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Evaluation of antioxidant and anti-proliferative efficacy of *Nostoc muscorum* NCCU-442

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ABSTRACT

Natural sources have always been the primary sources of bioactive principles. Cyanobacteria with its wide spectrum of metabolites represents a favourable source of compounds having medicinal properties like anti-oxidant, anti-proliferative etc. In this study we tested different organic extracts [Diethyl ether (DE), Dichloromethane (DM), Ethyl acetate (EA) and Heptane (HE) extracts] of cyanobacterium *Nostoc muscorum* NCCU-442 for the same. Antioxidant activity was evaluated through DPPH and FRAP assay. Phenolic content was estimated using FCR reagent as phenolic compounds are the well-established antioxidants. MTT assay was used to estimate anti-proliferative activity against cervical cancer cell line, SiHa. DE extract was found to have the highest Phenolic content, i.e. 96.71 ± 2.41 mgGAE/g as well as most antioxidant effect in DPPH assay (IC_{50} = 118 ± 0.34 μg/ml) and FRAP assay (EC_1 = 380.7 ± 2.43 μg/ml). The order of phenolic content of different extracts is: DE > EA > DM > HE. The order of antioxidant potential was same as the phenolic content. HE extract had the least antioxidant effect in the two assays (IC_{50} = 686 ± 3.3 μg/ml and EC_1 > 10 mg/ml for DPPH and FRAP respectively). All the extracts showed anti-proliferative activity against the cancer cell line in the order: DE (IC_{50} = 70 ± 0.22 μg/ml) > HE (116.5 ± 0.53 μg/ml) > EA (205 ± 0.77 μg/ml) > DM (190 ± 0.45 μg/ml). During Gas Chromatography-Mass Spectrometry (GC-MS), bioactives like Benzofuranone derivatives, Myristoleic acid, Resorcinol, Citronellyl butyrates, hydroquinone, hexadecanoic acid, farnesol were identified in the organic extracts. Thus, our study indicated that *Nostoc muscorum* NCCU-442 has an inherent capacity as a potential source of therapeutic compounds.

1. Introduction

Oxidation is a process involved in various metabolic reactions of our body, generating a lot of free radicals (hydroxyl radical, superoxide radical etc.) and reactive oxygen species (ROS). To a certain point our body can tolerate these free radicals and ROS but when production of free radicals exceeds this limit the homeostasis of our body is disturbed and these free radicals start reacting with cellular components like oxidising membrane lipids, cellular proteins, DNA and enzymes which causes interruption in the normal cellular functioning and causes apoptosis, cancer and also affect gene expression. Their production also influences normal cell signalling pathways (Vajragupta et al., 2004).

In our body, there are various counter mechanisms and enzymes to neutralise their effects, these are called antioxidants (Halliwell and Gutteridge, 1995). Antioxidants can also be described variably either as the moieties that inhibit lipid peroxidation (caused by the generation of free radicals) or any substance that when present in low concentration hampers the oxidation of the substrate- the oxidants (Halliwell and Gutteridge, 1989). They are either naturally produced by the cell (endogenously), or taken from the other sources (exogenously). Natural antioxidants in edible substances like fruits and vegetables are also speculated this prevent dreadful diseases like coronary heart disease and cancer (Eberhardt et al., 2000). On the contrary, many synthetic antioxidants like butylated hydroxytoluene (BHT) are reported to have

Abbreviations: DPPH, 2,2-Diphenyl-2-picrylhydrazyl; FRAP, Ferric reducing antioxidant power; FCR, Folin–Ciocalteu reagent; MTT, 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide; DE, Diethyl ether; DM, dichloromethane; EA, Ethyl acetate; HE, Heptane; ROS, Reactive oxygen species

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carcinogenic effect and have toxicity (Safer and Al-Nughamish, 1999). Thus, it is hypothesized that antioxidants from natural sources are safer, could be more bio-effective and thus needed to be studied more.

Abnormal proliferation of cells caused by various genetic and epigenetic alterations in the cell functioning can give rise to cancer. It is the second most lethal diseases of the mankind (Jemal et al., 2007). Despite of the discovery of so many anticancer drugs, this disease is still among the most non-curable illness of humans. However, naturally occurring compounds hold a great promise to be a source of diverse and more effective anti-proliferative drugs and many scientists are working in this direction.

Cyanobacteria (also known as "blue-green algae") are the gram-negative photosynthetic prokaryotic organisms that are found in diverse habitat. They possess a broad range of biodiversity and adaptability in diverse habitats including extreme conditions. Thus, they show the wide spectrum of secondary metabolites like terpenoids, phenolic compounds, chlorophyll and various other compounds like water-soluble pigments (phycobiliproteins), immunoactive products (e.g. Insulin) and antioxidant enzymes. (Duval et al., 2000; Abd El-Baky et al., 2004; Li et al., 2007; Anwer et al., 2012). These metabolites possess a wide spectrum of biological activities and have been used since ancient times to treat diseases. A number of compounds have been isolated from marine cyanobacteria and evaluated for its various biological activities like antioxidant, antimicrobial, anti-inflammatory, anticancer etc. Swain et al. (2015) reviewed about 144 compounds that were isolated from cyanobacteria as the source of antineoplastic agents, which were primarily screened with cancer cell lines. On the other hand, in 2015, Vijayakumar and Menakha (2015) reviewed about compounds like apratoxin, lyngbyabellin and curacin being one the most efficient antiproliferative drugs from marine cyanobacteria. Till date, only marine cyanobacteria have received much attention as a potential source of natural antioxidants and bioactives (Kuda et al., 2005), while only a limited information is available on the freshwater algae for the same (Herrero et al., 2005). As most of the work previously focussed on the marine cyanobacteria, this study addresses the antioxidant and anti-proliferative effect of various organic extracts of fresh water cyanobacterium *Nostoc muscorum* NCCU-442. Further, this study also attempts to estimate the phenolic contents of these extracts and used Gas chromatography-mass spectrometry to find out the probable composition of the extracts and presence of biologically active moieties.

2. Materials and methods

2.1. Chemicals and reagents

All chemicals used in this study were of analytical grade, purchased from the Hi-Media, Merck, SRL, GIBCO and Sigma. All buffers and reagents used in this study were prepared in double distilled water.

2.2. Maintenance of cyanobacterial culture

Nostoc muscorum NCCU-442 is freshwater heterocystous cyanobacterium that is why it was cultured in BG-11 (negative) medium in 250 ml to 2 L Erlenmeyer flasks (Stanier et al., 1971). The culture was raised under illumination under fluorescent tubes (Phillips), providing of 25 μmol m^{-2} s^{-1} light intensity following 12:12 h light/ dark regime at 30 ± 1 °C.

