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Bioactivity and Cytotoxic Effect of Cyanobacterial Toxin Against Hepatocellular Carcinoma

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Abstract

Cyanobacteria from exotic niches represent a rich resource of a wide array of unique bioactive compounds that are largely under explored, and proving to be potent source of anticancer drugs. A filamentous non-heterocystous isolate was identified by light microscopy and molecular methods using 23S rDNA as a marker was found to belong to *Plectonema* genus of Cyanobacteria. Organic extract of different cyanobacterial isolates was screened for their cytotoxicity against hepatocellular carcinoma cell line (HepG2). Extracts of (*Cyanothece* sp.) and (*Plectonema terebrans*) were found to have the most cytotoxic effect as they caused cell growth inhibition with IC_{50} value of 13.3% and 8.3% respectively. The cell viability, cell cycle analysis and caspase3 activity were measured. The cell viability of (*Cyanothece* sp.) and *(Plectonema terebrans*) showed high reduced (66.7% 57.4% respectively) compared with untreated cells (6.6%). Cell cycle analysis results showed significant arrest in G_0/G1 and G2/M phases in the cells treated with *Cyanothece* sp recorded (52.8%, 0.33%) respectively, low percentage in 2n phase recorded (46.4%), while cells treated with *Plectonema terebrans* showed (G_0/G1 recorded 63.3% and G2/M recorded 0.3 and 2n recorded 35.6%) compared to control which showed relative accumulation of cells in G_0/G1 and G2/M recorded (7.38% and 0.13%) respectively and aggressive accumulation of cells in 2n phase recorded (91.8%). Also, Caspase-3 activity increased in the cells treated with *Cyanothece* sp with highest activity at concentration 13.3% recorded 0.397 ± SD 0.02 and *Plectonema terebrans* with highest activity at concentration 4% recorded 0.402 ± SD 0.002 and with significant ($p<0.05$) compared to untreated cells (0.157 ± SD 0.05 nM/mL). These results indicated that two extracts of Cyanobacteria have a promising agent against hepatocellular carcinoma.

Keywords: Cytotoxicity; Antitumor activity; Cyanobacteria; Heptocellular carcinoma

Introduction

Hepatocellular carcinoma (HCC) is an increasingly common tumour with a poor prognosis and limited systemic treatment options; approximately 80% of patients die within a year of diagnosis due to hepatitis C infection and secondary cirrhosis [1]. In men, it is the fifth most common cancer worldwide and the third leading cause of cancer-related death [2,3]. Curative therapies such as resection, transplantation and ablation can improve survival in patients diagnosed at an early HCC stage and may offer a long-term cure with overall survival that may exceed 70% at 5 years. Patients with intermediate stage HCC benefit from chemoembolization and proper selection of candidates permits a 50% survival at 3-4 years. Finally, patients diagnosed at an advanced stage benefit from sorafenib, an oral available, multikinase inhibitor with anti-angiogenic and anti-proliferative effects [4]. Traditional therapies have many side effects on patients like mucositis skin rash, metabolic toxicities, hyperglycemia, hyperlipidemia, and hypophosphatemia [5]. So, researchers always try to find new agents with low side effects and more promising for Cancer. Natural products for biotherapy are being used in cancer treatment as an adjuvant therapy. In that regard, cyanobacteria come as a plausible candidate for such biotherapy [6]. Cyanobacteria (blue-green algae) are photosynthetic prokaryotes having applications in human health with numerous biological activities and as a dietary supplement. It is used as a food supplement because of its richness in nutrients and digestibility. Many cyanobacteria (*Microcystis* sp, *Anabaena* sp, *Nostoc* sp, *Oscillatoria* sp., etc.) produce a great variety of secondary metabolites with potent biological activities. Cyanobacteria produce biologically active and chemically diverse compounds belonging to cyclic peptides, lipopeptides, fatty acid amides, alkaloids and saccharides. More than 50% of the marine cyanobacteria are potentially exploitable for extracting bioactive substances which are effective in killing cancer cells by inducing apoptotic death. Their role as anti-viral, anti-tumor, antimicrobial, anti-HIV and a food additive have also been well established [7]. Several compounds with anticancer activities have been discovered from cyanobacteria and some of these have succeeded to enter the clinical trial. Humisto and his colleague have been reported that cyanobacteria harbor specific anti-leukemic compounds since several studied strains induced apoptosis against AML cells but keeping non-malignant cells like hepatocytes unaffected [8]. Also, a recent study postulated that Samoamide A, a pure compound isolated from American samoan marine cyanobacterium was shown to have *in vitro* cytotoxic activity against several human cancer cell lines in both traditional cell culture and zone inhibition bioassays and it was shown that although there was no particular selectivity between the cell lines tested for samoamide A, the most potent activity was observed against H460 human non-small-cell lung cancer cells (IC_{50}=1.1 μM) [2].

