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Comparative free radical scavenging potential and antiproliferative activity of crude methanolic extract and partially purified compounds isolated from *Tolypothrix* sp.

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Abstract
The aim of present study is to isolate significant bioactive secondary metabolites from methanol extract of cyanobacterium *Tolypothrix*sp. andto explore the antioxidant and anticancer activities of partially purified active bands,based on their antibacterial activity and further characterization by GC-MS analysis. The antioxidant and anti-proliferative property of the crude extract and active bands (bands exhibiting potent antibacterial activity) derived from *Tolypothrix*sp.were determined using DPPH radical scavenging assay and MTT assay respectively. Active band, Toly-5 was found to be most effective with the inhibition of 74% followed by Toly-6 with the inhibition of 65.43% at 250 µg/ml while the scavenging activity of crude extract was relatively less inhibitory (41.72%) at 250µg /ml. Anti-proliferative activity of active bands of *Tolypothrix* sp. were carried out with human lung cancer (A-549) and human normal kidney (HEK-293) cell lines. Toly-6 band compounds exhibited significant cytotoxic activity against the human lung cancer A-549 cell lines while almost no effect on the normal human HEK-293 cells giving a glimpse of the presence of "certain drug leads" in this Toly-6 band which can be developed as an effective anticancer drug. Presence of various compounds in different bands e.g., 2,6,10-trimethyl, 3,7,11,15-tetramethyl-2-hexadecen-1-ol, hexadecanoicacid, heneicosanoic acid and hexadecanoate has further enlighten the interest to isolate and purify these active compounds for further mechanism-based biological activity testing. Thus, the present study reflects a hope for the development of novel agents of bio-medical importance.

Key words: cyanobacteria, bioactive compounds, methanolic extract, MTT assay, antioxidant, anticancer activity.

Introduction

Emergence of multiple drug resistance to human pathogenic organisms due to indiscriminate use of commercial antimicrobial drugs has necessitated a search for new substances from other sources including cyanobacteria. This is of paramount importance to fight increasingly resistant pathogens and newly emergent diseases (1). The overwhelming available knowledge on the diversity and physiology of cyanobacteria serves as an excellent base for exploring their applications. In the last few years, cyanobacteria have gained much attention as a rich source of bioactive compounds and have been considered as one of the most promising groups of organisms to produce them (2, 3).The bioactivities exhibited by the cyanobacteria are due to the presence of novel metabolites which belong to the group of polyketides, phenols, alkaloids and peptides. These cyanobacterial metabolites include anti-inflammatory (4), antifungal (5), antiviral (6), antibacterial (7) and anticancer (8). These products are different from chemically synthesized pharmaceutical products, which are usually directly extracted from a biological source.

The major cause of most of today's disease is excess of ROS generated as a result of oxidative stress apart from the threats imposed by the microorganisms. Excess ROS in the body can lead to cumulative damage in proteins, lipids, and DNA which finally contributes to cancer, atherosclerosis, cardiovascular diseases, ageing and inflammatory diseases To combat the deleterious effects of ROS, cells develop complex antioxidant system. The antioxidant system includes enzymes with important scavenging functions, like superoxide dismutase (SOD), catalase and several peroxidases. Some other low molecular weight molecules also have crucial roles as antioxidants, such as glutathione, ascorbate or phenolic compounds (9).

Antibacterial and antioxidant potential of crude extracts of several cyanobacterial strains in different solvents is well documented (10,7,11). However, little information is available regarding the purification and identification of

compounds responsible for different bioactivities. Reports are available on the purification of compounds mainly from marine cyanobacterial strains. Amongst the freshwater cyanobacteria, literature regarding the identification of bioactive compounds in cyanobacteria like *Oscillatoria* and *Anabaena* is available. Organisms belonging to the genus *Tolypothrix* have been reported to produce several metabolites having commercial importance. Many strains of *Tolypothrix* are known to produce anti-inflammatory (4), antibacterial (7), and antifungal compounds (5). Literature data related to detailed analysis of the crude extract and partially purified compounds from *Tolypothrix* sp. and their antioxidant and antiproliferative activity is very limited. Therefore, an attempt has been made to partially purify the bioactive compound(s) present in the methanolic extracts of *Tolypothrix* sp. which were separated by TLC, evaluate the bioactive potential and characterize them by GC-MS analysis.