2.3. Growth curve

Growth curve of *Nostoc muscorum* NCCU-442 was obtained using colorimetric method and gravimetric method to determine the day of maximum growth. On this day, biomass was harvested and used for experimental work. For this, 50 ml culture was grown in 100 ml flasks by setting its absorbance = 0.3 at 700 nm. Ten such sets were prepared in triplicates. On every 3rd day, absorbance and weight of the harvested biomass dried in the oven at 50 °C were recorded.

2.4. Preparation of extract

Extracts were prepared using the method described previously (Yasin et al., 2018). At the maximum growth day, the culture was harvested and lyophilized. Freeze dried mass was grinded using pestle and mortar. One gram grinded biomass was taken in 50 ml falcon. Different solvents [ethyl acetate (EA), dichloromethane (DM), diethyl ether (DE) and heptane (HE)] were used as the extraction solvent and added to the pre-weigh biomass in ratio 1:10. Then, the mixtures were vortexed for 5 min to disperse the cell so that it gets evenly mixed with the solvent. This was followed by sonicating the sample by a sonicator (Sonics, Vibra cell) for 2 min. After sonication, the solution was centrifuged at 5000 rpm for 5 min. The supernatant (extract) was pooled and filtered using Whatman No. 1 filter paper and collected into 100 ml flask. All the steps were repeated until the biomass turned whitish. The extract was then vacuum dried using Buchi type rotary evaporator. The dried extract was weighed and dissolved in 0.2% DMSO to make the stock of strength 5 mg/ml, which was then filter sterilised using 0.45μ syringe filters.

2.5. Antioxidant assays

2.5.1. Estimation of total phenolic content

Phenolic content was estimated using Folin–Ciocalteu reagent (FCR) (Singleton et al., 1999). In 500 μl of sample of 1 mg/ml strength, 2.5 ml of FCR was added. The sample was incubated for 10 min, then 2 ml Na_2CO_3 (7.5%) was poured to the mixture and again incubated for 60 min at 50 °C in dark. The Absorbance of the final mixture was recorded at 765 nm. This experiment was carried out in triplicates. The results were expressed as mg gallic acid equivalents per gram of extract (mgGAE/g) using gallic acid standard curve (R^2 = 0.9851).

2.5.2. DPPH radical scavenging assay

The effect of extracts on DPPH (2,2-Diphenyl-2-picrylhydrazyl) radical was estimated by modified DPPH radical scavenging assay (Brand-Williams et al., 1995; Irshad et al., 2014). 300 μl of different concentrations of extracts were added to 900 μl DPPH solution. Each set of concentration was prepared in triplicates. The reaction mixture was kept at room temperature in dark for 30 min. Absorbance was recorded at 517 nm against their corresponding blanks.

$$\%\text{Scavenging of DPPH Radical} = \frac{A(\text{Control}) - A(\text{Test})}{A(\text{Control})} \times 100$$

Where, A(Control) = absorbance of the control (DPPH solution without extracts against ethanol as blank, A(Test)) = absorbance of the test samples. Results were expressed in terms of IC_{50} value i.e, the concentration at which 50% of the radicals were scavenged. Ascorbic acid was used as standard.

2.5.3. FRAP assay

FRAP (Ferric reducing antioxidant power) assay as described by Benzie and Strain (1996) was used. FRAP reagent is made by mixing 100 ml of Acetate buffer (300 mM; pH-3.6), 10 ml of TPTZ [10 mM dissolved in 40 ml HCl (40 mM)] and 10 ml of $FeCl_3$ (20 mM). To 1.5 ml of FRAP reagent 200 μl of sample was added in different concentrations. Absorbance was measured after four minutes at 593 nm using FRAP reagent as blank. The whole experiment was done in the dark room. Solutions of known Fe (II) concentrations ($FeSO_4{\cdot}7H_2O$) were used for the preparation of the calibration curve ($y = 1.4877x - 0.00986$, R^2 = 0.99223). Absorbance vs. concentration graph (Fig. 6, Supplementary data) was plotted for each sample including Ascorbic acid (Positive control) and $FeSO_4$. EC_1 value is estimated which is the parameter equivalent concentration that is defined as the concentration

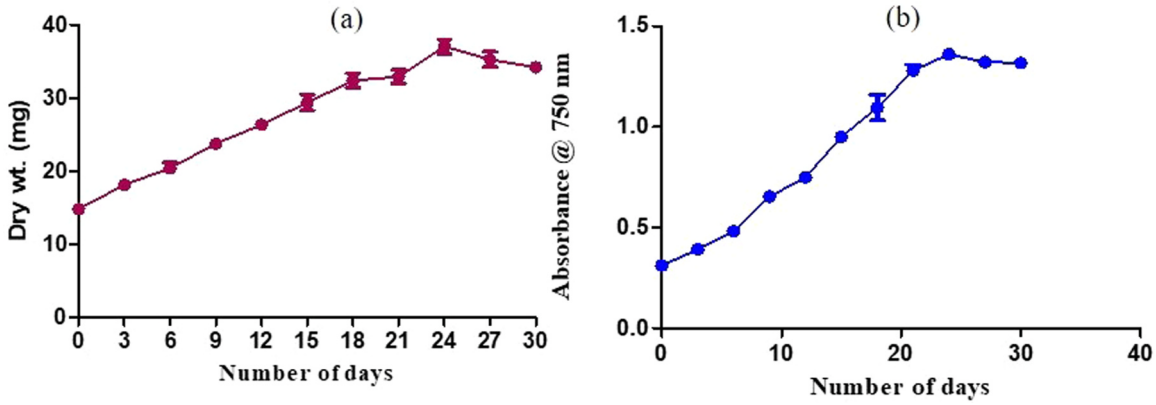

Fig. 1. Growth curve of *Nostoc muscorum* NCCU-442 by (a) gravimetric method and (b) colorimetric method.

of antioxidant having a ferric-TPTZ reducing ability equivalent to that of 1 mM $FeSO_4$·7H2O.

2.6. Antiproliferative assays

2.6.1. Maintenance of cell lines

Human cervical cancer SiHa cell line and non-cancerous Human Embryonic Kidney (HEK-293) cell line were procured from National Centre of Cell Sciences (NCCS), Pune and used in our study. These cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) with 1 g/L glucose, supplemented with 10% FBS and antibiotic mixture. Cells were cultured in 95% humid conditions and 5% CO_2 at 37 °C in CO_2 incubator.