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Cyanobacteria isolated from Egypt with its hot dry weather and different water bodies are proving to be a very rich source of bioactive compounds including antimicrobials [9,10]. as well as the recently discovered anticancer activity [11]. In that regard, several studies showed that the bioactive compounds derived from cyanobacteria had anticancer effect, e.g., [12,13]. Some compounds with anticancer activity from cyanobacteria were even identified including synthadotin [14], cryptophycin [15] and curacin [16]. Recently, the cyanobacteria isolated from extreme environments are proving to be potent source of anticancer drugs especially against new cancer types and the resisting existing ones [17]. Therefore, there is a need for extensive exploration of cyanobacterial isolates because of their unique bioactive metabolites [17]. In line with that, aqueous extracts from several filamentous cyanobacteria from Egypt proved to be very effective against diffrent cancer cell lines [18]. Interestingly, [6] Zanchett and Oliveira-Filho suggested the possible applications of toxins produced by cyanobacteria as anticancer agents such as the hepatotoxin microcystins that cause hepatic cellular damage and induce reactive oxygen species [6]. Clinical traditional therapies such as surgery, chemotherapy, radiotherapy have several side effects and that lead to searching for new more safe cancer treatment modality. And hence the need for searching new more safe cancer treatment modality as biological therapy; varying anticancer agents are needed to overcome increasing challenges in cancer treatments. Different search methods are used to reveal anticancer compounds from natural products that represent one of the most effective treatment modality with low side effects and low cost. The aim of this study is to evaluate the cytotoxic effect and anti-tumor activity of different cyanobacterial isolates against hepatocellular carcinoma.

Materials and Methods

Cyanobacterial strain identification and extract preparation

One unidentified cyanobacterium alongside other cyanobacterial strains was used in initial screening for their organic extract. The identified Isolates were *Leptolygya badia*, *Oscillatoria limentica, Phormidium uncinatum, Cyanothece* sp., *Phormidium pristleyi, Synechocystis salina and Cyanobacterium notatum.* The unidentified isolate was isolated and kept at Helwan culture collection and all selected isolates were tested for the anticancer activity of their extracts. The isolate was identified as non-heterocystous unbranched cyanobacterium belonging to section III of cyanobacteria. In order to identify this strain, the DNA was extracted using Promega DNA extraction kit. The large 23S subunit rDNA was used as a taxonomic marker [10]. The purified genomic DNA was used as a template for amplification of partial 23S rDNA using the primer pair p23SrV_f1: GGA CAG AAA GAC CCT ATG AA and p23SrV_r1: TCA GCC TGT TAT CCC TAG AG by Sherwood and Presting in 2007. The partial 23S rDNA sequence was deposited in the GenBank database under the accession number KM392421. Cyanbacterial extracts were obtained in a combined solvent system (hexane: methanol: water; 1: 1: 1; v: v: v) by sonicating about 0.5 gm fresh weight cyanobacterial biomass for nearly two minutes in this solvent system and incubating the mixture in the fridge. The mixture was then centrifuged and the supernatant was used in the cytotoxicity tests.

