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Antioxidant and Quantitative Estimation of Phenolic, Flavonoid and Carotenoids Contents of *Phormidium* Species

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Abstract: Cyanobacteria are more susceptible for oxidative stress and damage due to high contents of secondary metabolites. In this study, antioxidant activity of acetone and methanol extracts of *Phormidium* species was studied using *in vitro* assay. The antioxidant potential of the extracts was measured by free radical scavenging activity assay (DPPH method), Hydrogen peroxide radical scavenging activity and Ferric reducing antioxidant power assay. Total phenolic and flavonoid content was determined by using Gallic acid and Quercetin as a standard. In addition, total chlorophylls and carotenoids were also estimated. The results of the study showed that *Phormidium* sp. possesses significant antioxidant properties and may be considered as one of the natural sources for isolation of antioxidant compounds for pharmaceutical application.

Key words: *Phormidium* sp., Antioxidant activity, Phenolic compound and pigments.

Introduction

Antioxidants are naturally occurring or synthetic chemicals in variety of food that help to counter the detrimental effects of reaction oxygen species (ROS), free radicals which causes degenerative human diseases such as cancer, heart diseases and cerebrovascular diseases [23]. The search for novel antioxidants such as vitamins and phenol phytochemicals to prevent oxidative stress mediated diseases is significant since many diseases due to oxidative stress are developing resistance to routinely used antioxidants. In recent decades, Cyanobacteria, also known as blue-green algae with highly diverse group worldwide are proven as an excellent source for secondary metabolites. Secondary metabolites are quite diverse having different chemical structures. It includes steroids, terpenoids, alkaloids, polyketides, phenolic metabolites, carbohydrates, lipids and peptides and they can be classified on the basis of their biological function as vitamins, hormones, antibiotics, antioxidant, toxin and pheromones.

Most studies have been focused on microorganisms' especially cyanobacteria of fresh water, terrestrial and marine resources which still inadequate. Compounds with antioxidant, antibacterial, antiviral, algaecide, antifungal and Cytotoxic activities have been reported in these organisms are still not completely understood. Some few studies reported cyanobacteria to be a potential candidate to combat free radicals, which are harmful to our body and food systems [13]. The cyanobacteria species *Phormidium* belonging to family *Phormidiaceae*, is a photosynthetic prokaryote, widely found in various fresh water habitats. In the present study, efforts were made to reveal the total Phenolics and flavonoid contents and antioxidant activity in the isolates cyanobacteria by different mechanism such as Free radical scavenging assay (DPPH method), Hydrogen peroxide assay and Ferric reducing antioxidant power assay.

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Materials and methods

Isolation and maintenance of organisms

The blackish colour mats were collected in a sterilized polythene bag using sterile forceps from sewage drain, Siripur of Bhubaneswar, Odisha and transferred to Laboratory. The samples were initially washed with sterilized distilled water thoroughly to remove the impurities and subjected it to shade dry. Isolation and purification of axenic culture were followed using BG -11 media as described by Rippka *et al*., [14]. Identification of the axenic unialgal species was done by following monographs as described by Desikachary [1].

Preparation of crude extract

After 20 days of incubation the algal mass was collected by filtration through Buchner funnel with Whatman No. 1 filter paper and shade dried for 30 minutes in hot air oven and ground to fine powder with the help of glass homogenizer. 3 gms of the powdered samples of *Phormidium* species were extracted with methanol (210 ml) and acetone (210 ml) using soxhlet apparatus for 72 hours maintaining at temperature 60°C. The extract was collect in air tight container and stored at 4°C for further estimation.

Quantitative analysis of antioxidative compounds

Estimation of total phenolic contents

Total phenolic contents of methanol and acetone extracts were determined by the Folin - Ciocalteu method as described by Slinkard and Singleton [19].

Estimation of total flavonoid contents

Total flavonoid contents of the culture were determined by Aluminum chloride method as described by Zhishen *et al.*,[24].

