of hydrogen peroxide was calculated using the formula

$$\text{Percentage scavenging } (H_2O_2) = ((A_o - A_1) / A_o) \times 100$$

A_o - Absorbance of control; A_1 - Absorbance of sample

DPPH radical scavenging assay

The free radical scavenging activity of polysaccharide was measured by the 1-1-Diphenyl-2-picryl-hydrazyl (DPPH) following the method of Blois[27]. DPPH was used as a reagent which evidently offers a convenient and accurate method for titrating the oxidizable groups of natural (or) synthetic antioxidants. 0.1mM solution of DPPH in methanol was prepared and 1ml of this solution was added to 3ml of seaweed extracts of different concentration (100, 250, 500, 750 and 1000µg). After 10 minutes, absorbance was measured at 517nm. The percentage scavenging activity values were calculated using the following formula

Percentage of Scavenging = $((A_o-A_1) / A_o) \times 100$

Where A_o is absorbance of control and A_1 is absorbance of sample turbidity factor

ABTS inhibition assay

The ability of the extract to scavenge ABTS radical scavenging was determined by the method of Re *et al.*[28]. ABTS was generated by mixing 5ml of 7mM ABTS with 88µl of 140mM potassium per sulfate under darkness at room temperature for 16 hours. The solution was diluted with 50% ethanol and the absorbance at 734nm was measured. The ABTS radical cation scavenging activity was assessed by mixing 5ml ABTS solution (absorbance of 0.7±0.05) with 0.1ml polysaccharide (100, 250, 500, 750 and 1000µg). The final absorbance was measured at 743nm with a spectrophotometer. The percentage of scavenging was calculated by the following formula,

% of scavenging = $((A_o-A_1) / A_o) \times 100$

Where A_o- Absorbance of control; A_1 - Absorbance of sample

Hydroxyl radical scavenging assay

Hydroxyl radical scavenging activity was measured by studying the competition between deoxyribose and test compounds for hydroxyl radical generated by Fe^{3+}- Ascorbate EDTA H_2O_2 system (Fenton reaction) according to the method of Kunchandy and Rao[29]. The hydroxyl radicals attack deoxyribose that eventually results in TBARS formation. The reaction mixture contained in a final volume of 1.0 ml, 100µl of 2-deoxy-2-ribose (28 mM in potassium phosphate-potassium hydroxide buffer, pH 7.4), 500µl solutions of various concentrations of polysaccharide(100, 250, 500, 750 and 1000µg) and standard in KH_2PO_4-KOH buffer (20mM, pH 7.4), 200 µl of 1.04mM ethylene diamine tetra acetic acid and 200 µl of 200 µM Ferric chloride, 100 µl of 10 mM hydrogen peroxide and 100 µl of 1.0mM ascorbic acid was incubated at 37^oC for 1 hour. The free radical damage imposed on the substrate, deoxyribose (TBARS) was measured by the method of Yuan and Walsh[30]. 1.0 ml of thiobarbituric acid (1%) and 1.0 ml of trichloroacetic acid (2.8%) were added to the test tubes and were incubated at 100 ^oC for 30 minutes. After cooling, absorbance was measured at 535 nm against control containing deoxyribose and buffer. The percentage scavenging was determined by the comparing the result of the test compound and control using the following formula,

Radical scavenging activity (%) = $[(A_0-A_1/ A_0) \times 100]$

Where A_0- Absorbance of control; A_1- Absorbance of sample

Superoxide anion radical scavenging assay

Measurement of superoxide anion scavenging activity of the polysaccharide was done based on the method of Nishimiki *et al*.[31]. About 1 ml of nitro blue tetrazolium (NBT) solution (156µM NBT in 100mM phosphate buffer, pH 7.4), 1 ml of NADH solution (468µM in 100mM phosphate buffer, pH 7.4) and 0.1 ml of sample at various concentrations (100, 250, 500, 750 and 1000µg) were mixed and the reaction was started by

adding 100 µl of phenazine methosulphate (PMS) solution (60µM PMS in 100 mM phosphate buffer, pH 7.4). The reaction mixture was incubated at 25°C for 5 minutes and the absorbance at 560nm was measured against blank samples. The percentage scavenging value was determined as follows.

