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EVALUATION OF ANTIOXIDANT ACTIVITIES OF ALGAE AND EFFECT OF SOLVENT EXTRACTION SYSTEM

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ABSTRACT: The present communication is mainly focused to study the antioxidant activities of the four different extracts (benzene, chloroform, acetone and methanol) of five algae including *Aphanothece pallida, Anabena variabilis, Aphanothece saxicola, Lyngbya major, Nitella flagelliformis*. The total phenolic content varied from 2.99±0.23 to 25.38±0.22 mg/g, 4.20±0.135 to 14.10±0.13 mg/g, 3.07±0.22 to 27.03±0.11 mg/g and 2.34±0.03 to 4.87±0.14 mg/g dry material in the benzene, chloroform, acetone and methanol extracts of the algae respectively. The flavonoid content and flavonol content were found to be highest in the chloroform extract of *Nitella flagelliformis* and the least amount of flavonoids present in the acetone extract of *Lyngbya major* (4.88 ± 0.14 mg/g). The 1, 1-diphenyl-2-picryl hydrazyl (DPPH) radical scavenging effect of the extracts were determined spectrophotometrically. The highest radical scavenging was observed in the acetone extract of *Nitella flagelliformis* with IC_{50} = 0.15±0.01 mg dry material. The greater amount of phenolic compounds leads to more potent radical scavenging effect as shown by the acetone extract of *Nitella flagelliformis*. The reducing power of the extracts of the plants were also evaluated as mg AAE (ascorbic acid equivalent)/g dry material. The chloroform extract of *Nitella flagelliformis* showed the highest reducing power 231.08±2.01 mg AAE/g dry material. The results indicate that these algae can be utilized as natural antioxidant in order to prevent free radical toxicity

INTRODUCTION: Antioxidants are the molecules which can safely interact with free radicals and terminate the oxidation of other molecules and can prevent or slow the oxidative damage in our body. Antioxidant compounds like phenolic acids, anthocyanins, proanthocyanidins, flavonoids, flavonols, tannins etc. scavenge free radicals such as peroxide, hydroperoxide or lipid peroxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases such as such as atherosclerosis, parkinson's disease, alzheimer's disease, stroke, arthritis, chronic inflammatory diseases, cancers, and other degenerative diseases [1].

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Antioxidant compounds decelerates the production of free radical species that are induced by transition metal reactions and inhibit series of free radical reactions [2]. There is a great demand throughout the world in finding new natural sources for antioxidants to prevent oxidative damage to living cells and to reduce the deterioration of food by as tea, wine, fruits, vegetables and spices are used from the ancient days.

Plant materials are the rich source of active constituents like flavonoids and phenolic compounds which are responsible for exerting multiple biological activities like antioxidant, free radical scavenging abilities, anti-inflammatory, and anti-carcinogenic [3]. The complete extraction of active components from plant materials are strongly dependant on the nature of solvents used and therefore the antioxidant activity of the plants

are invariably affected by the polarity of the solvents [4].

The Cyanobacteria (blue-green algae) are the Gram negative photosynthetic prokaryotes found in almost all the ecological habitats. Cyanobacteria produce a vast array of different biologically active compounds, some of which are expected to be used in drug development. The fact that some of the active components from cyanobacteria potentially have anticancer, antimicrobial, antiviral, anti-inflammatory, and other effects is being used for marketing purposes [5].

Cyanobacteria are prokaryotic organism contains a wide variety of antioxidant pigments than the plants and most algal source. The alcoholic extracts of different cyanobacterial isolates including *Oscillatoria salina Synechcococcus, Oscillatoria annae, Oscillatoria chlorina, Spirulina sabsalsa and Spirullina platensis* were screened for their antioxidant property. The extract of *S. platensis* has the potent anti oxidant activity [6].

Hence, this present study is aimed to evaluate the *in-vitro* antioxidant activities of the four different solvent extracts of five different algae like *Aphanothece pallida, Anabena variabilis, Aphanothece saxicola, Lyngbya major* and *Nitella flagelliformis* and finding of this study would enable us to promote the usage and consumption of algae as natural food products and in order to prevent degenerative diseases.

