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Determination of antioxidant activities of algae from lentic ecosystems under anthropogenic stress: A comparative study

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ABSTRACT

The present study aim to investigate/compare the antioxidant activity of different species of algae collected specifically from water bodies under stress. Total phenolics and flavonoid content of the different algal extracts was determined using Folin Cio Calteau reagent and Quercetin as standard. Results indicate that the total phenolics is maximum (48.88 µgGAE/ml of extract) in Spirogyra fluviatilis and minimum (3.06 µgGAE/ml of extract) in Nitzschia amphibia, flavonoids content was highest (34.93 µgQE/ml of extract) in Klebsormidium flaccidum and lowest (4.75 µgQE/ml of extract) in Anabaena azollae. DPPH radical scavenging activity, Superoxide anion radical scavenging activity was performed in order to determine the antioxidant activities of the extracts prepared from the different species of algae. It was found that all the species of algae do possessed antioxidant activities. However, Spirogyra fluviatilis out performed all the other algal species in that it has a better ability to scavenge DPPH radicals, superoxide anion radicals and that it has a better reducing power in all the tested concentrations (500 µg/ml, 250 µg/ml, 125 µg/ml, 62.5 µg/ml and 31.25 µg/ml) of extract compared to the other algal species which indicate that it could therefore serve as a good and reliable source of natural antioxidants.

Keywords: Antioxidant activity, DPPH, Phenolics, Flavonoids, *Spirogyra fluviatilis*.

INTRODUCTION

Meghalaya is one of the hilly states situated in the North Eastern part of India. Meghalaya means *"the abode of clouds"*, it has a total area covering approximately 22,429 KM^2. The state received an average annual rainfall of approximately 2818 mm per year which contributed to its rich diversity of both flora and fauna. Most of its area are unaffected by human intervention and hence still remains in its pristine natural conditions. Meghalaya has a number of protected areas some of which include the Nokrek National Park, Balpakram Ridge National Park, Nongkhyllem wildlife Sanctuary, Siju Wildlife Sanctuary etc. In addition, Meghalaya is also blessed with a number of rivers, streams, lakes and reservoir which

all play a significant role in boosting the tourism sector's economy of the state. Until recently, Meghalaya had been relying on coal mining as one of the ways of improving the state economy. However, the impact of coal mining on rivers, lakes, streams and reservoir in the state was evidently deleterious and this has post a serious threat especially to aquatic life and ecosystems. Considering the impacts that coal mining practices (such as rat-hole mining) had posed on the safety of the natural environment in general, the National Green Tribunal (NGT) had ban all illegal coal mining activities in the state and also stated in its second interim report that there is a need to stop the practice of rat-hole mining and that efforts should be made to accessed loss to the environment and to develop environmental restoration practices with appropriate Disaster management plans to combat with any disastrous impact of coal-mining in the future (Katekey *et al.* 2019). Acid Mine Drainage (AMD), originating from coal mines and spoils, leaching of toxic heavy metals etc., is responsible for the acidic nature and high concentration of heavy metals in water bodies found near coal-mining sites (Hester *et al.* 1994). Earlier studies on the impact of coal-mining especially in the Jaintia Hills district of the state has linked Acid Mine Drainage (AMD) as one of the contributing factors leading to the degradation of water quality and the continued decline in aquatic biodiversity (Swer and Singh, 2004). In addition to coal-mining, Meghalaya also has a significant numbers of limestone quarries which also contributed fairly to the state's economy owing to its role in the manufacturing of cement in cement plant industries. However, limestone mining has also contributed to severe environmental degradation such as the loss of top fertile soils, deforestation and contamination of water bodies. In Meghalaya, limestone mining activities and cement plant industries has been the key reasons of elevated levels of pH, increase concentration of calcium and phosphate, total dissolved solids (TDS), alkalinity and hardness of water bodies (Lamare and Singh, 2014).

