

Evaluation of antimicrobial, antioxidant and cytotoxic activities and characterization of bioactive substances from freshwater blue-green algae

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Abstract

Organic solvent extracts of three cyanobacterial species (*Anabaena oryzae*, *Oscillatoria* sp. and *Stigonema ocellatum*) were tested for antimicrobial activity against human pathogenic fungal and bacterial strains as well as for antioxidant and cytotoxic activity against human breast adenocarcinoma (MCF-7). The acetone extract of *Anabaena oryzae* was found to be the most active one against tested fungal and bacterial strains. It showed a maximum antimicrobial activity against *Serratia marcescens* and *Candida albicans*. The methanol extract of *Oscillatoria* sp. exhibited the best total antioxidant capacity compared to the other solvents and algal species. Acetone and methanol extracts of *Anabaena oryzae* exhibited high toxicity against MCF-7 cell line with IC₅₀ of 45.1 and 44.4 µg/L, respectively. Acetone was the best solvent for extracting the active material. The acetone extracts were characterized by Gas Chromatography Mass Spectrophotometer (GC-MS) to identify the compounds responsible for such activities. Pharmaceutical important compounds in the acetone extract of cyanobacterial species like diacetone alcohol, acetic acid butyl ester mesityl oxide and heptadecane were present as a major component. These results indicate that extracts of studied cyanobacterial species exhibited appreciable antimicrobial, antioxidant and cytotoxic activity and could be a source of valuable bioactive materials for health products.

Keywords: Cyanobacteria, antimicrobial activity, antioxidant, anticancer, GC-MS.

1. Introduction

The organisms such as plants and animals as well as microorganisms (bacteria, algae, fungi) are well-known sources of compounds provided with interesting biological and therapeutic properties (Proksch *et al.*, 2003). For example, more than 75% of drugs utilized to treat

infectious diseases are derived from natural sources (Newman *et al.*, 2003; El Gendy *et al.*, 2016). From this point of view, algae and cyanobacteria have been demonstrated to produce secondary metabolites other than those produced by terrestrial organisms (Faulkner, 1994). Microalgae and Cyanobacteria with reference to their microbial activity and in pharmaceutical aspects have been studied by various workers (Shaieb *et al.*, 2014; Nermien and Fawzy, 2014; Abo-State *et al.*, 2015). Cyanobacteria are morphologically, physiologically, and metabolically very diverse group, which makes them as a promising group of organisms for research on drugs discovery (Abed *et al.*, 2011; Rama-Murthy *et al.*, 2012). Although cyanobacteria are still primarily viewed as an environmental nuisance or a source of toxins, hazardous to man and aquatic livestock, there are many potential benefits to research on chemicals produced by these organisms. Antibacterial, antiviral, antifungal, algicide and cytotoxic activities have been reported (Schaeffer and Krylov, 2000; Suikkanen *et al.*, 2004). The role of bioactive molecules in the producer organism itself is poorly understood, but, considering the wide spectrum of biological adaptations and tolerance to environmental stress revealed by cyanobacteria, some of these compounds can be produced in an attempt to confer advantages for their survival. Several studies showed that the bioactive compounds derived from cyanobacteria had an anticancer effect (Russo and Cesario, 2012). In Egypt, local cyanobacterial strains have proved to be a prolific source of antimicrobial agents (El Semary, 2012; El Semarya and Mabrouk, 2013). Cyanobacteria are one of the most promising groups of organisms for the isolation of novel and biochemically active natural products (Burja *et al.*, 2001). The cyanobacteria such as *Nostoc commune* (Jaki *et al.*, 2000), *Anabaena variabilis* (Ma and Led, 2000), *Nostoc spongiaeforme* (Hirata *et al.*, 1996), *Microcystis aeruginosa*, *Anabaena flos-aquae* (Khairy and El-Kassas, 2010), *Trichodesmium erythraeum*

