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Anticancer Activity of Some Filamentous Cyanobacterial Isolates against Hep-G2 and MCF-7 Cancer Cell lines

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ABSTRACT

Cyanobacteria represent a rich source of a wide array of bioactive metabolites that are largely unexplored, and some of these metabolic products are proved to be potent as anticancer drug. Five filamentous isolates namely Arthrospira platensis, Leptolyngbya boryana, Leptolyngbya sp, Nostoc punctiforme, and Oscillatoria sp were identified morphologically using scanning electron microscope and on molecular basis using 16S rRNA as a marker. Organic extracts of these isolates were screened for their cytotoxicity against human hepatocellular carcinoma cell line (Hep-G2) and human breast adenocarcinoma cell line (MCF-7). Extracts of four isolates namely Nostoc punctiforme, Oscillatoria sp, Arthrospira platensis, and Leptolyngbya sp were found to have cytotoxic effect against Hep-G2 cell line with IC_{50} value of 12.75,13.5,14, and 15 μg ml^{-1}, respectively. On basis of undiluted concentration used in this study (100 μg ml^{-1}) the crude extract of Leptolyngbya sp, showed the maximum inhibitory percentage compared to control against Hep-G2 cell line by 71.3%. The extracts of the same four isolates showed more cytotoxic effect against MCF-7 cell line with IC_{50} value of 10.15, 10.25, 12.1, and 14.5 μg ml^{-1} for Leptolyngbya sp, Nostoc punctiforme, Arthrospira platensis, and Oscillatoria sp, respectively. The maximum inhibitory percentage against MCF-7 cell line compared to control was 88.3% by undiluted concentration of Arthrospira platensis. Results obtained from this study revealed that four extracts of cyanobacteria are considered a source of promising compounds against liver and breast cancer, also this study is considered the first report of anticancer activity of these cyanobacterial isolates.

Introduction

Cyanobacteria are a diverse, primitive and ubiquitous group of photosynthetic prokaryotes, with a gram-negative bacteria cellular organization but resemble green plants in oxygenic photosynthesis (Stanier & Bazine, 1977). The long evolutionary history of cyanobacteria has led to a great physiological and morphological diversity, including the production of unique molecules with important biological activities (Mahdi & Fariba, 2012; Estela Silva-Stenico *et al*., 2013; LewisOscar *et al*., 2018).

Cancer is known as a malignant neoplasm. Cancer comprises a large number of diseases involving abnormal unregulated cell growth. In cancer, cells divide and grow uncontrollably, forming malignant tumors, and invade nearby parts of the body (Mandal & Rath, 2015). Hepatocellular carcinoma (HCC) is a common liver cancer with limited treatment options, about 80% of patients die within a year of diagnosis (Ahmed *et al*., 2017). Globally, liver cancer is the fifth commonest cancer responsible for 9.1% of all cancer deaths (Sangeetha *et al*., 2014). After lung cancer, breast cancer is the second most common type of cancer and the second cause of cancer deaths in women worldwide (Naman *et al*., 2017). In Egypt, liver and breast cancer are considered the first and the second commonest types of cancer (Ibrahim *et al*., 2014).

Approximately, 60% of the approved chemotherapeutic drugs for cancer are sourced from naturally produced compounds (Cragg *et al*., 1997). For decades, many pharmaceutical products have been discovered by screening bioactive molecules produced by a wide range of microorganisms (Da Rocha *et al*., 2001). Cyanobacteria produce a large number of

molecules with different biological activities as anti-viral, anti-tumor, antimicrobial and as a food additive (Liu & Rein, 2010).

Cyanobacteria produce several compounds which effective in killing cancer cells by inducing apoptotic death, compounds like synthadotin (Singh *et al*., 2011), cryptophycin (Tan, 2010) and curacin (Ahmed *et al*., 2017) were already identified with their anticancer effect, some of these compounds have succeeded to enter the clinical trial (Tan, 2007). More than 50% of marine cyanobacteria are regarded as a potential source for extracting bioactive compounds with anticancer properties (Tan, 2007), as a result of that, most recent studies focused on marine cyanobacterial strains.

In the present study and unlike the trend, we have screened five freshwater filamentous cyanobacterial strains for their anticancer potentialities against hepatocellular carcinoma cell line (Hep-G2) and breast cancer adenocarcinoma cell line (MCF-7).