Algae are a class of eukaryotic photosynthetic organisms that contribute to almost half of the world's oxygen supply. This group of organisms is also a good source of renewable biofuel and can also be a potential source of useful pharmaceutical drugs (Chapman, 2013). Algae are the main primary producers in all types of aquatic ecosystems and hence play a very significant and determining role in balancing the food chain of any aquatic habitat (Sen *et al.*2013). Algae are an interesting class of organisms which can withstand extreme environmental conditions (pH, nutrients, salinity, temperature etc.) and can thrive in complex natural as well as artificial habitats. This group of organisms have the ability to adapt to rapidly changing adverse environmental conditions by successfully and rapidly producing significant and diverse arrays of secondary metabolites that are biologically active (Welker *et al*. 2012). Antioxidants are one among numerous compounds found in algae that are of major interest in pharmaceutical industry (Gonulol *et al*. 2009). Antioxidants that are produce by algae include water-soluble compounds such as vitamins, poly-phenols and phycobiliproteins, fat soluble compounds such as Vitamin E (α-tocopherol) and carotenoids and several different enzymes (Shalaby, 2015).

Antioxidants are substances that prevents or delay oxidative damage cause by free radicals which are oxygen derivatives (superoxide anion, single state oxygen, hydroxyl radicals etc.) which may have degenerative effects on living cells, DNA, protein functions etc (Zahra *et al*. 2007). In order to reduce such oxidative damage caused by free radicals, several commercially synthesized antioxidant supplements such as α-tocopherol, butylated hydroxyl anisole (BHA), butylated hydroxyl toluene (BHT) were used (Gulcin *et al.* 2002). However, it was suspected that such commercially synthesized antioxidants are responsible for several side effects such as liver damage and carcinogenesis among others. Owing to these side effects while using synthetic antioxidants, investigators, industries, researchers and corporates have been extensively trying to seek natural antioxidants that may serve as alternative replacements of synthetic food and pharmaceutical products.

MATERIALS AND METHODS

Algal samples

Several species of algae were collected from polluted, limestone and coal mining affected water bodies of Meghalaya which were then observed under the microscope and identified using standard monograph and literatures (ADIAC, 1999; Algaebase; Desikachary, 1985; Fritsch, 1935; Gandhi, 1998; John *et al*. 2002). Analysis of pH, temperature, conductivity, dissolved oxygen, chlorophyll a content etc. of the collected water samples was done both on site and in the laboratory. Analysis of nutrients such as phosphate,

nitrate and nitrite was determined by following APHA, 2012. These species of algae were then cultured in liquid media using conical flasks and aquarium with continuous supply of oxygen (for *Klebsormidium*) using BG11 and Bold Basal media respectively followed by repeated sub-culturing in order to obtain a pure culture. After obtaining a pure culture, these algal species were then subjected to the following tests in order to determine their antioxidant properties.

Preparation of algal extract

1g of air dried algal material was extracted at room temperature using methanol. The extract was then filtered through a filter paper (Whatman No.1) and was evaporated to dryness. The crude extract was then dissolved in methanol and store in the refrigerator at -20°C until they were used in the test.

Quantitative analysis of antioxidant compounds

Determination of total phenolic content: Using gallic acid as standard and Folin & Ciocalteau reagent by following Slinkard and Singleton (1977), the total phenolic content of the algal extract was determined. In a volumetric flask 1ml of the extract (1mg/ml) was diluted with 46 ml of distilled water. 1 ml of Folin-Ciocalteau reagent was added and mixed thoroughly. After 3 minutes, 3 ml of 2% sodium carbonate was added and then the mixture was kept in the dark at room temperature for 2 hours. The mixture was shaken thoroughly and frequently every now and then. The absorbance of the mixture was then measured at 760 nm in a spectrophotometer against a blank that consist of all the reagents except the algal extract.

Using gallic acid a calibration curve was prepared and the results were expressed as µg GAE (gallic acid equivalent)/mg dry weight of extract.

Determination of total Flavonoid content

Using Quercetin as standard, the total flavonoid content was determined by spectrophotometric method (Quettier-Delue *et al*. 2000). 1ml of 2% methanolic $AlCl_3$ solution was mixed with with 1ml of 1mg/ml extract and its absorbance was determined at 415 nm. The mixture was incubated at room temperature for 10 minutes and the absorbance was measured at 415 nm. Negative control without extract was used as the blank. Using Quercetin, a calibration curve was prepared and the results were expressed as µg QE (Quercetin Equivalent)/mg dry weight of extract.

