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Studies on few fresh water green algal species reveals *Spirogyra triplicata* as the repository of high phenolic and flavonoid content exhibiting enhanced anti-oxidant property

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Abstract
This work explores the different antioxidant potential of methanolic extract of six different fresh water green algae of India. Among the six algal species studied *Spirogyra triplicata* shows the highest total phenolic content of 134.23 ± 4.45 mg GAE/g and total flavonoid content of 195.93 ± 0.41 mg QE/g extract. *Spirogyra triplicata* also showed the highest lipid peroxidation and DPPH scavenging activity, Superoxide anion scavenging, Nitric oxide and Catalase activity with an EC_{50} value of 7.2 ± 0.84 µg/ml, 50 ± 1.43 µg/ml, 17± 3.12 µg /ml, 30 ± 4.7 µg /ml and 15 ± 1.32 µg /ml respectively. Taken together date reflects that *Spirogyra triplicata* possesses highest Phenol and Flavonoid content exhibiting greatest DPPH scavenging and Lipid peroxidation activity in a pool of six green algal species studied. It also shows good anti-oxidant property as evidenced by different inhibition data there by suggesting presence of various efficacious compounds. Further analysis of these algae will help in identification and isolation of lead molecules for future drug development.

Keywords: Antioxidant, ROS, DPPH, SO, NO, Lipid peroxidation

1. Introduction

Plants are being recognized as an important biomedical resource since the prehistoric period of human civilization [1]. Starting from folk Ayurveda, Unani medicine to new generation Allopathic, Plants are being explored extensively for their efficacious property [2]. Approximate estimation reveals that 70-80% of people worldwide trust on traditional herbal medicine to fulfill their primary health care requirements (Farnsworth *et al.*, 1991) [3]. But lower grouped plants like thallophytes are still deprived and yet to widely unmask. Different cellular components in our body generate oxidant like Hydrogen peroxide (H_2O_2), Superoxide (O_2^-), hydroxyl radical (OH), Peroxyl radical (ROO), Nitric oxide (NO), Peroxynitrite ($ONOO^-$) and singlet oxygen radical ($^1O_2$) during metabolic process. These are commonly called as reactive oxygen species (ROS) [4] and failure to dissolve these ROS leads to the condition of oxygen stress [5]. These Free radicals which are generated during several cellular processes are hazardous for health. The consequences of mild to severe oxidative stress have been reported to encompass several life threatening diseases like cancer, diabetes, hypertension, atherosclerosis, acute respiratory distress syndrome, etc [6]; [7]; [8].

Algae and anti oxidant Nearly about 8000 algal species has been described to date [9] which represent 15.22% of Indian flora. Antioxidants present in various algal species neutralize those ROS of damaged cells and thus known as ROS scavengers. Sometimes it helps in preventing the growth of several cancer cell lines, [10-11] and act as potential repository of anti cancerous natural compounds [12]. So the detection of several antioxidant assays like DPPH, SOD, NO, lipid peroxidase of algal extract is very important as one can treat the cells with two different ways i.e. by up regulating the pro oxidant level in cancerous cells to kill the harmful cells or by the up regulation of antioxidant for increasing the population of healthy cells. *Rhizoclonium crassipellitum*, *Spirogyra triplicata*, *Spirogyra hymerae*, *Pithophora cleveana*, *Rhizoclonium fontinale* and *Hydrodictyon reticulatum* shown in Fig. 1 are the well-known green algae collected in fresh water of India. Methanolic fractions of these algae have been taken in this study for measuring their antioxidant property. Assessment of these six algae is exclusively novel and not done before. The antioxidant property of these thallophytic algae could take the challenge with the higher ones. Thus our aim in this study is to unravel the antioxidant nature of different green algal species and their correlation with cancer.

2. Material and methods

2.1 Reagents and chemicals

Gallic acid (Merck), Sodium Carbonate (Merck), Folin's Ciocalteau (Merck), Quercetin (SRL), Sodium nitrite (Hi media), Aluminium Chloride (Merck), Sodium hydroxide (SRL), 2, 2-diphenyl-1-picrylhydrazyl (DPPH) (SRL), Methanol (Merck), Nitroblue tetrazolium (SRL), NADH (SRL), Sodium phosphate buffer, PMS (SRL), Sodium nitropruside (Hi-Media), Phosphate buffer saline, Sulfannilic acid (Hi-Media), Naphthylene diamine chloride (Hi-Media), Ferrous Sulfate (Hi-media), Acetic acid(Merck), Trichloroacetate (TCA), 0.5% TBA Thiobarbituric acid diluted in 20% (w/v) TCA, n-Butanol (Hi-media), Hydrogen peroxide, Phosphate buffer, Dichromic acid.