HepG2 cell culture

Human hepatocellular carcinoma cell line Hep-G2 were obtained frozen in liquid nitrogen (-180°C) from the American Type Culture Collection. The cell lines were maintained in the National Cancer Institute, Cairo, Egypt, by serial sub-culturing. Cells were cultured in RPMI-1640 medium (Sigma–Aldrich, USA). The medium was supplemented with antibiotic-free 10% fetal bovine serum (FBS, Sigma, USA), 100 U/ml penicillin and 2 mg/ml streptomycin. The cells were sub cultivated after trypsination by Trypsin-EDTA (Cambrex, BioScience Verviers, Belgium) once or twice per week and resuspended in complete medium in a 1:5 split ratio. Cell lines were maintained as monolayer in T75 cell culture flasks with filter screw caps (TPP, Trasadingen, Switzerland) at 37°C in a humidified 5% CO_2 incubator.

Cytotoxicity analysis by MTT assay

The effects of different Cyanobactrium isolates (identified and unidentified) on cell viability were evaluated by MTT assay [19]. Briefly, after maintenance the cells with medium for 24 hrs, the medium of each well plate was removed, washed twice by PBS and replaced by 100 µl of each isolate at gradual concentrations (6.25% to 100%), incubated for 24 hrs. Then 10 µl MTT solution (5 mg/1 ml of PBS was added, incubated for 4 h at 37°C in a 5% CO_2 incubator. The medium was removed and 100 µl of DMSO was added to each well, mixed thoroughly using the pipette, and incubated in a dark room for 2 h. Afterwards absorbance of each well was read at 570 nm with ELISA reader. The prism program (Graph Pad prim 7) calculated percentage of relative viability and the half-maximal inhibitory concentration IC50.

Determination of cell viability under inverted microscope

Viability of cells was determined under inverted microscope ZeissAxio Vert.A1 inverted microscope, with magnification 40 X after incubation of HepG2 cell line with cyanobacteria extract (*Cyanothece* sp) and extract of (*Plectonema terebrans*) at gradual concentration (100% to 6.25%) from each extract.

Cell viability analysis by Cytell™ cell imaging system

The cells were treated by IC50 dose of both (*Cyanothece* sp) and (*Plectonema terebrans*) for 24 h then subjected to cytell™ cell imaging system after staining with Cytell™ cell viability kit (GE Healthcare Life Science, 29057496).

Assessment of Caspase-3 enzymatic activity

Caspase-3 activity was measured in HepG2 cells treated with extract of isolate *Cyanothece* sp at concentrations (6.5%, 13.3% and 26%) and *Plectonema terebrans* at concentrations (4%, 8.3% and 16%). Caspase-3 activity measured using colorimetric Bender Med System Caspase 3 assay kit; BMS2012INST.

Cell cycle analysis

The cells were treated by IC50 dose of both (*Cyanothece* sp) and (*Plectonema terebrans*) for 24 h then subjected to cytell™ cell imaging system after staining with Cytell™ cell cycle kit (GE Healthcare Life Science, 29057498).

Statical analysis

Data were statistically described in terms of mean ± standard deviation (SD), median and range. Comparison of numerical variables between the study groups was done using Mann Whitney *U* test for independent samples. *P* values less than 0.05 was considered statistically significant. All statistical calculations were done using computer program SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) release 15 for Microsoft Windows (2006).

Results

Molecular analysis revealed the non-heterocystous filamentous cyanobacterium to be *Plectonema* isolate with similarity 98% to

the closest-related isolate *Plectonema terebrans* reinforced by the statistical significance E. The other closely-related isolates were other filamentous cyanobacterial strains. Sequences in the Fasta format from representatives from different cyanobacterial sections were downloaded and aligned to allow phylogenetic tree reconstruction and sequence of partial 23S rDNA from *Cryptomonas curvata*, a eukaryotic microalga, was used as an outgroup taxon to root the tree (Figure 1). The evolutionary history was inferred using the minimum evolution method [20]. Optimal tree with the sum of branch length=0.45648245 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches [21]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the maximum composite likelihood method [22], and are in the units of the number of base substitutions per site. The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm [23] at a search level of 1. The Neighbor-joining algorithm was used to generate the initial tree [24]. All positions containing gaps and missing data were eliminated from the dataset. There was a total of 329 positions in the final dataset. Phylogenetic analyses were conducted in MEGA4 [25].