Antioxidant activity

DPPH radical scavenging activity

The algal extract DPPH radical scavenging activity was measured using the method described by Brand-Williams *et al.* (1995) with some modification. Two fold dilution of the extract was made to get a concentration of 500, 250, 125, 62.5 and 31.5 µg/ml. Diluted solution of extract (1ml) were mixed with 2ml of methanol solution of DPPH radical (0.05 mg/ml). The mixture was shaken vigorously and was allowed to stand for 30 minutes at room temperature. The absorbance was then measured at 517 nm against a blank solution that contained 2ml methanol and 1ml of algal extract. A solution containing 2ml DPPH and 1ml methanol was used as the control. Ascorbic acid was used as standard.

The ability of the algal extract to scavenge DPPH free radical was calculated using the following equation:

DPPH scavenging effect % = [(A0-A1)/A0] x 100

Where A0 is the absorbance of the negative control (2ml of methanol solution of DPPH radical + 1ml of methanol) and A1 is the absorbance of reaction mixture or standard.

The inhibition concentration at 50% inhibition (IC_{50}) was used to measure the radical scavenging activity. A lower IC_{50} value meant better radical scavenging activity.

Reducing Power

Using the method given by Oyaizu (1986), the reducing power had been determined. Two fold dilution of the extract was made to get a concentration of 500, 250, 125, 62.5 and 31.25 µg/ml. 1ml of these extract with different concentration was mixed with 2.5ml of phosphate buffer (0.2 M, pH 6.6) and potassium ferricyanide (2.5 ml, 1%). The mixtures were incubated at 50°C for 20 minutes. Trichloroacetic acid (10%, 2.5 ml) was then added to the mixture and centrifuged. Finally the upper layer (2.5 ml) was then mixed with distilled water (2.5 ml) and ferric chloride (0.5 ml; 0.1%.). The absorbance of the solution was then taken at 700 nm using a spectrophotometer. Blank was prepared with all the reaction agents without extract. Increased absorbance of the reaction mixture specified that the reducing power is high. Ascorbic acid was used as the positive control.

Superoxide anion radical scavenging activity

The algal extract sueroxide anion radical scavenging activity was measured following the methods given by Nishikimi *et al.* (1972). Two fold dilution of the extract was made to get a concentration of 500, 250, 125, 62.5 and 31.25 µg/ml. Each concentration (0.1 ml) was mixed with 1ml of Nitrobluetetrazolium (NBT) solution (156 µM in 0.1 M phosphate buffer, pH 7.4) and 1ml of nicotiamide adenine dinucleotide (NADH) solution (468 µM in 0.1 M phosphate buffer, pH 7.4). The reaction was started by adding 100µL of phenazinemetasulphate (PMS) solution (60µM in 0.1 M phosphate buffer, pH 7.4). The mixture was then incubated at room temperature for 5 minutes and the absorbance was measured at 560 nm. Phosphate buffer was used as the blank. Decreased absorbance readings indicated increased superoxide anion radical scavenging activity. Ascorbic acid was used as standard. The percentage inhibition of superoxide anion generation was calculated using the following equation:

Superoxide anion scavenging activity (%) = [(A0-A1)/A0] x 100

Where A0 is the absorbance of the negative control (All the reacting reagent except the extract) and A1 is the absorbance of the reaction mixture or standard.

The inhibition concentration at 50% inhibition (IC_{50}) was the parameter used to compare the radical scavenging activity. A lower IC_{50} meant better radical scavenging activity.

