Evaluation of Anti-Cervical Cancer Potential of Polypeptides from Blue-Green Algae

J. Swapna[1], G. Rajesh Kanna[2], Preethy P Raj[3], S. Ashok Kumar[3] S. Elumalai*[3] and T.Sangeetha*[4]

[1.] Department of Biotechnology, Loyola College (Autonomous), Chennai – 600054.

[2.] Department of Plant Biology and Plant Biotechnology, Presidency College (Autonomous), Chennai – 600005.

[3*.] Department of Biotechnology, University of Madras, Guindy Campus, Chennai – 600025.

Corresponding Email: ananandal@gmail.com

[4.] Department of Chemistry, Presidency College (Autonomous), Chennai – 600005.

Abstract: he polypeptides are short chain amino acid derivatives (secondary metabolites) with 5 or fewer amino acid residues. These polypeptides are not directly synthesized by ribosomes, instead they are synthesized by a specialized enzymes called Non-ribosomal polypeptide synthase. These non-ribosomal polypeptides can be synthesized in all the organismsfromprimitive prokaryotes to advanced eukaryotes. In this present study, we have extracted different polypeptides from three different cyanobacteria *Phormidiumpapyraceum*, *Nostocoryzae* and *Lyngbyawollei*isolated from fresh water lakes in Chennai, Tamil Nadu, India. Among the three different cyanobacteria, *Lyngbyawollei* has acquired high growth rate based on the biomass and low growth rate was observed in *Nostocoryzae*The LC-MS results revealed the presence of different polypeptides Anabaenopeptin and Hoiamide A in *Nostocoryzae*, Micropeptin A in *Phormidiumpapyraceum* and Microcystin in *Lyngbyawollei*The DPPH assay revealed that extract from *Nostocoryzae* exhibited high anti-oxidant activity (IC_{50}- 222 µg) when compared to *Lyngbyawollei* (IC_{50}-198 µg) whereas *Phormidiumpapyraceum* exhibited no activity. The anti-cancerous activity of all the three cyanobacterial extracts were analyzed by MTT assay using HeLa cervical cancer cell line. The 50 % inhibitory concentration of the HeLa cancer cells for *Nostocoryzae* was 560 µg and 610 µg for *Lyngbyawollei* whereas *Phormidiumpapyraceum* exhibited no activity. Hence non-ribosomal polypeptides could serve as an effective drug to kill cancerous cells.

Key words: Cyanobacteria; Polypeptides; Antioxidant; Anti-cancer.

1. Introduction

Cyanobacteria (blue-green algae) arethe prokaryotic photosynthetic microorganisms that are widely distributed in fresh,brackish and marine aquatic environments and in moist soil surfaces.Cyanobacteria have been considered a rich source of secondary metabolites with potential biotechnological applications in the pharmacological field. Lately, production of bioactive compounds with commercial and medical applications has also increased interest in studying these organisms (Tan, 2007). During recent decades, cyanoprokaryotes have attained a massive attraction among scientists due to their secondary metabolites called non-ribosomal polypeptides. These peptides are often cyclic and can have highly complex cyclic

structures, although linear non-ribosomal peptides are also common.These polypeptides are classified into cyclic peptides, Alkaloids, Polyketides, Amino acids. So far, more than 600 peptides or peptidic metabolites have been described from various taxa.Cyanobacterial secondary metabolites represent a vast diversity of structures (Moore, 1996; Burja*et al*., 2001; Staunton &Weissman, 2001; Harrigan& Goetz, 2002) isolated from a variety of taxa and geographic origins.

In vitro studies of soblidotin, a synthetic analog of dolastatin 10, showed promising results against human colon adenocarcinomas and this compound has progressed to phase II clinical trials. Synthadotin, derived from dolastatin 15, has shown promising results in phase II clinical trials of inoperable, locally advanced or metastatic melanoma (Liu and Rein, 2010).Oftedal*et al*. (2010) investigated the potential anticancer lineage of marine benthic cyanobacteria on the shores of the Baltic Sea and found that extracts from Anabaena sp. M27, M30 and M44 rapidly induceapoptosis in cells from acute myeloid leukemia (AML) in humans, compared to a higher-than-therapeuticconcentration of daunorubicin in these cells.

Cervical cancer is the second most common cause of female specific cancer next breast cancer, accounting for around 8% of both total cancer cases and total cancer deaths in women (World Cancer report, 2014).In India, approximately 6–29% of cervical cancer has been reported in women. Cervical cancer is mainly caused due to Human Papillomavirus, is the leading cancer in Indian women. The incidence of cervical cancer worldwide is approximately 510,000 annually, with approximately 288,000 cases of deaths reported (Sankaranarayanan and Ferlay, 2006).The drugs currently used to treat cervical cancer are Bevacizumab, Carboplatin, Cisplatin, Docetaxel Fluorouracil (5-FU), Gemcitabine, Ifosfamide, Mitomycin etc. whichcausesmany unwanted side effects and hence natural products from medicinal plants have gained significance in the treatment of this disease. About 50% of all drugs in clinical use in the world are natural products and their derivatives and 60% of drugs approved for cancer treatment are of natural origin (Singh *et al*., 2011).

The main objective of the present study is to evaluate the potential of polypeptides extracted from three different cyanobacteria for anti-cervical cancer activity.

2. Materials and Methods

2.1. Sample collection and isolation of blue-green algae

The fresh water and soil samples were collected from three different lakes Retteri lake (RL), Puzhallake (PL) and Chembarambakkam lake (SL) in and around greater Chennai. The physio-chemical parameters for the fresh water were analyzed including temperature, pH, salinity, total dissolved solids (TDS), electrical conductivity, dissolved oxygen, oxidation reduction potential of all the water samples were analyzed by YSI 650 Multiparameter display system (MDS) instrument (YSI Incorporater yellow spring, ohio, USA).Soil samples from three different localities were serially diluted and were cultured in BG11 agar medium (pH – 6.0) at room temperature under white fluorescent light illumination for 12 hours light and 12 hours dark conditions. From the spread plates, the predominantly grown blue-green algae colonies especially filamentous forms werechosen for the further study.The pure colonies were isolated and cultured separately. Further sub-cultures were maintained at the microalgal culture collection room for further studies. The 100X magnification photographs were taken using the compound microscope (OLYMPUS CH-20i).

2.2. Growth rate analysis

The growth rate were acquired by the biomass obtained from the blue-green algae for each and every day for up to 15 days of cultivation. About 15 conical flasks for each blue-green alga was taken with liquid BG11 medium from 1st day to 15th day with pH 6.0 and inoculated with 1 % of inoculum and incubated under culture conditions for 15 days. Both the dry and wet biomasses for the respective days were determined.

