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Characterization of Compounds in Flanonoidal Fraction of Methanolic Extract *Nostoc Muscorum* Cultured With & Without NH_4Cl

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Abstract

Nostoc muscorum is an oxygenic photosynthetic, obligate photo autotrophic, gram negative, free living green-brown coloured cyanobacteria found in both terrestrial and aquatic environmental condition. It is rich in secondary metabolites but least investigated. The present investigation was aimed at extraction of bioactive chemical mixture from *N.muscorum* grown in media with and without ammonium chloride. The species was used to culture in Chu no.10 medium then its dried fine powder was macerated methanol for extraction. The percentage yield of extraction of *N. muscorum* cultured in diazotrophic and Non-diazotrophic environment with methanol was reported as 4.8% and 3.9% respectively, while their TFC were reported as 1.014 and 1.136 mg/100mg respectively. Upon column chromatography, fraction 5 at 29-35 ml elution of *N.muscorum* grown in diazotrophic media while fraction 6 at 36-45 ml elution of *N.muscorum* grown in non-diazotrophic media were observe to have flavonoidal content with respect to $AlCl_3$ Test on TLC plate. With reference to IR, NMR and mass spectral analysis, the isolated compound from methanolic extract of *N.muscorum* grown in diazotrophic media was characterized as 3-(4-carboxy-3-(methoxymethyl)phenyl)-6-(methoxy-methyl)-2-oxo-4-((2-oxo-2H-pyran-6-yl)oxy)-2H-chromene-7-carboxylic acid while that which isolated from *N.muscorum* grown in non-diazotrophic media was characterized as 6-ethoxy-3-(4-ethoxyphenyl)-5,7-dihydroxy-4-oxo-2-phenoxy-4H-chromene-8-carboxylic acid. In order to evaluate biological and pharmaceutical potential of these compounds further extensive investigations are needed based *in vitro*, *in vivo* biological studies.

Key Words: *Nostoc muscorum*, Cyanobacteria, Metabolites, Non-diazotrophic, Diazotrophic

INTRODUCTION

Secondary metabolites have most likely been used as anti-infective drugs. Anti-infective secondary metabolites were worth 55 billion dollars in 2000, but by 2007, the antibiotic market had grown to 66 billion dollars (Barber, *et al*., 2004; Demain & Sanchez, 2009). Antibiotics have saved many lives and contributed to an increase in life expectancy. Antibiotics are abundant in nature, where they play an important role in regulating the microbial population of soil, water, sewage, and compost (Sethi,

et al., 2013). Antibiotics are typically obtained from bacteria, actinomyces, fungi, and chemically synthesised antibiotics, which are also used in the current scenario.

The diverse group of prokaryotic microorganisms known as cyanobacteria, or blue-green algae, is present in almost all terrestrial and freshwater habitats (Potts, 2002). It can perform oxygenic photosynthesis and is an important source of food for other organisms (Rizvi, *et al*., 2018).In this case, cyanobacteria are the least exploited species. Their secondary metabolites are effective against a wide range of pathogens. Metabolites active against pathogenic bacteria, fungi, and algae are produced by both marine and freshwater species. A few of them have antiviral properties (Singh, *et al*., 2020).

Nostoc muscorum is a green-brown, gram-negative, oxygenic photosynthetic, obligate photoautotrophic cyanobacterium that can be found in both terrestrial and aquatic environments (Blumwald and Tel-Or,1982; Komarek and Anagnostidis, 1989; Dodds,*et al*., 1995). *Nostoc muscorum* is heterocystic and filamentous organism. Cells are cylindrical, spherical, or ovoid in shape, and colonies can grow to be up to 20 cm in diameter (Rizvi,*et al*., 2018). Sugar products provide osmoregulatory activity in salt tolerance in *N. muscorum*, making it tolerant to saline environments. As a result, the ideal pH range for *N. muscorum* is 7.0 to 8.5, with a lower pH limit of 5.7. (Blumwald and Tel-Or, 1982). Although *N. muscorum* can continue to grow and fix nitrogen in the presence of glucose and no sunlight, it grows best in light intensities lower than those of direct sunlight (Allison,*et al*., 1937). Because it is high in protein and fibre, it is also used as a food supplement in the food industry and in biotechnological applications (Rizvi,*et al*., 2018).With reference to the facts and information gathered as above, the present investigation was aimed at characterization of flavonoidal bioactive compound from methanolic extract of a pre-isolated strain of *Nostoc muscorum* grown in media with and without ammonium chloride.

