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Utilisation potential of seaweeds from Goa coast

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ABSTRACT

Commonly growing seaweeds from the shores of Goa were screened for their nutraceutical potential barring those that caused hemagglutination. This study was focussed on the investigation of the property of antioxidant potential of three selected seaweeds as contenders to be deemed as nutraceutical sources. Methanolic extracts were analysed for DPPH scavenging activity and determination of reducing power. Further, in an attempt to identify the causative agent/ source of antioxidant property, qualitative and quantitative analyses were carried out for detecting Phenols and Flavonoids in the extracts. Total Phenolic content was precipitated and analysed by IR Spectroscopy. Total phenolic content (TPC) estimated was highest in the methanolic extract of *Sargassum tenerrimum* (0.0357 ± 0.004 GAE mg/g). The Total flavonoid content (TFC) also was highest in *Sargassum tenerrimum* (0.000119 ± 0.003 QE mg/g). All three methanolic extracts showed similar reducing power. However, their DPPH scavenging activity differed being highest in *Dictyota dichotoma* with an EC_{50} of 671.5 ìg/ml. Qualitative analysis confirmed the presence of Phenols and Flavonoids in the extracts. These two constituents may thus be contributing to the antioxidant activity of the extract. The IR spectrum displayed the organic and phenolic nature of the precipitate analysed. This study thus reports the antioxidant activity and the possible role of seaweeds as potential nutraceuticals.

Introduction

In the fourth century B.C. Hippocrates had proposed about the existing relation between diet and health (Kadam and Prabhasankar, 2010). An ever increasing global attraction on usage of natural resources for treating various ailments has led to the development of nutraceuticals and functional foods as an alternative to synthetic drugs (Plaza *et al.,* 2010). Seaweed is one such candidate, and a major component of diet in several Asian countries like Japan, China and Korea. It has been the reason for lower incidence of breast and prostate cancer in these countries compared to North America and Europe (Pisani *et al.,* 2002 and Sachindra *et al.,* 2010). Compounds extracted from seaweeds have been gaining the interest from pharmaceutical companies for their potential as nutraceuticals (Ly *et al.,* 2005 and Mak *et al.,* 2013). Seaweeds are a source of myriad biologically active phytochemicals, many of which are reported to possess biological activities beneficial for use in human healthcare. These compounds have been reported to be benefitting in control of hyperlipidemia, thrombosis, tumor and obesity (Plaza *et al.,* 2008).

In vitro antioxidant activity studies on methanolic extracts of seaweeds have shown their potency as natural antioxidants whose activity is dose dependent (Kumar *et al.,* 2008, Apostolidis and Lee, 2010, Kadam and Prabhasankar, 2010; Airanthi *et al.,* 2011**).** Several antioxidants have been reported from brown seaweeds such as pigments (Hosokawa *et al.,* 2009), polyphenols (Zou *et al.,* 2008), tocopherols etc. (Airanthi *et al.,* 2011). Despite of extensive research on the antioxidant potential of extracts from various types of marine seaweeds, very little information is available on the relationships between the active compounds and antioxidant activity of seaweeds. In the present study, the methanol extracts of three brown seaweeds *Sargassum tenerrimum, Dictyota dichotoma* and *Padina tetrastomatica* were analysed to determine their antioxidant activity and total phenolic and flavonoid contents.

