Research Paper
Impact Factor: 4.164
Refereed Journal

IJMDRR
E- ISSN –2395-1885
ISSN -2395-1877

PRELIMINARY PHYTOCHEMICAL PROFILING AND ANTIOXIDANT ACTIVITY OF *SARGASSUM WIGHTII*

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Abstract
Seaweeds are considered as source of bioactive compounds and produce a great variety of secondary metabolites characterized by a broad spectrum of biological activities and it's considered to be a rich source of antioxidants. The Antioxidant has the ability to trap free radicals, thus it delays or inhibits the oxidative mechanisms that lead to degenerative diseases. The present study was carried out to explore the phytochemical constituents and to determine the total antioxidant activities of Sargassum wightii. Secondary metabolites were extracted by Soxhlet assembly with various solvents such as chloroform, ethyl acetate and methanol. Qualitative analysis of extracted solvents showed methanol to have maximum number of compounds. Methanol extract exhibited Total antioxidant assay, Reducing power assay, and Lipid peroxidise activity that increased with increasing amount of the extract concentration, which was compared with Ascorbic acid. The study concludes that Sargassum wightii extract possess the highly active antioxidant substance which can be used for the treatment of oxidative stress-related diseases.

Key Words: Phytochemicals, Soxhlet Method, Antioxidant, Seaweeds, Sargassum Wightii.

Introduction

Marine macro algae, popularly known as seaweeds are potential renewable resources in the marine environment. Seaweeds are primitive non-flowering plants without true roots, stem and leaves. They grow in the intertidal, shallow and deep sea areas up to 180 meter depth and also in estuaries, backwaters and lagoons on solid substrates such as rocks, dead corals, pebbles, shells, mangroves, and other plants (Anantharaman et al., 2007). Seaweeds are classified as Rhodophyta (red algae), Phaeophyta (brown algae) and Chlorophyta (green algae) depending on their nutrient and chemical composition. It was estimated that about 90% of the species of marine plant are algae and about 50% of the global photosynthesis is contributed from algae (Dhargalkar VK et al., 2005). India ranks first among all countries bordering the Indian Ocean ahead of Australia and South Africa in the number of recorded specific and intra specific seaweed taxa (D.Sahoo et al., 2001). Seaweeds have been screened extensively to isolate life saving drugs or biologically active substances all over the world.

Phytochemicals are non-nutritive plant chemicals having protective or disease preventive properties. The phytochemical screening of plants reveals the presence of primary and secondary metabolites that suggest the plant might be of medicinal or industrial importance. Free radicals are unstable chemical species that cause damage to lipid, cells, proteins and DNA result due to imbalance between the generation of reactive oxygen species (ROS) and the antioxidant enzyme. Antioxidant are the substances which scavenge free radicals and they play an important role in the prevention of free radical-induced diseases by donating hydrogen radicals to the primary radicals which gets reduced to non-radical chemical compounds and then gets converted to oxidize antioxidant radicals (Yamaguchi et al., 1998).

Sargassum wightii is among the widely found marine brown algal species in India. It is dark brown to blackish in colour when dry. It belongs to the family Sargassaceae and order Fucales. It is found to be the most diverse genus among Phaeophyta in India and is represented by 38 species. It has tremendous biological applications and are known to be rich in sulphated polysaccharide content. It has been used traditionally for treating scrofula, goiter, tumor, edema, testicular pain and swelling (Liu et al., 2012). It has been reported that pharmacological activities of Sargassum include anticancer, anti-inflammatory, antibacterial and antiviral activities. Considering the chemical and immense pharmacological properties of brown algae, the present study was aimed to explore the phytochemical constituents of methanolic extract of *Sargassum wightii*.

Materials and Methods

Collection of Plant

Sargassum wightii was collected by handpicking from the coast of Rameshwaram Tamilnadu, India. The collected Seaweeds were cleaned well with sea water to remove all the extraneous matter such as epiphytes, sand particles, pebbles and shells and brought to the laboratory in plastic bags. Then the seaweeds were spread on blotting paper to remove excess water and dried. The shade dried samples were ground to fine powder using a tissue blender. The powdered samples were then stored in the refrigerator for further use.