(Thillairajasekar *et al.*, 2004), *Nodularia harveyana* (Pushparaj *et al.*, 1999) and *Calothrix brevissima* (Metting and Pyne, 1986) have been popularly reported to produce antimicrobial substances. Antimicrobial compounds have been previously detected in cyanobacterial extracts, such as Heptadecane and tetradecane from *Spirulina platensis* (Ozdemir *et al.*, 2004), phenolic compounds from *Nostoc muscorum* (El-Sheekh *et al.*, 2006), peptides, polypeptides, amides and alkaloids from *Fischerella ambigua* (Ghasemi *et al.*, 2004), lipopeptides from *Anabaena* spp. (Burja *et al.*, 2001), fatty acids, tetramine, spermine and piperazine derivative from *Anabaena* spp. (Shanab, 2007), laxaphycins from *Anabaena laxa* (Frankmolle *et al.*, 1992) and scytonemin from *Scytonema pseudohofmanni* (Ishibashi *et al.*, 1986), these compounds have been reported to possess antimicrobial activity. Most of these compounds are synthesized by ribosomal pathways or by nonribosomal pathways. Invasive fungal infections caused by *Candida* spp. and *Aspergillus* spp. are common, especially in immunocompromised patients. The increase of antifungal resistance indicates an urgent need for new antifungal compounds (Khan *et al.*, 2010). Cyanobacteria believed to be rich in antioxidants and phycobiliproteins (Mata *et al.*, 2010) (PBPs) which are the unique photosynthetic pigments of cyanobacteria. These pigments have been widely used as natural colorants in foods, cosmetics, and pharmaceuticals, particularly as substitutes for synthetic dyes (Singh *et al.*, 2005). Antioxidants play an important role in inhibiting and scavenging radicals, thus providing protection to humans against infections and degenerative diseases. The two most commonly used synthetic antioxidants; butylated hydroxyl anisole (BHA) and butylated hydroxyl toluene (BHT) are restricted because of their toxicity and DNA damage induction. Therefore, natural antioxidants from plant and algal extracts have attracted much attention because of safety. Algae generally have higher antioxidant activity due to higher contents of non-enzymatic antioxidant components, including ascorbic acid, reduced glutathione, phenols, and flavonoids (Wu *et al.*, 2010). This study was conducted to elucidate the cytotoxicity, antimicrobial and antioxidants properties of three species of cyanobacteria namely *Anabaena oryzae*, *Oscillatoria* sp. and *Stigonema ocellatum* for future applications in medicinal and pharmaceutical industries, along with identifying their chemical profiles using GC/MS.

2. Material and methods

2.1. Algae and culture conditions

Three isolates were used in this study. *Anabaena oryzae* was isolated from the soil of El-Kharga Oasis (New Valley) in Egypt, while *Oscillatoria* sp. and *Stigonema ocellatum* were isolated from cultivated soil of agriculture faculty farm at Assiut University (Egypt) and identified according to Prescott (1978). The cultures were grown in BG11 medium (Rippka and Herdman, 1993) for screening the biological activities. Cultures were maintained at $28 \pm 2^\circ\text{C}$ under continuous light intensity of $48 \mu\text{mol.m}^{-2} \text{s}^{-1}$.

2.2. Preparation of cyanobacterial extracts

At the end of log phase, algal cultures were centrifuged and the pellets were collected, weighted and used for extraction of antimicrobial agents. 0.3 g of each algal pellet was extracted separately in acetone, ethyl acetate and methanol in a mortar pestle and kept overnight at 4°C for complete extraction. The supernatant was collected after the centrifugation at $10000 \times g$ at 10 min. The solvent extracts were concentrated under reduced pressure at 40°C . Dry residue was redissolved in the different solvents to obtain final concentrations of 5 mg/ml, then the extract kept at 4°C until use for bioassay.

2.3. The growth of microorganisms set as microbial indicators

2.3.1. Bacterial strains and growth conditions

The Anti-bacterial spectrum of the different extracts of three blue-green algae were tested against seven isolates of bacteria as following: three isolates of Gram-positive bacteria namely: *Bacillus subtilis* (Ehrenberg) Cohn, *Micrococcus luteus* (Schroeter) Chon and *Staphylococcus aureus* (Rosenbach), in addition to four isolates of Gram-negative bacteria namely: *Escherichia coli* (Migula) Castellani & Chalmers, *Klebsiella pneumoniae* (Schroeter) Trevisan, *Pseudomonas aeruginosa* (Schroeter) Migula, and *Serratia marcescens* Bizio. Bacterial cultures were sub grown on fresh nutrient agar (NA) slants and incubated at 37°C for 24 h before used. All bacterial isolates were kindly obtained from the Clinical pathology Laboratories at Assiut University Hospital, Faculty of Medicine. Each bacterial biomass was prepared by inoculating 100 ml of nutrient broth medium. Bacterial cultures were shaken (250 rpm) at $32 \pm 2^\circ\text{C}$ for 24 h, resulting in suspension adjusted the turbidity to yield from 1×10^5 to 5×10^5 cells/ml (0.5 McFarland standard).

2.3.2. Candida species and growth conditions

Three isolates of pathogenic *Candida* species were kindly obtained from the Assiut Unit Mycological Center (AUMC). They were previously isolated from women complaining of vaginal candidiasis (Farhan, 2011). These isolates were *Candida albicans* (Robin) Berkhout, *Candida glabrata* (Anderson) S.A. Meyei & Berkhout and *Candida krusei* (Castellani) Berkhout. *Candida* cultures were grown on fresh Sabouraud dextrose agar medium (SDA) and incubated at $32 \pm 2^\circ\text{C}$ for 24 h before used. Inoculums of *Candida* strains were prepared by picking three distinct colonies of approximately 1 mm from 24 hours old culture grown on SDA and incubated at $35 \pm 2^\circ\text{C}$. Colonies were suspended in 2 ml of sterile 0.85% saline solution. Vortex the resulting suspension adjusted the turbidity to yield 1×10^5 – 5×10^5 cells/ml (0.5 McFarland standard).

