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Research article

EVALUATION OF THE ANTIOXIDANT AND ANTIMICROBIAL PROPERTIES OF *NOSTOC LINCKIA* ISOLATED FROM KUKKARAHALLI LAKE, MYSORE

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ABSTRACT: The present study evaluates the antioxidant and antimicrobial activity of *Nostoc linckia* which was isolated from Kukkarahalli lake, Mysore and maintained in BG-12 medium. The antioxidant potential of the *N. linckia* extract was investigated using 2,2- Diphenyl, 1- Picryl Hydrazyl and 2, 2´- Azino- bis-(3- ethylbenzothiazoline- 6- sulfonic acid) radical scavenging assays and ferric reducing power assay. It expressed DPPH radical scavenging activity at 1.58 mMTrolox Equivalent/g extract, ABTS radical scavenging activity at 3.8 mMTE/g extract and total ferric reducing power at 1.05mgButylated Hydroxy Anisole Equivalents/ g extract. The radical scavenging activity was compared with BHA as standard wherein it expressed DPPH and $ABTS^{+}$ radical scavenging activity of 2.8 mMTE/g and 4.3 mMTE/g respectively. *In vitro* bactericidal screening of ethanol extract of *Nostoc linckia* was carried out against six species of bacteria namely *Bacillus cereus, Bacillus subtilis, Escherichia coli, Klebsiella oxytoca, Proteus vulgaris* and *Staphylococcus aureus* wherein *B. cereus*, *B. subtilis* and *E.coli* expressed minimum bactericidal concentration values more than 1 mg/ml while *K. oxytoca*, *P. vulgaris* and *S. aureus* expressed MBC values of 0.51, 0.77 and 0.79 mg/ml respectiviely. The study also revealed minimum algicidal concentration of the extract at 0.625 mg/ml against *Nostoc* sp., *Spirullina* sp. *Synecocystis* sp., and 1.25 mg/ml against *Gleocapsia* sp by the 6th day after inoculation. The antimicrobial assay was carried out using micro titre plate method.

Key words: antibacterial activity, algicidal activity, antioxidant activity, free radicals

INTRODUCTION

Cyanobacteria are prokaryotic photoautotrophs which produce a wide variety of secondary metabolites that are accumulated in the cyanobacterial mass and are significant due to their unique structural features and biological activities (Chandra and Rajashekhar, 2013; Fish and Codd,1994) like antimicrobial, anticancer, antiplasmodial etc. activities (Gerwick et. al., 1994; Patterson et. al, 1994; Papendorf et. al., 1998; Jaki et. al., 2000; Mundt et.al, 2001). Therefore, these biologically active compounds, particularly cyanotoxins has received manifold interest (Volk, 2006). Two such cyanotoxins, borophycin (Hemscheidt et. al, 1994), an antibacterial agent and cyanotoxin LU-1 (Grmov et. al, 1991), an algicidal agent, have been isolated from marine strains of *Nostoc linckia*. β- carotene are naturally occurring carotenoids and is one of the few commercially used pigments having application as food colorant, cosmetics, nutrition and therapeutic relevance (Spolaore et. al., 2006). Their nutritional and therapeutic relevance owes to their ability to act as provitamin A which can be converted to vitamin A, quench relative oxygen species rendering anti- inflammatory properties and chemopreventive anticancer effect (Spolaore et. al, 2006). Poorly researched habitats have offered better prospects for discovering new natural products (Pramanik et. al, 2011). Kukkarahalli lake which is located in the heart of the Mysore city providing lung space to the city is one such habitat. No explicit investigation of the microalgae present in the lake has been reported. This is the first report of the microalga, *N. linckia*, present in the lake and its isolation. The study also extends to evaluate its potential antioxidant and antimicrobial activity.

MATERIALS AND METHODS

Chemicals

All chemicals including the antibiotics were obtained from Himedia, India. Methanol was obtained from Merck, India and ethanol was obtained from Changshu Yangyuan Chemical China, India.