Radical scavenging activity (%) = $[(A_0-A_1/ A_0) \times 100]$
Where A_0- Absorbance of control; A_1- Absorbance of sample

Nitric oxide radical scavenging assay

Nitric oxide radicals generated from sodium nitroprusside solution at physiological pH interacts with oxygen to produce nitrite ions which were measured by the Griess reaction[32]. 2 ml of sodium nitroprusside (10 mm) was mixed with 1 ml of the polysaccharide with varying concentrations (100, 250, 500, 750 and 1000µg) in phosphate buffer (pH 7.4). The mixture was incubated at 25°C for 150 minutes. 1 ml of sulphanilic acid reagent (0.33% sulphanilamide in 20% acetic acid) was added to 0.5 ml of the incubated solution and allowed to stand for 5 minutes for completing diazotization. Then,1 ml of 0.1% napthyl ethylene diamine dihydrochloride was added and incubated at room temperature for 30 minutes. Absorbance was read at 540 nm and percentage scavenging was calculated as follows.

Radical scavenging activity (%) = $[(A_0-A_1/ A_0) \times 100]$
Where A_0- Absorbance of control; A_1- Absorbance of sample

RESULTS

Estimation of chemical constituents of polysaccharide in C. racemosa

Chemical composition of the purified polysaccharide from *C. racemosa* was determined as carbohydrate content (47.43%), sulfate content (12.86%) and uronic acid content (4.9%).

Free radical scavenging activity of polysaccharide in C. racemosa

Total antioxidant capacity

The total antioxidant capacity of polysaccharide from *C.racemosa*was measured by phosphomolybdenum method. The antioxidant activities increased with increasing concentration of the sample. At the concentration of 1000µg/ml, the polysaccharide of *C. racemosa*exhibited higher antioxidant activity (80.24±0.56) which was found significant when compared with the standardascorbic acid (95.71±0.48) (Figure 1).

Reducing power

The reducing power of polysaccharide from *C. racemosa* [(0.243± 0.04) - (1.624 ± 0.07)] was compared with the standard ascorbic acid[(0.318± 0.01) - (1.921± 0.09)]. The reducing power of standard and samples increased with the increasing concentration.The reducing power of the samples is shown in figure 2.

Hydrogen peroxide scavenging assay

The hydrogen peroxide scavenging effect increased with the concentration of standard and samples. The polysaccharide from *C. racemosa* possessed (77.22±0.67%) scavenging activity.The hydrogen peroxide scavenging effect of samples is shown in figure 3.

DPPH radical scavenging assay

The DPPH scavenging effect increased with the concentration of standard (Gallic acid) and samples. The polysaccharide from *C. racemosa* possessed (71.42±0.74%) scavenging activity

where as gallic acid possessed (93.34± 0.48%). DPPH radical scavenging effect is shown in figure 4.

ABTS inhibition assay

The ABTS scavenging effect increased with the concentration of standard and samples. The polysaccharide from *C. racemosa* possessed (73.32±1.27%) scavenging activity on ABTS where as Gallic acid possessed (93.34±0.48%). The effect of polysaccharide and standard on ABTS cation was compared and shown in figure 5.

Hydroxyl scavenging activity

The scavenging effect of Hydroxylwas investigated using the Fenton reaction and the polysaccharide from *C. racemosa*exhibited the inhibition of about (68.29±1.03%). The result of hydroxyl scavenging activity is shown in figure 6.

Superoxide anion radical scavenging activity

The polysaccharide was subjected to be superoxide scavenging assay and it indicated that polysaccharide from *C. racemosa* exhibited the maximum Superoxide scavenging activity of (66.17±0.77 %).The result ofSuperoxide anion radical scavenging activity is shown in figure 7.

Nitric oxide scavenging activity

Suppression of Nitric oxide release may be attributed to a direct Nitric oxide scavenging effect.The polysaccharide decreased the amount of nitrite generated from the decomposition of sodium nitroprusside*in vitro*. The polysaccharide from *C. racemosa* had scavenging activity of (40.64 ±1.82%). The result of Nitric oxide scavenging activity is shown in figure 8.