2.2 Sample collection and identification

Rhizoclonium Crassipellitum, (CUH/AL/FW-213); *Spirogyra triplicata*, (CUH/AL/FW-212); *Spirogyra hymerae*, (CUH/AL/FW-214); *Pithophora cleveana*, (CUH/AL/FW-215); *Rhizoclonium fontinale*, (CUH/AL/FW-211); *Hydrodictyon reticulatum*, (CUH/AL/FW-217) were collected in between March 2015 to April 2016, from several lentic and mildly lotic water body of Kolkata and Jharkhand, India. The collected materials were botanically identified by phycology expert of University of Calcutta. Accession numbers were generated against each species (mentioned above with each species) and were deposited for further preservation.

2.3 Sample processing

The collected algae samples were washed well under tap water to avoid mud, epiphytes, and aquatic organisms entangled in the algae followed by distilled water. They were drained completely and placed over blotting paper for complete drying under shade. Then the samples were excised into very small pieces and ultimately pulverized well to make powder form to increase the surface area. The powdered samples were weighed, labeled and stored in air-tight packets to prevent fungus attack.

2.4 Extraction procedure

10 gram each algal powder mixed with sufficient amount of hexane to form hexane extract for 72 hours at room temperature. Sufficient amount of hexane extract was unavailable for most of the species except *H. reticulatum*. Then the sample is air dried to remove previous solvent. Methanol fractions are also prepared respectively following the same method. The ethyl acetate and methanol fractions were weighted, marked and stored in cliklok micro centrifuge tube by wrapping the cap with parafilm paper and freezed at 4 °C until use.

2.5 Antioxidant assay

2.5.1 Determination of total phenol content in algal extracts

Total phenol content of the methanol extract was analyzed using Folin-Ciocalteu reagent according to protocol Singleton VL *et al*. with slight modifications [13]. Briefly, 50 µl crude extract mixed with 50 µl, 0.2 N Folin-Ciocalteu reagent and allowed to stand for 3min. Then 1.4 ml, 10% Sodium Carbonate was added, followed by vortexing for 15 sec. The reaction mixture was incubated for 40 minutes in dark at room temperature. Then the O.D. was measured spectrophotometrically at 760 nm. Standard curve of Gallic acid was prepared. The calculation of the phenol content is measured according to the following formula. The assay repeated thrice. The total concentration of phenolic compounds in the methanolic algal extract was determined as mg of gallic acid equivalents (GAE) per gram of algal extract by using the following equation that was obtained from the standard gallic acid graph as prepared.

2.5.2 Determination of total flavonoid content in algal extract

Total flavonoid content was estimated using the aluminum chloride method according to Kamtekar *et al*. (2014) with slight modification [14]. 1 ml of aliquots and 1 ml standard quercetin solution of different concentration was taken in to test tubes and 4 ml of distilled water and 5% sodium nitrite solution was added into each. After 5 minute incubation, 0.3 ml of 10% aluminum chloride was added. At 6th minute, 2 ml of 1M NaOH was added and volume makes up to 10 ml with distilled water and thoroughly mixed. Orange yellowish colour was developed. The absorbance was measured at 510nm. The Blank was performed using distilled water. The assay repeated thrice. The data of total flavonoid content in the methanolic algal extract was expressed as mg of quercetin equivalents (QE) per gram of algal extract by using the estimated standard quercetin graph.

2.5.3 DPPH radical scavenging activity procedure

DPPH radical scavenging activity of methanolic extracts of the above mentioned algae were performed according to *Bloiss et al*. with slight modifications [15]. Each extract was diluted in methanol to make 10, 20, 40, 60, 80, 100 µg/mL dilutions. 200µl of each dilution were mixed with 800ul of DPPH solution (0.1mM/mL in methanol) and mixed thoroughly. The mixture was incubated at room temperature in dark for 1 hour. Absorbance was measured at 517 nm using spectrophotometer with methanol as blank. Each experiment was performed in triplicata at each concentration.

