In vitro Antioxidant Activities of Four Marine Green Algae Species of a Genus

Enteromorpha from Mandapam Coast, Tamil Nadu, India

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ABSTRACT

The present study focused on determining the antioxidant properties of the Enteromorpha compressa (Linnaeus) Nees, Enteromorpha flexuosa (Wulfen) J. Agardh, Enteromorpha intestinalis (Linnaeus) Nees, and Enteromorpha prolifera (Müller) J. Agardh, ethanol crude extracts, from the green algae. The evaluation of antioxidant properties was estimated and determined by using ten standard methods namely 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2,2′-azinobis 3ethylbenzthiazoline-6-sulphonic acid (ABTS), metal chelating activity, hydrogen peroxide radical assay, superoxide radical scavenging (SOD), reducing power, hydroxyl radical assay, nitric oxide (NO) scavenging assay, ferric reducing antioxidant power (FRAP) assay and total antioxidant activity assay. The tested green algae also had antioxidant activity like algae diatom which are showing good antioxidant potential. These algae had a lesser radical scavenging ability than standard ascorbic acid. This study suggests that ethanol crude extracts contain different potential antioxidant compounds capable to scavenge different types of free radicals.

Keywords: Enteromorpha, Antioxidant activity, ABTS, DPPH, FRAP.

I. Introduction

Seaweed is having the properties of antibacterial, antifungal, antiviral, and several phytochemicals[1] and also a good source of antioxidants. Nowadays, good food products are in demand without preservatives. Sherwin[2] reported that the preservatives are closely related to synthetic antioxidants namely like butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), etc., can cause liver damage, mutagenic, and neurotoxic. Oktay et al.,[1] reported that phytochemicals like flavonoids, polyphenols, etc., are broadly present in plants, which are found to be acting as free radical scavengers. Marine algal seaweeds contain high amounts of polyphenols and consequently can be used as effective natural antioxidants. These plants are utilized for their rich nutrient content and antioxidant properties in treating major degenerative and deficiency diseases. The food source for fishes, crustaceans, and gastropods are green algae like Ulva, Caulerpa, and Enteromorpha. These algal species are also used as salad and in soups. The bioactive compounds such as diterpenes, triterpenes, sesquiterpenes, and ceramides have been isolated from several genera particularly Caulerpa, Chaetomorpha and Ulva[3].

Aguilera et al.,[4] reported that the antioxidant substances in seaweeds are found to be an endogenous defense mechanism as a protection against oxidative stress due to tremendous environmental conditions. The Gulf of Mannar possesses abundant growth of more than 680 species of seaweed. Hence an attempt was undertaken to evaluate the antioxidant activity of selected seaweed from Mandapam coast.

II. Materials and Methods

2.1. Collection of Green Marine Algae

Green algae were collected from the Mandapam coast of Tamil Nadu, India. The freshly collected seaweeds were washed with clean seawater to remove salt, epiphytes, and sand attached to the surfaces of the samples and transported to the laboratory. The samples were carefully rinsed with tap water. The samples were pulverized into powder and stored at - 80 °C before extraction.

2.2. Preparation for Analysis

2.2.1. Sample Extract

5g of alga powder was used to extract overnight with 200 ml ethanol at room temperature and centrifuged at 3000 rpm for 10 min. The supernatant was collected in a separate labeled bottle after passing through a Whatman No.1 filter.
paper and the residue was re-extracted two times under the same conditions. The pooled extracts were dried, frozen, and kept on -80 °C until analysis. The freeze-dried extracts were re-dissolved in ethanol and used for the analysis.

2.3. Antioxidant Activity Assays

2.3.1. 1,1-diphenyl-2-picrylhydrazyl (DPPH) Radical Scavenging Activity

1,1-diphenyl-2-picrylhydrazyl radical was used in the evaluation of radical scavenging activity of the extracts, as described [5] with minor changes in the DPPH concentration. The reaction mixtures, containing 120 μL of 0.04 mg/ml DPPH solution in methanol, were mixed with 20μL of different concentrations of the extracts and shaken vigorously before being incubated in the dark for 20 min. Ascorbic acid was used as a positive control. Reduction in the absorbance of DPPH was measured against a blank at 517 nm. The radical scavenging activity was calculated using the following equation.

\[
\text{Percentage of DPPH radical scavenging} = \left( \frac{A_{\text{blank}} - A_{\text{samples/Positive Control}}}{A_{\text{blank}}} \right) \times 100.
\]

Where A blank and A sample/ positive control denote the absorbance of blank and absorbance of samples or positive control respectively.

