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Devaraj Isaac Dhinakaran
Department of Biotechnology, Ayya Nadar Janaki Ammal College, Sivakasi 626124.

Paramasivam Geetha
Department of Biotechnology, Ayya Nadar Janaki Ammal College, Sivakasi 626124.

Rajalakshmi
Department of Biotechnology, Ayya Nadar Janaki Ammal College, Sivakasi 626124.

Solomon Jeeva
Department of Botany, Scott Christian College, Nagercoil 629001.

Correspondence
Devaraj Isaac Dhinakaran
Department of Biotechnology, Ayya Nadar Janaki Ammal College, Sivakasi 626124.

Antioxidant activities of marine algae *Valoniopsis pachynema* and *sargassum swartzii* from the south east coast of India

Devaraj Isaac Dhinakaran, Paramasivam Geetha, Rajalakshmi, Solomon Jeeva

Abstract
Marine algae act as the vital source of marine ecosystem. To assess the antioxidant properties of marine algae *Valoniopsis pachynema* and *Sargassum swartzii.* Direct extraction method was used to prepare the algal extracts. Antioxidant activities has been evaluated using 2,2-diphenyl-1-picrylhydrazyl DPPH Radical Scavenging Assay, Hydrogen peroxide (H_2O_2) scavenging assay, Total phenolic content, β-carotene bleaching assay, Nitric oxide radical scavenging assay. High antioxidant activity was exhibited using *Sargassum swartzii* extracts. In Total phenolic content marine algae *Valoniopsis pachynema* showed high antioxidant activity. Comparative analysis results showed that both the algae could be used as antioxidant agents. The marine algae *Valoniopsis pachynema* and *Sargassum swartzii* methanol extracts exhibited effective antioxidant activities. Hence they could be used as antioxidants.

Keywords: *Valoniopsis pachynema*, *Sargassum swartzii,* DPPH, antioxidant activity, hydrogen peroxide

1. Introduction
Marine algae are an important source of medicinal products in the marine ecosystem. Particularly seaweeds are directly exposed and are susceptible to ambient microorganisms such as bacteria and fungi [1]. Marine algae are classified as red algae (Rhodophyta), brown algae (Phaeophyta) and Green algae (Chlorophyta) [2]. The methanol extracts of green and brown seaweeds showed better radical scavenging and reducing power ability with higher phenolic content present in the seaweed [3]. Algae are described as heterogeneous group of organisms with considerable metabolic diversities. It includes compounds such as, sterols, isoprenoid, terpenoids, steroid, phenolic compounds, fatty acids, acrylic acid and alkaloids. Exceptional sources act as antimicrobials, anticancer, antioxidants, antiviral, anti-inflammatory, *wound healing and* neuroprotective compounds [4]. Sulfated polysaccharides isolated from *Acanthohora spicifera* showed antitumor effect against the human leukemic cell lines. Ethanolic extract of *Acanthohora spicifera* subjected to the *In vitro* cytotoxic assay showed significant increase in the death rate of Ehrlich ascites carcinoma cell lines [5]. *Zostera marina* is an important species of algae in coastal ecosystems. It contributes nutrients and provides food stuffs and habitats of many marine organisms such as invertebrates and fishes. Antioxidant activity of the polysaccharide zosterine which was isolated from *Zoster marina* has been examined for the activation of free radicals peroxide oxidation in mice. It was compared with two antioxidant drugs [6]. Many phytochemicals are present in the marine algae. They protect the cell constituents against destructive oxidative damage. Inhibition of hydrolytic and oxidative enzymes including lipid peroxidation, thus limiting the risk of various degenerative diseases associated with oxidative stress [7].
Antioxidants found in many algae are important bioactive compounds that play a major role against various diseases and anti-ageing processes through protection of cells from oxidative damage. The total antioxidant activity from organic extracts of 37 algal samples, of Hawaiian algae from 27 different genera was determined [8]. All marine Brown-algae contain polyphenols and phlorotannins which act as antioxidants, antibacterial and anti-algal compounds. Antioxidant compounds from algae play an important role against various diseases (e.g., chronic inflammation, atherosclerosis, cancer and cardiovascular disorders) and ageing processes, which explains their considerable commercial potential in medicine, food