RESULTS AND DISCUSSION

Analysis of the water samples collected showed that the pH of all water bodies are acidic with the exception of those samples collected from the Nongtrai lime stone mining area (Stream) associated with the Lafarge mining Company which was alkaline. pH was maximum (8.76) in Nongtrai limestone mining area (stream) and minimum (3.58) in Mawksing Coal mining area (Stream). Conductivity was maximum (0.1277 mS) in Khliehriat coal mining area (stream) and minimum (0.0012 mS) in Nongtrai limestone mining area (stream), temperature of the water body was recorded to be maximum (23°C) in Nongtrai limestone mining area (stream) and minimum (20°C) in Umshyrpi River and Mawksing coal mining area (stream), phosphate was maximum (1.83 mg/l) in Umshyrpi River and minimum (0.88 mg/l) in Mawksing coal mining area (stream), Nitrate was maximum (2.56 mg/l) in Nongtrai limestone mining area (stream) and minimum (0.76 mg/l) in Mawksing coal mining area (stream) , Nitrite was maximum (2.25 mg/l) in Umshyrpi River and minimum (0.54 mg/l) in Khliehriat coal mining area (stream). Analysis of chlorophyll a content of the collected samples revealed that it was maximum (0.29 mg/l) in Nongtrai limestone mining area (stream) and minimum (0.12 mg/l) in Umshyrpi River. Similarly, the dissolved oxygen (DO) content was analysed and was found to be maximum (12.43 mg/l) in Nongtrai limestone mining area (stream) and minimum (4.21 mg/l) in Umshyrpi River (Table 1).

Table 1: Analysis of the water bodies

Sl. No.	Name of study site	pH	Conductivity (mS)	Tempe-rature (°C)	Phosphate (mg/l)	Nitrate (mg/l)	Nitrite (mg/l)	Chlorophyll a (mg/l)	Dissolved Oxygen (mg/l)
1	Umshyrpi River	6.32	0.0056	20	1.83	2.10	2.25	0.12	4.21
2	Mawksing Coal mining area (Stream)	3.58	0.1023	20	0.88	0.76	0.56	0.19	5.34
3	Nongtrai Limestone Mining area (Stream)	8.76	0.0012	23	1.45	2.56	1.13	0.29	12.43
4	Khliehriat Coal Mining area (Stream)	3.88	0.1277	21.5	0.99	0.83	0.54	0.15	4.33

After performing the above experiment, it was found that *Spirogyra fluviatilis* has the maximum (48.88 µgGAE/ml of extract) amount of total phenolic and *Klebsormidium flaccidum* has the maximum (34.93 µgQE/ml of extract) flavonoid content respectively whereas phenolics content was minimum (3.06 µgGAE/ml of extract) in *Nitzschia amphibia* and flavonoid content was minimum (4.75 µgQE/ml of extract) in *Anabaena azollae* (Table2, Fig.1). The smaller the value of DPPH and Superoxide anion radical scavenging activity at % inhibition IC_{50}, the better is the ability of an algal species to scavenge these radical and anions. Keeping this in mind, DPPH radical scavenging activity was maximum {551.45 IC_{50} (µg/ml)} in *Klebsormidium flaccidum* and minimum {189.98 IC_{50} (µg/ml)} in *Spirogyra fluviatilis* which suggested that *Spirogyra fluviatilis* can scavenge DPPH radical more effectively and *Klebsoridium flaccidum* can scavenge least effectively. Similarly, Superoxide anion scavenging activity was maximum {900.41 IC_{50} (µg/ml)} in *Nitzschia amphibia* and minimum {221.91 IC_{50} (µg/ml)} in *Spirogyra fluviatilis* which indicates that *Spirogyra fluviatilis* can scavenge superoxide anion more effectively and that *Nitzschia amphibia* has the least scavenging power (Table 3, Fig 2). The reducing power of these algal species was compared with the reference standard (Ascorbic acid) and it was found that taking all the concentrations into account *Spirogyra fluviatilis* has the best reducing power whereas *Chlorococcum humicola* has the least reducing power (Table 4, Fig 3).