MATERIALS AND METHODS:

Plant materials:

The five algae e.g *Aphanothece pallida , Anabena variabilis, Aphanothece saxicola, Lyngbya major* and *Nitella flagelliformis* were collected from different ponds of Hooghly district, West Bengal and authenticated in our office. The voucher specimens were preserved in the Plant Chemistry department of our office. The five algae were shed-dried, pulverized and stored in an airtight container for further extraction.

Chemicals:

1,1-Diphenyl-2-picrylhydrazyl (DPPH), butylated hydroxytoluene (BHT), ascorbic acid, quercetin were purchased from Sigma Chemical Co. (St. Louis, MO, USA)., Folin-Ciocalteus's phenol reagent, gallic acid, potassium ferricyanide, aluminium chloride, $FeCl_3$ and sodium carbonate were from Merck Chemical Supplies (Damstadt, Germany). All the chemicals used including the solvents, were of analytical grade.

Extraction of plant material (Benzene, Chloroform, Acetone and Methanol extract) :

One gram of each plant material were extracted with 20 ml each of benzene, chloroform, acetone and methanol at ambient temperature, with agitation for 18 -24 h. The extracts were filtered and diluted to 50 ml and aliquot were analyzed for their total phenolic, flavonoid and flavonol content, reducing power and their free radical scavenging capacity.

Estimation of total phenolic content:

The Folin-Ciocalteu procedure was used to estimate the amount of total phenolic content in different plant extracts [7]. The plant extracts (20 - 100 µl) were taken into test tubes ; 1.0 ml of Folin-Ciocalteu reagent and 0.8 ml of sodium carbonate (7.5%) were added. The plant extracts and other reagents were mixed well in the test tubes and allowed to stand for 30 min. The absorbance was measured at 765 nm in UV-visible spectrophotometer (Hitachi U 2000 Japan). The total phenolic content was expressed as gallic acid equivalent (GAE) using the equation based on the calibration curve: $y = 0.013x$, $R^2 = 0.9901$, where y was the absorbance and x was the GAE in miligram per gram (mg g^{-1}) of extract.

Estimation of total flavonoid content:

Total flavonoids were estimated using the method of Ordonez *et al*., 2006 [8]. The plant extracts (0.5 ml) were taken in a test tube and 0.5 ml of 2% aluminium chloride in ethanol solution was added in each tube allowed to stand for one hour at room temperature. The absorbance was measured at 420 nm in a UV-visible spectrophotometer (Hitachi U 2000 Japan). A yellow color indicated the presence of flavonoids. The total flavonoid contents were calculated as rutin (mg/g) equivalent using the equation based on the calibration curve: $y = 0.0182x - 0.0222$, $R^2 = 0.9981$, where y was the absorbance and x was the rutin equivalent in miligram per gram (mg g^{-1}) of extract.

Estimation of total flavonol content: The amount of total flavonols present in the tested plant extracts were estimated using the method of Kumaran and Karunakaran 2006 [9]. The tested extracts (2.0 ml), equal volume of 2% aluminium chloride in ethanol and 3.0 ml (50 g/L) sodium acetate solutions were taken in a test tube and allowed to stand for 2.5h at 20°C. The absorbance of the solution was read at 440 nm in UV-visible spectrophotometer (Hitachi U 2000 Japan). The total flavonol content in the extracts were calculated as quercetin equivalent (mg/g) using the equation based on the calibration curve: $y = 0.0049x + 0.0047$, $R^2 = 0.9984$, where y was the absorbance and x was the quercetin equivalent in miligram per gram (mg g^{-1}) of extract.

Measurement of reducing power:
The reducing power of the extracts was determined according to the method of Oyaizu 1986 [10]. The tested extracts (100 µl) were mixed with phosphate buffer (2.5 ml, 0.2 M, pH 6.6) and 1% potassium ferricyanide (2.5 ml). The mixtures were incubated at 50°C for 20 min. and 2.5 ml 10% trichloroacetic acid were added to the mixture, which was then centrifuged at 3000 rpm for 10 min. The upper layer of the solution (2.5 ml) was mixed with 2.5 ml distilled water and a 0.5 ml freshly prepared ferric chloride solution (0.1%).