The percentage scavenging of DPPH by the extracts was calculated according to the following formula:

Percentage DPPH radical scavenging = [(Control-Sample)/Control] ×100

2.5.4 Superoxide anion generation activity

Superoxide anion generating activity was measured according to the method of Kakkar *et al*. with slight modifications [16]. In brief, 200 µl methanolic extract (of different concentrations) was mixed with 200 µl Nitroblue tetrazolium (NBT), (50 µM); 200 µl of Phosphate buffer, 200 µl Nicotinamide adenine dinucleotide NADH (78uM). Then 200 µl PMS (10um) was added to start the reaction. The total 1ml reaction mixture was incubated at 37°C temperature in dark for five minutes. After that spectrophotometrically the absorbance was measured at 560 nm. Ascorbic acid was taken as a positive control. The following formula was used to determine the SO radical scavenging activity.

Percentage SO radical scavenging = [(Control-Sample)/Control] ×100

2.5.5 Nitric oxide scavenging assay

For the assessment of Nitric oxide scavenging activity the method of Hossain Hemay *et al*. was used with little modifications [17]. 200 µl of extract was mixed with 400 µl of (10mM) sodium nitropruside. Mixture was incubated at 25°C for 2.30hr. To this reaction mixture 600 µl Griess reagent (equal amount of 1% sulfannilic acid and 0.1% naphthylene diamine chloride (NED)) was added and again incubated at room temp for 30 min in dark. Absorbance was read at 540 nm. Different concentration of ascorbic acid was taken as positive control. % inhibition was calculated with the help of

following formula.
Percentage NO radical scavenging activity = [(Control-Sample)/Control] ×100

2.5.6 Lipid peroxidase activity assay
A modified thiobarbituric acid-reactive species (TBARS) assay [18] was used to measure the lipid peroxide formed, using egg yolk homogenates as lipid-rich media [19]. Malondialdehyde (MDA), a secondary end product of the oxidation of polyunsaturated fatty acids, reacts with two molecules of TBA yielding a pinkish red chromogen with an absorbance maximum at 532 nm. Egg homogenate (0.1ml of 20% v/v) and 0.1ml of extract were added to a test tube and made up to 1ml with distilled water. 0.005ml of $FeSO_4$ (0.07M) was added to induce lipid peroxidation and incubated for 30 min. Then 1.5ml of 20% acetic acid (pH adjusted to 3.5 with NaOH) and 1.5ml of 0.8% (w/v) TBA in 1.1% sodium dodecyl sulphate and 0.5ml 20% TCA were added and the resulting mixture was vortexed and then heated at 95°C for 50 min. After cooling, 700 µl of butanol were added to each tube and centrifuged at 3000 rpm for 10 min. The absorbance of the organic upper layer was measured at 532nm. Incubation of lipid peroxidation (%) by the extract was calculated according to [(1-E/C) x 100 where C is the absorbance value of the fully oxidized control and E is (Abs532+TBA – Abs532-TBA)].

2.5.7 Catalase assay
Catalase activity was determined according to Pari *et al*. (2004) was used [20]. The liver samples were homogenized in 0.01 M phosphate buffer (pH 7.0) and the reaction mixture centrifuged at 1530g for 5 min. After centrifugation, 0.4 ml of hydrogen peroxide (0.2 M) was added to the reaction mixture and incubated for 2–3 min. Two ml dichromate acetic acid reagent (5% aqueous solution of $K_2Cr_2O_7$ prepared in glacial acetic acid) was added to stop the reaction of reaction mixture. The absorbance of reaction mixture was measured at 620 nm and the inhibition percentage was calculated as follows:
Catalase inhibition (%) = Normal activity − Inhibited activity/ Normal activity×100
Where:
Normal activity = hydrogen peroxide + Phosphate buffer, inhibited activity = hydrogen peroxide + phosphate buffer + liver homogenate.

3. Results
3.1 Result of total phenol content
Total phenolic content (TPC) are found to be correlated with antioxidant property which act as free radical scavenger. The amount of total phenol was determined with the Folin-Ciocalteu reagent. Gallic acid was used as a standard compound and the total phenols were expressed as mg/g gallic acid equivalent using the standard curve equation: y = 0.0002x - 0.0125, where y = absorbance of algal methanolic extract; x = gallic acid concentration. R^2 = 0.997. The total phenolic contents of different algal methanolic extracts are given in Table 1. The result Indicates that *Spirogyra triplicata* has the highest TPC of 134.23 ± 4.45 mg GAE/g extract and *Spirogyra hymerae* possesses the second highest TPC of 107.29 ± 3.25 mg GAE/g at a particular concentration (Fig. 2).