Materials and methods

Cyanobacterial strains

Collection of cyanobacterial strains and growth conditions

*Tolypothrix*sp.a filamentous, heterocystous cyanobacterium was procured from Department of Biological Sciences, Allahabad Agricultural Institute, Deemed University, Allahabad. Axenic culture of *Tolypothrix* sp. was maintained in the culture room at 27 ± 2 °C. For regular experiments, cultures were grown in CHU-10 medium (pH 7.5) under photosynthetic photon flux density (PPFD) of 75 µmol m-2 s-1 and 14h photoperiod

Preparation of crude extracts

Solvent based extraction was carried out with the soxhlet apparatus. The 25 to 30 days old cyanobacterial cultures were filtered with sterile many folds muslin cloth and the pellets were air dried. Methanol was used for extraction. The above solvents were used in obtaining most of the compounds present in the cyanobacterial cells. The crude extracts were filtered by Whatman (No.1) paper. Extracts were dried at room temperature, and stored at 4°C until further use**.**

Extraction of the bioactive compounds

The methanol extract thus obtained was re-dissolved in 1 ml methanol. The yield after extraction was used for the detection of bioactive compounds such as flavonoids and total phenols by thin layer chromatography (TLC).For the TLC analysis, 25 µl of the extract was loaded on the TLC (Silica gel 60) plates (Merck, Germany). The solvent ratio used for the separation of the compounds was Chloroform:methanol (4:1, v:v). UV-trans-illumination of the plates at 365 nm revealed Seven bands of *Tolypothrix* sp. (Toly-1, Toly-2, Toly-3, Toly-4, Toly-5, Toly-6 and TOly-7). These bands were eluted separately with a minimum amount of methanol. All the bands were bio-assayed for antibacterial potential. Bands exhibiting potent antibacterial activity i.e., the active bands were selected for further studies.

Antibacterial Assay

The antibacterial assay was performed against *Staphylococcus epidermidis* (NCIM 2493) (National Chemical Laboratory, Pune, India) by disc diffusion method on Mueller-Hinton (MH) agar (12). The 0.1 ml of diluted inoculum (105 CFU/ ml) of test bacteria was spread on Mueller-Hinton (MH) agar plates. Wells of 5 mm diameter were punched into the agar medium and poured with 25 µl of extract prepared in DMSO (10 mg ml^{-1}). DMSO without extract was used as blank. After incubation for 24 h at 37 °C, antibacterial activity was evaluated by measuring the zone of inhibition against the test organism.

Determination of free radical scavenging activity or Antioxidant activity

In the present work we screened cyanobacteria for the antioxidant potential by using DPPH. The DPPH radical scavenging capacity of the crude methanol extract and TLC separated active bands was determined according to the method of (13)(Brand Williams 1995) modified by (14). DPPH radicals have an absorption maximum at 515 nm, which disappears with reduction by an antioxidant compound. The DPPH solution in methanol (6×10^{-5} M) was prepared freshly, and 3 mL of this solution was mixed with 100 *µ*L of various concentrations (0-0.5 mg/ml) of crude extract or TLC-eluted compounds. The samples were incubated for 20 min at 37 °C in a water bath, and then the decrease in absorbance at 515 nm was measured (*A*e). A blank sample containing 100 *µ*L of methanol in the DPPH solution was prepared and its absorbance was measured (*A*b). All tests and analyses were run in triplicates and the results obtained were averaged. Radical scavenging activity was calculated using the following formula: % inhibition = [(*A*b-*A*e)/*A*b] × 100

Where *A*b = absorbance of the blank sample, and *A*e = absorbance of the methanol extract.