2.3. Extraction of polypeptides from blue-green algae

The biomasses from three different blue-green algae were harvested and the biomasses from algae were well homogenized separately using a mortar and pestle for 20 minat room temperature with glass beads. The biomass was homogenized with 5 ml of absolute ethanol and sonication was performed at 53 kHz for 20 min.The samples were then subjected to vortex for a minute and stirred well for 2 to 3 hours. The samples were then store overnight in -4ºC and centrifuged at 8000 rpm for 10 min. at room temperature. The supernatant was conserved for characterization.

2.4.Estimation of Protein

Estimation of protein were carried out using (Bradford, 1976) method. The unknown protein concentration were estimated using the standard BSA (Bovine Serum Albumin). The

total reaction mixture (6 ml) was then incubated for 15 min. and the absorbance values were measured at 595 nm in a UV-visible spectrophotometer (Hitachi U-2900).

The unknown protein concentration was obtained based on the formula given below.

UPC (µg) = KPC / ABS of KPC from SC X ABS of UPC

Where,

UPC	:	Unknown protein concentration
KPC	:	Known protein concentration
ABS of KPC from SC concentration from standard graph	:	Absorbance value of known protein
ABS of UPC protein concentration	:	Absorbance value of unknown

2.5. Characterization of polypeptides

2.5.1. Liquid chromatography mass spectrum analysis of polypeptides

The crude extract from all the three cyanobacteria were analyzed on HPLC instrument (Shimadzu, Japan), using shim-pack CLCODS (4.6 × 15mm) column, and a 350 nm detector. Methanol and water (65:35, v/v) were adopted as a mobile phase with a flow rate of 1.0 ml/min at 25◦C. Filtered sample was injected into the column and the relative retention time was recorded. Then mass were further analyzed using a LC-MS instrument (Waters, Germany) consisting of Alliance separations module e2695; ACQUITY QDa detector, and a C18 reversed-phase column. Solvent A consisted of 0.01% (v/v) formic acid in water. Solvent B consisted of 0.01% (v/v) formic acid in acetonitrile. The mass spectrometer was operated in the positive-full- scan (m/z 0–1000) mode.

2.6. Antioxidant activity assay

2.6.1. DPPH free radical scavenging assay

DPPH free radical scavenging assay was carried out using (Blois, 1958) method. Polypeptides extracted from blue-green algae, about 100 - 500 µg of the crude extract was mixed separately with 0.5 ml of 0.5 mM of DPPH (2, 2-diphenyl-1-picrylhydrazyl) in methanol along with 0.5 ml of distilled water. After incubation in dark for 37 min. at room temperature the absorbance values were measured by UV visible spectrophotometer at 545

nm. Ascorbic acid was used as a positive control and the percentage of free radical inhibition can be estimated by the following formula:

$$\text{Percentage of Inhibition} = \frac{\text{Absorbance of Control} - \text{Absorbance of Sample}}{\text{Absorbance of Control}} \times 100$$

2.7. Evaluation of anti-cervical cancer potential of polypeptides using MTT assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay was carried out as per the procedure of (Mosmann, 1983).The MTT was dissolved in Dulbecco's phosphate buffered saline, pH – 7.4 at 5 mg/ml concentration. The solution was then filtered through 0.2 µM filter into a sterile, light protected container and stored at 4^{o}C devoid of light condition. The solubilisationsolution with 40 % of dimethyl formamide (DMF) in 2 % glacial acetic acid and 16 % sodium dodecyl sulphate (SDS) were dissolved and prepared (pH – 4.7). The HeLa cells and the test compounds in 96 well plates were prepared at final volume of 100µl/well. Then after incubation for four hours, 10 µl MTT solution per well were added to achieve a final concentration of 0.45 mg/ml and again incubated for 1 to 4 hours at 37°C. After addition of 100 µl solubilisation solution to each well to dissolve formazan crystals. The absorbance (Abs) were measured at 570 nm.

3.Results and Discussion

3.1.Collection of fresh water and soil samples

The topo-geographical view showing the map of the three fresh water lake sites in Chennai, Tamil Nadu, India; Puzhal Lake (PL) (13^{o} 09' 46.75" North and 80^{o} 11' 08.88" East), Retteri Lake (RL) (13^{o} 08' 40.83" North and 80^{o} 12' 51.05" East) and Chembarambakkam Lake (SL) (13^{o} 02' 04.80" North and 80^{o} 04' 10.87" East) where water and soil samples were collected (**Fig. 1**).

Fig. 1. The topographical view showing three different fresh water lakes, where water and soil samples were collected; a) Puzhal Lake; b) Retteri Lake and c) Chembarambakkam Lake, Greater Chennai, Tamil Nadu, India.

Among the physiochemical parameters of water samples collected from different lakes, pH was almost similar in all the sites ranging from 5.6 to 6.47 showing the water were near acidic to neutral in nature. Dissolved oxygen (%) was relatively high in Retteri Lake and low inChembarambakkam Lake. Salinity levels was high in the water samples of Retteri Lake and low in other two lakes, comparatively total dissolved solids was also high in concentration in Retteri Lake and low in the other two lakes. Resistance was very low in the Retteri Lake when compared to other two lakes. But specific conductivity was comparatively high in the water samples of Retteri Lake when compared with other two lakes (**Table 1**).

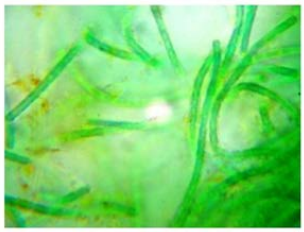
Table 1. Physiochemical properties of water samples collected from three different lakes; RL: Retteri Lake, SL: Chembarambakkam Lake and PL: Puzhal Lake, Greater Chennai, Tamil Nadu, India

Sample	Specific conductivity ms/cm[c]	Resistance ohm/cm	Total dissolved solids (TDS) g/l	Salinity (ppm)	Dissolved oxygen (DO) mg/l	pH	Oxidation reduction potential (ORP)	Temp. (°C)
RL-1	**1.462**	938.47	**0.946**	**0.74**	5.41	**5.6**	-147.8	34.35
RL-2	1.409	**931.466**	0.915	0.71	**6.47**	5.74	**-133.5**	35.55
SL-1	**0.327**	**3543.92**	**0.215**	**0.16**	5.99	**6.22**	**-152.6**	36.45
SL-2	0.410	2984.31	0.266	0.2	**5.63**	6.11	-146.6	**36.74**
PL	0.401	3076.48	0.271	0.2	5.65	5.8	141.8	34.67

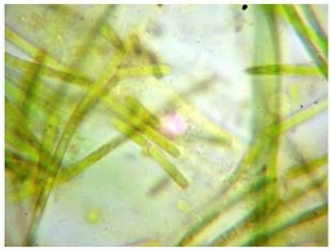
3.2. Isolation of blue-green algae

Based on the spread plate,*Nostocoryzae*was found to be predominant in Retter Lake at 10^{-6} dilution. *Phormidiumpapyraceum* was found to be the predominant algae obtained from Puzhal Lake at 10^{-5} dilution and*Lyngbyawollei* from Chembarambakkam Lake at 10^{-6} dilutions. The pure cultures obtained by frequent subcultures were maintained in solidified BG11 medium and subjected in liquid broth for further studies (**Fig. 2**).

