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# ANTIOXIDANT ACTIVITY IN THE FOUR SPECIES OF CYANOBACTERIA ISOLATED FROM A SULFUR SPRING IN THE WESTERN GHATS OF KARNATAKA

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## ABSTRACT

In the present study, efforts were made to reveal the total phenolics and flavanoid contents and antioxidant activity in the four cyanobacterial species viz., *Phormidium fragile*, *Lyngbya limnetica*, *Scytonema bohnerii* and *Calothrix fusca* isolated from a sulfur spring in the Western Ghats of Karnataka. The cultures were maintained in $BG_{11}$ medium. *In vitro* screening for antioxidant activity of ethanol and methanol extracts of these cultures was determined by total antioxidant capacity by phosphomolybdenum, DPPH free radical scavenging and ferric ion reducing assays. The study indicates the significant antioxidant potential in *Lyngbya limnetica* ($P<0.01$) followed by *Scytonema bohnerii* ($P<0.05$) and least activity was observed in *Calothrix fusca* ($P>0.05$). Therefore, mass cultivation of such strains for the production of valuable bioproducts may be a good strategy for future use. The study has revealed the utilization of certain strains of cyanobacteria for the production of biologically active compounds particularly, antioxidants.

**KEY WORDS : Cyanobacteria, Sulfur spring, Antioxidants, DPPH, Western Ghats, Karnataka.**

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## INTRODUCTION

Cyanobacteria are the potential source for food and pharmaceuticals.[1,2] Phytonutrients and pigments present in the cyanobacteria act as antioxidants which facilitate the formation of the body's defense against free radical damage to cells. Reactive oxygen species (ROS) are often generated either as byproducts of biological reactions.[3] Reactive oxygen species and free radicals formed during oxidation have been reported to contribute for diseases like cancer, diabetes, cardiovascular diseases and ageing.[4] Antioxidants have the ability to protect the body from oxidative damage by scavenging the free radicals and inhibiting peroxidation and other radical mediated processes[5]. In recent years, significant attention was given towards exploring plant-based natural antioxidants, especially the phenolics and tocopherols.[6,7,8] Natural antioxidants have the ability to neutralize reactive oxygen species which are implicated in the treatment of certain diseases.[9,10] There is a great demand throughout the world in finding new natural sources for antioxidants to prevent oxidative damage to living cells. Cyanobacteria have a highly evolved antioxidant system that catalyzes the harmful oxy radicals produced during photosynthesis.[11] Carotenoids of cyanobacteria take part in photosynthesis and possess potent antioxidant activity. Screening of cyanobacteria for antioxidants and other pharmacologically active compounds has received increasing attention as a potential source for new drugs. Recently, antioxidant property of *Phormidium tenue* (KMD 33) and *Oscillatoria annae* has been reported.[12,13] Cyanobacteria probably are the least explored group as far as their antioxidant properties are concerned.[14,15] In view of all these aspects, it is worth to study the antioxidant property of cyanobacteria from certain extreme environments and also to know the relationship of total flavonoid and phenol contents with antioxidant activity. Hence the present study was carried out to determine the total phenolics, flavanoid content and antioxidant activity in the four species isolated from a sulfur spring near Uppinangady in the Western Ghats of Karnataka.

## MATERIALS AND METHODS

### *Study area*

The sulfur spring is located at Panekal, about 22 km from Uppinangady (12°54' N, 75°17.5'S) in Dakshina Kannada District. It originates from crevices of rocks, and forms a small pond and later flows through paddy fields for about 0.5 km before joining river Nethravathi. The water temperature was 38$^{o}$C and water pH ranged between 8.8-9.2 throughout the year. The surrounding vegetation was sparse consisting of few trees species.