2.6.2. MTT assay

Anti-proliferative activity of all the extracts of cyanobacteria was tested on SiHa cells (cervical cancer cells) and HEK-293 cell line (non-cancerous Human embryonic kidney) using the MTT (3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) assay (Mosmann, 1983; Al-Fatlawi et al., 2014). In a 96-well tissue culture plate, 1×10^4 cells/well were seeded. The next day the cells were treated with different concentrations of the extracts (50 µg/ml-800 µg/ml). After 48 h the media was discarded and 50 µl fresh media was added to each well. To this 20 µl of MTT dye (5 mg/ml) was added. We incubated the plate for 4 h in dark so that blue formazan crystals were formed by reduction of dye by cellular dehydrogenases. After that, the entire content of the plate was discarded and 150 µl of DMSO was added in each well to dissolve the formazan crystals by keeping plate on the rocker for 15 min. The absorbance was read at 570 nm on ELISA plate reader (Bio-Rad). The percentage of the viable cell was calculated as:

$$\%\text{Viability} = 100 - \frac{\text{A(Control)} - \text{A(Test)}}{\text{A(Control)}} \times 100$$

Where A(Control) is Absorbance of the control (mean value) and A(Test) is Absorbance of the treated cells (mean value).

2.7. Chemical analysis of the extracts through GC-MS

A Gas Chromatography-Mass Spectrometry (GC MS) analysis of all the extracts were performed using Agilent 7890A series GC system which was equipped with an HP-5 column (30 m * 0.25 mm ID, 0.25 µm thickness, Varian) coupled with an Agilent 7000 QQQ MS. HP-5 column was used for good separation and for gas chromatography-mass spectroscopic detection. We used electron ionization system with ionization energy of 70 eV, 99.99% pure helium gas was used as a carrier gas at a constant flow rate of 1.1 ml/min. Mass transfer line and injector temperature were set at 220 °C and 250 °C respectively, and the oven temperature was programmed, initial temperature was 60 °C for 1 min then 5 °C/min to 180 °C for 1 min then 10 °C/min to 310 °C for 2 min 1 µl of sample injected in the split mode 5:1. the signals were recorded in full scan mode (*m/z* 20–600, 250 scans/ms). All components were identified by comparison of their mass spectra with those obtained from authentic samples and/or the NIST mass spectral database using AMDIS and mass hunter software.

2.8. Statistical analysis

All the experiments were done in triplicates (n = 3) and all values were expressed as mean ± SD. GraphPad Prism (2007) and Microsoft Excel (2010) were used to perform all the statistical analysis. Regression analysis was done to calculate IC_{50} and EC_1 values. Statistical significance was evaluated using *t*-test and p-values < 0.5 were regarded as significant.

3. Results and discussion

3.1. Growth curve

The growth of *Nostoc muscorum* was studied for 30 days. Twenty fourth day was found to be the day of maximum growth by both gravimetric and colorimetric methods (Fig. 1a and b). Hence, on every 24th day, the biomass was harvested for further study

3.2. Antioxidant assays

Phenolic content of the organic extracts of *Nostoc muscorum* was determined for its use as a natural antioxidant along with the determination of antioxidant potential using DPPH and FRAP assay.

3.2.1. Phenolic content

Phenolic compounds from natural sources are prominently antioxidants and have an important role in the prevention and treatment of cancer. These compounds from dietary sources may include flavonoids, phenolic acids, stilbenes, tannins, curcuminoids, lignans, coumarins, quinones etc. (Huang et al., 2010). These phenolic compounds show anti-inflammatory, UV protectant and chemopreventive properties and may also contribute to cell death by apoptosis induction. As an antioxidant they have prominent radical scavenging, metal chelating, reducing and hydrogen donating ability (Lamoral-Theys et al., 2010).

Gallic acid was used as a standard to calculate phenolic content (Fig. 2a). Among all the extracts, DE had the highest phenolic content i.e., 96.71 ± 2.41 mgGAE/g of extract and was followed by EA (78.57 ± 1.23 mgGAE/g) DM (29.4 ± 0.815 mgGAE/g) and HE (15.14 ± 0.91 mgGAE/g) (Fig. 2b). The presence of phenolics makes cyanobacteria a very good candidate for antioxidant activity. Ismaiel et al. (2014) also observed phenolic content in the aqueous extract of some cyanobacteria that exhibited good antioxidant activity.

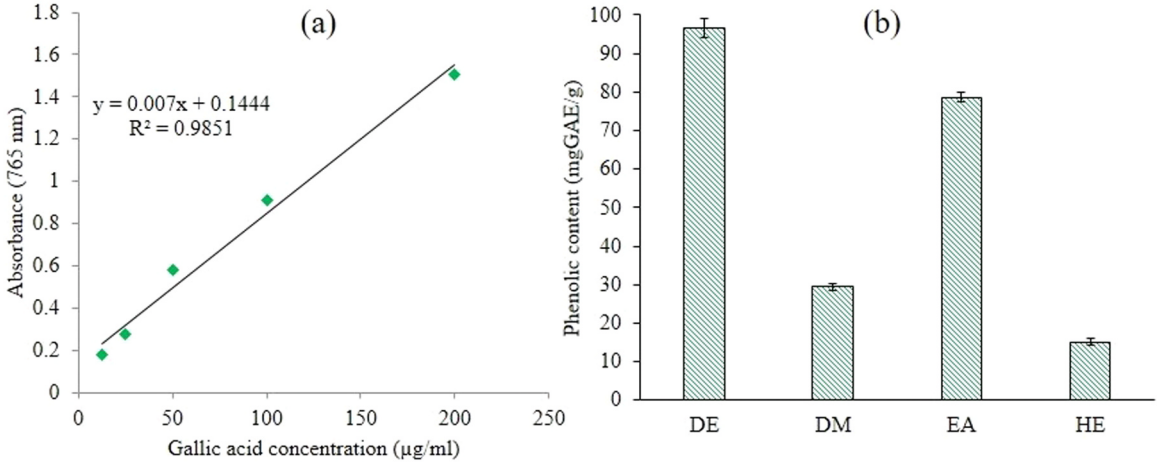

Fig. 2. Phenolic content. (a) Gallic acid standard curve. (b) Graph showing Phenolic content of *Nostoc muscorum* NCCU-442 extracts.

3.2.2. Ferric reducing antioxidant power (FRAP) assay

In FRAP assay the reducing potential of an antioxidant is measured by its ability to reacts with a ferric tripyridyltriazine (Fe^{3+}-TPTZ) complex to form blue coloured ferrous tripyridyltriazine (Fe^{2+}-TPTZ) complex by the action of electron donating antioxidants at low pH. The findings measure the reducing power of the extracts which is due to reductants that exert their effect by donating an electron. Increased absorbance was observed with increase in the concentration of extract. Regression curve of extracts and that of FeSO4 (y = 0.000986 +1.487; R^2 =0.99223) was used to calculate EC_1 value. Lower EC_1 value means better reducing/antioxidant ability. The *Nostoc muscorum* NCCU-442 extracts exhibited the dose-dependent reducing the ability for this assay ($p < 0.05$) (Fig. 3a). DE has the lowest EC_1 value (380.7 ± 2.43 µg/ml) (Fig. 3a) followed by EA (545.4 ± 10.3) and DM (2270 ± 35.87). Whereas HE was least potent for FRAP assay with highest EC_1 values (more than 10 mg/ml) (Fig. 3a). It should be noted that DE had the highest phenolic content and HE had the least. Thus, differential amounts of phenolic present in the extracts may be responsible for their differential reducing activity for FRAP assay. Ascorbic acid was used as the positive control with EC_1 value of 66.85 ± 0.21 µg/ml (Fig. 3). Sowndhararajan and Kang (2013) observed similar results in *Bauhinia vahlii* leaves where non-polar extracts exhibited lesser antioxidant potential and polar extracts showed the higher antioxidant potential during FRAP activity assay.