MATERIALS & METHODS

Sample Collection

A pre-isolated *Nostoc muscorum* strain, which was available in the Division of Microbiology, Department of Botany, Govt. M.V.M. Bhopal, M.P., India, was used as a sample for mass culture and extraction of bioactive chemical mixture of Cyanobacteria.

Mass Culture of *N. muscorum*

This cyanobacteria strain was cultured in Chu no.10 medium (Gerloff *et al*., 1950).According to the procedures used and recommended by Bilos *et al*., (2016), Tamburic *et al*., (2011), and Huang *et al*., (2017), mass cultivation of cyanobacteria species was carried out with some suitable modifications in light of the current situation.There were two variants of the Chu no. 10 medium was used for mass culture of *Nostoc muscorum*, one is with present of ammonium chloride (NH_4Cl) in the medium and other is without ammonium chloride in the medium. The pure cultures of *N.muscorum* species was inoculated first in sterilized liquid medium in test tubes in first step. The tubes were incubated on culture racks that provides 16/8 light and dark period with 2000 lux intensity supported by cool florescent tubes and maintained at 25±3^oC temperature for 15 to 20 days.Step by step upscaling procedure was followed to mass cultivate the *N. muscorum* species in higher volume flasks which took 3 to 4 months.

After that, filter screen cloth no. 200 and Whatman Filter paper no. 1 were used to re-filter the liquid medium containing mass-cultured *N. muscorum* cells. The collected biomass filter paper was then shade dried at room temperature to obtain *N. muscorum* flacks.

Extraction of Bioactive Chemicals

In present work, the dried flaks of *N. muscorum* were first pulverized or crushed into fine powder then was subjected maceration using pure chloroform and methanol as solvents separately for the extraction of bioactive chemicals present in it in accordance with Kokate, (1994); Khandelwal, (2005); Mukherjee, (2007). 50 gram of dried plant materials was exhaustively extracted with 100 ml of selected solvents for 24 to 48 hours. After the process, the marc was removed by filtration from liquid which is further subjected to evaporation in water bath at 50^{o}C to obtain a concentrate mixture of extracted chemical constituents in each case.

Estimation of Total Flavonoid Content

The total flavonoid content in methanolic extract of plant material was estimated using the methods described by Miliauskas *et al*., (2004) and Marinova *et al*., (2005), with references to the literatures, by aluminium chloride complexation followed by spectrophotometric analysis of the reaction mixture with some modifications suitable for the current experimental conditions (Chandra, *et al*., 2014).

0.6 ml of suitably diluted extract was mixed with 0.6 ml of 2% aluminum chloride followed by incubation for 60 minutes at room temperature. Thereafter absorbance was taken at 420 nm wavelength against blank in Single beam visible range digital microprocessed spectrophotometer (Electronic India model EI-2305). The absorbance readings were compared with concentration vs absorbance standard plot of standard flavonoid Quercetin (HiMedia India) starting from 25µg/ml upto5µg/ml concentration.

The concentration of total flavonoid content in the test samples was calculated from the calibration plot ($Y=0.030X + 0.039$, $R^2=0.999$) and expressed as µg/mg quercetin equivalent (QE)/mg of dried plant extract.

Fractionation of Bioactive Chemicals

Column Chromatography: Silica gel column chromatography was adapted to get the flavonoidal fraction of the methanolic extracts of *Nostoc muscorum* biomass cultured in medium with and without NH_4Cl. The column (40×4 cm) was prepared by first activating silica gel (60-120 mesh size) at 105^{o}C which was then suspended it in mobile phase (Toluene: Ethyl Acetate, 5:4v/v) and transfers it in glass column. After settling of silica gel 2.5 gm of extract was mixed with silica gel and sufficient amount of mobile phase in a beaker separately. This slurry was then introduced to column top over cotton plug. A mixture of Toluene: Ethyl Acetate (5:4) as mobile phase is used for elution of each 10 ml fraction which were collected, concentrated, stored and subjected to TLC for detection of desired fraction.

Identification of Flavonoindal Fractions: The different fractions of column chromatographic elution were monitored by TLC Toluene: Ethyl Acetate (7:3); using UV chamber and derivatization with specific reagent for identification of single isolated compound with comparison of reference compounds. The fractions which show similar fingerprinting profile on TLC were collected and mixed. Fraction showed single compound and have similar R_f value as compared to reference compound were dried, compounds were purified by recrystalization procedure.

Characterization of Isolated compound

The 10 ml fractions after column chromatography which was reported to be positive for presence of flavonoids in TLC analysis were collected and subjected to characterize by FTIR, NMR and Mass Spectra studies.