Anticancer (anti-proliferative) activity

Anti-proliferative activity of the methanolic crude extract of *Tolypothrix* sp. and active bands were carried out with human lung cancer (A-549) and human normal kidney (HEK-293) cell lines. Both A-549 and HEK-293 cell lines were obtained from the National Center for Cell Science, Pune, India. The A-549 cells and HEK cells were grown as monolayer cultures in H-12 K medium and Dulbecco's modified Eagle's medium, respectively and supplemented with 10% fetal calf serum. Cells were incubated at 37 °C in a humidified atmosphere of 5% CO_2 and 95% air. For MTT Assay (15), the cells (3 x 103/well) were cultured in 96-well plates treated with test sample dilutions for 48h and MTT was added to each well (1 mg/ml final concentration) for the detection of cytotoxicity.

GC-MS analysis

For the identification of metabolites showing antioxidant and anticancer potentials, the samples were subjected to GC-MS analysis from AIRF, JNU, New Delhi. The crude and active bands (2 mg/ml) dissolved in methanol (HPLC grade, Merck, India) were injected (1µl) with a split ratio of 1:10 into a RTX-5 column (60 m X 0.25 mm i.e., film thickness 0.25 µm) of GC-MS (model GC-MS-QP-2010 plus, Shimadzu Make). Helium was used as carrier gas at a constant column flow 1.2 ml/min at 173 kpa inlet pressure. Temperature programming was maintained from 100°C to 200°C with constant rise of 5°C/min and then held isothermal at 200°C for 6 min; further the temperature was increased by 10°C/min up to 290°C and again held isothermal at 290°C for 10 min. The injector and ion source temperatures were 270°C and 250°C, respectively. Mass spectra were taken at 70 eV; a scan interval of 0.5 s and fragments from 40 to 950 Dalton. The final confirmation of constituents was made by computer matching of the mass spectra of peaks with the Wiley and National Institute Standard and Technology (NIST) libraries mass spectral database.

Statistical analysis

All experiments were repeated three times to ascertain the reproducibility of the result. Values are represented as mean ± SE (n = 3). Statistical analysis was carried out by using the SPSS program (SPSS Inc., version 10.) $P<0.05$ was considered statistically significant.

Results

The present study tried to evaluate the bioactive potential(antioxidant and anticancer activity) of partially purified compounds isolated by preparative TLC on the basis of antibacterial activity from the *Tolyphothrixsp.* and their characterization by GC-MS analysis.

TLC analysis

TLC analysis of themethanolic extract from *Tolyphothrix sp.* showed the presence of seven bands (Toly-1, Toly-2, Toly-3, Toly-4, Toly-5, Toly-6 and Toly-7) under UV illumination (Figure 1).

Figure 1. TLC separation of methanolic crude extract of *Tolyphotrix sp.* using Chloroform: Methanol (4:1) as mobile phase, visualized under UV light 365 nm.

Antibacterial activity

The antibacterial activity of bands (Toly-1, Toly-2, Toly-3, Toly-4, Toly-5, Toly-6 and Toly-7) isolated from methanol extract of *Tolypothrix* sp. (data not shown). From all the bands isolated only three bands (Toly-4, Toly-5 and Toly-6) with R_f value 0.50, 0.72 and 0.86 respectively, were found to be active against *S. epidermidis*. The disc prepared from active band (Toly-6) showed maximum inhibition zone (13 mm) against *S. epidermidis* followed by Toly-5(10mm) and Toly-4 (7mm) respectively.

Antioxidant activity

Evaluation of antioxidant properties of crude extract and TLC eluted compounds of *Tolypothrix* sp. which exhibited potent antibacterial activity were determined using DPPH radical scavenging assay and the results are shown in Figure 2. DPPH was reduced with the addition of crude and active bands in a concentration dependent manner. Active band (Toly-5) was found to be most effective with the inhibition of 74% followed by Toly-6 with the inhibition of 65.43% at 250 µg/ml while the scavenging activity of crude extract was relatively less inhibitory (41.72%) at 250 µg/ml. Toly-6 showed relatively comparable inhibition to Ascorbic acid standard which was 85.45% at 250µg/ml.Toly-4 showed minimum inhibition.