### *Identification of cyanobacteria*

Identification was done using taxonomical keys according to Desikachary (1959)[16] and Anagnostidis and Komarek (1998).[17] The species isolated from sulfur spring were cultured in the laboratory using BG-11 growth medium with nitrate.[18]

### *Preparation of extracts*

The cultures were harvested at the stationary phase of growth (25 days). Spent media and biomass were separated by filtration. Biomass of cyanobacterial materials were lyophilized before the extraction procedure. The ethanol and methanol extracts were prepared by following the methods described by Kaushik and Chauhan (2009).[19]

### *Preliminary Phytochemical Screening*

To determine the broad classes of phytoconstituents present in cyanobacteria, samples of the ethanol and methanol extracts of all the four cyanobacteria were analyzed for the presence of tannins, flavonoids, saponins, steroids, terpinoids, glycosides,

anthraquinone glycosides, alkaloids, carotenoids, phycocyanins and cumarins of basic phytochemicals as described by Trease and Evans, (1984)[20] and Wagner et al., (1984).[21]

### *Determination of total phenolics*

Total phenolics were determined by Folin Ciocalteu reagent method.[22] A dilute extract of each cyanobacterial extract (0.5 ml of 1:10 g $ml^{-1}$) and tannic acid (standard phenolic compound) was mixed with Folin Ciocalteu reagent and aqueous $Na_2CO_3$ (4 ml, 1 M). The mixtures were allowed to stand for 15 min and the total phenolics were determined by spectrophotometer at 765 nm. The standard curve was prepared using 0, 50, 100, 150, 200, 250 mg $L^{-1}$ solutions of tannic acid in methanol: water (50:50, v/v). Total phenol values are expressed in terms of tannic acid equivalent (mg $g^{-1}$ of dry mass), which is a common reference compound.

### *Determination of total flavonoids*

Aluminum chloride spectrophotometric method of Chang et al., (2002)[23] was used for flavonoids determination. Each of the cyanobacterial extracts (0.5 ml of 1:10 g ml-1) in methanol was separately mixed with 1.5 ml of methanol, 0.1 ml of 10% aluminum chloride, 0.1 ml of 1 M potassium acetate and 2.8 ml of distilled water. It remained at room temperature for 30 min; the absorbance of the reaction mixture was measured at 415 nm using UV-Visible spectrophotometer (Systronics, Gujarat, India). The calibration curve was prepared by preparing quercetin solutions at concentrations 12.5 to 100 g $ml^{-1}$ in methanol.

### *Antioxidant activity*

#### *1. Evaluation of total antioxidant capacity*

This assay is based on the reduction of Mo (VI) to Mo (V) by the sample analyte and the subsequent formation of green phosphate/Mo (V) complex at an acidic pH.[24] Different concentrations of cyanobacterial extracts prepared in ethanol and methanol ranging from 10-500 µg/ml were pipetted out into a series of test tubes and combined with 1ml of reagent solution (0.6 M sulfuric acid, 28 mM sodium phosphate, and 4 mM ammonium molybdate). The tubes were capped and incubated in a water bath at 95°C for 90 min. After the whole set of samples were cooled to room temperature, the absorbance of the aqueous solution of each was measured at 695 nm against a blank. Tannic acid was used as standard antioxidant. The total antioxidant capacity was expressed as equivalents of tannic acid (µ moles /g of sample).

#### 2. *Free radical scavenging activity by DPPH method* [25]

Different concentrations (10µg, 100µg and 500µg) of extracts in ethanol/methanol and Butylated hydroxy anisole (BHA) were taken in different test tubes. The volume was adjusted to 500µl by adding respective solvent solution. Five milliliters of a 0.1 mM methanolic solution of 1, 1-diphenyl-2-picryl hydrazyl (DPPH) was added to these tubes and shaken vigorously. A control without the test compound, but with an equivalent amount of ethanol/methanol was maintained. The tubes were allowed to stand at room temperature for 20 min. The absorbance of the samples was measured at 517 nm.

Radical scavenging activity was calculated using the following formula:

$$\text{Free radical scavenging activity (\%)} = \frac{\text{control OD - sample OD}}{\text{control OD}} \times 100.$$

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### *3. Ferric reducing power assay*

Various concentrations of extract (10µg, 100µg and 500 µg) were mixed with 2.5 mL of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50ºC for 20 min. Next, 2.5 mL of 10% (w/v) trichloroacetic acid was added. 5 mL of above solution was mixed with 5 mL of distilled water and 1 mL of 0.1% of ferric chloride. The absorbance was measured spectrophotometrically at 700 nm. Butylated hydroxy anisole (BHA) was used as standard antioxidant.