Materials and Methods

Chemicals, Reagents and Instruments

Absolute methanol (RanKem, Avantor, Gujarat,

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India),1X PBS, Antisera (anti-A, B and D – Tulip Diagnostics, India), Blood samples (Rh+A, Rh+B and Rh+O), commercially sold *Spirulina platensis* tablets(Sanat Products Ltd, Delhi, India), Conc. H_2SO_4 (Merck, Mumbai, India), Conc. HNO_3(Sd-Fine, Mumbai, India), Deionised water, $FeCl_3$(HiMedia, Mumbai, India), Quercetin (Sigma-Aldrich, Steinheim Germany), Phloroglucinol (HiMedia, Mumbai, India), Folin-Ciocalteau's (FC) reagent (SRL, Mumbai, India) , Gallic Acid (Sigma-Aldrich, Steinheim Germany), 2,2-diphenyl-1-picrylhydrazyl (DPPH-Sigma-Aldrich, Steinheim Germany) and KBr (HiMedia, Mumbai, India). Chemicals used were of analytical grade and reagents used were prepared fresh. UV-Vis Spectrophotometer (Shimadzu-Japan), FT-IR (Shimadzu-Japan) and Rotary Evaporator (Equitron- Roteva, Mumbai, India).

Collection of seaweed samples

Three abundantly growing seaweeds *Sargassum tenerrimum (St), Padina tetrastomatica (Pt)* and *Dictyota dichotoma (Dd)* were collected from the rocky coast of Anjuna, Goa (15°35´04.14"N, 73°44´13.21" E) during the post monsoon months of November and December, 2015. The seaweeds were rinsed in seawater in the collection locality and transported to the laboratory in clean polythene bags with seawater. Samples were again washed with tap water and distilled water to remove associated sand and epiphytes. The seaweed species were identified using the expertise of a botanist and taxonomist, Dr. Vijaya Kerker (Dept. Of Botany, Goa University).These seaweeds were shade dried for 96 hrs, packed in clean polythene bags and stored in -20°C till further use.

Preparation of extracts

Two different solvent extracts were prepared. 1X PBS extracts were prepared (Kumar and Barros, 2010) and used for initial screening. Absolute methanolic extracts were prepared, by modifying the method used by Souza *et al.* (2011) for estimations and analyses. The extraction was carried out over a period of 24-30 hours on a magnetic stirrer at 4°C. Extracts after filtration were centrifuged and stored at -20°C till further use.

Hemagglutination slide test

The seaweed samples were screened using a hemagglutination spot test (Kumar and Barros, 2010) with minor modifications. Positive controls used were the anti-sera procured from Tulip Diagnostics. Seaweeds testing positive for hemagglutination were discontinued from further study.

Qualitative chemical analysis

Qualitative tests were used to confirm presence of phenols and flavonoids in the chosen seaweed candidates. Presence of Phenols was confirmed with the modified neutral $FeCl_3$ test (Furniss *et al.,* 1989) using Phloroglucinol as a positive control. Seaweeds were also checked for the presence of flavonoids using modified protocol. (Harborne, 1998 and Isaac *et al.,* 2011). Quercetin was used as the control for Flavonoids.

Determination of Total Phenolic Content

The Total phenolic content was determined using Folin-Ciocalteu's (FC) reagent (Wang *et al.,* 2009) and absolute methanolic extracts. A caliberation curve using Gallic Acid was prepared to determine the concentrations. The absorbance was recorded at 725 nm. Total phenolic content of *Spirulina* (*Spirulina platensis* tablets) was compared with that of the seaweeds. Total phenolic content was calculated as follows and expressed as milligrams of Gallic Acid equivalents (GAE) per gram of extract.

$$C = (c \times V)/M$$

C is the total content of phenolic compounds, (mg GAE/g extract), c is the concentration of phloroglucinol established from the calibration curve (mg/ml), V is the volume of extract (ml) and M is the weight of extract (g).

Estimation of Total Flavonoid Content

Using the method of Liu *et al.* (2009) and Kannan *et al.,* 2014) with minor modifications, the total Flavonoid content was estimated. Absorbance was recorded using a spectrophotometer at 510 nm. *Spirulina* extract was used for the purpose of comparison. The calibration curve was plotted using 0.5mg/ml Quercetin. Total flavonoid content was expressed as milligrams of Quercetin equivalents (QE) per 100 gram of extract.