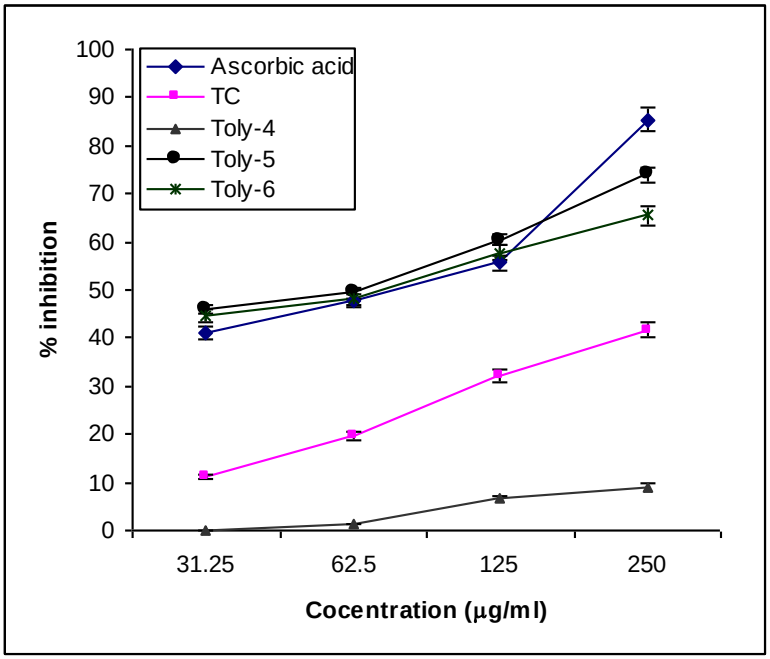

Figure 2.DPPH radical scavenging activity of methanolic crude extracts and active bands (Toly-4, Toly-5 and Toly 6) isolated from *Tolypothrix* sp.

Anticancer (anti-proliferative) activity

Anti-proliferative activity of the methanolic crude extract of *Tolypothrix* sp. and active bands (Toly-4, Toly-5 and Toly-6) were carried out with human lung cancer (A-549) and human normal kidney (HEK-293) cell lines. *In vitro* studies suggest the potential of crude extract and the active bands (Toly-4,Toly-5 and Toly-6) on cell line A-549 and HEK -293 (Table 1). The comparison of cytotoxicity clearly indicated that active band (Toly-6) at 100µg/ml has low toxicity (0.15%) towards normal human cells, HEK-293. At the same time, it is cytotoxic (31.93%) at 100 µg/ml towards A-549 lung cancer cell lines.

Table 1. Antiproliferative activity of *Tolypothrix* sp.

Compound	Concentrations (µg/ml)	Inhibition of growth in comparison to control (%)	
		A-549	HEK-293
Toly Crude	100	34.77±0.68	21.26±0.45
	50	22.85±0.30	13.01±0.18
	25	11.97±0.10	11.22±0.09
	12.5	2.51±0.05	0.82±0.01
	6.25	0.21±0.01	ND*
	100	11.97±0.05	0.37±0.017
	50	4.5±0.035	0.25±0.017
	25	2.51±0.03	0.13±0.002
	12.5	0.31±0.02	ND*
Toly-4	6.25	ND*	ND*
	100	38.72±0.62	23.86±0.86
	50	24.19±0.32	14.05±0.15
	25	18.17±0.14	4.5±0.1
	12.5	16.52±0.13	ND*
Toly-5	6.25	6.579±0.09	ND*
	100	31.93±0.11	0.15±0.01
	50	23.63±0.07	ND*
	25	20.54±0.04	ND*
	12.5	7.23±0.02	ND*
Toly-6	6.25	3.64±0.02	ND*

ND* Not detected at this concentration. Value are mean ± SD (*n*= 3).

GC-MS analysis

For the identification of metabolites showing antioxidant and anticancer potentials, the samples were subjected to GC-MS analysis. The GC-MS analysis of crude and active band as shown in Figures 3-6 indicates the presence of different components. The major components present in the methanolic crude extract of *Tolyothrix*sp. were Citronellyl formate (RT: 9.096), 2,6,10-Trimethyl (RT: 18.293), 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (RT: 18.942), Hexadecanoic acid (RT: 19.654) and Heneicosanoic acid (RT:22.295) (Table 2).Six components were identified in active band (Toly-4), out of which Hexadecanoate (RT: 19.573) and Hexadecatrienoic acid (RT: 21.918) were the major compounds (Table 3). Five components were detected in the active band (Toly-5), however, Acetic acid (RT: 9.073) and Hexadecanol (RT: 17.603) were the major components (Table 4). Four components were identified in active band(Toly-6), out of which Methyl stearidonate (RT: 21.799) and 6,9,12-Octadecatrienoic acid (RT: 21.690) were the major compounds (Table 5).