### *Statistical Analysis*

Data were expressed as mean of the triplicate values ± standard deviation. The data was statistically analyzed by student's t test. P values < 0.05 were considered as significant.

## RESULTS AND DISCUSSION

### *Phytochemical Screening*

The preliminary phytochemical screening of cyanobacteria was carried out in order to determine the presence of various phytoconstituents in the four species of cyanobacteria (Table 1). The study has shown the presence of certain phytoconstituents viz., tanins, flavonoids, alkaloids, terpinoids, steroids, carotenoids, phycocyanins and phenolic compounds.

**Table 1**
***Preliminary screening of cyanobacterial species for the presence of certain phytoconstituents***

| Test | Species | | | |
|---|---|---|---|---|
| | Phormidium fragile | Lyngbya limnetica | Scytonema bohnerii | Calothrix fusca |
| Alkaloids | + | + | + | - |
| Saponins | - | - | - | - |
| Tannins | + | + | + | + |
| Phenolic compounds | + | + | + | + |
| Steroid/ Triterpines | + | + | + | - |
| Flavonoids | + | + | + | + |
| Anthraquinone Glycosides | - | - | - | - |
| Carotenoids | + | + | + | + |
| Phycocyanins | + | + | + | + |
| Coumarin | - | - | - | - |

***+ : present - : absent***

The total phenolic content in the ethanol and methanolic extracts of cyanobacteria is shown in Fig.1. It was noticed that maximum quantity of phenolics was in *Lyngbya limnetica* (3.5%) followed by *Calothrix fusca* (2.6%), whereas *Scytonema bohnerii* (1.2%) showed the least

quantity in both extracts. Similarly, total flavonoids in the four cyanobacterial species is shown in Fig.2. The study revealed that high content of flavonoids was in *Lyngbya limnetica* (2.38%) followed by *Phormidium fragile* (1.4%) and it was minimum in *Scytonema bohnerii* (0.25%) in both the extracts.

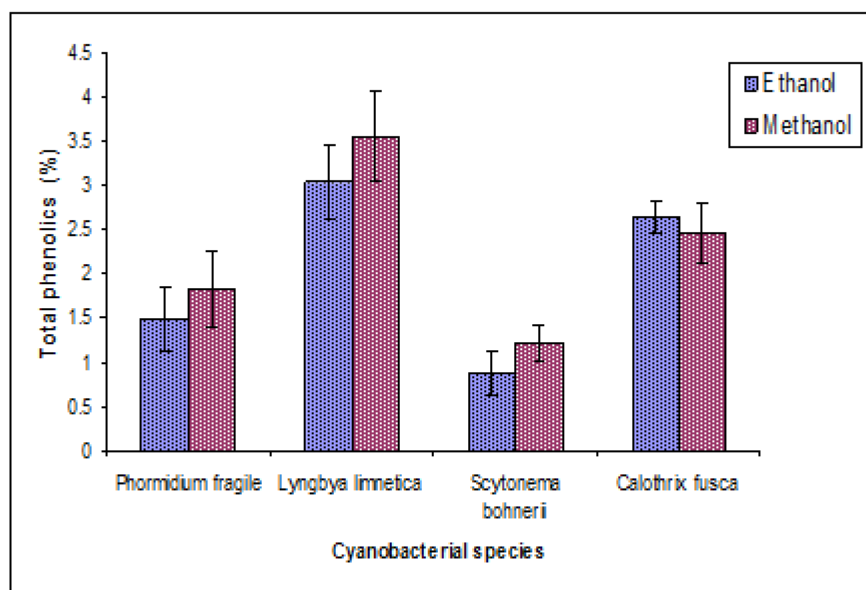


**Figure 1**
**Total phenolics contents respectively in the ethanol and methanol extracts (100 µg/ml) of cyanobacteria isolated from the sulfur spring.**

