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Anticancer activity of *Cyanothece* sp. strain extracts from Egypt: First record

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ABSTRACT

Objective: To assess the anticancer activity of eight cyanobacterial hydrophilic extracts on Ehrlich ascites carcinoma cell line.
Methods: The cyanobacterial strains used in the investigation were collected from diverse habitats in Egypt. The initial cytotoxicity test of cyanobacterial hydrophilic extracts was carried out by MTT assay. The *in vitro* anticancer activity of the four most active extracts was performed on MCF-7 cells using sulforhodamine B assay. Morphological and molecular techniques were used to characterise identity of the isolate from which the most potent cytotoxic extract was obtained.
Results: Extracts from four cyanobacterial strains had higher cytotoxic activities scoring 76.68%, 77.70%, 76.70% and 74.45%, respectively. A considerable anticancer effect was only detected when the concentrated extracts were used. One cyanobacterial extract gave the highest anticancer activity on human breast adenocarcinoma cell line (57.6% of inhibition) as compared to control. The isolate was best-matched to *Cyanothece* sp. with sequence resemblance 98% to *Cyanothece* sp. strain PCC7564 and the phylogenetic analysis confirmed its close identity to the *Cyanothece* genus.
Conclusions: This is the first study to report the anticancer effect of aqueous extracts derived from the unicellular *Cyanothece* sp. from Egypt and its potential as a plausible candidate for future mass biotechnological applications.

1. Introduction

Cyanobacteria are reported to be a promising source of a wide range of rather unique yet underexplored bioactive metabolites that requires further exploration and gene mining [1,2]. Several studies showed that the bioactive compounds derived from cyanobacteria had anticancer effect [3,4]. In Egypt, local cyanobacterial strains have proved to be a prolific source of antimicrobial agents [5,6]. However, in an effort to further explore the anticancer activity of local strains, this study was conducted to investigate the anticancer activity of their extracts. In addition, the identity of one local cyanobacterial strain that gave us cytotoxic activity was investigated to accurately describe its taxonomic position in order to reveal some of its physiological aspects. Overall, we aim to highlight the potential of using local underreported and unexploited cyanobacteria that are easily cultivated and extracted as a promising source of anticancer agents.

2. Materials and methods

2.1. Cyanobacterial cultures

The cyanobacterial cultures used are all collected from different locatities in Egypt including Wadi El Rayan, Oyoun Mousa, the Nile and Wadi El Natroun in 2013 and 2014. There were identified and kept at Helwan Culture Collection. Nevertheless, the identity of the isolate understudy is yet to be investigated. The isolate under study is oval or cylindrical unicellular cyanobacterium which was kept as a clonal culture pending identification. All cultures were kept in modified BG-11 medium [7] at room temperature. Cyanobacterial fresh biomass was harvested by centrifugation at 6000 r/min for 10 min, supernatant was decanted and fresh weight was determined. Ten milliliter of distilled water was added to the fresh biomass and the cells were sonicated using an ultrasound sonicator at a

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pulse speed of 20000 Hz for 10 s. The sonication was repeated until all cells were broken. The homogenized cell lysate was centrifuged at 6000 r/min for 20 min to obtain cell-free supernatant which represents the cyanobacterial hydrophilic extract. The supernatant was collected and used for cytotoxicity test.

2.2. Cell lines

Ehrlich ascites carcinoma (EAC) cell line was obtained from the National Cancer Institute, Cairo University. The cells were maintained in the ascitic form *in vivo* in Swiss albino mice by means of sequential intraperitoneal transplantation of 2×10^6 cells/mouse after every 10 days. Human breast adenocarcinoma cell line (MCF-7) was obtained from the American Type Culture Collection (ATCC, Minnesota, USA). The tumor cell line was maintained at the National Cancer Institute, Cairo, Egypt, by serial sub-culturing.

2.3. Initial cytotoxicity test of cyanobacterial hydrophilic extracts by MTT method

The anticancer activity was judged by MTT assay [8]. Briefly, 0.2 mL of freshly prepared EAC cell suspension was seeded in each well of 24-well plates. Cells were incubated with 0.2 mL from the eight cyanobacterial hydrophilic extracts at a concentration of 1 mg/mL for 24 h at 37 °C, 5% CO_2 with 98% relative humidity. A fresh medium was used containing 0.5 mg/mL of MTT for 2 h. The supernatant was aspirated and MTT formazan crystals were dissolved in 0.5 mL of a mixture of iso-propanol and 0.1 mol/L HCl. Absorbance was measured at 560 nm by using a spectrophotometer. The effect of extract on the proliferation of EAC cells was expressed as the percent of cell viability, using the following formula:

$$\%\ \text{of cell inhibition(death)} = 100 - (\text{Absorbance of sample}/\text{Absorbance of control} \times 100)$$

