Original Article

Phytochemical Analysis And Antimicrobial Activity Of Nostoc Muscorum Extracts Against E.Coli And S.Bongori

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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 *Nostoc 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 chloroform and methanol for extraction. The extracts were tested for various phytochemical groups present in there according to Haborne. The percentage yield of extraction of *N. muscorum* cultured in Non-diazotrophic and Diazotrophic environment with chloroform was reported as 3.6% & 4.5% respectively while that extracted in methanol was 4.8% and 3.9% respectively. All extracts are rich in alkaloids, flavonoids, carbohydrates and diterpenes in particular upon preliminary chemical test. The total flavonoidal content of *N. muscorum* cultured in Non-diazotrophic and Diazotrophic environment of chloroform was reported as 0.954 and 0.897 mg/100mg respectively, while that extracted in methanol was 1.014 and 1.136 mg/100mg respectively. The antimicrobial activity for chloroform extract of *N.muscorum* cultured in Non-diazotrophic environment showed 6±0, 7±0.47 and 8±0.47 mm zone of inhibition against *E. coli* (MTCC-1687) at 25, 50 and 100 mg/ml dilutions used respectively. While against *S. bongori* (MTCC-3858) it was 8±0.47, 10±0.47 and 12±0.47 respectively. Again, for chloroform extract of *N.muscorum* cultured in Diazotrophic media showed 7±0.47, 9±0.47 and 9±2.49 mm zone against *S. bongori* (MTCC-3858) whereas its methanolic extract showed 6±0.23, 8±0.47 and 9±0.47 mm zone against *E. coli* (MTCC-1687) at 25, 50 and 100 mg/ml dilutions used respectively. In order to have profound biological activity of *N. muscorum* in order to develop novel therapeutic compounds with least side effects, further extensive investigations are needed based on high end techniques.

Keywords: *Nostoc muscorum*, Cyanobacteria, Antimicrobial activity, Non-diazotrophic, Diazotrophic

INTRODUCTION

Probably, the most important use of secondary metabolites has been as anti-infective drugs. In the year 2000, anti-infective secondary metabolites marketed 55 billion dollars, but in the year 2007, the market for antibiotics increased to 66 billion dollars (Barber, *et al*., 2004; Demain & Sanchez, 2009). Antibiotics have saved a large number of lives and also contributed to the increase in life expectancy. The antibiotics are widely distributed in the nature, where they play an important role in regulating the microbial population of soil, water, sewage, and compost (Sethi, *et al*., 2013). The antibiotics are being usually obtained from bacteria, actinomyces, fungi and chemically synthesized antibiotics also used in present scenario.

Cyanobacteria, also known as blue-green algae include a highly diverse group of prokaryotic microorganisms and widely distributed in nature and can be found in most terrestrial and freshwater habitat (Potts, 2002). It can able to perform oxygenic photosynthesis and used as an important food for other organisms (Rizvi, *et al*., 2018). Cyanobacteria are the least exploited species in this case. Their secondary metabolites are active against many known pathogens. Both marine and freshwater species synthesize metabolites active against pathogenic bacteria, fungi, and algae. A few of them have an antiviral effect (Singh, *et al*., 2020).

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 (Blumwald and Tel-Or, 1982; Komarek and Anagnostidis, 1989; Dodds,*et al*., 1995). *Nostoc muscorum* is filamentous and having heterocyst. The shape of cells is cylindrical, spherical or ovoid and size of colonies are reached upto 20 cm in diameter (Rizvi, *et al*., 2018). *N.*

muscorum are tolerant to saline environments, with sugar products providing osmoregulatory activity in salt tolerance. So, the ideal environment for *N. muscorum* on the pH in the range of 7.0 to 8.5, with a lower pH limit of 5.7 (Blumwald and Tel-Or, 1982). *N. muscorum* grows best when light intensity is less than that of direct sunlight, but can continue to grow and fix nitrogen in the presence of glucose and absence of sunlight (Allison, *et al*., 1937). It is rich in protein and fiber content so it is also used as food supplements in food industries and in biotechnological applications (Rizvi, *et al.,* 2018). With reference to the facts and information gathered as above, the present investigation was aimed at extraction of bioactive chemical mixture from a pre-isolated strain of *Nostoc muscorum* grown in media with and without ammonium chloride followed by screening their antimicrobial activity against Gram –ve enteric pathogenic bacteria.