Table 2: Total phenolics and flavonoids content

Name of algal species	Total phenolics content (µgGAE/ml of extract)	Total flavonoids content (µgQE/ml of extract)
Anabaena azollae	7.13	4.75
Chlorococcum humicola	19.79	15.74
Scenedesmus communis	29.05	23.48
Spirogyra fluviatilis	48.88	31.19
Chaetophora elegans	30.58	29.92
Klebsormidium flaccidum	38.77	34.93
Spirulina agilissima	15.12	32.08
Gomphonema parvalum	6.17	9.18
Nitzschia amphibian	3.06	9.61
Mougeotia viridis	34.14	33.58

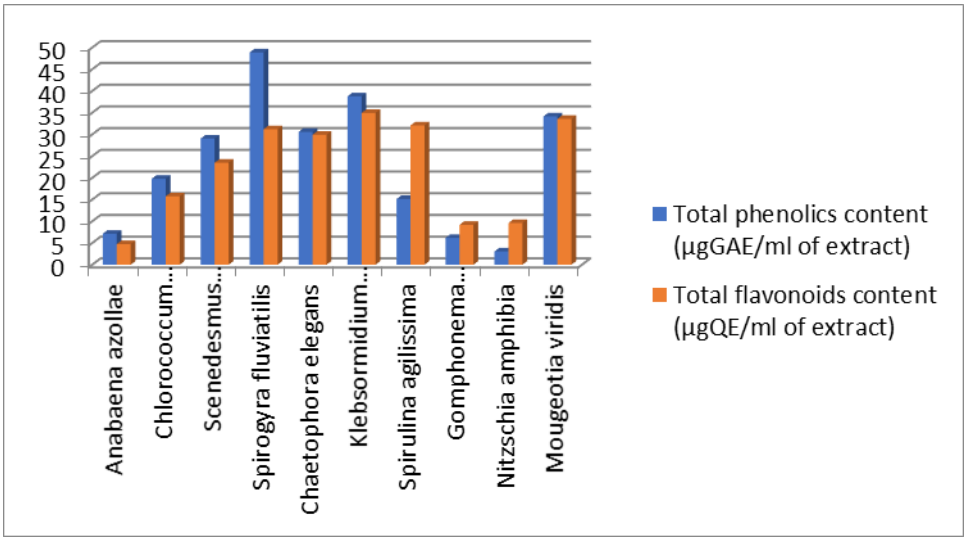

Fig 1: Comparison of total phenolics and flavonoid content in the 10 algal species

Table 3: DPPH radical scavenging activity and Superoxide anion radical scavenging activity of methanol extract of 10 algal species

Name of algal species	DPPH Radical scavenging activity IC_{50} (µg/ml)	Superoxide anion scavenging activity IC_{50} (µg/ml)
Anabaena azollae	321.18	543.28
Chlorococcum humicola	367.39	562.76
Scenedesmus communis	339.24	553.87
Spirogyra fluviatilis	189.98	221.91
Chaetophora elegans	276.93	296.77
Klebsormidium flaccidum	551.45	536.97
Spirulina agilissima	295.60	534.21
Gomphonema parvalum	478.52	800.32
Nitzschia amphibia	485.76	900.41
Mougeotia viridis	323.63	559.07