Preparation of Extract

Crude Sample extract was prepared by Soxhlet extraction method. About 20gm of powdered sample material was uniformly packed into a thimble and extracted with 250ml of different solvents such as methanol, ethyl acetate, chloroform. The process of extraction has to be continued for 24 hours or till the solvent in siphon tube of extractor become colorless. Then the extracts were collected separately and stored at the refrigerator for further studies. The extracts were analyzed for the presence of phytochemical constituents using standard procedure.

Phytochemical Screening

Preliminary phytochemical analysis was carried out for all the extracts of *Sargassum wightii* as per standard methods described by Brain and Turner 1975 and Evans 1996.

Detection of alkaloids

1. Extracts were dissolved individually in dilute hydrochloric acid and filtered. The filtrate was used to test the presence of alkaloids.
2. Mayer's test: Filtrates were treated with Mayer's reagent. Formation of a yellow cream precipitate indicates the presence of alkaloids.
3. Mayer's reagent: Mercuric chloride (1.358g) is dissolved in 60ml of water and potassium iodide (5g) is dissolved in 10ml of water. The two solutions are mixed and made up to 100ml with water.

Detection of Flavonoids: A few drops of 1% NH_3 solution was added to the extract in a test tube. A yellow coloration was observed for the presence of flavonoids.

Detection of Steroids: Liebermann-Burchard Test: 2ml of acetic anhydride was added to 0.5g of the extracts, each with 2ml of H_2SO_4. The color changed from violet to blue or green in some samples indicate the presence of steroids.

Detection of Terpenoids: Salkowski's test: 0.2g of the extract was mixed with 2ml of chloroform and concentrated H_2SO_4 (3ml) was carefully added to form a layer. A reddish brown coloration of the inner face was indicates the presence of terpenoids.

Detection of Anthroquinones: Borntrager's test: About 0.2g of the extract was boiled with 10% HCl for few minutes in a water bath. It was filtered and allowed to cool. Equal volume of $CHCl_3$ was added to the filtrate. Few drops of 10% NH_3 were added to the mixture and heated. Formation of pink color indicates the presence anthraquinones.

Detection of Phenols: Ferric chloride test: Extracts were treated with few drops of 5% ferric chloride solution. Formation of bluish black color indicates the presence of phenol.

Detection of Saponins: Froth test: About 0.2g of the extract was shaken with 5ml of distilled water. Formation of frothing (appearance of creamy stable persistent of small bubbles) shows the presence of saponins

Detection of Tannins: Ferric chloride test: A small quantity of extract was mixed with water and heated on water bath. The mixture was filtered and 0.1% ferric chloride was added to the filtrate. A dark green color formation indicates the presence of tannins.

Detection of Carbohydrates

1. Fehling's test: 0.2gm filtrate is boiled on water bath with 0.2ml each of Fehling solutions A and B. A red precipitate indicates the presence of sugar.
2. Fehling's solution A: Copper sulphate (34.66g) is dissolved in distilled water and made up to 500ml using distilled water.
3. Fehling's solution B: Pottassium sodium tartarate (173g) and sodium hydroxide (50g) is dissolved in water and made up to 500ml.

Detection of Oils and Resins: Spot test: Test solution was applied on filter paper. It develops a transparent appearance on the filter paper. It indicates the presence of oils and resins.

Quantitative Phytochemical Analysis

Estimation of Alkaloids

Alkaloid determination is done using Harborne (1973) method. 5g of the sample was weighed into a 250 ml beaker and 200 ml of 10% acetic acid in ethanol was added and covered and allowed to stand for 4 hours. This was filtered and the extract was concentrated on a water bath to one quarter of the original volume. Concentrated ammonium hydroxide was added drop wise to the extract until the precipitation was complete. The whole solution was allowed to settle and the precipitated was collected and washed with dilute ammonium hydroxide and then filtered. The residue is the alkaloid, which was dried and weighed.

Alkaloid content (%) = weight of alkaloids extracted/weight of sample

Estimation of Flavonoids

10g of sample was repeatedly extracted with 100ml of 80% aqueous methanol at room temperature. The mixture was then filtered through a filter paper into a pre-weighed 250ml beaker. The filtrate was transferred into a water bath and allowed to evaporate to dryness and weighed. The percentage flavonoid was calculated by difference (Krishnaiah et al, 2009).

