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IN-VITRO EVALUATION OF ANTIMICROBIAL AND ANTICANCER ACTIVITY OF TWO DIAZOTROPIC CYANOBACTERIA FROM BILASPUR CHHATTISGARH, INDIA

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Abstract

Cyanobacteria are potential source of biologically active compounds with anticancer, antioxidant, antimicrobial activities. In present investigation, the organic solvent extract of two cyanobacterial species *Nostoc* sp. (DGN2) and *Revularia* sp. (NDR5) were tested for antimicrobial activity against human pathogenic bacteria and fungal strains as well as anticancer activity against human breast cancer (MCF-7) and human colon cancer HT - 29. The methanol extract of *Nostoc* sp. (DGN2) was found to be most active against tested bacterial and fungal strains. It showed a maximum antimicrobial activity against Bacillus subtilis (26.00±0.816) and Candida krusei. (28.00±0.816). *In vitro* cytotoxicity activity against human breast cancer MCF7 and human colon cancer HT - 29 cell lines by Sulforhodamine B assay method. The Growth inhibition of 50% (GI_{50}) was analyzed by comparing it with standard drug Adriamycin. The extracts did not show any activity when compared to Adriamycin at different concentrations (µg/ml) on both the cell lines MCF7 and HT - 29. Thus, authors have attempted to provide importance to these cyanobacterial strains by subjecting them to anticancer studies. In future, new cell lines may provide relevance to these species and they can be actual put in therapeutic role Methanol was the best solvent for extracting the active material. These results indicate that extracts of studied cyanobacterial species exhibited appreciable antimicrobial and cytotoxic activity and could be a source of valuable bioactive materials for health product.

Key words : Cyanobacteria; *Nostoc* sp., *Revularia* sp., Anticancer activity, Antimicrobial activity.

Introduction

Cyanobacteria (also known as blue-green algae) are Gram-negative prokaryotes that are able to perform oxygenic photosynthesis similar to plants. Fossil records indicate that cyanobacteria have populated the earth for around 3.5 billion years and ancient cyanobacteria (BGA) are believed to have been instrumental in the creation of the Earth's oxygen rich atmosphere (Schopf, JW. 2000), Cyanobacteria are nature's unique gift to mankind, as they possess several innate properties that make them ideal organisms with potential multifaceted bio-prospective applications. Cyanobacteria are considered a promising source for new pharmaceutical bioactive compounds and a large number of chemically diverse metabolites have been obtained from cyanobacteria the organisms such as eukaryotic (plants and animals) as well as microorganisms (bacteria, micro 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; Nermien *et al.*, 2019). From this point of view cyanobacteria have been demonstrated to produce secondary metabolites other than those produced by terrestrial organisms (Faulkner, 1994). 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, Skoèibušiæ *et al.*, 2019, Bhaskar K.D., *et al.* 2020). Cyanobacteria are ecologically, morphologically, physiologically and metabolically excellent diverse group, which makes them as a promising group of organisms for research on pharmaceutical drugs discovery (Abed *et al.*, 2011; Rama-Murthy *et al.*, 2012). 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 blue green algae, 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 blue green algae had an anticancer effect (Russo and Cesario, 2012,). Cyanobacteria (BGA) 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 *Calothrix brevissima* (Metting and Pyne, 1986), *Nostoc spongiae forme* (Hirata *et al.*, 1996), *Anabaena variabilis* (Ma and Led, 2000), *Nostoc commune* (Jaki *et al.*, 2000), *Microcystis aeruginosa* and *Anabaena flos-aquae* (Khairy and El-Kassas, 2010), have been popularly reported to produce antibacterial and antifungal substances. The increase of antifungal resistance indicates an urgent need for new antifungal compounds (Khan *et al.*, 2010). Antimicrobial compounds have been previously studies in cyanobacterial extracts, such as lipopeptides from *Anabaena* sp. (Burja *et al.*, 2001), Heptadecane and tetradecane from *Spirulina platensis* (Ozdemir *et al.*, 2004), peptides, polypeptides, amides and phenolic compounds from *Nostoc muscorum* (El Sheekh *et al.*, 2006), fatty acids, tetramine, spermine and piperazine derivative from *Anabaena* sp. (Shanab, 2007), and laxaphycins from *Anabaena laxa* (Frankmolle *et al.*, 1992), these bioactive compounds have been reported to possess antibacterial and antifungal activity. This study was conducted to elucidate the cytotoxicity and antimicrobial properties of two species of cyanobacterial strains namely *Nostoc* sp. (DGN2) and *Revularia* sp. (NDR5) for future applications in medicinal and pharmaceutical industries.