Collection, isolation and identification of Nostoc linckia:

Isolation and purification of *N. linckia* was conducted following the antibiotic treatment method where a pool of antibiotics; ampicillin, neomycin and streptomycin with a concentration range of 100µg/ml as antibacterial agents, nystatin as antifungal agent and cycloheximide to inhibit the growth of eukaryotic organisms with the concentration range 100µg/ml were used. 10µl aliquots of the freshwater sample was diluted into 100 ml of sterile distilled water and vacuum filtered through a sterile 47 mm diameter nylon membrane filter (0.2 µm pore diameter, Nucleopore). While in one set of petriplates containing BG12 and BG0 media (Table 1) the filters were aseptically transferred, inoculums side up, in another set of plates containing solid agar media 10 µl aliquots of sample was transferred and spread over the media using sterilized glass spreader. Both sets contained 100 µg/ml of nystatin and cycloheximide. Single colonies from mixed population of cyanobacterial colonies growing on the surface of agar media and glass fibre plates were separately picked and transferred into 20 ml of BG12and BG0 medium containing antibiotic solution in order to overcome heterotropic bacterial contamination. The cultures were incubated at 25ºC and illuminated with cool white fluorescent lamp at an irradiation of 3-5 klux. After 3 to 4 weeks, sufficient biomass was obtained and 400 µl of sterile nutrient solution containing sucrose (2.5% w/v), yeast extract (0.5% w/v), peptone (0.5% w/v) and antibiotic solution was added, incubated in dark at 18- 20ºC for 18- 24 hours and harvested by centrifugation at 14000 rpm for 15 minutes at 25ºC. The cells were washed twice and finally suspended in 1/10 of the original volume of media. The cell suspension was plated on BG-12 and BG0 agar containing nystatin and cycloheximide plates and incubated for 2 to 4 weeks. Purified colonies of *Nostoc linckia* were picked and transferred to plates of BG-12 and BG0 media and mass cultured in 5.0 L BG12 and BG0 media. *Nostoc linckia* was identified by Dr. Shankar P. Hosamani, Professor and Head of Biotechnology Department, SBRR Mahajana First Grade College, Mysore, India based on its morphological charateristics. BG-12 media containing 1.5% sodium nitrate was selected as the best media suitable for mass culturing.

Inocula

Test bacteria: Standard bacterial strains which included Gram +ve bacteria such as *Bacillus cereus* MTCC 430, *Bacillus subtilis* MTCC 121, *Staphylococcus aureus* MTCC 96 and Gram -ve bacteria such as *Escherichia coli* MTCC 1304, *Proteus vulgaris* MTCC 426 and *Klebsiella oxytoca* MTCC 2275 were procured from cell repository of National Centre for Cell Studies, Pune.

Test Cyanobacteria: The standard cyanobacterial culture procured from cell repository of Bharathi Dasan University were used as test organisms which included *Nostoc* BDU 40302, *Spirullina* BDU 40302, *Synechocystis* BDU 30311 and *Gloeocapsa* BDU 130192.

Extraction of Nostoc linckia

Extraction of *Nostoc linckia* was carried out as described by Kaushik and Chauhan (2009) with slight modifications. The mass culture of *Nostoc linckia* was collected and centrifuged at 5000 rpm for 15 mins. The collected pellet was dried *in vacuo.* 10g of dried material was sonicated (Sonics Vibra-cell CV188) in 5.0 ml of 0.9% NaCl solution for 60s at 1500 Hz and subjected to extraction in 100 ml ethanol with vigorous agitation for 15 min. The extract was centrifuged at 5000 rpm for 15 min. The collected supernatant was dried *in vacuo* and the dried extract was used for further analysis.