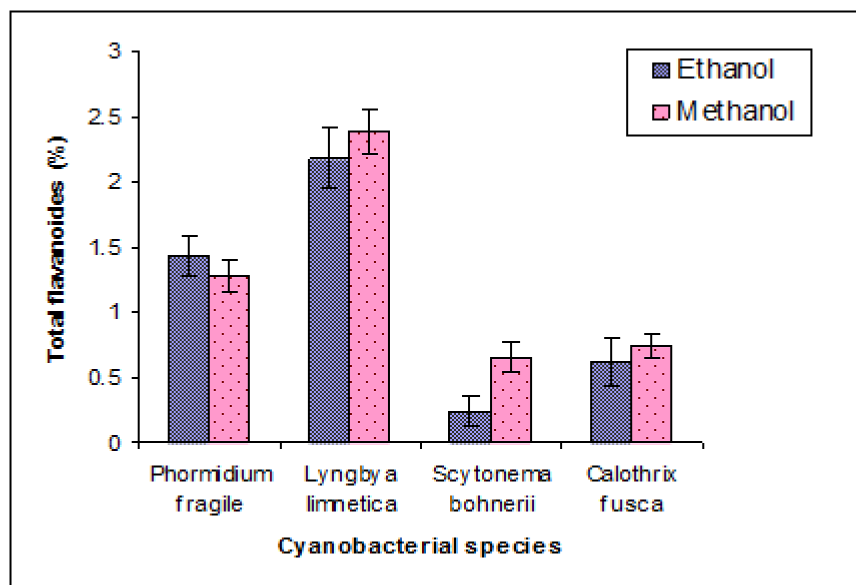


**Figure 2**
**Total flavonoid contents respectively in the ethanol and methanol extracts (100 µg/ml) of cyanobacteria isolated from the sulfur spring.**

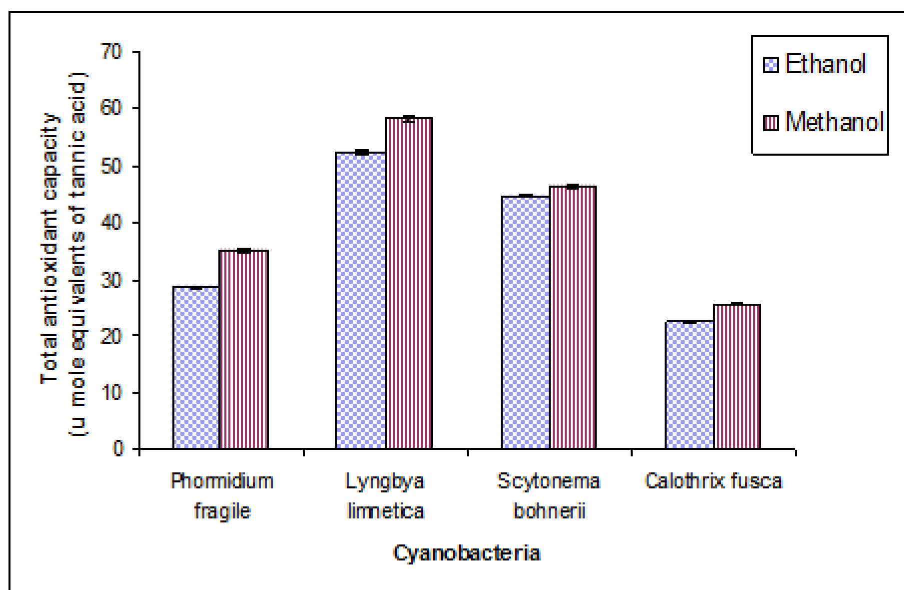


**Figure 3**
**Total antioxidant capacity in ethanol and methanol extracts (100 µg/ml) of four species of cyanobacteria.**

