Pak. J. Bot., 52(4): 1481-1490, 2020. DOI: http://dx.doi.org/10.30848/PJB2020-4(24)

SCREENING OF ANTIMICROBIAL AND CYTOTOXIC ACTIVITIES OF TWO MARINE CYANOBACTERIA COLLECTED FROM MANGROVE BACKWATER AT SANDSPIT, PAKISTAN

YASMEEN ZAMIR AHMED[2], SEEMA SHAFIQUE[1], ZAIB-UN-NISA BURHAN[1] AND PIRZADA JAMAL AHMED SIDDIQUE[1]

[1]Centre of Excellence in Marine Biology, University of Karachi, Karachi-75270, Pakistan
[2]Aquatic Diagnostic Laboratory, National Centre for Maritime Policy Research, Bahria University, Karachi, Pakistan-75260
**Corresponding author e-mail: seema.shafique@uok.edu.pk*

Abstract

Two strains of cyanobacteria form backwaters mangrove forest were isolated and examined for potential antagonist activity against clinical and environmental strains of bacteria and yeast. The cyanobacterial fractions (from sea and distilled water, ethanol, sodium hydroxide) exhibited variable activity. Results from agar spot assay revealed that the ethanolic fraction of *Phormidium breve* and seawater extract of *Aphanocapsa litoralis*, exhibited positive antagonistic activity against *Candida* albicans. Most of bacterial strains were resistant to test extracts. The cytotoxicity test was employed using *Artemia salina*. The probit analysis (95% confidence interval) revealed that the bioassay was highly sensitive against *Phormidium breve* ethanolic extract and moderately sensitive against *Aphanocapsa litoralis* sea water fraction. The undiluted crude fractions of ethanolic and seawater were found to be lethal and effective. Median lethal concentration (LC_{50}) values of *Phormidium breve* ethanolic extract was 0.02 mg/ml (20 ppm) and *Aphanocapsa litoralis* sea water fraction was found to be 6.2 mg/ml (6200 ppm) after 24-hours respectively. These findings indicate the fractions were biologically active and provide a baseline for further antifungal protein research of mangrove associated cyanobacterial strains.

Key words: Mangrove, Microbial mat, antagonistic substances, *Phormidium breve, Aphanocapsa litoralis.*

Introduction

Cyanobacteria are ubiquitous in moist environments and are highly adaptable under extreme conditions (Golubic, 1994; Golubic, 2000). They are commonly found in soils and saline environments (Bhatnagar *et al*., 2008; Abed *et al*., 2011) and are responsible for microbial mat formation on mangrove forest floors (Stal, 2000; Ahmed *et al*., 2016). Cyanobacteria serve as a natural source of bioactive substances. Some cyanobacteria have nutritional health benefits (Morton & Steve, 2008; Keming *et al*., 2018), others are treated for first generation biofuel production (Bigogno *et al*., 2002). Several cyanobacteria produce metabolites that are agriculturally, pharmaceutically and ecologically significant (Schwartz *et al*., 1990; Schaeffer & Krylow, 2000; Kim & Lee, 2006; Prabakaran, 2011). Marine cyanobacteria are among the most extensively researched organisms and are a prolific source of novel marine natural products of pharmaceutical interest (Gerwick & Moore, 2012). In Pakistan, several studies on marine cyanobacteria have been performed. While most studies have assessed the identity of cyanobacteria from coastal areas (Mansoor *et al*., 2000; Zaib-un-Nisa *et al*., 2000; Shameel, 2001; Siddiqui & Bano, 2001; Saifullah & Ahmed, 2007), fewer have focused on the bioactivity and properties of marine and freshwater cyanobacteria. Aftab & Shameel, (2006) analyzed the metabolite composition of *Microcystis aeruginosa* whereas Hameed, (2009) reported the antimicrobial and cytotoxic activity of some marine cyanobacteria. To date there are no reports on the detailed metabolite composition or activity of mangrove associated cyanobacteria. Many strains of cyanobacteria in the genera *Microcystis* and *Oscillatoria* are known to produce extracellular and intracellular metabolites with antibacterial properties (Ishida *et al*., 1997; Mundt *et al*., 2003; Prabakaran, 2011; Sakthivel & Kathiresan, 2012; Abd *et al*., 2015). Moreover, metabolites derived from *Mycrocystis* affect the feeding pattern and reproduction of *Daphnia* (Hietala *et al*., 1995; Herrera *et al*., 2015) and are cytotoxic or hepatotoxic (Gullege *et al*., 2002; Hameed, 2013) it also adversely affects the quality of fish in marine (Harke *et al*., 2016) and aquaculture pond systems (Soares *et al*., 2004) and compromise human health (Carmichael & Boyer, 2016). Sandspit mangrove backwaters in the Karachi coast harbor extensive microbial mats (Ahmed *et al*., 2016) which may be a source of bioactive compounds of commercial and economical interest. As part of our ongoing research, this study is an initial approximation towards the study of natural products from estuarine cyanobacteria from Pakistan.

The aim of the present study was to evaluate the cytotoxic and antagonistic activity of crude organic extracts from cyanobacteria present in the microbial mat belonging to the genera *Microcystis* and *Oscillatoria* against common clinical bacterial and yeast strains. Two environmental bacterial strains *Klebsiella pneumoniae* SSC14011 and *Proteus* sp. SSC1407 (native of microbial mats of test cyanobacterial cultures) were also evaluated.

Material and Methods

Site and sampling: Sampling was conducted at the Sandspit mangrove backwaters forest (24º49'05.63" N, 66º56'37.21" E) in Karachi, Pakistan (Fig. 1). Samples were collected from the top soil of mangrove forest floor during the monsoon season. 5cm sections of green microbial mats were collected with a glass slide. They were preserved in sterile seawater (autoclaved at 15 lb pressure, 121ºC for 20 minutes) and modified ASN III medium (Rippka, 1988). Samples were kept in an ice box during transport and stored in the laboratory samples at 4ºC.

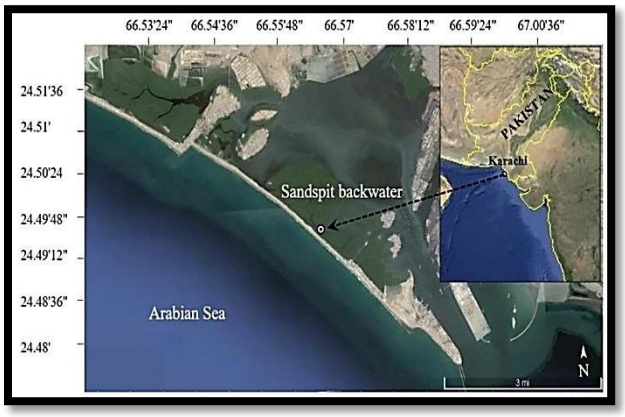

Fig. 1. Map of experiment site, Sandspit Mangrove Area, Karachi (showing the position of mangrove Backwater channel and the location of the sampling area).

Pure culture isolation: Modified culture medium BG-11(Stiner *et al*., 1971, adjusted with seawater or 25g/L of NaCl,) was used to culture filamentous *Phormidium* and modified ASN III was used for isolation of *Aphanocapsa*. Cyanobacteria were isolated and purified as follows, field samples were transferred into BG-11 or ASN III media and observed periodically for two weeks. After this period, sub samples were transferred into fresh media under sterile conditions. This method was repeated for 3 months. Later, to facilitate cyanobacterial purification the streak plate method (Phang & Chu, 1999) was used. Agar plates of the two respective media were prepared and the liquid cultures previously established were serially diluted and aseptically spread over the agar plates and incubated at 28ºC under continuous 2500 Lux light regimen, for 15 days. After successive sub culturing for 3 months, isolated colonies (in case of the unicellular form-*Microcystis*) and single filament (in case of the filamentous form-*Oscillatoria*) was picked by the capillary isolation method (Acreman, 1994; Andersen & Kawachi, 2005; Bui, 2014). Pure cultures were further recultured for 3 months. Cultures were then inoculated in Erlenmeyer flasks containing fresh 100o ml of fresh culture media and recultured (1:10 culture/media) after 1 month until further analysis (Bui, 2014).

