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Original Article

FATTY ACID PROFILING AND ANTIOXIDANT POTENTIAL OF TOTAL POLAR LIPID CONTENT OF CYANOBACTERIUM *NOSTOC MUSCURUM*

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ABSTRACT

Objective: In recent years cyanobacteria has gained much importance due to the presence of secondary metabolites possessing several biological activities like antibacterial, antifungal, anti algal, antiprotozoal, and antiviral activities. *Nostoc muscurum, a* member of family Nostocaceae, known to have components with potent bioactive properties. The present investigation deals with the analysis of total polar lipids of *Nostoc muscurum* and its antioxidant potential.

Methods: Culture of *Nostoc muscurum* was grown under lab conditions. Cells were harvested in earlier stationary phase and total polar lipid extracted by modified Bligh and Dyer method. Antioxidant analysis of total polar lipid was done by DPPH free radical method. Finally, total polar lipid was transesterified, and components were identified by GC-MS analysis.

Results: Highest percentage inhibition of free radical (65%) was detected at concentration 500 µg/ml which might be due to the presence of bioactive principles hexadecanoic acid. Total twenty-nine compounds were identified through GC-MS analysis. GC-MS analysis revealed that Phthalic acid and 9-Octadecenoic acid, ethyl ester was found in higher concentration in the total polar lipid of *Nostoc muscurum* as compared to other bioactive compounds. Former is known to possess antimicrobial property while later was found to have bioactive properties like anti-inflammatory, anticancer, hypocholesterolemic, 5-alpha reductase inhibitor and anti androgenic activity.

Conclusion: Identified compounds with bioactive properties were mostly organic acids. Also, higher percentage of hydrocarbon was found in the fraction which might be used in the fuel industry. The biological activities of some of the components ranged from antimicrobial, antioxidant and antitumoral activities and can be used in pharmaceutical formulations.

Keywords: Cyanobacteria, *Nostoc muscurum*, Gas chromatography, DPPH, Total Polar Lipid.

INTRODUCTION

Cyanobacteria are gram negative photosynthetic bacteria known for its classical activities like nitrogen fixation, which is a most prevailing function of cyanobacteria. Cyanobacteria also known by other names like cyano prokaryotes, cyanophytes, and blue-green bacteria because of the presence of a blue-green pigment, c-phycocyanin (C-PC), which is involved in photosynthesis and have fossil record from 3.3 to 3.5 billion years [1]. Cyanobacteria inhabit almost all the habitats on earth; from bare rock to soil and from water to air. They have unique food storage compounds, myxophycean starch, and cyanophycin. Cyanobacteria perform water-oxidizing photosynthesis by using both Photosystem I and Photosystem II (Z-scheme), but under anaerobic conditions they are capable of using only photosystem I like purple bacteria and use same electron transport machinery for both photosynthesis and respiration. The morphological characteristic of cyanobacteria varies from unicellular to filamentous or colonial forms. Depending on the environmental conditions colonies are often surrounded by a mucilaginous or gelatinous sheath. Some of the filamentous cyanobacteria have three types of cells namely: vegetative cells, climate-resistant akinetes, and thick-walled heterocysts.

In spite of several approaches and efforts being made towards the discovery of the newer drug, drug development has not gained much pace due to lack of proper lead in Biomolecules, which is crucial to designing newer drugs [2]. Hence, there is an increasing demand to switch over natural resources for the screening of new bioactive compounds and this lead to the researchers to cyanobacteria for discovering new drugs. Cyanobacteria are eubacteria leading to the development of natural biopharmaceuticals due to the presence of structurally diverse groups of compound [3, 4]. The cyanobacterial secondary metabolites include several biological activities like antibacterial, antifungal, antialgal, antiprotozoal, and antiviral activities [5]. During the last two decades, secondary metabolites from cyanobacteria have attracted the attention of researchers mainly due to two reasons; (i) acute toxicity of toxins produced by several bloom-forming cyanobacteria in freshwater system and their harmful effect on animals and human health, and (ii) potential therapeutic use of several secondary metabolites [6-7].