Analysis of antioxidant potential by reducing power assay

Reducing power assay was done using protocols of (Vijayabaskar and Vaseela, 2012; Farvin and Jacobsen, 2013). The reducing power of the methanolic seaweed extracts was measured over a range of 0.2 - 1.0 mg/ml concentrations. The absorbance was recorded on the spectrophotometer at 700 nm. 1mg/ml methanolic extract of Turmeric used as reference and was checked for the reducing property in comparison to that of the seaweeds due to its efficient reducing property.

Analysis of Antioxidant Potential by DPPH Free Radical Scavenging Assay

The methanolic seaweed extracts analysed for their ability to scavenge 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radicals. Their scavenging activity was calculated based on the extent of the DPPH radicals scavenged (Blois, 1958 and Souza *et al.,* 2011). The methanolic extracts were checked over a range of 0.1-1mg/ml. Absorbance was measured at 517 nm. 0.2 mg/ml. Ascorbic acid was used as a reference. EC_{50} values extract was found using the statistical software GraphPad Prism 5. Scavenging activity was expressed as;

$$\% \text{ Scavenging Activity} = \frac{\text{Absorbance of Control} - \text{Absorbance of Test Sample}}{\text{Absorbance of Control}} \times 100$$

Infrared (IR) spectral analysis of the phenolic residue

The approach for IR analysis used was as per a modified approach developed by combining the protocols of Lim *et al.* (2002) and Sabrine *et al.* (2014). Poly-phenols from the seaweed were extracted by gentle heating in 0.1N NaOH. The 0.1N NaOH extract was filtered and the filtrate obtained was used to precipitate the phenolic component by addition of conc. HCL. This solid phenolic precipitate obtained by the chemical test was washed, dried and subjected to IR spectroscopy using potassium bromide (KBr) disks as the background and carrier.

Statistics

All studies were carried out in triplicates **(n=3)** and their standard deviation (±SD) was calculated and reported. For the DPPH radical scavenging assay, the EC_{50} values for the seaweed extracts were statistically calculated (GraphPad Prism 5). Two tailed '*t*-test' (GraphPad Prism 5) was used to calculate the significance in reducing power of each seaweed extract concentration in comparison to that of methanolic extract of Turmeric. The significance was reported on basis their P-values ≤ 0.05, 0.01 or 0.001 and the most significant P-value being ≤ 0.001.

Results

The seaweeds were collected from the intertidal region of the rocky coast of Goa. The three collected seaweeds belonge to the Class Phaeophyceae and were identified as *Sargassum tenerrimum, Dictyota dichotoma* and *Padina tetrastomatica.* They were than screened for the property of hemagglutination which is undesirable if the seaweed is to be chosen for nutraceutical studies. Ix PBS extracts of seaweeds were used for this screening. None the three seaweeds that were collected displayed hemagglutination with the human blood. Thus all the three brown seaweeds were studied further. Table-1 displays the results of the hemagglutination assay.

Presence of Phenols and Flavonoids was qualitatively detected in the three Methanolic extract of *St, Dd* and *Pt* because of their known associated bioactivities. By quantitative estimations, the total Phenolic contents was found to be 0.0357 ± 0.004 GAE mg/g for *St,* 0.0312 ± 0.002 GAE mg/g for *Dd* and 0.0191± ± 0.002 GAE mg/g for *Pt*. When compared with methanolic extract of *Spirulina platensis* (0.0159 ± 0.002 GAE mg/g), the three seaweeds had higher TPC. The total flavonoid content was found to be 0.000119 ± 0.003 QE mg/g for *St,* 0.000063 ± 0.004 QE mg/g for *Dd* and 0.000028 ± 0.004 QE mg/g for *Pt*. The TFC for *Spirulina platensis* tablets was 0.000015 ± 0.002 QE mg/g, which again was lower in comparison to that of the seaweeds.