Material and methods

Area of study

For isolation of cyanobacterial species, water samples were collected from different localities in the Damietta branch of the Nile river in Dakahlia governorate, Egypt (31°2'12"N 31°20'29"E) and Wadi-elnatrun brackish bonds in Beheira governorate, Egypt
(30°35′00″N 30°20′00″E) in September 2017.

Isolation and culturing of cyanobacteria

Cyanobacterial strains were isolated from the Nile river ecosystem. Only *Arthrospira platensis* was isolated from wadi-elnatrun brackish ponds. The isolated cyanobacteria were enriched initially in a 250 ml Erlenmeyer conical flask using BG-11 medium (Rippka *et al*., 1979) except *A. platensis* which was cultivated in spirulina medium (Aiba & Ogawa, 1977). All culture media were kept at 25 ± 2°C, under 1.2 klux light intensity and photoperiod of 16:8 hour for 7 days. The isolated species were separately grown in 2L culture flask, each containing 1000 ml of medium, incubated under same growth conditions for 21 days to obtain biomass.

Morphological and molecular identification of cyanobacterial isolates

The isolated cyanobacteria were identified morphologically according to features described by Anagnostidis and Komark (1988, 1989) using light microscope and JEOL JSM 6510 scanning electron microscope. For molecular identification purpose, the DNA of the isolates was isolated according to method described by Sambrook *et al*. (1989). PCR reaction was performed using 0.2 µl Taq polymerase (5 Uμl^{-1}) in a 25 ml reaction. The PCR mixture contained 1x Taq polymerase buffer, 1ul of 10 Pico moles ul^{-1} of forward and reverse primer, 2ul of 2.5 mM dNTPs, and 100 ng template DNA. For the molecular identification of the isolated cyanobacteria specific 16S rRNA primer used (16Sf 5’-AGAGTTTGATCMTGGCTCAG-3’ and 16S r 5’ TACGGYACCTTGTTACGACTT-3’). The sizes of amplified products were visualized by gel assessed by comparison with DNA molecular weight marker (Fisher –Canada) Consisted of 16 DNA fragment ranging in size from 5000 bp to100 bp and stored at -20°C. PCR clean up column kit (Maxim biotech INC, USA) was used to purify the amplified products. The purified PCR products were subjected to DNA sequencing using forward primer in the sequence reaction. Sequencing was performed using BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, Foster City, CA, USA) and model 3130xl Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Preparation of Cyanobacterial crude extracts

Extraction was performed using 21 days old batch cultures. The harvested biomass of each cyanobacterial species was frozen at -20°C for a week. 1 g of each freeze-thawed sample was extracted twice with 10 mL followed by 10 mL methanol (100%) according to Iloki-Assanga *et al*. (2015) with sonication using an ultrasound sonicator at a pulse speed of 20000 Hz for 10 s. The sonication was repeated until all cells were broken. The extracts were kept for 24 h at room temperature and was then centrifuged at 6000 r/min for 20 min to obtain cell-free supernatant. The extracts concentrated in a rotary vacuum evaporator at 40°C. The dried residue was dissolved in 3 ml of dimethyl sulfoxide (DMSO) and kept at 4°C until further use.

Gas chromatography-mass spectrometry (GC–MS) Analysis

Chemical composition of the crude extracts was analyzed using Varian Chrompack CP-3800 GC/MS/MS-2000 equipped with split-splitless injector and DB-5.625 GC column (30 m × 0.25 mm i.d., 0.25 µm film thickness) was used. Helium was used as carrier gas with a flow rate of 1 ml/min. The mass detector was set to scan ions between 50 and 550 m/z using full-scan fixed mode electron impact (EI: 70 eV). The compounds were identified by matching their recorded spectra with the data bank mass spectra (Saturn and NIST library databases) provided by the instrument software, the databases were compiled using more than 80,000 electron impact (EI) mass spectra.

Cell lines

Both Human hepatocellular carcinoma cell line (Hep-G2) and Human breast adenocarcinoma cell line (MCF-7) were obtained from the American Type Culture Collection (ATCC, Minnesota, USA). The cell lines were maintained in the National Cancer Institute, Cairo, Egypt, by serial sub-culturing. Hep-G2 cells were cultured in RPMI-1640 medium (Sigma–Aldrich, USA) while, MCF-7 cells were cultured in Dulbecco's Modification of Eagle's Medium (DMEM, Sigma Aldrich, USA). Both media were supplemented with antibiotic free 10% Fetal Bovine Serum (FBS, Sigma., USA), 100 Uml^{-1} penicillin and 2 $mgml^{-1}$ streptomycin. The cells were maintained at 37°C in 95% relative humidified atmosphere containing 5% CO_2.