Besides the use of cyanobacteria in pharmaceutical industries they have also been recognized as an excellent source of vitamins and proteins and are also reported to be a source of fine chemicals, renewable fuel and bioactive compounds [8]. Cyanobacteria contain both polar, and non-polar lipids, and some of them are rich in essential fatty acids such as linoleic and gamma-linolenic acids. The common storage lipids found in cyanobacteria are triglycerides which constitute 80% of total lipid content of cyanobacterial lipids while other lipids found in cyanobacteria are sulpho-quinovosyl-diglycerides (SQDG), mono galactosyl-diglyceride (MGDG), digalactosyl-diglyceride (DGDG) and phosphatidylglycerol (PG) [9-10]. Cyanobacterial lipids also play an important role in providing protection against stress conditions such as salt-induced damage, low temperature, and high light induced photoinhibition, desiccation and lipid peroxidation. The mechanism behind stress tolerance depends on phospholipid bilayers stabilized by sugars (trehalose) and unsaturation of fatty acids [11].

Nostoc muscorum is a species of cyanobacterium belonging to the family Nostocaceae of subgroup 4, section A, of the oxygenic phototrophic bacteria and share characteristics of both gram-negative bacteria and photosynthetic eukaryotes [12]. *Nostoc muscurum* contains various bioactive components like phenolics, phycocyanin, triterpenoids amino acids, polyunsaturated fatty acids, sulphated polysaccharides, and carotenoids. These components have bioactive properties like antimicrobial, antioxidant and antibacterial activity. The analysis of the overall fatty acid profile, as well as the

occurrence of fatty acids in different lipid classes in microalgae, is an emerging field which is expected to reveal the identification of novel fatty acids with a variety of new functional groups [13]. Mass spectrometry, coupled with Gas chromatography (GC/MS) is normally used for the analysis of non-polar components and volatile essential oil, fatty acids, lipids [14] and alkaloids [15]. The presence of lipids, hydrocarbons, and other complex oils is dependent on the algal species [16]. The aim of present study is to determine the fatty acid profile and elucidate the antioxidant potential of total polar lipid of *Nostoc muscurum*.

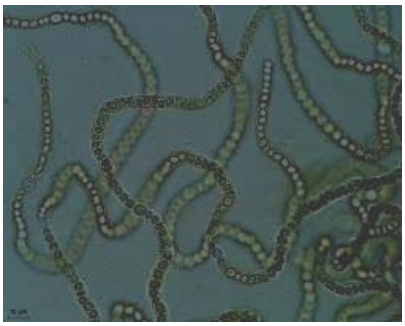

Fig. 1: *Nostoc muscurum* under compound microscope

Materials and methods

Maintenance of *Nostoc muscurum* under lab conditions

Cultures of *Nostoc muscurum* were grown in the N-free BG-11(pH-8) at 26±2 °C, illuminated with white fluorescent tubes providing an intensity of 75 mol/m^2/s and a photoperiod of 10:14 light and dark.

Growth behavior in terms of chlorophyll an estimation

Chlorophyll a was estimated by the method of McKinney [17]. Cells were harvested on day 5, 10, 15, 20, 25 and 30. On each subsequent 5^{th} day, 5 ml of cultures were pelleted down by centrifugation at 5000 rpm for 10 min at 4 °C. Supernatant was discarded. Chlorophyll a was extracted in 3 ml of 100% chilled methanol through freezing and thawing of samples overnight and centrifuged again for 10 min at 4°C. The absorbance of the supernatant was recorded at 680 nm against methanol serving as blank. This process was repeated in triplicates. Chlorophyll a was calculated using the equation: A = KclA=Kcl, where: A-absorbance at 680 nm (A680), K-Molar extraction coefficient, which is constant (13.42), C–Concentration (mg/ml), L-Path length which is 1 cm.