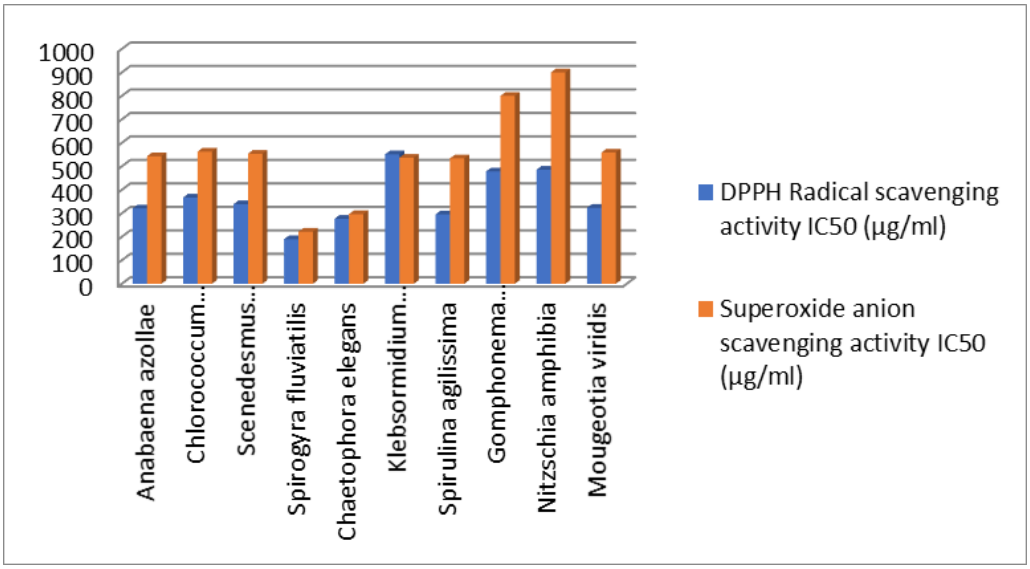

Fig 2: Comparison between DPPH and Superoxide anion scavenging activity IC_{50} (µg/ml) of the 10 algal species.

Table 4: Reducing power of methanol extract of 10 algal species

Name of algal species	500 µg/ml	250 µg/ml	125 µg/ml	62.5 µg/ml	31.25 µg/ml
Anabaena azollae	0.48	0.38	0.32	0.20	0.19
Chlorococcum humicola	0.302	0.300	0.257	0.221	0.180
Scenedesmus communis	0.75	0.61	0.53	0.44	0.32
Spirogyra fluviatilis	0.97	0.82	0.71	0.68	0.58
Chaetophora elegans	0.58	0.51	0.47	0.41	0.37
Klebsormidium flaccidum	0.41	0.37	0.33	0.30	0.27
Spirulina agilissima	0.50	0.47	0.45	0.41	0.39
Gomphonema parvalum	0.33	0.30	0.28	0.24	0.20
Nitzschia amphibia	0.32	0.29	0.26	0.24	0.19
Mougeotia viridis	0.43	0.39	0.36	0.33	0.30
Reference standard					
Ascorbic acid	2.02	2	1.88	0.94	0.67