Table-1. Screening of seaweeds by blood hemagglutination activity assessment. (n=3)

Sr.No	1X PBS Extract	Hemagglutnation Observed		
		RhA^+	RhB^+	RhO^+
1.	*S. tenerrimum*	-	-	-
2.	*D. dichotoma*	-	-	-
3.	*P. tetrastomatica*	-	-	-

PBS - Phosphate Buffered Saline; RhA^+ - Rhesus Blood Group A positive; RhB^+ - Rhesus Blood Group B positive; RhO^+- Rhesus Blood Group O positive

Further the methanolic extracts were checked for their antioxidant potential by assessing its reducing power and ability to scavenge DPPH free radicals. On the basis of the recorded absorbance for the reducing power, *Pt* had the highest value of 0.4396 ± 0.001 followed by *Dd* (0.413 ± 0.005) and *St* (0.394 ± 0.001). However the values were similar and indicative that each methanolic seaweed extract is equally efficient in its reducing function. Reducing power of turmeric was 0.397 ± 0.005 which was lower than that of *Pt* and *Dd.* As seen in Fig. 1 and Fig. 2, the reducing power of the three seaweeds was significantly higher than that of Turmeric.

The DPPH free radical scavenging activity of the three seaweed extracts was measured over a range of concentrations (0.1 -1.0 mg/ml) and found to be concentration dependent. The EC_{50} values for each methanolic seaweed

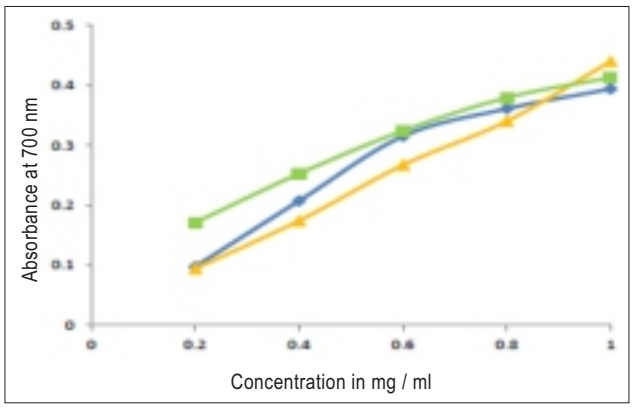
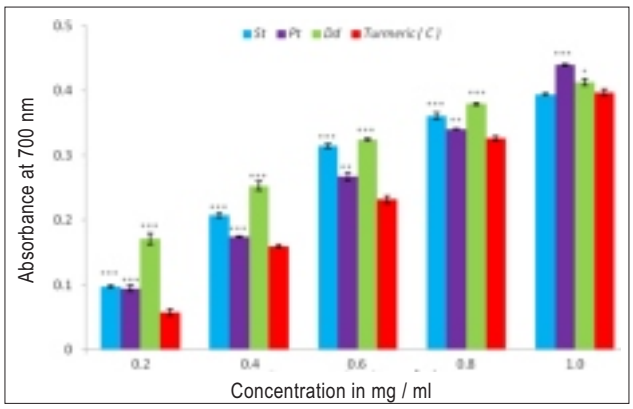


Fig. 1. *Reducing power of three methanolic seaweed extracts*



Note: P-value ⩽ 0.05*, P-value ⩽ 0.01**, P-value ⩽ 0.001***

Fig. 2. *Reducing power - seaweed extracts vs turmeric extracts*

Table-2. Presence Of Phenols And Flavonoids, Total Phenolic Content, Total Flavonoid Content & EC_{50} For DPPH Radical Scavenging. (n=3)

Sr.No	Species	Presence of Phenol	Presence of Flavonoids	Total Phenolic Content GAE mg/g	Total Flavonoid content QE mg/g	EC_{50} for DPPH Radical Scavenging ìg/ml
1.	*S. tenerrimum*	+	+	0.0357 ±0.004	0.000119 ±0.003	864.7 ±9.51
2.	*D. dichotoma*	+	+	0.0312 ±0.002	0.000063 ±0.004	671.5±12.44
3.	*P. tetrastomatica*	+	+	0.0191 ±0.002	0.000028 ±0.004	732.4±8.60