Biomass harvest

The biomass was harvested by filtration and then concentrated by centrifugation at 15,000 rpm for 15 min. The cell pellets were suspended in deionized water and re-centrifuged two times to remove residual medium.

Extraction of lipid

Total polar lipid from *Nostoc muscurum* was extracted according to the Bligh and Dyer standard method with some modifications [18]. Briefly, methanol–chloroform 2:1 (v/v) was added to extract the lipids from the cells of *Nostoc muscurum*, the mixture was shaken for 15 min, and then centrifuged and the supernatant was transferred by means of a pipette to another tube. The precipitated residual material was then re-suspended in methanol–chloroform–water 2:1:0.8 (v/v) and the mixture was again shaken for 15 min and centrifuged. The combined extracts were diluted with chloroform–0.2 M KCl 1:1 (v/v) in order to have a final mixture of methanol–chloroform–water 1:1:0.9 (v/v). Then, phase separation was obtained by centrifugation, and the lower chloroform phase was withdrawn, and chloroform was added to chloroform–water phase to optimize the lipid recovery. The combined chloroform phases were dried in a rotatory evaporator and the lipids and pigments obtained were re-dissolved in chloroform and stored at −20 °C.

Preliminary identification of lipid by thin layer chromatography

Thin layer chromatography was carried out on silica plate of size 20 cm X 20 cm from MERCK. Plates were activated at 120°C for 2 h in hot air oven. After the application of extracts about 10 µl of the sample, the plates were developed in the chromatographic tank saturated with the solvent. The solvent systems used in TLC development for glycolipids and phospholipids were chloroform: methanol: 25% ammonia solution (65:25:4, v/v/v) [19]. After development, the plates were removed from the solvent chamber and dried at room temperature.

Visualization of lipids by Iodine vapours

Plates were exposed to iodine vapours in an airtight tank. All lipids appeared as brownish yellow to yellow spots[20] and then the R_F value was calculated by the following formula:-

$$R_F \text{ value} = \frac{\text{Distance travelled by sample}}{\text{Distance travelled by solvent}}$$

Trans-esterification of total polar lipid

The dried crude lipid was transesterified using a modified method [21, 22]. 1 ml of 3-N methanolic HCl was added to the dried lipid in a test tube and heated in a water bath at 85 °C for 2.5 h. After cooling the mixture to room temperature, 0.5 ml double ionized water and 1 ml of hexane were added and mixed well by shaking the contents by hand. The trans-esterified FAME containing the hexane layer was collected and 1 ml hexane added again twice to the remaining methanol/ water to pool rest of FAME.

GC-MS analysis of fatty acid methyl esters

The fatty acid profile of total polar lipid of *Nostoc muscurum* was done by Gas chromatography coupled with mass spectrometry (GC-MS). Chromatography was performed using a Shimadzu Mass Spectrometer-2010 series system (AIRF, JNU, New Delhi) equipped with a RTX-5 MS. qg. For GCMS detection, an electron ionization system with ionization energy of 70eV was used. Helium gas was used as a carrier gas at a flow rate of 1.2 ml per minute. Injector and mass transfer line temperature were set at 270 and 280 °C. The compounds were identified by comparing them with the standards, or the mass spectra were matched with the inbuilt library National Institute of Standard and technology (NIST).

DPPH radical scavenging activity assay

Quantitative measurement of the radical scavenging activity of total polar lipid obtained from *Nostoc muscurum* was carried out according to the method described by Blois [23]. 1 ml of 0.1 mM 2, 2-dipheny-l-picrylhydrazyl (DPPH) solution in methanol was added to 3 ml of the methanolic extract prepared at different concentrations (50–500µg/ml). Ascorbic acid was used as a positive control. Discoloration was measured at 517 nm after incubation for 30 min. The capacity to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect}\ (\%) = [\text{control OD} - \text{sample OD/control OD}]$$

RESULTS AND DISCUSSION

Growth behavior of *Nostoc muscurum* in terms of chlorophyll a

Growth behavior of *Nostoc muscurum* was determined in terms of chlorophyll 'a' content [24, 25]. An increase in chlorophyll 'a' content was observed from day 5 (0.009±0.0015 mg/ml) to day 30 (0.0157±0.0007 mg/ml). However, higher content of chlorophyll 'a' was found on 25^{th} day of its growth (0.0214±0.0023 mg/ml) (fig. 2). Decrease in chlorophyll 'a' content after day 25, might be due the reason that cells have reached in stationary phase which resulted in reduced photosynthesis and reduced growth.

