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ORIGINAL ARTICLE

Antioxidant and Antiproliferative Activity of *Asparagopsis taxiformis*

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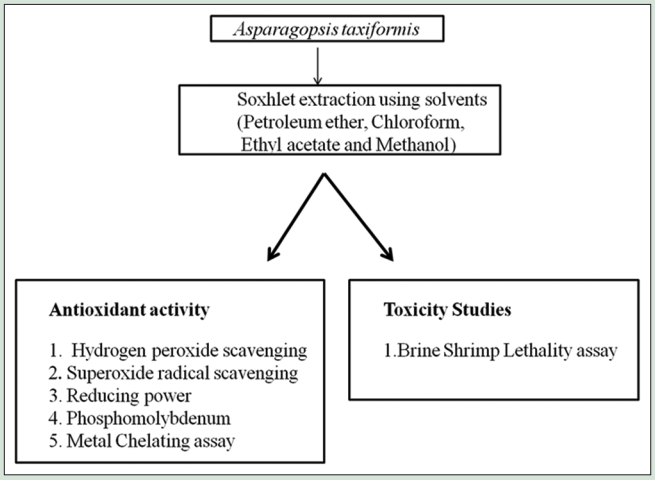
ABSTRACT

Background: *Asparagopsis taxiformis* (*Rhodophyta*) is a species of red algae belonging to the family *Bonnemaisoniaceae*. The objective of the present study was to evaluate antioxidant and antiproliferative activity of four fractions (petroleum ether, chloroform, ethyl acetate, and methanol) of *A. taxiformis*. **Materials and Methods:** The red seaweed, *A. taxiformis* was collected from Mandapam Coastal Region, Gulf of Mannar, Tamil Nadu. Epiphytes present in algal extracts were cleaned and washed with seawater and fresh water. *In vitro* antioxidant activity was determined by hydrogen peroxide scavenging, ferric reducing antioxidant power, superoxide radical, metal-chelating activity, and phosphomolybdenum reduction assay. Further, the cytotoxic activity was evaluated using brine shrimp lethality assay. This method is rapid, reliable, inexpensive, and convenient as compared to other cytotoxicity assays. Gallic acid, ethylenediaminetetraacetic acid, ascorbic acid, and quercetin were used as reference antioxidant compounds. **Results:** Reducing power of chloroform extract increased with increasing concentration of the extract. The radical scavenging activity of extracts was in the following order: ascorbic acid > methanol > chloroform > petroleum ether > ethyl acetate. Highest metal-chelating activity was observed in petroleum ether fractions (63%). Reduction of Mo (VI) to Mo (V) increased in methanol extract (27%) at 100 µg/ml. Moreover, all fractions had an inhibitory effect on the formation of hydroxyl radicals. Results showed that ethyl acetate, methanol, and petroleum ether fractions exhibited potent cytotoxic activity with median lethal concentration values of 84.33, 104.4, and 104.4 µg/ml, respectively. **Conclusion:** Thus, the results showed that red algae possess strong antioxidant and cytotoxic activity that suggests their possible use in the development of pharmaceutical drugs.

Key words: Antioxidant activity, *Asparagopsis taxiformis*, cytotoxic, free radical scavenging

SUMMARY

- Various fractions of red algae *Asparagopsis taxiformis* was evaluated for *in vitro* antioxidant and antiproliferative studies. All results indicate potential use of red algae for drug development.



Abbreviations Used: Mo: Molybdenum, $AlCl_3.H_2O$: Aluminum chloride, $NaNO_2$: Sodium nitrite, NaOH: Sodium hydroxide, H_2O_2: Hydrogen peroxide, NADH: Nicotinamide adenine dinucleotide, NBT: Nitroblue tetrazolium chloride, PMS: Phenyl methanesulfonate, $FeCl_2$: Ferrous chloride.

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INTRODUCTION

Seaweeds or macroalgae are potential renewable resources in the marine environment.[1] About 20,000 species of seaweeds are widely distributed throughout the world, of which 221 species are commercially utilized, including 110 species for phycocolloid production and 145 species for food consumption.[2] The marine floral diversity in India comprises 844 species of seaweeds (in 217 genera), 14 species of seagrasses, and 69 species of mangroves.[3] Based on their pigmentation, marine macroalgae are classified as red (*Rhodophyta*), green (*Chlorophyta*), and brown (*Phaeophyta*).[4] Seaweeds are used as an important dietary component in the eastern cuisine.[5] Apart from these, they are widely used in industries as thickeners and gelling agents and as cosmetics and fertilizers.[6] Further, seaweeds have also demonstrated chelators of heavy metals.[7] Recent report suggests that seaweeds or their extracts possess bioactive compounds of great medicinal value.[8] However, seaweeds have received great attention in previous years as potential natural antioxidants.[9] Apart from these, they also possess numerous health promotive effects such as anti-inflammatory,[10] antimicrobial,[11] and anticancer[12] properties. However, scientific reports on antioxidant studies of seaweed extracts in India are very limited. Hence, the objective of the present study was to determine antioxidant and antiproliferative activity of different fractions of *Asparagopsis taxiformis*. Reactive oxygen species (ROS) are a primary cause of biomolecular oxidation that results in significant damage to cell structure, contributing to various diseases such as diabetes, cancer, aging, and atherosclerosis.[13] During recent years, antioxidant-based drug therapies are found for the treatment of oxidative damage induced by ROS.[14] Although several synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, and tert-butylhydroquinone are available, they are toxic and can cause carcinogenic effects.[15] Recent