Anticancer activity of cyanobacterial extracts using sulforhodamine B (SRB) method

The *in vitro* anticancer assay of the cyanobacterial extracts againstHep-G2 and MCF-7 cells was performed using SRB assay as it is a sensitive method for evaluating cytotoxic activity (Skehan *et al*., 1990). Cells were seeded in 96-well microtiter plates at initial concentration of 3×10^3 cell/well in

a 150-mL fresh medium and left for 24 h to attach to the plates in CO_2 incubator at 37°C. Later, test extracts were added to wells in a broad concentration range (0, 12.5, 25, 50, and 100 μgmL^{-1}) and incubated for 48 h. Fixation was performed using 50 mL of 50% trichloroacetic acid at 4°C for 1 h. The plates were washed with distilled water using automatic washer and stained with 50 mL 0.4% SRB dissolved in 1% acetic acid for 30 min at room temperature. The excess of dye was removed by washing 4 times with 1% acetic acid. The dye was solubilized with 100 mL of 10 $mmolL^{-1}$Tris-base (pH10.4) and optical density of each well was measured spectrophotometrically at 570 nm with an ELISA microplate reader. Percentage of relative viability and the half-maximal inhibitory concentration IC_{50} calculated using prism program (Graph Pad prim 7). Also, percent of cell death was calculated using the following formula:

% of Cell inhibition (death) = 100 – (Absorbance of sample/Absorbance of control × 100)

Results

Morphological and Molecular Identification of Cyanobacterial isolates

Five filamentous cyanobacterial isolates were obtained. According to their morphological appearance, four of them were non-heterocyst filamentous species and identified as *Arthrospira platensis, Leptolyngbya boryana*, *Leptolyngbya* sp, and *Oscillatoria* sp, only one isolate was a heterocyst filamentous species and identified as *Nostoc punctiforme.* Scanning electron micrographs of the cyanobacterial isolates were shown in Figure 1. The result of molecular identification proves the morphological identification as the obtained DNA nucleotide sequences showed high similarity to closely related organisms. The resulting phylogenetic tree was shown in Figure 2 on the basis of maximum parsimony analysis using MEGA 7 software (Kumar *et al*., 2016). The maximum parsimony tree was obtained using the close-neighbor-interchange algorithm. There were 550 positions in the final dataset, out of which180 were parsimony informative sites. Bootstrap support values greater than 50% were reported. The obtained sequence was added to the GenBank data base with different accession numbers as following, *Arthrospira platensis* MH285264, *Leptolyngbya boryana* MH155317, *Leptolyngbya* sp MH285263, *Nosto cpunctiforme* MH155318, and *Oscillatoria* sp MH155316.