production and the cosmetic industry. *Halimeda opuntia* and *Sarconema filiforme* are the source of pharmacological benefits. Methanol extract of these two seaweeds showed significant cytotoxicity (LC_{50}<500µg) on brine shrimp. *Halimeda opuntia* showed highest cytotoxic activity (LC_{50}=192.3µg) than *Sarconema filiforme* [9]. Natural antioxidants such as α-tocopherol, phenols and β-carotene found in higher plants are being used in the food industry to inhibit lipid peroxidation. They can protect the human body from free radicals and retard the progress of many chronic diseases [10]. Marine algae contain various bromophenols that have been shown to possess a variety of biological activities, including antioxidant, antimicrobial, anticancer, anti-diabetic, and anti-thrombotic effects [11]. Seaweed (*Ascophyllum nodosum*) a plant growth regulator, showed increased activity of the antioxidant superoxide dismutase (SOD) and has specific vitamin precursors. Seaweed act as antioxidants have potential role as health promoting ingredients in the pharmaceutical and functional food industries [12]. Therefore the present study is focused on whether the algae extracts act as antioxidants. These findings helps us to progress for the next level of research.

2. Materials and methods

2.1. Collection and identification of seaweeds

Two different seaweed samples were collected during the low tidal conditions at depths of 1 to 3m from Rasta god region, in Kanyakumari district, Tamilnadu. The collected samples were transferred to the laboratory and the taxonomic position was identified at Department of Marine and Coastal Studies, Madurai Kamaraj University, Tamil Nadu, India. Algal samples were cleaned of epiphytes and extraneous matter, and necrotic parts were removed. Plants were washed with sea water and then in fresh water. The samples were rinsed with sterile distilled water and were shade dried, cut into small pieces and powdered in a mixer grinder.

2.2. Preparation of extracts

The algae after drying were weighed and powdered. Algae extracts were prepared by using direct extraction method. The samples were weighed and dissolved in methanol. It was kept for 24 h at room temperature and mixed at regular intervals. After 24hr the dissolved samples were filtered using Whatman filter paper and stored for further use.

2.3. DPPH Radical Scavenging Assay

Free radical scavenging ability of the extracts was tested by DPPH radical scavenging assay as described [13]. Different concentration of samples at 20µg/ml, 40µg/ml, 80 µg/ml and 160 µg/ml of the extracts were taken in the test tubes. 3 ml of 0.1mM DPPH in ethanol was added to each tube and incubated in dark at room temperature for 30minutes.The absorbance was read at 517nm using UV-visible spectrophotometer. Ascorbic acid was used as the standard. The % inhibition (I%) was calculated using the formula

I% = [Abs (control) - Abs (sample)] x100/Abs (control)

2.4. Hydrogen peroxide (H_2O_2) scavenging assay (HRSA)

H_2O_2 scavenging activity was determined by using seaweed extracts. A solution of H_2O_2 (10mM) was prepared in phosphate buffer (pH7.4). Reaction mixture containing 2.5ml of H_2O_2 solution and 50µg/ml, 100µg/ml, 250µg/ml, and 500µg/ml of varying concentration of algal extracts. It was made up of two 3ml with phosphate buffer. The volume of water was added in different aliquots 950 µl, 850 µl, 750 µl, 500 µl. The absorbance was measured at 0min at 230nm. Ascorbic acid was used as the standard. Total H_2O_2 scavenging activity was expressed in %.

HRSA (%) = [Abs (control) – Abs (sample)/Abs (control)] x 100

2.5. Total Phenolic Content

Total phenolic content was determined with Folin and Ciocalteau reagent and by using gallic acid as standard. [14]. The concentration range 10µg/ml, 20µg/ml, 30µg/ml, 40µg/ml of the sample was mixed with 9 ml of distilled water and 1 ml of Folin-Ciocalteu reagent and 10 ml of stock of 7% sodiumcarbonate each tube 2ml was added. After 90 min of incubation the absorbance was determined at 760 nm. The phenolic content was expressed as GAE (Gallic acid equivalent) in mg/ml. It is calculated in %.

2.6. β-carotene bleaching assay.