Culture identification: Two pure strains were selected for further analysis and were identified according to Desikachary (1959), Komárek & Anagnostidis, (1999) and Komárek & Hauer, (2013). 0.1 ml culture samples were examined using an Olympus 40X microscope. 50 fields were examined to assess the dimensions of a single cell (unicellular form) or filament (filamentous form). Photographs were taken with a digital camera (Olypmpus-100, 12M.P).

Preparation of cyanobacterial extracts: Pure cultures were placed in fresh media and incubated for 15 days. Biomass was harvested by filtration (Whatman No.1) and weighed. The following protocol was used for protein based extraction (Barbarino & Lourenço, 2005; Aniket, 2012). Once prepared, all extracts were stored at 4ºC until further analysis (Fig. 2).

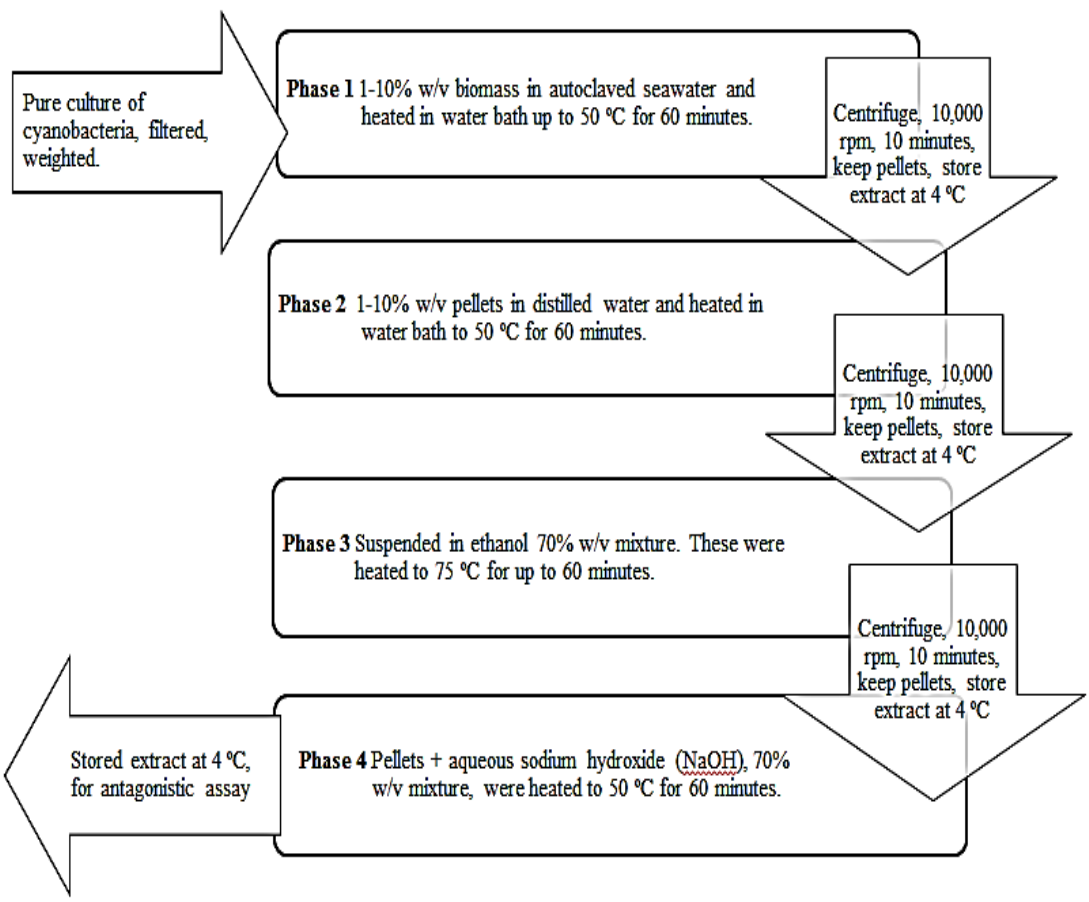

Fig. 2. Flow chart of extraction of bioactive protein extracts from cyanobacterial strains.

Step 1: The aqueous extracts from *Aphanocapsa* and *Phormidium* were obtained 1-10% w/v biomass in an autoclaved seawater and heated in water bath up to 50ºC for 60 minutes. The samples slurries were centrifuged (10,000 rpm, 10 minutes) to obtain extract in supernatant (liquid phase). The pellets (sediment phase) were used for the following phase. These aqueous extracts were named MCE01 (*Aphanocapsa*), OCE01 (*Phormidium*).

Step 2: After step 1, the resulting pellets were resuspended 1-10% w/v in distilled water and heated in water bath to 50ºC for 60 minutes. Slurries were then centrifuged (10,000 rpm, 10 minutes) and a second set of aqueous crude extracts (MCE02, OCE02) was obtained. The pellets were used in the third step.

Step 3: Pellets from Step 2 were suspended in ethanol (Sigma), 70% w/v mixture. They were heated at 75 ºC for 60 minutes and centrifuged (10,000 rpm, 10 minutes). The supernatant was collected and stored (MCE03, OCE03). Sediments were used for the final step.

Step 4: In the final extraction phase, the pellets were suspended in an alkaline of aqueous sodium hydroxide (NaOH, pH 9). The slurries 70% w/v mixture, were heated at 50ºC for 60 minutes and centrifuged (10,000 rpm, 10 minutes). The resulting liquid phase (MCE04, OCE04) was stored for further analysis.

Screening for antagonistic activity: To evaluate the antimicrobial potential of selected cyanobacterial species, common clinical strains were obtained from Microbiology Reference culture collection laboratory, Department of Microbiology and Department of Biotechnology, University of Karachi. Two environmental microbial strains, *Klebsiella pneumoniae* SSC14011 and *Proteus* sp. SSC1407 were isolated by the author from a cyanobacterial mat. This was achieved by serial dilutions and subsequent sub culturing on NaCl-LB agar and broth. The bacterial strain was maintained and grown for tests on LB (Luria-Bertani) broth and agar at 37ºC. The yeast strain was cultured and maintained on Sabouraud dextrose agar at 30ºC. 0.5 MacFarland turbidity index was used to assess the bacterial population for all test strains.

Spot agar method for screening of antagonistic activity: 50µl extracts of two cyanobacterial cultures were spot inoculated on LB/SD agar plates seeded with test cultures and incubated for 18 hours at 37ºC. The spot lawn plates were then incubated for further 24 hours (for all bacterial test strains, at 37ºC) and 72 hours (for yeast strain, at 30ºC) respectively. The antagonistic activity was examined by measuring the inhibition zone diameter in mm (Schlegel *et al*., 1998; Pawar & Puranik, 2008).

Protein determination by Bradford assay: As the cyanobacterial extracts in this research are protein in nature, the protein contents present in the crude extracts were measured using Bradford assay (Bradford, 1976). Bovine serum albumin (BSA) was used to obtain standard curve. The protein was estimated both in mg/ml and µg/ml.