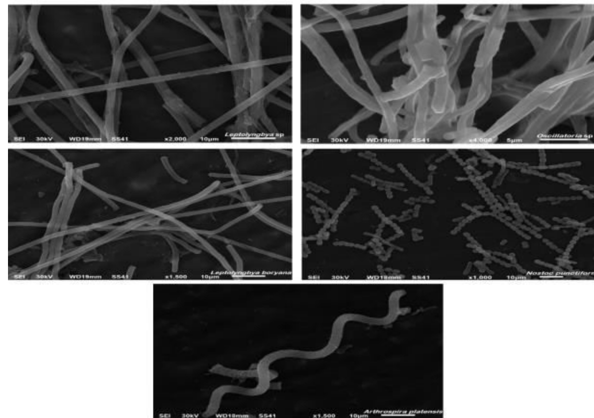

Figure 1: Scanning electron micrographs for cyanobacterial isolate

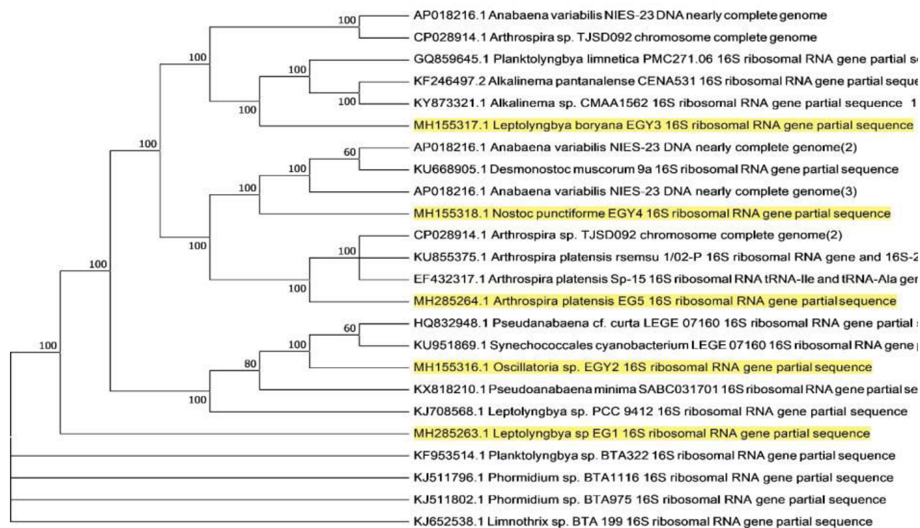

Figure 2: Phylogenetic bootstrapped tree based on maximum parsimony method.

Gas Chromatography Mass Spectrum

A total of 32 different compounds were identified in the five crude extracts. The identified compounds with their IUPAC name, retention time, and % peak area was shown in Table 1. The chromatograms of different crude extracts are shown in Figure 3. The identified chemical groups classified into 8 main groups including ketones, terpenes, fatty acids, fatty alcohols and esters derived fatty acids and/or dicarboxylic acid, hydrocarbons, steroids, and others (Table 1). The lowest number of compounds (9 compounds) was identified in the crude extracts of *Leptolyngbya* sp and *Oscillatoria* sp, while the highest one (18 compounds) was identified in the methanolic extract of *Arthrospira platensis*. A large number of the identified compounds displayed very important biological activities including antitumor, antioxidant, antimutagenic and anti-infective effects.

Table 1: Bioactive compounds recorded in the methanolic extracts of tested cyanobacterial isolates.

No	Compound	Molecular formula	Retention time (Min)	%Peak Area				
				a	b	c	d	e
1	**Esters** Benzoic acid, methyl ester	$C_8H_8O_2$	21.1	5.83	3.93			
2	4-Hydroxymandelic acid, ethyl ester, di-TMS	$C_{16}H_{28}O_4Si_2$	30.9	23.25	23.35	18.43	1.66	
3	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester (Diisobutyl phthalate)	$C_{16}H_{22}O_4$	35.1	5.80	3.14	5.02	0.55	10.77
4	Pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$	35.6	4.01	5.58			
5	1,2-Benzenedicarboxylic acid, butyl octyl ester	$C_{20}H_{30}O_4$	36.4	9.3	7.87			
6	1,2-Benzenedicarboxylic acid, diisooctyl ester (Isooctyl phthalate)	$C_{24}H_{38}O_4$	45.6	10.46	14.17			2.15
7	7-Hexadecenoic acid, methyl ester, (Z)-	$C_{17}H_{32}O_2$	35.9			5.58	8.33	
8	Hexadecanoic acid, methyl ester (Methyl palmitate)	$C_{17}H_{34}O_2$	37			5.02	27.7	
9	11-Octadecenoic acid, methyl ester	$C_{19}H_{36}O_2$	38.8			7.80	13.34	
10	6,9,12-Octadecatrienoic acid, methyl ester	$C_{19}H_{32}O_2$	38.5				17.23	
11	9,12-Octadecadienoic acid, methyl ester (Methyl linoleate)	$C_{19}H_{34}O_2$	38.9				15.00	
12	Octadecanoic acid, 1-[(tetradecyloxy)carbonyl]pentadecyl ester	$C_{48}H_{94}O_4$	25.1					2.15
13	Pentadecanoic acid, 14-methyl-, methyl ester	$C_{17}H_{34}O_2$	35.3					6.46
14	Ethaneperoxoic acid, 1-cyano-1-[2-(2-phenyl-1,3-dioxolan-2-yl)ethyl]pentyl ester	$C_{19}H_{25}NO_5$	35.7					7.32
15	1,2-Benzenedicarboxylic acid, diisooctyl ester	$C_{24}H_{38}O_4$	39.1					2.15
16	**Fatty acid** Pentanoic acid, 4-methyl- (Isocaproic Acid)	$C_6H_{12}O_2$	26			15.08		
17	**Fatty Alcohol** 3-Methyl-2-(3-methylpentyl)-3-buten-1-ol	$C_5H_{10}O$	27.5					1.72
18	**Hydrocarbon** Eicosane	$C_{20}H_{42}$	32.5	6.92			1.66	1.29
19	Nonadecane	$C_{19}H_{40}$	34	11.6				
20	4-Dodecene, (E)-	$C_{12}H_{24}$	26.1		17.32			
21	Pentadecane	$C_{15}H_{32}$	33.8		6.29	16.2		
22	Pentadecane, 7-methyl-	$C_{16}H_{34}$	30.8					18.96
23	**Keton** Propiophenone, 2'-(trimethylsiloxy)-	$C_{12}O_{18}O_2Si$	21					1.72
24	1,4-Benzenediol, 2,6-bis(1,1-dimethylethyl)-	$C_{14}H_{22}O_2$	18.2					13.94
25	Acetophenone, 2-chloro-	C_8H_7ClO	32			3.93	15.08	
26	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,8-dione	$C_{17}H_{24}O_3$	33.4					4.3
27	**Steroids** Cholesterol	$C_{27}H_{46}O$	51.8					4.64
28	**Terpens** 3,7,11,15-Tetramethyl-2-hexadecen-1-ol (Phytol)	$C_{20}H_{40}O$	34.7	8.78	4.72	4.46	2.22	10.34
29	Phytol	$C_{20}H_{40}O$	38.6				5.55	3.44