This experiment was carried out by measuring the oxidation of β -carotene and linoleic acid system, β-carotene solution in chloroform (10mg/1 ml) was added to a mixture of linoleic acid and Tween 20. The extracts at the range of 10µg/ml, 20µg/ml, 30µg/ml, 40 µg/ml were used. The chloroform in the preparation was gently removed under a rotary evaporator. Distilled water was added and mixed well with the residue to prepare an emulsion. β -carotene/linoleic acid emulsion were mixed with the methanol solutions of the extracts and commercial antioxidant (vitamin c), and incubated in a water bath at 50 °C for120min. Inhibition of the oxidation of the β -carotene/linoleic acid emulsion system by the extracts and the commercial antioxidants was monitored spectrophotometrically by measuring the absorbance at 470 nm.

2.7. Nitric oxide radical scavenging assay

Various concentrations of the extract and sodium nitroprusside (10mM) in standard phosphate buffer solution (0.025 M, pH 7.4) in final volume of 3 ml was incubated at 37 °C for 150 min. Control experiments without the test compounds but with equivalent amount of buffer were prepared in the same manner as done for the test. The extracts at the range of 20µg/ml, 40µg/ml, 80µg/ml, 160 µg/ml were used. Thereafter, 0.5 ml of incubation solution was removed and diluted with 0.5ml Griess' reagent (1%sulphanilamide, 2% Ortho Phosphoric acid and 0.1% naphthyl ethylenediamine dihydrochloride) and allowed to react for 30 min. The absorbance of the chromophore formed during diazotisation of nitrite with sulphanilamide and subsequent coupling with naphthyl ethylenediamine dihydrochloride was read at 565nm. The percentage inhibition was calculated.

% inhibition = $[(A_0 – A_1) / A_0] \times 100$

Where, A_0 = Absorbance of control. A_1 Absorbance of sample

3. Results

3.1. DPPH method

Table 1 shows the antioxidant activity of marine algae crude extracts in *Valoniopsis pachynema* and *Sargassum swartzii* using DPPH method. The absorbance was measured at 517nm. The extracts were taken as 20µg, 40µg, 80µg, 160µg concentrations. Figure 1 shows the comparative analysis of *Valoniopsis pachynema* and *Sargassum swartzii* in DPPH method. Maximum activity was seen in *Sargassum swartzii* extracts.

Table 1: Comparative analysis between marine algae *Valoniopsis pachynema* and *sargassum swartzii* using DPPH method

Concentration of test samples µg/ml	Volume of DPPH	Optical density at 517nm % Inhibition (I %)	
		Valoniopsis pachynema	***Sorgassum swartzii***
20µg	2960µl	55	75.34
40µg	2960µl	68.20	79.45
80µg	2960µl	76.32	87.54
160µg	2960µl	88.30	98.70

3.2. Hydrogen peroxide assay

Table 2 represents the antioxidant activity of seaweeds *Valoniopsis pachynema* and *Sargassum swartzii* using H_2O_2 method. The absorbance was measured at 230nm. Figure 2 shows the comparative analysis of the marine algae crude extracts such as *Valoniopsis pachynema* and *Sargassum swartzii* using H_2O_2 method. *Sargassum swartzii* showed effective and maximum antioxidant activity than *Valoniopsis pachynema*

Table 2: Comparative analysis for the H_2O_2 assay using marine algae *Valoniopsis pachynema* and *sargassum swartzii*

S.No	Concentration of extract µg/ml	Volume of distilled water	Volume of H_2O_2	Optical Density at 230nm and HRSA (%)	
				Valoniopsis pachynema	***Sargassum swartzii***
1.	50 µg	950 µl	2 ml	62.08	70.02
2.	100 µg	850 µl	2 ml	75.12	75.32
3.	250 µg	750 µl	2 ml	83.70	88.31
4.	500µg	500µl	2 ml	93.44	98

3.3 Total phenolic content

Table 3 suggests the antioxidant activity of seaweeds such as *Valoniopsis pachynema* and *Sargassum swartzii* using TPC method. The absorbance was measured at 760nm. This also shows the comparative analysis of total phenolic content in the extracts of *Valoniopsis pachynema* and *Sargassum swartzii*. Maximum activity was seen in *Valoniopsis pachynema*

Table 3: Comparative analysis for the Total phenolic content using marine algae *Valoniopsis pachynema* and *sargassum swartzii.*