Cytotoxic assay: *Artemia salina* is a highly euryplastic organism widely used for assessing the cytotoxic activity of various natural and synthetic substances (Panagoula *et al*., 2002). For the assay, 50mg of *Artemia salina* cysts were hatched in 500 ml of filtered (0.45 µm, Whatman) and autoclaved aged seawater for 24-48 hours at ambient temperature in a dark round bottom flask and aerated with an automated aerator motor. Within 48 hours, nauplii were observed. The nauplii were collected by beaming a light source at one side of the dark flask. The nauplii were gathered towards the light source and transferred into test vials (10 in each vial) using sterile Pasteur pipettes. 2-fold serial dilutions of each cyanobacterial extract were prepared in autoclaved seawater. 1 ml of each dilution was transferred into each vial. The tests were conducted in duplicate. Seawater was used as positive control and distilled water was used as negative control. Final test concentrations were determined in µg/ml dose (Table 1). The results obtained were expressed as medium lethal concentrations LC50 (Finney, 1971).

Table 1. Cytotoxic assay of crude extracts of cyanobacteria.

Cyanobacteria	Sample concentration (µg/ml)	Concentration (Log)	Mortality (%)	LC_{50} (µg/ml)
Aphanocapsa litoralis MCE01*[1]	0	0.0	0	5900.7
	779	2.9	10	
	1558	3.2	40	
	3116	3.5	30	
	6232	3.8	60	
	12465	4.1	40	
	24930	4.4	90	
Phormidium breve OCE03*[2]	0	0	0	20.3
	23	1.4	80	
	47	1.7	100	
	94	2.0	100	
	188	2.3	100	
	375	2.6	100	
	750	2.9	100	
	1500	3.2	100	

where, *[1] is the fraction from seawater extract, *[2] is the fraction from Ethanolic extract. The data of extracts showing brine shrimp mortality are presented in tabular form

Statistical analysis: Experiments were performed in triplicate except for the cytotoxicity assay which was conducted in duplicate. Data are presented as the arithmetic mean (± standard error). To assess the LC50 of cyanobacterial extracts of two test species, a Probit analysis was performed using the Minitab 17 statistical software. Microsoft Excel 2016 was used to analyze raw data and draw graphs and tables.

Results and Discussions

Two cyanobacterial strains were isolated from a mixed microbial mat culture thriving at Sandspit mangrove forest. Two culture media were used to obtain the pure cultures. For the coccoid/circular form, ASN III medium worked best. For the filamentous form, BG- 11 was most favorable medium for primary isolation and sub culturing. After pure cultures were successfully established, ASN-III medium was used for long term maintenance of both growth forms. Cyanobacterial strains were grown in high salinity (25 to 35 PSU) media. The optimum temperature for growth was 30±2ºC. Cyanobacterial strains were identified on the basis of their shape, color, dimensions, colony morphology and motility.

(1) Kingdom: Eubacteria, Phylum: Cyanobacteria, Class: Cyanophyceae, Order: Synechococcales, Family: Merismopediaceae

Aphanocapsa litoralis (Hansgirg) Forti 1907: 89 (Basionym: *Aphanocapsa litoralis* Hansgirg, 1892) (Desikachary, 1959, Komárek & Anagnostidis, 1999, Guiry & Guiry, 2016). Identification characters: Colonies macroscopic, long, irregularly arranged, covered with transparent and amorphous mucilage. Cells unicellular, dark green in color, spherical, 5 µm broad, non-motile, cells divide by binary fission, gas vacuoles absent. Found with other cyanobacteria throughout year (author observation). Bano & Siddiqui, (2003) described this specie (as *Microcystis litoralis*) from the intertidal rocky shores of Buleji, Karachi (Fig. 3. A, B, C, D).

(2) Kingdom: Eubacteria, Phylum: Cyanobacteria, Class: Cyanophyceae, Order: Oscillatoriales, Family: Oscillatoriaceae.

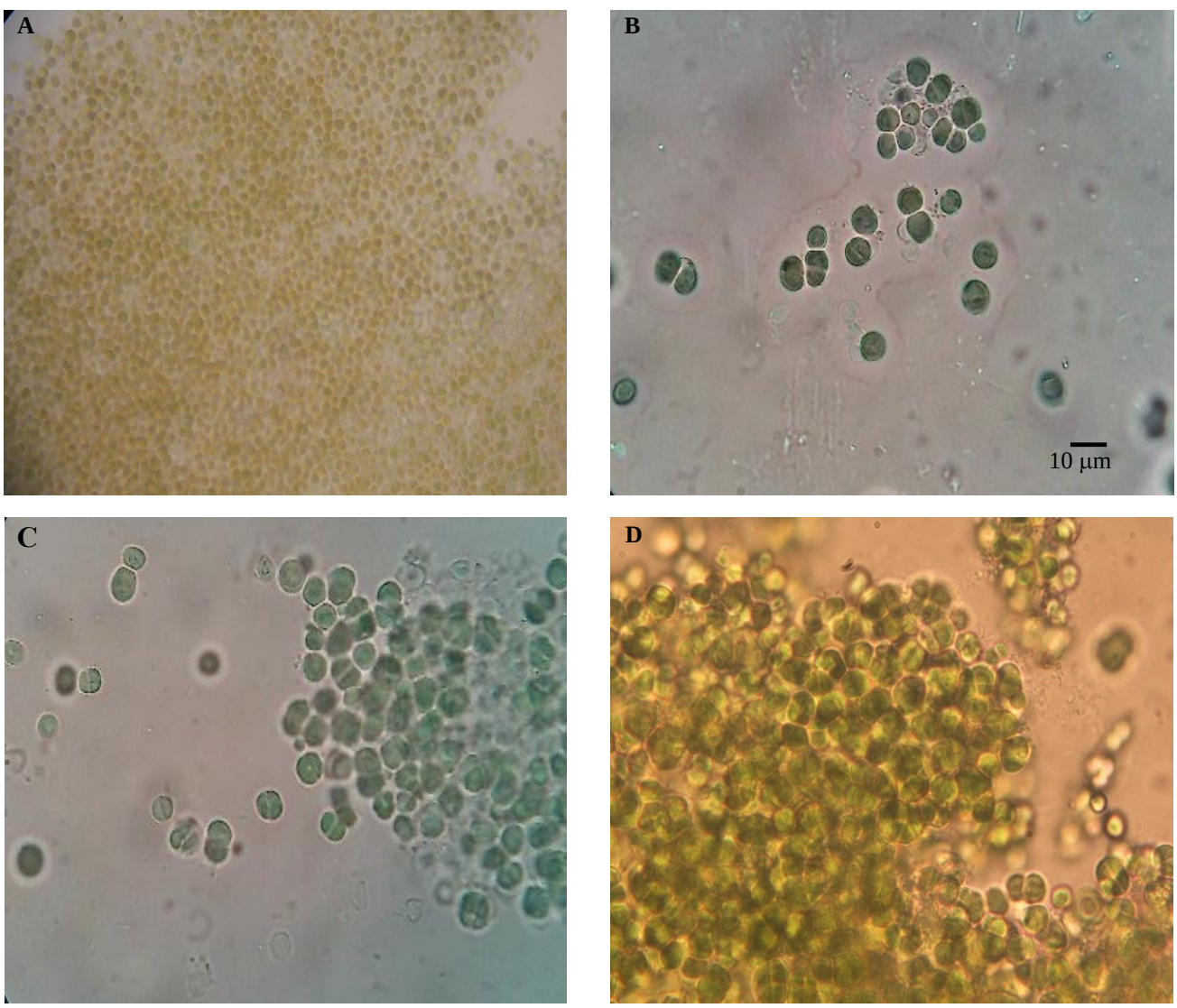

Fig. 3. *Aphanocapsa litoralis* circular cyanobacteria found in the microbial mat at Sandspit mangrove area. (A) at stationary stage, 40x, (B) and (C) at exponential stage, 100x, (D) at l ate exponential stage, 100x.