RESULTS & DISCUSSION

Extraction Yield and Total Flavonoidal Content

The percentage yield of extraction of *Nostoc muscorum* extract is depicted in table 2 which indicates that in case of *N.muscorum* grown in media without NH_4Cl have higher yield of extraction as 4.8% compared to that grown in media containing NH_4Cl which is 3.9% when extracted with methanol by maceration.

Table 2: Results of percentage yield of extraction in methanol for *Nostoc muscorum* cultured in diazotrophic and Non-Diazotrophic environment in present study

Extracts of *N. muscorum*	Percentage yield (%) in Methanolic Extraction
Grown in diazotrophic	4.8%
Grown in Non-Diazotrophic	3.9%

Total flavonoids content in extracts was calculated as quercetin equivalent (µg/mg)by comparing the absorbance of reaction mixture with calibration curve based on the standard concentrations whose reading are mentioned in table 3 and its respective calibration curve as depicted in figure 1. The calibration curve equation used was as follows:

$Y=0.030X + 0.039$, $R^2=0.999$,

Where: X = absorbance and Y = quercetin equivalent (QE).

Table 3: Quercetin as standard concentration vs absorbance at 420 nm to plot standard curve for estimation in samples Using $AlCl_3$ precipitation Method.

S.N.	Concentration (µg/ml)	Absorbance (λ)
1	5	0.191
2	10	0.348
3	15	0.514
4	20	0.652
5	25	0.812

Instrument Used: Single beam visible range digital microprocessed spectrophotometer from Electronic India model EI-2305.

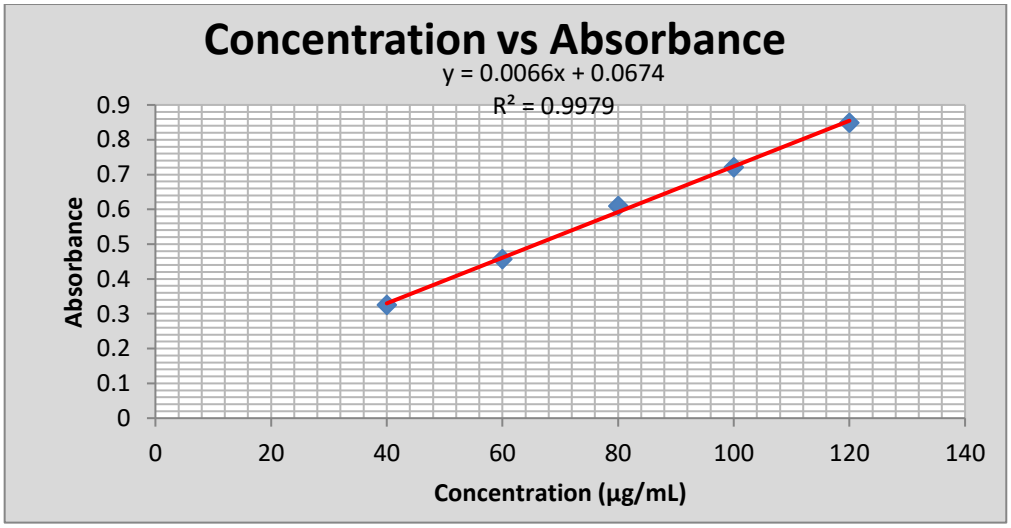


Figure 2: Standard Plot for known concentration of *Quercetine Standard*. The Graph is obtained from Excel 2013 linear regression function

In order to fit the absorbance reading in the standard curve, the 1 mg/ml concentration working solution of each extract of *N.muscorum* biomass culture in non-diazotrophic and diazotrophic medium was 100 times diluted in reaction mixture with distilled water. The total flavonoidal concentration in mg/100 mg of each extract is depicted in table 4 and figure 2 in a comparative manner based on the absorbance reading compared to the standard curve plot.

The quercetin equivalent total flavonoid concentration was observed to be highest in methanolic extracts of both *N.muscorum* cultured in both non-diazotrophic and diazotrophic medium according to earlier investigation done connected to this study. However, the quercetine equivalent flavonoidal content in methanolic extract of *N.muscorum* cultured in non-diazotrophic medium was reported to have higher compared to that culture in diazotrophic i.e. without NH_4Cl where the the TFC values are reported to be 1.136 and 1.014mg/100 mg extract respectively.

Table 4: Comparative values of total flavonoid content in methanolic extracts of *N.muscorum* biomass culture in diazotrophic and non-diazotrophic mediumin present study.