3.2 Results of Total flavonoid content
Quercetin was used as a standard compound and the total flavonoid were expressed as mg/g Quercetin equivalent using the standard curve equation Y = 0.0004x + 0.0325 where y = absorbance of algal methanolic extract at 510nm and x = quercetin concentration and R^2 = 0.9892. The result shows the contents of total flavonoid that were measured by $AlCl_3$ reagent in terms of Quercetin acid equivalent. The total flavonoid varied from 21.423611±0.73 mg QE/g extract to 195.9375±0.41 mg QE/g extract in the methanolic algal extracts (Table 2). The maximum flavonoid content was found in the methanol extract (195.9375±0.41 mg QE/g extract) of *Spirogyra triplicata*. The results obtained from present study showed in Fig. 3 that the extract of *Spirogyra triplicata*, which contain maximum amount of flavonoid compounds, followed by *Spirogyra hymerae* (128.50 ± 0.98 mg QE/g extract). These two algal extracts exhibited the greatest antioxidant activity and thus can be used to explore for new drug discovery against cancer.

3.3 Result of DPPH
DPPH is a highly stable free radical with purple colour. After reacting with an antioxidant it turned into a stable yellow colour compound (diphenyl-picrythydrazine). The perishing of the DPPH radical absorption at a characteristic wavelength was monitored by decrease in optical density. Reduction in the colour was measured by spectrophotometer (λ_{max} 517). The disappearance of the DPPH radical absorption at a characteristic wavelength was monitored by decrease in optical density. There was a direct positive relationship between antioxidant activity and increasing concentration of the extracts. For this purpose, different concentrations of Ascorbic acid solution were prepared as standard. In this study, differential DPPH reduction activity of six different algal methanolic extracts were evaluated. Methanolic extract of *Spirogyra triplicata* indicated high DPPH radical scavenging activity with an EC_{50} value 50µg/mL followed by methanolic extract of algae *Spirogyra hymerae* with an EC_{50} value more than 100 µg/ml (Fig. 4). EC_{50} is a useful parameter for measuring the antioxidant activity and compare the antioxidant capacity of various samples the free radical scavenging activity was found to be increasing with increasing concentrations. The results are expressed as percentage inhibition of DPPH (n=3) and depicted in Fig. 4.

3.4 Results of Superoxide anion scavenging activity
Superoxide anion generation results with the presence of the different methanolic algal extracts has been studied. All samples treated in this experiment indicated potential antioxidant activities (Fig. 5). Results depicted in Fig. 5 showed superoxide anion scavenging activity of methanolic extracts of six above mentioned algal species. The scavenging activity was dose-dependent. The EC_{50} value of the *Spirogyra triplicata* was 17 ± 3.12 µg/ml was found to be highest followed by *Spirogyra hymerae*. Others algal species showed modest superoxide scavenging activity. Data taken together suggests that the extract effectively scavenges ROS and could protect against oxidative damage. In summary we observed the superoxide scavenging activity of six algal species studied is *Spirogyra triplicata* > *Spirogyra hymerae* > *Rhizoclonium fontinale*> *Hydrodictyon reticulatum* > *Pithophora cleveana* > *Rhizoclonium Crassipellitum*.

3.5 Results of Nitric oxide scavenging assay
Nitrite radical scavenging assay was carried out on the methanol extracts of six above mentioned algae from a concentration of 10 to 100 µg/mL. Percentage free radical scavenging was plotted against concentration of the extracts as shown in Fig. 6. The algae exhibited antioxidant activity through competing with oxygen to scavenge for the nitrite

radical which was generated from sodium nitropruside at physiological pH in an aqueous environment. The antioxidant activity increased with an increase in concentration of the extracts. Increasing the concentration of the methanolic extracts resulted in an increase in the nitrite radical scavenging activity. Nitric oxide scavenging activity leading to the reduction of the nitrite concentration in the assay medium. The maximum free radical scavenging activity and potency were. *Spirogyra triplicata* methanol extract was the most efficient nitric oxide scavenger as it removed the nitrite radical at a lower concentration as compared to the other algal extracts with EC_{50} value 30 ± 4.7 µg /ml followed by *Hydrodictyon reticulatum which shows* EC_{50} value 40 ± 3.9 µg /ml. Other algal species studied showed minimal nitric oxide scavenging activity