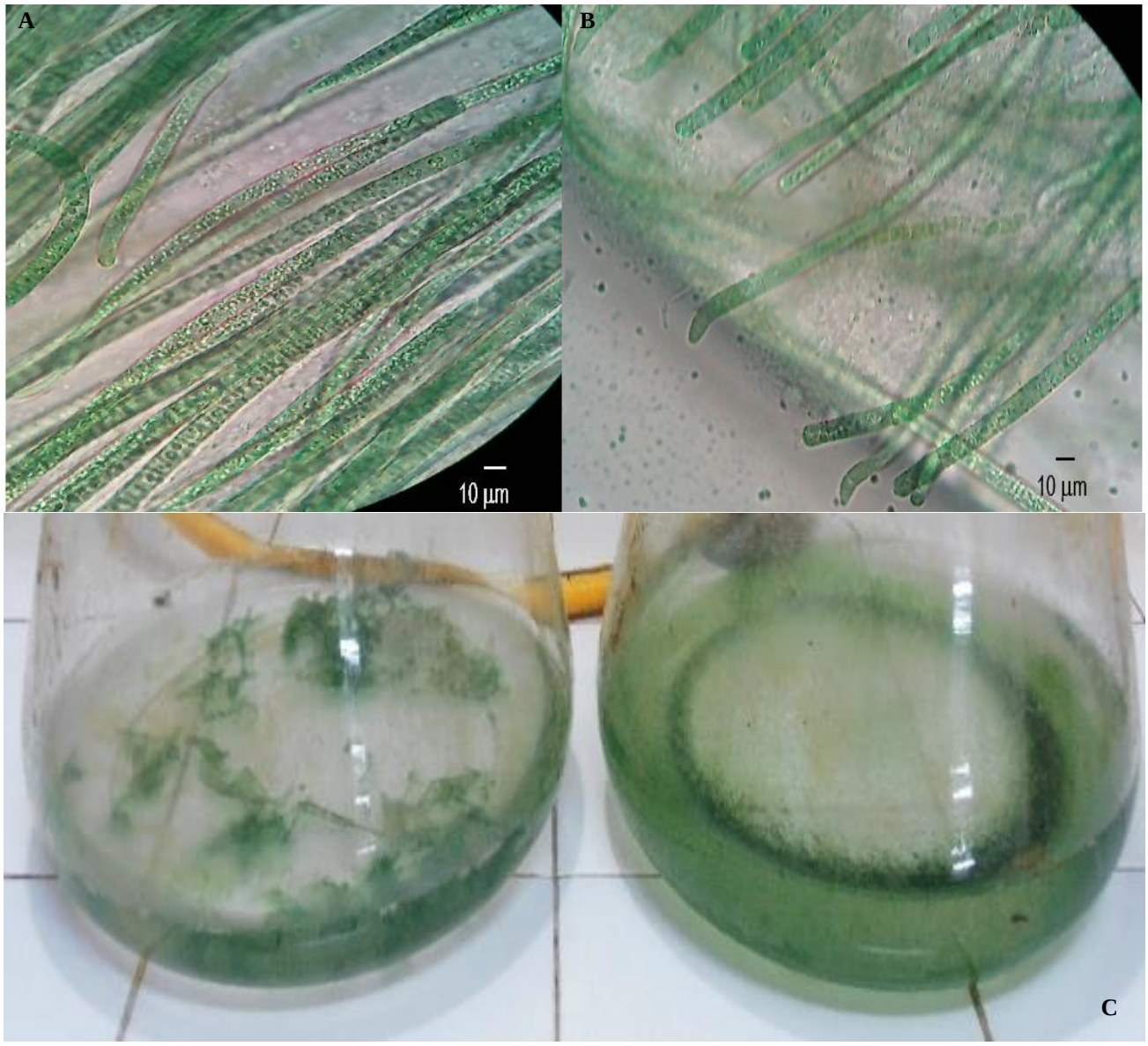

Fig. 4. *Phormidium breve* filamentous cyanobacteria found in the microbial mat at Sandspit mangrove backwaters. (A) at early stationary phase, 100x, (B) at mid exponential phase and close to stationary phase, 100x, (C) left flask containing *Phormidium breve* culture, forms sheath like mats, right flask containing *Aphanocapsa litoralis* culture, forms fine granular suspension. Both flasks contain modified ASN III medium.

Phormidium breve Kützing ex Gomont 1892 (Desikachary, 1959; Komárek & Anagnostidis, 2005; Komárek & Hauer, 2013; Guiry & Guiry, 2016). Identification characters: Colonies macroscopic, transparent mucilage. Filament green, unbranched, densely aggregated, filament straight composed of disc like cells stacked together in filament, cell 5 µm broad, end filament slightly bent, terminal region cell attenuated, cells with granules, calyptra absent, heterocystes absent, motile resembles waving motion. Found with other cyanobacteria abundantly in all seasons throughout the year (author observation). Bano & Siddiqui, (2015) observed this cyanobacterium from the tide pool of Buleji, Karachi (Fig. 4 A, B).

The results obtained from the antagonistic activity test revealed that only two extracts MCE01 (*Aphanocapsa litoralis*, seawater extract) and OCE03 (*Phormidium breve*, ethanol extract) were found to have significant inhibitory effect against *Candida albicans* (Table 2). MCE01 showed minimal zone of inhibition against Staphylococcus aureus (2 mm) (LH) and SSC14011 (3 mm). MCE02 (*Aphanocapsa litoralis*, distilled water extract) exhibited minor zone of inhibition (2 mm) against Staphylococcus aureus (LH). OCE03 also revealed a small zone of inhibition (5 mm) against SSC14011 strain respectively. It was observed that the antagonistic activity against test microorganisms differed with respect to cyanobacterial species and extraction method (Rao, 1994). For instance, the ethanolic fractions of filamentous *Phormidium breve* were highly potent against *Candida albicans* than other fractions and form clearer zones of inhibition. (Fig. 5 A, B, C). On the other hand, *Aphanocapsa litoralis* extracts exhibited lower potency and formed turbid zones of inhibition. The sodium hydroxide extracts were least effective. Most of the clinical test organisms did not respond to all the extracts. This may be due the presence of some inhibitory substances in extract that effect the antibacterial activity against clinical strains (Martins *et al*., 2008).