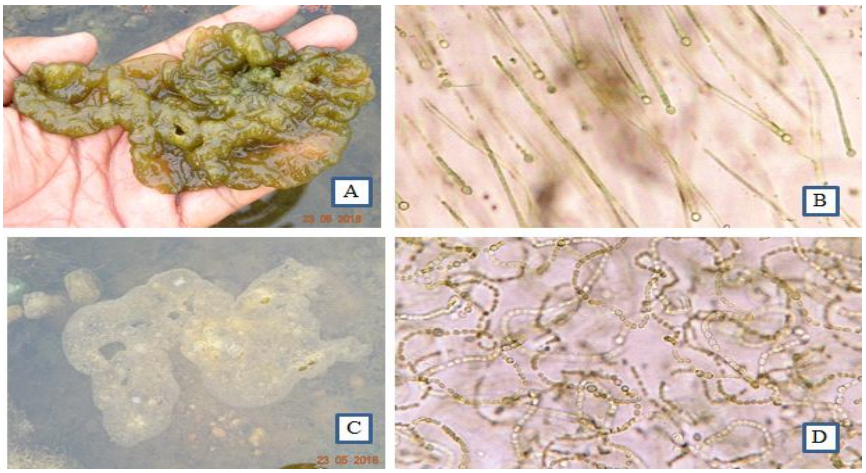

Fig. 1: Photographs of hetrocystous filamentous cyanobacteria A. *Revularia* colony on water surface; B. *Revularia* sp.; C. *Nostoc* colony on water surface; D. *Nostoc* sp.

Materials and Methods

Sample Collection and morphological analyses

Cyanobacterial sample was collected from Bilaspur division (Chhattisgarh), India. Two isolates were used in this study *i.e.* DGN2 and NDR5. *Nostoc* sp. *(*DGN2*)* was isolated dispatches water the Arpa river of Darry Ghat from Bilaspur district while *Revularia* sp. (NDR5) were isolated from pond water of Negurdih from Janjgir-champa district of Chhattisgarh, India. The longitude & latitude of location is being identified using GPS locator as N-22°01'29.61", E-42° 13'40.35" and N-21°49'10.87", E-82°38'58.19", Elevation ft 815ft. and 785ft. Morphological observations [presence and absence of sheath, shape and size of the vegetative cells, heterocyst's, akinetes (if present)], of the axenic cultures of blue green algae ere made using an Olympus KIC22809 microscope fitted with a digital camera as described by (Desikachary 1959)., (Komarek and Anagnostidis 1998, 2005), Cyanobacteria (BGA) images were captured at 100x magnification (Fig. 1).

Pure culture isolation & mass cultivation

Nostoc sp. (DGN2) *and Revularia* sp. (NDR5) isolate were inoculated in BG11 solid medium and incubated at 28±2æ%C temperature under controlled light intensity for optimizing growth under laboratory conditions. *Nostoc* sp. (DGN2) *and Revularia* sp. (NDR5). were grown in laboratory condition for 12-14 days along with germanium dioxide & cycloheximide to control diatom & green algae contamination. Filaments were picked up selectively and inoculated in to BG11 liquid media for mass cultivation. Cyanobacterial cultures were initially grown in 250 ml conical flasks BG11 medium with pH 7.5±0.2 (Rippka and Herdmann 1993) followed with 1000ml BG11 medium for mass cultivation.

Preparation of cyanobacterial extracts

At the end of log phase, cyanobacteria cultures were centrifuged and the pellets were collected, weighted and used for extraction of antimicrobial agents. 0.3 g of each cyanobacteria pellet was extracted separately in acetone and methanol in a mortar pestle and kept overnight at 4°C for complete extraction. The supernatant was

collected after the centrifugation at 20000×g at 5 min. The solvent extracts were concentrated under reduced pressure at 40°C. Dry residue was again dissolved in the different solvents to obtain final concentrations of 5 mg/ml, then the extract was kept at 4°C until use for bioassay.