The absorbance was measured at 700 nm in a UV-visible spectrophotometer (Hitachi U 2000 Japan). The reducing power was expressed in ascorbic acid equivalent (AAE) using the equation based on the calibration curve: $y = 0.0023x - 0.0063$, $R^2 = 0.9918$, where y was the absorbance and x was ascorbic acid equivalent in miligram per gram (mg g^{-1}) of extract.

Determination of free radical scavenging activity:
The free radical scavenging activity of the plant extracts and butylated hydroxyl toluene (BHT) as positive control was determined using the stable radical DPPH (1,1-diphenyl-2-picrylhydrazyl) [11]. The plant extracts (20 - 100 µl) were placed in the test tubes and 3.9 ml of freshly prepared DPPH solution (25 mg L^{-1}) in methanol was added in each test tube and mixed and kept for 30 min in dark. The absorbance was measured at 517 nm in a UV-visible spectrophotometer (Hitachi U 2000 Japan). The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenged (\%)} = \{(Ac - At)/Ac\} \times 100$$

Where Ac is the absorbance of the control reaction and At is the absorbance in presence of the sample of the extracts. The antioxidant activity of the extract was expressed as IC_{50}. The IC_{50} value was defined as the concentration in mg of dry material per ml (mg ml^{-1}) that inhibits the formation of DPPH radicals by 50%. Each value was determined from regression equation.

Values are presented as mean ± standard error mean of three replicates. The total phenolic content, flavonoid content, flavonol content, reducing power and radical scavenging activities of each plant material were calculated by using Linear Regression analysis.

RESULTS AND DISCUSSION:
The antioxidant activities of some selected algae were carried out by employing different free radical generating system. The antioxidant activity and the extraction of active constituents from algae were dependant on the nature of the solvents. Therefore, solvents with different polarities (benzene, chloroform, acetone and methanol) were used for the extraction of active compounds from algae.

Total phenol, flavonoid and flavonol content in the different solvent extracts of algae:
The total phenolic content of five algae were evaluated as gallic acid equivalents (GAE) in miligram per gram (mg g^{-1}) of extract.

The screening of the four different solvent (benzene, chloroform, acetone and methanol) extracts of five algae revealed that there was a wide variation in the amount of total phenolics ranging from 2.34±0.03 to 27.03±0.11 mg GAE/g dry material **(Table 1)**

The highest amount of phenolic content was found in the acetone extract of *N. flagelliformis* (27.03±0.11 mg GAE/g dry material), while least amount was observed in the methanol extract of *L. major* (2.34±0.03 GAE).

TABLE 1: TOTAL PHENOLIC CONTENT IN THE ALGAE USING DIFFERENT SOLVENT EXTRACTS

Sl No	Name of the plant	Total phenolic content (GAE mg / g dry material)			
		Benzene	Chloroform	Acetone	Methanol
1	*Aphanothece pallida*	2.99±0.23	5.38±0.14	3.07±0.22	4.87±0.14
2	*Anabena variabilis*	5.00±0.22	4.35±0.12	7.69±0.44	3.63±0.06
3	*Aphanothece saxicola*	6.15±0.11	9.74±0.33	8.37±0.22	2.36±0.07
4	*Lyngbya major*	15.89±1.47	4.20±0.135	6.30±0.08	2.34±0.03
5	*Nitella flagelliformis*	25.38±0.22	14.10±0.13	27.03±0.11	4.39±0.04

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM

The benzene extract of *N. flagelliformis*, *L. major*, the chloroform extract of *N. flagelliformis* and *A. saxicola* were also found to contain a very good amount of phenolic compounds.