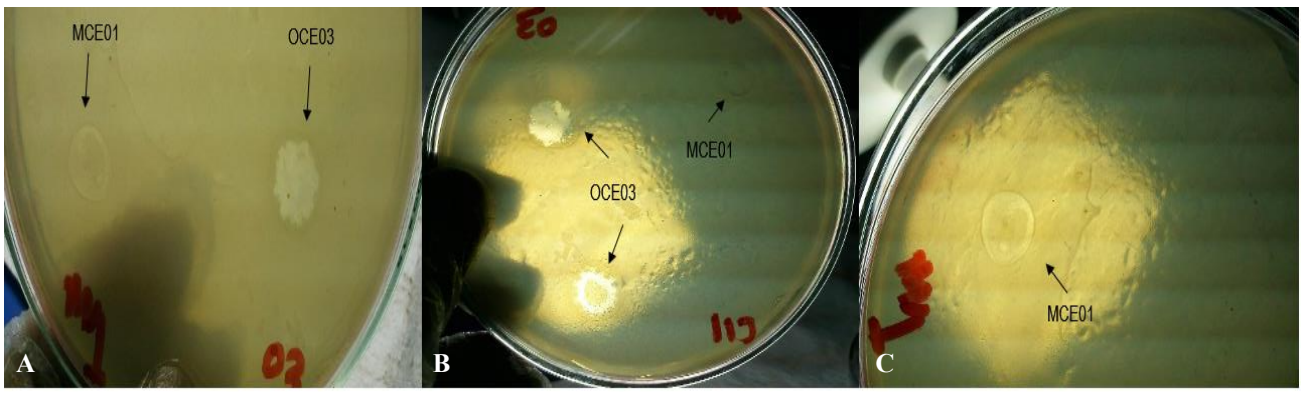

Fig. 5. Spot agar test of cyanobacterial extracts on potato dextrose agar plates seeded with clinical strain of *Candida albicans*. (A) (B) (C), plates showing zone of inhibition formed, MCE01 is the *Aphanocapsa litoralis* seawater extract, OCE03 is the *Phormidium breve* ethanolic extract. 50 µl Undiluted crude extracts were tested.

Table 2. Antagonistic activity of mangrove associated cyanobacteria (crude extracts) against different strains.

Cyanobacterial fractions/ Test strains	*Aphanocapsa litoralis*				*Phormidium breve*			
	Sea water	Distilled water	Ethanol	NaOH	Sea water	Distilled water	Ethanol	NaOH
Clinical strains								
Escherichia coli 1030	-	-	-	-	-	-	-	-
Staphylococcus aureus 1161	-	-	-	-	-	-	-	-
Staphylococcus aureus (LH)	+	+	-	-	-	-	-	-
Klebsiella pneumoniae	-	-	-	-	-	-	-	-
Beta Haemolytic Streptococci G	-	-	-	-	-	-	-	-
Pseudomonas aeruginosa	-	-	-	-	-	-	-	-
Micrococcus sp.	-	-	-	-	-	-	-	-
Acinetobacter sp.	-	-	-	-	-	-	-	-
Proteus O	-	-	-	-	-	-	-	-
Candida albicans	++	-	-	-	-	-	++	-
Environmental strains								
SSC14011	+	-	-	-	-	-	+	-
SSC1407	-	-	-	-	-	-	-	-

where, (+) zone of inhibition 2-5(mm) , (++)zone of inhibition 10 mm, (-) No zone of inhibition, (LH) This Localized strain was isolated form civil hospital, Karachi and provided by department of biotechnology, University of Karachi, (*Klebsiella pneumoniae* SSC14011) gram negative, short rods isolated from microbial mat of native cyanobacteria, (*Proteus* sp. SSC1407) gram negative, short rods isolated from microbial mat of native cyanobacteria. Concentrations of crude extracts were 300 µg/ml

Bradford assay revealed that *Phormidium breve* was found to contain 1.49 mg/ml protein whereas, *Aphanocapsa litoralis* contained 24.93 mg/ml protein content. In both cyanobacterial extracts the mortality rate was lower in diluted fractions as compared to undiluted fractions. In case of cyanobacterial culture of *Aphanocapsa litoralis*, out of four concentrated crude extracts only one extract MCE01 (Sea water fraction) exhibited cytotoxic activity. The toxicity of *Aphanocapsa litoralis* (MCE01) towards *Artemia salina* showed an LC_{50} of 6.2 mg/ml (6237.3 µg/ml) (Table 1). The remaining extracts were not cytotoxic and showed 100% survival rates in fractions of MCE02 (distilled water), MCE03 (ethanol) and MCE04 (NaOH). In case of *Phormidium breve*, out of four extracts, OCE03 was found to be the most effective and showed cytotoxic activity against *Artemia* nauplii LC50 0.02 mg/ml (20.3 µg/ml). The crude undiluted OCE03 (Ethanol fraction) demonstrated 100% lethality which remained constant upon subsequent dilutions. The LC_{50} of OCE03 was 0.02 mg/ml (20.3µg/ml). The other fractions which were eluted in seawater (OCE01), distilled water (OCE02) and NaOH (OCE04) showed no lethality to *Artemia*. Probit analyses revealed that between the two most active extracts, OCE03 exhibited an increased potency compared to MCE01. This suggests that there might be highly toxic compounds in the extract that were lethal to *Artemia salina* in increased dilutions. The Probit results also suggest that at stress level of 0.9 log dose, 99% of test of the *Artemia* nauplii exposed to MCE01 and 100% exposed to OCE03 might be killed (Figs. 6 and 7, Tables 3 and 4).

In this study two members of cyanobacteria, a filamentous form and coccoid form belonging to the genera *Phormidium* and *Aphanocapsa* respectively, were evaluated for their antibiotic and cytotoxic activity. It was found that the filamentous form was more potent compared to the coccoid cyanobacterium against the yeast *Candida albicans*. The same was also true for the cytotoxic activity against *Artemia* nauplii. Our results agree with an earlier study by Scholz & Liebezeit, (2012) where most of the bioactivity was detected in biomass

extracts. As temperature and light intensity also play a significant role in the antagonistic protein production in both cyanobacterial genera (Sivonen, 1990; Utkilen & Gjolme, 1992), it was observed in the current investigation that and increased amount of biomass was obtained when the cultural conditions were at pH 6.8, even light source 2000 lux, temperature 29-30ºC, 10-15 days incubation period and 2 minutes agitation/day respectively. Under natural field conditions it was observed that the monsoon period was more favorable, in terms of biomass production for both strains as compared to the pre-monsoon and post- monsoon seasons. Both strains were observed to be among the primary members of the green microbial mat at Sandspit mangrove forest. *Aphanocapsa litoralis* was also reported earlier by Bano & Siddiqui, (2003) on the rocky shores of Buleji, Karachi. Present research showed that cyanobacterial strains of Sandspit mangrove forest were found to be halotolerant and retain bioactive substances. These strains were selected because there are several studies related to the natural products chemistry of *Aphanocapsa* and *Phormidium* demonstrating the production of compounds that can be effective against microorganisms of clinical and ecological significance. *Aphanocapsa* litoralis can be found in benthic mats under stressed environments (Abed & Pichel, 2001; Srivastava *et al.,* 2013). It has been taxonomically studied (Shah *et al.,* 2001; Silambarasan *et al.,* 2012); Sakthivel & Kathiresan, 2013) but there was some antagonistic activity research on this strain by Veerabadhran *et al.,* (2014). On the other hand, studies on *Phormidium breve* both from fresh water and saline water sources are not rare. This cyanobacterial species has been investigated for its antimicrobial activity (Metting & Pyne, 1986; Scholz & Liebezeit, 2012), taxonomic assessment (Nagarkar, 2002), presence of odorous compounds such as geosmin and 2-methylisoborneol (Berglind, 1983; Naes *et al*., 1985) and heavy metal tolerance (Tong *et al*., 2002) respectively. This reinforces the importance of investigating cyanobacterial strains from native mangrove areas. Hameed, (2009) evaluated the antimicrobial and cytotoxic activities of *Synechocystis* sp., *Chroococcus* sp., *Pseudoanabaena* sp. and *Geitlerinema* sp. from Pakistan's coastal region but there are no recent studies related to the currently tested cyanobacterial strains.