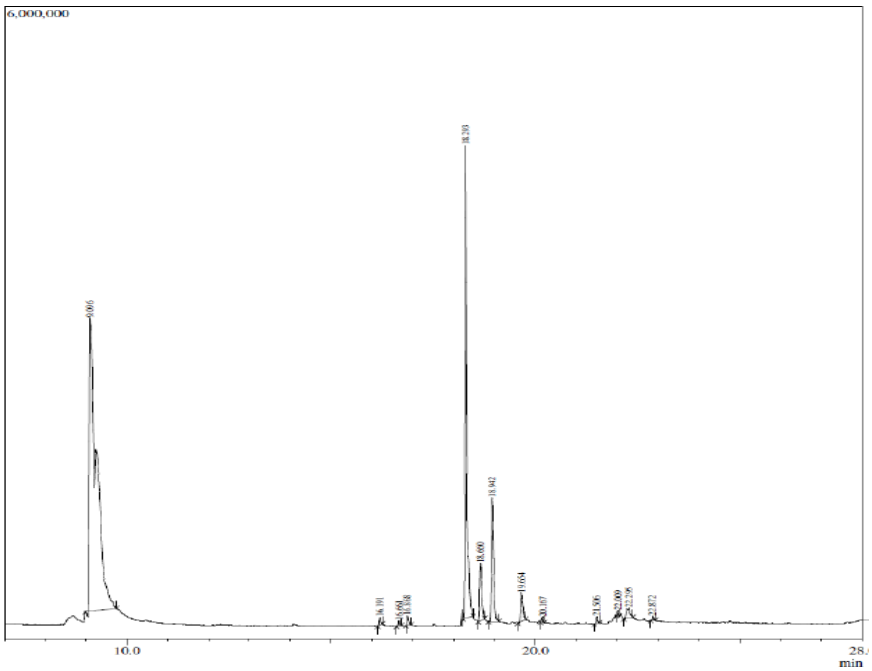

Figure 3.GC-MS chromatogram of crude methanolic extract of *Tolypothrix* sp.

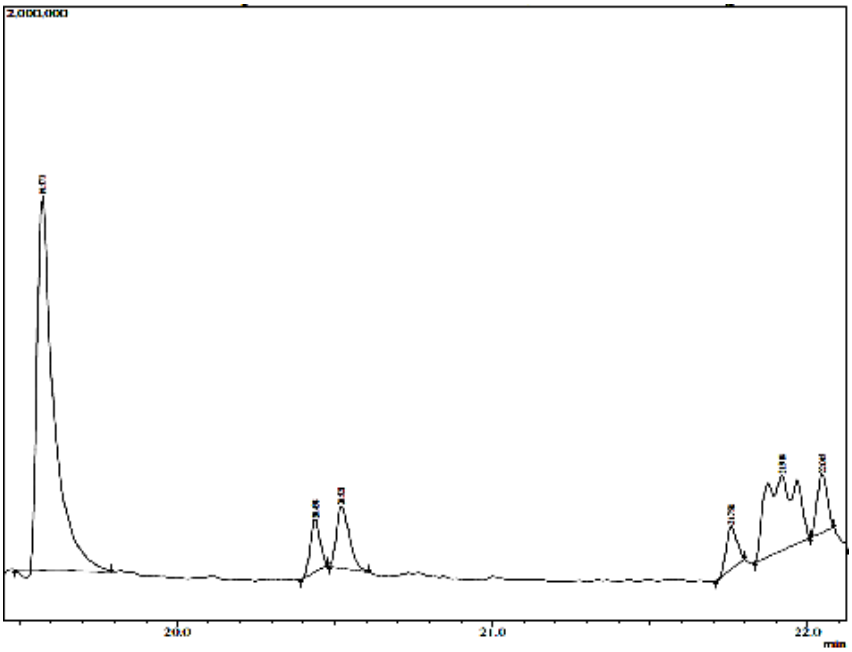

Figure 4.GC-MS chromatogram of Active Band (Toly-4) isolated from crude *Tolypothrix* sp.