ANTIOXIDANT ACTIVITY OF POLYSACCHARIDE

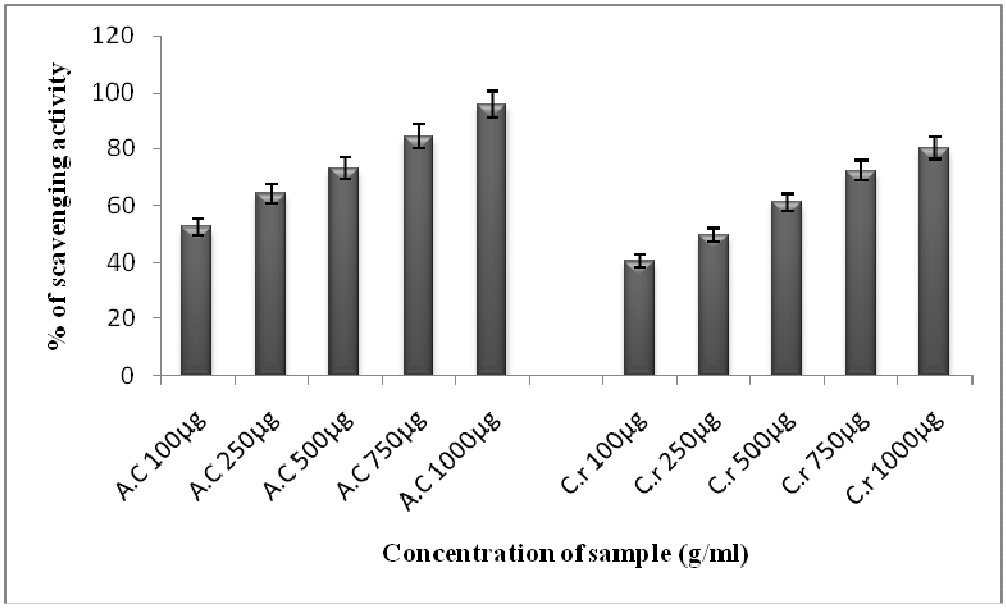

Figure 1
Total antioxidant activity of polysaccharide from C. racemosa compared with standard ascorbic acid (AC).

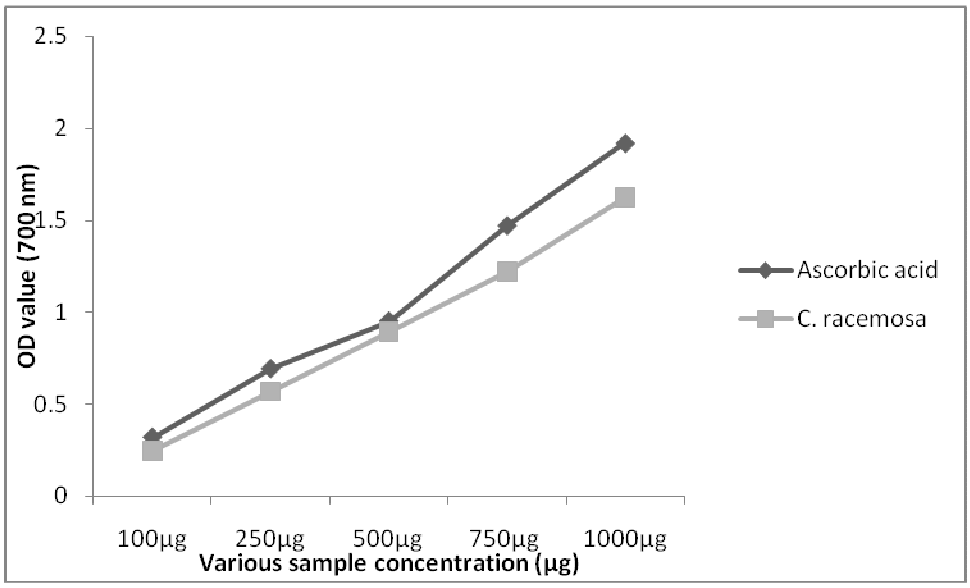

Figure 2
Reducing power of polysaccharide from C. racemosacompared with standard ascorbic acid (AC).