MATERIALS & METHODS

Sample Collection

For mass culture and extraction of bioactive chemical mixture of Cyanobacteria, a pre-isolated *Nostoc muscorum* strain was used which was available in Division of Microbiology Department of Botany Govt. M.V.M. Bhopal, M.P., India. While the test MTCC bacterial cultures of *Escherichia coli* (MTCC-1687) and *Salmonella bongori* (MTCC-3858) were resourced from IMTECH Chandigarh.

Mass Culture of *N. muscorum*

This strain of cyanobacteria was cultured in Chu no.10 medium (Gerloff *et al*., 1950).The mass cultivation of cyanobacteria species was done as per the methods followed and suggested by Bilos, *et al.,* (2016), Tamburic, *et al*., (2011), and Huang, *et al*., (2017) with some suitable modification as per the present condition.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.

The liquid medium containing mass cultured *N. muscorum* cells were then filtered by first passing it through filter screen cloth no. 200 followed by re-filtration by Whatman Filter paper no.1. The collected biomass filter paper was then used to shade dry at room temperatures to get the flacks of *N. muscorum*.

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). 50gram 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^oC to obtain a concentrate mixture of extracted chemical constituents in each case.

Preliminary Chemical Analysis of *N. muscorum* Extract

Chemical tests were carried out for different extracts to detect the presence of bioactive components in them by using standard methods described by Harborne (1973; 1998) and Sofowora, (1993). A small portion of the dry extracts were subjected to test the presence of carbohydrates, proteins, alkaloids, terpenoids, saponins, phenols, flavonoids and glycosides (Tenguria,*et al*., 2012; 2014).Following tests were conducted in preliminary analysis;

Test for alkaloids: About 100 µl of stock was diluted with distilled water and few drops of Dragendorff's reagent added orange red precipitate indicates the presence of alkaloids.

Test for tannins: small quantity of extracts mixed with water, heated, filtered and ferric chloride added. A dark green solution indicates the presence of tannins.

Test for terpenoids: About 100 µl of stock was diluted with distilled water followed by mixed with 2ml chloroform ($CHCl_3$) and concentrated H_2SO_4 (3ml) was carefully added to form a layer. A reddish-brown coloration of the interface formed indicating the presence of terpenoids.

Test for saponins: About 100 µl of stock was diluted with 5 ml of distilled then warmed a little then shaken with 5ml of distilled water and then heated to boil frothing (appearance of creamy mix of small bubbles) shows the presence of saponins.

Test for flavonoids: About 100 µl of stock was diluted with distilled water they 2-3 drops of 10% lead acetate was added. A creamy white dirty precipitate indicates the presence of flavonoids.

Test for glycosides: Benedict reagent was used to check the presence of glycosides in diluted extract. The diluted extract was first warmed they 2-3 drops of Benedicts reagent were added to the reaction tube. The appearance of yellow or orange precipitate indicates the presence of glycoside in extract.

Estimation of Total Flavonoid Content

The estimation of total flavonoid content in ethanolic extract of plant material was done in accordance with the methods described by Miliauskas, *et al*., (2004) and Marinova, *et al*., (2005) with reference to the literatures by aluminum chloride complexation followed by spectrophotometric analysis of reaction mixture with some modification suitable for present 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 micro processed 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.

In vitro Antimicrobial Activity of Extract

The antimicrobial activity of extracted chemical mixture of cyanobacteria in present study were performed by most widely used agar well diffusion method for antimicrobial activity (Magaldi, et al., 2004). For assay, Nutrient Agar media plates were prepared (Himedia). The already prepared standard inoculum broth of test bacterial cultures were applied on to the solid media plates with the help of sterile swabs all over in a pattern to raise lawn culture. After this, well of approx. 6 mm diameter were aseptically punched by wide open side of sterile microtip of 100 µl volume capacity. Now the wells were filled with 20 µl of extracts from stocks or its dilutions according to the test to be performed. In present study 3 different dilutions of each extract were used which were 25, 50 and 100 mg/ml.
The plates were than place in an incubator for 24 to 48 hours at 37ºC for incubation, after which the plates were observed for zone of inhibition which were measured in millimeter (mm) with the help of zone scale (Himedia). The antimicrobial sensitivity assay using agar well diffusion method as described by Vedhanarayanan, *et al.*, (2013). Separate plate with each pathogenic microorganism was used for each extract.