The growth of microorganisms set as microbial indicators

Bacterial strains and Nutrient Agar Media/broth

The Anti-bacterial spectrum of the different extracts of two blue-green algae were tested against two isolates of bacteria as following: one isolates of Gram-positive bacteria namely: *Bacillus subtilis* (MTCC619) and one Gram-negative bacteria namely: Escherichia coli (MTCC119) The medium was prepared by dissolving 28 g of the commercially available Medium (Hi Media) in 1000ml of distilled water. The dissolved medium was autoclaved at 15 lbs pressure at 121°C for 16 minutes. The autoclaved medium was mixed well and poured on to 100mm Petri plates (25- 30ml/plate) while still molten. One litter of nutrient broth was prepared by dissolving 13 g of commercially available nutrient medium (Hi Media) in 1000ml double distilled water and boiled to dissolve the medium completely. The medium was sterilized by autoclaving at 15 lbs pressure (121ºC) for 16 minutes.

Candida species and SDS Media/broth

Two isolates of pathogenic Candida species were *Candida krusei* (MTCC9215), *Candida albicans* (MTCC227). They were previously isolated from women complaining of vaginal candidiasis (Seddek *et al.,* 2019) These isolates were Candida albicans (Robin) Berkhout, Candida glabrata (Anderson) S.A. Meyei & Berkhout and Candida krusei (Castellani) Berkhout. The medium was prepared by dissolving 65 g of the commercially available Sabouraud Dextrose Agar Medium (Hi Media) in 1000ml of distilled water. The dissolved medium was autoclaved at 15 lbs. pressure at 121°C for 16 minutes. The autoclaved medium was mixed well and poured onto 100mm Petri plates (25-30ml/plate) while still molten. One litter of nutrient broth was prepared by dissolving 30 g of commercially available medium (Hi Media) in 1000ml distilled water and boiled to dissolve the medium completely. The medium was sterilized by autoclaving at 15 lbs pressure (121ºC) for 16 minutes.

Testing antimicrobial activity by the agar well diffusion method

Agar well diffusion method (Sleigh *et al.,* 2004), 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. Petri plates containing 20ml Muller Hinton medium were seeded with 24hr culture of bacterial strains. Wells were cut and 20µl of the given sample (of different concentrations) were added. The plates were then incubated at 37°C for 24 hours. The antibacterial activity was assayed by measuring the diameter of the inhibition zone formed around the well. Ofloxacin, Amphotericin, Ampicillin were used as a positive control.

SBS assay method

Investigations of different cyanobacterial extracts were carried out on Human breast cancer (MCF-7) based on the colorimetric MTT assay method (Vanicha *et al.,* 2006) which was conducted essentially according to the manufacturer's protocol. The cell lines were grown in RPMI 1640 medium containing 10% fatal bovine serum and 2 mM L-glutamine. For present experiment, cells were inoculated into 96 well micro liter plates in 100 µL at plating densities as shown in the study details above, depending on the doubling time of individual cell lines. After cell inoculation, the micro liter plates were incubated at 37° C, 5 % CO2, 95 % air and 100 % relative humidity for 24 h prior to addition of experimental drugs.

Nostoc sp. (DGN2) *and Revularia* sp. (NDR5) extracts standard drugs were initially solubilized in dimethyl sulfoxide at 100mg/ml and diluted to 1mg/ml using water and stored frozen prior to use. At the time of drug addition, an aliquot of frozen concentrate (1mg/ml) was thawed and diluted to 100 µg/ml, 200 µg/ml, 400 µg/ml and 800 µg/ml with complete medium containing test article. Aliquots of 10 µl of these different drug dilutions were added to the appropriate micro liter wells already containing 90 µl of medium, resulting in the required final drug concentrations *i.e.* 10 µg/ml, 20 µg/ml, 40 µg/ml, 80 µg/ml.

Nostoc sp. (DGN2) *and Revularia* sp. (NDR5) extracts standard drugs addiction, plates were incubated at standard conditions for 48 hours and assay was terminated by the addition of cold TCA. Cells were fixed *in situ* by the gentle addition of 50 µl of cold 30 % (w/v) TCA (final concentration, 10 % TCA) and incubated for 60 minutes at 4°C. The supernatant was discarded; the plates were washed five times with tap water and air dried. Sulforhodamine B (SRB) solution (50 µl) at 0.4 % (w/v) in 1 % acetic acid was added to each of the wells, and plates were incubated for 20 minutes at room temperature. After staining, unbound dye was recovered and the residual dye was removed by washing five times with 1 % acetic acid. The plates were air dried. Bound stain was subsequently eluted with 10 mM trizma base,

and the absorbance was read on a plate reader at a wavelength of 540 nm with 690 nm reference wavelength.