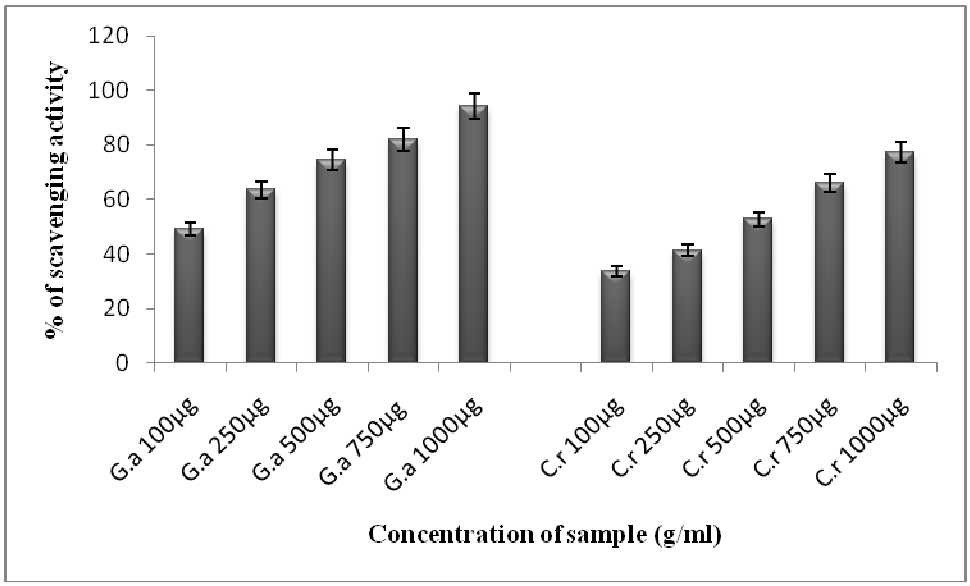

Figure 3
Hydrogen peroxide scavenging assay of polysaccharide from C. racemosacompared with standard gallic acid (GA).

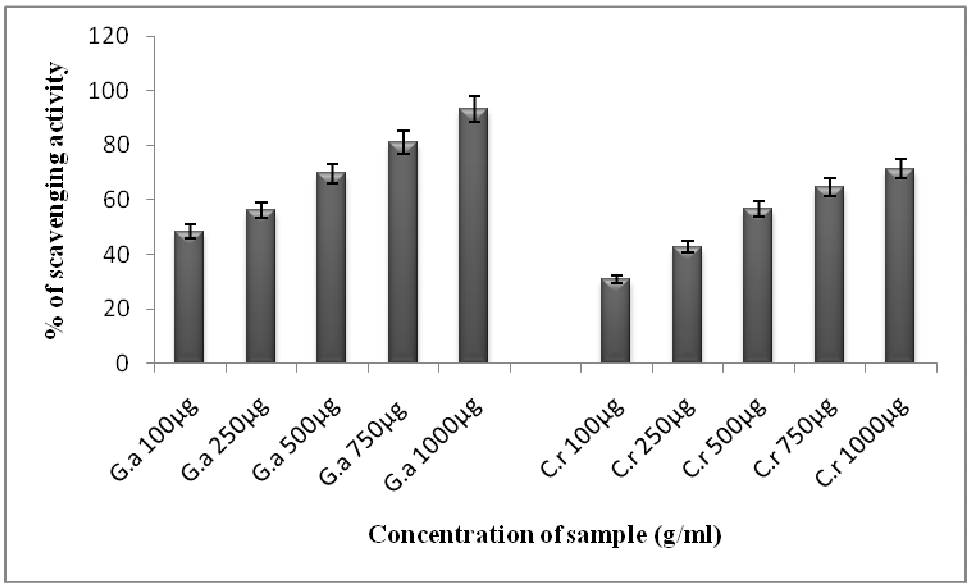

Figure 4
DPPH scavenging assay of polysaccharide from C. racemosacompared with standard gallic acid (GA).