S. No	Concentration of extract	Volume of Folin Ciocalteau reagent	Volume of Na_2Co_3	Optical density at 760nm GAE mg/ml	
				Valoniopsis pachynema	***Sargassum swartzii***
1.	10µg/ml	1 ml	2 ml	50	30
2.	20µg/ml	1 ml	2 ml	65	48
3.	30µg/ml	1 ml	2 ml	78	56
4.	40µg/ml	1 ml	2 ml	86	62
5.	50µg/ml	1 ml	2 ml	98	74

3.4. β-carotene bleaching assay

Table 4 shows the antioxidant activity in seaweeds like *Valoniopsis pachynema* and *Sargassum swartzii* using β-Carotene method. The absorbance was measured at 470nm. It also denotes the comparative analysis of marine algae crude extracts in *Valoniopsis pachynema* and *Sargassum swartzii* using β-Carotene method. Maximum activity was seen in *Sargassum swartzi*.

Table 4: Diagrammatic representation of β-carotene bleaching assay using marine algae Valoniopsis pachynema an sargassum swartzii

S.No	Concentration of extract µg/ml	Optical density in regular intervals (minutes)	Optical density at 470nm	
			Valoniopsis pachynema	***Sargassum swartzii***
1.	10µg	15	0.291	0.399
2.	20µg	30	0.280	0.295
3.	30µg	45	0.260	0.288
4.	40µg	60	0.259	0.278

3.5. Nitric oxide radical scavenging assay

Table 5 reviews the antioxidant activity of seaweeds *Valoniopsis pachynema* and *Sargassum swartzii* using Nitric oxide assay. The absorbance was measured at 565nm. It also shows the comparative analysis of the marine algae crude extracts such as *Valoniopsis pachynema* and *Sargassum swartzii* using Nitric oxide assay method. Maximum activity was seen in *Sargassum swartzi*

Table 5: Graphical representation of the Nitric oxide radical scavenging assay using marine algae *Valoniopsis pachynema* and *sargassum swartzii.*

S.No	Concentration of extract µg/ml	Volume of Nitroprusside	Optical density at 517nm % inhibition	
			Valoniopsis pachynema	***Sorgassum swartzii***
1.	20µg	2.0 ml	62	74
2.	40µg	2.0 ml	76	80
3.	80µg	2.0 ml	85	87.20
4.	160µg	2.0 ml	95.32	98

4. Discussion

In the present study antioxidant activities were observed in the marine algae *Valoniopsis pachynema* and *Sargassum swartzii* using methanol extracts. Previous studies indicate that the extract from *S. siliquastrum* showed antioxidant activity [15]. DPPH radical scavenging assay was performed to identify antioxidant properties of the extracts. It was shown that *Sargassum swartzii* exhibited a potent proton-donating ability to DPPH. Phenolic compounds in methanol extract were able to transfer labile hydrogen atoms to DPPH. Bleaching of purple-coloured DPPH was observed with increasing sample concentration. Formation of dark purple colour slowly bleached into stable compound (yellow) by reacting with an antioxidant agent.

The H_2O_2 radical scavenging activity of the *Sargassum baccularia* extract increases with increase in concentration [16]. The methanolic extract of *Sargassum baccularia* was found to be effective than hexane extract. Similarly the Nitric oxide scavenging activity of the algal extract increases with increasing concentration at 89.85% in the presence of 1 mg/ml BHT. Hence it is noted that marine algae *Sargassum swartzii* have high antioxidant activity in Nitric acid scavenging assay, beta carotene assay, DPPH and H_2O_2 except in TPC where *Valoniopsis pachynema* showed maximum antioxidant activity than *S. Swartzii*. The phenolic compounds were seen at high concentrations in brown algae *A. taxiformis* and *S. vulgare* [17]. Here the marine algae *Valonopsis pachynema* and *Sargassum swartzii* are rich in phenolic compounds. Previous studies states that algae *Sargassum baccularia* and *Cladophora patentirameaare* considered as the good sources for antioxidants [18].