30	3',8,8'-Trimethoxy-3-piperidyl-2,2'-binaphthalene-1,1',4,4'-tetrone	$C_{28}H_{25}NO_7$	46.7	11.17	
31	Squalene	$C_{30}H_{50}$	38.8		6.46
32	**Others** Benzoic acid, hydrazide	$C_7H_8N_2O$	23.5		0.86

*Where **a**: Leptolyngbya sp **b**: Oscillatoria sp **c**: Leptolyngbya boryana **d**: Nostoc punctiforme **e**: Arthrospira platensis*

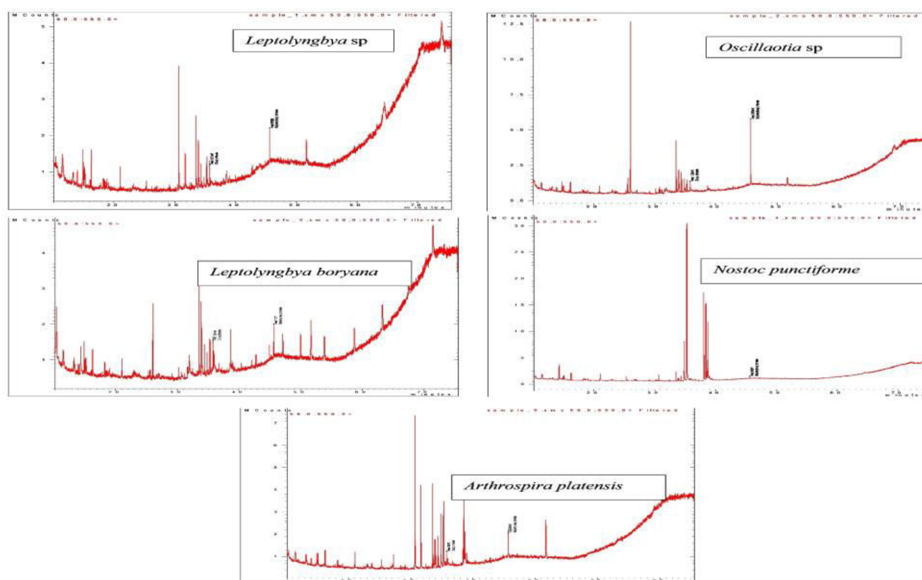

Figure 3: Chromatograms of tested cyanobacterial crude extracts.