Nostoc oryzae (JS-1) from Retteri lake (RL-1)



Phormidium papyraceum (JS-2) from Puzhal lake (PL-1)



Lyngbya wollei (JS-3) from Chembarambakkam lake (SL-2)

Fig. 2. The 100 X magnification of microscopic images showing isolated pure cultures of Cyanobacteria (Adapted from Presidency College (Autonomous), Chennai.

3.3. Growth curve analysis for the selected three different blue-green algae

The growth curve analysis clearly indicates that the biomass was obtained high during 13^{th}, 14^{th} and 15^{th} days of incubation under controlled culture conditions from all the three cyanobacteria sp. studied. At the same time, the accumulation of high biomass was obtained from JS-3 *Lyngbyawollei* followed by JS-2 *Phormidiumpapyraceum* and the least amount of biomass was recorded in *Nostocoryzae* JS1. Therefore, the results shows that the growth rate was comparatively high in*Lyngbyawollei* when compared to other cyanobacterial forms (**Fig. 3 to7**).

Fig. 3. The conical flasks showing the growth of *Nostocoryzae* (JS-1) for 15 days of *in vitro* cultivation

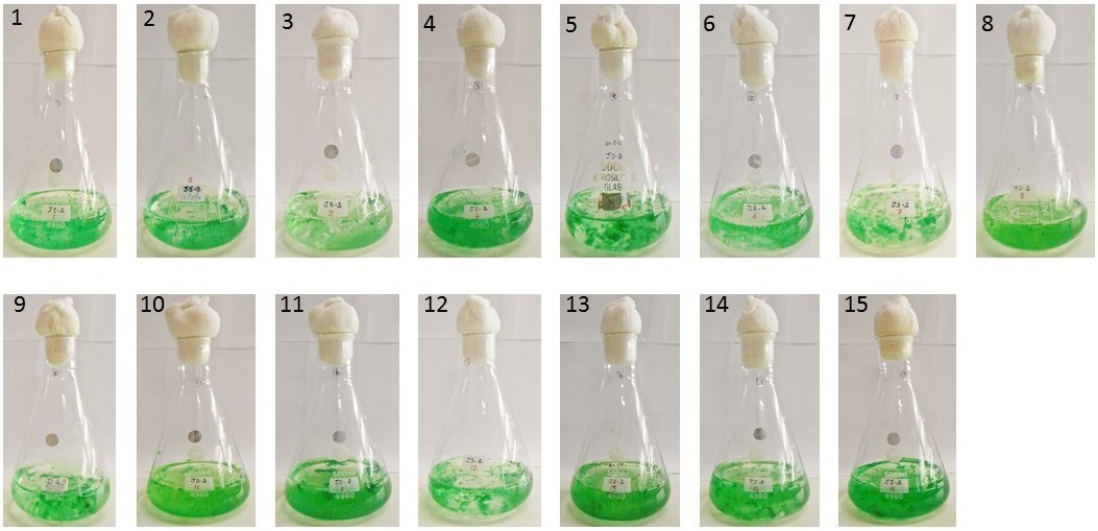


Fig. 4. The conical flasks showing the growth of *Phormidiumpapyraceum* (JS-2) for 15 days of *in vitro* cultivation

Fig. 5. The conical flasks showing the growth of *Lyngbyawollei* (JS-3) for 15 days of *in vitro* cultivation

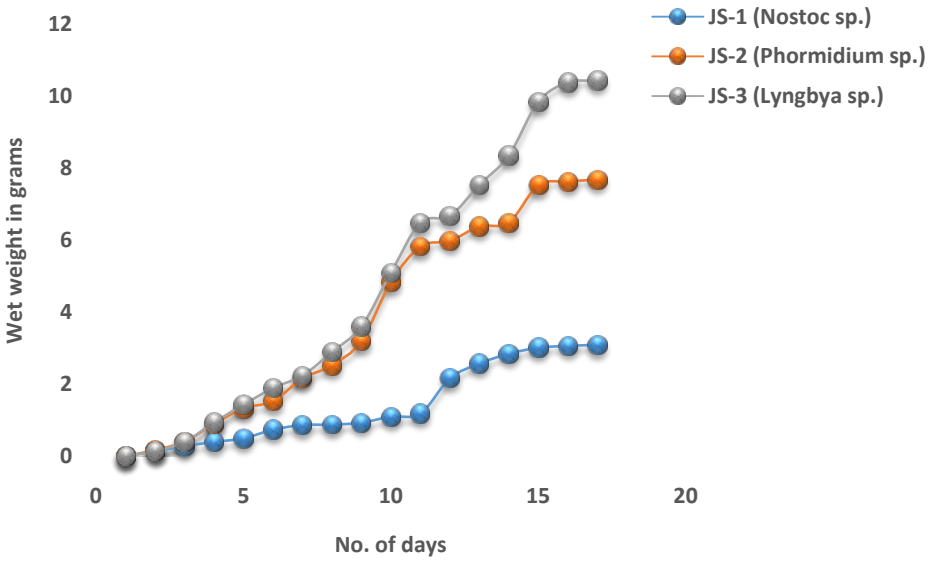


Fig. 6. The graph showing growth curve based on wet weight of three different filamentous cyanobacteria isolated from three different lakes

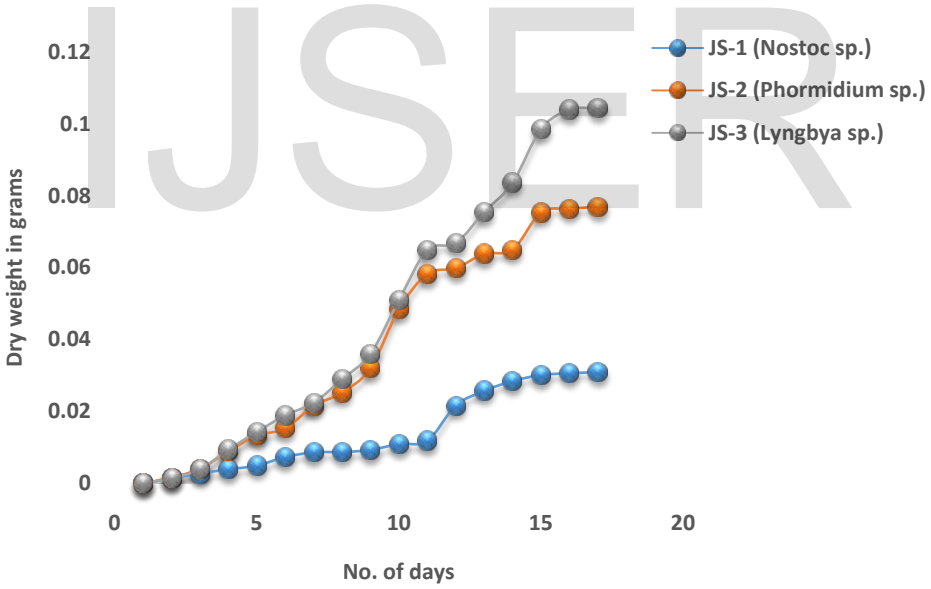


Fig. 7. The graph showing growth curve based on dry weight of three different filamentous cyanobacteria isolated from three different lakes