2.3.3. Filamentous fungi and growth conditions

Two isolates of filamentous fungi namely; *Scopulariopsis brevicaulis* and *Penicillium aurantiogresium* were kindly obtained from the AUMC. A modified Czapek's agar medium was used for growing these isolates. Seven days old of pure isolates were prepared to be as an indicator of the antifungal activities of all algal extracts. An inoculum of previous two filamentous isolates was prepared by

taking the fungal growth of approximately 1 cm² and suspended in 5 ml distilled water supplemented with two drops of Tween 80 followed by vigorous shaking to get the spore suspension.

2.4. Testing antimicrobial activity by the disc diffusion method

Disc diffusion method (Sleigh and Timburg, 1981) is the powerful and widespread method used to measure Antimicrobial activities. A cell suspension of each test organism 10⁵ colony-forming units (CFU)/ml for bacteria or yeast cells were streaked on the surface of NA or SAD medium using a sterile cotton swab. In the case of filamentous fungi, 1 ml fungal spore suspension was transferred into sterile Petri dishes before the suitable agar medium was poured, swirled and allowed to solidify. Sterilized filter paper discs (0.5 cm diameter) Wattman No.1 were saturated with approximately 100 µl of each tested extract were placed on the surface of the inoculated medium (3 discs per plate). All plates which inoculated with bacteria or *Candida* were incubated at 35 ± 2°C for 24 hours while the others were incubated at 28 ± 2°C for five days.

The inhibited diameter (average inhibition zones in mm) of each indicator organism was measured and recorded as the antimicrobial activity of the algal extract. These inhibition activities were assessed by the diameter of average inhibition zones in relation to those of positive and negative controls. Standard antibiotics like Chloramphenicol (Cidostein 250 g – CID company) and Ampicillin (Ampicid 250 gm – CID company) were co-assayed as positive controls for antibacterial activities while Ketoconazole and Itraconazole discs (HiMedia Company, India) were co-assayed as positive controls for Anti-fungal and anti-*Candida* activities. Sterilized filter paper saturated with each solvent were used as a negative control for all microbial indicators. All results were recorded as mean values of three replicates ± standard deviation.

2.5. Estimation of total antioxidant capacity

Total antioxidant capacity (TAC) was measured in the different algal extracts using suitable kits according to guidelines of (Koracevic *et al.*, 2001). The main principle of the determination of antioxidative capacity is performed by the reaction of antioxidants in the sample with a definite amount of exogenously provided hydrogen peroxide (H₂O₂). The antioxidants in the sample eliminate a certain amount of the provided hydrogen peroxide. The residual H₂O₂ determined colorimetrically by an enzymatic reaction.

2.6. Cytotoxicity assay

Cytotoxicity investigations of all algal extracts were carried out on Human breast adenocarcinoma (MCF-7) based on the colorimetric MTT assay method which was conducted essentially according to the manufacturer's protocol. MCF-7 cells at a concentration of 2x10⁵ cell/ml were plated and grown in 96-well plates containing DMEM medium supplemented with 10% fetal bovine serum (FBS), incubation occurred at 37°C under

conditions of 5% CO₂, and 95% air for 24 hour prior to the addition of tested extracts. All studied algal extracts were dissolved in different solvents and diluted in the respective medium containing 1% FBS. Then, cells were treated with different concentrations of the different studied extracts for the next 24 hours; cells were treated with the same amount of the used solvents as a control. MTT (0.5 mg/ml) was added to each well and incubated for 4 hours. The insoluble formazan was dissolved in 100 µL of DMSO at room temperature for 20 min. Cell viability was assessed by measuring the absorbance at 540 nm using a microplate reader. The results were analyzed by means of cell viability curves and expressed with average of three measurements of IC₅₀ values ± standard deviation in Table 4 and Figure 2.

2.7. Cell culture

Human breast adenocarcinoma (MCF-7) cancer cell line was obtained from the Institute of Vaccine and Serum (Cairo, Egypt). The MCF-7 cell line was cultured in PRMI (Invitrogen, Carlsbad, USA) with 10% heat-inactivated fetal bovine serum and 2% antibiotics (streptomycin + penicillin). All cell lines were maintained under standard cell culture conditions at 37°C in an atmosphere of 5% CO₂. The medium was changed every two days.