The flavonoid contents of the extracts in terms of rutin equivalent were between 4.88± 0.14 to 527.30±0.04 mg/g dry material **(Table 2).**

TABLE 2: TOTAL FLAVONOID CONTENT IN THE ALGAE USING DIFFERENT SOLVENT EXTRACTS

Sl No	Name of the plant	Total flavonoid content (Rutin equivalent mg / g dry material)			
		Benzene	Chloroform	Acetone	Methanol
1	*Aphanothece pallida*	7.97±0.22	8.46±0.10	10.86±0.24	5.88±0.20
2	*Anabena variabilis*	14.98±0.24	11.38±0.12	22.45±0.48	10.35±0.43
3	*Aphanothece saxicola*	5.29±0.04	15.43±0.47	13.77±0.21	10.46±0.25
4	*Lyngbya major*	8.57±0.31	7.60±0.12	4.88±0.14	6.64±0.02
5	*Nitella flagelliformis*	141. 26±1.56	527.30±0.04	123.09±1.01	150.62±0.03

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM

The highest amount of flavonoid content was observed in the chloroform extract of *N. flagelliformis* (527.30 ± 0.04 mg/g). A very good amount of flavonoid was also found in the benzene, acetone and methanol extract of this plant. The moderate amount of flavonoid was found in the acetone extract of *A. variabilis* , *A. saxicola* and *A. pallida*.

In case of flavonol, the highest amount was observed in the chloroform extract of *N. flagelliformis* (186.69±1.39 mg/g) followed by the benzene and methanol extract of the same plant (132.27±1.79 mg/g and 98.94±1.11 mg/g respectively). A very good amount of flavonol was also found in the different extracts of *A. variabilis* , *A. saxicola* and *L. major* **(Table 3).**

TABLE 3: TOTAL FLAVONOL CONTENT IN THE ALGAE USING DIFFERENT SOLVENT EXTRACTS

Sl No	Name of the plant	Total flavonol content (Quercetin equivalent mg / g dry material)			
		Benzene	Chloroform	Acetone	Methanol
1	*Aphanothece pallida*	11.08±0.78	17.89±0.78	8.12±0.89	27.06±0.47
2	*Anabena variabilis*	51.66±2.38	37.22±0.44	86.32±2.35	43.91±0.68
3	*Aphanothece saxicola*	42.15±0.44	91.12±1.76	39.65±0.78	48.29±4.21
4	*Lyngbya major*	59.82±2.90	41.75±0.47	19.71±0.47	27.65±0.07
5	*Nitella flagelliformis*	132.27±1.79	186.69±1.39	93.97±6.17	98.94±1.11

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM

It has been established that phenolic compounds are the major plant compounds with antioxidant activity and this activity is due to their redox properties. Phenolic compounds are a class of antioxidant agents which can adsorb and neutralize the free radicals [12]. Flavonoids and flavonols are regarded as one of the most widespread groups of natural constituents found in the plants. It has been recognized that both flavonoids and flavonols show antioxidant activity through scavenging or chelating process [13]. The results strongly suggest that phenolics are important components of these plants. The other phenolic compounds such as flavonoids, flavonols, which contain hydroxyls are responsible for the radical scavenging effect in the plants. According to our study, the high content of these phenolic compounds in the different extracts of *N. flagelliformis*, *A. variabilis* , *A. saxicola* and

L. major can explain their high radical scavenging activity and reducing power.

Reducing power of the different solvent extracts of algae:

The reducing power of the algae under investigation were evaluated as ascorbic acid equivalent (AAE mg/g) as shown in **Table 4**).

TABLE 4: REDUCING POWER (ASCORBIC ACID EQUIVALENT) IN THE ALGAE USING DIIFERENT SOLVENT EXTRACTS

Sl No	Name of the plant	Reducing power (Ascorbic acid equivalent mg / g dry material)			
		Benzene	Chloroform	Acetone	Methanol
1	*Aphanothece pallida*	27.48±2.10	58.40±1.67	63.69±2.51	70.11±0.76
2	*Anabena variabilis*	43.40±1.91	34.38±0.95	62.17±2.51	54.63±0.74
3	*Aphanothece saxicola*	51.41±0.62	170.21±2.51	49.22±2.10	34.76±1.38
4	*Lyngbya major*	71.66±1.91	44.31±2.26	28.66±1.53	19.30±0.42
5	*Nitella flagelliformis*	133.98±2.61	231.08±2.01	123.78±0.65	22.38±0.58

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM

The highest reducing power was exhibited by the chloroform extract of *N. flagelliformis* (231.08 ± 2.01 mg/g AAE) which is also high in flavonoid content (527.30±0.04 mg /g dry material) and flavonol content (186.69 ± 1.39 mg /g dry material) and the methanol extract of *L. major* showed lowest activity in terms of ascorbic acid equivalent (19.30±0.42 mg/g). The different extracts of *A. variabilis*, *A. saxicola*, *A. pallida* and *L. major* showed potent reducing activity. In this assay, the presence of antioxidants in the extracts reduced Fe^{+3}/ferricyanide complex to the ferrous form. This reducing capacity of the extracts may serve as an indicator of potential antioxidant activities through the action of breaking the free radical chain by donating hydrogen atom [14].