2.4. Anticancer activity of the four selected cyanobacterial hydrophilic extracts by sulforhodamine B (SRB) method

The *in vitro* anticancer activity of the most active extracts was performed on MCF-7 cells using SRB assay as it is a sensitive method for evaluating cytotoxic activity [9]. Cells were seeded in 96-well microtiter plates at initial concentration of 3×10^3 cell/well in a 150 μL fresh medium and left for one day 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, 250, 500, 750 and 1000 μg/mL) and incubated for 48 h. Fixation was performed using 50 μL of 50% trichloroacetic acid at 4 °C for 1 h. The plates were washed with distilled water using automatic washer (Tescan, Germany) and stained with 50 μL 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 μL of 10 mmol/L Tris-base (pH 10.4) and optical density of each well was measured spectrophotometrically at 570 nm with an ELISA microplate reader (Sunrise Tecan reader, Germany). Percent of cell death was calculated using following formula:

$$\%\ \text{of cell inhibition(death)} = 100 - (\text{Absorbance of sample}/\text{Absorbance of control} \times 100)$$

2.5. Cyanobacterial morphological and molecular characterization

The cyanobacterial cells were unicellular, oval with cylindrical appearance when dividing with no common gelatinous sheath. Cells possessed granular appearance. The DNA was extracted using Promega DNA extraction kit. The large 23S subunit rRNA gene 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 [10]. The partial 23S rDNA sequence was deposited in the GenBank database under the accession number KM392420.

3. Results

3.1. Initial cytotoxicity test of cyanobacterial hydrophilic extracts using MTT method

The cyanobacterial hydrophilic extracts of isolates number 1, 4, 5 and 6 showed higher cytotoxic activities in this MTT assay (Table 1). The maximal inhibition of cell growth was 76.68%, 77.70%, 74.45% and 76.70% respectively and obtained with 1 mg/mL of the extracts. Whereas the other isolates extracts showed lower inhibitions which are 71.46%, 69.30%, 66.26% and 74.20% for isolates number 2, 3, 7 and 8, respectively.

3.2. Anticancer activity of the potent extracts by SRB method

The cytotoxic activity of the most active four hydrophilic extracts on the growth of the human breast cancer MCF-7 cell line was presented in Table 2. Anticancer activity was analyzed after 48 h. The undiluted concentration used in the study was 1000 μg/mL. When the undiluted concentrated hydrophilic extracts were used, different inhibition percentages for different extracts were obtained. The highest inhibition percentage of which was 57.6% for the isolate understudy, whereas the extract from *L. badia* isolate displayed weak inhibition of only 12.1%. Interestingly, the other remaining two extracts scored similar

Table 1
Percent cell inhibition of eight cyanobacterial hydrophilic extracts on EAC cell line.

Isolate number	Cyanobacterial isolates	% Cell inhibition on EAC
1	*L. badia*	76.68
2	*Oscillatoria limentica*	71.46
3	*Phormidium uncinatum*	69.30
4	(Isolate under study)	77.70
5	*P. pristleyi*	74.45
6	*P. terebans*	76.70
7	*Cyanobacterium notatum*	66.26
8	*Synechocystis salina*	74.20

L. badia: *Leptolyngya badia*; *P. pristleyi*: *Phormidium pristleyi*; *P. terebans*: *Plectonema terebans*.

Table 2
Percent cell inhibition of the most active four cyanobacterial hydrophilic extracts on MCF-7 cell line. %.

Concentration (μg/mL)	Isolate under study	*L. badia*	*P. terebans*	*P. pristleyi*
0	0.0	0.0	0.0	0.0
250	15.3	12.6	18.4	0.0
500	24.5	21.2	20.8	7.6
750	50.6	17.0	30.8	34.6
1 000	57.6	12.1	46.7	46.7

results; 46.7% for *P. pristleyi* and the same for *P. terebans*. The different dilutions made to the hydrophilic extract did not show any anticancer activity indicating the importance of the application of the concentrated extract directly.

3.3. Molecular analysis

The molecular analysis revealed the unicellular cyanobacteria to be *Cyanothece* isolate with a similarity 98% to the closest-related isolate *Cyanothece* sp. PCC78801. Statistical significance E was 0.0 indicating the null possibility of random similarity. The other closely-related isolates were other *Cyanothece* and unicellular strains with similarity 93% or less. 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* (*C. curvata*), a eukaryotic microalgae, was used as an out-group taxon to root the tree.

3.4. Phylogenetic inference and tree reconstruction

The evolutionary history was inferred using the maximum parsimony method [11]. The maximum parsimony tree was obtained using the close-neighbor-interchange algorithm [12]. There were 336 positions in the final dataset, out of which 180 were parsimony informative sites. Phylogenetic analyses were conducted in molecular evolutionary genetics analysis software version 4.0 [13]. Bootstrap support values greater than 50% were reported (Figure 1). The eukaryotic alga *C. curvata* was used as an out-group taxon.