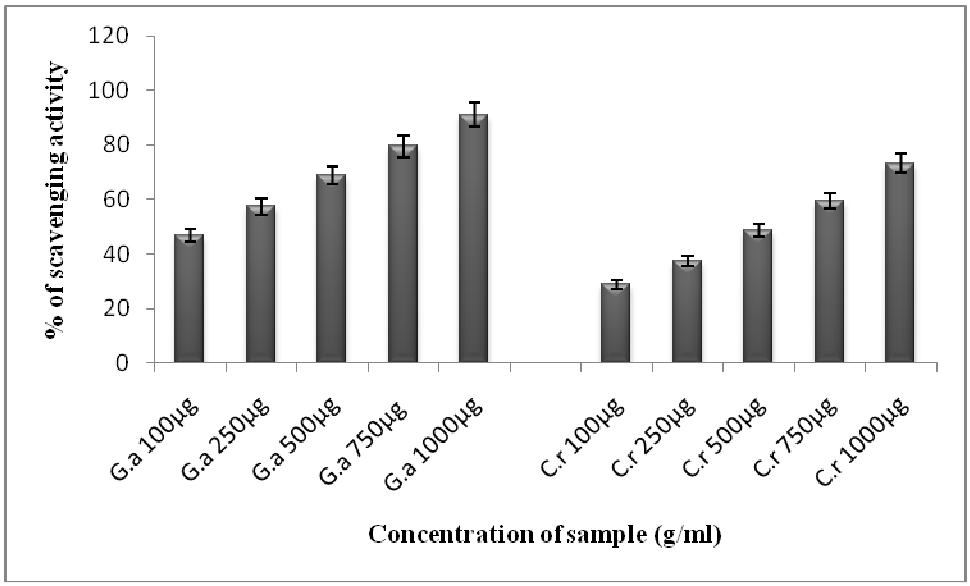

Figure 5
ABTS scavenging assay of polysaccharide from C. racemosacompared with standard gallic acid (GA).

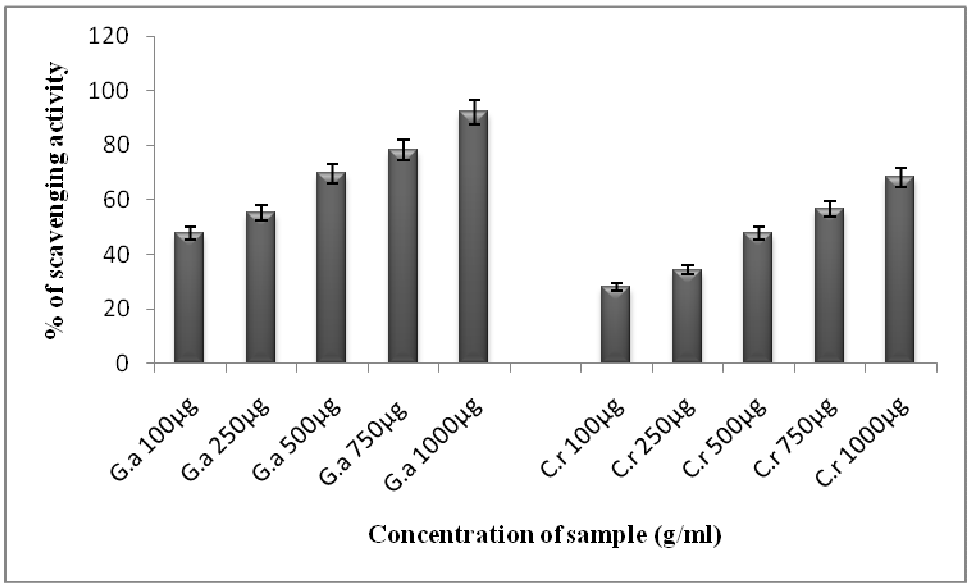

Figure 6
Hydroxyl scavenging assay of polysaccharide from C. racemosacompared with standard gallic acid (GA).

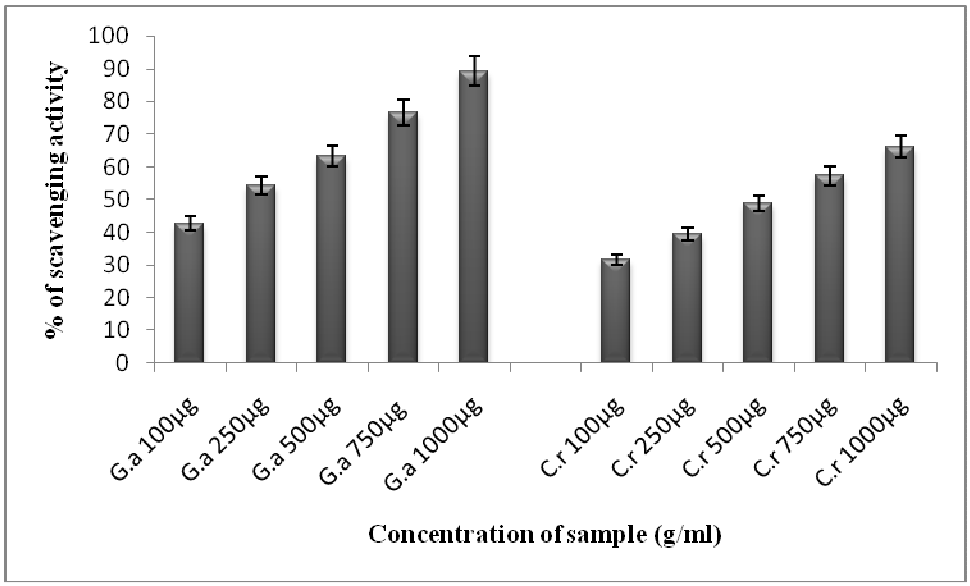

Figure 7
Superoxide scavenging assay of polysaccharide from C. racemosacompared with standard gallic acid (GA).