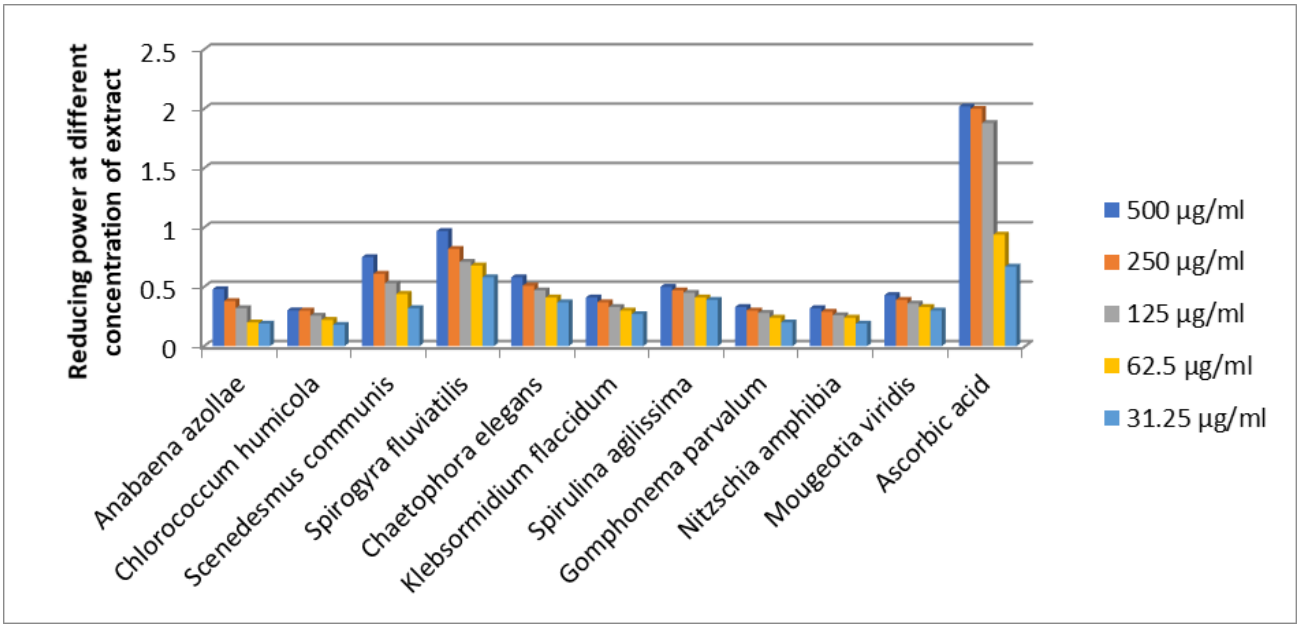

Fig 3: Reducing power of algal species at different concentration of extract compared with that of the reference standard (ascorbic acid) at different concentration.

DISCUSSION

The present comparative and investigative study of the antioxidant activities under *in-vitro* conditions of 10 species of algae belonging to different classes collected from several disturbed sites revealed that all the species investigated do possessed different levels of antioxidants activities. *Spirogyra fluviatilis* had been found to have the maximum phenolic contents and better antioxidants activity in this study which is supported by earlier reports which indicated that phenolics content is closely correlated to the antioxidative activities of algae (Demirel *et al.* 2011; Siva Kumar and Rajagopal, 2011). Moreover, *Nitzschia amphibia* had been found to have the minimum phenolic contents and least antioxidants activity which further supported earlier findings. This is also true for the remaining 8 algal species under observations with minor differences. By preventing radical formation and chelating heavy metal ions, phenolic compounds can therefore act as antioxidants (Al-Azzawie and Mohamed Saiel, 2006). In addition to phenolics, flavonoids content has also been known to play a significant role in determining the antioxidative activities of algae (Pietta, 2000; Rice-Evans *et al.*1996). Earlier reports indicated that flavonoids, the largest group of phenolic compounds (which include anthocyanidins, catechins, flavones, flavonols, isoflavonoids and proanthocyanidins) contains a diverse spectrum of biological and chemical activities including free radical scavenging properties and antioxidant activities (Kahkonen *et al.* 1999; Ndhlala *et al.* 2007). In addition to phenolic and flavonoid compounds, vitamins and minerals which may be present in these microalgae play a significant role in their recorded antioxidant activities. The extraction of vitamins and minerals from natural sources has received tremendous attention in recent years because these compounds are associated with high antioxidant activity. Moreover, earlier reports suggested that natural sources of these compounds are more effective than synthetic ones (Kelman *et al.* 2012). From a biological point of view, algae therefore represent one of the most promising natural sources for new products which has huge potential in industrial application (Pulz and Gross, 2004). Due to their stable and balanced chemical compositions, algae can be used to enhance the effectiveness and nutritional values of animal feed and food products. In addition to the presence of antioxidant constituents, algae is also a good source of compounds with antibacterial, antifungal and antiviral activity (Christaki *et al.* 2011; Lee *et al.* 2013; Molina Grima *et al.* 2003,) Natural antioxidants derived from algae are useful bioactive compounds that play an important role in the prevention of various diseases which may be of bacterial, viral or fungal origin mainly by protecting the cells from oxidative stress. Synthetic products can also be substituted by more reliable, effective and safer products derived from algae.

CONCLUSION

This study concluded that different algal species exhibited different antioxidant activities although some are better candidates than others. Besides the well documented carotenoids, phenolics and flavonoids also play a significant contributory role towards the antioxidant properties of algae. From this study, it may also be concluded that filamentous algae like *Spirogyra fluviatilis, Chaetophora elegans, Klebsormidium flaccidum* etc. seem to possessed better antioxidant properties than their non-filamentous counterparts. Therefore, further researches are needed in developing fast *in-vitro* culturing methods especially for filamentous algae which hold great promises in the production of biological antioxidants compounds that could replace the synthetic ones. Since, water bodies are the primary habitats of algae, there is a need to conserve them owing to algae's usefulness in providing us with valuable compounds of diverse significance. Lastly, anthropogenic disturbances and environmental stress does not seem to stop nor hinder the ability of algae to exhibit antioxidant properties.

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