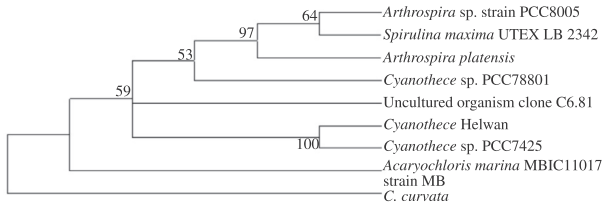

Figure 1. Phylogenetic bootstrapped tree based on maximum parsimony method showing evolutionary relationships of other related taxa to our isolate.

We arbitrarily designated our isolate as *Cyanothece* Helwan (accession number KM392420). Other taxa with accession numbers included: *Arthrospira* sp. strain PCC8005 (FO818640); *Arthrospira platensis* (JN831263); *Cyanothece* sp. PCC78801 (NR_076616); *Acaryochloris marina* MBIC11017 strain MB (AY518279); *C. curvata* strain CNUCRY 19 (KF907414); Uncultured organism clone C6.81 (EU342081) and *Spirulina maxima* UTEX LB2342 (JN831262).

4. Discussion

Cancer is a condition in which cells divide uncontrollably and may spread to other tissues unlike normal cells which divide in a controlled manner [14]. Programmed cell death (apoptosis) is important to prevent cancer but cancerous cells for several reasons cannot enter apoptotic phase [15]. Efforts are focused on making cancer cells enter apoptotic stage. In that context, it is reported that microalgal extracts can be effective in inducing apoptosis in cancer cells [15]. In that regard, cyanobacteria are considered as a promising source of anticancer agents regardless of their geographical origin, genera and climate [16]. They may induce cancerous cell death through causing the condensation of chromatin and the fragmentation of the nucleus in addition to and release of apoptotic bodies [17]. We screened some of the local strains for anticancer agents of which *Cyanothece* sp. gave the highest anticancer activities. The identity of this unicellular cyanobacterium was confirmed by both molecular and phylogenetic analyses and was grouped with other *Cyanothece* strain in one subclade with 100% bootsrap values. This unicellular cyanobacterium is unique in terms of physiological activity. It is capable of alternating photosynthesis during the day with nitrogen fixation during the night thereby contributing to both nitrogen and carbon cycle [18]. This regulation is important for photosynthesis generating oxygen which can cause irreversible inhibition of the nitrogenase enzyme responsible for nitrogen fixation [1]. The regulation of those two rather contradicting processes is therefore enigmatic. Genome sequencing studies revealed the adaptability of this organism's genome to different environmental conditions [18]. The use of combined molecular and morphological approach for the description of prokaryotes is necessary due to lack of morphological diagnostic phenotypic characters especially in unicellular prokaryotes [6]. Interestingly, *Cyanothece* sp. was reported to possess sulphated polysaccharides that are capable of inhibiting the adhesion of pathogenic bacteria *Helicobacter pylori* to gastric epithelial cells [19]. Interestingly, extracts derived from *Cyanothece* sp. strain had high anticancer impact on T-lymphoma cells but not against myelogenic leukemia cells [16]. In that context, unicellular cyanobacteria are reported to be a promising source of anticancer compounds [20]. Moreover, it was reported that cyanobacterial extracts contain long-lasting effective apoptotic compounds [16]. Their apoptotic effects may be attributed to causing the cancerous cells to undergo cell cycle arrest, mitochondrial dysfunctions and drastic changes in certain enzymes and proteins levels as well as changing membrane sodium dynamics [21–24]. In addition some of the known cyanobacterial toxins were suggested to have anticancer effects such as the hepatotoxin microcystins that cause hepatic cellular damage and induces reactive oxygen species [22,25]. These toxins are being transported by organic anion transporting polypeptides [22]. As cancer cells are already vulnerable to reactive oxygen species, microcystins and their analogues can selectively kill cancer cells that express certain organic anion transporting polypeptides without adversely affecting normal cells [22,26]. In addition, some compounds with anticancer activity from cyanobacteria were identified including synthadotin [27], cryptophycin 1 [28] and curacin [24]. Recently, the cyanobacteria isolated from extreme environments are proved to be potent source of anticancer drugs especially against new

cancer types and resist existing ones [29]. Therefore, there is a need for extensive exploration of those isolates because of their unique bioactive metabolites [29]. In line with that, aqueous extracts from several filamentous cyanobacteria from Egypt were proved to be very effective against cancer cell lines [30]. In that regard, the current study represents the first report to show the anticancer effect of aqueous extracts derived from the unicellular *Cyanothece* sp. from Egypt. It is noteworthy that more attention is wanted to shed some light on microflora isolated from that subtropical part of the world whose biological wealth is largely underexplored let alone exploited despite their unique metabolic products [5]. The promising results together with the simple and cost-effective culturing and extraction technique make this isolate quite plausible candidate for future mass biotechnological applications.

Conflict of interest statement

We declare that we have no conflict of interest.

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