2.3.2. 2,2’-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical scavenging assay

The ABTS radical scavenging assay was performed according to Re et al.[6]. 7 mM concentration of ABTS was prepared by using deionized water. ABTS radical cation (ABTS+·) produces by the ABTS solution is reacting with 2.45mM potassium persulfate solution at a 1:1 ratio. It was kept in the dark at room temperature for 12-16 h before use. For the study, the ABTS solution was diluted with ethanol. An aliquot of 100μL of a solvent solution containing different extracts/ standard concentrations was added to 1900μL of ABTS solution. The absorbance was measured at 734 nm exactly 6 min after initial mixing. All assays were determined in triplicate and the inhibition percentage was calculated as follows:

\[
\text{Inhibition} = \left( \frac{A_{c} - A_{s}}{A_{c}} \right) \times 100.
\]

Where Ac is ABTS absorbance of the control reaction and As is the ABTS absorbance in the presence of the sample/ standard. Ascorbic acid was used as a positive control.

2.3.3. Superoxide anion radical scavenging activity

The superoxide anion scavenging activity was assayed according to a method described earlier[7] with slight modifications in the nitroblue tetrazolium (NBT) and phenazine methosulphate (PMS) concentrations. The reaction mixture containing 25μL of NBT solution (150μM NBT in 100mM phosphate buffer, pH 7.4), 2μL of PMS solution (60μM PMS in 100mM phosphate buffer, pH 7.4) and 20μL of NADH solution (468μM in 100mM phosphate buffer, pH 7.4) was added to different concentrations of extracts. The mixture was incubated in the dark for 10 min at 25 °C and the absorbance read at 560 nm. Ascorbic acid was used as a positive control. All experiments were done in triplicate and results were expressed as percentage inhibition of superoxide anion radical using the following equation.

\[
\text{Percentage of superoxide anion radical scavenging} = \left( \frac{A_{\text{blank}} - A_{\text{samples/Positive Control}}}{A_{\text{blank}}} \right) \times 100.
\]

2.3.4. Nitric oxide scavenging activity

The nitric oxide scavenging activity was determined using a previously described method [8]. 10μL of 10mM sodium nitroprusside (SNP) in phosphate buffer was mixed with 10μL of different concentrations of extracts. The mixture was incubated in the dark at room temperature for 2.5 h. Ascorbic acid was used as a positive control. 40μL of sulphanilic acid reagent (0.33 % sulphanilic acid in 20 % glacial acetic acid) was added to the mixture, after the incubation period and further incubated for 5 min, after which 40μL of 0.1 % naphthyl ethylene diamine dihydrochloride (NEDA·2HCl) was added, mixed and incubated for 30 min at 25 °C. The absorbance of the chromophore formed was read at 540 nm. All determinations were performed in triplicate and results were expressed as a percentage of nitric oxide scavenged by using the following equation.

\[
\text{Percentage of nitric oxide scavenging} = \left( \frac{A_{\text{blank}} - A_{\text{samples/Positive Control}}}{A_{\text{blank}}} \right) \times 100.
\]
2.3.5. Hydroxyl radical scavenging activity

The site-specific hydroxyl radical scavenging assay was determined as described [9]. The reaction mixture containing 23.8μL of 100mM FeCl₂ solution, 23.8μL of 1.25mM H₂O₂ solution, 23.8μL of 2.25mM deoxyribose and 23.8μL of 100mM ascorbic acid was added to 5μL of different concentrations of extracts. The mixture was incubated at 37 °C for 1 h, after which 100μL of 0.5 % of thiobarbituric acid (TBA) in 25mM NaOH and 100μL of 2.8 % trichloroacetic acid (TCA) was added. The resulting mixture was then boiled at 100 °C for 15 min and subsequently cooled on ice before taking the absorbance readings at 550 nm. Ascorbic acid was used as a positive control. All determinations were performed in triplicate and results were expressed as the percentage of hydroxyl radical scavenging activity as calculated by the following equation.