Anticancer activity of cyanobacterial crude extracts against Hep-G2 and MCF-7

Cytotoxicity was assessed by SRB assay that expresses the survival fraction of treated cells compared with untreated cells. Anticancer activity was measured after 48 h. Hep-G2 and MCF-7 cells were treated with crude extracts of five cyanobacterial isolates. Treated Hep-G2 cells showed that crude extracts of *Nostoc punctiforme* and *Oscillatoria* sp isolates had the most cytotoxic effect as they caused inhibition of cell growth in a dose-dependent manner, with IC_{50} value of 12.75 and 13.5 µg ml^{-1} (Fig 4). The undiluted concentration used in the study was 100 µg ml^{-1}. When the undiluted concentrated extracts were used, different inhibition percentages for different extracts were obtained (Table 2). The highest inhibition percentage of which was 71.3% by *Leptolyngbya* sp extract, whereas the extract from *Leptolyngbya boryana* isolate displayed weak inhibition of only 14.9%. Crude extracts of *Leptolygnbya* sp and *Nostoc punctiforme* showed the most cytotoxic activity against MCF-7 cells with IC_{50} value of 10.15 and 10.25 µg ml^{-1} (Fig 5). The undiluted concentration of different extracts exhibited different inhibitory percentages against MCF-7 (Table 3). The highest inhibition percentage was 88.3% by *Arthrospira platensis* isolate extract, also the extract of *Leptolyngbya boryana* isolate showed weak inhibition against MCF-7 cells with 20.6%.

Table 2: Percent cell inhibition (%) of tested cyanobacterial extracts against Hep-G2

Concentration µg/ml	**Cyanobacterial extracts**				
	Arthrospira platensis	***Leptolyngbya boryana***	***Leptolyngbya* sp**	***Nostoc punctiforme***	***Oscillatoria* sp**
0	0	0	0	0	0
12.5	4.3	1.1	4.3	14.9	4.3
25	6.4	3.2	34.8	40.4	10.7
50	47.9	14.17	46.4	48.9	48.9
100	66	14.9	71.3	62.8	69.1

Table 3: Percent cell inhibition (%) of tested cyanobacterial extracts against MCF-7

Concentration µg/ml	**Cyanobacterial extracts**				
	Arthrospira platensis	***Leptolyngbya boryana***	***Leptolyngbya* sp**	***Nostoc punctiforme***	***Oscillatoria* sp**
0	0	0	0	0	0
12.5	6.2	13.7	12.1	15.4	29.9
25	24.5	17.5	33.2	39.3	44.5
50	52	18.8	62.3	56.5	46.6
100	88.3	20.6	70.9	80.9	68.7