S.N.	**Extract *N. muscorum***	**Total Flavonoid Content in Methanlic Extracts (in mg/100mg dried extract)**
1.	culture in diazotrophic medium	1.014
2.	culture in non-diazotrophic medium	1.136

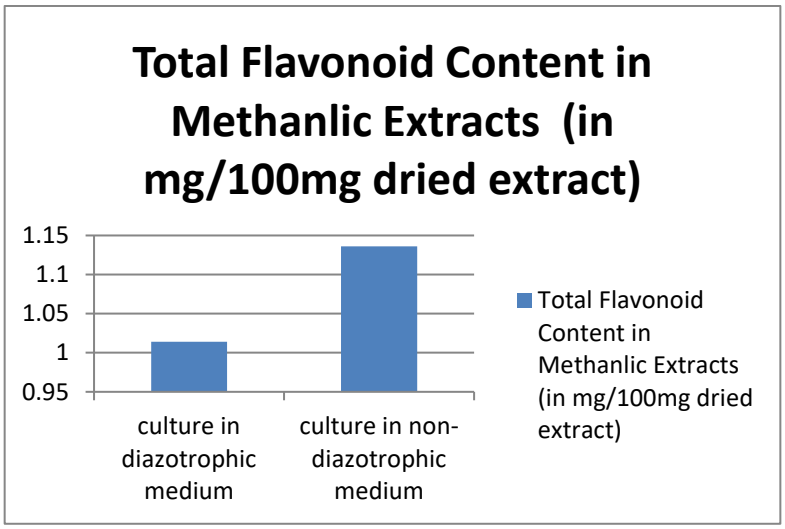


Figure 2: Graphical comparison of total flavonoidal content in methanolic extracts of *N.muscorum* biomass culture in diazotrophic and non-diazotrophic medium.

Characterization of Flavonoidal Fraction of Extracts

It was observed that the 10 ml fraction 5 at 29-35 ml elution of methanolic extract of *N.muscorum* biomass grown in diazotrophic medium *i.e.*, without NH_4Cl while 10 ml fraction 6 at 36-45 ml elution of methanolic extract of *N.muscorum* biomass grown in non-diazotrophic medium were positive for$AlCl_3$ Test on TLC plate which is the indicator of presence of flavonoid in the fraction. The two fractions from both types of extracts were used to characterization of compound with the help of IR, NMR and Mass Spectra analysis. The spectral analysis of IR, NMR and Mass Spectra analysis of fractionsare depicted in figure 3, figure 4, figure 5, figure 6, figure 7, and figure 8 respectively.The interpretation of the data of different spectra of isolated compound, their structure and nomenclature in two fractions are depicted in table 5 and table 6.

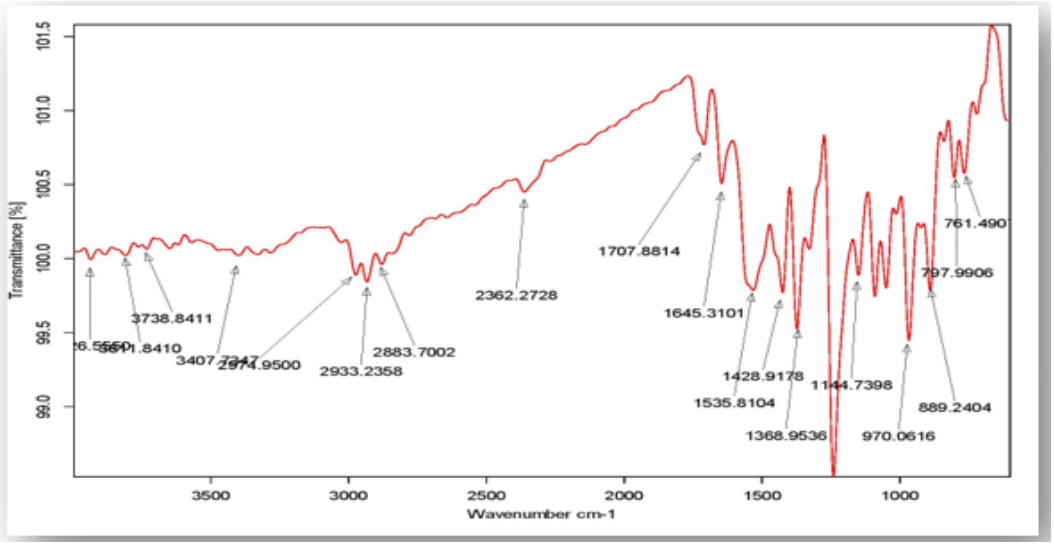


Figure 3: IR Spectra of fraction 5 at 29-35 ml elution for methanolic extract of *Nostoc muscorum* biomass, cultivated in diazotrophic medium.