Antioxidant assay methods

Free radical scavenging activity (DPPH method)

The effect of extracts on DPPH radical scavenging activity was performed according to the method of Shimada *et al*.,[17]. The methanol and acetone extract of species was diluted to make volume up to 25, 50, 100, 150, 200, 250, 300 and 350 µg/ml dilutions. Two ml of each dilution was mixed with 1ml of DPPH solution (0.2 mM/ml in methanol) and mixed thoroughly. The mixture was incubated in dark at 20°C for 40mins. Absorbance was measured at 517 nm using UV-Vis spectrophotometer with methanol and acetone as blank. Each experiment was performed in triplicates at each concentration. The percentage of the DPPH radical scavenging by the extracts was calculated according to the following formula:

$$\% \text{ Inhibition of DPPH radical scavenging} = [(A_c - A_t)/A_c] \times 100$$

(Where; A_c - absorbance of the control (DPPH) A_t - absorbance of test sample)

Hydrogen peroxide scavenging activity

Scavenging strength of extract was determined by the method of Ruch *et al*.,[16]. A solution of H_2O_2 was prepared in phosphate buffer (P^H=7.4). Reaction mixture contained 10 mM of H_2O_2 at different concentration of test samples and the absorbance values were measured at 10 mins and after 60 mins at 240 nm. Ascorbic acid was taken as standard. The percentage of hydrogen peroxide scavenging was calculated as follows:

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

(Where, A_i was the absorbance of control and A_t was the absorbance of test sample)

Ferric reducing antioxidant power assay

Reducing power of different crude extract was determined by Oyaizu [10]. Briefly 1.0 ml of different solvent extract containing different concentration of samples were mixed with 2.5 ml of phosphate buffer (0.2M, P^H 6.6) and 2.5 ml of potassium ferric cyanide (1 %). Reaction mixture was kept in a water bath at 50°C for 20 mins. After incubation, 2.5 ml of trichloroacetic acid (10 % TCA) was added and centrifuged at 650 rpm for 10 mins. From the upper layer, 2.5 ml solution was mixed with 2.5 ml distilled water and 0.5 ml of $FeCl_3$ (0.1 %). Absorbance was measured at 700 nm. Ferric reducing antioxidant power is expressed as the number of equivalents of ascorbic acid.

Estimation of pigments

The estimation of pigments i. e Chlorophyll-*a* and Carotenoids were estimated according to the method of McKinney [8] and Jenssen [5].

Statistical analysis

All tests were conducted in triplicate. Data are reported as means ± standard deviation (SD).

Results and discussion

Algae are worldwide in distribution in different habitats varying from terrestrial to aquatic. They have gained a lot of attention in recent years due to their ability to synthesize novel biologically active compounds [4], which offer a wide range of therapeutic possibilities such as antibacterial, antifungal, antiviral, anti-inflammatory, antioxidant, cancer and cardiovascular as chronic disease [21]. Recent researchers have been interested in the use of cyanobacteria as novel antioxidants source to prevent reactive oxygen species mediated disease. Thus the present study, were carried out to investigate in *vitro* antioxidant activities of aquatic cyanobacteria isolated from sewage drain water, Siripur, Bhubaneswar, Odisha. The culture of Cyanobacteria was purified on BG-11 medium and the microscopic structure was observed. The microscopic observations showed the presence of vegetative cell filaments, thin firm sheath and apical pointed tip that confirms the presence of *Phormidium* species (Fig. 1).

Antioxidant compounds

In the current study, total phenolic content of extracts was determined with the folin-ciocalteu reagent in terms of Gallic acid, used as a standard compound for both acetone and methanol solvent and the total phenols were expressed as mg/gm Gallic acid equivalent using the standards curve equation: $Y = 0.760 x + 0.050$, $R^2 = 0.906$. Where Y is the absorbance at 760 nm and x is the total phenolic content of in the *Phormidium* extract expressed in mg/g. The cyanobacteria isolates showed total phenolic contents 22.55 ± 1.96 mg/g and 10.19 ± 2.03 mg/g dry weight in the extracts of acetone and methanol respectively as shown in Table 1. Nagasathya and Thajuddin [9], analyzed the phenolic content in different hypersaline cyanobacteria isolates namely *Phormidium tennue* (KMD 33), *Phormidium fragile* and *Phormidium angustissimum* and indicate that *Phormidium tennue* (KMD 33) showed potent antioxidant property. The amount of total flavonoid was determined by Aluminum chloride reagents in terms of Quercetin, used as a standard compound and the total flavonoid were expressed as mg/g Quercetin equivalent using the standard curve equation: $Y = 0.99 x + 0.047$, $R^2 = 0.996$, Where Y is the absorbance at 510 nm and x is the total flavonoid content in the extract of *Phormidium* species expressed in mg/g. The total flavonoid content in acetone and methanol extracts was 87.9 ± 2.06 mg/g and 1.89 ± 1.95 mg/g dry weight as shown in Table 1. Acetone extracts exhibited highest amount of phenolic and flavonoid