3.2.3. DPPH radical scavenging assay

Nostoc muscorum NCCU-442 extracts (EA, DCM, DE and HE) were tested if they could scavenge DPPH free radicals. Our study revealed appreciable free radical scavenging activity/antioxidant capacity of these extracts in a dose-dependent manner ($p < 0.05$) (Fig. 3b). The DPPH radical scavenging activity of extracts was detected and compared with standard antioxidant – ascorbic acid. IC_{50} value of ascorbic acid was found to be 110 µg/ml (Fig. 3b). IC_{50} value of the DE was the lowest (118 ± 0.34 µg/ml) indicating that it has the maximum radical scavenging activity than the other extracts. IC_{50} values of EA and DM was found to be 205.1 ± 0.6 µg/ml and 627.2 ± 2.1 µg/ml respectively. Among all the extracts HE was least potent for DPPH radical scavenging with highest IC_{50} value (686 ± 3.3 µg/ml).

The radical scavenging activity of the extracts can be correlated with their respective phenolic contents (Piluzza and Bullitta, 2011). Phenolics are the potent hydrogen donators to DPPH as exhibited by their excellent structural chemistry (Von Gadow et al., 1997). Hossain et al. (2016) also studied the antioxidant activity of many cyanobacteria from Sri Lanka using DPPH and FRAP.

Free radicals are generated in our body regularly via different pathways of cellular metabolism. These free radicals include superoxide anion ($O_2^{\bullet}$), per hydroxyl radical ($HO_2^{\bullet}$), hydroxyl radical ($^{\bullet}OH$), nitric oxide and other species such as hydrogen peroxide (H_2O_2), singlet oxygen ($^1O_2$), hypochlorous acid (HOCl) and peroxynitrite ($ONOO^-$) etc. generated via. Normally, these free radicals also involved in many

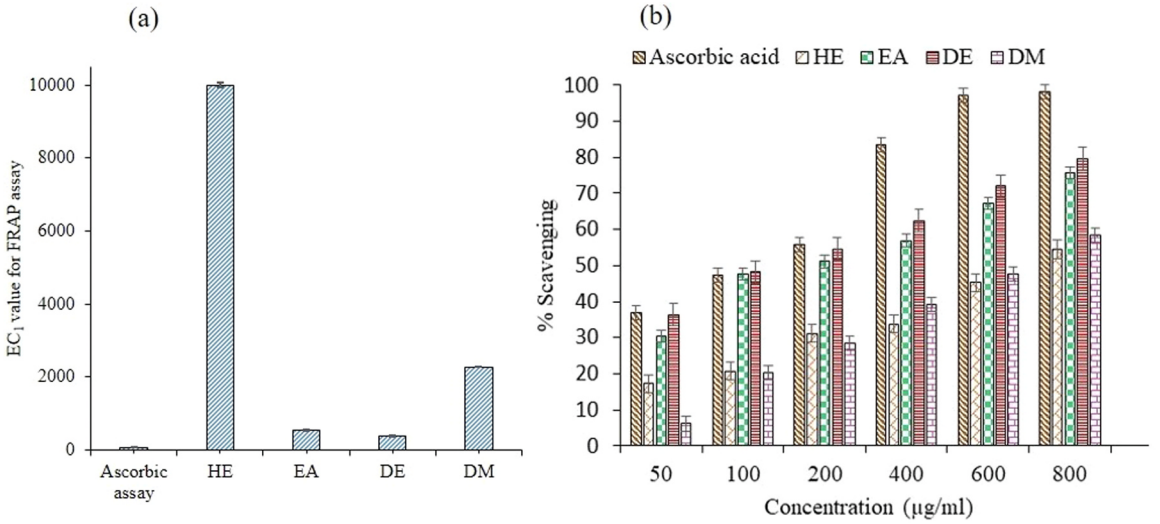

Fig. 3. Antioxidant assay. (a) FRAP activity and (b) DPPH radical Scavenging activity of *Nostoc muscorum* NCCU-442 extracts.

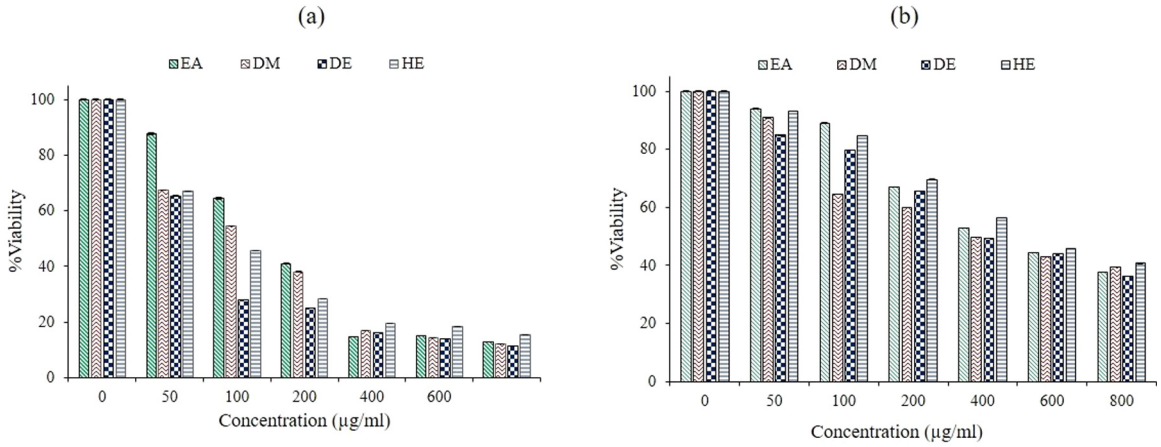

Fig. 4. Graph showing effect of ethyl acetate (EA), dichloromethane (DM), diethyl ether (DE) and heptane (HE) extracts of *Nostoc muscorum* NCCU-442 *on* cell viability of (a) SiHa cells and (b) HEK-293 cell using MTT Assay.