\[
\text{Percentage of hydroxyl radical scavenging (\%) = \left( \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right) \times 100.}
\]

2.3.6. Hydrogen Peroxide Scavenging Assay

The hydrogen peroxide radical scavenging assay was performed with ethanol extract of the seaweed sample. The ability of the seaweed extracts to scavenge H₂O₂ was revealed, according to the method of Ruch et al. [10] with slight modification. About 40mM H₂O₂ was prepared in phosphate buffer (pH 7.4) and the H₂O₂ concentration was determined spectrophotometrically. Ethanol extracts with different concentrations in distilled water and ascorbic acid (20 ~ 100μg/ml) were added to 0.6 ml of 40 M H₂O₂ solution. It was kept for 10 min incubation against a blank solution containing phosphate buffer without H₂O₂ and then the absorbance of H₂O₂ was determined at 230 nm. The percentage of scavenging of H₂O₂ was calculated using the following formula:

\[
\text{H₂O₂ radical scavenging activity (\%) = } \left( \frac{A_{\text{blank}} - A_{\text{sample}}}{A_{\text{blank}}} \right) \times 100.
\]

2.3.7. Reducing power radical scavenging activity

The reducing power was determined as described by Yen and Chen [11]. Briefly, 0.13 ml of sample different concentration (10-50μg/ml) in phosphate buffer (0.2 M, pH 6.6) were mixed with 0.125 ml of potassium ferricyanide (1%, w/v) and incubated at 50 °C for 20 min. Afterward, 0.125 ml of TCA (10%, w/v) was added to the mixture to terminate the reaction. Then, the solution was mixed with 1.5 ml ferric chloride (0.1%, w/v) and the absorbance was measured at 700 nm.

2.3.8. Metal ion chelating activity

The reaction mixture contained 1.0 ml of various concentrations of Enteromorpha species sample, 0.1 ml of 2mM FeCl₃, and 3.7 ml ethanol [12]. All the reaction reagent mixture was used as a control, without sample. The reaction was initiated by the addition of 2.0 ml of 5mM frozen. After 10 minutes at room temperature, the absorbance of the mixture was determined at 562nm against a blank. The lower absorbance of the reaction mixture indicated a higher iron-chelating ability. The capacity to collate the ferrous ion was calculated by % chelation = [1-(ABS sample/ABS control)] ×100.

2.3.9. Ferric-reducing antioxidant power (FRAP) assay

10mM of 2, 4, 6-tripyridyl-s-triazine (TPTZ) stock solution in 40mM HCL, 20mM FeCl₃,6H₂O and 0. 3M acetate buffer (pH 3.6) was prepared [13]. The FRAP reagent was a mixture of 2.5 ml TPTZ solution, 2.5 ml ferric chloride solution, and 25 ml acetate buffer. It was freshly prepared and warmed to 37°C. FRAP reagent (900 ml) was mixed with 90 ml distilled water and 30 ml of the sample and standard antioxidant solution. The reaction mixture was then incubated at 37 °C for 30 minutes and the absorbance was recorded at 595 nm. An intense blue color complex was formed when ferric tripyridyltriazine (Fe₃⁺-TPTZ) complex was reduced to ferrous (Fe₂⁺) form. The absorption at 540 nm was recorded

2.3.10. Total Antioxidant Assay

The total antioxidant capacity of seaweed extracts was estimated by the phosphomolybdenum method [14]. A reagent solution was prepared by combining 0. 6M H₂SO₄, 28mM Na₂HPO₄, and 4mM (NH₄)₆MoO₄·24. Ethanol extracts with different concentrations were combined with 3 ml of reagent solution. Then the tubes containing the reaction solution were incubated at 95°C for 90 min in a water bath. The tubes were kept for cooling at room temperature. At 695 nm, the absorbance was measured against blank after cooling to room temperature. Ethanol was used in the place of extracts as the blank. The total antioxidant activity was interlocked as the number of gram equivalent of ascorbic acid. The calibration curve was prepared by mixing ascorbic acid with ethanol.
2.4. Statistical analyses

All the data were expressed as means ± standard deviation (SD). The experiments were carried out in triplicates. The statistical analyses were performed using the SPSS statistical package (SPSS Inc., Version 16.).