3.6 Results of lipid peroxidase assay

Lipid peroxidation is the oxidative degradation of lipids. In this process free radicals "steal" electrons from the lipids in cell membranes, resulting in cell damage. Lipid peroxidation recognized as primary toxicological event and a well-established mechanism of cellular injury, is used as an indicator of oxidative stress. The TBARS (Thiobarbeturic acid reactive substance) assay has been used to determine the degree of lipid peroxidation. TBA reacts specifically with malondialdehyde (MDA), a secondary product of lipid peroxidation to give a red chromogen, which may then be determined spectrophotometrically at 532nm. The result of lipid peroxidation assay is represented in fig. 7. Species *Spirogyra triplicata* shows maximum inhibition of lipid peroxidation. The EC_{50} value of *S. triplicata* species is 7.2 ± 0.84 µg/ml followed by *Rhizoclonium crassipellitum* with EC_{50} value 10 ± 1.98 µg/ml. Other algal species exhibited minimal Lipid peroxidation activity. In summary we observed the lipid peroxidation activity of six algal species studied is *Spirogyra triplicata* > *Rhizoclonium Crassipellitum* > *Rhizoclonium fontinale* > *Pithophora cleveana* > *Spirogyra hymerae* > *Hydrodictyon reticulatum*.

3.7 Results of Catalase activity

The effect of six methanolic algal extracts on enzymatic antioxidant activities in goat liver slices exposed to *in vitro* H_2O_2 is shown in fig. 8. The effect in Catalase activity was observed in H_2O_2-exposed liver slices. Co-administration of algal methanolic extract with H_2O_2 causes an increase in Catalase activity. *Spirogyra triplicata* shows moderate amount of catalase inhibition activity, with an EC_{50} value 15 ± 1.32 µg /ml. Other alga exhibits fair amount of inhibition activity. In summary we observed the lipid peroxidation activity of six algal species studied is *Rhizoclonium fontinale* > *Pithophora cleveana* > *Spirogyra hymerae* > *Hydrodictyon reticulatum* > *Rhizoclonium Crassipellitum* > *Spirogyra triplicata*.

Table 1: Levels of total phenol content (TPC) in different algal extract (Mean[¥] ±SD[Φ]) (mg GAE/g)

Species of algae	TPC mg GAE/g ¥, Φ
Rhizoclonium Crassipellitum	30.06 ± 0.25
Spirogyra triplicata	134.23 ± 4.45
Spirogyra hymerae	107.29 ± 3.25
Pithophora cleveana	26.18 ± 0.24
Rhizoclonium frontenale	46.59 ± 1.048
Hydrodictyon reticulatum	23.95 ± 1.5

[¥] Values are means of triplicata data at specific concentration; [Φ]SD, standard deviation

Table 2: Levels of total flavonoid content (TFC) in different algal extract (Mean[¥] ±SD[Φ]) (mg QE/g)

Species of algae	TFC mg QE/g ¥, Φ
Rhizoclonium Crassipellitum	54.82 ± 1.18
Spirogyra triplicata	195.93 ± 0.41
Spirogyra hymerae	128.50 ± 0.98
Pithophora cleveana	48.36 ± 0.93
Rhizoclonium frontenale	21.42 ± 0.73
Hydrodictyon reticulatum	54.96 ± 0.86

[¥] Values are means of triplicata data at specific concentration; [Φ]SD, standard deviation.

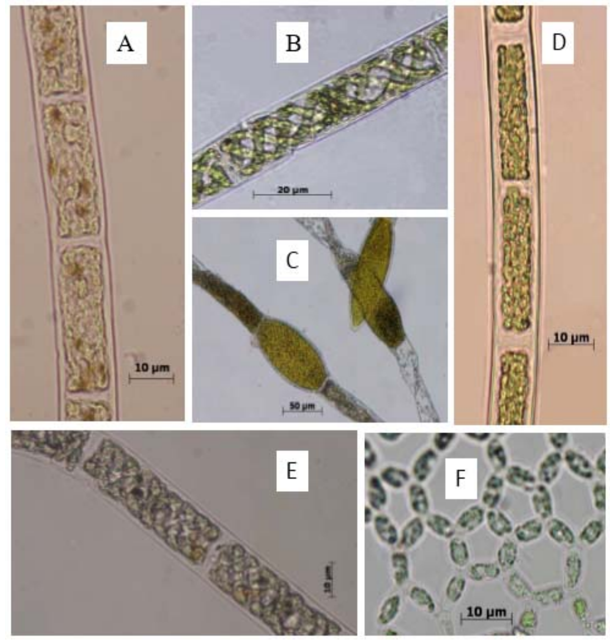

Fig 1: Microscopic structure of six different algae. A-*Rhizoclonium crassipellitum,* B- *Spirogyra triplicata,*C-*Pithophora cleveana,* D-*Rhizoclonium fontinale,* E- *Spirogyra hymerae,* F-*Hydrodictyon reticulatum.*