3.4. Extraction of polypeptides from blue-green algae

The polypeptide extracts from all the three cyanobacteria *Nostocoryzae* (JS-1), *Phormidiumpapyraceum* (JS-2) and *Lyngbyawollei*(JS-3) were shown in **Fig.8**. Where the

ethanolic extract showing yellowish green coloured extract solution for *Nostocoryzae* and green extract solution for the rest *Phormidiumpapyraceum* and *Lyngbyawollei*

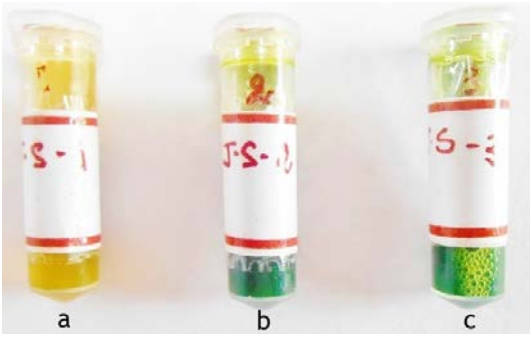


Fig. 8. The microcentrifuge tubes showing the extracts from three different cyanobacterial cultures; a) *Nostocoryzae* (JS-1), b) *Phormidiumpapyraceum* (JS-2) and c) *Lyngbyawollei*(JS-3).

3.5. Estimation of Protein (Bradford, 1976)

The total protein content estimated from the extract of all the three cyanobacteria obviously shown that the protein concentration was high in the extract of *Lyngbyawollei* (JS-3) followed by *Phormidiumpapyraceum* (JS-2) and was least in *Nostocoryzae* (JS-1) with 4.24 mg, 2.71 mg and 1.34 mg respectively (**Fig. 9**).

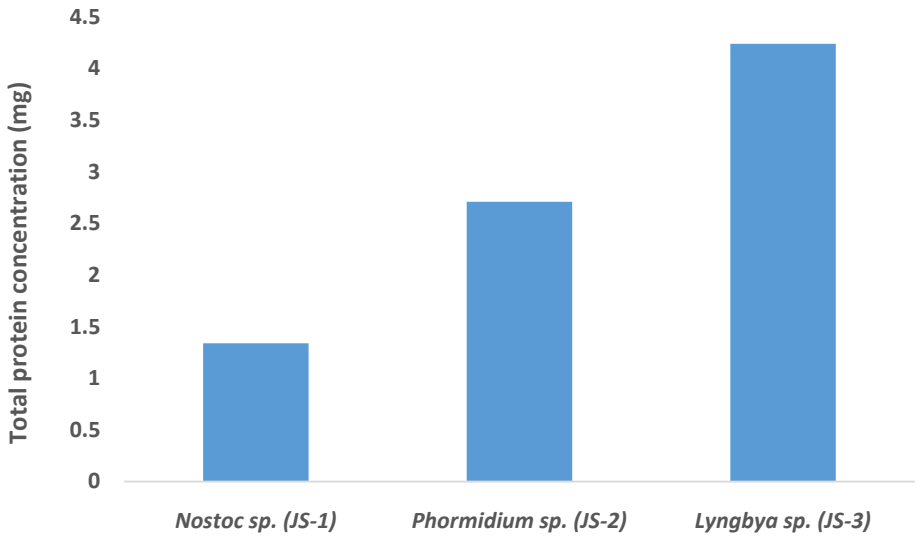


Fig. 9. The bar graph showing the total protein concentration of the extracts from three different cyanobacteria

3.6 Characterization of polypeptides

3.6.1. Liquid chromatography mass spectrometry analysis of polypeptides

Based on the LC-MS results obtained from the extracts of three cyanobacteria, *Nostocoryzae* consists of two different types of polypeptides Anabaenopeptin (Fig. 10) (Spoof *et al*., 2016) and Hoiamide A (**Fig. 11**) (Pereira *et al*., 2009) followed by *Phormidiumpapyraceum* with Micropeptin A (**Fig. 12**) (Czarneckiet *al*., 2006) and Microcystin (**Fig. 13**) (Christiansen *et al*., 2003) in *Lyngbyawollei* All these polypeptides were found based on their m/z from the LC-mass spectrum matched with the m/z of their respective compound.

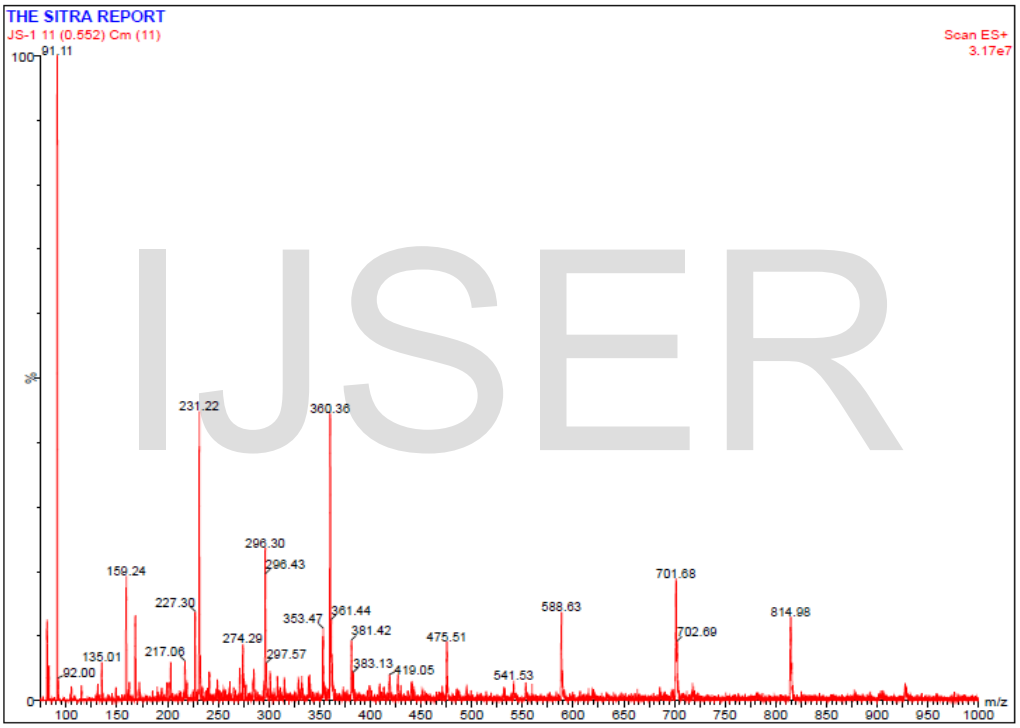


Fig. 10. The LC-MS of Anabaenopeptin from the extract of *Nostocoryzae* (JS-1)