Therefore, the present research acknowledges the antibiotic potential of estuarine cyanobacteria from the Sandspit mangrove backwaters. Further molecular and natural product chemistry studies according to the works by Engene *et al.,* 2013, Gerwick & Moore, 2012, Golubic *et al*., (2010), Esquenazi *et al.,* 2008, Richert *et al*., (2005) and Abed *et al*., (2003) are recommended to fully examine the pharmaceutical potential of these two cyanobacteria.

Table 3. Probit analysis of *Aphanocapsa litoralis* (seawater fraction).

S. No.	Parameters				
1)	**Statistics log dose µg/ml**				
	Mean	3.7709			
	St. Dev	0.871227			
	Median	3.7709			
	IQR	1.17527			
2)	**Regression**				
	Variable	**Coefficient**	**Standard error**	**Z**	**P**
	Constant	-4.32826	0.417732	-10.36	0.00
	log Dose	1.14781	0.11285	10.17	0.00
3)	**Goodness-of-fit tests**				
	Method	**Chi-Square**	**DF**	**P**	
	Pearson	55.9524	5	0.00	
	Deviance	55.8714	5	0.00	
4)	**Tolerance distribution**				
	Parameter	**Estimate**	**Standard error**	**95.0% CI**	
				Lower	**Upper**
	Mean	3.7709	0.048538	3.67577	3.86603
	St. Dev	0.871227	0.085658	0.718526	1.05638
5)	**Table of percentiles**				
	Percent	**Percentile**	**Standard error**	**95.0% CI**	
				Lower	**Upper**
	10	2.65438	0.110579	2.39089	2.83953
	50	3.7950	0.048538	3.67776	3.87182
	90	4.88742	0.128783	4.67289	5.19585
6)	**Table of survival probabilities**				
	Stress	**Probability**	**95.0%CI**		
			Lower	**Upper**	
	0.9	0.999508	0.997217	0.999976	

where, St. Dev = Standard deviation, IQR = Interquartile, Z= Z-Value, DF = Degree of freedom, P= Probability, CI= Confidence interval

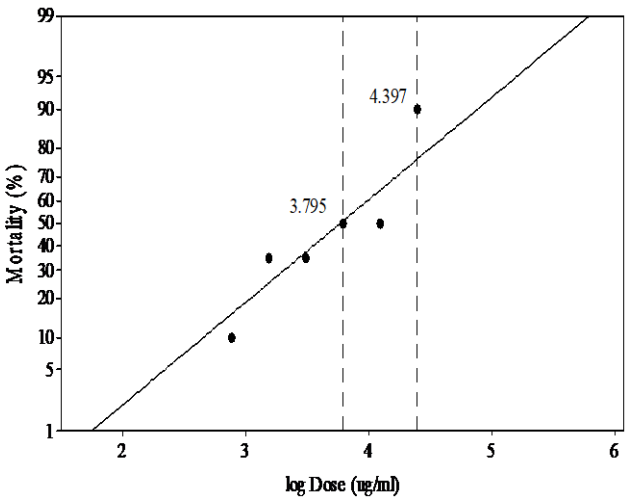

Fig. 6. Probit analysis plot showing effect of *Aphanocapsa litoralis* seawater extract towards *Artemia salina* (brine shrimps). 50% mortality is expected at log dose ≅ 3.8 µg/ml whereas, 90% mortality is expected at ≅ 4.4 µg/ml of log dose. LC_{50} is computed as 10^ (log dose50) µg/ml.

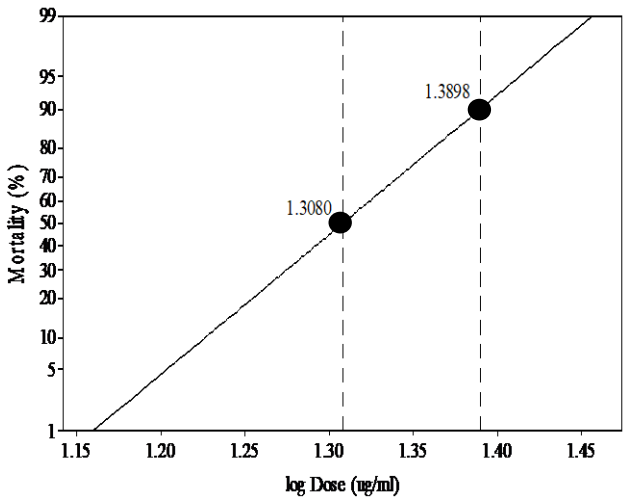

Fig. 7. Probit analysis plot showing the cytotoxic effect of *Phormidium breve* ethanol fraction towards *Artemia salina* (brine shrimps). 50% mortality is expected around log dose ≅ 1.3 µg/ml whereas, 90% mortality is expected at ≅ 1.4 µg/ml of log dose respectively. LC_{50} is computed as 10^ (log dose50) µg/ml.

Table 4. Probit analysis of *Phormidium breve* (Ethanolic fraction).

S. No.	Parameters				
	Statistics log dose µg/ml				
1)	Mean	1.30803			
	St. Dev	0.063806			
	Median	1.30803			
	IQR	0.086073			
	Regression				
2)	**Variable**	**Coefficient**	**Standard error**	**Z**	**P**
	Constant	-20.5002	996.518	-0.02	0.98
	log Dose	15.6726	731.804	0.02	0.98
	Goodness-of-fit tests				
3)	**Method**	**Chi-Square**	**DF**	**P**	
	Pearson	6E-07	6	1.00	
	Deviance	1.2E-06	6	1.00	
	Tolerance distribution				
4)	**Parameter**	**Estimate**	**Standard error**	**95.0% CI**	
				Lower	**Upper**
	Mean	1.30803	2.50746	-3.6065	6.22256
	St. Dev	0.063806	2.97929	0	-
	Table of percentiles				
5)	**Percent**	**Percentile**	**Standard error**	**95.0% CI**	
				Lower	**Upper**
	10	1.22626	6.32557	-	-
	50	1.30803	2.50746	-	-
	90	1.3898	1.31071	-	-
	Table of survival probabilities				
6)	**Stress**	**Probability**	**95.0%CI**		
			Lower	**Upper**	
	0.9	1.00	-	-	

where, St. Dev= Standard deviation, IQR= Interquartile, Z= Z-value, DF= Degree of freedom, P= Probability, CI= confidence interval

Acknowledgement

The authors are thankful to Higher Education Commission (HEC) of Pakistan for financial support to carry out the present research work through HEC-NRPU proect # 6550.

References

Abd, G.H., N.H. Zaki and A.S. Merthad. 2015. The effect of some extracted compounds from the algae *Oscillatoria tenuis* against pathogenic bacteria. *J. Pharm. Sci. Innov.*, 4(1): 36-39.

Abed, R.M. and F. Garcia-Pichel. 2001. Long-term compositional changes after transplant in a microbial mat cyanobacterial community revealed using a polyphasic approach. *Environ. Microbiol.,* 3: 53-62.

Abed, R.M., S. Dobrestov, S. Al-Kharusi, A. Schramm, B. Jupp and S. Golubic. 2011. Cyanobacterial diversity and bioactivity of inland hypersaline microbial mats from a desert stream in the Sultanate of Oman. *Fottea*, 11(1): 215-224.

Abed, R.M., S. Golubić, F. Garcia-Pichel, G. Camoin and S.J. Lee. 2003. Identity and speciation in marine benthic cyanobacteria: The Phormidium-complex. *Algol. Stud.*, 109(1): 35-56.

Acreman, J. 1994. Algae and cyanobacteria: isolation, culture and long-term maintenance. *J. Ind. Micro.Biotech.*, 13(3): 193-194.