Antioxidant Activity

2, 2- Diphenyl, 1- Picryl Hydrazyl Free Radical Scavenging Activity: The assay was performed following the procedure described by Brand-Williams et. al.(1995), with minor modifications. 0.1 ml of 1mg/ml extract was pipetted into 1.0 ml of DPPH solution to initiate the reaction. The absorbance was read every 5 minute at 517 nm for 20 min using UV-1800 Shimadzu UV Spectrophotometer and methanol as blank.Under these conditions, the decrease in absorbance indicated the scavenging activity of extracts on DPPH radical. Trolox (0- 200 µM) was used to obtain the standard curve while Butylated Hydroxy Anisole was used as positive control. The free radical scavenging activity was expressed as micromolar Trolox equivalent per gram of sample (µM TE/g extract). The analysis was carried out in replicates of three.

2, 2´- Azino- bis-(3-ethylbenzothiazoline- 6- sulfonic acid) ($ABTS^{\cdot+}$) Free Radical Scavenging Activity:

The $ABTS^{\cdot+}$ radical scavenging activity was estimated essentially as per the method described by Loganayki, Rajendran and Manian (2010). 7mM $ABTS^{\cdot+}$ solution was mixed with 2.45 mM potassium persulphate and left in the dark at room temperature for 12- 15 hours. This was carried out in order to oxidize $ABTS^{\cdot+}$ by the action of potassium to produce the $ABTS^{\cdot+}$ radicals. After consistent absorbance of the $ABTS^{\cdot+}$ free radical solution at 734 nm, the solution was diluted till the absorbance measured was 0.7 ± 0.02. The radical scavenging activity of the hydrophilic fractions was determined by a procedure reported by Miller and Rice-Evans (1997) wherein fresh $ABTS^{\cdot+}$ solution was prepared for each analysis. Antioxidant or standard solutions, 50 µl, were mixed with 1 ml of diluted $ABTS^{\cdot+}$ solution and incubated at 30^0 C. The absorbance at 734 nm was read every minute for 20 min using UV Spectrophotometer with water as a blank. Trolox with concentrations from 0 to 200 µM was used as a standard while BHA was used as positive control. The free radical scavenging activity was expressed as micromolar Trolox equivalent per gram of sample (µM TE/g extract) . The experiment was conducted in triplicates.

Ferric reducing assay: Various concentrations of extract (10- 200µg/ml) were mixed with 2.5 ml of 200mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide (2002). The mixture was incubated at 50^0C for 15 min and 2.5 ml of 10% (w/v) trichloroacetic acid was added. 5ml of above solution was mixed with 5ml of distilled water and 1ml of 0.1% of ferric chloride. The absorbance was measured spectrophotometrically at 700 nm. Butylated hydroxy anisole (BHA) was used as standard antioxidant to obtain standard curve.

Antimicrobial Activity

The assay was performed following the procedure described by Volk and Furkert (2005), with minor modifications. The bacteria were cultivated at 37°C in Nutrient broth for 24 h and each culture was rinsed with 2 ml of the NaCl solution. The resulting suspensions were transferred to sterile test tubes. The suspensions were diluted with NaCl solution in order to achieve a number of 1×10^4 colony forming units per ml. The assays were performed as serial dilution tests as described in instructions for the determination of the minimum bactericidal concentration (MBC), (Volk and Furkert, 2005). According to the broth micro dilution method the tests were carried out in micro titer plates (Nunc-Immun 96 MicroWell Plate), covered with a Nunc Standard Lid (both Nunc GmbH & Co. KG, Wiesbaden, Germany).

10 mg/ml concentration solution of cyanobacteria extract was prepared and aliquots of each of these solutions were serially diluted by half with NaCl solution to obtain seven 50 µl aliquots with decreasing concentrations. Then 50µl nutrient broth and the same volume of the inoculums were added into each well. As positive controls, mixtures of 50 µl aliquots of NaCl solution, nutrient broth and inoculums were prepared. Negative controls contained 100 µl NaCl solution and 50 µl nutrient broth each. For all microorganisms the test was performed in triplicate. The plates were incubated for at least 24 h at 37° C for bacteria. Thereafter the plates were shaken and the turbidity of the suspensions was measured at 620 nm using the ELISA plate reader. 0.1 mg/ml of concentration of ampicillin and streptomycin were used as standard antibiotics.