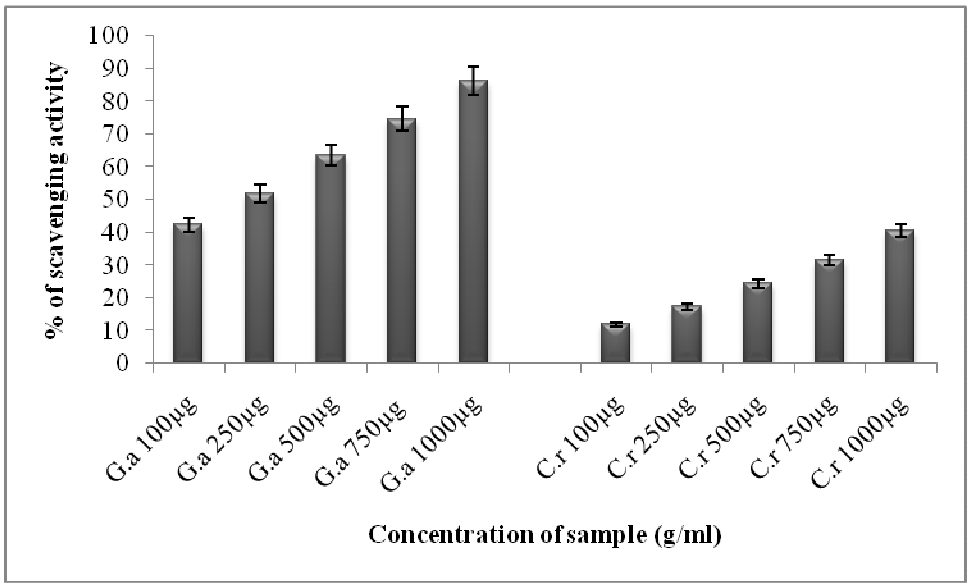

Figure 8
Nitric oxide scavenging assay of polysaccharide from C. racemosacompared with standard gallic acid (GA).

DISCUSSION

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[33].The antioxidant activity of several naturally occurring compounds have been known for decades. Seaweeds can be used as food additives and can also provide protection against tissue oxidation[34].In the present study the polysaccharide from green seaweed *C. racemosa* was isolated and purified by ethanol precipitation and DEAE-cellulose ion exchange chromatography. The employment of DEAE-cellulose as a matrix has been widely reported for polysaccharide separation and also to reveal the characteristics of different algae species, such as on *Gelidium crinale*[35], *Ecklonia cava*[36], *Champia feldmannii*[37] and *Halymenia pseudofloresia*[38]. In the present investigation the total carbohydrate content (47.43%), sulfate content (12.86%) and uronic acid content (4.9%) of the polysaccharide from *C. racemosa*was determined. Ye *et al.*[39] reported that chemical composition of the purified water-soluble polysaccharide from*Capsosiphon fulvescens* was composed of neutral sugars (49.8%), sulfate (5.7%), uronic acid (4.8%) and protein (0% in mass).Nirmal *et al.*[40]reported the carbohydrate content ofgreen seaweeds *Cladophora fascicularis* (43.4%),*Caulerpa racemosa* (41%) and *Ulva lactuca* (36.2%). Ghosh *et al*.[5]reported that extraction of the marine green alga *C. racemosa* with hot water yielded a crude polysaccharide-containing fraction (named CrHWE) having activity against Herpes simplex virus.The sugar composition analysis of hot water extracted fraction showed the presence of galactose, glucose, arabinose and xylose, together with smaller amounts of mannose and rhamnose and traces of fucose residues. In addition to neutral sugars, 4% of uronic acid residue, 9% of sulfate hemiester group and 5% (w/w) protein were found.