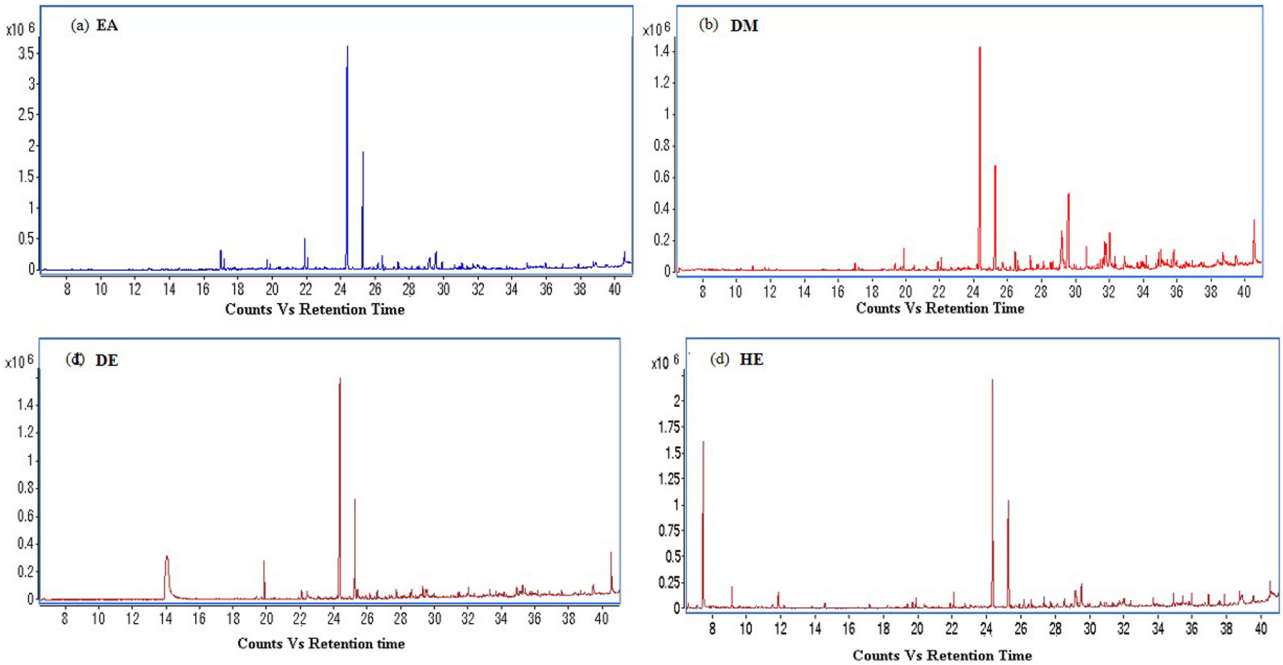

Fig. 5. GC chromatogram of ethyl acetate (EA), dichloromethane (DM), diethyl ether (DE) and heptane (HE) extracts of *Nostoc muscorum* NCCU-442.

beneficial activities like cellular defence (Droge, 2002; Young and Woodside, 2001) but excessive production of these radical can cause harmful effects. These effects can be neutralised by the antioxidants. The results obtained in by DPPH assay and FRAP assay clearly supported the idea that organic extracts of *Nostoc muscorum* NCCU-442 has prominent antioxidant capability and could protect us from harmful effects of free radicals.

3.3. Antiproliferative assay (MTT assay)

MTT assay was performed for the assessing anti-proliferative effect of *Nostoc muscorum* NCCU-442 against cervical cancer cell line SiHa and normal human embryonic kidney 293 cell line (Fig. 4). All the extracts had shown anti-proliferative activity against the cancer cell line in a dose-dependent manner ($p < 0.05$) with the DE extract being most effective (IC_{50} = 70 ± 0.22 µg/ml) followed by HE (116.5 ± 0.53 µg/ml) while IC_{50} values EA was 205 ± 0.77 µg/ml and that of DM was 190 ± 0.45 µg/ml (Fig. 4a). The extracts have shown a very little toxicity on the normal cell line HEK-293 killing only about 40% cells at the maximum concentration i.e., 1 mg/ml (Fig. 4b). This indicated that these extracts have the potential to kill cancer cells specifically causing little damage to the normal cells, thus they can be considered biocompatible. The findings may be attributed to the phenolic content and antioxidant ability as shown by different extracts of *Nostoc muscorum* but this attribute is not absolute in this case. Though the HE extracts had least phenolic content and antioxidant ability yet it showed the appreciable anti-proliferative activity, this indicated that there could other compounds (citronellyl butyrate or pentanoic acid as detected in GC-MS) present in the extracts that may not have the antioxidant potential but could kill cells more efficiently. Srivastava et al. (2015) studied extracts of various freshwater cyanobacteria and found that *Arthrospira* sp. CCC729 and *Geitlerinema* sp. CCC728 were active against human colon adenocarcinoma (HT29) and human kidney adenocarcinoma (A498) cancer cell lines. Various potent anticancer compounds have been isolated from the marine cyanobacteria but none has reached the clinical trials. Moreover, freshwater cyanobacteria are not extensively researched for the same, thus they hold a lot of hope as a source of chemotherapeutic agents.

Table 1
List of compounds identified in different organic extracts of *Nostoc muscorum* NCCU-442.