Preliminary identification of lipids by thin-layer chromatography

Preliminary identification of total polar lipid of *Nostoc muscurum* by thin layer chromatography shown the presence of six bands of R_f value 0.81, 0.74, 0.62, 0.45, 0.33 and 0.19 (Table. 1, Fig.3) The bands

of Rf value 0.74 and 0.62 might be the bands of glycolipids belonging to the classes steryl-glucoside and monogalactosyl-diacylglycerol while the bands of Rf value 0.33 and 0.19 might be bands of phospholipid corresponding to the classes phosphatidyl-ethanol-amine and phosphatidylcholine, rest three bands of Rf value 0.81, and 0.45 were not determined [19].

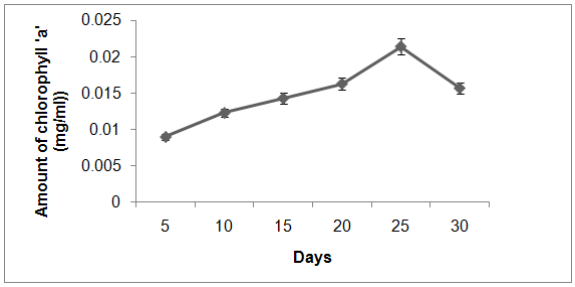

Fig. 2: Growth behavior of *Nostoc muscurum*

Table 1: Resolved lipid bands and their Rf values

S. No.	Rf value	Color
1.	0.19	±
2.	0.33	±
3.	0.45	++
4.	0.62	++
5.	0.74	±
6.	0.81	+

*'**++**' symbolizes yellowish brown color; *'+' symbolizes light brown color; * '±' symbolizes pale brown color.

GC-MS analysis of total polar lipid of *Nostoc muscurum*

Twenty-nine compounds in the total polar lipid of *Nostoc muscurum* were identified by GC-MS analysis. The active principles with their retention time (RT), molecular formula (MF), and concentration (%) are presented in Table. 2 and fig. 3. The prevailing compounds were docosane (23.11%), pthalic acid (9.65%), tetracontane (8.53%), hexacosane (8.06%), hexatriacontane (7.25%), pentacosane (6.09%), heptacosane (4.87%), tetracontane (3.77%), tetracosane (3.53%), tetra-triacontane (2.51%), 9-octadecanoic acid (2.40%), and hexadecane (2.16%).

Fig. 3: Thin layer chromatogram of total polar lipid of *Nostoc muscurum*

Compounds with potent bioactive potentials present in the total polar lipids fraction of *Nostoc muscurum* were 2-pentadecyl-1,3-dioxolane (0.74%), phenol, 2,4-bis (1,1-dimethylethyl (1.40%), hexadecanoic acid methyl ester (0.71%), hexadecanoic acid ethyl ester (1.18%), tetradecanoic acid ethyl ester (0.09%), phthalic acids(9.65%), 9-octadecenoic acid (Z)-ethyl ester (2.40%) (Table.3). Pthalic acid was found in higher concentration (9.65%) compared to other bioactive compounds and known to possess antimicrobial activity as reported in the literature [26]. 9-Octadecenoic Acid (Z)-Ethyl Ester was also present in a comparatively good amount (2.40%) and possesses various bioactive properties like anti-inflammatory, anticancer, hypocholesterolemic, 5-Alpha reductase inhibitor and anti androgenic activity [27].