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reports suggest that algal extracts scavenge free radicals and active oxygen and are able to inhibit cancer cell proliferation.[16,17] Hence, antioxidants from natural sources are of great demand for the treatment of human ailments. *A. taxiformis* (*Bonnemaisoniales*, *Rhodophyta*) is a red algae distributed across tropical and temperate marine ecosystem.[18] It exhibited strong invasive behavior and was included in the list of "Worst invasives in the Mediterranean Sea."[19] Recent studies show that this species consists of number of cryptic species, as well as other marine algae.[20] It is also a rich source of halogenated compounds such as methanes, ketones, acetates, acrylates, and haloforms.[21] Members of *Bonnemaisoniaceae* family, in which *Asparagopsis* species belong, form "vesicle" or "gland cells."[22] The pungent aroma of this algae is due to the presence of essential oil composed of bromoform along with fewer amounts of ethane, bromine, ethanol, chlorine, acetones, acetaldehydes, 2-acetoxypropanes, propenes, epoxypropanes, acroleins, butenones, and iodine-containing methane stored in vacuoles within gland cells.[23] Various reports suggest antibacterial, antimicrobial, antimethanogenic, anticoagulant, and antifungal activities[24,25] of *A. taxiformis*, but antioxidant and toxicology studies have not been performed till date. Therefore, we assessed brine shrimp lethality assay (BSLA) of *A. taxiformis*. Brine shrimp assay is a preliminary cytotoxic assay for the detection of natural and synthetic drugs before advanced studies.[26] Brine shrimp has been used as model organism for analyzing the cytotoxic potential of seaweeds and plants.[27] A number of studies have demonstrated cytotoxic activities of red algae using BSLA. Hence, cytototoxic effect of *A. taxiformis* on brine shrimp was carried out.

MATERIALS AND METHODS

Sample collection

Fresh species of seaweeds were collected from Mandapam Coastal Region (Latitude 9°17"N; Longitude 79°11"E), Gulf of Mannar, Tamil Nadu, South India. The red algal samples were washed with seawater and then fresh water to remove epiphytes, sand, and other extraneous matter. The algae were brought to the laboratory in plastic bags under ice. Before extraction, the samples were blotted on the blotting paper and air-dried under shade. After shade drying for 3 days, they were cut into small pieces and kept in oven at 45°C. The samples were then powdered and stored in air-tight containers at −20°C.

Identification of *Asparagopsis taxiformis*

The unique feature of *A. taxiformis* includes dull pink up to 15 cm tall attached to the root surfaces by creeping rhizomatous portion bearing the holdfast, rhizoidal portion much branched, erect portion naked up to the middle, upper portion densely covered with branches and branchlets, delicate branches often tip covered, uniaxial. Central cavity runs through the main stem and primary axis. Width of the thallus is 1000–1250 μm. Peripheral cells are 30–43 μm long and 16–43 μm wide and covered with chromatophores. Cortex is pseudo-parenchymatous and colorless with 70–134 μm wide. Spermatangia are in dense cylindrical clusters with 333–500 μm width. Cystocarps terminal is on short branchlets, subspherical to pyriform: 567–667 μm diameter with a small ostiole. Stalk of the cystocarp is 467–750 μm length and 133–167 μm width.[28]

Preparation of extracts

The dried seaweed samples (30 g) were extracted using 250 ml of solvents of increasing polarity, including petroleum ether (60°C), chloroform (61.2°C), ethyl acetate (77.1°C), and methanol (64.7°C) in Soxhlet apparatus for 8 h. The solvents were evaporated and residue was dried using rotary evaporator (Super Fit, Rotavap, model, PBU-6, India). The samples were lyophilized using a freeze dryer (Lark, Penguin Classic Plus, India) and stored in a refrigerator at 2–8°C for further experiments.

Phytochemical analysis

The concentrated residues from the different extracts were used to detect the secondary metabolites including alkaloids, steroids, phenolics, flavonoids, and anthraquinones.[29]

Test for tannins (ferric chloride test)

Five milligrams of extract was taken and added to a tube containing 0.2 ml of distilled water and then boiled for an hour. A few drops of ferric chloride was added and allowed to stand for proper color development. A blue-black coloration indicated the presence of tannins.[30]

Test for saponins (frothing test)

Saponins were tested by dissolving 5 mg of the extract in a test tube containing 0.05 ml of hot distilled water, and then, the mixture was shaken vigorously for 1 min and persistent foaming observed, indicating the presence of saponins.[31]

Test for phenolics

To 500 μl of the extract, one drop of 5% $FeCl_3$ (w/v) was added. Formation of greenish precipitate indicated the presence of phenolics.

Test for flavonoids

For the confirmation of flavonoid, 5 mg of each extract was added in a test tube and 0.1 ml of distilled water and 0.05 ml of dilute ammonia solution were added to a portion of the aqueous filtrate of each plant extract, followed by addition of 0.01 ml concentrated sulfuric acid (H_2SO_4). Indication of yellow color shows the presence of flavonoid in each extract.

Test for alkaloids

Five milligrams of the selected samples was added to each test tube and 75 μl of hexane was mixed in it, shaken well, and filtered. One hundred and twenty-five microliters of 2% HCl was poured into a test tube having the mixture of extract and hexane. Test tubes having the mixture were heated and filtered, and few drops of picric acid were poured into the mixture. Formation of yellow precipitate indicated the presence of alkaloids.

Test for reducing sugar (Fehling's solution)

The aqueous extract (100 μl) was added to boiling Fehling's solution (A and B) in a test tube. The mixture was shaken and heated in a water bath for 10 min. A brick-red precipitate indicates presence of reducing sugar.

Test for anthraquinones

Five milligrams of the extract was boiled with 0.1 ml of H_2SO_4 and filtered while hot. The filtrate was shaken with 0.05 ml of chloroform. The chloroform layer was pipetted into another test tube, and 0.01 ml of dilute ammonia was added. The resulting solution was observed for color changes.