Cytotoxicity was assessed by MTT assay that expresses the survival fraction of treated cells compared with untreated cells. HepG2 cells treated with eight cyanobacterial isolates, showed that isolates of (*Cyanothece* sp) and *(Plectonema terebrans*) have the most cytotoxic effect as they caused inhibition of cell growth in a dose-dependent manner, with IC_{50} value of 13.3% and 8.33% respectively (Table 1); where IC_{50} values of *Leptolygya badia, Oscillatoria limentic, Phormidium uncinatum* and *Synechocystis salina* were (44.5% and 44. 5% and 97.5% and 90%) respectively, in other hand both of *Phormidium pristleyi* and *Cyanobacterium notatum* have no effect on HepG2 cell line as shown in (Figure 2).

Morphological appearance of HepG2 cell lines treated with *Plectonema terebrans* and *Cyanothece sp*

It was clear that *Plectonema terebrans* and *Cyanothece sp* have the most cytotoxic effect on HepG2 cells; the cells markedly affected seemed to undergo apoptosis. They showed condensation, shranked and aggregation after 24 h treatment (Figure 3).

The result showed a significant increase in Caspase-3 activity on HepG2 treated cells after incubation with (*Cyanothece* sp) at concentration; (6.5%, 13.3% and 26%) and *(Plectonema terebrans*) at concentrations (4% and 8.3% and 16%) for 24 h compared to control which recorded 0.157 ± SD 0.003, where *Cyanothece* sp has the highest activity of Caspase 3 at concentration 13.3% recorded 0.397 ± SD 0.02 nM/ml compared to concentration at 6.5% which recorded 0.290 ± SD 0.01 nM/ml and at concentration 26% which recorded 0.275 ± SD 0.005 nM/ml, while *Plectonema terebrans* showed highest activity of Caspase-3 at concentration 4% recorded 0.402 ± SD 0.002 nM/ml compared to concentration at 8.3% which recorded 0.352 ± 0.003 nM/ml and concentration at 16% which recorded 0.280 ± SD 0.005 nM/ml with significant ($p<0.05$) (Figure 4).

Cell viability results revealed that *Cyanothece* sp and *Plectonema terebrans* marked inhibition in cell viability (66.7% and 57.4%) respectively compared to un-treated cells (6.6%) as shown in (Table 2 and Figure 5).

The result of cell cycle analysis revealed aggressive accumulation of cells treated with *Cyanothece* sp. in G_0/G1 (52.8%) and G2/M (0.33) and relative decrease in 2n phase (46.4%). Meanwhile cells treated with (*Plectonema terebrans*) showed a high percent of HepG2 cells in G_0/G1 (63.3%) and G2/M (0.3%) and low persent of cells in 2n phase (35.6%) compared to untreated cells (Table 3 and Figure 6).

Discussion

The development of effective therapeutic agents for HCC is necessary to improve current chemotherapy, where it has many side effects and is limited in treating cancer, thus we are in great need of finding new agents with limited side effects. A number of studies suggest that agents derived from herbal plants either inhibiting or reversing the development of cancer [4,26,27]**.** Natural products have been used as traditional medicines where they have fewer side effects and were shown to act as anti-tumor agents. Cyanobacteria (blue-green algae) are photosynthetic prokaryotes having numerous biological activities. Cytotoxicity potential and anti-tumor activity of different eight extracts of cyanobacterial isolates including one, whose identity