DPPH radical scavenging activity of total polar lipid of *Nostoc muscurum*

Antiradical properties of total polar lipid of *Nostoc muscurum* was evaluated by stable DPPH free radicals. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule [28]. Results obtained by DPPH free radical reduction method at different concentrations of total polar lipid ranging from 50µg/ml to 500µg/ml (Fig.4). Total polar lipid of *Nostoc muscurum* showed the dose-dependent antioxidant potential. It was maximal at a concentration of 500µg/ml with 65% inhibition of DPPH radical. This activity may be due to bioactive principles hexadecanoic acid, present in the total polar lipid of *Nostoc muscurum.* The ascorbic acid has shown higher percentage inhibition at low concentration as compared to the total polar lipid of *Nostoc muscurum.* (Fig.5). A growing market for identification of novel antioxidants from non-expensive resources explains educated screening of microalgae for their potential antioxidant features. Evidence gathered in a large number of worldwide researches supports the involvement of antioxidants in the prevention and control of growth of certain tumors, as well as in the incidence and severity of cardiovascular and degenerative diseases [29-31]. Furthermore, microalgae are known to have adaptive responses towards oxidative stresses, via stimulation of their antioxidant defense system [32], consisting of both enzymatic and non-enzymatic mechanisms. Superoxide dismutase, catalase, glutathione reductase and ascorbate peroxidase are key enzymes in the former, whereas the non-enzymatic counterpart includes such mediator compounds as ascorbic acid, reduced glutathione, tocopherols, carotenoids and phycocyanin [33]. In this present study GC-MS analysis confirmed the presence of bioactive components with antioxidant activity in the total polar lipid of *Nostoc muscurum.*