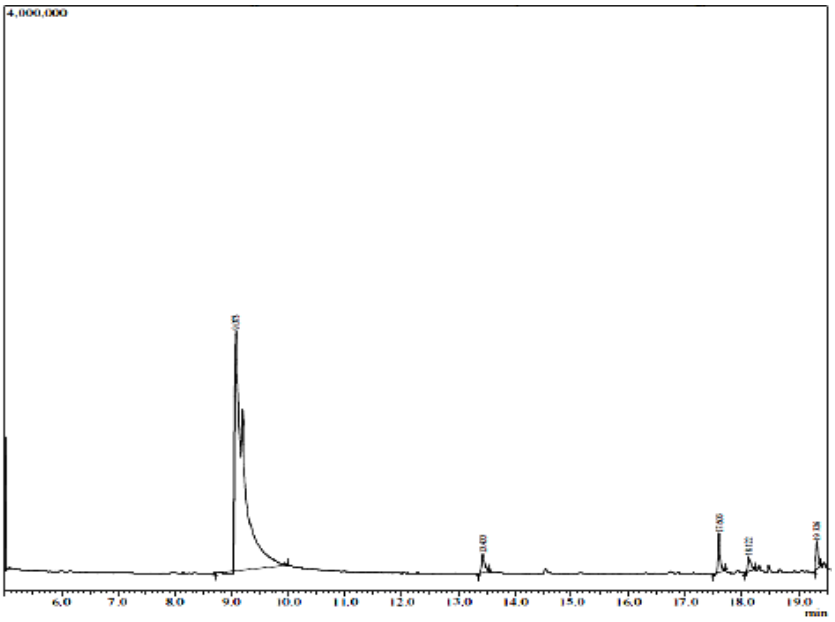

Figure 5.GC-MS chromatogram of Active Band (Toly-5) isolated from crude *Tolypothrix* sp.

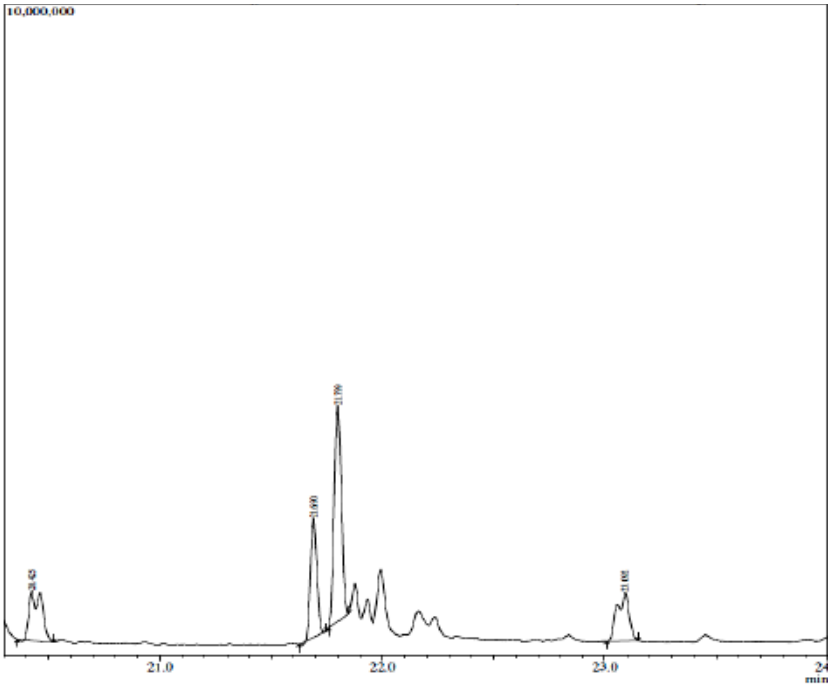

Figure 6.GC-MS chromatogram of Active Band (Toly-6) isolated from crude *Tolypothrix* sp.