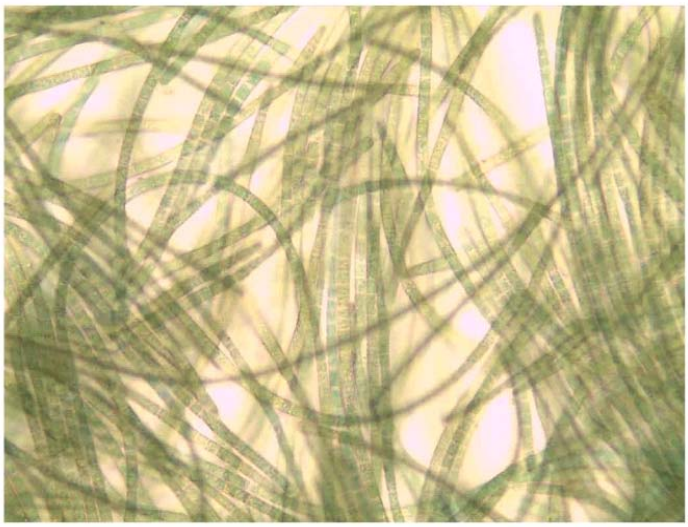

Fig. 1. Microscopic photographs of *Phormidium* sp. isolated from sewage drain water, Siripur, Bhubaneswar, Odisha

Table 1. Total Phenolic and Flavonoid contents in isolated *Phormidium* sp.

Organism	Organic solvent	Total Phenol content (mg/ml DW)	Total Flavonoid content (mg/ml DW)
Phormidium species	Acetone	22.55 ± 1.96	87. 9 ± 2.06
	Methanol	10.19 ± 2.03	1.89 ± 1.95

Results were expressed as mean ± Standard deviation

contents as compared to methanol extract.

Phenolic compounds are the major chemical substances that served as important antioxidants because of their ability to donate a hydrogen atom or an electron in order to form stable radical intermediates as reported by Roya and Fatemeh [15]. These values were compared with the values reported by Rai and Rajashekhar [12] for phenolic and flavonoid content of *Phormidium corium* (5.41mg GAE/g and 0.74 mg QE/g), and *Phormidium tenue* (9.22 mg GAE/g and 1.44 mg QE/g) respectively. The data with present study showed great difference in the quantities estimated i, e presence of phenolic and flavonoid contents in *Phormidium* sp. compared to *Phormidium corium* and *Phormidium tennue*. The findings of this estimation exhibited the greatest antioxidant activity and thus can be used to explore new drugs.

Antioxidant assay

DPPH radical scavenging activity

The antioxidant potential of acetone and methanol extracts were measured by DPPH radical scavenging activity, Hydrogen Peroxide activity and Ferric reducing antioxidant power activity compared with standard antioxidant Ascorbic acid. The results were expressed as percentage inhibition of ascorbic acid and reported in Figure 2 - 4. Acetone extract resulted in high DPPH radical scavenging activity with IC_{50} value of 60.4 % as compared to methanol (55.4 %) and the scavenging activity was found to be increasing with dose (Fig. 2). Pumas *et al*., [11] reported the DPPH radical scavenging potential of the *Phormidium* sp. PD40 -1. The results showed that the methanol extract of the *Phormidium* sp. PD40-1 resulted into 6.61mg GAE/g dry weight DPPH radical scavenging activity. Earlier, the methanol extract of *Anabaena* PCC 7119 and its fractions were reported to possess efficient radical scavenging activity. The effect of antioxidants on DPPH is thought to be due to their proton donating ability and could serve as free radical inhibitors or scavengers, acting possibly as primary antioxidants reported by Mukund *et al*., [7]. This activity may be due to phenolic compounds and flavonoid present in the extract as also indicated by Velioglu *et al*., [22].