Algicidal Activity

The test cyanobacteria were cultivated under conditions as described for *Nostoc linckia* cultivation. The *Nostoc linckia* extract dilutions were prepared as prepared for the anti bacterial activity. The test was carried out in Nunc-Immuno 96 Micro Well plates which were covered with a Nunc Standard Lid. The optical density at 440 nm of a 21 day old culture of each test organism was determined and a part of this suspension was concentrated by centrifugation (5000 *g*, 10 min) and adjusted to an optical density of 2.0 ±0.2 to achieve 5×10^6 cells ml^{-1} and higher. 50 µl of the serially diluted extracts were tested against 100 µl of test algal suspension to each test well of the plate. The cultures were maintained in the same condition applied to culture the microalgae. Visual monitoring of the test algae was conducted day wise. Discolouration of the green test algae indicated the algicidal activity of the sample. The assay was conducted according to Volk and Franker (2005) in triplicates.

Thin Layer Chromatography

N. linckia ethanol extract: Thin Layer Chromatography was carried out using preparative thin layer chromatography plates with a mixture of ethylacetete: methanol: water (100: 16.5: 13.5; v: v: v) (Volk, 2006). To prepare test solutions 100 mg of *Nostoc linckia* extract was dissolved in 200 µl of mobile phase and was spotted on the plates where β- carotene was used as reference compound. The spots which had R_f values matching to that of the β- Carotene were scrapped, reconstituted in methanol and centrifuged at 5000 rpm for 15 minutes. The supernatant was aspirated and dried *in vacuo*. The collected compound was subjected to HPLC analysis.

High Performance Liquid Chromatography

100 µg/ml of stock solution of β-carotene was prepared using n-hexane. 1 mg of the TLC purified compound was prepared in methanol. HPLC analysis, conducted at Azymes Biosciences Pvt. Ltd., Bangalore, was carried out on Waters HPLC (Model 510) system using a Kromasil C-18 column (250mm× 4.6mm, 5µm particle size) equipped with a photodiode array detector with 450 nm as the detecting wavelength at a column temperature 27°C using acetonitrile/water (88: 15) as mobile phase in isocratic elution with flow rate of 1ml/min. The pressure of the column was kept 2300 psi. Standard solution (20 µl) of beta carotene and TLC purified ethanol extracts was injected. The standard beta carotene peak was achieved at the retention time of 4.26 minutes.

Statistical Analysis

All assays were conducted in replicates of three. Data is represented as mean ± standard deviation. Correlation analytical data was obtained using the software Origin 5.0.

RESULTS

Distinct morphological features were observed in the *Nostoc* sp. isolated from Kukkarahalli lake to characterize it as *Nostoc linckia*. The thallus was variable in size, from globosa to irregularly expanding, gelatinous and dark green or brown colour. The filaments were densely entangled. Trichomes were pale blue- green, cells were short and barrel shaped, heterocysts were sub- spherical and the akinetes were subspherical with a smooth epispore. $ABTS^{\cdot+}$ and DPPH radical scavenging activity of *N. linckia* ethanol extract is given in Figure 2. The ethanol extract of *Nostoc linckia* expressed $ABTS^{\cdot+}$ radical scavenging activity at 3.8 mMTE/g extract while DPPH radical scavenging activity of ethanol extract was 1.58 mMTE/g extract. BHA expressed DPPH and $ABTS^{\cdot+}$ radical scavenging activity of 2.8 mMTE/g and 4.3 mMTE/g respectively. The total ferric reducing activity was found to be 1.05 µgBHAE/ mg extract. Reducing power of the extract increased with increasing concentrations. To examine the antibacterial activity of the *N. linckia* ethanol extracts the minimum bactericidal concentrations (MBC) (Table 2) against selected Gram +ve and Gram -ve bacteria were determined using a micro titre well plate method. The ethanol extract had significant inhibitory activity against *Klebsiella oxytoca Proteus vulgaris* and *Staphylococcus aureus* at 0.51, 0.77 and 0.79 mg/ml respectively. The extract however, had MBC more than 1.0 mg/ml for *Escherichia coli, Bacillus cereus* and *Bacillus subtilis.* In the assay the antialgal effect of extract was time dependent in most cases. After a period of 6 days lower concentrations of the test compounds led to a decolouration of the test organism in comparison to the second day (Table 3). After the sixth day no changes were observed. TLC of the ethanol extract of *N. linckia* was conducted to detect the presence of β- carotene using an internal standard (Fig. 3), and extract the compound. R_f value of β-carotene separated from the ethanol extract was found to be 0.85 which corresponded to that of the standard. The TLC purified β-carotene from *N. linckia* was injected to confirm the carotenoid using HPLC.The retention time of the extracted β- carotene was 4.377 min which corresponded with that of the internal standard (R_t= 4.26 min; Fig.5A and 5B). Extraction yield of studied compound was found to be 2.8% β-carotene per 100g dry weight.