Test for terpenoids

An amount of 5 mg of sample was taken in a test tube, and then, 0.1 ml of methanol was poured into it, shaken well, and filtered to take 0.05 ml extract of sample. Then, 0.02 ml of chloroform was mixed with the extract of selected sample and 0.03 ml of H_2SO_4 was added with the selected sample extract. Formation of reddish brown indicates the presence of terpenoids in the selected plants.[32]

Test for steroids (Liebermann-Burchard test)

About 5 mg of the extract was dissolved in 0.5 ml dichloromethane to give a dilute solution, and then 5 μl of acetic anhydride was added, followed by three drops of concentrated H_2SO_4. A blue-green coloration indicated the presence of steroids.[33]

Test for fatty acids

The plant extracts were mixed with 5ml of ether. These extracts were allowed to evaporate and then filter paper was dried. The transparency appeared on filter paper indicated the presence of fattyacids.

Determination of total phenolic content

Total phenolic content was estimated by the Folin–Ciocalteu method.[34] Two hundred microliters of diluted sample was added to 1 ml of 1:10 diluted Folin–Ciocalteu reagent. After 4 min, 800 µl of saturated sodium carbonate (75 g/l) was added. After 2 h of incubation at room temperature, the absorbance at 765 nm was measured. Gallic acid (20–100 µg/ml) was used for the standard calibration curve. The results were expressed as gallic acid equivalent/g dry weight of macroalgae and calculated as mean ± standard deviation.

Determination of total flavonoids

Total flavonoid content was determined according to the method of Zhishen *et al.*[35] Briefly, a 250 µl aliquot of each extract was mixed with 1.25 ml of double distilled water and 75 µl of 5% $NaNO_2$ solution. After 6 min, 150 µl of 10% $AlCl_3.H_2O$ solution was added. After 5 min, 0.5 ml of 1M NaOH solution was added and then the total volume was made up to 2.5 ml with double distilled water. Following through mixing of the solution, the absorbance against blank was determined at 510 nm. Quercetin was used to prepare the standard curve, and results were expressed as mg quercetin equivalents/g extract.

In vitro antioxidant assay

Hydrogen peroxide scavenging activity

Hydrogen peroxide (H_2O_2) scavenging activity of extract was determined according to the method given by Ruch *et al.*[36] A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). Extracts (125–500 µg/ml) were added to H_2O_2 solution (0.6 ml). Absorbance of H_2O_2 at 230 nm was determined after 10 min against a blank solution containing phosphate buffer without H_2O_2 and compared with ascorbic acid, the reference compound. The percentage of H_2O_2 scavenging of both *A. taxiformis* extracts and standard compounds was calculated.

$$\% \text{ scavenged } (H_2O_2) = ([A_C - A_S]/A_C) \times 100$$

Where A_C = Absorbance of the control and A_S = Absorbance in the presence of the sample of *A. taxiformis* or standards.

Ferric reducing antioxidant power assay

Reducing power of *A. taxiformis* was determined by the method described by Oyaizu.[37] Briefly, 1.0 ml of extract was mixed with 2.5 ml of phosphate buffer (0.2 M, pH 6.6) and 2.5 ml potassium ferricyanide (1%). Reaction mixture was incubated at 50°C for 20 min. After incubation, 2.5 ml of trichloroacetic acid (10%) was added and centrifuged (650 g) for 10 min. From the upper layer, 2.5 ml solution was mixed with 2.5 ml of distilled water and 0.5 ml $FeCl_3$ (0.1%). Absorbance of all the sample solutions was measured at 700 nm. Ascorbic acid is used as positive control.

Superoxide radical scavenging assay

The nonenzymatic generation of superoxide anion was measured according to the method of Robak and Gryglewski.[38] The sample solution (0.1 ml) was mixed with 0.1 ml of 0.1 M phosphate buffer (pH 7.2), 0.025 ml of 2 mM nicotinamide adenine dinucleotide, and 0.025 ml of 0.5 mM nitroblue tetrazolium chloride, and absorbance at 550 nm was measured as a blank value. After a 3-min incubation with 0.025 ml of 0.03 mM phenyl methanesulfonate, the absorbance was measured.

Phosphomolybdenum assay

The antioxidant activity of seaweed extracts was evaluated by the phosphomolybdenum method.[39] The assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and by the formation of green complex at acidic pH. Different concentration of extract (20–100 µg/ml) was combined with 1 ml of reagent solution (0.6 M H_2SO_4, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes containing the reaction mixture were capped and incubated in water bath at 95°C for 90 min. The sample mixture was cooled and absorbance was noted at 695 nm against blank. Ascorbic acid was used as a reference antioxidant.

Metal-chelating assay

The Fe^{2+}-chelating activity of the extracts was estimated according to the method reported by Liu *et al.*[40] Briefly, the reaction mixture containing different concentrations of samples (20–100 µg/ml), ferrous chloride (0.1 ml, 2 mM) and ferrozine (0.4 ml, 5 mM), was shaken well and incubated for 10 min at room temperature. The absorbance of the mixture was measured at 562 nm against a blank. The ability of different concentrations of samples to chelate ferrous ion was calculated using the following equation:

$$\text{Chelating ability } (\%) = ([A_0 - A_1]/A_0)$$

Where A_0 = Absorbance of the control reaction and A_1 = Absorbance of the sample.

Toxicity studies of *Asparagopsis taxiformis* extracts

Brine shrimp lethality assay

Brine shrimp cytotoxicity assay was performed according to the standard protocol described by Turker and Camper.[41] About 10 g of *Artemia salina* (Linnaeus) cysts (obtained from Bonneville Artemia International) was aerated in 1 L capacity glass cylinder (jar) containing seawater prepared by dissolving 36 g of sea salt in 1 L of distilled water. The airstone was placed in the bottom of the jar to ensure complete hydration of the cysts. After 10–12 h of incubation at room temperature (27–29°C), newly hatched free swimming pink-colored nauplii were harvested from the bottom outlet. Two days was allowed for the shrimp to mature as nauplii (shrimp can be used 48/72 h after the initiation of hatching). Since the nauplii are positively phototrophic (attracted to light), illumination was provided on one side to attract newly hatched larvae.