Percent growth was done on a plate-by-plate basis for test wells relative to control wells. Percent Growth was expressed as the ratio of average absorbance of the test well to the average absorbance of the control wells * 100. Using six absorbance measurements [time zero (Tz), control growth (C), and test growth in the presence of drug at the four concentration levels (Ti)], the percentage growth was calculated at each of the drug concentration levels.

Percentage growth inhibition was calculated as: [Ti/C] ×100 %

Results and Discussion

In vitro antimicrobial activities of different studied cyanobacteria Antimicrobial activity of acetone and methanol of two species of cyanobacteria *Nostoc* sp. (DGN2) *and Revularia* sp. (NDR5) against tested different bacterial and fungal species was shown in Table 1. The cyanobacterial extracts showed a different degree of antimicrobial activity and the intensity of inhibitory action varied depending on the species of microorganism, cyanobacterial species and the type of solvent. Concerning the anti-bacterial activities of the investigated different cyanobacterial extracts, we found that the methanol *Nostoc* sp. (DGN2) extract was the most active one. Its inhibition activity was ranged from 26.00±0.816 against *Bacillus subtilis* to 21.67±0.471against *E. coli*, it succeeded on the inhibition of growth Gram +ve and Gram –ve bacteria as Gram –ve bacteria as showing Table 1.

Table 3: Growth concentration value LC_{50} (µg/ml), total growth inhibition (TGI) and mediangrowth inhibition (GI_{50}) for tested methanolic extract of *Nostoc* sp. (DGN2) *and Revularia* sp. (NDR5) and adriamycin.

Drug concentrations (µg/ml) calculated from graph			
MCF-7	**LC50**	**TGI**	**GI50***
DGN2	NE	NE	>80
NDR5	NE	NE	>80
ADR	NE	<10	<10

The present findings on the antifungal activities of different cyanobacterial extracts were represented as anti-Candida fungi. The results indicated that *Candida krusei* was more sensitive than *Candida albicans* to all tested extracts. *Candida species* were inhibited by all tested cyanobacterial extracts methanol extract of *Nostoc* sp. (DGN2) their effect ranged between 28.00±0.816 *Candida krusei* and 28.33±1.247 against *Candida albicans*. When closely observed in Table 1 which showing the anti- Candida activities of studied cyanobacterial extracts, all different extracts of *Nostoc* sp. (DGN2) exhibited attractive anti- Candida properties. It worth to mention that, methanol extracts of *Revularia* sp. (NDR5) demonstrated to be inhibitory against two different species of Candida. Whereas, their maximum inhibitory action was 25.00±0.816 against *Candida krusei* and minimum inhibitory action was 24.67±0.471 against *Candida albicans*. *Where* acetone extracts of *Revularia* sp. (NDR5) demonstrated to be inhibitory against two different species of Candida. Whereas, their maximum inhibitory action was 25.00±1.414 against *Candida krusei*

Table 1: Antibacterial and antifungal activities of the cyanobacterial extracts.

	Inhibition of growth expressed as diameter of inhibition zone (mm)			
	Nostoc sp. ***(DGN2)***		*Revularia* sp. ***(NDR5)***	
	Acetone	**Methanol**	**Acetone**	**Methanol**
Gram +ve bacteria				
Bacillus subtilis	24.33±0.471	26.00±0.816	22.33±0.471	30.00±0.816
Gram -ve bacteria				
E. coli	21.33±0.471	21.67±0.471	21.67±1.247	23.00±0.816
Candida species				
Candida albicans	28.33±1.247	25.00±0.816	21.00±0.816	23.67±0.943
Candida krusei	23.67±2.055	28.00±0.816	25.00±1.414	22.00±1.633

Note: Mean+SD (n=2).

Table 2: % Control Growth against Human Breast Cancer Line MCF-7.