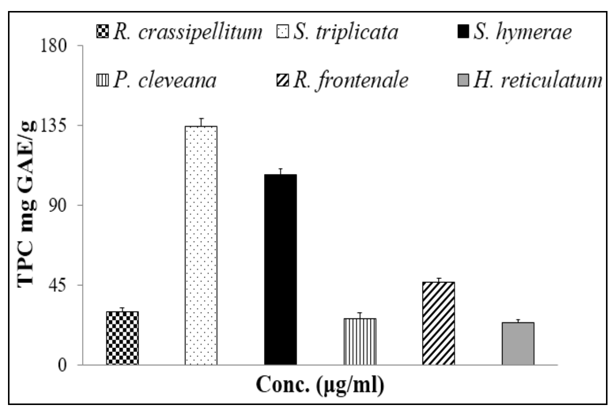

Fig 2: Measurement of total phenol content of different algal extract. Here *Rhizoclonium crassipellitum* (A), *Spirogyra triplicata* (B), *Pithophora cleveana* (C), *Rhizoclonium fontinale* (D), *Spirogyra hymerae* (E), *Hydrodictyon reticulatum* (F) has been used at concentration at 3 mg/ml. The TPC was determined as mg of gallic acid equivalents (GAE) per gram of algal extract by using the previously mentioned equation that was obtained from a standard gallic acid graph.

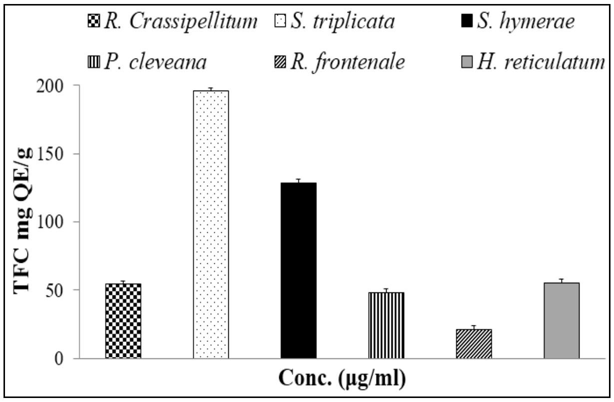

Fig 3: Measurement of total flavonoid content of different algal extract. Here *Rhizoclonium crassipellitum* (A), *Spirogyra triplicata* (B), *Pithophora cleveana* (C), *Rhizoclonium fontinale* (D), *Spirogyra hymerae* (E), *Hydrodictyon reticulatum* (F) has been used at concentration at 12 mg/ml. The TFC was determined as mg of quercetin acid equivalents (QE) per gram of algal extract by using the previously mentioned equation that was obtained from a standard quercetin acid graph.

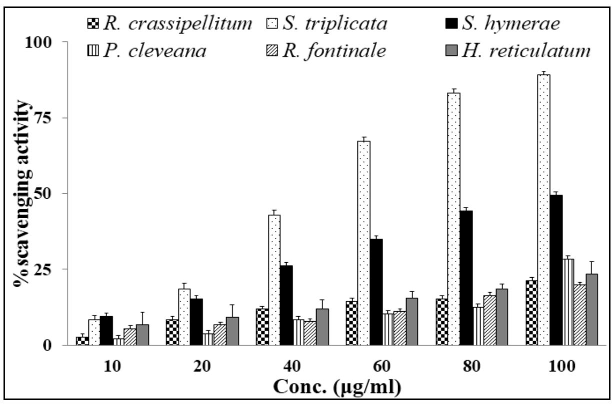

Fig 4: Antioxidant activity of methanolic extracts of different algae as measured by DPPH. Here *Rhizoclonium crassipellitum* (A), *Spirogyra triplicata* (B), *Pithophora cleveana* (C), *Rhizoclonium fontinale* (D), *Spirogyra hymerae* (E), *Hydrodictyon reticulatum* (F) has been used at concentrations 10 to 100 µg/ml. Results shown in bar graph indicate atleast mean ± standard deviation of three separate experiments.