GAE mg/g—Gallic Acid Equivalents mg/g; QE mg/g—Quercetin Equivalents mg/g; EC_{50} for – 50 % of the Effective Concentration

extract was calculated, the lowest being *Dd* with an EC_{50} of 671.5 ± 12.44 μg/ml followed by *Pt* (732.4 ± 8.60 μg/ml) and *St* (864.7 ± 9.51 μg/ml) respectively, thus making the methanolic extract of *Dd* the most efficient radical scavenging extract among the three. Further study was made to confirm the presence of phenols in the seaweeds using IR spectral studies by the detection of the poly-phenol associated –OH groups. Signals specific for –OH group were detected for the analysed IR spectra of *St, Dd* and *Pt*.

Lectins belong to the superfamily of proteins that bind to specific carbohydrates reversibly without their covalent structure being altered (Van Buul and Brouns, 2014). Their ability to agglutinate red blood cells is well known and used for blood typing - hence also commonly referred to as hemagglutinins (Liu *et al.,* 2010). Nutrition literature suggests possible role of lectin in inducing adverse health effects by binding to the epithelium in the gut, damaging the cells, resulting in a leaky gut epithelium, thus leading to reduced nutrient-uptake (Biesiekierski *et al.,* 2010). Thus the hemagglutination parameter was used to screen for nutritionally safe (non-agglutinating) seaweeds. PBS extracts of seaweeds were used so as to maintain the stability of the extracted lectins components if any, thus facilitating an effective screening further. The PBS extracts of none of the three brown seaweeds caused agglutination of human RBC and therefore, all three were studied further.

Marine brown seaweeds have been reported to accumulate a variety of poly-phenols which could be used as functional ingredients in nutraceuticals, with potential health benefitting effects (Wijesekara *et al.,* 2010; Ngo *et al.,* 2011). Seaweeds also possess other components like pigments, tocopherols, sulphated polysaccharides etc. which are also associated to the antioxidant, antibacterial, anti inflammatory, antitumor, anti-diabetic and other therapeutic bioactivity or benefits. Often these activities, especially, antioxidant activity is attributed to the poly-phenolic content of seaweed (Dellai *et al.,* 2013; Rengasamy *et al.,* 2015). Thus, the occurrence of Phenols and Flavonoids being detected qualitatively, directed the study towards their quantification in the methanolic extracts. By quantitative estimations *St,* was found to possess the highest TPC (0.0357 ± 0.004 GAE mg/g) as well as the highest TFC (0.000119 ± 0.003 QE mg/g). It should also be noted that the methanolic extract of each of the three seaweeds had a higher TPC and TFC in comparison to that of the methanolic extract of *Spirulina platensis* Thus, having phenols and flavanoids present in them, the seaweeds could be well studied for its antioxidant bioactivity.

Synthetic and commercially available natural antioxidants in recent times have been found to be inefficient in some foods and tend to have side effects. Thus the importance of replacing them with natural alternatives has increased greatly (Farvin and Jacobsen, 2013). On analysing the methanolic extracts for their antioxidant potential it was observed that each of the extract posessed reducing power and ability to scavenge DPPH free radicals.

The presence of reducing molecules or compounds in the methanolic extracts of the three seaweeds were responsible for conversion of the $Fe(III)^{+}$ or ferricyanide complex to its Perl's Prussian blue coloured, ferrous form in solution, which was recorded at 700nm and in accordance to reports (Isabel *et al.,* 2007). The principal behind the reducing power assay is, "greater the absorbance, greater is the reducing power of the sample being tested". The reducing property indicates that the compounds with antioxidant properties are electron donors and can reduce the oxidation intermediates, thus acting as primary and secondary antioxidants (Yen and Chen, 1995). Therefore eventhough, *Pt* had the highest absorbance value (0.4396 ± 0.001), the three seaweed extracts were similar in their reducing function. This could be either due to the similar active molecules or due to the existence of similar antioxidant mechanism. The reducing power of the extracts of three seaweeds over the concentrations of 0.2 - 1.0 mg/ml, by the two tailed '*t*-test' was found to be significant. Till the concentration of 0.8 mg/ml the reducing power of the three seaweeds was highly significant, while at 1.0 mg/ml *Pt* gave the highest significance with P-value < 0.001 followed by *Dd* (P-value < 0.05).