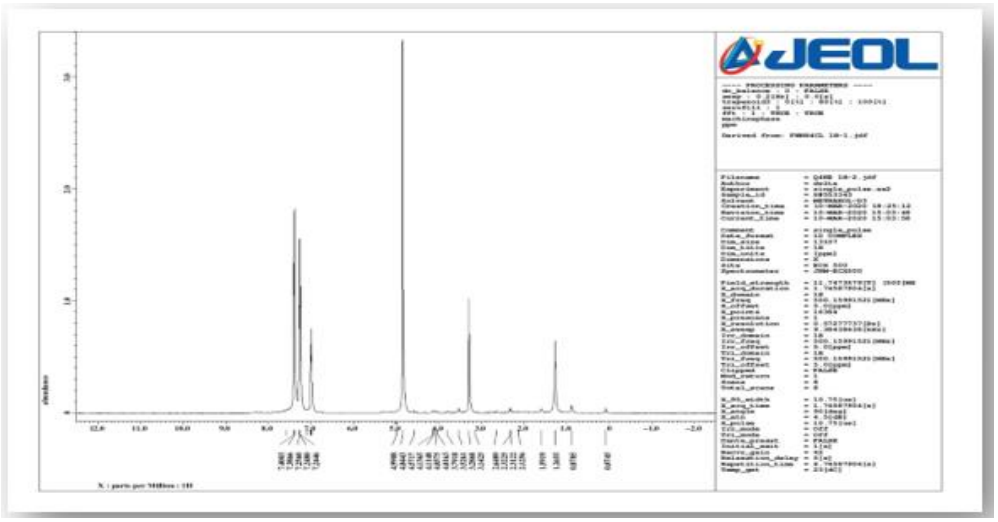


Figure 4: NMR Spectra of fraction 5 at 29-35 ml elution for methanolic extract of *Nostoc muscorum* biomass, cultivated in diazotrophic medium.

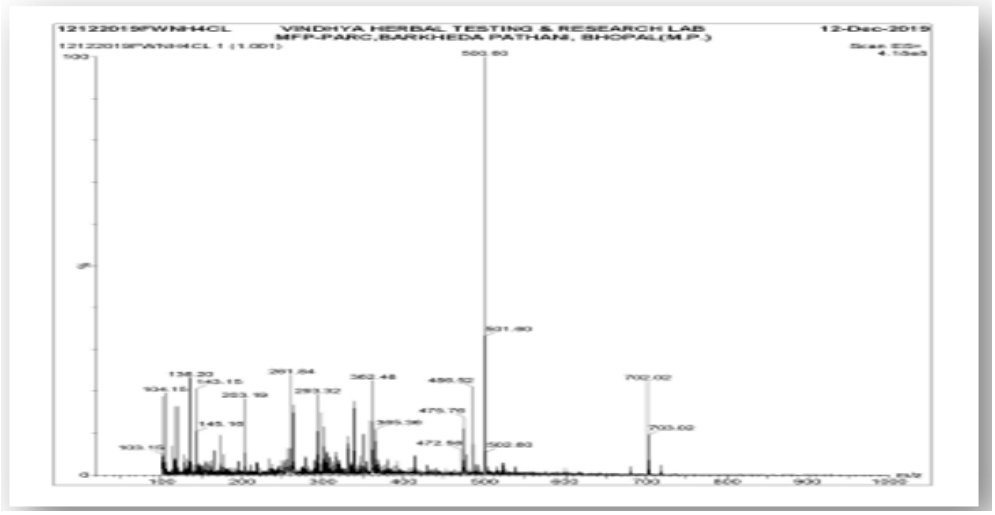
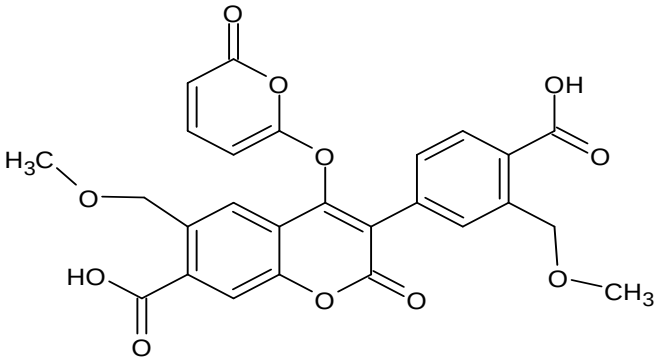


Figure 5: Mass Spectra of fraction 5 at 29-35 ml elution for methanolic extract of *Nostoc muscorum* biomass, cultivated in diazotrophic medium.