Flavonoid content (%) = weight of flavonoid extracted/weight of sample

Determination of Total phenols

5 ml of the extract was pipetted into a 50 ml flask then 10 ml of distilled water was added. 2 ml of ammonium hydroxide solution and 5ml of concentrated amyl alcohol were also added. The methanol extract were made up to mark and left to react for 30 minutes for colour development. This was measured at 505nm (Siddhuraju and Decker, 2003).

Phenol content (%) = weight of phenols extracted/weight of sample

Antioxidant Study

DPPH Radical Scavenging Activity

DPPH radical scavenging activity was carried out by the method of Molyneux (2004). To 1.0 ml of 100.0µM DPPH solution in methanol, equal volume of the sample in methanol of different concentration was added and incubated in dark for 30 minutes. The change in coloration was observed in terms of absorbance using a spectrophotometer at 514 nm. 1.0 ml of methanol instead of test sample was added to the control tube. The different concentration of ascorbic acid was used as reference compound. Percentage of inhibition was calculated from the equation [(Absorbance of control - Absorbance of test)/ Absorbance of control)] x 100. IC50 value was calculated using Graph pad prism 5.0.

Evaluation of Total Antioxidant Activity

An aliquot of 0.1ml of the sample solution containing a reducing species in DMSO was combined in an Eppendorff tube with 1ml of reagent solution (0.6M Sulphuric acid, 28mM sodium phosphate, and 4mM ammonium molybdate). The tubes were capped and incubated in water bath at 95°C for 90 minutes. The samples were cooled to room temperature, and the absorbance of each solution was measured at 695nm. The total antioxidant capacity was expressed as mM equivalent of ascorbic acid (Mojca et al., 2005).

Lipid peroxidation Inhibitory Activity

Lipid peroxidation (Ohkawa et al., 1979) was evaluated by estimating the thiobarbituric acid reactive substances using the standard method. Briefly, different concentrations of the extract (100 – 500 microgram / ml in saline) were added to the liver homogenate (0.5ml). Lipid peroxidations were initiated by adding 100µl of 15mM of $FeSO_4$ solution and then incubate at 37^0C for 30 minutes. After 30 minutes, 1.0ml of 10% TCA was added and centrifuged. After 10 minutes to the supernatant, 1.0 ml of thiobarbituric acid was added. The tubes were then boiled for 20 minutes and the pink colour developed was read at 535 nm. Butylated hydroxyanisole were at different concentrations (1-5 nanomoles) was used as the standard. Control tube contain all chemicals except methanol extract.

Reducing power assay (Oyaizu, 1986)

The sample together with Ascorbic acid solutions were spiked with 2.5ml of phosphate buffer (0.2 M, pH 6.6) and 2.5ml of 1% potassium ferricyanide. The mixture was kept in a 50°C water-bath for 20min. The resulting solution was cooled rapidly, spiked with 2.5ml of 10% trichloroacetic acid, and centrifuged at 3000 rpm for 10 minutes. The supernatant (5ml) was mixed with 5ml of distilled water and 1ml of 0.1% ferric chloride and incubated for 10 minutes. The absorbance was detected at 700nm on spectrophotometer. The extract concentration providing the absorbance was calculated from the graph of absorbance at 700nm against extract concentration. Ascorbic acid was used as standard. Higher absorbance indicates higher reducing power.

Results and Discussion

Qualitative phytochemical analysis

In the present study, the phytochemical screening was performed with methanol, ethyl acetate, and chloroform extracts of *Sargassum wightii*. phytochemical analysis of ten different chemical compounds (alkaloids, terpenoids, steroids, tannins, saponins, flavonoids, phenols, anthroquanine, oil resin, and carbohydrates) were tested in three different extracts. The results of the preliminary phytochemical investigation of methanol, ethyl acetate and chloroform extract of *Sargassum wightii* are summarized in Table 1. Of the three different extracts, methanol extract showed the presence of maximum number i.e., 8 compounds. Next to that, Ethyl acetate extracts showed seven compounds and Chloroform extract showed three compounds.