2.8. Analysis of algal crude extracts using gas chromatography mass spectrometry (GC-MS)

Identification of the chemical constituents of acetone extracts of cyanobacterial species was analyzed by Gas Chromatography-Mass Spectrometer (GC-MS), Agilent Model 7890A-5975B [Column, DB 5 ms, Agilent form (30 m x 250 µm x 0.25 µm)] in the Unit of Analytical Chemistry, Department of Chemistry, Faculty of Science, Assiut University. The column was initially maintained for 2 min at 40°C, and then the temperature was increased to 50°C at a rate of 4°C/min and held for 3 min, then increased to 150°C at a rate of 10°C/min and held for 3 min, then increased to 220°C at a rate of 10°C/min and held for 6 min, finally increased to 280°C at a rate of 15°C/min and held for 10 min. Helium (purity 99.999%) was used as the carrier gas with a flow rate of 0.5 ml/min for 10.9 min, then 1 ml/min per min to 1 ml/min for 30 min. Neither internal nor external chemical standards were used in this chromatographic analysis. Interpretation of the resultant mass spectra were made using a computerized library-searching program (Willey 9 and NIST library) for the identification of the chemical constituents of algal extract.

3. Experimental results

3.1. In vitro antimicrobial activities of different studied blue-green algae

Antimicrobial activity of acetone, ethyl acetate and methanol of different species of blue-green algae (*Anabaena oryzae*, *Oscillatoria* sp. and *Stigonema ocellatum*) against tested different bacterial and fungal species was shown in Table 1. The algal extracts showed a different degree of antimicrobial activity and the intensity of inhibitory action varied depending on the species of

microorganism, algal species and the type of solvent. Concerning the anti-bacterial activities of the investigated different algal extracts, we found that the acetone *Anabaena* extract was the most active one. Its inhibition activity was ranged from 30 ± 0.7 against *Serratia marcescens* to 4.0 ± 0.0 against *Bacillus subtilis*, it succeeded on the inhibition of growth all bacterial indicators except *Staphylococcus aureus* as shown in Table 1. It followed by acetone extract of

Stigonema ocellatum, it prevents the growth of all positive and negative bacterial indicator except *Staphylococcus aureus* and *E.coli*. Out of seven types of bacterial indicators, only *Serratia marcescens* was completely inhibited by all tested algal extracts with an average inhibition zone range (5.0 ± 0.1 to 30 ± 0) while *E. coli* was fierce microbe where it was sensitive only to acetone *Anabaena* extract.

Table 1. Antibacterial and antifungal activities of the cyanobacterial extracts

	Inhibition of growth expressed as diameter of inhibition zone (mm)								
	<i>Anabaena oryzae</i>			<i>Oscillatoria sp.</i>			<i>Stigonema ocellatum</i>		
	Acetone	Ethyl acetate	Methanol	Acetone	Ethyl acetate	Methanol	Acetone	Ethyl acetate	Methanol
Gram +ve bacteria									
<i>Bacillus subtilis</i>	4.0 ± 0.0	-	-	-	-	-	6.0 ± 0.1	-	-
<i>Micrococcus luteus</i>	4.0 ± 0.1	-	-	-	-	2.0 ± 0.2	27.0 ± 0.2	27.0 ± 0.3	-
<i>Staphylococcus aureus</i>	-	4.0 ± 0.1	3.0 ± 0.3	-	-	-	-	-	-
Gram -ve bacteria									
<i>E. coli</i>	20 ± 0.4	-	-	-	-	-	-	-	-
<i>Klebsiella pneumoniae</i>	11 ± 0.3	-	-	-	-	-	4.0 ± 0.2	4.0 ± 0.1	3.0 ± 0.0
<i>Pseudomonas aeruginosa</i>	8.0 ± 0.0	-	-	10.0 ± 0.1	-	-	3.0 ± 0.05	-	-
<i>Serratia marcescens</i>	30 ± 0.7	9.7 ± 0.05	10.0 ± 0.17	30.0 ± 0.0	6.0 ± 0.1	11.0 ± 0.1	19.0 ± 0.1	8.0 ± 0.05	5.0 ± 0.1
Candida species									
<i>Candida albicans</i>	30 ± 0.0	3.0 ± 0.3	13.0 ± 0.2	25.0 ± 0.2	6.0 ± 0.1	19.0 ± 0.2	6.0 ± 0.1	6.0 ± 0.1	-
<i>Candida glabrata</i>	10 ± 0.1	3.5 ± 0.2	3.0 ± 0.0	4.0 ± 0.1	-	6.0 ± 0.0	-	-	-
<i>Candida krusei</i>	25 ± 0.2	10.0 ± 0.4	13.0 ± 0.15	17.0 ± 0.5	-	11.0 ± 0.1	7.0 ± 0.2	3.0 ± 0.3	-
Filamentous Fungi									
<i>Scopulariopsis brevicaulis</i>	7.0 ± 0.1	-	-	-	-	-	-	-	-
<i>Penicillium aurantiogresium</i>	5.0 ± 0.0	-	-	-	-	-	-	-	-