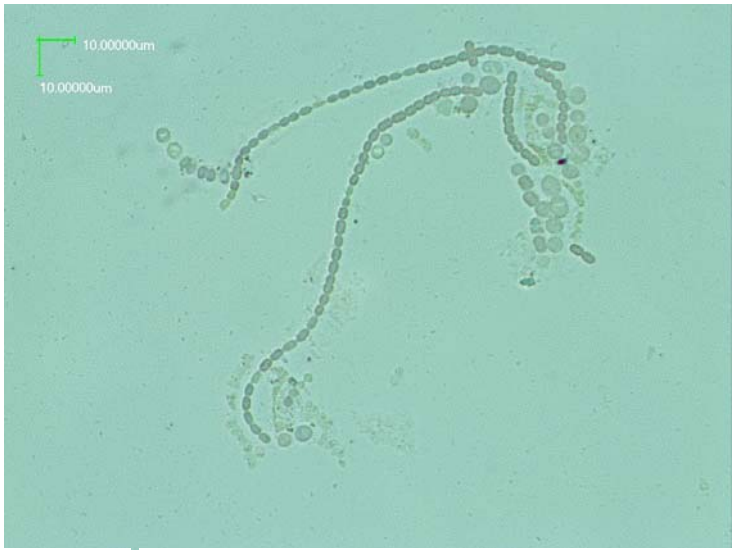

Fig. 1 Microscopic view of *Nostoc linckia* under phase contrast microscope (Primus) at 40X magnification.