Cyanobacterial strains	IC_{50}
Plectonema terebrans	8.33%
Cyanothece sp	13.3%

Table 1: IC_{50} value of *Cyanothecesp* and *Plectonematerebranson* HepG2 cells

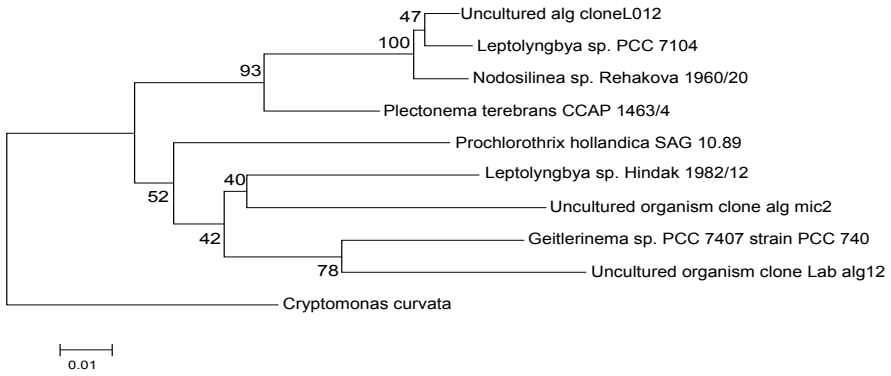

Figure1: Evolutionary relationships of 10 taxa using minimum evolution method.