Table 1: Qualitative phytochemical of different extracts

Phytochemicals	Methanol	Ethylacetate	Chloroform
Carbohydrates	Present	Present	Present
Tanins	Present	Present	Absent
Saponins	Present	Absent	Absent
Flavonoids	Present	Present	Absent
Alkaloids	Present	Absent	Absent
Terpenoids	Present	Absent	Absent
Phenol	Present	Present	Absent
Steroids	Present	Present	Present
Anthroquanin	Absent	Absent	Absent
Oil resin	Absent	Present	Present

Estimation of identified phytochemicals

The seaweed *Sargassum wightii* showed the presence of Flavanoids, Phenols and Alkaloids at Percentage of 20.92, 29.20 and 77.58 respectively. It has been confirmed that pharmacological effect of flavonoids is correlating with their antioxidant activities. These secondary metabolites are reported to have many biological and therapeutic properties. so this species is expected to have many medicinal uses.

Table 2: Estimation of Phytochemicals

Phytochemicals	Gram	Percentage
Flavonoid	1.046	20.92
Phenols	1.460	29.20
Alkaloids	3.879	77.58

Antioxidant activity

Reducing power Assay

The antioxidant can donate an electron to free radicals, which leads to the neutralization of the radical. Reducing power was measured by direct electron donation in the reduction of $Fe^{3+}(CN^-)_6$-$Fe^{2+}(CN^-)_6$. The product was visualized by forming the intense Prussian blue color complex and then measured at 700nm. As shown in Fig.1 a higher absorbance value indicates a stronger reducing power of the samples. *Sargassum wightii* extract showed concentration-dependent reducing power. However, its reducing power was weaker than that of BHT, which exhibited the strongest reducing power. Antioxidant compounds are able to donate electrons to reactive radicals, reducing them into more stable and unreactive species (Gulcin et al., 2003). The inhibition concentration percentage for reducing power assay for the methanol extract is 505.14µg/ml and the standard value is 196.38µg/ml.

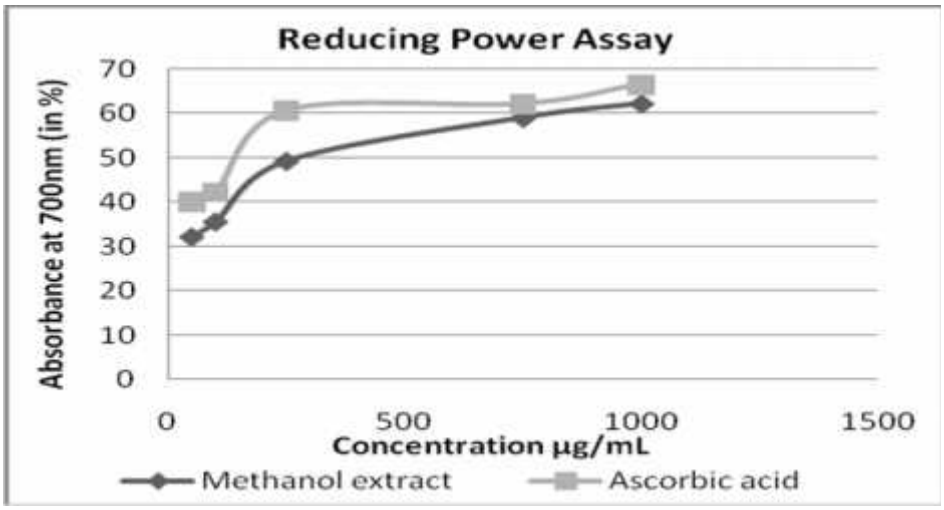

Fig. 1: Reducing power activities of *Sargassum wightii* in comparison with ascorbic acid.

DPPH Assay

Total free radical scavenging method is based on the reduction of DPPH, a stable free radical and any molecule that can donate an electron or hydrogen to DPPH can react with it and thereby bleach the DPPH absorption. Because of its odd electron, DPPH gives a strong absorption maximum at 514nm by visible spectroscopy (purple colour). As the odd electron of the radical becomes paired off in the presence of a hydrogen donor, that is, a free radical scavenging antioxidant, the absorption strength is decreased and the resulting decolorization is stoichiometric with respect to the number of electrons captured (Blios, 1958). As shown in Fig.2 the *Sargassum wightii* exhibited strong radical scavenging activity with inhibition percentage at 792.13µg/ml while the standard value was 162.04µg/ml.