1.00	**Human Breast Cancer Cell Line MCF-7**							
	% Control Growth							
	Drug Concentrations (µg/ml)							
	Experiment 1				**Experiment 2**			
	10	**20**	**40**	**80**	**10**	**20**	**40**	**80**
DGN2	37.8	48.4	71.4	126.5	86.1	89.2	87.9	94.2
NDR5	42.9	40.7	51.7	89.4	91.2	89.3	82.0	86.1
ADR	-50.0	-56.9	-61.0	-46.4	-45.9	-52.1	-56.7	-41.1
	Experiment 3				**Average Values**			
	10	**20**	**40**	**80**	**10**	**20**	**40**	**80**
DGN2	81.9	86.7	91.3	101.0	68.6	74.8	83.5	107.2
NDR5	85.5	87.8	93.2	87.3	73.2	72.6	75.6	87.6
ADR	-40.5	-52.1	-10.5	-53.7	-45.5	-53.7	-42.7	-47.1

Table 4: % Control Growth against Human colon Cancer Line HT – 29.

2.00	Human Colon Cancer Cell Line HT-29							
	% Control Growth							
	Drug Concentrations (µg/ml)							
	Experiment 1				Experiment 2			
	10	20	40	80	10	20	40	80
DGN2	113.3	127.8	139.4	143.1	124.8	124.6	126.3	119.4
NDR5	124.0	149.0	150.5	131.1	121.8	136.8	137.3	111.7
ADR	2.7	7.7	9.3	5.8	-7.3	-6.0	-3.1	-6.6
	Experiment 3				Average Values			
	10	20	40	80	10	20	40	80
DGN2	141.9	137.8	138.1	134.8	126.7	130.1	134.6	132.4
NDR5	132.0	143.4	125.1	131.8	125.9	143.1	137.6	124.9
ADR	-2.5	8.3	4.2	-3.9	-2.4	3.3	3.5	-1.6

Table 5: Growth concentration value LC50 (µg/ml), total growth inhibition (TGI) and median growth inhibition (GI50) for tested methanolic extract of *Nostoc* sp. (DGN2) *and Revularia* sp. (NDR5) and adriamycin.

Drug concentrations (µg/ml) calculated from graph			
HT-29	LC50	TGI	GI50*
DGN2	NE	NE	>80
NDR5	NE	NE	>80
ADR	NE	<10	<10

and minimum inhibitory action was 23.67±0.943 against *Candida albicans*In all cases, methanol extract was more effective than acetone extract against both studies bacteria and fungi.

Anticancer activities

The extracts did not produce significant effect on the human breast cancer MCF7 and human colon cancer HT- 29 cell lines used in these studies as depicted in Tables 2- 5 and Figs. 3-6. Cyanobacteria can be screened for their potential with various extraction techniques. Researchers citing the paper should try to explore different cell lines at different concentrations.

The present study is a strive towards the production of antibacterial, antifungal and anticancer by the blue-green algae namely; *Nostoc* sp. (DGN2) *and Revularia* sp. (NDR5) This screened for their antimicrobial activities against different species of pathogenic two bacteria and two fungi and anticancer activities. Finding an antimicrobial activity for tested cyanobacteria *In vitro* experiments is predictive of their capacity to produce new compounds which act as antibacterial and antifungal compounds. In our knowledge, no literature about antimicrobial activities of *Nostoc* sp. (DGN2) *and Revularia* sp. (NDR5). They screened antimicrobial activity of *Nostoc* sp. (DGN2) *and Revularia* sp. (NDR5). by using the Agar disc diffusion method against pathogenic bacteria and fungi and we found that the methanol *Nostoc* sp. (DGN2), extract was the most active one. Its inhibition activity was ranged from 29.00±0.816 against *Bacillus subtilis* to 25.00±0.816 against *E. coli*, it succeeded on the inhibition of growth Gram +ve and Gram –ve bacteria as Gram – ve bacteria. Also, they recorded the antifungal activity of