The assay system was prepared with 2.5 ml of filtered seawater prepared containing chosen concentration of extract (125, 250, and 500 µg/ml) in six-well plates. In each, 10 nauplii were transferred and the setup was allowed to remain for 24 h, under constant illumination. After 24 h, the dead nauplii were counted. Based on the percent mortality, the median lethal concentration (LC_{50}) value of the plant extract was determined. Three replicates were prepared for each concentration. The same saline solution used to prepare the stock test sample solution was used as a negative control. The lethal concentration value is then calculated using a computer program "Biostat-2006" or simple calculation using regression equation (y = mx + c). The mortality of the shrimp was calculated by the formula:

$$\text{Mortality rate } (\%) = (\text{dead nauplii/total nauplii}) \times 100\%$$

RESULTS

Phytochemical screening of *Asparagopsis taxiformis*

In the present study, phytochemical analysis of four extracts of *A. taxiformis* was determined. Important phytochemicals, i.e., tannins, saponins, steroids, flavonoids, phenols, alkaloids, reducing sugar, anthraquinones, terpenoids, and fatty acids, were screened for their presence and are presented in Table 1 and Figure 1.

Total phenolic content

Total phenolic content of *A. taxiformis* extracts was expressed in terms of mg gallic acid per gram of extract [Tables 2 and 3]. The fractions were evaluated at various concentrations (20–100 µg/ml). Among all the

extracts, methanol showed highest amount of phenolic content (25%), followed by chloroform (23%), petroleum ether (11%), and ethyl acetate (4%) [Figure 2].

Total flavonoid estimation

Total flavonoid content of *A. taxiformis* was presented in Tables 4 and 5. Among the fractions, chloroform extracts showed maximum flavonoid content (85%), followed by methanol (76%), petroleum ether (56%), and ethyl acetate (47%) at 100 µg/ml [Figure 3]. All the tested extracts were compared with standard, gallic acid.

Antioxidant activity

Hydrogen peroxide scavenging activity

The ability of *A. taxiformis* to scavenge H_2O_2 is shown in Table 6. The extracts showed scavenging activity in a concentration-dependent manner. Maximum scavenging activity was shown by chloroform extract (11.07% ± 0.151% inhibition) at 500 µg/ml, followed by petroleum ether (10.88% ± 0.139% inhibition), methanol (10.77% ± 0.131% inhibition), and ethyl acetate (10.25% ± 0.136% inhibition) extracts. However, it was observed that positive control (ascorbic acid) exhibited 17% scavenging activity [Figure 4].

Superoxide radical scavenging assay

The effect of *A. taxiformis* extract on superoxide radical scavenging assay was compared with standard [Table 7]. It was observed that percentage of scavenging increases with concentration of extract. Of the tested extracts, methanol (85%) and chloroform (79%) exhibited highest scavenging activity at 500 µg/ml, followed by petroleum ether (47%) and ethyl acetate (45%). The reference compound (ascorbic acid) also showed similar scavenging effect (87%) to that of methanol extract [Figure 5].

Ferric reducing antioxidant power assay

The reducing capacity of the extracts was determined by ferric reducing antioxidant power (FRAP) method [Table 8]. The FRAP assay revealed maximum antioxidant activity in chloroform extract (67%) at 500 µg/ml. The activity was found similar to methanol (65%), petroleum ether (64%), and ethyl acetate (54%) fractions. Ascorbic acid (standard) showed more or less similar effect to reduce Fe^{3+} as compared to all fractions [Figure 6].

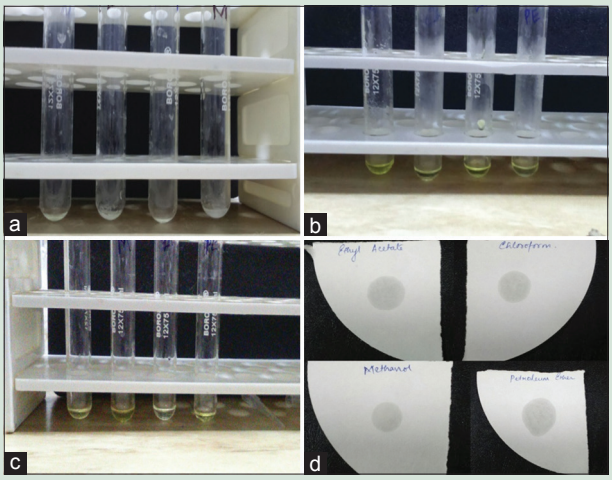

Figure 1: Phytochemical analysis of *Asparagopsis taxiformis* (a) curdy white precipitate (presence of anthraquinones), (b) yellow color (presence of flavonoids), (c) solution turned colorless giving positive results for alkaloids, (d) filter paper turned oily (presence of fatty acids)

Phosphomolybdenum assay

The total antioxidant activity of *A. taxiformis* was evaluated, and the results were expressed as mg ascorbic acid/g [Table 9]. In phosphomolybdenum assay, Mo (VI) is reduced to Mo (V) followed by the formation of green phosphate/Mo (V) complex. An increase in the inhibition (%) was observed for both standard (ascorbic acid) and extracts. Among these extracts, methanol showed maximum inhibition equivalent to standard (36%) at 100 µg/ml. Further, chloroform, petroleum ether, and ethyl acetate showed 26%, 23%, and 19% inhibition, respectively [Figure 7].