Table 2. Bioactive compounds identified in the methanolic crude extract of *Tolyothrix* sp.by GC-MS

Peak	R. Time	Area%	Compound Name
1	9.096	59.90	Citronellyl formate
2	16.191	0.35	Tetradecane
3	16.661	0.24	Dichloroacetic acid
4	16.868	0.51	5,6-DIPROPYLDECANE
5	18.293	24.72	2,6,10-TRIMETHYL
6	18.660	3.06	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
7	18.942	7.70	3,7,11,15-Tetramethyl-2-hexadecen-1-ol
8	19.654	1.75	Hexadecanoic acid
9	20.167	0.16	1,5-HEPTADIEN-4-OL
10	21.506	0.26	2,3-Dimethyl-undec-1-en-3-ol
11	22.009	0.11	13-DOCOSENOIC ACID
12	22.295	1.07	Heneicosanoic acid
13	22.872	0.17	Phytol
		100	

Table 3.Bioactive compounds identified in the active band (Toly-4) isolated from methanolic crude extract of *Tolyothrix* sp. by GC-MS

Peak	R. Time	Area%	Compound Name
1	19.573	56.70	Hexadecanoate
2	20.438	4.76	1-Heptadecene
3	20.521	7.26	HEXADECANOIC ACID
4	21.758	4.28	HEXADECATRIENOIC ACID
5	21.918	21.42	HEXADECATRIENOIC ACID
6	22.045	5.59	9,12,15-OCTADECATRIENOIC ACID
		100.00	

Table 4.Bioactive compounds identified in the active band (Toly-5) isolated from methanolic crude extract of *Tolyothrix* sp. by GC-MS

Peak	R. Time	Area%	Compound Name
1	20.425	17.87	1-Nonadecene
2	21.690	21.51	6,9,12-OCTADECATRIENOIC ACID
3	21.799	44.30	Methyl stearidonate
4	23.092	16.33	HEPTADECANOIC ACID
		100.00	

Table 5.Bioactive compounds identified in the active band (Toly-6) isolated from methanolic crude extract of *Tolyothrix* sp. by GC-MS

Peak	R. Time	Area%	Compound Name
1	9.073	89.67	Acetic acid
2	13.433	2.50	3,5-bis(1,1-dimethylethyl)-phenol
3	17.603	3.35	Hexadecanol
4	18.122	1.95	Tetradecanal
5	19.326	2.52	9-HEXADECENOIC ACID
		100.00	

Discussion

The aim of the present study was to identify the potential bioactive compounds in the cyanobacterium, *Tolypothrix* sp. which has not been much explored much in context with the bioactive compound production. Polar solvent such as methanol has ability to extracts tannins, saponins, flavonoids, steroids and most of the other bioactive compounds (16).

During the growth microorganisms synthesized metabolites of different nature that is accumulated in the cells or excreted into the medium for the support in growth and metabolic activities. The antimicrobials production ability may be significant not only as a defensive instruments for algae but also as a good source of novel bioactive compounds from a pharmaceutical point of view (17).The antibacterial activity of the extracts could be due to the presence of different chemical agents that may include flavonoids and triterpenoids and other compounds of phenolic nature or free hydroxyl group (18). The presence of certain metabolites such as tannin, alkaloids, protein, and flavonoids in the extract of cyanobacteria was also reported (19) The species *Phormidium* and *Microcoleus* demonstrated the effective ethanol extracts for antibacterial activity against *Streptococcus enteritidis* and *Escherichia coli*. The extracts of *Phormidium* sp. showed highest inhibition zone to *Streptococcus enteritidis*(11 mm) at 0.2 g/ml demonstrated by

(7).Active bands (Toly-5 and Toly-6) of *Tolyphothrixsp.*was found to have more antioxidant activity than methanol crude extract of above strains while Toly-4 exhibited less antioxidant potential as compared to the crude extract. The higher activity of crude extract may be due to the synergistic effect of a number of compounds present and identified by GC-MS analysis. The antioxidant potential of the active bands (Toly-4, Toly-5 and Toly-6) and crude extract may be attributed to differences in their chemical composition such as polyphenyl compounds, phenolic compounds, like hexadecanoic acid, squalene and others (20).(21) also reported the active constituents of cell extracts of various algae which might be used in various pharmaceutical industry.Antioxidant potential of methanol extracts of different cyanobacteria (*Nostoc*sp*, Osillatoria* sp.,*Plectonema boryanum*, *Chroococcus* sp., *Scytonema* sp. and *Anabaena variabilis*) was also studied by (22). Recently good antioxidant activity of methanolic extract of *Lyngbyalimnetica*and *Scytonema bohnerii* has been reported with the notable presence of polyphenols like carotenoids, phycocyanins and some phytoconstituents (23).