RESULTS AND DISCUSSION

Preliminary Chemical Analysis

The percentage yield of extraction of *Nostoc muscorum* extract is depicted in table 2 and its comparative graphical representation is depicted in figure 1

Table 2: Results of percentage yield of extraction in 2 different solvents for *Nostoc muscorum* cultured in Non-diazotrophic and Diazotrophicenvironment in present study

Extracts of *N. muscorum*	**Percentage yield (%) in different solvent extractions**	
	Chloroform Extract	**Methanolic Extract**
Grown in Non-diazotrophic	3.6%	4.8%
Grown in Diazotrophic	4.5%	3.9%

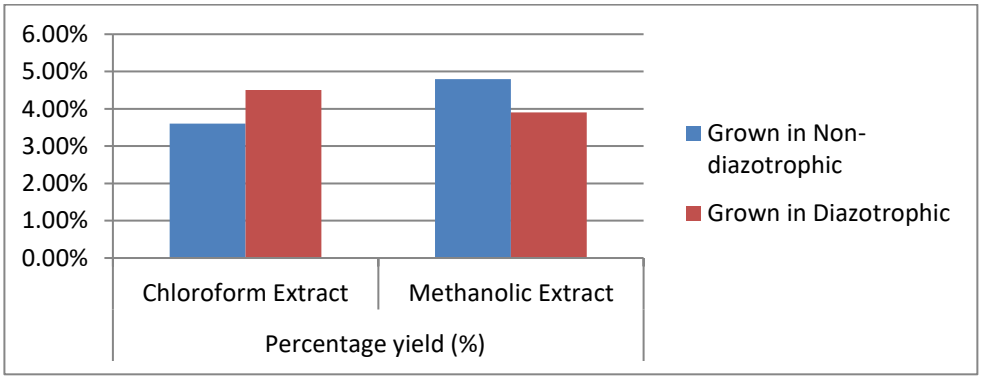

Figure 1: Comparative graphical representation of percentage yield of extraction of *Nostoc muscorum* cultured in Non-diazotrophic and Diazotrophicenvironment

Referring to table 2 and figure 1 it is clearly seen that the percentage yield of extraction from both Non-diazotrophic and Diazotrophically cultured *Nostoc muscorum* was observed be highest in methanolic extract of *N.muscorum* grown in Non-

diazotrophic environment, while lowest among them is of chloroform extracts. When we compare solvent wise, chloroform extract of *N.muscorum* grown in diazotrophic environment has higher percentage yield as 4.5% than thechloroform extract of *N.muscorum* grown in non-diazotrophic environment with3.6%.However, the inverse is true for the case of methanolic extacts.

Preliminary Chemical Analysis

Due reference to the tests applied according to Harborne (1973; 1998) and Sofowora, (1993). to detect the presence of bioactive components, the observed results of preliminary chemical analysis are depicted in table 3 and table 4in *N.muscorum* biomass cultured in non-diazotrophic and diazotrophic environment respectively. According to the outcomes of preliminary biochemical profile, both the bio masses are rich in variety of phytochemical groups especially alkaloids, flavonoids, carbohydrates and diterpenes for the case of *Nostoc muscorum* cultured in both diazotrophicand non-diazotrophic environment.

Table 3: Results of chemical screening of solvent extracts of *Nostoc muscorum* cultured in Non-Diazotrophicenvironment in present study

S.N.	Constituents Tested	Chloroform extract	Methanol extract
1.	**Alkaloids**	+ve	+ve
2.	**Glycosides**	–ve	–ve
3.	**Flavonoids**	+ve	+ve
4.	**Saponins**	–ve	+ve
5.	**Phenolics**	–ve	–ve
6.	**Carbohydrate**	+ve	+ve
7.	**Proteins**	–ve	–ve
8.	**Diterpenes**	+ve	+ve

Table 4: Results of chemical screening of solvent extracts of *Nostoc muscorum* cultured in Diazotrophic environment in present study

S.N.	Constituents Tested	Chloroform extract	Methanol extract
1.	**Alkaloids**	+ve	+ve
2.	**Glycosides**	–ve	–ve
3.	**Flavonoids**	+ve	+ve
4.	**Saponins**	–ve	+ve
5.	**Phenolics**	–ve	–ve
6.	**Carbohydrate**	+ve	+ve
7.	**Proteins**	–ve	–ve
8.	**Diterpenes**	+ve	+ve

Since the solvent extracts of Cyanobacteria were rich in variety of polyphenols hence, they have the ability to impart the pharmacological activity.

Estimation of Total Flavonoidal Content

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 5 and its respective calibration curve as depicted in figure 2. The calibration curve equation used was as follows:

$$\mathbf{Y=0.030X + 0.039,\ R^2=0.999},$$

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

Table 5: 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 micro processed spectrophotometer from Electronic India model EI-2305.