Previous report suggested that the antioxidant activity were observed in the three species of seaweeds *Sargassum dentifolium*, *Laurencia papillosa* and *Jania corniculata* collected from Egypt [19]. In our study marine algae *Valonopsis pachynema* and *Sargassum swartzii* exhibited antioxidant activities and were collected from the south east coast of India. Similarly the extracts from 48 marine macroalgae species (17 Chlorophyta, 8 Phaeophyta and 23 Rhodophyta) from the coasts of Yucatan and Quintana Roo (Mexico) were evaluated their antioxidant activity [20]. The hexane, petroleum ether, chloroform, acetone and methanol extracts of *Kedrostis foetidissima* revealed the presence of flavonoids, steroids, tannins, triterpenoids, phenols, steroids, glycosides and cardiac glycosides [21]. The *invitro* screening of secondary metabolites like, alkaloids, carbohydrates, saponins, glycosides protein and aminoacids, phytosterol, phenolic compound, flavonoids, terpinoids, and tannins [22]. The ant oxidative activities were detected from the species of Chlorophyta and Phaeophyta in Jeju Island. Especially, *Sargassum thunbergii* showed active results [23]. The polysaccharides seen in the brown alga *Sargassum fusiforme*, and *S. fusiforme* (SFPS) were extracted in hot water. They exhibited good antioxidant activities [24]. There are numerous reports of macro algae like *Caulerpa taxifolia*, *Caulerpa racemosa* derived chemical compounds that have a broad range of biological activities, like antioxidant and antitumor. Some of which have been used in pharmaceutical. Industries. It includes *Chlorophyceae*, *Phaeophyceae*, *Rhodophyceae* from all over the world [25]. Marine algae *Valonopsis pachynema* and *Sargassum* swartzii used in this study showed significant antioxidant activity in Nitric oxide assay, and beta carotene assays. Fucoidan is a class of Sulfated polysaccharides enriched with fuses in the extracellular matrix of brown algae. Fucoidan have demonstrated various biological activities including antiviral, anti-inflammatory, anticoagulant, antiangiogenic, immunomodulatory, and anti-adhesive activity [26]. *Biomphalaria glabrata, Ulva lactuca* (Chlorophyta), *Padina gymnospora, Sargassum vulgare* (Phaeophyta), *Hypnea musciformis,* and *Digenea simplex* (Rhodophyta) collected from Brazil showed larvicidal activity gainst *Aedes aegypti*, molluscicidal activity against *H. musciformis* and *P. gymnospora* [27]. It is revealed that the marine algae *Valonopsis pachynema* and *Sargassum swartzii* used in this study were collected from Indian Oean

5. Conclusion

The present study demonstrated the antioxidant properties of marine algae *Valonopsis pachynema* and *Sargassum swartzii* using methanol extracts. It is also observed that the algae could be used as effective antioxidants for the prevention of diseases.

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7. References

1. Pandian P, Selvamuthukumar S, Manavalan R, Parthasarathy V. Screening of antibacterial and antifungal activities of red marine algae. *Acanthaphora spicifera* (Rhodophyceae). J Biomed Sci Res. *2011*; 3(3):444-448.
2. Cox S, Abu-Ghannam N, Gupta S. An assessment of the antioxidant and antimicrobial activity of six species of edible Irish seaweeds. Inter F Res J. 2010; 17:205-220.
3. Anandhan S, Kumara HS. Biorestraining potential of marine macro algae collected from Rameshwaram. Tamil nadu J res. boil. 2011; 1:385-392.
4. Seung HL, Jeon YJ. Anti-diabetic effects of brown algae derived phlorotannins, marine polyphenols through diverse mechanisms. *Fitoterapia* 2013; 86:129-136.
5. Meenakshi SM, Sathiyamoorthi M, Udhayachandran T, Aravind S. *In vitro* cytotoxicity and molecular docking studies on *Acanthophora spicifera.* Der Phar Chem 2014; 6(1):411-417.
6. Stirk WA, Reinecke DL, Staden JV. Seaasonal variation in antifungal, antibacterial and acetylcholinesterase activity in seven South African seaweeds. J Appl Phycol. 2007; 19:271-276.
7. Vinery RP, Prakash RP, Sushil SK. Antioxidant activity of some Selected medicinal plants in Western Region of India. Adv Biol Res 2010; 4(1):23-26.
8. Dovi K, Posner EK, Karla J, Dermid, Tabandera KN *et al*. Antioxidant Activity of Hawaiian Marine Algae. *Mar Drugs* 2012; 10:403-416.
9. Samy A, Selim S. Antimicrobial, Antiplasmid and Cytotoxicity Potentials of Marine Algae *Halimeda opuntia* and *Sarconema filiforme* collected from Red Sea Coast, *WASET* 2012; 1:61.
10. Mayer AMS, Hamann MT. Marine pharmacology in 2001-2002. Marine compounds with anthelmintic, antibacterial, anticoagulant, antidiabetic, antifungal, anti-inflammatory, antimalarial, antiplatelet, antiprotozoal, Antituberculosis and antiviral activities: affecting the cardiovascular immune and nervous systems and other miscellaneous mechanisms of action. *Comp Biochem Physiol* 2005; 140:265-286.