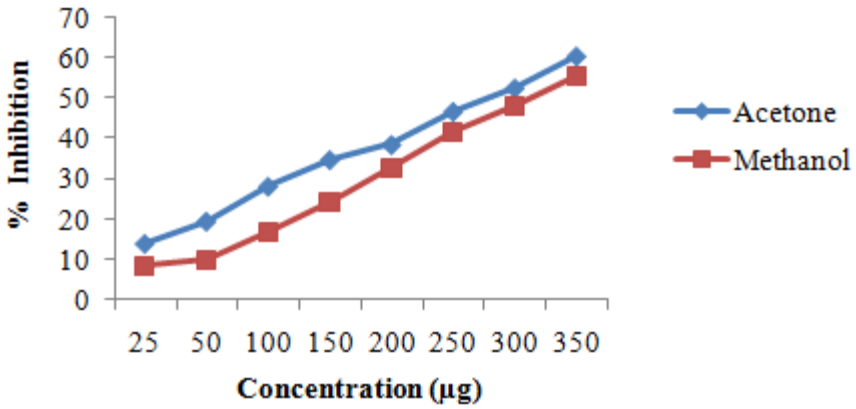

Fig. 2. DPPH radical scavenging activity of the varying concentrations (µg/ml) of acetone and methanol extract of *Phormidium* sp. Data is expressed in percentage (n = 3)

Hydrogen peroxide scavenging activity

From figure 3, Acetone extract of *Phormidium* sp. showed moderately good Hydrogen peroxide scavenging activity between 50 and 400 µg/ml. At a concentration of 350 µg/ml, the scavenging activity of the acetone extract of *Phormidium* sp. was 77.79 %. The percentages of inhibitions were increased with increasing concentration of the extracts (Fig. 3). This result indicates that, scavenging activity of extracts may be attributed to their Phenolics, which can donate electrons to Hydrogen Peroxide, thus neutralizing it to water. Both the extracts had the capacity of scavenging Hydrogen Peroxide in a concentration dependent manner. Hydrogen peroxide itself is not very reactive, but can sometimes may be toxic or damage to cell because it may give rise to hydroxyl radical in the cells as reported by Shirwaikar, *et al.*,[18].

Ferric reducing antioxidant power activity

On the other hand, reducing power of the extracts displayed the increased trend with increasing concentration as indicated by the increases in the absorbance of reaction mixture. Acetone extract of *Phormidium* sp. showed good ferric reducing antioxidant power than the methanol extract (Fig. 4). In this study it was noticed that acetone extract showed higher antioxidant potentiality when compared to methanol extracts. Earlier, the methanol extract of *Phormidium tenue* were reported to possess significant reducing power capacity reported by Rai and Rajashekhar [12] with value 19.06 µmol GAE/g. Thus reducing

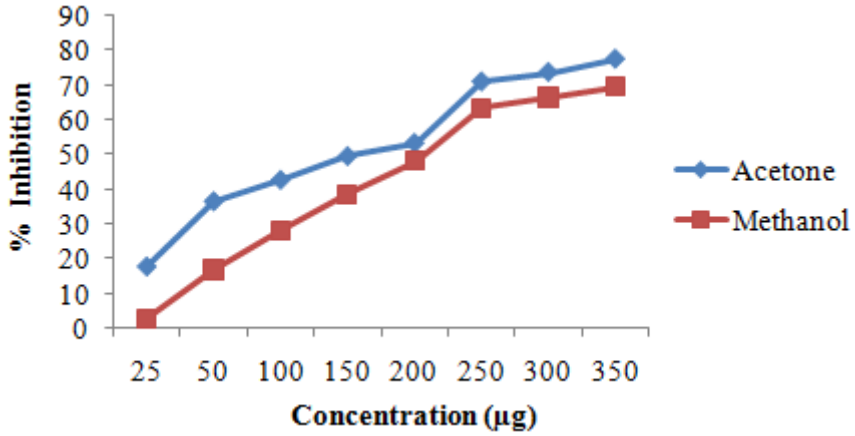

Fig. 3. Hydrogen peroxide scavenging activity of the varying concentrations (µg/ml) of acetone and methanol extracts of *Phormidium* sp. Data is expressed in percentage (n = 3)