Note: Mean±SD (n=3), '-': no activity

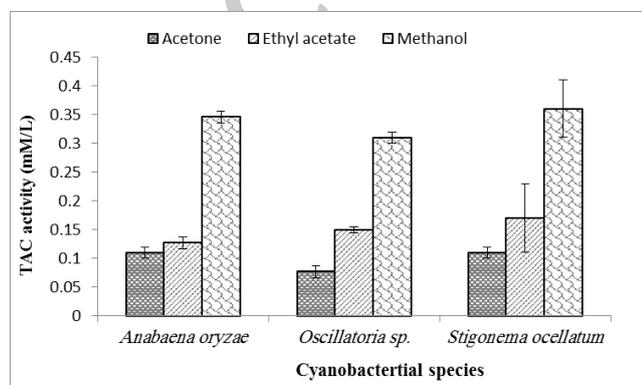


Figure 1. The total antioxidant capacity of different cyanobacterial extracts

The present findings on the antifungal activities of different algal extracts were represented as anti-*Candida* and anti-filamentous fungi. The results indicated that *C. albicans* was more sensitive than *C. glabrata* and *C. krusei*

to all tested extracts. *Candida* species were inhibited by all tested algal extracts except methanol extract of *Stigonema*, their effect ranged between 3 ± 0 against *C. albicans* and 0.3 ± 0 against *C. glabrata*. When closely observed in Table 1 which showing the anti-*Candida* activities of studied algal extracts, all different extracts of *Anabaena* exhibited attractive anti-*Candida* properties. It worth to mention that, Acetone and methanol extracts of *Oscillatoria* demonstrated to be inhibitory against all different species of *Candida*. Whereas, their maximum inhibitory action was 25.0 ± 0.2 and 19.0 ± 0.2 against *C. albicans* while the minimum inhibitory action was 4.0 ± 0.1 and 6.0 ± 0 against *C. glabrata*, respectively. Concerning to anti-*Candida* activity of *Stigonema*, only acetone and ethyl acetate extracts achieved the desired aim against only *C. albicans* and *C. krusei* (Table 1). The antifungal activities of different algal extracts were also determined against *Scopulariopsis brevicaulis* and *Penicillium aurantiogresium*

as filamentous fungal indicators. In general, algal extract showed similar antifungal activities, they had not any activity except acetone extract of *Anabaena* which was almost as active against the tested fungi. The antifungal inhibitory effect of this extract exhibited by 7.0 ± 0.1 and 5.0 ± 0 respectively against both filamentous fungal indicators (Table 1).

3.2. Total antioxidant capacity (TAC):

Data of the total antioxidant capacity in Figure 1 showed marked variation among both the different tested algal species and extracts. The methanol extract showed the best TAC activity for the three tested algae. In methanol extract, the best TAC Activity value was recorded in *Oscillatoria* sp. while the less value was obtained in *Anabaena oryzae*. In ethyl acetate extract, the arrangement of the three tested algae according to their total antioxidant capacity differed from the previous. The higher TAC activity was at *Stigonaema ocellatum* and the less TAC was at *Anabaena oryzae*. On the other hand,

acetone extract of *Anabaena* and *Stigonaema* gave the better TAC activity while *Oscillatoria* sp. gave the less total antioxidant capacity (Figure 1).

3.3. Anticancer activities

The cytotoxicity of the different extractions by using three different extraction solvents (acetone, methanol and ethyl acetate) were represented in Figure 2. The data showed that for the three extracts that obtained from *Anabaena oryzae* by using acetone, ethyl acetate and methanol show high toxicity against MCF-7 cell line with IC_{50} of 45.1, 58.7 and 44.4 $\mu\text{g/L}$, respectively. Also, three types of extracts from *Oscillatoria* showed toxicity activity with different IC_{50} of about 62.3, 30.8 and 130.7 $\mu\text{g/L}$ for extracted that formed by using acetone, ethyl acetate and methanol, respectively. In addition, the acetone, ethyl acetate and methanol extract of *Stigonaema ocellatum* have good cytotoxicity activities with IC_{50} values of about 49.1, 56.7 and 59 $\mu\text{g/L}$, respectively (Figure 2).

Table 2. GC-MS analysis of the active components (in % Relative Content) in acetone extract of blue green algal species

Active compounds	<i>A. oryzae</i>	<i>Oscillatoria</i> sp.	<i>S. ocellatum</i>
Mesityl oxide	5.081	3.079	5.45
Diacetone alcohol	89.247	59.562	89.26
2,6-Dimethyl-6-nitro-2-hepten-4-one	0.107	-	0.10
Heptadecane	1.836	0.150	3.42
pentadecane	0.872	-	-
Neophytadiene	0.195	0.108	-
Phytol	2.62	0.591	0.70
1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester (CAS)	0.042	-	0.41
Acetic acid, butyl ester	-	31.041	-
m-Xylene	-	0.306	-
2,6,10,14-Tetramethyl heptadecane	-	0.326	-
2-Ethoxy-2-methylbutane	-	0.228	-
Propanedioic acid, dimethyl ester	-	0.143	-
Hexaethylene glycol dimethyl ether; 2,5,8,11,14,17,20-Heptaohaheneicosane	-	0.278	-
Propylene Glycol Trimer 3	-	0.278	-
Phthalic acid, mono-(2-ethylhexyl) ester	-	1.037	-
2,6,10-trimethyl,14-ethylene-14-pentadecne	0.195	-	0.23
(3E)-3-Icosene	-	-	0.11
(Z)-14-Tricosenyl formate	-	-	0.07
6-Octen-1-ol, 3,7-dimethyl-, acetate	-	-	0.08
9-Hexadecenoic acid octadecyl ester	-	-	0.06
Hexadecanoic acid, methyl ester	-	0.074	0.05