Table 5: Interpretation of different spectra of fraction 5 at 29-35 ml elution for methanolic extract of *Nostoc muscorum* biomass, cultivated in diazotrophic medium.

Analysis	Spectral Interpretation
IR	2974, 2933(doublet) & 2831(C-H str.), Alkane 1707(C=O) Ketone 1645 (C=O str.) Carboxylic acid (dimerization) 1614 (C=Cstr.) α-β Unsaturated Ketone 1428 (O-H) bend Carboxylic acid 1144 (O-C) Aryl alkyl ether
^{1}HNMR (ppm)	**^{1}H NMR (METHANOL-D3 - 500Mz) δ:**3.28(s, 6H, O-C**H**$_3$), 4.84 (s, 4H, C**H**$_2$), 6.99-7.00 (m, 3H,C**H**-Ar), 7.24-7.25 (m, 2H,*J*= 1.7, 9, C**H**-Ar), 7.40 (m, 2H,C**H**-Ar).
MASS (m/z %)	**ESI-MS (m/z):**502
Structure	
Empirical Formula	**C$_{26}$H$_{20}$O$_{11}$**
Nomenclature	3-(4-carboxy-3-(methoxymethyl)phenyl)-6-(methoxymethyl)-2-oxo-4-((2-oxo-2H-pyran-6-yl)oxy)-2H-chromene-7-carboxylic acid

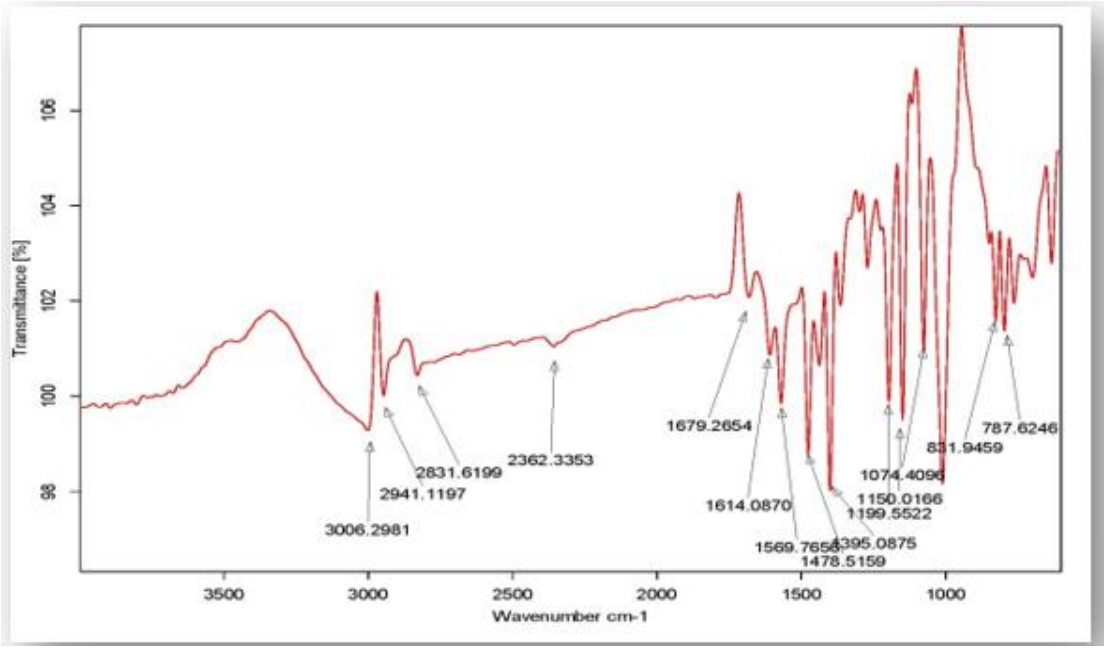


Figure 6: IR Spectra of fraction 6 at 36-45 ml elution for methanolic extract of *Nostoc muscorum* biomass, cultivated in non-diazotrophic medium.