III. Results and Discussion

DPPH is an abbreviated form of an organic chemical compound of 2, 2-diphenyl-1-picrylhydrazyl. It is a standard and a stable form of a free radical molecule with a dark-colored crystalline powder. The DPPH has been used enormously as a free radical chemical to evaluate reducing substances\(^1\). The salient feature of DPPH is the purple color, usually disappeared while an antioxidant compound is present in the medium. Thus, molecules with antioxidant can quench DPPH free radicals and convert them to a colorless product, resulting in a decrease in absorbance at 517 nm. So, the more rapidly the absorbance decreases the more potent antioxidant activity of the extract. The ethanol extracts of four species of *Enteromorpha* exhibited significant concentration-dependent inhibition of DPPH activity. These algae had a lesser activity than the standard of ascorbic acid. The results showed (Fig. 1, and 1a) that *E. compressa*, *E. flexuosa*, *E. intestinalis* and *E. prolifera* were showed 23.56 µg/ml, 23.93 µg/ml, 27.34 µg/ml, and 30.25 µg/ml of IC\(_{50}\), respectively which were lesser behavior than standard ascorbic acid (21.77µg/ml of IC50).

[Image of DPPH Scavenging Activity graph]

ABTS assay is a simple indirect method for determining the activity of natural antioxidants. ABTS radical is rather stable, but it reacts energetically with an H-atom donor, such as phenolics, been converted into a non-colored form of ABTS. The ABTS radical cation-scavenging assay performed showed that the antioxidant activity increases with an increase in the concentration\(^2\). The results showed (Fig.2, and 2a) that *E. compressa*, *E. flexuosa*, *E. intestinalis* and *E. prolifera* were showed 7.22 µg/ml, 8.21µg/ml, 13.74µg/ml, and 8.94 µg/ml of IC\(_{50}\), respectively which were lesser behavior than standard ascorbic acid (5.23µg/ml of IC50).

[Image of ABTS assay graph]

Superoxide anion radical is one of the strongest reactive oxygen species among the free radicals that are generated after the oxygen is taken into living cells. Srikanth et al., \(^3\) stated that superoxide anion changes to other harmful ROS and free radicals such as hydrogen peroxide and hydroxyl radicals, which induce oxidative damage. The decrease in the absorbance at 560 nm with the *E. compressa*, *E. flexuosa*, *E. intestinalis* and *E. prolifera* thus indicate the consumption of superoxide anion in the reaction mixture. The antioxidant activity of the ethanol crude extract determined by superoxide anion radical assay varied as seen in Fig.3, and 3a. The reducing power was found to be higher in ethanol extract. At a concentration of 12.19µg/ml, 11.89µg/ml, 18.48µg/ml, and 28.68µg/ml of IC\(_{50}\) with *E. compressa*, *E. flexuosa*, *E. intestinalis* and *E. prolifera*.
E. prolifera in superoxide anion radical generated which showed lesser scavenging activities than the standard ascorbic acid (9.05µg/ml of IC\textsubscript{50} value).

The nitric oxide radical scavenging assay was also performed with ethanol crude extract of the seaweed samples. The scavenging of nitric oxide by E. compressa, E. flexuosa, E. intestinalis and E. prolifera was increased in a dose-dependent manner as illustrated in Fig. 4, and 4a. At a concentration of 11.02\( \mu \)g/ml, 11.33 \( \mu \)g/ml, 25.86 \( \mu \)g/ml, and 17.21 \( \mu \)g/ml of IC\textsubscript{50} value nitric oxide generated scavenging abilities with E. compressa, E. flexuosa, E. intestinalis and E. prolifera. The IC\textsubscript{50} value of ascorbic acid was 9.14\( \mu \)g/ml. Nitric oxide and seaweed ethanol extract react and prevent the formation of nitrite. These compounds alter the structure and function of many cellular components, either natural or synthetic compounds with antioxidant properties might contribute towards the partial or total alleviation of this damage\textsuperscript{[18]}.