Table 3. The total antioxidant capacities of different cyanobacterial extracts with ANOVA, Means with different superscripts (letters) are significantly differ ($P \leq 0.05$)

Algal Species	Extract	TAC activity (mM/L)	replicates
<i>Anabaena</i> sp.	Acetone	$0.11^a \pm 0.01$	0.11, 0.10, 0.112
	Ethyl acetate	$0.127^a \pm 0.01$	0.127, 0.137, 0.117
	Methanol	$0.346^{ab} \pm 0.01$	0.346, 0.356, 0.336
<i>Stigonaema</i> sp.	Acetone	$0.11^a \pm 0.01$	0.11, 0.12, 0.10
	Ethyl acetate	$0.17^a \pm 0.06$	0.17, 0.23, 0.11
	Methanol	$0.36^{ab} \pm 0.05$	0.36, 0.41, 0.31
<i>Oscillatoria</i> sp.	Acetone	$0.077^b \pm 0.01$	0.077, 0.78, 0.76
	Ethyl acetate	$0.15^a \pm 0.005$	0.15, 0.155, 0.145
	Methanol	$0.37^{ab} \pm 0.01$	0.37, 0.38, 0.36

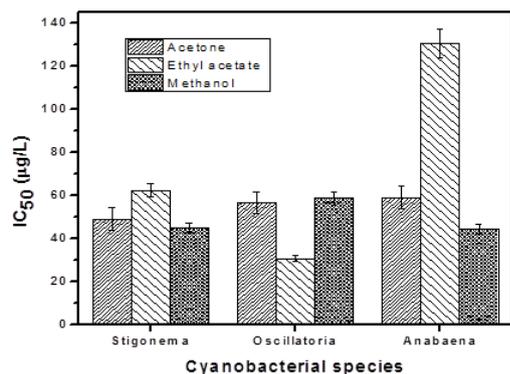


Figure 2. The values of IC₅₀ (µg/L) of different cyanobacterial extracts against human breast adenocarcinoma (MCF-7)

Table 4. The values of the average IC₅₀ (µg/L) ± standard deviation (SD) of different cyanobacterial extracts against human breast adenocarcinoma (MCF-7)

Algal Species	solvent	IC ₅₀ (µg/L) ± SD
Stigonema	Acetone	49.1±1.25
	Ethyl acetate	56.7±1.45
	Methanol	59±1.51
Oscillatoria	Acetone	62.3±1.59
	Ethyl acetate	30.8±0.79
	Methanol	130.7±3.33
Anabaena	Acetone	45.1±1.15
	Ethyl acetate	58.7±1.497
	Methanol	44.4±1.13

3.4. GC-MS Analysis of active algal extracts

In this work, acetone extract of the target species was found to be most active when compared to the other extracts. So, this extract was further selected for its GC-MS analysis. A total of 22 chemical compounds were found from the acetone extract of the cyanobacterial species. The chemical composition of the extract varied from alga to other with the highest number of compounds was found in *Oscillatoria* sp. (14 compounds) followed by *Stigonema ocellatum* (12 compounds) and *Anabaena oryzae* (9 compounds), however a few of them were predominant (Table 2). The results showed that the most bioactive compounds produced in major amounts by *Oscillatoria* sp. was diacetone alcohol, acetic acid butyl ester and mesityl oxide representing 59.56%, 31.04% and 3.08%, respectively. The components of *Anabaena oryzae* also consisted of diacetone alcohol (89.25%) and mesityl oxide (5.08%) as major components. Similarly, diacetone alcohol (89.26%), mesityl oxide (5.45%) and heptadecane (3.42%) were abundantly present in the acetone extract of *Stigonema ocellatum*. Other compounds such as 2,6-Dimethyl-6-nitro-2-hepten-4-one, neophytadiene, phytol, 1,2-Benzenedicarboxylic acid bis(2-ethylhexyl) ester, 2,6,10-trimethyl,14-ethylene-14-pentadecne and hexadecanoic acid methyl ester, have been reported to be a common minor volatile component in some cyanobacterial species. A small amount of pentadecane

was only detected in *Anabaena oryzae* (0.872%). While, m-Xylene, 2,6,10,14-Tetramethylheptadecane, 2-Ethoxy-2-methylbutane, propanedioic acid dimethyl ester, hexaethylene glycol dimethyl ether, propylene glycol trimer 3 and phthalic acid mono-(2-ethylhexyl) ester were detected only in small amounts by *Oscillatoria* sp. On the other hand, (3E)-3-Icosene, (Z)-14-Tricosenyl formate, 6-Octen-1-ol 3,7-dimethyl- acetate and 9-Hexadecenoic acid octadecyl ester were estimated only in *Stigonema ocellatum* (Table 2).