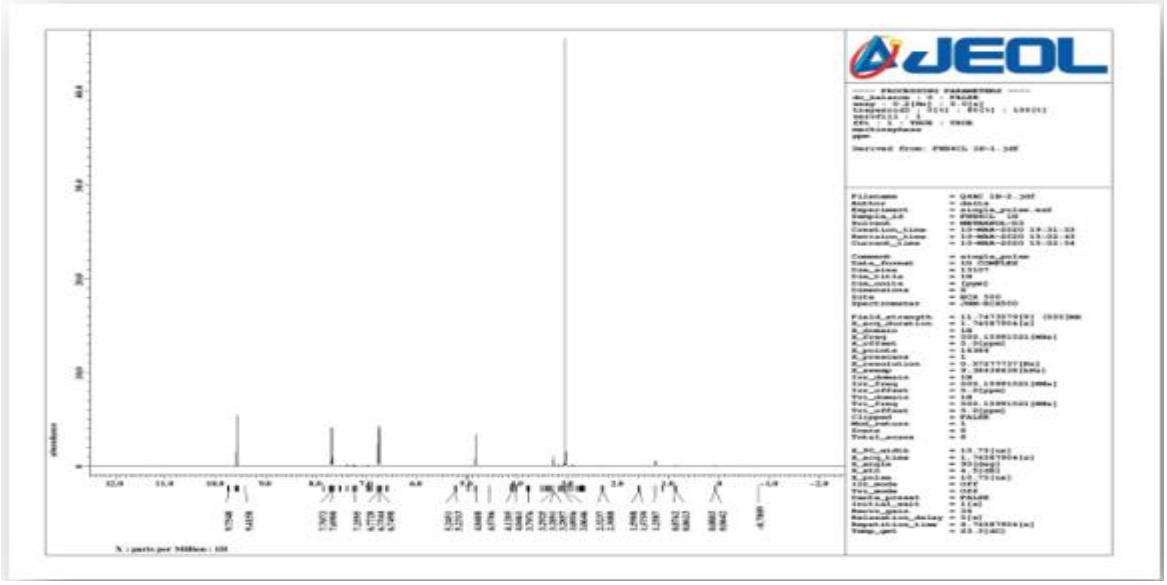


Figure 7: NMR Spectra of fraction 6 at 36-45 ml elution for methanolic extract of *Nostoc muscorum* biomass, cultivated in non-diazotrophic medium.

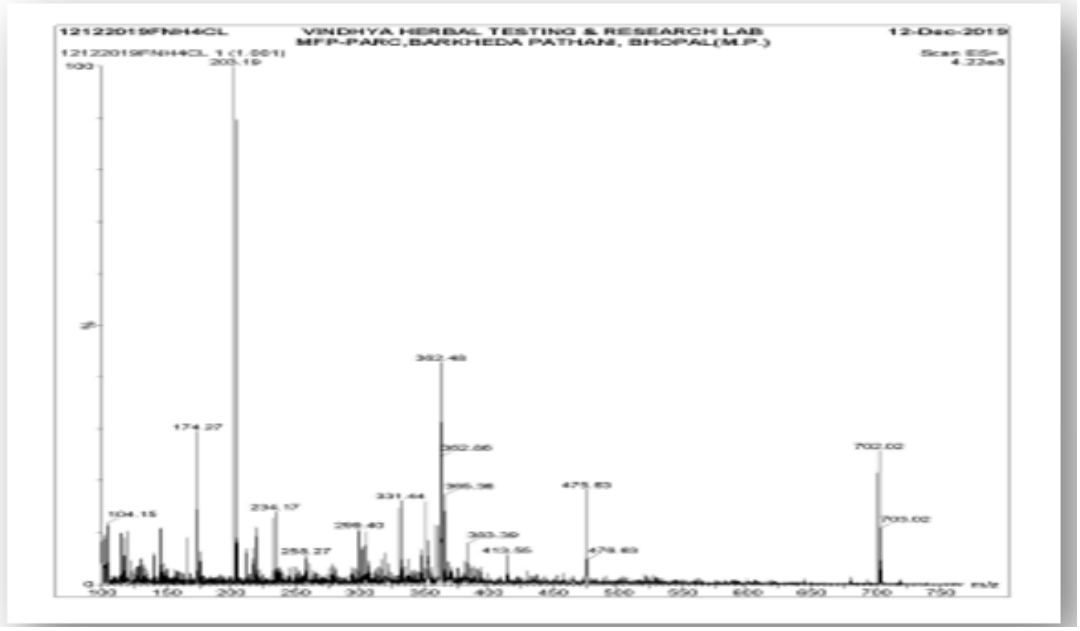
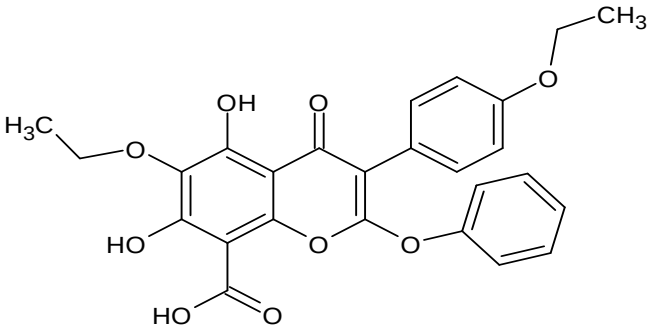