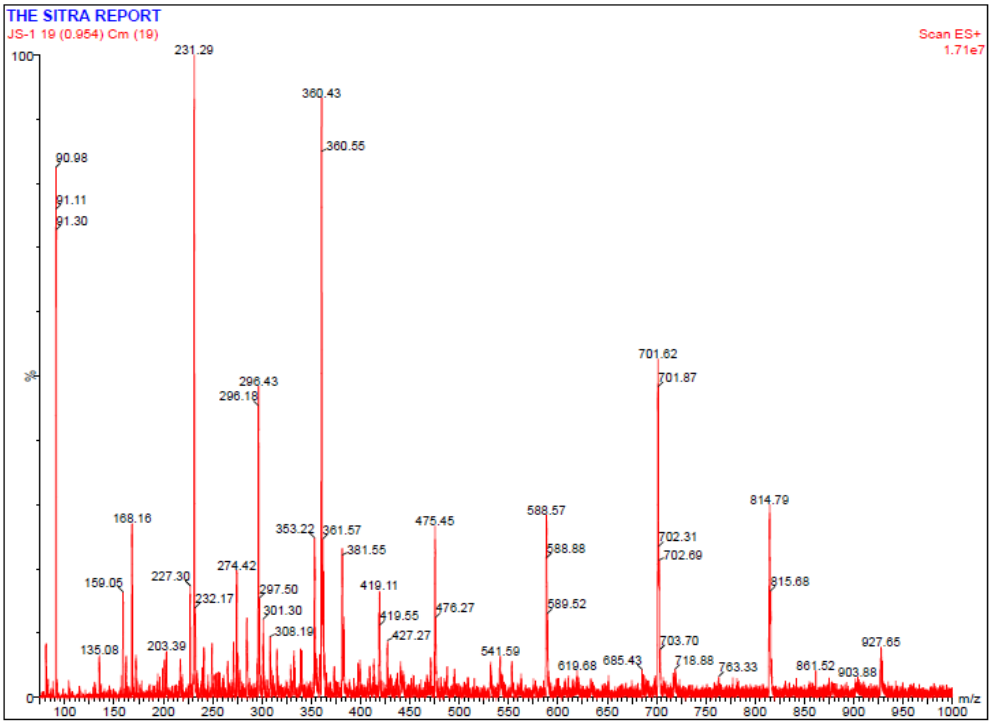


Fig. 11. The LC-MS of Hoiamide A from the extract of *Nostocoryzae*(JS-1)

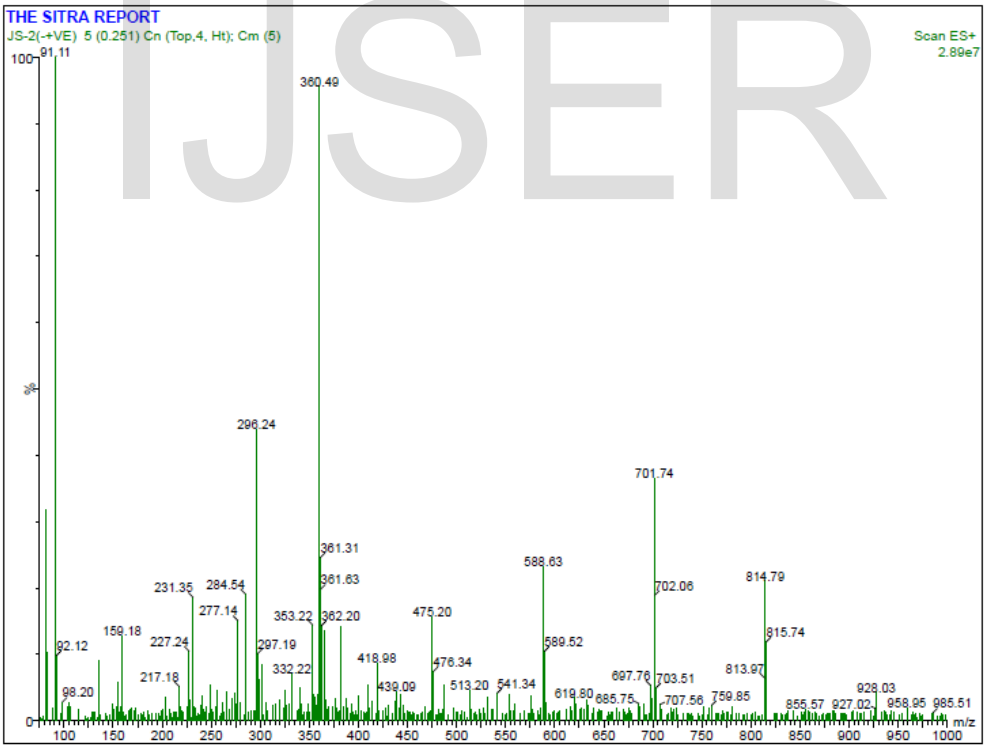


Fig. 12. The LC-MS of Micropeptin from the extract of *Phormidiumpapyraceum* (JS-2)