The extracts also possessed the ability to scavenge DDPH free radicals. The mechanism of function involves to conversion of the purple solution of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) to 2, 2-diphenyl-1-picrylhydrazin which is brownish/pale yellow solution. The antioxidant molecules donate an H^{+} to the 2, 2-diphenyl-1-picrylhydrazyl radical having unpaired electrons in order to stabilise it (Singh and

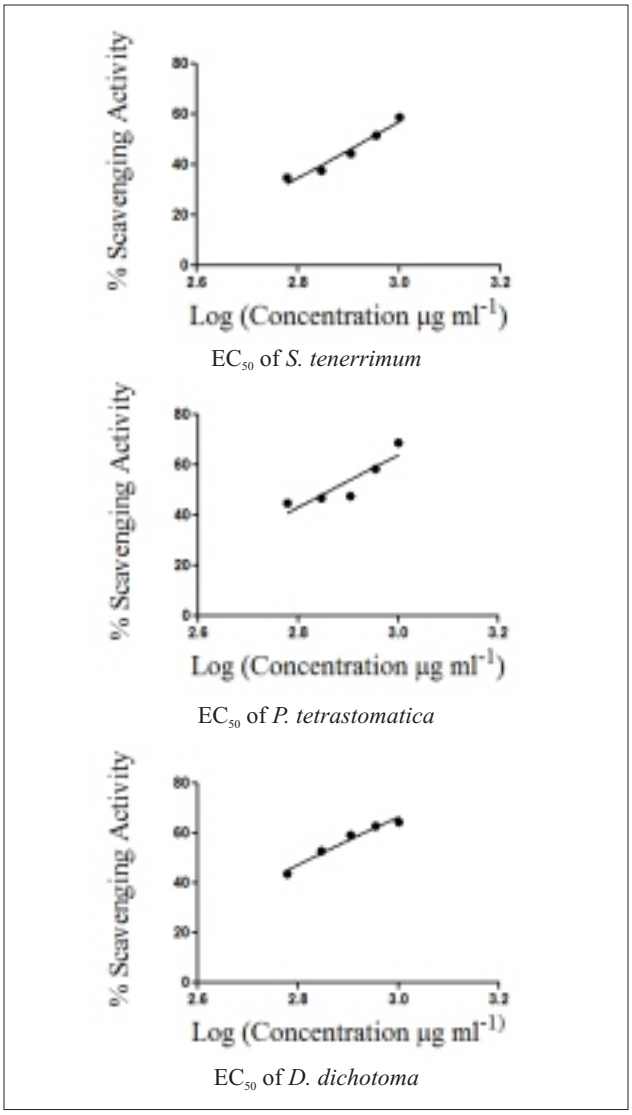


Fig.3. *EC_{50} for DPPH radical scavenging activity of the seaweed extracts*

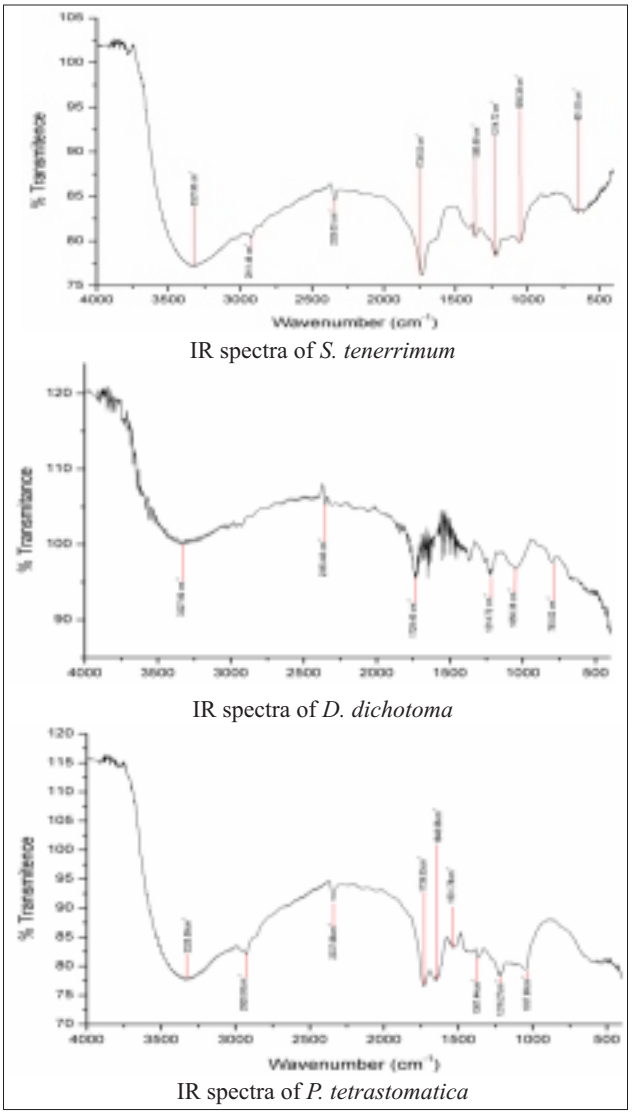


Fig.3. *IR spectra of Phenolic Residues of three seaweeds*

Rajini, 2004). The, methanolic extracts of *St, Pd* and *Dd* seems to have followed the radical scavenging mechanism as they efficiently scavenged the DPPH radicals. On statistically calculating the EC_{50}, the extract of *Dd* was identified as having the lowest effective concentration and therefore most potent DPPH radical scavenging activity. Thus, the observable differences in activity of seaweed extract with respect to its reducing power and DPPH radical scavenging, was an indicative of the multi antioxidant mechanisms involved and hints at the role they could play in case of failure in single antioxidant mechanism, consequently highlighting the prospects of seaweed antioxidant.

The IR spectral studies provide data about the complexity and organic/ inorganic nature of the molecules or compound being analysed based on the detection of functional groups across the IR spectrum. The region between 2800-3300 nm of the IR spectra is specific for the –OH groups (Vijayabaskar and Vaseela, 