Table 1: Phytochemical analysis of different extracts of *Asparagopsis taxiformis*

Phytochemicals	Petroleum ether	Chloroform	Ethyl acetate	Methanol
Tannins	-	-	-	-
Saponins	-	-	-	-
Steroids	-	-	-	+
Flavonoids	+	+	+	+
Phenols	+	+	+	+
Alkaloids	+	+	+	+
Reducing sugar	-	-	-	-
Anthraquinones	+	+	+	+
Terpenoids	-	-	-	-
Fatty acids	+	+	+	+

Presence = (+), Absence = (-)

Table 2: Total phenol content of the standard (gallic acid)

Concentration (µg/ml)	Absorbance at 725 nm (standard)
20	0.116±9.92
40	0.242±19.87
60	0.438±29.77
80	0.508±39.74
100	0.597±49.70

The experiments are carried out in triplicates (n=3) and the results are expressed as mean±SD. SD: Standard deviation

Table 3: Total phenolic content of *Asparagopsis taxiformis* extract

Concentration (µg/ml)	Extract	Absorbance at 725 nm	Total phenol content
20	Ethyl acetate	0.029±0.002	4.55
40	Petroleum ether	0.076±0.009	11.97
60	Methanol	0.162±0.008	25.63
80	Chloroform	0.146±0.006	23.10

The experiments are carried out in triplicates (n=3) and the results are expressed as mean±SD. SD: Standard deviation

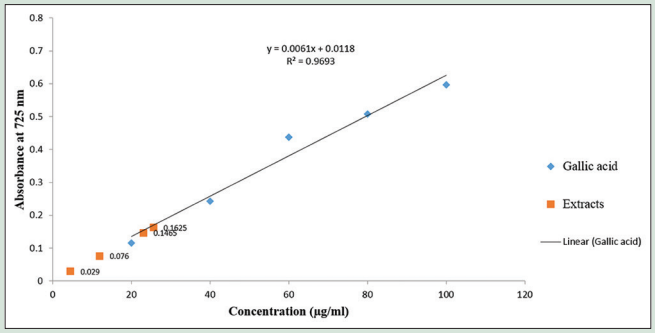

Figure 2: Graphical representation of total phenol content of solvent extracts equivalent to standard (gallic acid) at 415 nm

Metal-chelating assay

The chelating effect of *A. taxiformis* and metal chelator ethylenediaminetetraacetic acid (EDTA) at various concentrations (20–100 µg/ml) was examined and is presented in Table 10. The highest chelating effect was observed in petroleum ether (63%), which was more or less similar to EDTA (67%), and the least was found to be ethyl acetate (46%). The metal-chelating effect increased as concentration of extracts increased in a dose-dependent manner [Figures 7 and 8].

Table 4: Total flavonoid content of the standard (quercetin)

Concentration (µg/ml)	Absorbance at 415 nm (standard)
20	0.094±9.95
40	0.12±19.94
60	0.182±29.91
80	0.237±39.88
100	0.267±49.87

The experiments are carried out in triplicates (n=3) and the results are expressed as mean±SD. SD: Standard deviation

Table 5: Total flavonoid content of *Asparagopsis taxiformis* extract

Concentration (µg/ml)	Absorbance at 415 nm	Extract	Total flavonoid content
20	0.134±0.005	Ethyl acetate	47.53725
40	0.1545±0.0012	Petroleum ether	56.47034
60	0.2±0.004	Methanol	76.29744
80	0.2215±0.0055	Chloroform	85.66629

The experiments are carried out in triplicates (n=3) and the results are expressed as mean±SD. SD: Standard deviation

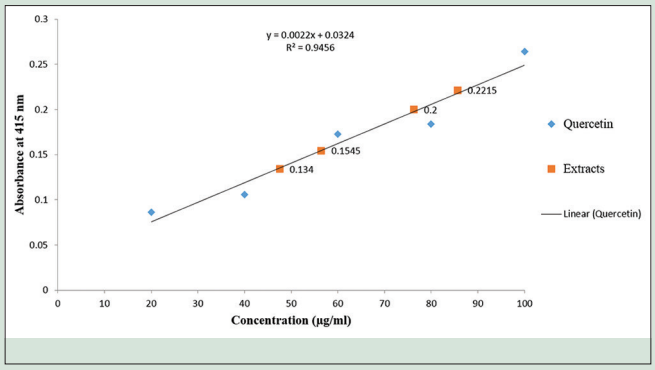

Figure 3: Graphical representation of total flavonoid content of solvent extracts equivalent to standard (quercetin) at 415 nm

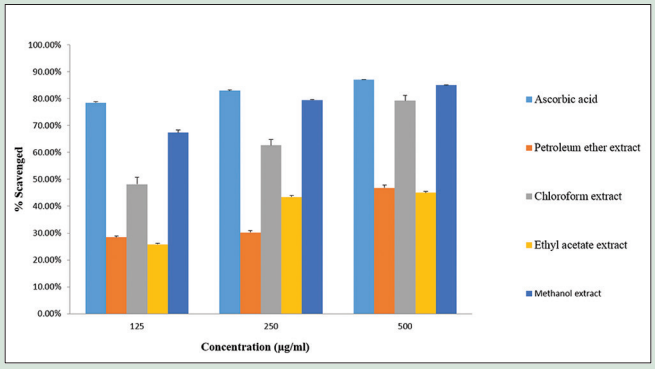

Figure 5: Superoxide radical scavenging activity of *Asparagopsis taxiformis* extract compared with standard (ascorbic acid)

Toxicity studies

Brine shrimp lethality assay

The toxicity of extracts was evaluated after 24 h of exposure. In the brine shrimp assay, the degree of hatching inhibition is directly related to concentration of *A. taxiformis* extract. The dose level (500 µg/ml) showed 100% hatching inhibition in petroleum ether, methanol, and ethyl acetate fractions, whereas at low concentration (125 µg/ml), it showed detrimental effects on brine shrimp eggs [Table 11] ethyl acetate fractions [Figure 9].