Aftab, J. and M. Shameel. 2006. Phycochemistry and bioactivity of Microcystis aeruginosa (Chroocophyceae Shameel) from Miani Hor, Pakistan. *Int. J. Phyco. & Phycochem*., 2(2): 137-148.

Ahmed, Y.Z., S. Shafique, B. Zaib-un-nisa and P.J.A. Siddique. 2016. Seasonal abundance of six dominant filamentous cyanobacterial species in microbial mats from mangrove backwaters in Sandspit Pakistan. *Pak. J. Bot.*, 48(4): 1715-1722.

Andersen, R.A. and M. Kawachi. 2005. Traditional microalgae isolation techniques. In: Andersen, R.A. (Ed.), *Algal culturing techniques*. Physological Society of America, Elsevier academic Press, pp. 83-100.

Aniket, K. 2012. Extraction of proteins by a two solvent method. *U.S. Patent No. US8211309 B2*. Heliae Development, LlcWashington, DC: U.S. Patent and Trademark Office.

Bano, A. and P.J.A. Siddiqui. 2003. Intertidal cyanobacterial diversity on a rocky shore at Buleji near Karachi, Pakistan. *Pak. J. Bot.*, 35(1): 27-36.

Barbarino, E. and S.O. Lourenço. 2005. An evaluation of methods for extraction and quantification of protein from marine macro-and microalgae. *J. Appl. Phycol.*, 17(5): 447-460.

Berglind, L., I.J. Johnsen, K. Ormerod and O.M. Skulberg. 1983. *Oscillatoria brevis* (Kutz.) Gom. and some other especially odouriferous benthic cyanophytes in Norwegian inland waters. *Water Sci. Tech.*, 15(6-7): 241-246.

Bhatnagar, A., M.B. Makandar, M.K. Garg and M. Bhatnagar. 2008. Community structure and diversity of cyanobacteria and green algae in the soils of Thar Desert (India). *J. Arid Env.*, 72: 73-83.

Bigogno, C., I.K. Goldberg, S. Boussiba, A. Vonshak and Z. Cohen. 2002. Lipid and fatty acid composition of the green oleaginous alga *Parietochloris incisa*, the richest plant source of arachidonic acid. *Phytochem.*, 60(5): 497-503.

Bradford, M.M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, 72(1-2): 248-254.

Bui, T.H. 2014. *Cyanobacteria as source of new antifungals* (Doctoral dissertation). http://d-nb.info/1051958008/34

Carmichael, W.W. and G.L. Boyer. 2016. Health impacts from cyanobacteria harmful algae blooms: Implications for the North American Great Lakes. *Harm. Algae.*, 54: 194-212.

Desikachary, T.V. 1959. *Cyanophyta*. Indian Council of Agricultural Research, New Dehli. 686.

Engene, N., S.P. Gunasekera, W.H. Gerwick and V.J. Paul. 2013. Phylogenetic inferences reveal a large extent of novel biodiversity in chemically rich tropical marine cyanobacteria. *Appl. Environ. Microbial.*, 79(6): 1882-1888.

Esquenazi, E., C. Coates, L. Simmons, D. Gonzalez, W.H. Gerwick and P.C. Dorrestein. 2008. Visualizing the spatial distribution of secondary metabolites produced by marine cyanobacteria and sponges via MALDI-TOF imaging. *Mol. BioSyst.*, 4(6): 562-570.

Gerwick, H.W. and S.B. Moore. 2012. Lessons from the past and charting the future of marine natural products drug discovery and chemical biology. *Chem. Biol*., 19(1): 85-98.

Golubic, S. 1994. The continuing importance of cyanobacteria. In: Bengtson, S. (Ed.), *Early Life on Earth*. New York: Nobel Symposium 84. Columbia University Press. pp. 334-340.

Golubic, S., R.M. Abed, K. Palińska, S. Pauillac, M. Chinain and D. Laurent. 2010. Marine toxic cyanobacteria: diversity, environmental responses and hazards. *Toxicon*, 56(5): 836-841.

Golubic, S., S.J. Lee and K. Browne. 2000. Cyanobacteria: Architects of sedimentary structures. In: (Eds.): Riding, R. and S. Awramik. *Microbial Sediments*. Berlin, Heidelberg, New York: Springer-Verlag. pp. 57-67.

Guiry, M.D. and G.M. Guiry. 2016. *AlgaeBase*. World-wide electronic publication, National University of Ireland, Galway. http://www.algaebase.org.

Gulledge, B. M., .B. Aggen, H.B. Huang, A.C. Nairn and A.R. Chamberlin. 2002. The Microcystins and Nodularins: Cyclic Polypeptide Inhibitors of PP1 and PP2A. *Curr. Med. Chem.*, 9: 1991-2003.

Hameed, S. 2009. *Isolation, identification, screening of toxicity and oligopeptides of some marine and brackish cyanobacteria from Norwegian and Pakistani waters, in the search for bioactive natural compounds* (Master's Thesis). Norway.

Hameed, S. 2013. *Investigation of the production and isolation of bioactive compounds from cyanobacteria* (Doctoral dissertation). http://openair.rgu.ac.uk

Herrera, N. A., L.F. Echeverri and A.S. Ferrao-Filho. 2015. Effects of phytoplankton extracts containing the toxin microcystin-LR on the survival and reproduction of cladocerans. *Toxicon*, 95: 38-45.

Hietala, J., M. Reinikainen and M. Walls. 1995. Variation in life history responses of *Daphnia* to toxic *Microcystis aeruginosa. J. Plank. Res.*, 17: 2307-2318.

Ishida, K., H. Matsuda, M. Murakami and K. Yamaguchi. 1997. Kawaguchipetin B, an antibacterial cyclic undecapeptide from the cyanobacterium *Microcystis aeruginosa*. *J. Nat. Prod.*, 60: 724-726.

Keming, Z., L. Kaixia, L. Juan, X. Shuo, S. Zafar, and T. Xiaoli. 2018. The Cyanobacterium hemoglobin (CHb) promotes the growth of bacteria, yeast and *Brassica napus*, and enhances submergence resistance of *Brassica napus*. *Pak. J. Bot*., 50(6): 2429-2434.

Kim, J.D. and C.G. Lee. 2006. Diversity of heterocystous filamentous cyanobacteria (blue-green algae) from rice paddy fields and their differential susceptibility to ten fungicides used in Korea. *J. Micro. Biotech.*, 16(2): 240-246.

Komárek, J. and K. Anagnostidis. 1999. Cyanoprokaryota. 1. Chroococcales. In*:* (Eds.): Ettl, H., G. Gärtner, H. Heynig and D. Mollenhauer. *Süßwasserflora von Mitteleuropa. Begründet von A. Pascher. Band 19/1.* Heidelberg and Berlin: Spektrum, Akademischer Verlag, 1-548.

Komárek, J. and K. Anagnostidis. 2005. Cyanoprokaryota: 2. Teil/2nd Part: Oscillatoriales. In: (Eds.): Büdel, B., L. Krienitz, G. Gärtner and M. Schagerl. *Süsswasserflora von Mitteleuropa 19/2. München*. Elsevier Spektrum Akademischer Verlag, pp. 1-759.

Komárek, J. and T. Hauer. 2013. *CyanoDB.cz. On-line database of cyanobacterial genera*. Word-wide electronic publication, University of South Bohemia and Institute of Botany ASCR [online]. Website: http://www.cyanodb.cz

Mansoor, S.N., P.J.A. Siddiqui, A. Bano and Zaib-un-Nisa. 2000. Microbial flora associated with mangroves. In: (Ed.): Ahmed, V.U. *The Arabian Sea as a source of Biological Diversity.* Proceedings of the National O.N.R. Symposium. H.E.J. Institute of Chemistry, University of Karachi, pp. 157-164.