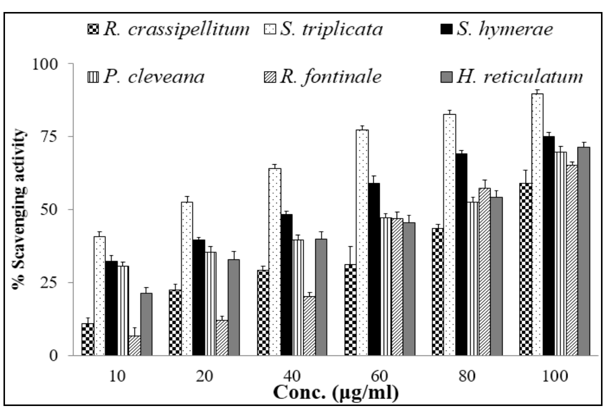

Fig 5: Comparison of superoxide anion scavenging activity of six algal species. Here *Rhizoclonium crassipellitum* (A), *Spirogyra triplicata* (B), *Pithophora cleveana* (C), *Rhizoclonium fontinale* (D), *Spirogyra hymerae* (E), *Hydrodictyon reticulatum* (F) has been used at concentrations 10 to 100 µg/ml. Each column indicates mean ± standard deviation value of three independent experiments.

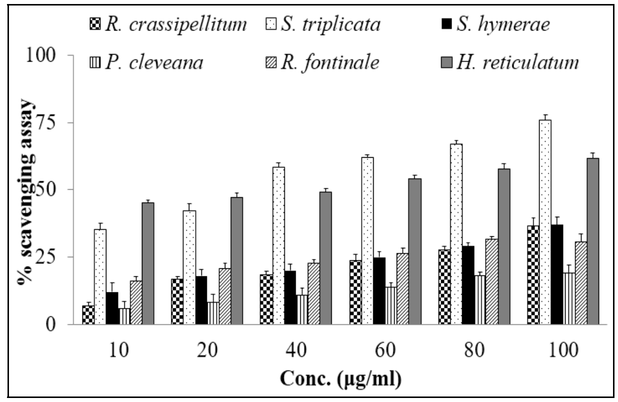

Fig 6: Comparison of nitric oxide scavenging ability of *Rhizoclonium crassipellitum* (A), *Spirogyra triplicata* (B), *Pithophora cleveana* (C), *Rhizoclonium fontinale* (D), *Spirogyra hymerae* (E), *Hydrodictyon reticulatum* (F) has been used at concentrations 10 to 100 µg/ml. Each column denotes mean ± standard deviation value of three independent experiments.

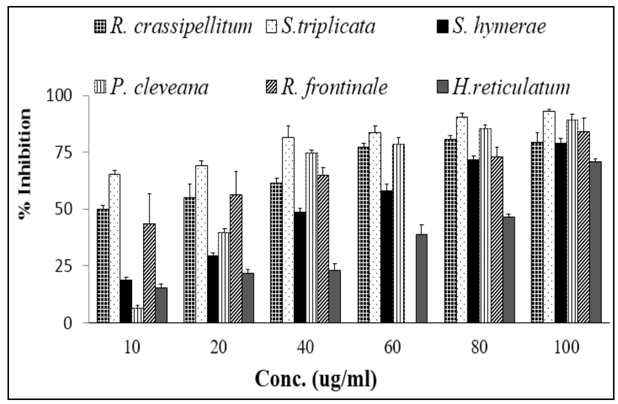

Fig 7: Comparisn of lipid peroxidation assay of *Rhizoclonium crassipellitum* (A), *Spirogyra triplicata* (B), *Pithophora cleveana* (C), *Rhizoclonium fontinale* (D), *Spirogyra hymerae* (E), *Hydrodictyon reticulatum* (F) has been used at concentrations 10 to 100 µg/ml. Each column indicates mean ± standard deviation value of three independent experiments.

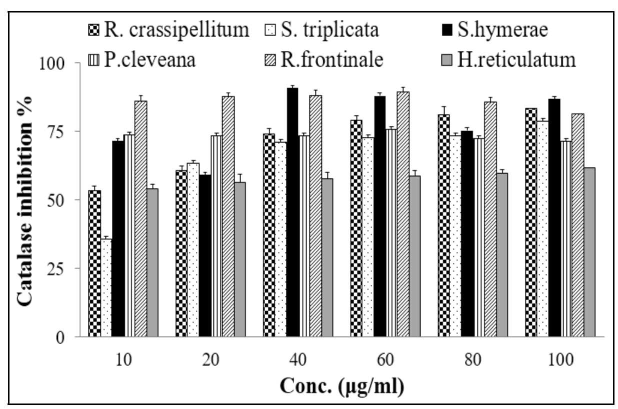

Fig 8: Comparison of catalase scavenging ability of *Rhizoclonium crassipellitum* (A), *Spirogyra triplicata* (B), *Pithophora cleveana* (C), *Rhizoclonium fontinale* (D), *Spirogyra hymerae* (E), *Hydrodictyon reticulatum* (F) has been used at concentrations 10 to 100 µg/ml. Each column refers mean ± standard deviation value of three independent experiments.