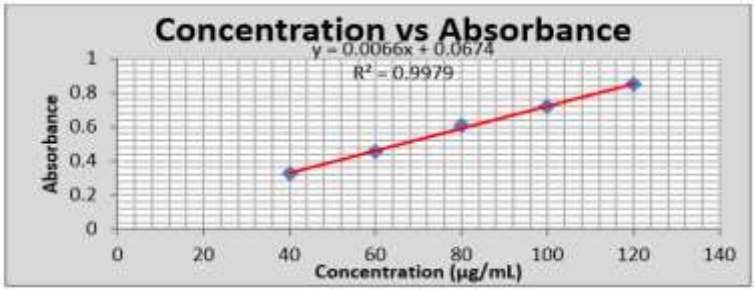

Figure 2: Standard Plot for known concentration of Quercetine Standard. The Graph is obtained from Excel 2013 linear regression function

For estimation of flavonoidal concentration, the1 mg/ml concentration working solution of each extract of *N.muscorum* biomass culture in non-diazotrophic and diazotrophic mediumwas 100 times diluted in reaction mixture with distilled water in order to fit the absorbance reading in the standard curve. Based on the absorbance reading compared with standard curve plot, the total flavonoidal concentration in mg/100 mg of each extract is depicted in table 6 and figure 3 in a comparative was.

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. The *N.muscorum* cultured in diazotrophic medium was reported to have higher TFC (1.136mg/100 mg) than non-diazotrophic (1.014mg/100 mg) in case methanolic extract. The lowest of TFC was observed in chloroform extracts *N.muscorum* cultured in diazotrophic medium which is reported to be 0.897mg/100 mg while that of which was grown in non-diazotrophic medium has 0.954mg/100 mg values as TFC.

Table 6: Comparative values of total flavonoid content in 2 types of extracts of *N.muscorum* biomass culture in non-diazotrophic and diazotrophic mediumin present study.

S.N.	Extract	Total Flavonoid Content in Extracts (in mg/100mg dried extract)	
		TFC in extracts of *N. muscorum* culture in non-diazotrophic medium	TFC in extracts of *N. muscorum* culture in diazotrophic medium
1.	**Chloroform**	0.954	0.897
2.	**Methanol**	1.014	1.136

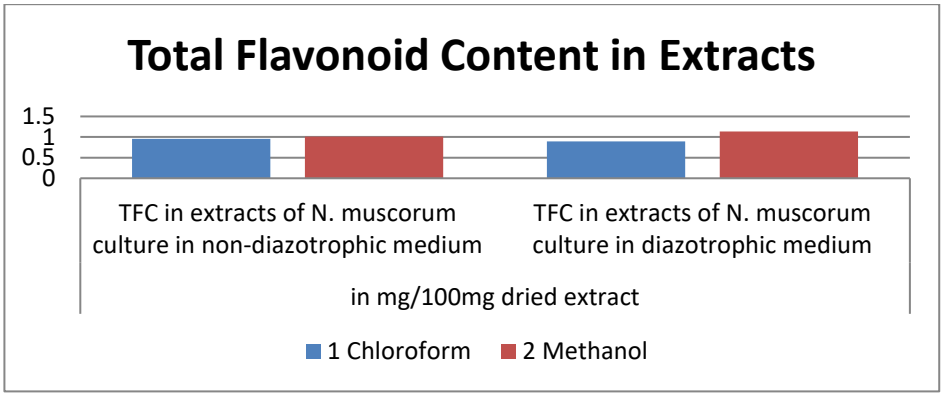

Figure 3: Graphical comparison of total flavonoidal content in 4 different extracts of *N.muscorum* biomass culture in non-diazotrophic and diazotrophic medium.

In vitro Antimicrobial Activity

The results of antimicrobial activity of chloroform and methanolic extracts of *N.muscorum* biomass cultured in non-diazotrophic and diazotrophic environment are depicted table 7 and table 8 respectively. It is clearly observed from the results as depicted in table 7 that the chloroform extracts of *N.muscorum* biomass, imparted inhibitory action against the both the test bacterial strains used in present investigation.