Uncontrolled cell division in case of cancers can be prevented by Programmed cell death (apoptosis) but cancerous cells for several reasons cannot enter apoptotic phase. In that context, microalgal extracts are reported to be effective in inducing apoptosis in cancer cells (24). Cyanobacteria are considered as a promising source of structurally novel bioactive secondary metabolites and have been reported to modulate cell death through condensation of chromatin and the fragmentation of the nucleus in addition to release of apoptotic bodies. Furthermore the majority of these potent biomolecules target eukaryotic cytoskeleton, such as tubulin and actin microfilaments, as well as target enzymes such as histone deacetylase making them an attractive source of potential anticancer drugs (25). (26) also reported anticancer activity of *Phormidium* sp. using HT 29 and HeLa cell line. In addition, species specific and concentration dependent growth inhibition of above cell line was exhibited by crude extract of selected cyanobacterial strain. They adopted 12.5 µg mL^{-1} as the concentration of the potent band elutes, compared with 100 µg mL^{-1} of the crude extracts, in anticipation of increased effectiveness after separation from the crude materials. The purified fractions also had anticancer potential, although with lower impact.
MTT study done by (27) also showed significant and dose dependent inhibitory activities of crude extracts of *lyngbya officinales* and *Oscillatoria* sp. against HeLa cell line. (28) reported anticancer activity of *Cyanothece*sp. on human breast adenocarcinoma cell line (57.6% of inhibition) as compared to control.
Similar results were reported by (29), while investigating the protective effect of aqueous extract from *Spirulina platensis* against cell death induced by free radicals and concluded that a mixture of compounds was more active than a single pure one. This might be due to the synergistic effect of a number of compounds present in crude extract.

GC-MS analysis of the crude extract of *Tolypothrix* sp. and active bands (Toly-4, Toly-5 and Toly-6) showed presence of certain metabolites (Tables 2-5).In the present study bioactive potential of the crude extract of *Tolypothrix* sp. and active bands (Toly-4 and Toly-5) could be due to presence of hexadecanoic acid which have antioxidant activity (30).While the antioxidant potentials of the active band (Toly-5) could be attributed to the presence of 3,5-bis(1,1-dimethylethyl) Phenol. Phenolic compounds with less complex structures, have also shown to exhibit fungicidal and bactericidal activities (20).

However, the higher anticancer activity of crude extract and active band (Toly-6) may be in connection with the compound present 2,6,10-Trimethyl and Heptadecatriynoic acid respectively. Our results are in accordance with (31), who showed that higher antiproliferative activity of selected fraction only at higher concentration of 100 µg mL-1 was due to the presence of 9,19-cyclo- 25,26 epoxyergostan-3-ol, 4,4,14-trimethyl-, acetate and 5,8,11-heptadecatriynoic acid. The presence of broad range of bioactive constituents as observed in the partially purified bands together with the simple and cost-effective culturing and extraction technique make this isolate quite plausible candidate for future mass biotechnological applications. Thus, the present study reflects a hope of novel compounds which have wide array of biomedical importance.

Conclusion

The presence of wide range of mixture of bioactive compounds as observed in the partially purified bands indicates that further studies are required to completely purify the active compounds with maximum pharmacological potential to reveal their mechanism of actions. The toxicity of these active compounds could also be conducted. On the basis of activity and relative toxicity, better analogues could also be synthesized using medicinal chemistry pharmacophore

approach with no or least toxicity without compromising on their biological activities. Thus, the present study reflects a hope for the development of novel agents of biomedical importance.

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Declaration of Interest section
The authors report no declarations of interest.

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