4. Discussion

The present study is a strive towards the production of antibiotics, antifungals and antioxidants by the blue-green algae namely; *Anabaena oryzae*, *Oscillatoria* sp. and *Stigonema ocellatum* which screened for their antimicrobial activities against different species of pathogenic bacteria and fungi as well as antioxidant and anticancer activities. Finding an antimicrobial activity for tested algae *in vitro* experiments is predictive of their capacity to produce new compounds which act as antibacterial and antifungal compounds. It was found that *Anabaena oryzae* has high biological activity against all Gram positive and negative bacterial strains in addition to three species of *Candida* and species of filamentous fungi. Also, acetone extract of *Anabaena* was the most active one; similar results were described earlier by Rao (2015) who found that a acetone culture crude extract of *Anabaena oryzae* exhibited the antibacterial activity with a maximum zone of inhibition 15.50 mm against the strain of *Salmonella typhi* followed by 10.33 mm inhibition zone was found in methanol extract against *Klebsiella aerogenes*. Acetone extract of *Stigonema ocellatum* also exhibited an inhibitory action; it prevents the growth of all positive and negative bacteria indicators except *Staphylococcus aureus* and *E. coli*. In our knowledge, no literature about antimicrobial activities of *Stigonema ocellatum* extracts except Malathi *et al.* (2016). They screened antimicrobial activity of *Anabaena circinalis*, *Nostoc muscorum*, *Stigonema ocellatum* and *Hapalosiphon welwitschii* by using the Agar disc diffusion method against pathogenic bacteria and fungi and they found that chloroform and hexane extracts of *S. ocellatum* culture were expressed good antibacterial activity with 19.3 ± 0.88 mm and 10.3 ± 0.33 mm zone of inhibition against *Klebsiella pneumoniae* and *E. coli*, respectively. Also, they recorded the antifungal activity of *S. ocellatum* extract against *Aspergillus fumigatus*.

Total antioxidant capacity was found to be higher in the methanolic extract than in ethyl acetate extracts and the lowest values showed at acetone extracts of the three tested algae. Similarly, Sivakumar and Rajagopal (2011) reported that the highest antioxidant activity was observed in the methanol extract from eight green algal species. Uma *et al.* (2011) observed that the methanolic extracts displayed greater potential in all antioxidant assays when compared to ethanolic and acetone extract of green microalgae *Desmoccoccus olivaceous* and

Chlorococcum humicola. Concerning to the methanol extract the highest antioxidant activity was recorded in the methanolic extract of *Oscillatoria* sp. (0.37 ± 0.01 mM/L). Methanolic extracts showed antioxidant activity in the order of *Oscillatoria* sp. > *Stigonema ocellatum* > *Anabaena oryzae*. Methanol is a good multifunctional solvent for preliminary extraction (Harborne, 2006), so that it has been used for marine algal antioxidant extraction (Khumar *et al.*, 2008). Methanol concentration for extraction influences the total phenol content (Chew *et al.*, 2008) and the antioxidant activity of the marine algae (Damongilala *et al.*, 2013). The antioxidant activity of the alga *Anabaena oryzae* was low, almost among the different tested extracts. This low antioxidant activity can be attributed to the low, and perhaps the lack of, antioxidant compounds with high antioxidant property such as phenol and flavonoid in the non-polar extracts of the algae (Muret *et al.*, 2007). Phenolic compounds (in marine algae) are correlated with antioxidant activity and also dependent on the solvent and algal species used for extraction (Horincar *et al.*, 2011).