In this present study, the algal sample was found to scavenge O\textsubscript{2} significantly and in a dose-dependent manner and may protect the DNA, protein, and lipid from damage. The results for the hydroxyl radical scavenging assay were shown in Fig. 5, and 5a. The concentrations for 50\% inhibition were found to be 32.92 \( \mu \)g/ml, 34.59 \( \mu \)g/ml, 37.4 \( \mu \)g/ml, 41.28 \( \mu \)g/ml and 25.49 \( \mu \)g/ml of IC\textsubscript{50} value with hydroxyl radical scavenging abilities of E. compressa, E. flexuosa, E. intestinalis and E. prolifera and ascorbic acid, respectively. Hydroxyl radical iron is the major active oxygen and is causing lipid peroxidation.
in enormous biological damage. The highly reactive hydroxyl radical can cause oxidative damage to macromolecules like DNA, lipid, and protein\(^{[19]}\).

Many species of seaweeds possess scavenging ability of hydrogen peroxide had been reported by Srikanth et al.\(^{[17]}\). It can cross membranes and may slowly oxidize many compounds. Hydrogen peroxide itself is not very reactive, but sometimes it can be toxic to cells because of the rise in the hydroxyl radicals in the cells. The $\text{H}_2\text{O}_2$ radical scavenging assay was also performed with the ethanol crude extract of the seaweed samples. From the results of the selected green algae $E.\text{ compressa}$, $E.\text{ flexuosa}$, $E.\text{ intestinalis}$ and $E.\text{ prolifera}$ were had $27.69\, \mu\text{g/ml}$, $35.34\, \mu\text{g/ml}$, $35.17\, \mu\text{g/ml}$, and $41.93\, \mu\text{g/ml}$ of IC50 value of hydrogen peroxide scavenging ability respectively. These results were lesser activities than the standard ascorbic acid ($21.91\, \mu\text{g/ml}$ of IC50 value of hydrogen peroxide scavenging activity) (Fig. 6 and 6a).

Reducing power capacity is considered as a significant indicator of potential antioxidant activity of a compound or sample\(^{[20]}\). The presence of antioxidants makes the conversion of the $\text{Fe}^{3+}/\text{ferricyanide}$ complex into the ferrous form. Therefore, by measuring the formation of Perl’s Prussian blue at 655 nm, the amount of Fe$^{2+}$ can be monitored. Higher absorbance indicated higher reducing power\(^{[21]}\). The reduced capabilities of $Enteromorpha$ species compared to ascorbic acid (Fig.7). The reducing power of the sample was increased in its quantity. From the results of the selected green algae
*E. compressa*, *E. flexuosa*, *E. intestinalis* and *E. prolifera* were had 35.33 µg/ml, 46.71 µg/ml, 43.91 µg/ml, and 41.14 µg/ml of IC50 value of reducing power radical scavenging ability respectively, These results were lesser activities than the standard ascorbic acid (31.04 µg/ml of IC50 value of reducing power radical scavenging ability) (Fig. 7 and 7a).

Siriwardhana et al., [22] reported that the metal iron chelating ability of seaweeds may be ascribed to the presence of endogenous chelating agents, mainly phenolic because certain phenolic compounds properly orient functional groups, which can chelate metal ions. Ferrozine can cognitively form complexes with Fe^{2+}. In the presence of chelating agents, the red color of the complex is decreased, because, the complex formation is disrupted. The concentrations for 50% inhibition were found to be 7.37 µg/ml, 8.37 µg/ml, 9.89 µg/ml, 16.68 µg/ml and 6.2 µg/ml of IC50 value with metal ion chelating activity of *E. compressa*, *E. flexuosa*, *E. intestinalis* and *E. prolifera* and ascorbic acid, respectively.(Fig. 8 and 8a).