DISCUSSION

Marine organisms are rich sources of secondary metabolites that might represent enormous resource for the development of therapeutic drugs.[42] Among them, marine macroalgae possess active ingredients responsible for diverse biological activities such as antifungal,[43] antimicrobial,[44] antiviral,[45] and antioxidant activities.[46] Hence, the present study was designed to evaluate antioxidant and antiproliferative activities of *A. taxiformis*.

ROS is generated in living organisms in the form of superoxide anion (O_2), hydroxyl radical (OH), H_2O_2 and nitric acid during metabolism.[47] It is produced from molecular oxygen due to oxidative enzymes of the body or nonenzymatic reactions such as auto-oxidation by catecholamines.[48] Excessive amounts of ROS in the body cause biomolecular oxidations which lead to cell injury and death and create oxidative stress which results in the development of various chronic disorders such as cancer, liver cirrhosis, atherosclerosis, and a number of neurodegenerative diseases.[49] Overproduction of ROS causes lipid peroxidation, oxidative stress that leads to redox imbalance and harmful consequences. Hence, in the past decade, algal extracts

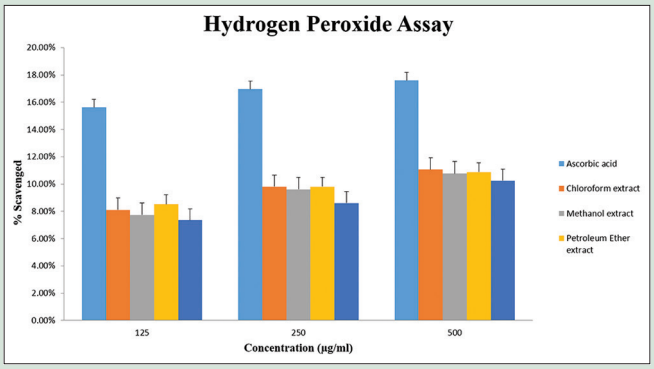

Figure 4: Hydrogen peroxide scavenging activities of *Asparagopsis taxiformis* compared with standard antioxidant (ascorbic acid)

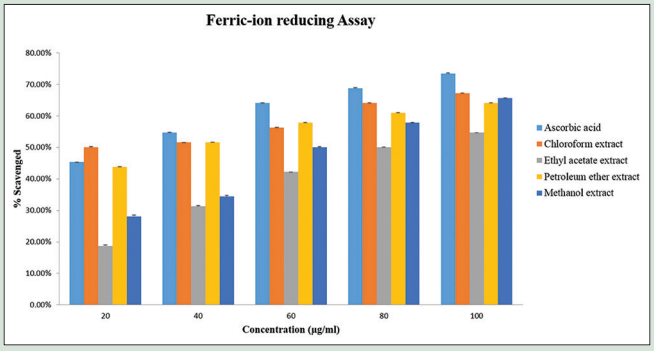

Figure 6: Ferric ion-reducing property of *Asparagopsis taxiformis* compared with standard (ascorbic acid)

as natural antioxidants have drawn attention for the prevention of various diseases, in which free radicals are implicated. Previous reports indicate that antioxidants have been considered as potential source of ROS inhibitors.[50] In the current study, we evaluated four fractions of *A. taxiformis* extract and were shown to have significant radical scavenging effect in a dose-dependent manner. The methanol extract (85%) showed maximum scavenging effect at 500 µg/ml, followed by chloroform (79%), petroleum ether (47%), and ethyl acetate (45%). The radical scavenging effect of extract could be directly related to nature and amount of phenolics and flavonoids and their hydrogen-donating ability.[51] In the present study, antioxidant activity

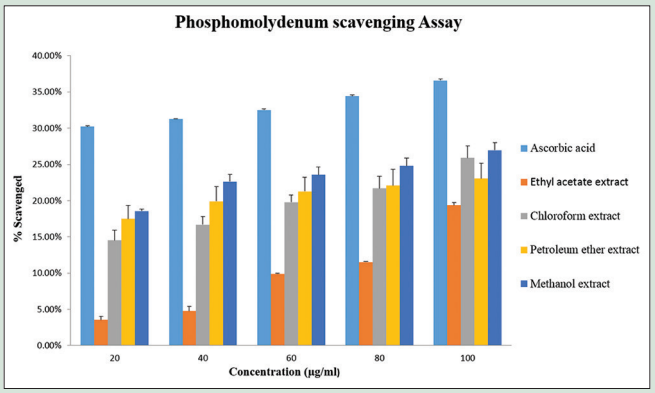

Figure 7: Phosphomolydenum scavenging activity of *Asparagopsis taxiformis* compared with standard (ascorbic acid)

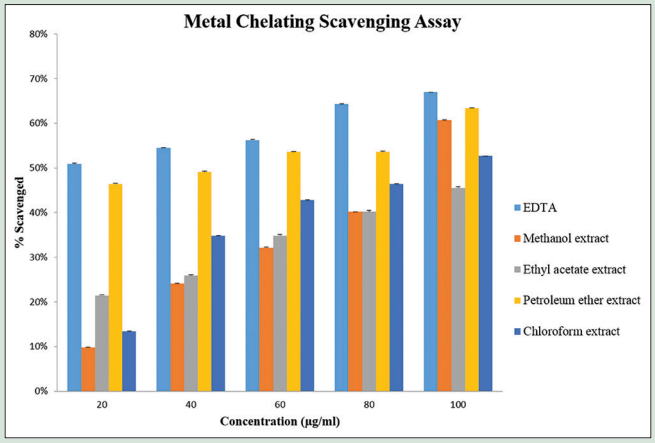

Figure 8: Comparative metal chelating scavenging activities of *Asparagopsis taxiformis* compared with standard (ethylenediaminetetraacetic acid)