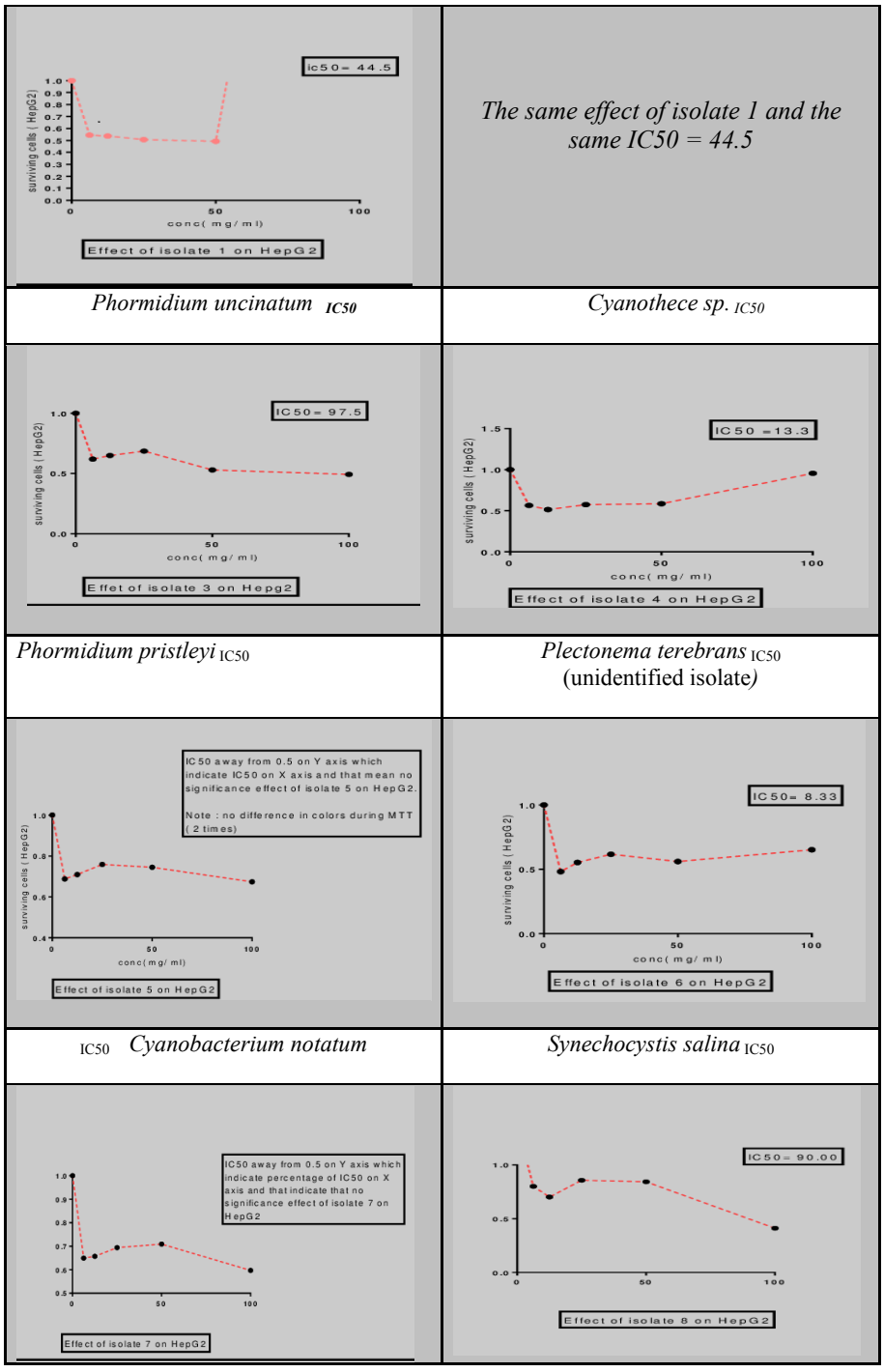

Figure 2: Growth response curve for HepG2 cells after treatment with eight different cynobacterial extracts.

was here in revealed to be *Plectonema terebrans,* were investigated against HepG2 cell line. Cyanobacterial organic extracts belong to *Plectonema* genus and another one which belongs to *Cyanothece* sp. reduced HepG2 cells proliferation and had cytotoxic effect at IC50% (8.33% for *Plectonema terebrans* and 13.3% for *Cyanothece* sp). This result was agreeing with C. Benjamin et al. in 2017 who report in the recent study that a bioactive cyclic octapeptide; samoamide A isolated from Marine Cyanobacterium was shown a cytotoxic activity against human non-small-cell lung cancer cells (H460). Results of Caspase-3 revealed that *Cyanothece* sp has the highest activity of Caspase 3 at concentration 13.3% recorded 0.397 ± 0.02 nM/ml compared to another two concentration (6.5% and 26%) which recorded (0.290 ± 0.01 nM/ml and 0.275 ± 0.05 nM/ml) and control which recorded (0.157 ± 0.05 nM/ml), in other side *Plectonema terebrans* has the highest activity of Caspase-3 at concentration 4% recorded (0.402 ± 0.002 nM/ml) compared to two another (8.3% and 16%) recorded (0.352 nM/ml and

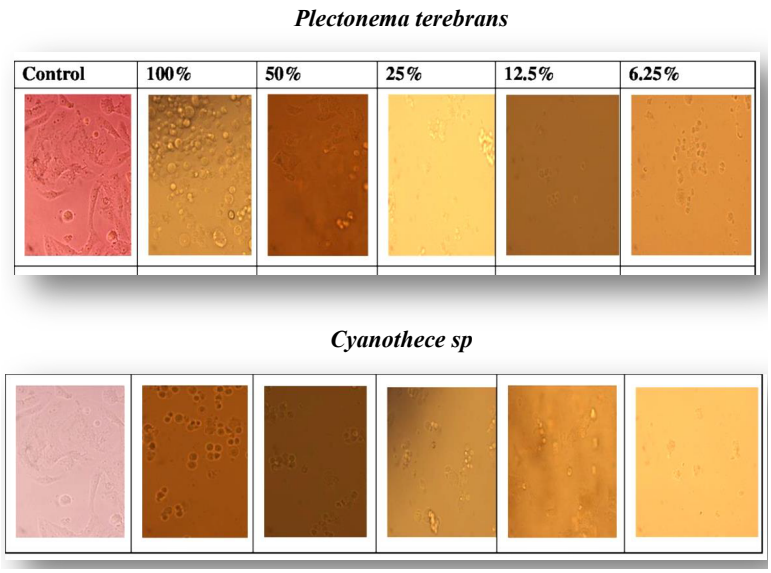

Figure 3: Microscopic examination of HepG2 cells treated with different concentrations of *Plectonema terebrans* and *Cyanothece* sp.