Table 7: Observations of sensitivity of test bacterial strains towards Chloroform and Methanolic Extract of *N.muscorum* biomass cultured in Non-diazotrophic environment

S.N.	Extract Concentration Used	Zone of inhibition (in mm) due to Chloroform Extract of *N.muscorum* against		Zone of inhibition (in mm) due to Methanolic Extract of *N.muscorum* against	
		E. coli	***S. bongori***	***E. coli***	***S. bongori***
1.	**25mg/ml**	6±0	8±0.47	**nil**	**nil**
2.	**50 mg/ml**	7±0.47	10±0.47	**nil**	**nil**
3.	**100mg/ml**	8±0.47	12±0.47	**nil**	**nil**

***(n=3, mean ± SD)**

Table 8: Observations of sensitivity of test bacterial strains towards Chloroform and Methanolic Extract of *N.muscorum* biomass cultured in diazotrophic environment

S.N.	Extract Concentration Used	Zone of inhibition (in mm) due to Chloroform Extract of *N. muscorum* against		Zone of inhibition (in mm) due to Methanolic Extract of *N. muscorum* against	
		E. coli	*S. bongori*	*E. coli*	*S. bongori*
1.	**25mg/ml**	**nil**	7±0.47	6±0.23	**nil**
2.	**50 mg/ml**	**nil**	9±0.47	8±0.47	**nil**
3.	**100mg/ml**	**nil**	9±2.49	9±0.47	**nil**

***(n=3, mean ± SD)**

The Chloroform Extract of *N. muscorum* biomass cultured in non-diazotrophic environment at lowest test concentration of 25mg/ml showed its lowest inhibitory activity against *E.coli* and *S. bongori* with a zone size of 6±0 mm and 8±0.47 mm respectively. 50 mg/ml of extacts imparted a zone of inibhition as 7±0.47 and 10±0.47 mm towards *E.coli* and *S. bongori* respectively while at 100 mg/ml of extact concentration, the zone of inhibition as 12±0.47 mm and 8±0.47 mm was observed against *S. bongori* and *E.coli* respectively. On the other hand the methanolic extracts were observed to have no inhibition of test bacterial strains at all. The antimicrobial efficacy of chloroform extract of *N. muscorum* biomass culture in diazotrophic environment is observed only in *S. bongori*. At the test concentration 25mg/ml, 50 mg/ml, and 100mg/ml of extract the zone of inhibition against the against *S. bongori* were 7±0.47 mm, 9±0.47 mm and 9±2.49 mm respectively. Again, with reference to table 8the methanolic extract of *N. muscorum* biomass culture in diazotrophic environment showed inhibitions towards *E. coli* only with 6±0.23 mm, 8±0.47 mm and 9±0.47 mm sized zone of inhibitions respectively.

El-sheikh *et al*., (2006) used BG-11 medium for cultivation of *N.muscorum* at pH 8.0 and 35ºC temperature whereas in present study Chu no 10 medium worked well for cultivation of *N.muscorum* successfully in environment with and without NH_4Cl. There are variety of bioactive chemical groups reported in present study in both type of *N.muscorum* biomass culture in environment with and without NH_4Cl including alkaloid, flavonoids, and diterpenes in particular as well as all extracts were reported to have enough amount of quercetin equivalent flavonoids to impart any biological activity. *Nostoc muscorum* synthesizes polymers such as the polyhydroxybutyrate thermoplastic that is used for clinical and industrial applications (Haase *et al*., 2012; Ansari and Fatma, 2016); and, unsaturated fatty acids of pharmaceutical interest like α-linolenic acid (18:3) (Kim *et al*., 2015). Additionally, the ultrastructure of *N. muscorum* can be exploited for the production of based-hydrogen fuel (Shah *et al*., 2003; Singh *et al.*, 2017).

Production of antimicrobial active substance under various growth conditions from the cyanobacterium *Nostoc muscorum* and the purification of the active components and elucidation of its chemical structure were done by El-sheikh and coworkers (2006). Bloor and England (1989) also report that the antibiotic produced by *N.muscorum* that inhibit the growth of bacteria, moreover, the increase in nitrate concentration of the medium led to an increase in the antimicrobial production. This is what observed in present study also. Arun *et al*., (2012) reported that the antimicrobial activity of methanolic extract of *N. muscorum* showed maximum zone of inhibition of 2.0 cm against *P. aeruginosa*, whereas it was 0.8 cm for B. cereus, *M. luteus* and *S.aureus*. On the other hand, *B.subtilis* and *K. pneumonia* were resistant to methanolic extract of *N. muscorum* in their study.

CONCLUSIONS

Cyanobacteria are one of the important organisms in terms of producing food, feed and pharmaceutical products however their different species are least investigated particularly *N. muscorum* (Pedersen and Dasilva,1973; Nowruzi, *et al*., 2018).Also the production of extract cellular metabolites is affected by variation in nutritional composition of the medium in which *N. muscorumis* grown (Bloor and England, 1991). 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 chloroform and methanolic extracts have show considerable antimicrobial potential against the test pathogenic strains. In order to have profound biological activity of *N. muscorum* in order to develop novel therapeutic compounds with least side effects, further extensive investigations are needed based on high end techniques.

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