Ferric-reducing antioxidant power (FRAP) is another important indicator of the antioxidant potential of either a compound or an extract with a solvent [23]. The ability to reduce ferric ions indicates that the antioxidant compounds are electron donors, which could be reduced the oxidized intermediate compounds of lipid peroxidation processes, hence acting as primary and secondary antioxidants [24]. The antioxidant activity of the ethanol crude extract determined by FRAP assay varied
as seen in Fig. 9 and 9a. From the results of the selected green algae *E. compressa, E. flexuosa, E. intestinalis* and *E. prolifera* were had 12.01 μg/ml, 15.51 μg/ml, 19.99 μg/ml, and 28.77 μg/ml of IC50 value of FRAP assay respectively, These results were lesser activities than the standard ascorbic acid (10.94 μg/ml of IC50 value of FRAP assay).

![Fig. 8: Metal Ion Chelating Activity of Enteromorpha Species](image)

**Fig. 8:** Metal Ion Chelating Activity of *Enteromorpha Species*

The value is expressed as mean ± SD (n=3)

![Fig. 8a: IC50 value of Ethanol Extracts of Enteromorpha Species and Ascorbic acid (Standard) for Metal IonChelating Activity](image)

**Fig. 8a:** IC50 value of Ethanol Extracts of *Enteromorpha Species* and Ascorbic acid (Standard) for Metal IonChelating Activity

![Fig. 9: FRAP Assay of Enteromorpha Species](image)

**Fig. 9:** FRAP Assay of *Enteromorpha Species*

The value is expressed as mean ± SD (n=3)

![Fig. 9a: IC50 value of Ethanol Extracts of Enteromorpha Species and Ascorbic acid (Standard) for FRAP Assay.](image)

**Fig. 9a:** IC50 value of Ethanol Extracts of *Enteromorpha Species* and Ascorbic acid (Standard) for FRAP Assay.

Total antioxidant activity, which is a quantitative method to evaluate water-soluble and fat-soluble antioxidant capacity (total antioxidant capacity), the extract demonstrated high electron-donating capacity showing its ability to act as chain terminators, transforming relative free radical species into more stable non-reactive products [25]. In the ranking of the antioxidant capacity was obtained by this method (Fig.10). The concentrations for 50% inhibition were found to be 3.21 μg/ml, 4.01 μg/ml, 5.71 μg/ml, 10.39 μg/ml and 2.53 μg/ml of IC50 value with total antioxidant activity of *E. compressa, E. flexuosa, E. intestinalis* and *E. prolifera* and ascorbic acid, respectively (Fig. 10 and 10a).

Meenakshi *et al.,* [26] reported that the maximum total antioxidant activity present in *Sargassum* sp. compared to the other algae. The maximum antioxidant activity was exhibited by the ethanol extract of *C. serrulata* 12.8 mg of ascorbic acid/g of seaweed extract and the lowest activity was recorded in the green algae *Chaetomorpha linum* [27].
Kannan Karthikeyan et al., [28] reported that the highest antioxidant potential was exhibited in the ethanol extract of a brown alga Sargassum swartzii followed by Gracilaria corticata, Enteromorpha sp., Caulerpa taxifolia, Cystosperma indica and Caulerpa racemosa. Similarly, the present findings also showed that the total antioxidant activity was remarkable in ethanol extract. Hence it can be confirmed that the E. compressa, E. flexuosa, E. intestinalis and E. prolifera possess good antioxidant properties compared to other green algae [29].

IV. Conclusion

In the present study, the antioxidant activity of E. compressa, E. flexuosa, E. intestinalis and E. prolifera with ethanol solvent for extraction and Ascorbic acid as a standard antioxidant has been studied and the results indicate that these algae can be used as a natural antioxidant agent. The experimental findings proved that these algae extracts are an excellent source of bioactive compounds with a wide variety of applications as natural antioxidants in different food and pharmaceutical industrial products.

References