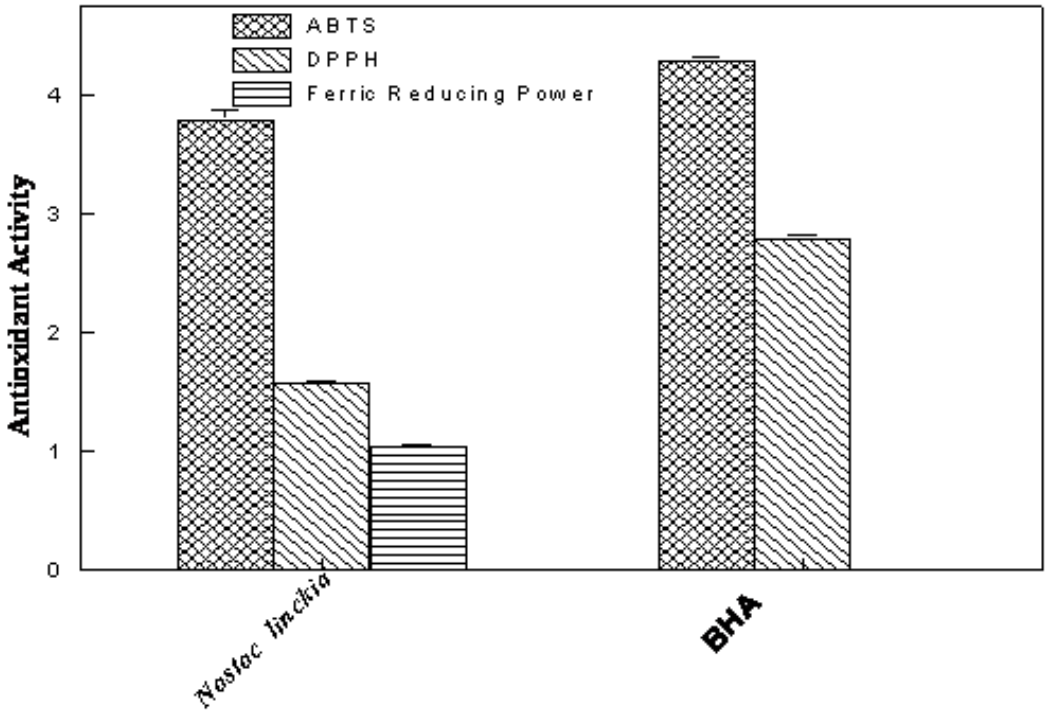

Fig 2 Antioxidant activity of *Nostoc linckia* ethanol extract compared with BHA as standard. $ABTS^{\cdot+}$ and DPPH Radical Scavenging Activity is expressed as µMTrolox Equivalent/g extract (r^2 = 0.987, p < 0.1, r^2 = 0.980, p < 0.01) while ferric reducing power is expressed as µgBHA Equivalents/mg extract (r^2 = 0.987, p < 0.01)