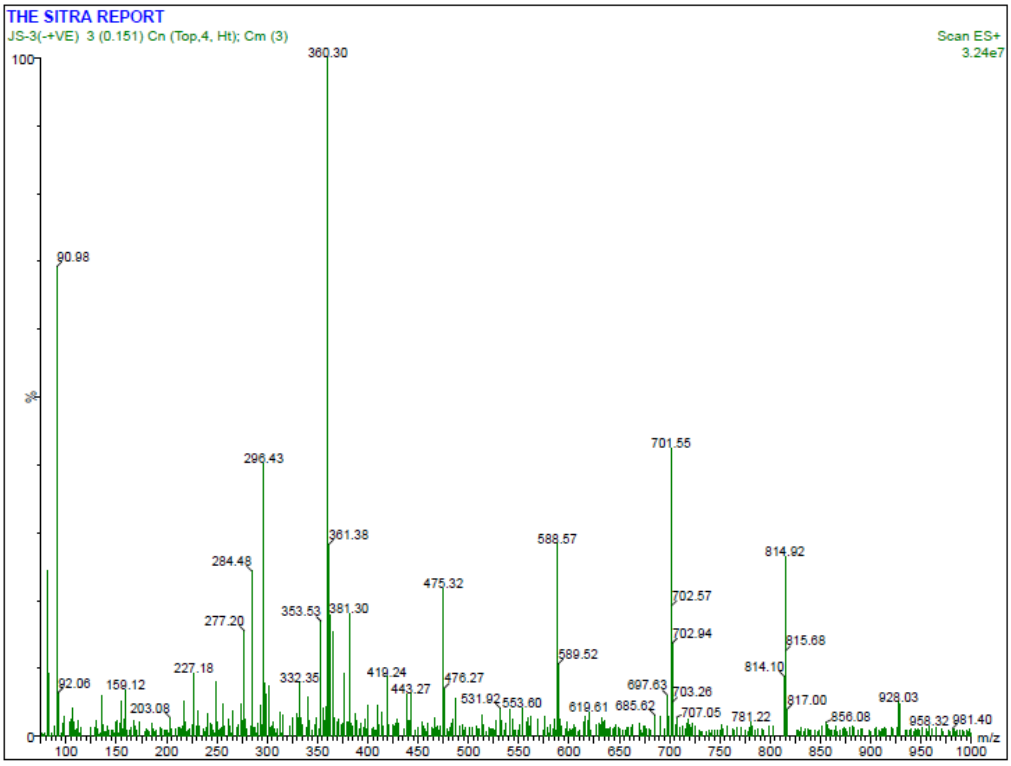


Fig. 13. The LC-MS of Microcystin from the extract of *Lyngbyawollei* (JS-3)

3.7. DPPH free radical scavenging assay (Blois, 1958)

The 50 % inhibitory concentration of free radicals by the extract were determined and are 222 µg for the extract from *Nostocoryzae* (JS-1) and 198 µg for the extract from *Lyngbyawollei* (JS-3) (**Table 2**) (**Fig. 14 and 15**). No antioxidant activity was found in the extract of *Phormidiumpapyraceum* (JS-2) (**Fig. 4.16**). The antioxidant activity of the extracts were increasing while increasing the concentration of the extract.

Table 2. Evaluation of DPPH free radical scavenging activity of the extract from Cyanobacteria

S. No.	Sample	50 % Inhibitory concentration (IC_{50})
1.	*Nostocoryzae*(JS1)	222 µg
2.	*Phormidiumpapyraceum* (JS2)	Nil activity
3.	*Lyngbyawollei* (JS3)	198 µg

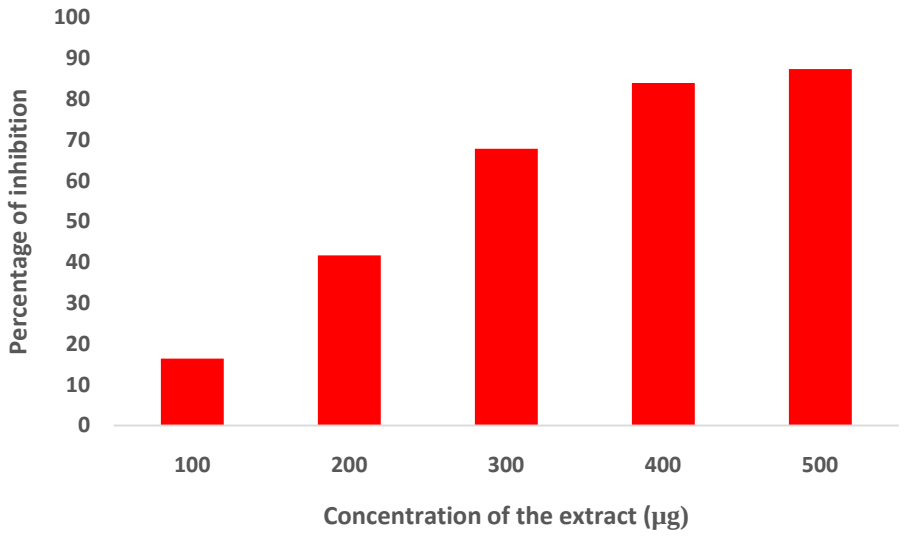


Fig. 14. Evaluation of antioxidant activity of the extract from *Nostocoryzae* (JS-1)

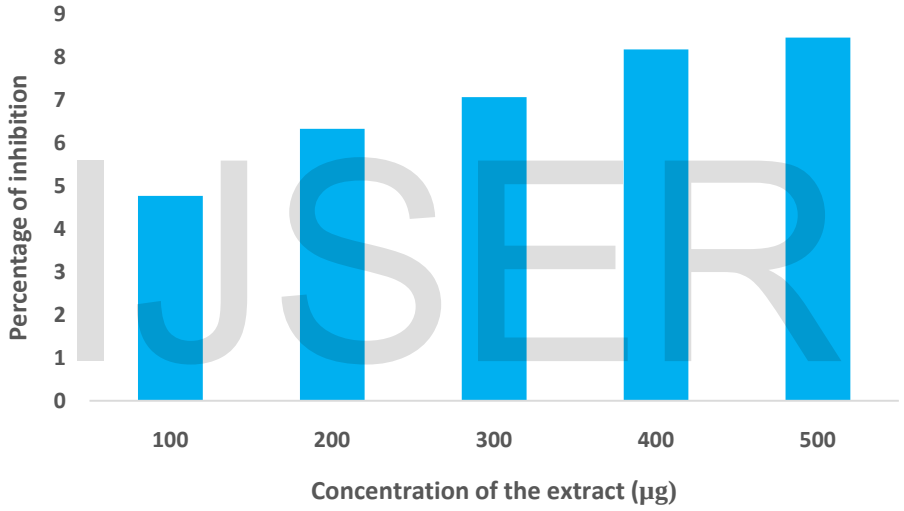


Fig. 15. Evaluation of antioxidant activity of the extract from *Phormidiumpapyraceum* (JS-2)