The ethanol and methanolic extract of *Lyngbya limnetica* showed more antioxidant capacity followed by *Scytonema bohnerii* whereas, *Calothrix fusca* showed the least activity (fig.-3.). The study has shown that the methanolic extract is having higher potentiality of phenols, flavonoids and total antioxidant capacity than ethanolic extract. It was also noticed that, among the four strains of cyanobacteria *Lyngbya limnetica* is more potent and suitable strain having all the above mentioned properties in better range compared to other species. *In vitro* screening for antioxidant activity of ethanol and methanollic extracts of cyanobacteria were carried out at different concentrations (10, 100, 500 µg $mL^{-1}$). The free radical scavenging activity of these cyanobacteria was determined by DPPH method (Table 2.). Free radical scavenging activity in ethanolic and methanolic extracts of cyanobacteria was compared with standard antioxidant butylatedhydroxy anisole (BHA). Among the four species *Lyngbya limnetica* (up to 25.20 % in ethanolic extract and 28.32 % in methanolic extract at a concentration of 100 µg/ml, $P< 0.01$) has shown the higher scavenging activity followed by *Scytonema bohnerii* (up to 16.52 % in ethanolic extract and 19.50 % in methanolic extract, $P< 0.05$) and it was lower in *Calothrix fusca* (up to 8.55 % in ethanolic extract and 9.75 % in case of methanolic extract, $P> 0.05$)). By this study it was found that, an increased trend in the activity was observed with increasing concentration of the sample extract.

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**Table 2**
***Antioxidant activity in the four species of cyanobacteria isolated from a sulfur spring***

| Antioxidant assay | Extract type | Extract concentration (µg/ml) | *Phormidium fragile* | *Lyngbya limnetica* | *Scytonema bohnerii* | *Calothrix fusca* | BHA (Control) |
|---|---|---|---|---|---|---|---|
| Free radical scavenging activity (%) | Ethanol | 10 | 4.50 ±0.4 | 8.80±0.60* | 6.22±0.80 | 2.40± 0.22 | 25.5± 1.22 |
| | | 100 | 9.85 ±0.44 | 25.20±0.65** | 16.52±0.90* | 8.55±0.85 | 45.25±1.20 |
| | | 500 | 22.95±0.65* | 32.15±0.85** | 26.35±0.90* | 19.35±1.0* | 65.45±1.40 |
| | Methanol | 10 | 6.10±0.35 | 10.20±0.55* | 8.40±0.70 | 4.30±0.90 | 30.4±1.10 |
| | | 100 | 10.25±0.35 | 28.32±0.66* | 19.50±0.76* | 9.75±0.95 | 51.45±1.0 |
| | | 500 | 25.33±0.45* | 34.45±0.55** | 30.78±0.80* | 21.25±0.9* | 70.20±1.80 |
| Ferric reducing power | Ethanol | 10 | 0.034 ±0.02* | 0.05±0.01* | 0.032±0.001 | 0.02±0.001 | 0.12±0.02 |
| | | 100 | 0.06±0.01* | 0.18±0.01** | 0.09±0.01* | 0.04±0.01 | 0.51±0.04 |
| | | 500 | 0.12±0.02* | 0.35±0.06** | 0.18±0.03** | 0.11±0.01 | 0.80±0.08 |
| | Methanol | 10 | 0.04±0.01* | 0.088±0.02** | 0.035±0.01* | 0.03±0.01 | 0.18±0.04 |
| | | 100 | 0.07±0.02 | 0.27±0.04** | 0.12±0.03* | 0.045±0.02 | 0.65±0.08 |
| | | 500 | 0.15±0.04* | 0.48±0.06** | 0.21±0.08* | 0.13±0.05 | 0.92±0.10 |