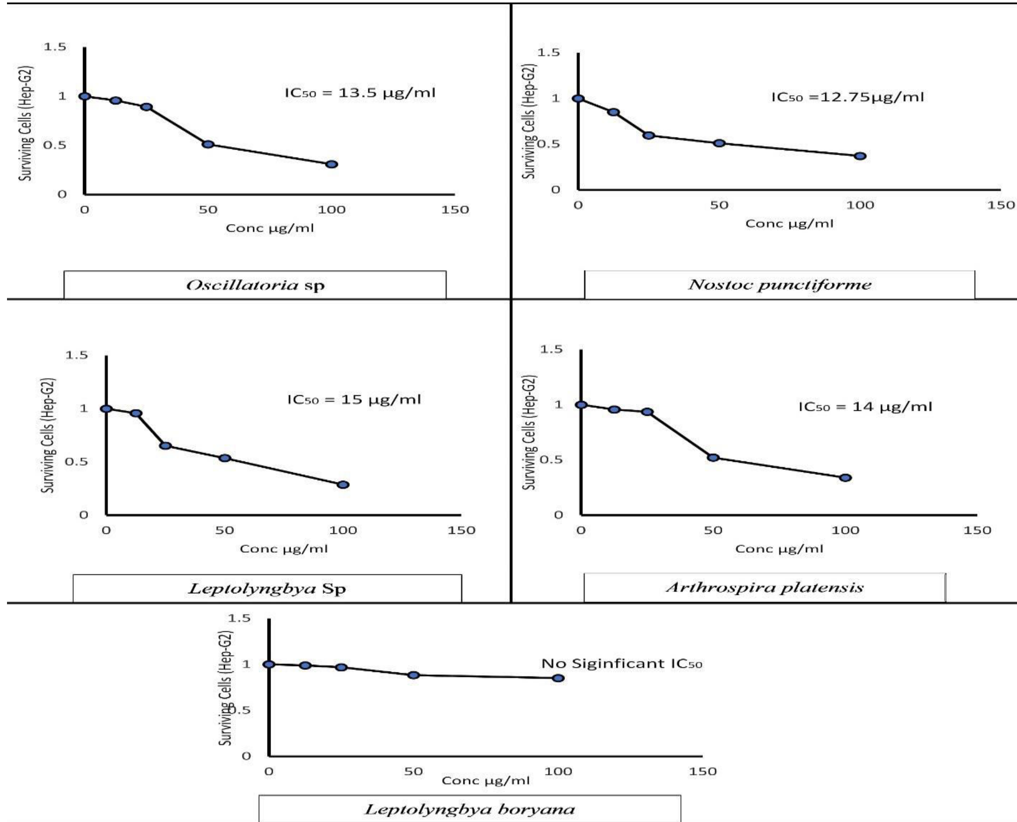

Figure 4: Growth response curve for Hep-G2 cells after treatment with the tested cyanobacterial extracts.

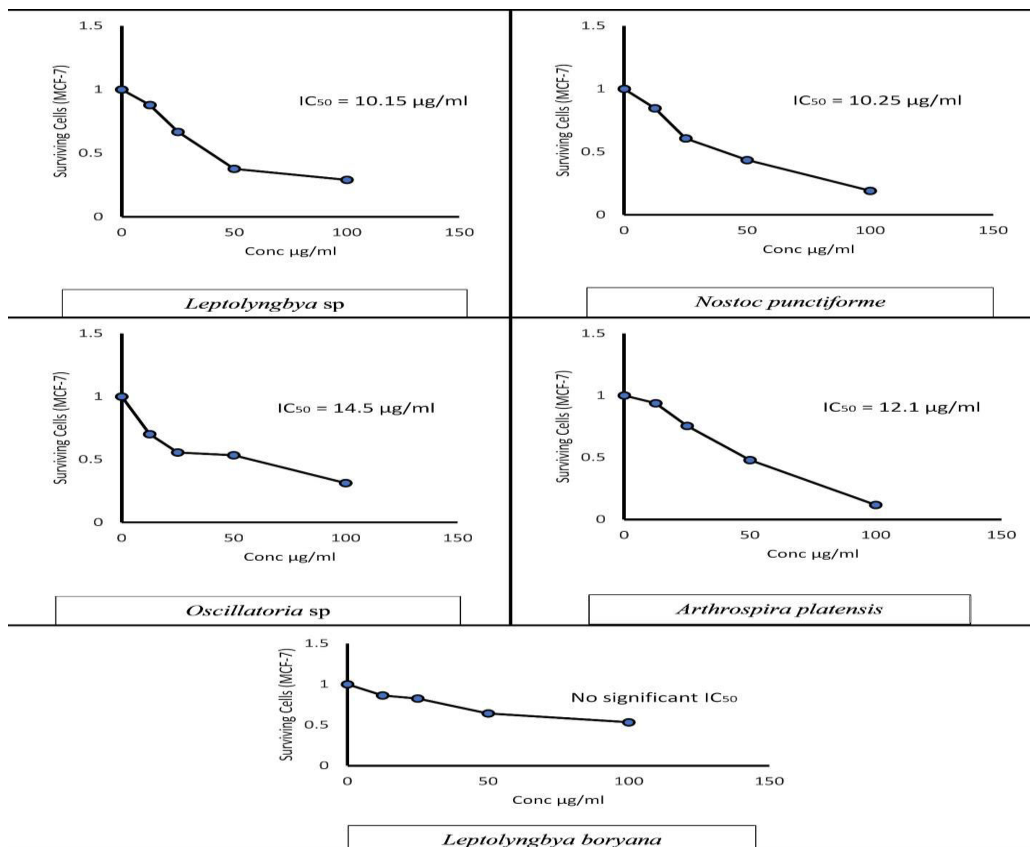

Figure 5: Growth response curve for MCF-7 cells after treatment with the tested cyanobacterial extracts.

Discussion

Being the second leading cause of death globally, cancer is considered to be one of the most public health threats which humanity faces nowadays (Cohen *et al*., 2017). The development of new therapeutic agents against cancer is a necessity to improve current therapeutic strategies, where it has many side effects and is limited in treating some types of cancer, thus there is a great need to find new therapeutic agents with limited side effects (Ahmed *et al*., 2017).