S. no.	Compound name	Nature	RT	Abundance%			
				DE	DM	EA	HE
1	Undecane, 4,7-dimethyl-	Branched alkane	6.5785	–	–	–	2.4
2	Heptane, 2,5,5-trimethyl-	Branched alkane	7.085	–	–	–	4.2
3	2-Pyrrolidinone, 1-methyl-	5- membered Lactam	7.4815	–	–	–	0.06
4	1H-1,2,4-Triazol-3-amine, 5-methyl-	Alkaloid	7.6744	–	6.20	–	–
5	Undecane	Alkane	9.12	–	–	–	0.57
6	Naphthalene	polycyclic aromatic hydrocarbon	11.48	–	–	–	1.7
7	Dodecane	Alkane	11.842	–	–	–	0.72
8	1H-Pyrrole-2,5-dione, 3-ethyl-4-methyl-	Triazole Alkaloid	12.756	–	–	3.60	–
9	Resorcinol	phenolic compound	14.0235	1.5	–	–	–
10	Hydroquinone, acetate	Phenolic derivative	14.052	0.82	–	–	–
11	*n*-Tridecan-1-ol	long chain fatty alcohol	16.9596	–	–	0.95	–
12	Tetradecane	Alkane	17.1695	–	–	0.65	–
13	Naphthalene, 1,3-dimethyl-	polycyclic aromatic hydrocarbon	17.7795	–	–	1.8	–
14	Alanine, N-methyl-n-butoxycarbonyl-, octyl ester	ester	18.1875	–	–	–	2
15	Pentadecane	alkane	19.6675	–	–	0.79	–
16	Pentanoic acid, 5-hydroxy-, 2,4-di-*t*-butylphenyl esters	Lipid	19.831/19.844/19.841/ 19.841/19.842	0.25	3.00	0.79	0.68
17	2(4H)-Benzofuranone, 5,6,7,7a-tetrahydro-4,4,7a-trimethyl-, (R)-	Hetrocyclic compound with Furan ring stucture	20.36	–	–	2.8	–
18	Naphthalene, 1,6,7-trimethyl-	polycyclic aromatic hydrocarbon	20.7685	–	–	6	–
19	3-Octadecene, (*E*)-	Olefins	21.8644	–	3.70	–	–
20	Sulfurous acid, 2-ethylhexyl hexyl ester	ester	22.043/22.04/19.35	2.00	3.80	–	4.7
21	Hexadecane	Alkane	22.049/22.0436	–	–	0.71	0.86
22	D-Proline, n-butoxycarbonyl-, decyl ester	Amino acid esters	22.36	3.8	–	–	–
23	5-Nonanone, 2-(dimethylamino)-1-phenyl-	Alkyl benzene	22.374	3.00	–	–	–
24	Oxirane, decyl-	epoxide	22.5095	–	–	7.6	–
25	Dodecane, 2-methyl-6-propyl-	Branched alkane	23.0494	–	–	4.1	–
26	zzz	alkane	24.3705	–	–	0.03	–
27	Hexacosane	acyclic alkanes	24.33/24.3205/24.34	0.09	0.10	–	0.06
28	3-Isoxazolecarboxylic acid, 5-[1-(acetylamino)-1-methylethyl]-, ethyl ester	Alkaloid	24.3415	–	–	0.89	–
29	Tetracosane	alkane	24.3705	–	–	0.03	–
30	4,6-di-*tert*-Butylresorcinol	Phenolics	24.669	–	–	3.20	–
31	Undecane, 2,3-dimethyl-	Branched alkanes	25.2346			–	
32	9-methylheptadecane	Branched alkane	25.2376/25.241/25.2665/ 25.248	0.23	0.20	0.08	0.14
33	Naphthalene, 1,7-dimethoxy-	polycyclic aromatic hydrocarbon	25.389	3.30	–	–	–
34	Dodecane, 5-methyl-	Branched alkane	25.408	–	5.70	–	–
35	Pentane, 3-ethyl-2,4-dimethyl-	Branched alkane	25.4132	–	–	–	5.3
36	Allyl(n-pentyl)dimethylsilane	TMS derivative	25.6655	–	8.10	–	–
37	3-Nonanol, 3-methyl-	Branched alkane	25.6695	–	–	–	13
38	*n*-Decanoic acid	Fatty acid	25.6695	–	–	8.20	–
39	Nonane, 3,7-dimethyl-	Branched alkane	25.8856	6.20	–	–	–
40	Octane, 2,7-dimethyl-	Branched alkane	25.8862	–	–	–	4.8
41	Cyclopropane, 1-methyl-1-(2-methylpropyl)-2-nonyl-	Cycloalkane	26.136/26.137/26.138	8.9	10	–	7
42	9-Octadecene, (*E*)-	Olefin	26.138/21.8595	–	–	2.9	5.9
43	1,1,1,5,7,7,7-Heptamethyl-3,3-bis(trimethylsiloxy) tetrasiloxane	TMS derivative	26.1545	–	–	–	2.5
44	5-Octadecene, (*E*)-	Olefin	26.4105	–	–	–	9.4
45	E-14-Hexadecenal	Aldehyde	26.4115/21.8745	–	3.40	0.57	–
46	Hexane, 2,2,3,3-tetramethyl-	Branched alkane	26.5645	–	5.00	–	–
47	Dodecane, 2,7,10-trimethyl-	Branched alkane	26.57	2.4	–	–	1.9
48	Bicyclo[4.1.0]heptane, 7-pentyl-	Cyclic alkane	27.112	–	–	11	–
49	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	diterpene alcohol	27.3165/27.3175/27.3135	–	1.60	2.3	3
50	Phthalic acid, hept-4-yl isobutyl ester	Phthalates	27.7125	1	–	–	–
51	Phthalic acid, cyclobutyl isobutyl ester	Phthalates	27.7145/27.7172	–	1.20	1.4	–
52	Phthalic acid, hex-3-yl isobutyl ester	Phthalates	27.716	–	–	–	1.1
53	Bicyclo[2.2.1]heptane, 1,3,3-trimethyl-	Cyclic alkane	27.78	–	7.80	–	–
54	Citronellyl butyrate	monoterpene alcohol	28.107	–	–	–	9.1
55	7-Hexadecenoic acid, methyl ester, (*Z*)-	Fatty acid esters	28.4275	–	4.80	–	–
56	Decane, 6-ethyl-2-methyl-	Branched alkane	28.4858/26.5715/29.982	5.7	–	4	3.5
57	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	Heterocyclic ketones	28.5125	–	–	–	7.4
58	Tetradecanoic acid, 12-methyl-, methyl ester, (S)-	Fatty acid esters	28.8655	–	–	3.4	–
59	Hexadecanoic acid, methyl ester	Fatty acid esters	28.8705	–	1.80	–	–
60	Trisiloxane, 1,1,1,5,5,5-hexamethyl-3,3-bis[(trimethylsilyl) oxy]-	TMS derivative	28.91	–	–	–	2.6
61	1-Hexyl-2-nitrocyclohexane	Nitroalkane	29.147	–	–	6.3	–
62	Myristoleic acid	omega − 5 fatty acid	29.161	–	–	2.00	–
63	E-2-Octadecadecen-1-ol	Alcohol	29.168	–	–	–	2.1
64	cis-9-Hexadecenoic acid	omega − 7 monounsaturated fatty acid	29.1775	–	1.20	–	–
65	Phthalic acid, 4-cyanophenyl nonyl ester	Phthalates	29.2845	–	–	–	2.2
66	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	Phthalates	29.29	0.85	–	–	–

(*continued on next page*)

Table 1 (*continued*)