2012), whose presence could be due to moisture or phenolic molecules, while any detected signal in the mid IR region suggest about the compounds organic nature due to associated aromatic rings. In the study IR analysis was carried out together with qualitative tests for phenol so as to confirm the signals specific for –OH group was due to phenols present in the extracts and not due to moisture. That along with the multiple signals detected in the mid IR region confirm the organic and phenolic nature of the compounds tested.

Brown seaweeds in majority are usually used for obtaining phycocoloid alginates. Given the abundance of these seaweeds, and their associated bioactivities *S. tenerrimum, D. dichotoma* and *P. tetrastomatica* have immense potential as a nutraceutical in the future. The present study has dealt with

antioxidants activity attributed to polyphenolic and flavonoid content of the seaweeds. It also confirms the organic nature of the poly-phenols through thorough analysis of the IR spectral data for each of the three seaweeds. The study hints at a multi-mechanism antioxidant system being involved, on basis of variations observed in the reducing power assay and DPPH radical scavenging assay. This being a preliminary study focuses only on antioxidant potential of the seaweeds. However further exploration of these seaweeds in a meticulous manner would facilitate the finding of more bioactive molecules and their associated bioactivities. The findings portray *Sargassum tenerrimum, Dictyota dichotoma* and *Padina tetrastomatica* as promising nutraceutical candidates.

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