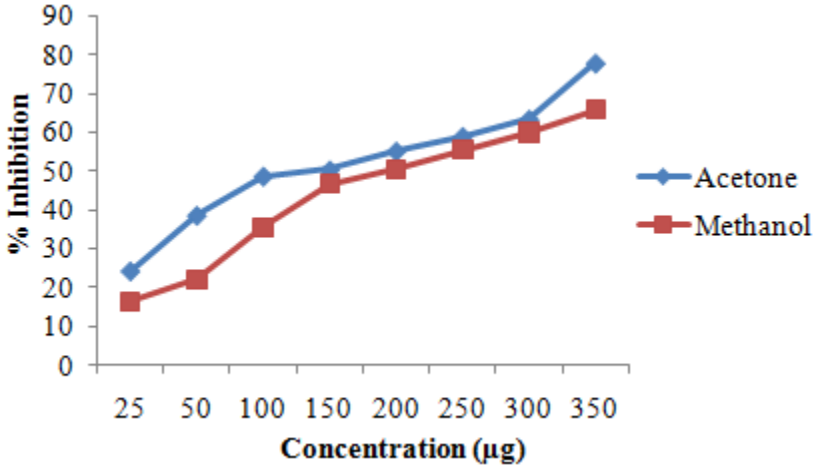

Fig. 4. Ferric reducing antioxidant power activity of the varying concentrations (µg/ml) of acetone and methanol extracts of *Phormidium* sp. Data is expressed in percentage (n = 3)

power capacity of a compound may serve as a significant indicator of potential antioxidant activity. Reducing power is an ability to reduce or give electrons to free radicals and change them into the stabilized form, which can halt the free radical chain reaction reported by Oyaizu [10].

The radical scavenging activity, hydrogen peroxide activity and reducing power capacity were proved that the crude extracts of cyanobacteria contained both the electron and hydrogen atom donating ability. The extracts containing antioxidants such as phenolic compounds are able to donate hydrogen atom to the free radical which may create as an obstructin towards damaging of cells. The overall study has shown that antioxidant potential of *Phormi-dium* species was found to be statically significant.

Quantitative estimation of pigments

Dufossé *et al*., [2] reported that phytoplankton is an excellent source of natural pigments. Johnson and Schroeder [6] reported natural pigments plays role in protection against photo-oxidative damage. In the present study, the total chlorophyll-*a* pigment was found to be high in acetone extract i, e. 22.85 µg/ml as compared to methanol extracts (12.02 µg/ml). Similar results to be obtained in the total carotenoids (1.48 µg/g and 0.73 µg/g) respectively as shown in Table 2. The total chlorophyll-*a* and carotenoids content of a *Phormidium corium* and *Phormidium tenue* reported by Rai and Raja-shkhar [12] were lower than the values obtains in present study and the values was 1.08 mg/g DW and 3.95 mg/g DW. The chlorophyll is a primary photosynthetic pigment and they produce various secondary pigments, such as PBPs and a wide range of carotenoids. Several studies have demonstrated that carotenoids contribute significantly to the total antioxidant capacity of phyto-plankton [3,20].

Conclusion

The present study represented that *Phormidium* sp. isolates contain potent antioxidants activity with the notable presence of polyphenols like phenolics, flavonoids, chlorophyll and carotenoids, which exhibit antioxidant activities by different mechanisms such as scavenging and reducing. The acetone extracts of *Phormidium* species exhibits higher antioxidants potentiality than the methanol extracts. On the other hand, pigments too contribute significantly to the total antioxidant of the cyanobacteria. Further scientific investigation on these active molecules would results in biotechnological exploitation of Cyanobacterial species.

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Table 2. Total Chlorophyll and β-Carotene contents in isolated *Phormidium* sp.

Organism	Organic solvent	Total Chlorophyll content (mg/ml DW)	Total β-Carotene content (mg/ml DW)
Phormidium species	Acetone	22.85 ± 0.96	1. 48 ± 0.02
	Methanol	12.02 ± 1.03	0.73 ± 1.95

Results were expressed as mean ± Standard deviation

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