DPPH radical scavenging activity of the different solvent extracts of algae:

The evaluation of anti-radical properties of five algae were performed by DPPH radical scavenging assay. The 50% inhibition of DPPH radical (IC_{50}) by the different plant materials were determined **(Table 5)**, a lower value would reflect greater antioxidant activity of the sample.

DPPH stable free radical method is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extracts [15]. The antioxidant effect is proportional to the disappearance of the purple colour of DPPH in test samples. Thus antioxidant molecules can quench DPPH free radicals by providing hydrogen atom or by electron donation and a colorless stable molecule 2, 2- diphenyl-1-hydrazine is formed and as a result of which the absorbance (at 517 nm) of the solution is decreased. Hence the more potent antioxidant, more decrease in absorbance is seen and consequently the IC_{50} value will be minimum.

In the present study the highest radical scavenging activity was shown by the acetone extract of *N. flagelliformis* (IC_{50} = 0.15±0.01 mg dry material), whereas the methanol extract of *A. saxicola* showed lowest activity (IC_{50} = 1.26±0.08 mg dry material). Strong inhibition was also observed for the acetone extract of *A. variabilis*, *A. pallida* (IC_{50} = 0.16±0.01 mg dry material and 0.15±0.01 mg dry material respectively).

TABLE 5: FREE RADICAL SCAVENGING ABILITY OF THE BLUE GREEN ALGAE BY THE USE OF A STABLE DPPH RADICAL (ANTIOXIDANT ACTIVITY EXPRESSED AS IC_{50}) USING DIFFERENT SOLVENTS

Sl No	Name of the plant	Free radical scavenging ability IC_{50} mg / g dry material)			
		Benzene	Chloroform	Acetone	Methanol
1	*Aphanothece pallida*	0.97±0.11	0.59±0.03	0.19±0.01	0.47±0.01
2	*Anabena variabilis*	0.75±0.17	0.79±0.06	0.16±0.01	0.68±0.02
3	*Aphanothece saxicola*	0.58±0.01	0.33±0.01	0.44±0.01	1.26±0.08
4	*Lyngbya major*	0.27±0.01	0.78±0.01	0.69±0.01	1.18±0.01
5	*Nitella flagelliformis*	0.30±0.01	0.50±0.01	0.15±0.01	0.57±0.01

Each value in the table was obtained by calculating the average of three experiments and data are presented as Mean ± SEM

The high radical scavenging property of *N. flagelliformis* and moderate antioxidant properties of the different extracts of *A. pallida*, *A. variabilis*, and *L major* may be due to the hydroxyl groups existing in the phenolic compounds chemical structure that can provide the necessary component as a radical scavenger. The different extracts of all algae under investigation exhibited different extent of antioxidant activity and thus provide a valuable source of nutraceutical supplements. Depending on the values, some plants are more important than some others.

CONCLUSION: The result of present study showed that the benzene, chloroform, acetone and methanol extracts of *N. flagelliformis* which contain highest amount of phenolic compounds and appreciable amount of flavonoids and flavonols exhibited the greatest reducing power and also showed strong radical scavenging activity. The highest radical scavenging activity and very strong reducing power of the different extracts of *N. flagelliformis* may be due to the presence of a very good amount of total phenolics, flavonoids and flavonols contents in this plant. The radical scavenging activities of the selected algal extracts are still less effective than the commercial available synthetic like BHT. As the plant extracts are quite safe and the use of synthetic antioxidant has been limited because of their toxicity, therefore, these algae could be exploited as potential sources of natural antioxidants or as nutritional supplements.

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