Martins, R.F., M.F. Ramos, L. Herfindal, .A. Sousa, K. Skærven and V.M. Vasconcelos. 2008. Antimicrobial and cytotoxic assessment of marine cyanobacteria-*Synechocystis* and *Synechococcus*. *Mar. drugs*, 6(1): 1-11.

Metting, B. and J.W. Pyne. 1986. Biologically active compounds from microalgae. *Enzyme Microb. Technol.*, 8(7): 386-394.

Morton, T. and L. Steve. 2008. *Modern Uses of Cultivated Alga*. Ethnobotanical Leaflets. Southern Illinois University Carbondale, pp. 12-26.

Mundt, S., S. Kreitlow and R. Jansen. 2003. *Oscillatoria redekei* HUB 051. *J. Appl. Phycol.*, 15(2-3): 263-267.

Naes, H., H. Aarnes, H.C. Utkilen, S. Nilsen and O.M. Skulberg. 1985. Effect of photon fluence rate and specific growth rate on geosmin production of the cyanobacterium *Oscillatoria brevis* (Kütz.) Gom. *Appl. Environ. Micro.*, 49(6): 1538.

Nagarkar, S. 2002. Morphology and ecology of new records of cyanobacteria belonging to the genus Oscillatoria from Hong Kong rocky shores. *Bot. Mar.*, 45(3): 274-283.

Panagoula, B., M. Panayiota and J. Iliopoulou-Georgudaki. 2002. Acute toxicity of TBT and IRGAROL in *Artemia salina*. *Int. J. Toxicol.*, 21: 231–233.

Pawar, S.T. and P.R. Puranik. 2008. Screening of terrestrial and freshwater halotolerant cyanobacteria for antifungal activities. *World J. Micro. and Biotech.*, 24(7): 1019-1025.

Phang, S.M. and W.L. Chu. 1999. *Catalogue of Strains*. *University of Malaya, Algae culture Collection.* Institute of Post Graduate Studies and Research, University of Malaya, Kuala Lumpur, Malaysia, pp. 77.

Prabakaran, M. 2011. *In vitro* antimicrobial potentials of marine *Oscillatoria* species. *Asian J. Plant Sci. Res.*, 1: 58-64.

Rao, C.S.V.R. 1994. Antimicrobial activity of cyanobacteria. *Int. J. Mar. Sci.,* 23: 55-56.

Richert, L., S. Golubic, R. Le Guédès, J. Ratiskol, C. Payri and J. Guezennec. 2005. Characterization of exopolysaccharides produced by cyanobacteria isolated from Polynesian microbial mats. *Curr. Micro.*, 51(6): 379-384.

Rippka, R. 1988. Isolation and purification of cyanobacteria. In: (Eds.): Packer, L. and N.G. Alexander. *Methods in Enzymology*. Academic Press, UK, pp. 3-27.

Saifullah, S.M. and W. Ahmed. 2007. Epiphytic algal biomass on pneumatophores of mangroves of Karachi, Indus Delta. *Pak. J. Bot.*, 39(6): 2097-2102.

Sakthivel, K. and K. Kathiresan. 2013. Cyanobacterial diversity from mangrove sediment of south east coast of India. *Asian J. Biodiver.*, 4: 190-203.

Schaeffer, D.J. and V.S. Krylov. 2000. Anti-HIV activity of extracts and compounds from algae and cyanobacteria. *Ecotoxicol. Environ. Safety*, 45: 208-227.

Schlegel, I., N.T. Doan, N. de Chazal and G.D. Smith. 1998. Antibiotic activity of new cyanobacterial isolates from Australia and Asia against green algae and cyanobacteria. *J. Appl. Phycol.*, 10(5): 471-479.

Scholz, B. and G. Liebezeit. 2012. Screening for biological activities and toxicological effects of 63 phytoplankton species isolated from freshwater, marine and brackish water habitats. *Harm. Algae*, 20: 58-70.

Schwartz, R.E., C.F. Hirsch and D.F. Sesin. 1990. Pharmaceuticals from cultured algae. *J. Ind. Micro.*, 5: 113-124.

Shah, V., N. Garg and D. Madamwar. 2001. Record of the marine cyanobacteria from the rocky shores of Bet-Dwarka and Okha, India. *Acta Bot. Mal.*, 26: 188-194.

Shameel, M. 2001. Diversity exhibited by marine benthic algae inhabiting the coast of Balochistan, Pakistan. *Pak. J. Mar. Sci.*, 10(2): 87-103.

Siddiqui, P.J.A. and A. Bano. 2001. Distribution of Epiphytic cyanobacteria on macroalgae occurring at a rocky shore (Buleji), Karachi, Pakistan. *Pak. J. Mar. Bio*., 7(1&2): 251-259.

Silambarasan, G., T. Ramanathan and K. Kathiresan. 2012. Diversity of marine cyanobacteria from three mangrove environment in Tamil Nadu Coast, south east coast of India. *Curr. Res. J. Biol. Sci.*, 4(3): 235-238.

Sivonen, K. 1990. Effects of light, temperature, nitrate, orthophosphate and bacteria on the growth of the hepatotoxin by *Oscillatoria agardhii* strains. *Appl. Env. Micro.*, 56: 2658-2666.

Soares, R.M., V.F. Magalhães and S.M. Azevedo, 2004. Accumulation and depuration of microcystins (cyanobacteria hepatotoxins) in Tilapia rendalli (Cichlidae) under laboratory conditions. *Aqua. Toxicol.*, 70(1): pp.1-10.

Srivastava, A.K., A.N. Rai and B.A. Neilan. 2013. *Stress biology of cyanobacteria: molecular mechanisms to cellular responses*. CRC Press.

Stal, L.J. 2000. Cyanobacterial mats and stromatolites. In: (Eds.): Whitton, B.A. and M. Potts. *The ecology of cyanobacteria: Their diversity in time and space*. Dordrecht, Kluwer Academic Publishers, pp. 61-120.

Stanier, R.Y., R. Kunisawa, M. Mandel and G. Cohen-Bazire. 1971. Purification and properties of unicellular blue-green algae (order Chroococcales). *Bacteriol. Rev.*, 35(2): 171.

Tong, L., S. Nakashima, M. Shibasaka, M. Katsuhara and K. Kasamo. 2002. A novel histidine-rich CPx-ATPase from the filamentous cyanobacterium *Oscillatoria brevis* related to multiple-heavy-metal cotolerance. *J. Bacterial.*, 184(18): 5027-5035.

Utkilen, H.A. and G.N. Jolme. 1992. Toxin Production by *Microcystis aeruginosa* as a Function of Light in Continuous Cultures and Its Ecological Significance. *Appl. Environ. Microbiol.*, 58: 1321-1325.

Veerabadhran, M., N. Manivel, D. Mohanakrishnan, D. Sahal and S. Muthuraman. 2014. Antiplasmodial activity of extracts of 25 cyanobacterial species from coastal regions of Tamil Nadu. *Pharma. Biol.*, 52(10): 1291-1301.

Zaib-un-Nisa, B., S.N.Mansoor and P.J.A. Siddiqui. 2000. Species diversity of cyanobacteria growing on pneumatophores and in the adjacent surface sediment in mangrove swamp at Sandspit backwaters Karachi. *Pak. J. Mar. Biol.*, 6(1): 59-68.

(Received for publication 2 December 2018)