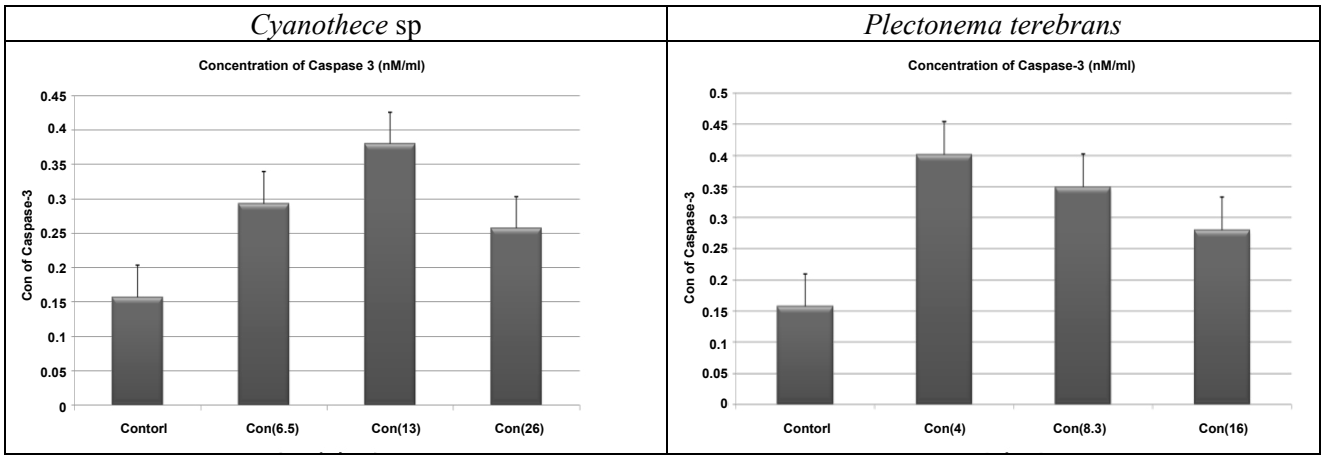

Figure 4: Caspase3 activities on HepG2 cell treated with *Cyanothece sp* at concentrations (6.5%, 13.3% and 26%) and *Plectonema terebrans* at concentrations (4%, 8.3% and 16%) VS control and it is showed that Caspase-3 activity increased in cell treated with *Cyanothece* sp specifically at concentration13.3 recorded 0.397 ± 0.02 and *Plectonema terebrans* specifically at concentration 4% recorded 0.402 ± 0.002 and with significant ($p<.0.05$) compared to control.

Cell viability/Drug	Dead cell	Viable cell
Control (untreated cells)	6.60%	93.40%
(*Cyanothecesp*)	66.70%	33.30%
(*Plectonema terebrans*)	57.40%	42.60%

Table 2: Effect of (*Cyanothece* sp) and *(Plectonema terebrans)* on cell viability in HepG2 treated cells after 24 h incubation.

Cell cycle phases/Drug	2n	G_0/G1	S	G2/M
Control	91.8	7.38	0.39	0.13
Cyanothece sp	46.4	52.8	3.5	0.33
Plectonema terebrans	35.6	63.6	0.5	0.3

Table 3: Cell cycle analysis for HepG2 cell treated with *Cyanothece sp* and *Plectonema terebranson* extracts.

0.280 nM/ml) and control which recorded (0.157 nM/ml). This agreed with results of Zhang et al. [28] who found that CYN induce apoptosis on steroli cells represented by the elevated level of caspase 3 activity on steroli cells treated with CYN toxin. Also, our results found to be agree with Humisto who reported that several cyanobacteria strain induce anti-leukemic effect through enhancement of apoptosis while non-malignant cells like hepatocytes keept unaffected [8]. The result also comes consistence with a very recent research that demonstrate that natural oxadiazine nocuolin A (NoA) compound was isolated as a positive hit during screening for apoptotic inducers in crude cyanobacterial extracts [29].