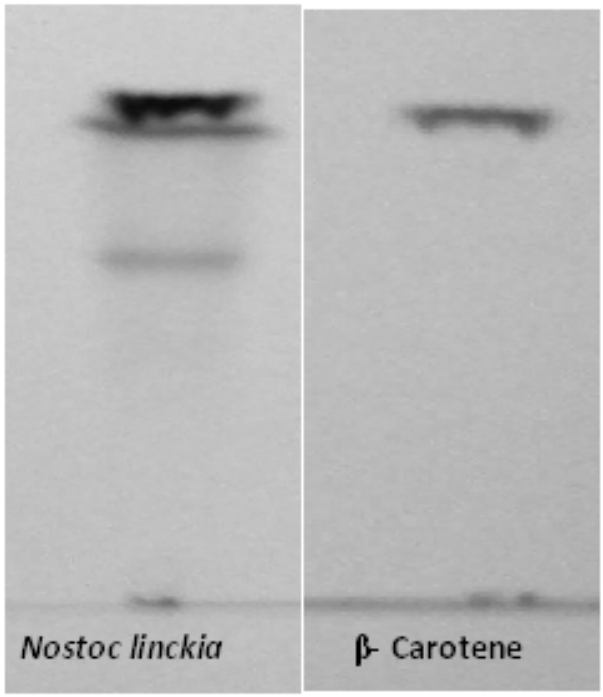

Fig. 3: Thin Layer Chromatography of *Nostoc linckia* with standard β-carotene

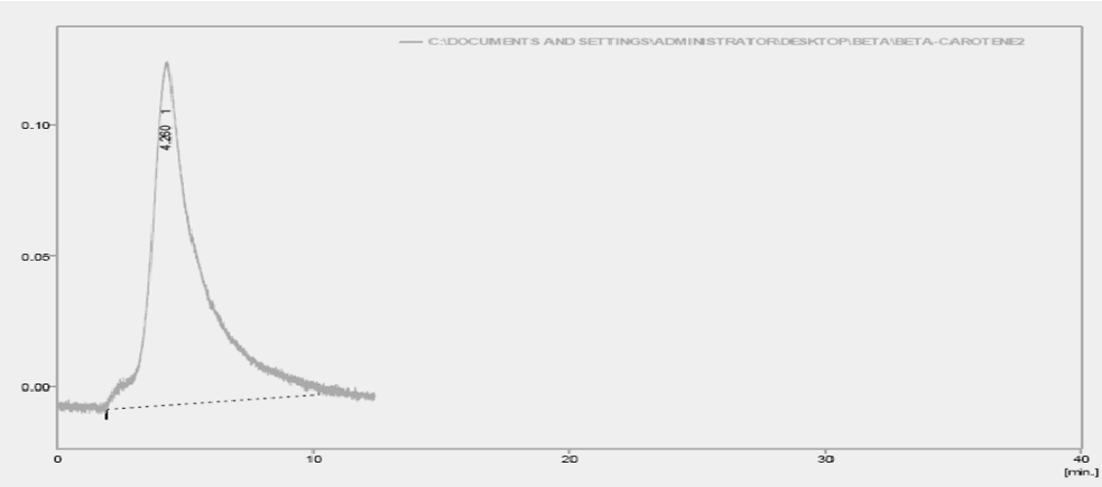

Fig:4A-HPLC of standard β carotene

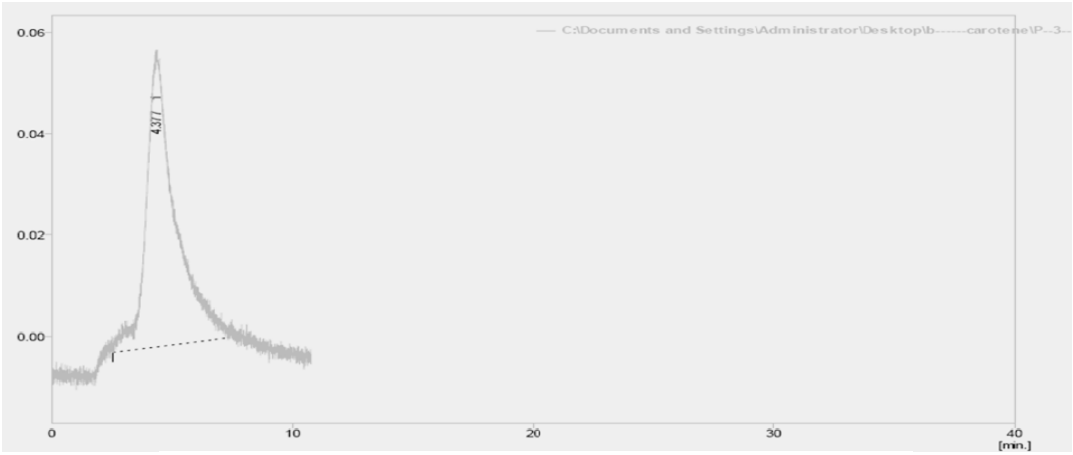

Fig: 4B- HPLC of partially purified extract of *Nostoc linckia*

Table 1A: Composition of BG0 and BG12 media (HEPES 1.2g)

Name of Chemical	BG- 12 (g/L)
NaNO3	1.5
K_2HPO_4	0.04
$MgSO_4$.7H20	0.075
$CaCl_2.2H_2O$	0.036
Citric Acid Ferric ammonium citrate	0.006
Disodium magnesium EDTA	0.006
Na_2CO_3	0.02
4- (2- Hydroxyethyl)- 1- piperazine- ethansulfonic acid (HEPES) buffer	0.001 1.2g
Trace Elements	1ml

Table 1B Composition of Trace Elements

Component	Stock Solution	Quantity
H3BO3	------	2.860 g
Mncl2 • 4H2O	------	1.810g
ZnSO4 • 7H2O	------	0.220 g
CuSO4 • 5H2O	79.0 g L-1 dH_2O	1 mL
Na2MoO4 • 2H2O	------	0.391g
Co(NO3)2 • 6H2O	49.4 g L-1 dH_2O	1 mL

Table 2: Minimum Bactericidal Concentration (mg/ml) of *Nostoc linckia* extracts against selected test bacteria.

Test organisms	MBC of *Nostoc linckia* ethanol Extract (mg/ml)
Escherichia coli	>1.0
Bacillus cereus	>1.0
Bacillus subtilis	>1.0
Staphylococcus aureus	0.79 ± 0.003
Klebsiella oxytoca	0.51 ± 0.012
Proteus vulgaris	0.77 ±0.009

Table 3:The time dependent minimum cytotoxicity concentration (mg/ml) of *Nostoc linckia* ethanol extracts against selected test cyanobacteria.