4. Discussion

It is widely recognized that many of today's diseases are due to the oxidative stress that results from an inequity between formation of ROS and their neutralization when endogenous antioxidant mechanisms are incapable to quench the free radicals. The free radicals are known to be scavenged by synthetic antioxidants, but due to their adverse side effects leading to carcinogenicity and various other health hazards, search for effective and natural antioxidants has become crucial [21]. Natural antioxidants are believed to be safer and bioactive component thus our target was to analyze the bio-properties of different algal species of India.

Algae exhibit a very good stress tolerance capacity like very high light intensity and oxygen concentration throughout their life cycle which leads to generation of free radicals and several other effective oxidizers. The bio-resistance of algal cellular and structural components against this oxidative challenge proclaims that the intracellular bioactive antioxidants may be responsible for the preventive role as an intrinsic mechanism for sustenance. [22] Algae represents the repository of many biologically active molecules including phenolic and flavonoid content, which deserve attention because of the many physiological benefits they provide. Experimental outcome proclaims that algal product also have considerable amounts of total phenolic and flavonoid content which are comparable to phenolic content in black tea. [23] Our result reflects that the highest total phenol content as well as total flavonoid content is exhibited by *Spirogyra triplicata* of the different algal species studied. DPPH free radical scavenging assay is a basic simple widely used assay which is considered most accurate screening method used to evaluate the antioxidant activity of biological samples. [24] *Spirogyra triplicata* also exhibits greatest DPPH scavenging activity in a pool of six green algal species studied. So it can be assumed that the strong scavenging capacity of methanol extract on DPPH might probably due to the phenolic compounds which could act as a hydrogen donor antioxidant. The antioxidant potential of polyphenols has been correlated to the capacity of donating hydrogen radicals [25].

Superoxide anions (O_2^-) are the most frequent free radicals whose concentration increases at oxidative stress and are produced either by auto oxidation processes or by enzymes and produces other cell damaging free radicals and oxidizing agents [26]. Differential superoxide anion generation was observed in six different algal species tested with *Spirogyra triplicata* showing the most effective antioxidant properties. Nitric oxide acts as an important chemical mediator generated by macrophages, neurons, endothelial cells etc. and engaged in the relation of various physiological processes [27]. Nitric oxide is classified as a free radical because of its unpaired electron and displays important reactivity with certain types of proteins and other free radicals. The toxicity of NO becomes adverse when it reacts with superoxide radical, forming a highly reactive peroxynitrite anion ($ONOO^-$). The methanolic extract of *spirogyra triplicata* along with five other alga shows a huge scavenging of nitric oxide and acting as efficient antioxidant. Lipid peroxidation undergoes the formation and propagation of membrane lipid radicals, which eventually destroy membrane lipids [28]. The TBARS formation assay was used to evaluate the inhibition of Fe^{2+} induced lipid peroxidation by the extract. The methanolic extract of *Spirogyra triplicata* showed a very effective concentration-dependent inhibition of lipid peroxidation followed by other algal species. Catalase is an important enzyme that activates the decomposition of free radical hydrogen peroxide to water and oxygen. This enzyme possesses the protective nature that down regulates the oxidative damage of the cells caused by ROS [29]. The depletion of this enzyme activity in cellular level may cause toxic effects due to the accumulation of hydrogen peroxide in animal cells. Though *Spirogyra triplicata* showed very efficient superoxide, lipid peroxidation, DPPH and Nitric oxide scavenging activity but in our study it showed very modest catalase activity.

5. Conclusion

This study dealing with six different algal species of India and antioxidant properties reveals that methanolic extract of *Spyrogyra triplicata* along with other methanolic algal extract acts as good repository of phyto-chemicals having good anti oxidant activity. Further analysis of these algae will aid in characterization and isolation of lead molecules that could help in future drug development.

6. Conflict of interest statement Authors declare that they don’t have any conflict of interest.

7. Author contributions SP conceived and designed the study.AM collected the sample from field and CN, RP identified them. AM performed the laboratory tests. SP, AM analyzed data and wrote the manuscript. All authors approved the final manuscript.

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9. Reference

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