Previous studies have reported high antioxidant activity in the genus *Caulerpa*[41, 42]. In the present study the total antioxidant capacity of polysaccharide of *C. racemosa* was found to be (80.24±0.56%). Costa *et al.*[8] reported that total antioxidant activity of total polysaccharides from the macroalgae *Dictyota cervicornis, Dictyopteris delicatula, Dictyota menstrualis, Dictyota mertensii,*

Sargassumfilipendula,Spatoglossum schroederi, Gracilaria caudata, Caulerpa cupressoides, Caulerpaprolifera, Caulerpa sertularioides and *Codium isthmocladum*. The total antioxidant activity of polysaccharides from above seaweeds ranged from 53.9 to 9.2 mg/g of acid ascorbic equivalents. In the present study the reducing power assay from polysaccharide of *C. racemosa* was found to be [(0.243±0.04) - (1.624±0.07)].The reducing properties are generally associated with the presence of reductions. Vijayabaskar and Shiyamala[43] reported that the reducing power of *Turbinaria ornata* increases with the increasing concentration. The reducing power of the samples from*Turbinaria ornata* was (0.2± 0.04) - (0.72± 0.07). The extent of H_2O_2 scavenging activity is one of the useful methods for determining the ability of antioxidants to decrease the level of pro-oxidants[44].In the present study hydrogen peroxide scavenging activity of polysaccharide of *C. racemosa* was found to be (77.22±0.67%). Antonio *et al.*[45] reported that the H_2O_2 production by *Caulerpa taxifolia* was significantly higher (52%). Collen and Pedersen[46] reported that *Caulerpa taxifolia* when epiphytized by *Lophocladia lallemandii*showed increased lipid peroxidation which could be related to the increased H_2O_2 production to compete against *Lophocladia* rather than a marker of oxidative damage. In this situation of competence, the release of H_2O_2 could have no direct autotoxic effect. In the present study the DPPH and ABTS assay from polysaccharide of *C. racemosa* was found to be(71.42±0.74% and73.32± 1.27%).Zubia *et al.*[47] reported that phenolic compounds of algal extracts of *Halimeda tuna, Caulerpa cupressoides* and *Caulerpa paspaloides*also exhibited relatively high DPPH radical scavenging activities (6.17±0.10, 6.35±0.15 and 7.36±0.16 mg ml^{-1} respectively). Subash*et al.*[20] reported that the total antioxidant ability of crude polysaccharide (TCP) from a brown alga *Turbinaria ornata* was compared with Trolox standard. ABTS radical scavenging activity of crude polysaccharide from *Turbinaria ornata* was 89.69% whereas Trolox showed 82.39%.

There are two types of hydroxyl antioxidant mechanism: one suppresses the generation of the hydroxyl radical and the other scavenges the hydroxyl radicals generated. In the former, the antioxidant activity may link to the metal ions which H_2O_2 to give the metal complexes. The metal complexes thus formed cannot further react with H_2O_2 to give a hydroxyl radical[48].In the present study the hydroxyl scavenging assay from polysaccharide of *C. racemosa* was found to be (68.29±1.03). Costa *et al.*[8] reported only the sulfated polysaccharides from *D. mertensis, D. menstruallis, G. caudata* and *C. sertularioides* have shown activity in hydroxyl radical scavenging. Sulfated polysaccharides from *C. sertularioides* at 0.5 mg/ml showed 11.8% scavenging activity whereas those from *D. mertensis, D. menstruallis* and *G. caudata* at the same concentration showed 8.7, 7.5 and 8.0% scavenging ability respectively. In the present study the superoxide anion radical scavenging activity from polysaccharide of *C. racemosa* was found to be(66.17±0.77 %).Costa *et al.*[8] reported the presence of the sulfated polysaccharides and commercial antioxidant in *C. sertularioides.*The superoxide radical scavenging activity of *C. sertularioides* was (23.3% of scavenging at 0.5 mg/ml).Fucoidans (sulfated polysaccharides) from the seaweed *Ulva pertusa*[48]and *Laminaria japonica*[49]have much stronger scavenging activity on superoxide radical than vitamin C. In the present study the nitric oxide scavenging activity from polysaccharide of *C. racemosa* was found to be (40.64± 1.82%).Vijayabaskar and Shiyamala[43] reported that the suppression of nitric oxide release may be attributed to a direct nitric oxide scavenging effect. The *Turbinaria ornata* had scavenging activity of (39.8± 2.52%). The polysaccharides of the *C. racemosa* had potent antioxidant activity which clearly indicates the beneficial effects of green seaweed polysaccharides as antioxidants.Further studies are needed to investigate the biological activities such as immune modulation, antitumor and anti-inflammatory activities.

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