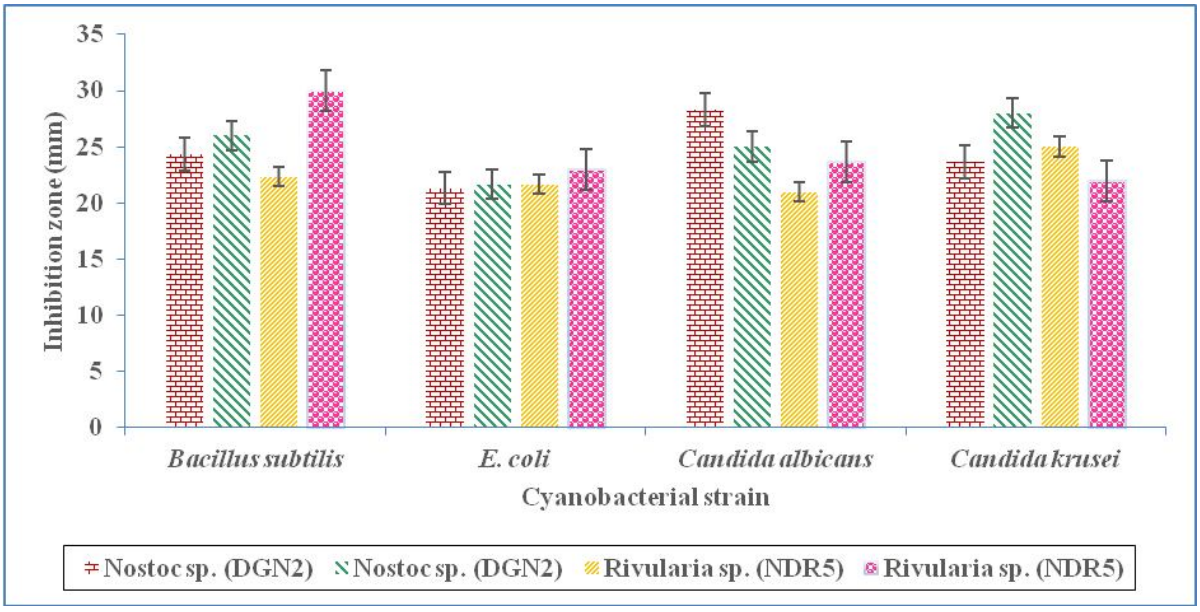

Fig. 2: Antibacterial and antifungal activities of the cyanobacterial extracts.

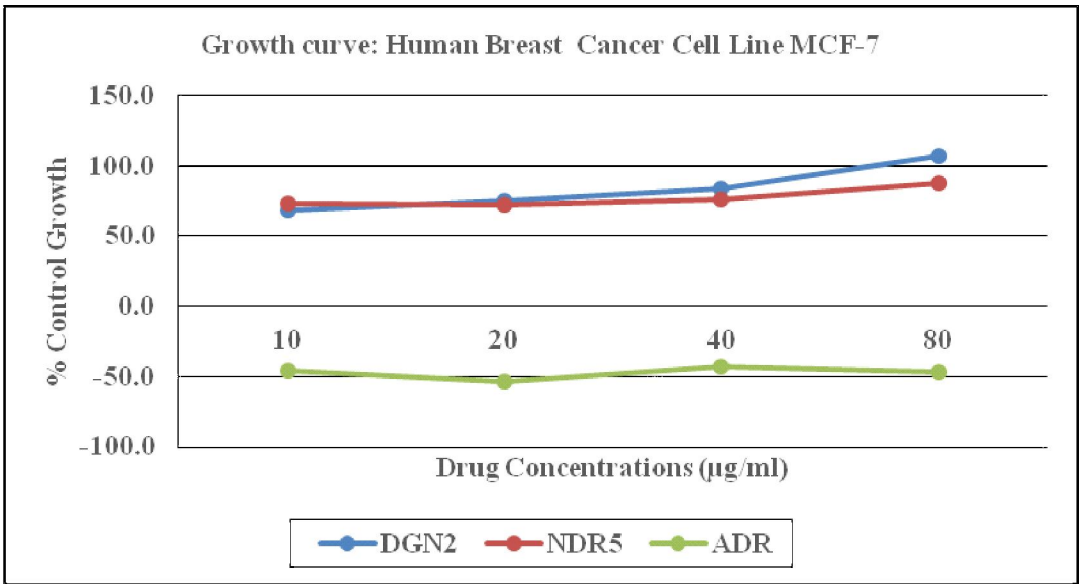

Fig. 3: Growth Curve: Human Breast Cancer Line MCF-7 of cyanobacterial strais methanol extracts of *Nostoc* sp. (DGN2) *and Revularia* sp. (NDR5) and ADR (Adriamycin).