S. no.	Compound name	Nature	RT	Abundance%			
				DE	DM	EA	HE
67	Dibutyl phthalate	Phthalates	29.294	0.86	–	–	–
68	Phthalic acid, 2-chloropropyl isobutyl ester	Phthalates	29.2985	–	–	1.5	–
69	1-Cyclohexylheptene	Olefin	29.4785	–	–	3.00	–
70	4-Nonenal, (*E*)-	alkenal	29.4795	8.5	–	–	–
71	1,15-Pentadecanediol	Fatty alcohol	29.4835	–	–	–	3.4
72	Z-10-Tetradecen-1-ol acetate	Alkene acetate	29.489	–	1.90	1.5	3.3
73	Nonanoic acid	Fatty acid	29.525	6.96	–	–	–
74	*n*-Hexadecanoic acid	Fatty acid	29.5345/29.53235	–	1.10/1.2	–	4.2
75	*n*-Hexadecanoic acid	Fatty acid	29.5355	6.3	–	0.93	–
76	3-Eicosene, (*E*)-	Olefin	29.891	–	7.60	2.6	–
77	Octane, 2,6,6-trimethyl-	Branched alkane	29.978	5.9	–	–	–
78	6-Tridecene	Olefins	30.6145	–	4.90	–	–
79	1-Trifluoroacetoxy-10-undecene	Olefins	30.615	–	–	–	6.3
80	E-11,13-Tetradecadien-1-ol	alcohol	30.619/31.703	–	–	3.7	6
81	Silane, [[4-[1,2-bis[(trimethylsilyl)oxy]ethyl]-1,2-phenylene] bis(oxy)]bis[trimethyl-	TMS derivative	30.852	–	–	–	2.8
82	Oxalic acid, allylpentadecyl ester	ester	30.9445	–	–	4.5	–
83	Hexadecen-1-ol, trans-9-		31.059	–	–	2.8	–
84	Phytol	diterpene alcohol	31.3425/31.3465	–	2.00	2.8	–
85	Cyclohexanol, 1-methyl-4-(1-methylethyl)-	Cycloalkanol	31.351	–	–	–	4.4
86	Bicyclo[4.1.0]heptane, 3-methyl-	Cycloalkane	31.625	–	10	–	–
87	Cyclooctaneacetic acid, 2-oxo-	Fatty acid ester	31.711/31.705	–	6.10	4.1	–
88	Cyclooctane, methyl-		31.7575	–	–	5.8	–
89	2,3-Dimethyl-5-oxohexanethioic acid, S-t-butyl ester	esters	32.0105	4.2	–	4.5	–
90	Decanoic acid, 2-oxo-, methyl ester	Fatty acid methyl esters	33.294	7.4	–	–	–
91	Hexasiloxane, tetradecamethyl-	TMS derivative	33.679/35.9256	–	–	2.2	1.2
92	5-Hepten-2-one, 7-phenyl-	aromatic ketone	34.9035/34.901	3.5	4.50	–	–
93	5-Ethyl-3-nonanol	Branched alcohol	35.029	–	–	6.40	–
94	1H-Indene, 1-hexadecyl-2,3-dihydro-	Indene derivative	35.0585/35.0625	4.1	4.10	–	–
95	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	Fatty acid ester	35.241	5.1	–	–	–
96	Undecanoyl chloride	Acyl chloride	35.2425	–	5.40	–	–
97	Phthalic acid, di(2-propylpentyl) ester	phthalates	35.3935/35.395	2.20	1.20	–	–
98	1,5-Heptadiene, 2,6-dimethyl-	Olefins	37.561	4.4	–		–
99	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-, (*E*,*E*)-/ Farnesol	acyclic sesquiterpene alcohol	37.563/37.5655	–	5.30	–	3.6
100	Tetracosamethyl-cyclododecasiloxane	TMS derivative	37.8415/37.837	–	–	2.5	1.4
101	Silane, dichloromethylpentyl-	TMS derivative	39.434	–	–	7.6	–
102	1,3-Disilacyclopent-4-ene, 1,1,3,3-tetramethyl-	TMS derivative	39.4595	–	–	6.8	–
103	Butanamide, 3-hydroxy-N-(2-oxo-3-piperidinyl)-	Alkaloid	39.4615	3.1	–	–	–
104	3′,4′-Difluoroacetophenone	Fluorinated Aromatic ketone	39.478	–	–	8.3	–
105	Phosphonofluoridic acid, ethyl-, nonyl ester	esters	40.5205/40.5215	–	1.30	–	1.2
106	2-Furancarboxylic acid, heptadecyl ester	Ester of furoic acid	40.5355	0.84	–	–	–
107	2-Pentenamide, N-(2-oxo-3-piperidinyl)-	Alkaloid	40.5375	–	–	0.99	–

3.4. Analysis of extract by GC-MS

The mass spectrometer in GC-MS analyzes the compounds eluted at different retention times to identify the nature and structure of the compounds by comparing the obtained mass spectra with the spectra of compounds present in the NIST database. **45, 31, 29, and 39**, compounds were detected in EA, DM DE, and HE extracts respectively on the basis of the peaks obtained in the chromatogram [Fig. 5(a-d); Table 1]. Various bioactive compounds were identified in these extracts.

2(4 H)-Benzofuranone, 5, 6, 7, 7a-tetrahydro-4,4,7a-trimethyl-(R)-; Tetradecanoic acid and Myristoleic acid were found only in EA (Table 1) extract. Various **benzofuranone** derivatives have been observed to have shown antioxidant activities (Meng et al., 2016). Myristoleic acid is a monounsaturated fatty acid. It is cytotoxic and appreciable cell death inducing activity, it is prescribed to be used for the treatment of prostate cancer (Iguchi et al., 2001). Tetradecanoic acid is a fatty acid compound and it may act as an antioxidant, cancer preventive, cosmetic, hypercholesterolemic, nematicide and lubricant (Devi and Muthu, 2014).

Resorcinol; Hydroquinone; 1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester and 2-Furancarboxylic acid, heptadecyl ester were probable bioactive constituents that were found exclusively in DE (Table 1) extract. Resorcinol was purified from Awa-ban tea and reported to have an antioxidant activity. It was found to exhibit radical-scavenging activity in vitro at levels equivalent to those of (−)-epigallocatechin gallate (Hiasa et al., 2013). Hydroquinone: or 1,4-benzenediol is a hydroxylated benzene metabolite with many biological activities. In one study it was shown to have antiproliferative effect against A431, SYF, B16F10, and MDA-MB-231 cells, along with synergistic effect on A431 cell death with other anti-cancer drugs, like adenosine-20, -dialdehyde and buthionine sulfoximine (Byeon et al., 2018). Resorcinol derivatives were isolated from the roots of *Ardisia brevicaulis* and had shown the inhibitory effect against the proliferation of human pancreatic PANC-1, human lung A549, human gastrointestinal carcinoma SGC 7901, human breast MCF-7, and human prostate PC-3 cancer cells (Chen et al., 2011). Heirridin B, which is structurally similar to resorcinol was isolated from the marine picocyanobacterium *Cyanobium* sp. LEGE 061132-had exhibited antitumor activity against eight human cell lines (Leão et al., 2013). Furancarboxylic acid, heptadecyl ester is a furan derivative. Furan is a five-member aromatic heterocyclic compound, which is a common structural motifs in many natural products (Keay and Dibble, 1996). The furan derivatives have been reported to have shown numerous biological activities including antibacterial (Matsuura et al., 1996) and antioxidant (Malmstrom et al., 2001). On the other hand, 1, 2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester was reported to have detected in the volatile oils of *Fagopyrum esculentum, Fagopyrum*

tataricum and *Fagopyrum cymosum* flowers which exhibited antioxidant properties (Zhao et al., 2018)