Figure 8: Mass Spectra of fraction 6 at 36-45 ml elution for methanolic extract of *Nostoc muscorum* biomass, cultivated in non- diazotrophic medium.

Table 6: Interpretation of different spectra of fraction 6 at 36-45 ml elution for methanolic extract of *Nostoc muscorum* biomass, cultivated in non-diazotrophic medium.

Analysis	Spectral Interpretation
IR	3006 (O-H str.) Carboxylic acid, 2941& 2831(C-H str.), Alkene 1679 (C=C str.) Alkene 1614 (C=Cstr.) α-β Unsaturated Ketone 1478 (C-H) Methylene 1199 (O-C) Aryl alkyl ether
^{1}HNMR (ppm)	**^{1}H NMR (METHANOL-D3 - 500Mz) δ:**3.09(t, 9H, O-C**H$_3$**), 4.84 (d,d, 4H, C**H$_2$**), 6.77 (d, 2H, *J*= 9.2, C**H**-Ar), 7.70 (d, 2H,*J*= 8.6, C**H**-Ar), 9.58 (s, 1H,C**H**-Ar).
MASS (m/z %)	**ESI-MS (m/z):** 480
Structure	
Empirical Formula	**C$_{26}$H$_{22}$O$_9$**
Nomenclature	6-ethoxy-3-(4-ethoxyphenyl)-5,7-dihydroxy-4-oxo-2-phenoxy-4H-chromene-8-carboxylic acid

It is important to note that the biological activity of both known and undiscovered cyanobacteria metabolites is relevant to many applications, including those in the medical industry. As a result, there is an increasing interest in detecting these molecules in various sample species and determining the dynamics of their production (Macário, *et al*., 2022). The ability to detect, identify and quantify a wide variety of metabolites in intricate biological matrices makes metabolomics methods effective for this purpose. Mass Spectrometry (MS), which is usually preceded by some kind of chromatographic separation, and Nuclear Magnetic Resonance (NMR) spectroscopy are the most commonly used analytical techniques in metabolomics (Schwarz, *et al*., 2013; Macário, *et al*., 2022). Macário, *et al*., (2022) utilized high resolution 1H NMR to evaluate the metabolic constituents of the freshwater cyanobacterium *N.muscorum* were they spotted numerous sugars and oligosaccharides, lipids (e.g., glycolipids, ω-3 and ω-6 fatty acids), amino acids, along with mycosporin-like, peptides, and pigments (e.g., chlorophyll a and carotenoids) like fascinating metabolites.With the help of Gas Chromatography-Mass Spectrometry (GC-MS)Yasin, *et al*., (2018)identified bioactives like Benzofuranone derivatives, Myristoleic acid, Resorcinol, Citronellylbutyrates, hydroquinone, hexadecanoic acid, and farnesol in the Diethyl ether, Dichloromethane, Ethyl acetate and Heptane like organic extracts of cyanobacterium *Nostoc muscorum*NCCU-442. A direct search for biologically active compounds as well as their production by algal or cyanobacterial culture requires some understanding of the culture conditions favouring their production. Almost all of the biologically active compounds of interest are secondary metabolites and thus are usually most abundant in stationary phase or in slow-growing cultures or the type of nutritional supplements present in the culture medium (Borowitzka, 1995). In present investigation, it was observed that, *N.muscorum* when cultured in different environmental conditions like with and without NH_4Cl in medium when extracted with methanol could produce structurally diverse type of bioactive metabolites.

CONCLUSION

Based on present experimental work, it is again cleared that the *N. muscorum* is grown in non-diazotrophic and diazotrophic environment are the rich sources of bioactive compounds where methanolic extracts of *N. muscorum* culture in non-diazotrophic medium have considerably higher total flavonoidal content. However *N.muscorum* when cultured in different environmental conditions like with and without NH_4Cl in medium when extracted with methanol could produce structurally diverse type of bioactive metabolites. In order to evaluate biological and pharmaceutical potential of these compounds further extensive investigations are needed based *in vitro*, *in vivo* biological studies.

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