In addition, results of cell viability which applied by Cytell™ cell imaging system revealed that decrease in cell viability of cells after treated with both *Cyanothece* sp which recordrd 66.7% and *Plectonema terebrans* recorded 57.4% compared to control which recordrd 6.6%. Also results of cell cycle analysis which also applied by Cytell™ cell imaging system showed that the presence of aggressive accumulation of cells in G_0/G1 recorded 52.3% and G2/M recorded 0.33% phases of the cell cycle in cells treated with *Cyanothece* sp with a relative decrease of cells in 2n phase recorded 46.4% [30,31]. While cells treated with *Plectonema terebrans* showed a significant increase of the percent of HepG2 cells in both of G_0/G1 recorded 63.6% and G2/M recorded

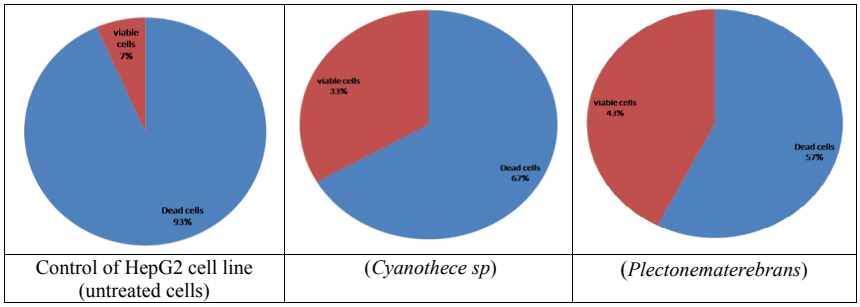

Figure 5: cell viability of HepG2 cell treated with *Cyanothece* sp and *Plectonematerebranson* organic extract as detected by CytellTM cell imaging system after staining by flurecent cytell cell viability kit.

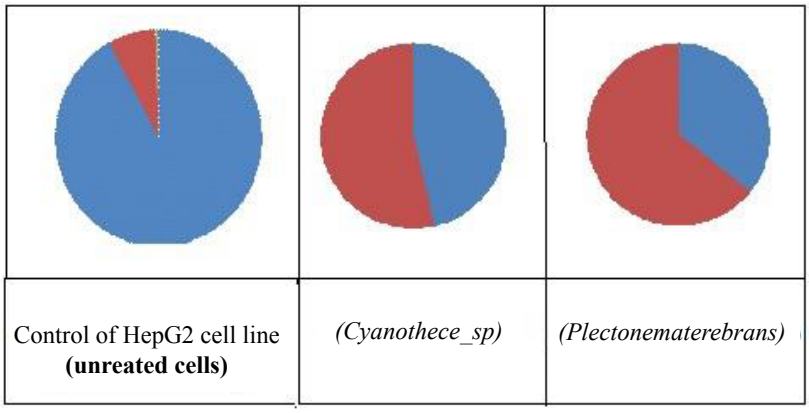

Figure 6: Cell cycle analysis for HepG2 cells treated with *Cyanothece sp* and *Plectonematerebranson* extracts.

0.3% and relative decrease of cells in 2n phase recorded 35.6% this in comparable to control untreated cells where showed that relative accumulation in G_0/G1 recorded 7.38% and G2/M recorded 0.13% and significant accumulation of cells in 2n recorded 91.8%, our results indicated cell cycle arrest specially by *Cyanothece* sp, and this is in agreement with other research by Alja et al. [32]. who proved that CYN is genotoxic for HepG2 cells reduced cell-proliferation of HepG2 cells by induction of cell cycle arrest in G0/G1 phase after 24 h of exposure and in S phase after prolonged exposure (72 h and 96 h) [33].

Conclusion

The *Plectonema and Cyanothece sp. of cyanobacteria* have a great value of cytotoxic effect against hepatocellular carcinoma through inhibition of cell viability, induction of apoptosis and cell cycle arrest at different phases.

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