Test organisms	*Nostoc linckia*	
	Time	
	2 Day	6 Day
Nostoc	1.25	0.625
Spirullina	1.25	0.625
Synecocystics	1.25	0.625
Gleocapsia	5.0	1.25

DISCUSSION

Cyanobacteria and other microalgae are known to produce a wide variety of biological active organic compounds. Most of those compounds are accumulated in the microalgal biomass, others are excreted during growth into the environment as exometabolites (Sharathchandra and Rajashekar, 2013; Abd- El et. al, 2008). Therefore, in the present screening for antioxidant, antibacterial and algicidal metabolites, microalgal biomass was tested. The need for simple and reliable *in vitro* antioxidants test is widely acknowledged. The ability to quench free radical by hydrogen donation and the ability to transfer an electron are two of the mechanisms most widely used in *in vitro* assays to determine antioxidant (Olson and Krinsky, 1995). The overall study has shown that antioxidant potential in *Nostoc linckia* was found to be statistically significant. In the antioxidant activity of the ethanol extract of *N. linckia* the scavenging activity against $ABTS^{\cdot+}$ was on par with the standard antioxidant, BHA. BHA, however, expressed better DPPH radical scavenging activity than the ethanol extract. This could be because the absorption maximum of the crude extract, which was between 400-450 nm, has interfered with maximum absorption of DPPH at 517 nm. $ABTS^{\cdot+}$ however, having maximum absorption of 734 nm had a broader spectrum which was not interfered by the maximum absorption of the crude extract. The DPPH and $ABTS^{\cdot+}$ radical scavenging activity and reducing capacity of a compound may serve as significant indicators of potential antioxidant activity as the reducing ability of a compound generally depends on the presence of reductones which break the free radical chain and donate a hydrogen atom (Jaki et. al, 2000). The antibacterial activity studied was an endeavour towards antibacterial agent production by the microalgae. The ethanol extract had no significant activity against *B.subtilis*, *B. cereus* and *E. coli*. In order to quantify the algicidal (anticyanobacterial) activity detected for the extract, microwell plate assay developed by Volk and Furkert (2005) that was easy to perform and was less substance consuming, and which allowed the estimation of the minimum cytotoxic concentration against selected cyanobacteria, was used. Growth inhibiting activities against cyanobacteria could not be detected because of reduced aeration in the microwell plates causing a delayed growth of the cyanobacteria. In addition, autoinhibition observed for the test *Nostoc* species must be emphasised. Whether this property is involved in a self regulation mechanism of populations of these species must be determined in the future. It was observed that concentrations of biomass extract resulting in antibacterial activity were lower than concentrations necessary for algicidal activity rendering it a better antibacterial agent. Spectroscopic analysis of extracts of *N. linckia* revealed maximum absorption to be 435nm (data not shown) which indicated that β-carotene was present in the extract as the maximum absorption of standard β-carotene was confirmed to be 445 nm. This method revealed that β-carotene was predominant as compared to other secondary metabolites since the maximum absorption lied between 400-500nm. The HPLC analysis allowed the separation and estimation of β-carotene of *N. linckia*. β-carotene is one among the carotenoids identified from marine strains of cyanobacteria that have shown an antioxidant effect in reducing oxidative markers stress rendering it as a dietary phytochemical product (Rioccini, 2012). The significant concentration of β-carotene present in the fresh water strain of *N. linckia* extract could be responsible for the antioxidant and antimicrobial effect. Thus future analysis of *N. linckia* as a source of nutritional supplement and therapeutic value is underway.

CONCLUSION

The present study attempted to determine the antioxidant and antimicrobial potential of fresh water strain of *Nostoc linckia*. It was observed that the metabolites concentrated within the biomass contributed significantly as biologically active agents. β-carotene was identified as predominant secondary metabolite.

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