Furthermore, the cytotoxicity of all extractions demonstrated that although all extractions show good cytotoxicity, however, their cytotoxicity activity was different based on the type of algae and the solvent of the extraction. The three types of extracts from *Oscillatoria* showed the highest cytotoxicity in comparing to the all different extractions from the three different bacteria types and the extract that was obtained by using ethyl acetate have the lowest IC_{50} highest cytotoxicity activities. Also, the three extracts from *Stigonema* showed almost equal cytotoxicity activities that are near to the cytotoxicity activity of extraction from *Oscillatoria* by using methanol or acetone. In addition, the two extracted from *Anabaena* by using methanol or acetone have shown good cytotoxicity activities almost like that from *Oscillatoria* or from *Stigonema*, however the extract from *Anabaena* by using ethyl acetate showed the lowest cytotoxicity activity in comparing with all the other extracts. The result of the compound assay from the acetone extract of cyanobacterial species using GC-MS shows that there were several compounds contained in the extract. Based on the identification result using GC-MS, the compounds that were extracted belonged to benzene, aromatic hydrocarbon, ketones, alcohols and fatty acid groups. High amounts of diacetone alcohol were detected in all tested cyanobacterial species. Maligan *et al.* (2013) reported that *Tetraselmis chuii* extract using acetone solvent was also contained high amounts of diacetone alcohol (53.617) which is in good agreement with our results. Alcohol groups have a toxic effect on microorganism (Sikkema *et al.*, 1995). Mesityl oxide is a ketone has been reported to be common in all studied cyanobacterial species. Hydrocarbons, alcohol, and ketones have inhibitory activity against mold, yeast, and bacteria. Whereas the antifungal activity of the alcohol groups was delayed the growth of fungal conidia (Helal *et al.*, 2006). The majority of cyanobacterial species also contains small amounts of short-chain hydrocarbons (Murata and Nishida, 1987) like

heptadecane. In this study, small amounts of heptadecane (1.836%, 0.150%, 3.42%) were detected in *Anabaena oryzae*, *Oscillatoria* sp. and *Stigonema ocellatum*, respectively. Previous records reported that *Spirulina platensis* consisted of heptadecane (39.70%) and tetradecane (34.61%) which can inhibit some Gram +ve and Gram -ve bacteria and *Candida albicans* (Ozdemir *et al.*, 2004). Khairy and El-Kassas (2010) stated that the most bioactive compound produced by *Anabaena flos-aquae* was heptadecane and 7-methylheptadecane representing 26.11, 18.65%, respectively. The phytols are an acyclic diterpene alcohol, also known as farnesol were present as lower contents in the tested cyanobacterial species. These compounds have proven antibacterial action against *Staphylococcus aureus*, MRSA (Methicillin Resistant *S. aureus*) & MSSA (Methicillin Susceptible *S. aureus*) (Jabra-Rizk *et al.*, 2006) and fungal growth inhibition (Sato *et al.*, 2004). Farnesol has the ability to sensitize *S. aureus* to antimicrobials when used in synergy with antimicrobials. Therefore, it has been suggested for a potential application as an adjuvant therapeutic agent against biofilm related infections involving MRSA and thereby counteracting drug resistance. Farnesol is also known to possess chemopreventive and anticancerous activity by unknown mechanisms. Small contents of 1,2-Benzenedicarboxylic acid, bis(2-ethylhexyl) ester was present in the acetone extract of *Anabaena oryzae* and *Stigonema ocellatum*. This compound is known as phthalate derivative; it exhibit a pronounced antibacterial action whereas, it can react with a hydrophobic side of the cell membrane which causes disruption of the permeability of the cell membrane (Uyeda *et al.*, 1990). Bharat *et al.* (2013) reported that, this compound has potent antibacterial activity and was present in *Chroococcus* sp. (0.56%), *Aulosira fertilissima* (10.64%), *Anabaena variabilis* (4.55%) & *Westiellopsis prolifera* (1.58%) which also showed the higher zone of inhibitions (16.06, 30.04, 33.36 & 25.93 mm) and lower MIC of 64 to 128 μ g/ml against *S. aureus* 25923. Fatty acids largely known in cyanobacteria are Octadecanoic, Octadecenoic, hexadecanoic acids, hexadecenoic acids, hexadecadienoioc (Murata and Nishida, 1987). The fatty acids hexadecanoic acid methyl ester was present in the acetone extract of *Oscillatoria* sp. and *Stigonema ocellatum* as well as 9-hexadecenoic acid octadecyl ester was only estimated in *Stigonema ocellatum* extract affording antimicrobial and cytotoxic action. Several types of fatty acid compounds that have an antimicrobial activity such as linoleic acid, n-hexadecanoic acid, and 9 hexadecenoic acid (Agoramoorthy *et al.*, 2007). 9-hexadecenoic acid has an effect on membrane permeability and ion partitioning on the cell membrane layer of microorganisms (Langner and Hui 2000). Antibacterial, antioxidant, and cytotoxic activity were due to the synergistic effect of all above compounds.

5. Conclusions and future prospects

Cyanobacteria are known to be the spearhead organisms in major habitats and along with their diversity in

structure and ability to produce a wide range of compounds. Despite their omnipresence and a large body of obtainable information on cyanobacterial metabolites and their biological properties, there is a paucity of application-oriented research regarding their roles in drug development and production of new industrial compounds. Collective efforts are needed to scout the chemical, biological and genetic diversity of cyanobacteria for controlling Plant, human and animal diseases that hinder advances in agriculture, drug industry or proliferation of human life. Future research also needs to be lead to bioprospecting cyanobacterial diversity in symbiotic associations for novel chemicals. Also, efforts need to be taken to identify novel genes/molecules or drug discovery from isolates belonging to new habitats.

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