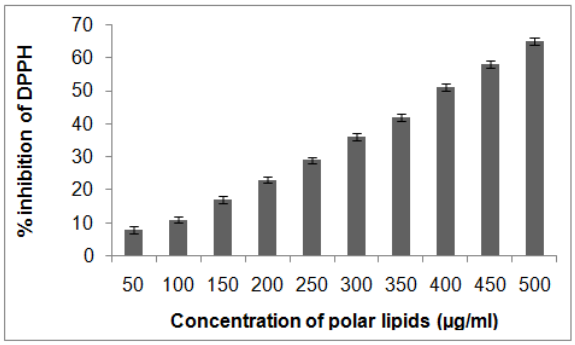

Fig. 4: % inhibition of DPPH by total polar lipid of *Nostoc muscurum*

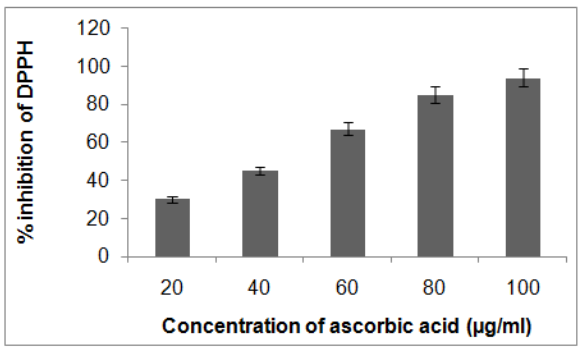

Fig. 5: % inhibition of DPPH by ascorbic acid

Table 2: GC-MS chromatogram of total polar lipid of *Nostoc muscurum*

S. No.	Compound name	Retention time(min)	Area (%)	Molecular formula
1	2-Pentadecyl-1,3-Dioxolane	3.646	0.74	$C_{18}H_{36}O_2$
2	Tetradecane	5.089	1.29	$C_{14}H_{30}$
3	Phenol, 2,4-Bis(1,1-Dimethylethyl)	7.722	1.40	$C_{14}H_{22}O$
4	Hexadecane	9.819	2.16	$C_{16}H_{34}$
5	Not Determined	12.455	0.27	-
6	Octadecane	15.076	1.54	$C_{18}H_{38}$
7	Hexadecanoic Acid, Methyl Ester	18.314	0.71	$C_{17}H_{34}O_2$
8	Hexadecanoic Acid, Ethyl Ester	19.971	1.18	$C_{18}H_{36}O_2$
9	Dotriacontane	20.052	0.41	$C_{32}H_{66}$
10	Not Determined	23.844	1.99	-
11	9-Octadecenoic Acid (Z)-, Ethyl Ester	23.964	2.40	$C_{20}H_{38}O_2$
12	Tetradecanoic Acid, Ethyl Ester	24.543	0.09	$C_{16}H_{32}O_2$
13	Octatriacontane	24.614	0.40	$C_{38}H_{78}$
14	Hentriacontane	26.749	1.38	$C_{31}H_{64}$
15	Tetracosane	28.798	3.53	$C_{24}H_{50}$
16	Pentacosane	30.767	6.09	$C_{25}H_{52}$
17	Hexacosane	32.664	8.06	$C_{26}H_{54}$
18	Phthalic Acids	31.829	9.65	$C_{24}H_{38}O_4$
20	1,4-Epoxynaphthalene-1(2H)-Methanol	34.222	1.72	$C_{23}H_{36}O_2$
21	Docosane	34.490	23.11	C22 H46
22	1,4-Epoxynaphthalene-1(2H)-methanol	34.694	1.52	$C_{23}H_{36}O_2$
23	Not Determined	35.602	0.34	-
24	Tetracontane	36.256	8.53	$C_{40}H_{82}$
25	4,8,13,17,21-Pentamethyl-4,8,12,16,20-Docosapentaenal	36.820	0.35	$C_{27}H_{44}O$
26	Heptatriacontane	37.324	0.35	$C_{37}H_{76}$
27	Hexatriacontane	37.957	7.25	$C_{36}H_{74}$
28	Not Determined	38.890	0.10	-
29	Nonatriacontane	38.98	0.50	$C_{39}H_{80}$
30	Heptacosane	39.607	4.87	$C_{27}H_{56}$
31	Tetracontane	41.283	3.77	$C_{40}H_{82}$
32	Tetratriacontane	43.245	2.51	$C_{34}H_{70}$
33	Octacosane	45.603	1.47	$C_{28}H_{58}$

Table 3: Bioactive properties of compounds of *Nostoc muscurum*

S. No.	Compound name	Compound nature	Activity
1.	2-Pentadecyl-1,3-Dioxolane	Aromatic compound	Antibacterial and Antifungal activity
2.	Phenol, 2,4-Bis(1,1-Dimethylethyl)	Organic compound	Antifungal activity, Antimicrobial, UV stabilizer and an antioxidant for hydrocarbon-based product
3.	Hexadecanoic Acid, Methyl Ester	Fatty acid	Antioxidant, Hypercholesterolemic, Pesticide
4.	Hexadecanoic Acid, ethyl Ester	Fatty acid	Antioxidant, Hypocholesterolemic, Nematicide, Pesticide, Lubricant, Anti androgenic, Flavor, Hemolytic 5-Alpha reductase inhibitor
5.	Tetradecanoic Acid, Ethyl Ester	Fatty acid	Hypercholesterolemic
6.	Phthalic Acids	Organic acid	Antimicrobial activity
7.	9-Octadecenoic Acid (Z)-, Ethyl Ester	Fatty acid	Anti-inflammatory, Anticancer, hypocholesterolemic, 5-Alpha reductase inhibitor, and anti androgenic activity

CONCLUSION

In the present study, twenty nine compounds were identified by GC-MS analysis in the total polar lipid content of *Nostoc muscurum*. Identified compounds with bioactive properties were mostly organic acids. A higher percentage of hydrocarbons was also found in the fraction which might draw attraction from fuel industries. Several research findings have also shown that fatty acids and lipid of several strains of cyanobacteria has various pharmacological applications ranged from antimicrobial, antioxidant and antitumor activities. However, isolation and screening of the individual bioactive components to biological activity and toxicity profile will give more fruitful results.

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CONFLICT OF INTERESTS

Declared none

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