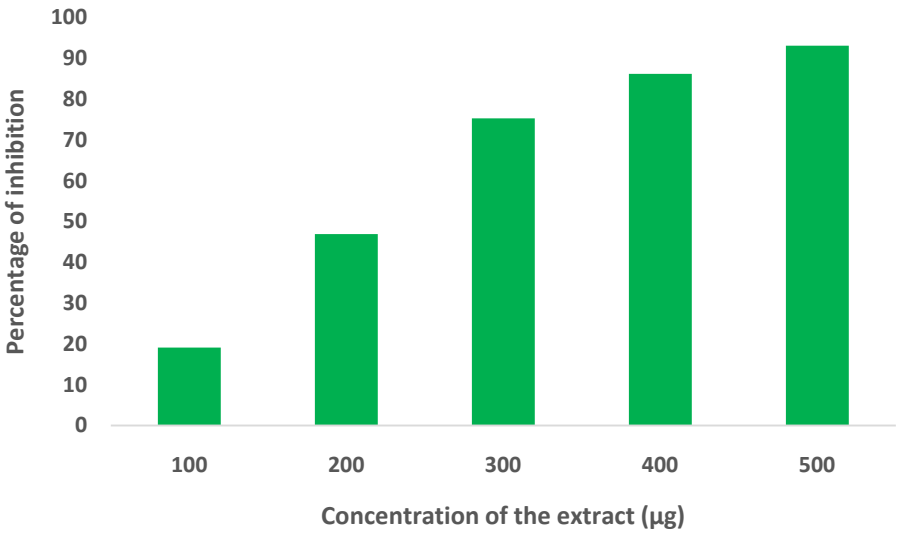


Fig. 16. Evaluation of antioxidant activity of the extract from *Lyngbyawollei*(JS-3)

3.8. Evaluation of anti-cervical cancer potential of polypeptides using MTT assay (Mosmann, 1983)

The evaluation of anti-cervical cancer activity of the extract from all three different filamentous cyanobacteria have shown that the viability of the cancer cells were inhibited well at the maximum concentration of the extract (500 µg). The viability percentage of the cancer cells were inhibited for both the extracts of *Nostocoryzae* (JS-1) and *Lyngbyawollei* (JS-3). But there was no any considerable changes in the cancer cells while evaluating the extract of *Phormidiumpapyraceum* (JS-2). The 50 % inhibitory concentration of the cancer cells by the extract of cyanobacteria were determined evaluated, which was 560 µg for *Nostocoryzae* and 610 µg for *Lyngbyawollei*(**Fig. 17 to 20**). No such anticancer activity was obviously visible by the extract from *Phormidiumpapyraceum.*

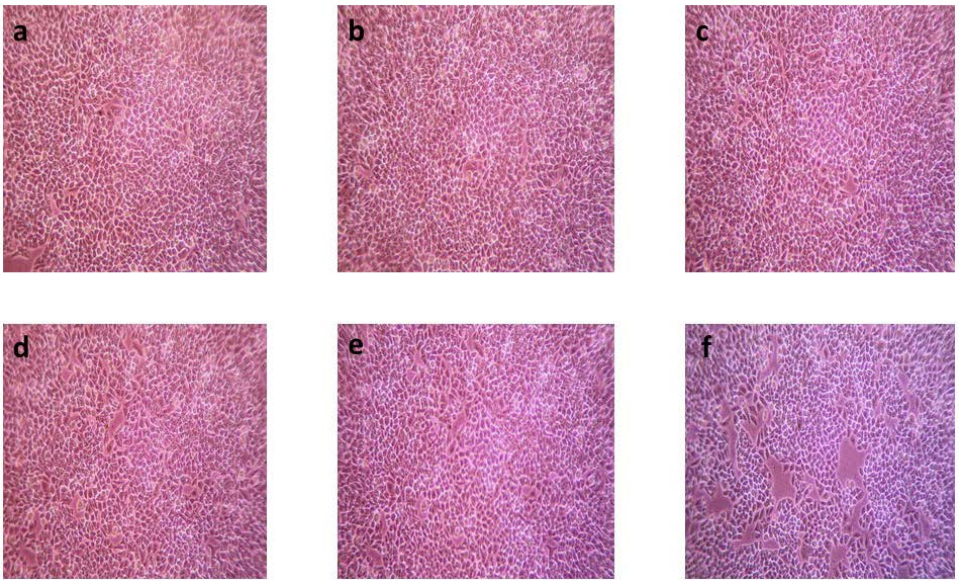


Fig. 17. Evaluation of anti-cervical cancer activity of the extract from *Nostocoryzae* (JS1) using HeLa cell line; a) Control, b) 1µg of the extract, c) 10µg of the extract, d) 100µg of the extract, e) 250µg of the extract and f) 500µg of the extract

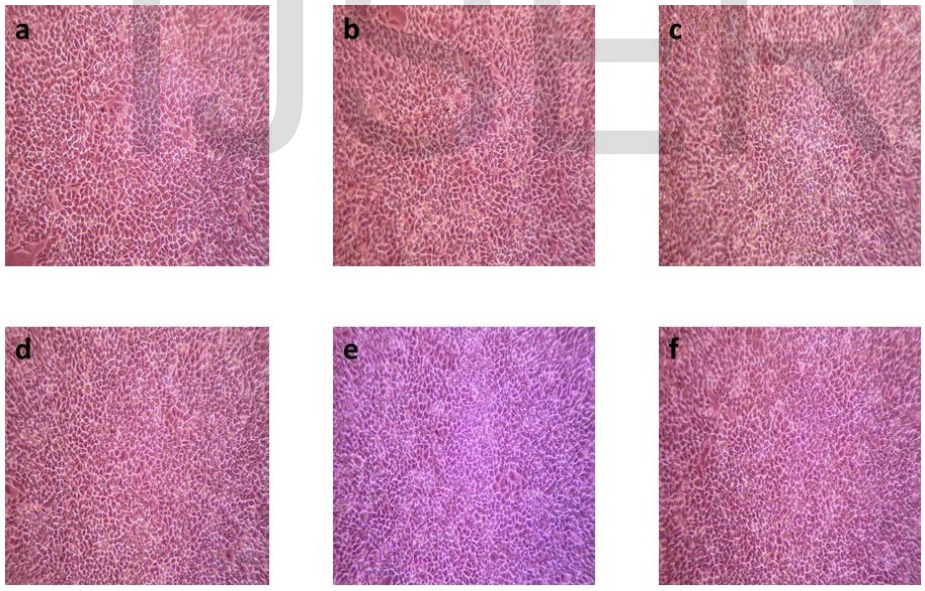


Fig. 18. Evaluation of anti-cervical cancer activity of the extract from *Phormidiumpapyraceum* (JS2) using HeLa cell line; a) Control, b) 1µg of the extract, c) 10µg of the extract, d) 100µg of the extract, e) 250µg of the extract and f) 500µg of the extract