***** P<0.01, * P<0.05 when compared with control. Values are expressed as mean ± SEM. BHA: butylated hydroxy anisole***

Similarly the ferric reducing power assay (Table 2.) of these isolates revealed the maximum absorbance in *Lyngbya limnetica* (P< 0.01) followed by *Scytonema bohnerii* (P< 0.05) where as it was least in *Calothrix fusca* (P>0.05). Reducing power of the extracts displayed an increased trend with increasing concentrations, as indicated by the increase in the absorbance of reaction mixture. The reducing capacity of a compound may serve as a significant indicator of potential antioxidant activity. The reducing ability of a compound generally depends on the presence of reductones, which have exhibited antioxidative potential by breaking the free radical chain and donating a hydrogen atom.[26] The overall study has shown that antioxidant potential in *Lyngbya limnetica* and *Scytonema bohnerii* were found to be statistically significant. In this study it was noticed that methanolic extract showed higher antioxidant potentiality when compared to ethanolic extract. Recently, much attention is given for cyanobacteria due to the presence of biologically active compounds such as phycobilins, phenols, antioxidants, terpenoids, steroids and polysaccharides.[27] Phenolic compounds are a class of antioxidant agents which act as free radical terminators.[28] Phenolic compounds have been extensively studied for their antioxidant properties not only in fruits and vegetables but also in cyanobacteria.[29,30] Miranda et al. (1998)[31] studied the antioxidant activity of carotenoids, phenolics and tocopherols extracted from *Spirulina maxima* and found that the phenolic compounds responsible for the antioxidant properties of their extracts this hypothesis was agreed with our results where all the four species containing phenolics and flavanoids exhibited the antioxidant activity. The occurrence of phenolic compounds in cyanobacteria was less documented than that in higher plants.[32] Algal

phenolic compounds were reported to be potential antioxidants to combat free radicals, which are harmful to our body and food systems.[33] Several epidemiological studies revealed that phenolic compounds present in diet are helpful in treating coronary heart disease.[34] Further, phenols have been reported to exhibit pharmacological properties such as anticarcinogenic, antiviral, antimicrobial, anti-inflammatory or anti tumoral.[35] Flavonoids are a group of polyphenolic compounds with known properties which include free radical scavenging, inhibition of hydrolytic and oxidative enzymes and anti-inflammatory action.[36] Some evidence suggests that the biological actions of these compounds are related to their antioxidant activity.[37]

Development of suitable antioxidant molecule is gaining more importance in present days as it plays a key role, in preventing or delaying some of the pathological consequences like hepatotoxicity, heart diseases and cancer.[38] The antioxidant potential of the methanol extracts of different cyanobacteria was determined by Shazia et al., (2011)[39], among the extracts of different cyanobacteria, *Plectonema boryanum* and *Scytonema* sp. exhibit greater antioxidant activity as it was 30% and 27% inhibition of DPPH than the positive control ascorbic acid (25 %) at 50 mg $ml^{-1}$.Similar to this Abd El-Baky *et al*., (2008)[27] observed pronounced anti-oxidant activity in a crude extracts of *Spirulina maxima*. *Spirulina* and its antioxidant activity were documented.[27,40,41] The potent antioxidant activity of the extract of different cyanobacteria might be due to the total phenolics, phycocyanin, triterpenoids present in the extracts.[42] Phycocyanin is a water soluble pigment, which is known to exhibit antioxidant, anti-inflammatory, hepato protective effects.[43,44] These findings support our results and some of the phytoconstituents were also found in the present species. There are restrictions on the use of synthetic antioxidants like ascorbic acid and BHA, as they are suspected to be carcinogenic.[45] Natural antioxidants therefore, attain much importance in recent days due to their easy availability and lesser side effects. The reduction capability of DPPH radicals was estimated by the decrease in its absorbance at 517 nm, which is induced by antioxidants present in the extract. The significant decrease in the concentration of the DPPH radical is due to the scavenging ability of the phytoconstituents present in the extract. The reducing capacity of the extract may serve as a significant indicator of its potential antioxidant activity. This activity may be due to phenolic compounds and flavonoids present in the extract as also indicated by Velioglu et al., (1998).[46]

## CONCLUSION

The study has shown that, of the four species of cyanobacteria - *Lyngbya limnetica* and *Scytonema bohnerii* have good antioxidant activity with the notable presence of polyphenols like phenolics, flavonoids, carotenoids, phycocyanins and some phytoconstituents. In the study it was also found that methanolic extract has higher antioxidant potentiality than the ethanolic extract. The findings open a new aspect for further study on cyanobacteria, particularly the species isolated from unusual habitats like sulfur spring. This would be a turning point for pharmaceutical sciences in determining a novel antioxidant compound from cyanobacteria and its optimum utilization.

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