Natural product extracts continue to be the most promising source of new chemotherapeutic drugs for cancer (Al-Awadhi *et al*., 2018; Ravikumar *et al*., 2012). Cyanobacteria are considered a source of anticancer products regardless of their geographical origin, genera and climate (Oftedal *et al*., 2011). Unlike most studies which focused on cyanobacteria isolated from marine environments, the present study was made as an attempt to find out the anticancer property from crude extracts of freshwater cyanobacterial isolates. On this regard, the identity of the isolated cyanobacteria was confirmed by molecular, phylogenetic analyses in addition to morphological characterization using scanning electron microscope. Organic extracts of isolated *Nostoc punctiforme, Oscillatoria* sp, *Arthrospira platensis*, and *Leptolyngbya* sp reduced Hep-G2 cells proliferation and had cytotoxic effect at IC_{50} of 12.75,13.5, 14 and 15 µg ml^{-1}, respectively. This result was agreed with Ahmed *et al.* (2017) who reported the cytotoxic effect of *Plectonema terebrans* and *Cyanothece* sp against Hep-G2 cell line. Using the undiluted concentration (100 µg ml^{-1}) crude extracts of *Leptolyngbya* sp, *Oscillatoria* sp, *Arthrospira platensis*, and *Nostoc punctiforme* caused growth inhibition percentage compared to control of 71.3, 69.1, 66 and 62.8% against Hep-G2 cell line. This result revealed that crude extracts of isolated cyanobacteria decrease cell viability of Hep-G2 and this comes consistence with results obtained by Ahmed *et al.* (2017) and also results of Alja *et al.* (2013) who proved that *Cylindrospermopsis* extract is genotoxic for Hep-G2 cells reducing cell-proliferation of Hep-G2 cells.

In line with that, the organic extracts of *Leptolyngbya* sp, *Nostoc punctiforme*, *Arthrospira platensis*, and *Oscillatoria* sp had more cytotoxic effect against MCF-7 cell line with IC_{50} value of 10.15, 10.25, 12.1 and 14.5 µg ml^{-1}, respectively. Extracts of *Arthrospira platensis*, *Nostoc punctiforme*, *Leptolyngbya* sp, and *Oscillatoria* sp Also showed high percentage inhibition of 88.3, 80.9, 70.9 and 68.7%, respectively against MCF-7 using the undiluted concentration. These results were agreed with results obtained by Elsamary and Fouda (2015) who reported cytotoxic effect of *Cyanothece* sp against MCF-7 cell line, and results obtained by Felczykowska *et al.* (2015) who reported cytotoxic effect of some cyanobacterial isolates against MCF-7.

The GC-MS analysis of the crude extract of different cyanobacterial isolates showed the presence of chemical substances with previously known anticancer effect. 1,4-Benzenediol, 2,6-bis(1,1-dimethylethyl)-a phenolic compound found in *Leptolyngbya* sp and *Oscillatoria* sp was found to enhance apoptotic action in different type of cancer cells (Selassie *et al*., 2015). Phytol a Diterpene compound found in almost all crude extracts of isolated cyanobacteria known with its anticancer property (Netscher, 2007; McGinty *et al*., 2010). Hydrocarbon pentadecane and the fatty acid Pentanoic acid, 4-methyl- are known with their antitumor activity (Essien *et al*., 2012; Clombo*et al*.,2005). Squalene is a terpene which recently used in colon cancer treatment and well known with its antitumor properties was found in crude extract of *Arthrospira platensis* (Vera *et al*., 2009; Kotelevets *et al*., 2017). Cyanobacteria possess their cytotoxic effect against cancer through different mechanisms. Only in case of few cyanobacterial cytotoxic compounds the mode of action has been explained. Some cyanobacterial secondary metabolites affect cytoskeletal structures, and some interact with different eukaryotic enzymes, other affect DNA of cancer cell (Hoa *et al*., 2011). Along with that, some cyanobacterial metabolic products induce programmed cell death (Apoptosis) which is important to prevent cancer, as cancerous cells for several reasons cannot enter apoptotic phase (Humisto *et al*., 2016). Humisto reported that several cyanobacteria strains induce anti-leukemic effect through enhancement of apoptosis while non-malignant cells like hepatocytes kept unaffected (Humisto *et al*., 2016).

Conclusion

The present study proved that organic extract of four local filamentous cyanobacterial isolates possess effective cytotoxic effect against Hepatocellular carcinoma cell line (Hep-G2) and breast adenocarcinoma cell line (MCF-7). The promising results together with the simple and cost-effective culturing and extraction technique make these isolates quite plausible candidate as a potential source for anticancer chemotherapeutic drugs for future, using biotechnological applications.

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