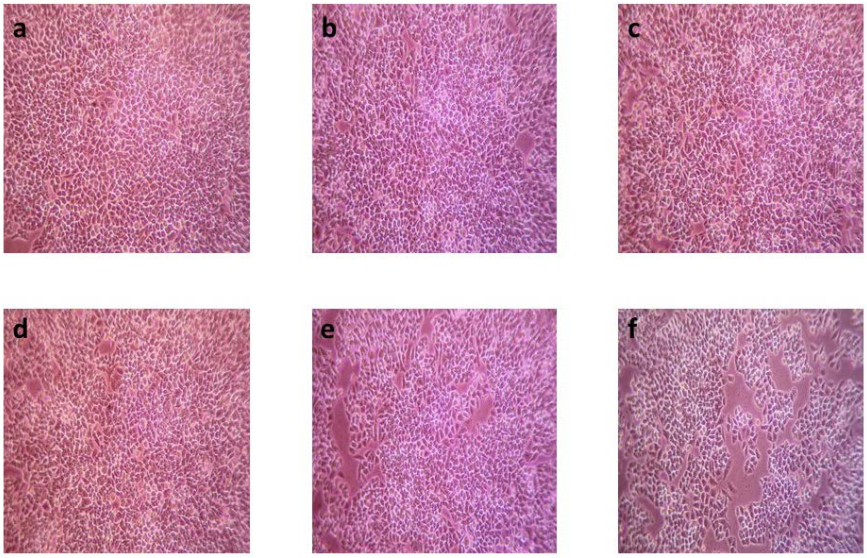


Fig. 19. Evaluation of anti-cervical cancer activity of the extract from *Lyngbyawollei*(JS3) using HeLa cell line; a) Control, b) 1µg of the extract, c) 10µg of the extract, d) 100µg of the extract, e) 250µg of the extract and f) 500µg of the extract

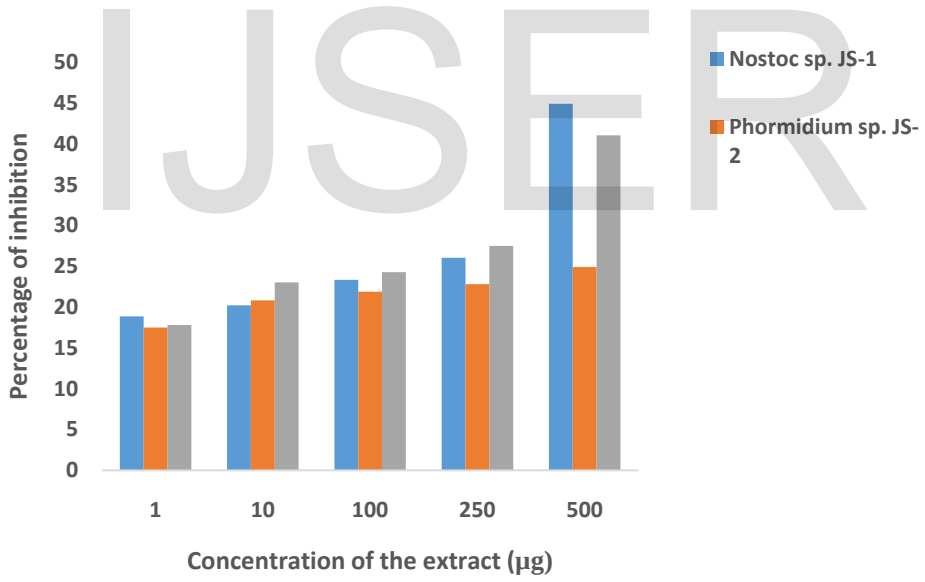


Fig. 20. Bar graph showing the comparison of percentage of inhibition of cancer cells in treating with all the three different extracts of different range of concentration

4. Discussion

Cyanobacteria (*Nostocoryzae*) were first used to treat gout and several forms of cancer in 1500 BC and the modern approach in this field started in the year 1990 by Moore and Gerwick. The Non-ribosomal polypeptides from cyanobacteria have been reported with antibacterial, antifungal, antiviral and anticancer activities. In the present study three different cyanobacteria were isolated from the fresh water lakes (*Nostocoryzae, Phormidiumpapyraceum*and *Lyngbyawollei*)among them *Nostocoryzae*and *Lyngbyawollei* exhibited high anti-oxidant activity. Inhibitory concentration(IC)-50 values were similar for both the extracts and the LC-MS analysis revealed the presence of anabaenopeptin and hoiamideA in *Nostocoryzae* whereas in *Lyngbyawollei* and *Phormidiumpapyraceum*Microcystin and micropeptin A were observed respectively. Anabaenopeptins are cyclic hexapeptides which are N-methylated and consists of conserved uredo linkage which makes it a potent protease inhibitors. These peptides are synthesized by several cyanobacteria such as *Nodularia, Microcystis, Anabaena and Planktothrix*(Sivonen and Börner, 2008 and Welker and von Döhren, 2006).

The apoptotic cell undergoes morphological changes such as cell shrinkage, nuclear or chromatin condensation, membrane blebbing etc.(Morita *et al*., 2001). HoiamideA induced neuronal apoptosis by stimulating caspase-3 and nuclear condensation (Cao *et al*., 2015).Micropeptin A (cyanopeptolins) is a serine protease inhibitors and it showed potent inhibitory activity to crustacean and mammalian serine proteases. Similarly microcystin (cyanoginosins) are toxins produced by *Microcystis aeruginosa, Anabaena, Oscillatoria, Nostoc, Planktothrix*etc. since they are cytotoxic it could be used to induce apoptosis in cancer cells. Hence these polypeptides could be used to treat cervical cancer (Gademann*et al*., 2010).

About 70% of the cervical cancer occurs in developing countries. Human Papilloma Virus (HPV) is one of the major cause of cervical cancer (Canavan and Doshi, 2000).The vaccines Gardasil and Cervarix reduces the risk of cancer by 93% and 62% respectively. These drugs are effective for 8 years but it is effective only if given prior to the infection and the high cost of the vaccines are of another concern (Medeiros *et al*., 2009).The extracts from all the three cyanobacteria were tested in HeLa cell lines and the 50 % inhibitory concentration (IC_{50}) of *Nostocoryzae*were observed to be 560 µg and 610 µg for *Lyngbyawollei*and absence of anticancer activity were observed in

*Phormidiumpapyraceum*Hence NRPSs from cyanobacteria could prove to be a cost effective drug to treat cervical cancer.

5. Conclusion

The cyanobacteria are the potent source of new and novel kinds of secondary metabolites. Recently, research on pharmaceutical properties of these new types of polypeptides have increased among scientists due to the neurotoxic, hepatotoxic and cytotoxic properties. Most of these polypeptides are protease inhibitors and thus could inhibit the proliferation of cancer causing retroviruses. In our present study, four different polypeptides Anabaenopeptin, Hoiamide A, Micropeptin A and Microcystin were extracted and characterized by LC-MS from *Nostocoryzae*, *Phormidiumpapyraceum*and*Lyngbyawollei* respectively. The antioxidant activity was relatively high in the extracts of *Nostocoryzae* and *Lyngbyawollei*Similarly, anticancer activity was also observed to be high in both the species. Therefore, it has been concluded that the polypeptide extracts from cyanobacteria has potential antioxidant and anticancer activities.

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