3-Nonanol, 3-methyl-, 7,9-Di-tert-butyl-1-oxaspiro [4.5] deca-6,9-diene-2,8-dione and Citronellyl butyrate are the potential bioactives spotted in HE (Table 1) extract. 3-methyl-3-nonanol-was found in *Artocarpus hirsutus* exhibiting DPPH radical scavenging properties (Jeyam et al., 2013). Citronellyl butyrate is an inhibitor of the mitogenic activity of epidermal growth factor (EGF), it is also antifungal in nature (Upgade and Bhaskar, 2013). 7,9-Di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione is an antioxidant. It is naturally found in aerial parts of *Gmelina asiatica Linn (Verbenaceae)* and in essential oils of some *Stachys* species from Mediterranean area (https://www.trc-canada.com/product-detail/?D493755).

Phytol is found in DM and EA extracts. Phytol is a diterpene alcohol that has been reported to have shown anti-proliferative activity against MCF-7 cell line (Sheeja et al., 2016) HeLa, HT-29, A-549, Hs294T MDA-MB-231 and prostate adenocarcinoma PC-3 cells (Pejin et al., 2014). It was also observed to induce apoptosis in human gastric adenocarcinoma AGS cells. (Woo and Cho, 2015).

1H-Indene, 1-hexadecyl-2,3-dihydro- is detected in DE and DM extract. 1H-Indene, 1-hexadecyl-2,3-dihydro- was found in marine red seaweeds that possess the radical scavenging capacity (Mohy and El-Ahwany, 2016).

2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-, (*E,E*)-/ Farnesol is detected in HE and DM extracts. Farnesol was reported to have inhibited tumor growth and enhanced the anticancer effects of bortezomib in multiple myeloma xenograft mouse model through the modulation of STAT3 signalling pathway (Lee et al., 2015).

3-Eicosene, (E)- is observed in DM and EA extracts. It is frequently observed in the extracts having antioxidant and anticancer activities (Manoj et al., 2012; Adeosun et al., 2013).

Hexacosane is observed in DE, DM and HE extracts. It is reported to have found in essential oil from *Cotula coronopifolia* L. that exhibited antioxidant activity (Kether et al., 2012).

Hexadecanoic acid is a potential bioactive compound that is found in all the extracts. *Cis*-hexadecanoic acid (Palmitoleic acid) is also cancer preventive and was reported to be effective against ehrlich ascite tumor (Ito et al., 1982). n-Hexadecanoic acid- is reported to have activities like antioxidant, hypocholesterolemic, nematicide, anti-androgenic, as flavoring agents, hemolytic, antibacterial and cytotoxic and as 5-alpha reductase inhibitor (Lalitharani et al., 2009; Dineshkumar and Rajakumar, 2015; Rajeswari et al., 2013).

Pentanoic acid, 5-hydroxy-, 2,4-di-*t*-butylphenyl esters is also observed all extracts. This compound was reported from *Calendula officinalis* exhibited cytotoxic effects against Hep2 cell lines (Jalill, 2014).

Presence of all the above-mentioned compounds (summarised in Table 2) indicate that they might be responsible for the observed bioactivity of *Nostoc muscorum* extracts.

4. Conclusion

The results obtained from the present study clearly indicated that *Nostoc muscorum* NCCU-442 has promising antioxidant (evaluated by DPPH assay and FRAP assay) and anti-proliferative efficacy (evaluated by MTT assay). Diethyl ether extract was found to be most effective for all the bioactive assays. Several bioactive compounds were detected in different extracts of *Nostoc muscorum* NCCU-442 by GC-MS (Table 2). These compounds might be the reason behind its antiproliferative and antioxidant activity. Thus, our study provides an insight into the direction that beside marine cyanobacteria, freshwater cyanobacterium like *Nostoc muscorum* NCCU-442 can also be used in future to develop various therapeutic drugs. Though, the research in the direction of purification of biologically active molecules from these cyanobacteria can be done in future, yet, if the biocompatibility and efficacy of these extracts could be established in in vivo experiments, these crude

Table 2
Summary of all the probable bioactive compounds detected in different organic extracts of *Nostoc muscorum* NCCU-442.

S. no.	Compound	Extract Detected in	Activity	Reference
1	1,2-Benzenedicarboxylic acid, butyl 2-methylpropyl ester	DE	Antioxidant	Zhao et al. (2018)
2	1H-Indene, 1-hexadecyl-2,3-dihydro	DE, DM	Antioxidant	Mohy, El-Ahwany (2016)
3	2-(4H)-Benzofuranone, 5, 6, 7, 7a-tetrahydro-4,4,7a-trimethyl-(R)-	EA	Antioxidant	Meng et al. (2016)
4	2,6,10-Dodecatrien-1-ol, 3,7,11-trimethyl-, (*E,E*)-/ Farnesol	HE, DM	Anticancer	Lee et al. (2015)
5	2-Furancarboxyllic acid, heptadecyl ester	DE	Antibacterial, antioxidant	Matsuura et al. (1996); Malmstrom et al. (2001)
6	3-Nonanol, 3-methyl-,	HE	Antioxidant	Jeyam et al. (2013)
7	7,9-Di-tert-butyl-1-oxaspiro[4.5]deca-6,9-diene-2,8-dione,	HE	Antioxidant	https://www.trc-canada.com/product-detail/?D493755).
8	Citronellyl butyrate	HE	Inhibitor of mitogenic activity	Upgade, Bhaskar (2013)
9	Hexadecanoic acid	All	Antioxidant, hypocholesterolemic, nematicide, anti-androgenic, cancer preventive	Ito et al. (1982)
10	Hydroquinone;	DE	Anti-proliferative	Byeon et al. (2018)
11	Myristoleic acid	EA	Antiproliferative, anticancer	Iguchi et al. (2001)
12	Phytol	DM, EA	Antiproliferative, Anti-apoptotic	Sheeja et al. (2016); Pejin et al. (2014); Woo, Cho (2015).
13	Resorcinol	DE	Antioxidant, Antiproliferative	Hiasa et al. (2013); Chen et al. (2011)
14	Tetradecanoic acid	EA	Antioxidant, cancer preventive, cosmetic, hypercholesteromic	Devi and Muthu (2014)

extracts could work as an alternative medicine that would save us the cost of purifying compounds from these organisms. However, in future, the mechanism behind the bioactivities of these extracts would require to be understood to establish their exact effect.

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Conflict of interest statement

The authors declare to have no conflict of interest.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.bcab.2018.12.001.

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