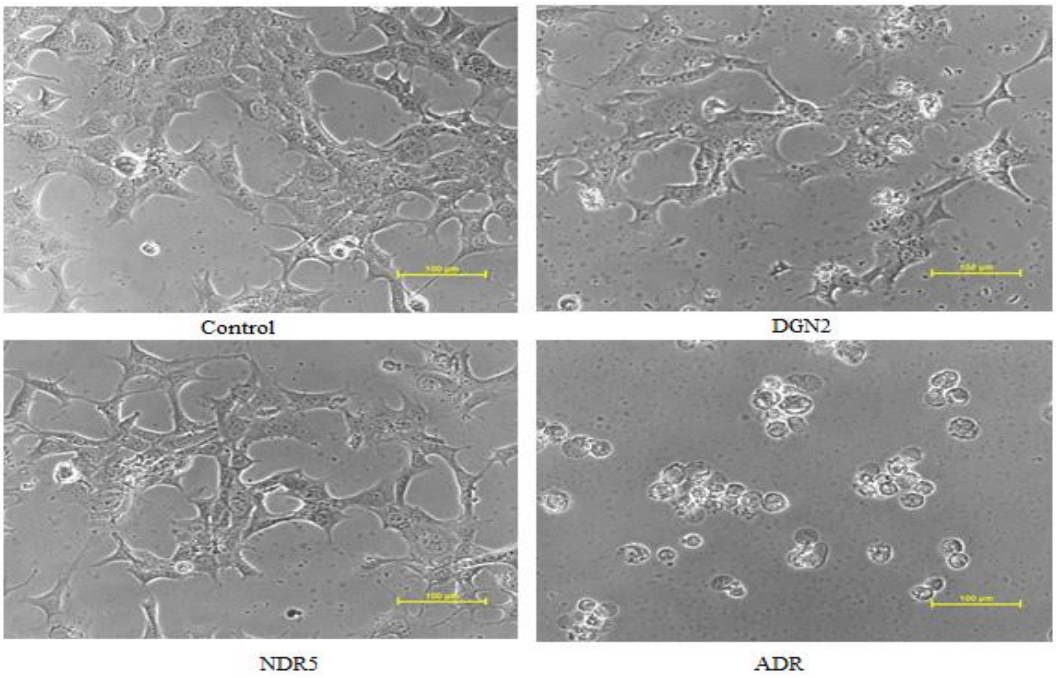

Fig. 4: Image of Human Breast Cancer Cell Line MCF7.

Nostoc sp. (DGN2) *and Revularia* sp. (NDR5) extract against *Candida krusei*. The extracts did not produce significant effect on the human breast cancer MCF7 and human colon cancer HT- 29 cell lines used in these studies as depicted in (Tables 2- 5 and Figs. 2-6). Cyanobacteria can be screened for their potential with various extraction techniques.

Conclusions

It can be concluded that the analysis of bioactive compounds from blue green algae and its importance of extraordinary performance against human pathogens. The diazotrophic cyanobacteria are important components of the ecosystem and their distribution may indicate the health of environment and contributing to the society. Future research also needs to be led to bio-prospecting 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 habitat.

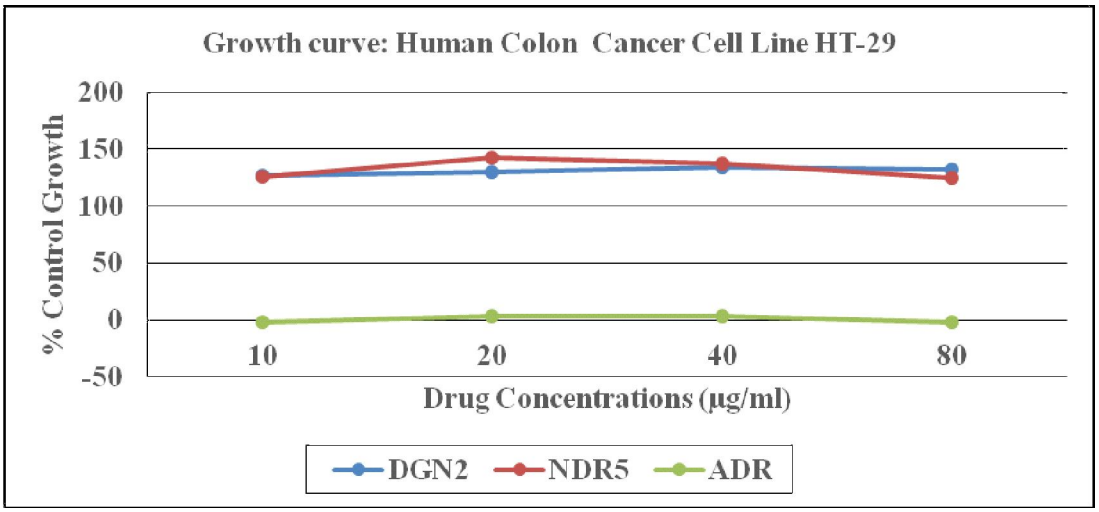

Fig. 5: Growth Curve: Human Colon Cancer Line HT – 29 of cyanobacterial strais methanolic extracts of *Nostoc* sp. (DGN2) *and Revularia* sp. (NDR5) and ADR (Adriamycin).