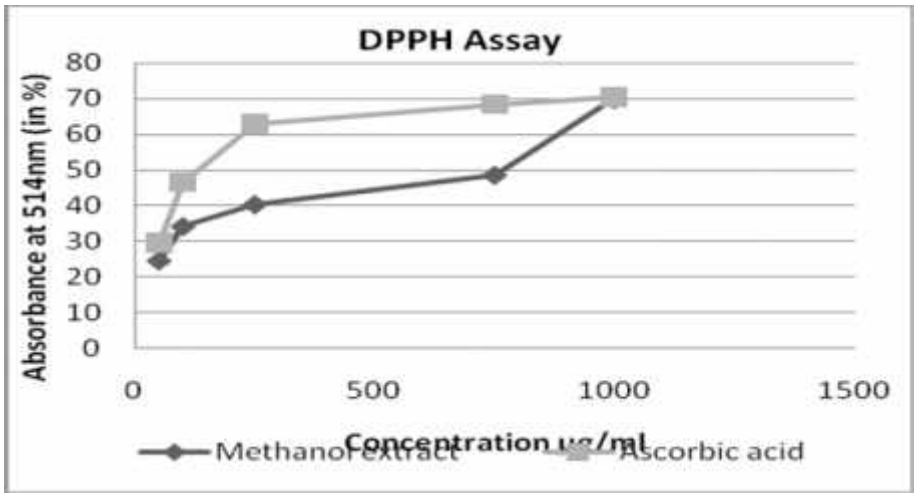

Fig. 2: Radical scavenging activities of *Sargassum wightii* in comparison with ascorbic acid.

Lipid Peroxidase Assay

Antioxidant activity of *Sargassum wighti* extracts is measured to inhibit lipid peroxidation (LPO) by FTC and TBA methods. The tested methanol extract exhibited strong antioxidant activity or differential capacity to inhibit LPO by FTC and TBA method which is indicated by their low absorbance values. The FTC method measures the amount of peroxide produced during the initial stages of lipid oxidation. Subsequently, at a later stage of lipid oxidation, peroxide decomposes to form carbonyl compounds that are measured by the TBA method (Mackeen et al., 2000). As shown in Fig.3, higher activity was recorded in the extracts of *Sargassum wightii* surpassing the activity of the standard commercial antioxidants, α- tocopherol and butylated hydroxy toluene. In general, the antioxidant by TBA method is higher than that of FTC method. This might suggest that the amount of peroxide in the initial stage of lipid per oxidation is less than the amount of peroxide in the secondary stage. Furthermore, the secondary product is much more stable for a period of time.

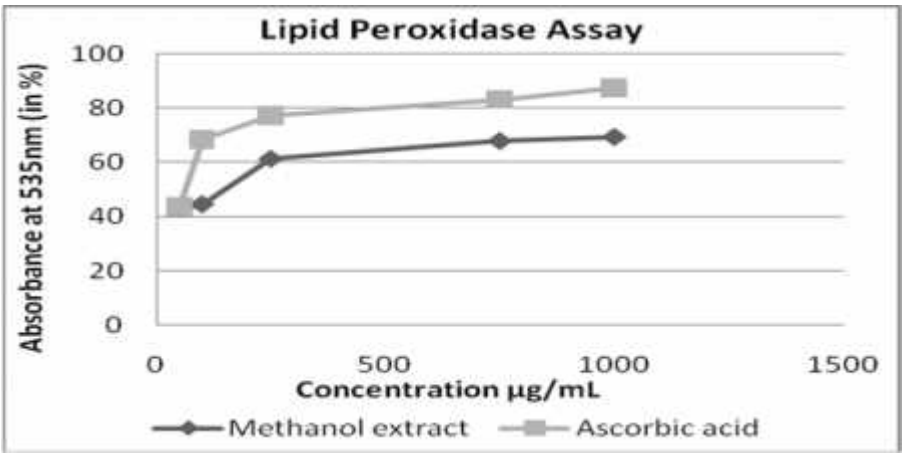

Fig. 3: Lipid peroxidase Assay activities of *Sargassum wightii* in comparison with ascorbic acid.

Conclusion

The present study concludes that phytochemical extraction of *Sargassum wightii* using methanol to be much productive than other solvents. Total antioxidant activity, Reducing power assay, DPPH radical scavenging activity and Lipoxygenase activity that increased with increasing amount of the extract concentrations. With these antioxidant properties, the *Sargassum wightii* can be effectively used in the development of new pharmaceutical medicine for oxidative stress mediated problems. Accomplished study is a milestone to affirm the opportunity for future research to throw light on the biological activity of the *Sargassum wightii* and its bioactive compounds against various diseases.

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