11. Leven MM. *Escherichia coli* that cause diarrhea: Enterotoxiogenic, enteropathogenic, enteroinvasive, enterohem orrhagic and enteroadherent. J Infect Dis. 2008; 155:41-47.
12. Fabricant DS, Farnsworth NR. The value of plants used in traditional medicine for drug discovery. Environmental Health Perpectives 2011; 109:69-75.
13. Blois MS. Antioxidant determinations by the use of a stable free radical. *Nature* 1958; 181:1199-1200.
14. Singleton VL, Orthofer R, Lamuela- Raventos RM. Analysis of total phenols and oxidation substrates and antioaxidants by means of Folin- Ciocalteau reagent. *Meth Enzymol* 1999; 299:152-177.
15. Lima HC, Costa FHF, Sampaio AH, Neves SA, Benevides NMB *et al*. Induction and inhibition of human lymphocyte transformation by the lectin from the red marine algae Amansia multifida. J Appl Phyco. 1998; 10:153-162.
16. Gluder TA, Moore BS. Chasing the treasure of the sea bacterial marine natural products. Curr Opn Micro 2009; 12:252-260.
17. Zahra R, Mehrnaz M, Farzaneh V, Kohzad S. Antioxidant activity of extract from a brown alga, Sargassum boveanum. Afr J Biotechnol 2007; 6(24):2740-2745.
18. Ismail A, Marjan ZM, Foong CW. Total antioxidant activity and phenolic content in selected vegetables. *Food Chemistry* 2004; 87:581-586.
19. Khan S, Kong C, Him S. Protective effect of *Amphiroa dilatata* on ROS induced oxidative damage and MMP expressions in HT1080 cells. Biotech Biopr Eng 2010; 15:191-198.
20. Yolanda FP, Morales JL. Antibacterial activity in marine algae from the coast of Yucatan, *Botanica Marina* 2004; 47:140-146.
21. Vasanth NI, Chaykina EL, Busarova NG, Anisimov MM. Antimicrobic and hemolytic activity of low molecular metabolites of brown seaweeds Laminaria cichorioides. Appl Biochem 2012; 2:22-26.
22. Hebsibah E, Dhana Rajan MS. Evaluation of antimicrobial activity and phytochemical screening of Gelidium acerosa. J Pharm Sci Res. 2010; 2(11):704-707.
23. Heo SJ, Park EJ, Lee KW, Jeon YJ. Antioxidant activities of enzymatic extracts from brown seaweeds. *Bioresour Technol* 2005; 96:1613-1623.
24. Mori JM, Iwashima H, Wakasugi H, Saito H et al. New plastoquinones isolated from the brown alga, Sargassum micracanthum. Chem Pharm 2005; 53:1159-1163.
25. Manjula E, Narasimha R. In vitro study of antimicrobial activity in marine algae caulerpa taxifolia and caulerpa racemosa. IJABPT 2014; 5(2).
26. Luis J, Gomaz V, Irma E, Mercardo S, Graciela G *et al*. Antibacterial and anticancer activity of seaweeds and bacteria associated with their surface. Revista de Bio Marina y oceanografia 2010; 45(2):267-275.
27. Guedes EAC, Silva TG, Aguiar JS, Barros LD, Pinotti LM, Sant' Ana AEG. Cytotoxic activity of marine algae against cancerous cells. Revista Brasileira de Pharmacognosy. 2013; 23(4):668-673.