Table 6: Hydrogen peroxide scavenging activity of *Asparagopsis taxiformis*

Concentration (µg/ml)	Ascorbic acid	Chloroform	Methanol	Petroleum ether	Ethyl acetate
125	15.62±0.222	8.11±0.137	7.73±0.121	8.52±0.123	7.74±0.109
250	16.96±0.218	9.81±0.144	9.62±0.125	9.81±0.122	8.60±0.122
500	17.59±0.222	11.07±0.151	10.77±0.131	10.88±0.139	10.25±0.136

The experiments are carried out in triplicates (n=3) and the results are expressed as mean±SD. SD: Standard deviation

Table 7: Superoxide radical scavenging activity of *Asparagopsis taxiformis*

Concentration (µg/ml)	Ascorbic acid	Petroleum ether	Chloroform	Ethyl acetate	Methanol
125	78.42±0.006	28±0.009	48.16±0.008	25.79±0.007	67.37±0.016
250	82.89±0.004	30±0.0105	62.63±0.007	43.42±0.009	79.47±0.003
500	87.11±0.0005	47±0.018	79.21±0.006	45.00±0.008	85.00±0.002

The experiments are carried out in triplicates (n=3) and the results are expressed as mean±SD. SD: Standard deviation

Table 8: Ferric reducing antioxidant power scavenging activity of *Asparagopsis taxiformis* extract with standard (ascorbic acid)

Concentration (µg/ml)	Ascorbic acid	Chloroform	Ethyl acetate	Petroleum ether	Methanol
20	45.31±0.005	50±0.003	18.75±0.004	43.75±0.003	28.31±0.006
40	54±0.002	51.56±0.0005	31.25±0.004	51.56±0.0015	34.38±0.006
60	64.06±0.021	56.25±0.001	42.19±0.0005	57.81±0.0015	50±0.004
80	68.75±0.003	64.06±0.0005	50±0.001	60.94±0.0015	57.81±0.0015
100	73.44±0.002	67.19±0.0005	54.69±0.0005	64.06±0.0005	65.63±0.001

The experiments are carried out in triplicates (n=3) and the results are expressed as mean±SD. SD: Standard deviation

Table 9: Phosphomolybdenum scavenging activity of *Asparagopsis taxiformis* and standard (ascorbic acid)

Concentration (µg/ml)	Ascorbic acid	Ethyl acetate	Chloroform	Petroleum ether	Methanol
20	30.22±0.0025	4±0.009	14±0.024	17±0.032	19±0.004
40	31.30±0.0005	5±0.011	17±0.0195	20±0.034	23±0.017
60	32.52±0.003	10±0.0015	20±0.017	21±0.0335	24±0.019
80	34.42±0.003	12±±0.015	22±0.029	22±0.0385	25±0.0185
100	36.59±0.004	19.6±0.0065	26±0.029	23±0.037	27±0.0175

The experiments are carried out in triplicates (n=3) and the results are expressed as mean±SD. SD: Standard deviation

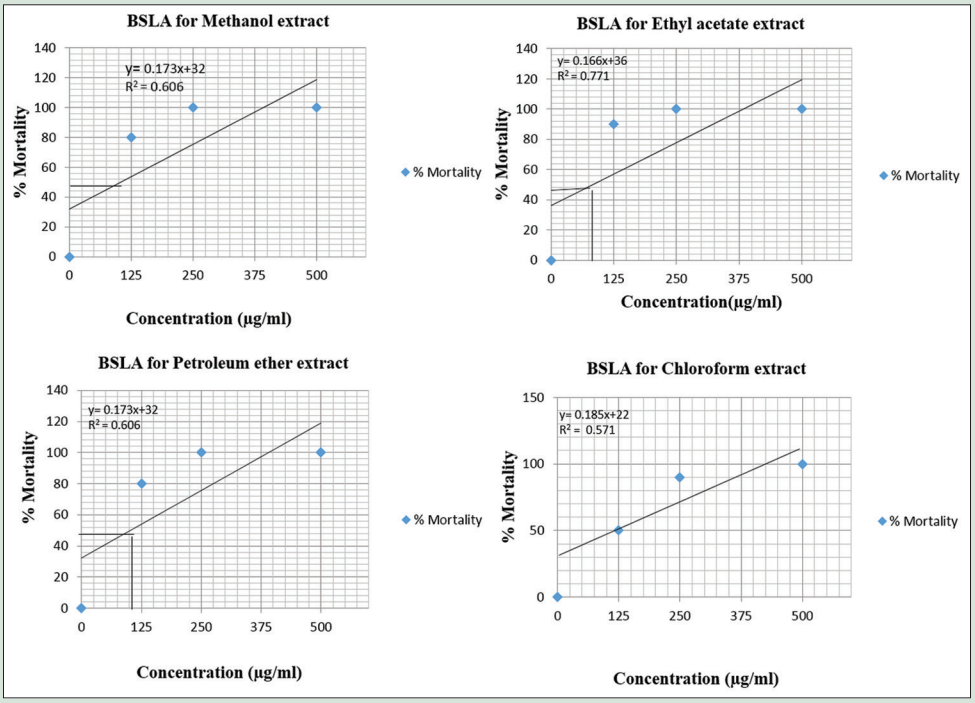

Figure 9: Graph representing the brine shrimp lethality assay of the four extracts

Table 10: Metal-chelating scavenging activity of *Asparagopsis taxiformis*

Concentration	EDTA	Methanol	Ethyl acetate	Petroleum ether	Chloroform
20	51±0.0025	10±0.0015	21±0.002	46±0.003	13±0.0015
40	54±0.0025	24±0.0005	26±0.002	49±0.0025	35±0.0015
60	56±0.0025	32±0.002	35±0.004	54±0.001	43±0.001
80	64±0.001	40±0.0005	40±0.0055	54±0.003	46±0.001
100	67±0.0005	61±0.001	46±0.0045	63±0.0005	53±0.0005

The experiments are carried out in triplicates (n=3) and the results are expressed as mean±SD. SD: Standard deviation; EDTA: Ethylene diamine tetraacetic acid