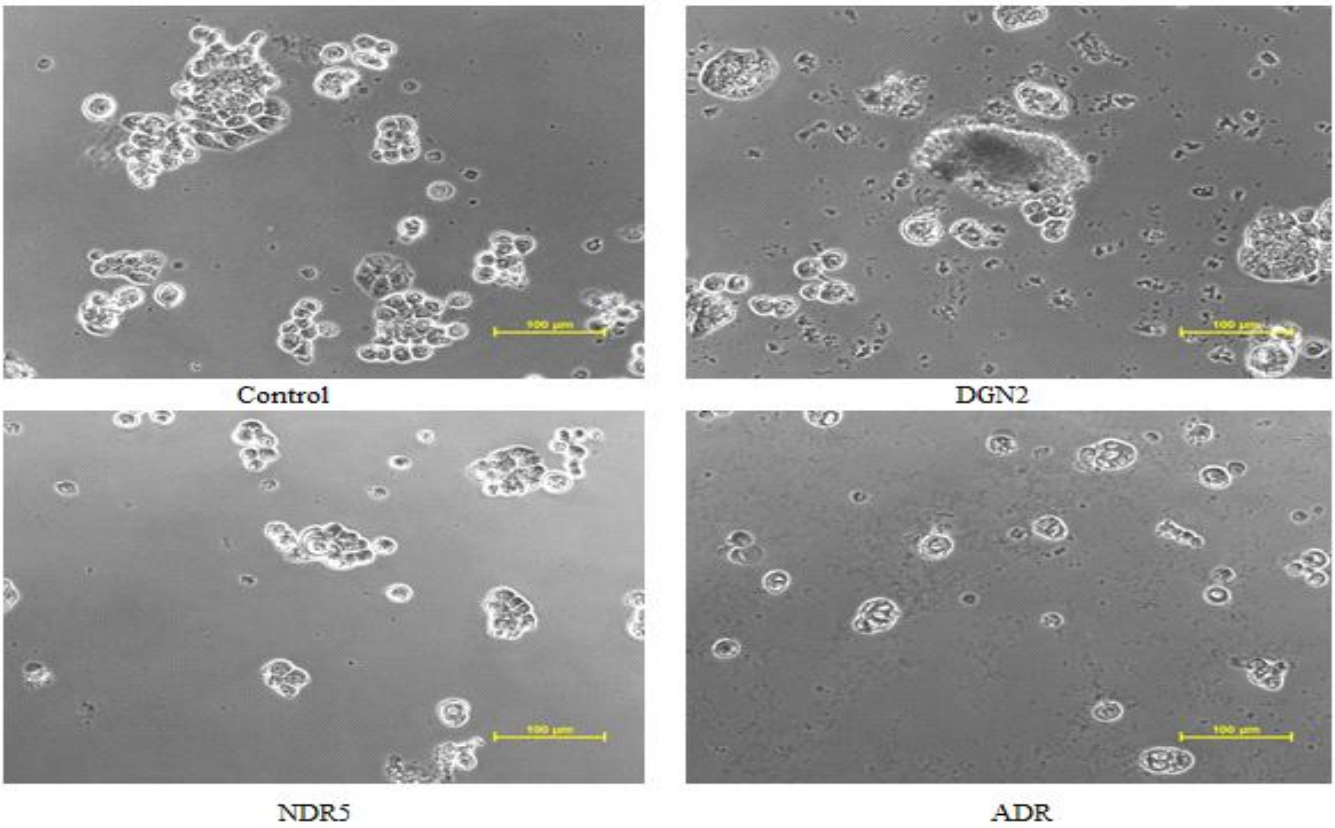

Fig. 6: Images of Human colon Cancer Cell Line HT - 29.

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Conflict of interest

The authors hereby declare that there is no conflict of interest.

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