Table 11: Determination of brine shrimp cytotoxicity of the different extracts of *Asparagopsis taxiformis*

Extract	Concentration (µg/ml)	Number of nauplii taken	Dead nauplii	Percentage mortality	LC50 value (µg/ml)
Petroleum ether	125	10	8	80	104.04
	250	10	10	100	
	500	10	10	100	
Methanol	125	10	8	80	104.04
	250	10	10	100	
	500	10	10	100	
Ethyl acetate	125	10	9	90	84.33
	250	10	10	100	
	500	10	10	100	
Chloroform	125	10	5	50	125
	250	10	9	90	
	500	10	10	100	
Control	-	10	1	10	-

LC50: Lethal concentration 50

of *A. taxiformis* was related to amount of phenols and flavonoids present in the extract. The total phenol content was determined by Folin–Ciocalteu method. This method determines total phenols, blue color formation by reducing heteropolyphosphomolybdate–tungstate ions. Among four fractions studied, maximum phenol content was observed in methanol extract of *A. taxiformis* (25%) in a dose-dependent manner. Reports indicate close relationship between phenols and antioxidant activity of seaweed extracts.[52] Radical scavenging activity increases with increasing flavonoid content.[53] As shown in Table 5, chloroform extract exhibited maximum total flavonoid content (85%). Thus, these results show some correlation between antioxidant activity and phenols and flavonoid content of *A. taxiformis.* Antioxidant activity of extracts of *A. taxiformis* was determined by the reduction of Fe^{3+}/ferricyanide complex to the ferrous form. The reducing ability depends on the

presence of reductones,[54] which exhibits antioxidant[55] potential by donating hydrogen atom for breaking a free radical chain. In the present study, all the extracts exhibited good reducing capacity. Among them, chloroform fraction was shown to have high reducing power (67%). Further, there were no significant differences between reducing power of extracts and standard (73%). The reducing power of the extracts increased with increase in concentration of extracts. These results show that extracts of *A. taxiformis* may possess redox potential that can serve as potential antioxidant. Similar reports of reducing power of red algae were reported in previous years.[56,57] The total antioxidant activity of seaweeds was evaluated by phosphomolybdenum method. The method is based on the reduction of Mo (VI) to Mo (V) in the presence of antioxidant compounds and the formation of a green phosphate Mo (V) complex at acidic pH.[58] Our results demonstrate that methanol extract (27%) showed maximum antioxidant activity as compared to positive control, ascorbic acid (36%). Hence, our findings suggest that antioxidant activity of *A. taxiformis* may be attributed to phenolic compounds present in the extract. Moreover, reports suggest that many seaweed species possess scavenging ability of H_2O_2.[59] H_2O_2 is an important ROS that has the ability to cross biological membrane and can oxidize number of compounds.[60] Although H_2O_2 is not reactive, it can be toxic to the cell if it gives rise to OH in the cells.[61] The inhibitory activity of *A. taxiformis* extract on H_2O_2 is less as compared to other antioxidant assays.

BSLA is a simple and inexpensive method for testing cytotoxicity of extracts. Reports related to cytotoxic activity of *A. taxiformis* against *A. salina* is scanty. Here, a simple zoological organism (*A. salina*) was used as a convenient method for screening.[62] The brine shrimp lethality bioassay is widely used in the evaluation of toxicity of heavy metals, pesticides, medicines, especially natural plant extracts.[63] Further, it is also used for cell line toxicity and antitumor activity.[64] This test has several advantages as compared to other toxicity assays such as rapidness, simplicity, low requirements (no aseptic techniques, no special equipment, and relatively small amount of test sample is needed), robustness, cost-effective, and high degrees of repeatability. In the current study, ethyl acetate of *A. taxiforms* showed highest brine shrimp lethality at very low concentration with LC_{50} value of 84.33 µg/ml, followed by methanol and petroleum ether fractions with LC_{50} values of 104.04 µg/ml. The lowest lethal activity was found in chloroform extract (LC_{50} value = 125 µg/ml). Based on the brine shrimp lethality potential, LC_{50} = 250 µg/ml extrapolated from the graph indicated that *A. taxiformis* extract was moderately toxic. The dose level 500 µg/ml elicited 100% hatching inhibition after 24 h, while at 125 µg/ml, the hatching rate was reduced a bit [Table 11]. The statistical analysis for petroleum ether extract and methanol extract showed the same level of significance $R^2 = 0.606$, whereas for ethyl acetate, the significance level was found to be 77% ($R^2 = 0.70$) and chloroform showed 57% ($R^2 = 0.571$). Reports suggest that many algae and land plants are considered to be cytotoxic using brine shrimp as a model organism.[65,66] Similarly, methanol extracts of *Halimeda opuntia* showed significant cytotoxicity with LC_{50} values of 192.3 µg/ml.[67] In another experiment reported by Manilal *et al.*,[68] the active fraction of *Laurencia brandenii* was found to be most cytotoxic with LC_{50} value of 93 µg/ml. Hence, our study showed that *A. taxiformis* extracts had toxic activity on brine shrimp. The cytotoxic action of a drug is due to the presence of antitumor compounds.[69] This may be due to the presence of several cytotoxic compounds such as fucoidans, terpenoids, and laminarins stated to possess anticancer, antiproliferative, and antitumor properties.[70] Study on the phytochemical analysis of *A. taxiformis* revealed the presence of flavonoids, alkaloids, phenols, anthraquinones, terpenoids, and fatty acids responsible for biological activities such as antioxidant and cytotoxic activities.

CONCLUSION

The present study revealed that red algae, *A. taxiformis* possess strong antioxidant and cytotoxic properties. Hence, further work is necessary to isolate the active compounds from *A. taxiformis* and to study the mechanism of action.

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Conflicts of interest

There are no conflicts of interest.

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