3. MATERIALS AND METHODS

3.1. Description of sampling sites

The inky, blue waters of the Bay of Bengal and the sea around Andaman and Nicobar Islands, once dreaded as ‘Kalapani’ (the dark waters) and the overwhelming grandeur of cast, cloud-ridden skies make these islands, a domain of unsullied nature, within their perimeters they present panoramic views, sheltered fairy nooks, sun-kissed seascapes and unmatched scenic beauty embracing lush-green tropical forests, serpentine creeks and backwater channels, radiant valleys, enthralling hill slopes, co-existing with coastal rides and beaches making this area quite in comparable with any other part of India. Very appropriately it is called the’ Land of the yellow sun’.

The present study was carried out in the marine environment of South Andaman Island starting from Chidyatapu, the southernmost tip of the south Andaman (Lat 11° 27’ N and Long 92° 44’E) and few small islands nearby South Andaman such as Ross, Red skin and North Bay island.(Plate 1 & 2).

Plate 1. Map showing the sampling stations
3.2. Analysis of water samples

3.2.1. Physico – chemical analysis

Physico – chemical parameters of the water samples such as pH, temperature, Electrical conductivity, dissolved oxygen, carbonate, bicarbonate, chloride, calcium, total dissolved soil, salinity (ppm), zinc, copper, iron, manganese, boron, molybdenum, chromium, magnesium, total phosphorous, inorganic phosphorous, total nickel, total cadmium, total cobalt, total mercury, total arsenic, total cyanide, total lead, selenium, total silver, nitrate, nitrite, ammonia, inorganic sulphide, sulphate, calcium, magnesium (mg/l) were analysed using the standard methods (Strickland and Parsons, 1972; Wetzel and Likens, 1979).

3.2.2. Correlation between the physico-chemical and biological parameters with distribution of cyanobacteria

Spearman rank test was used for the correlation analysis to compare the distribution of cyanobacterial population with respect to its physico-chemical parameters and the study areas (Biodiversity PRO software). The findings were considered as significant if p value was < 0.005 by using ANOVA correlations method.
3.3. Isolation of cyanobacteria (Rippka, 1988)

The identifi cyanobacterial cultures were isolated by using spread plate method in ASN III medium. A portion of cyanobacterial mat was taken in an eppendorf tube and crushed with the help of sterile micropestle, and 0.1 ml of this suspension was spread over the plate. It containing ASN III medium. Then the plates were incubated at 2000 – 3000 lux light intensity for 10 – 15 days.

Composition of ASN – III Medium (g l⁻¹)

<table>
<thead>
<tr>
<th>Chemicals</th>
<th>Grams/Litre</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>25</td>
</tr>
<tr>
<td>MgCl₂.6H₂O</td>
<td>2</td>
</tr>
<tr>
<td>KCl</td>
<td>0.5</td>
</tr>
<tr>
<td>NaNO₃</td>
<td>0.75</td>
</tr>
<tr>
<td>K₂HPO₄.3H₂O</td>
<td>0.02</td>
</tr>
<tr>
<td>MgSO₄ 7H₂O</td>
<td>3.5</td>
</tr>
<tr>
<td>CaCl₂.2H₂O</td>
<td>0.5</td>
</tr>
<tr>
<td>Citric acid</td>
<td>0.003</td>
</tr>
<tr>
<td>Ferric ammonium citrate</td>
<td>0.003</td>
</tr>
<tr>
<td>EDTA (disodium salt)</td>
<td>0.00055</td>
</tr>
<tr>
<td>Na₂CO₃</td>
<td>0.02</td>
</tr>
<tr>
<td>Trace metal mix</td>
<td>1 ml</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
<tr>
<td>pH</td>
<td>7.5</td>
</tr>
</tbody>
</table>

Trace metal mix

- H₃BO₃ - 2.86
- MnCl₂.4H₂O - 1.81
- ZnSO₄̃ 7H₂O - 0.222
- Na₂MoO₄.2H₂O - 0.39
- CuSO₄.5H₂O - 0.079
- Co (No₃)₂.6H₂O - 0.0494
3.4. Identification of cyanobacteria

Small pieces of microbial colonies of sea water cyanobacterial isolates were picked out from the ASN – III medium. The semi permanent slides were prepared and observed under the Nikon microscope at oil immersion (400X and 1000X) objective. Different cyanobacterial morphotypes such as, nature of filament, shape, size of vegetative cells, akinetes and heterocysts were identified and photographs were taken using Nikon Digital Microscopic Camera (Japan).

3.5. Purification methods

It is often possible to obtain axenic cultures from field material by performing isolation and the subsequent purification stress on plates as described sometimes, however this is not the case particularly if the natural sample is highly contaminated by bacteria and the cyanobacterial species are non motile and incapable of self purification by gliding away from their contaminations or of the single filaments micro colonies do not grow on solid media purification of such cyanobacteria should be attempted after the establishment of cyanobacterial isolates in liquid cultures.

3.6. Repeated liquid subculture

This technique has been successfully used when a natural collection particularly rich in a specific cyanobacterium. Marine liquid medium was prepared and distributed it to sterilized flask containing ASN III medium and made frequent subcultures to the fresh medium to get rid of the other contaminating bacteria.

Based on the predominant occurrence and ease cultivability eight cyanobacterial isolates (Phormidium angustissimum (SGBRA 01), Phormidium sp. (SGBRA 02), Phormidium tenue (SGBRA 03), Oscillatoria subbrevis (SGBRA 04), Oscillatoria sp. (SGBRA 05), Oscillatoria amphibia (SGBRA 06), O. chlorina (SGBRA 07) and Microcoleus sp. (SGBRA 08)) were selected for further studies in view to exploit them for commercial utilization.
3.7. Extract preparation

The mid log phase cyanobacterial cells were separated and rinsed with sterile water to remove any associated debris. According to the methods of Khan et al., (1988) and Vlachos et al., (1996) pyredine, n – butanol, ethyl acetate, and aqueous were used to extract cyanobacterial metabolites and were preserved at ±4°C. The selected pathogenic cultures were represented in Table 1.

Table 1. Strains used in the study

<table>
<thead>
<tr>
<th>Pathogenic strains</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bacterial strains (Gram positive and Gram negative bacteria)</td>
<td></td>
</tr>
<tr>
<td>Bacillus cereus</td>
<td>MTCC 6909</td>
</tr>
<tr>
<td>Enterobacter sp.</td>
<td>Personal isolate</td>
</tr>
<tr>
<td>Enterococcus faecalis</td>
<td>ATCC – BAA 2128</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>ATCC – BAA 1720</td>
</tr>
<tr>
<td>Salmonella typhi</td>
<td>Personal isolate</td>
</tr>
<tr>
<td>Fungal strains</td>
<td></td>
</tr>
<tr>
<td>Aspergillus flavus</td>
<td>MTCC 6323</td>
</tr>
<tr>
<td>A. koeningii</td>
<td>Personal isolate</td>
</tr>
<tr>
<td>A. quercinus</td>
<td>Personal isolate</td>
</tr>
<tr>
<td>A. oryzae</td>
<td>Personal isolate</td>
</tr>
<tr>
<td>A. wentii</td>
<td>Personal isolate</td>
</tr>
<tr>
<td>Candida albicans</td>
<td>MTCC 6954</td>
</tr>
<tr>
<td>Helminthosporium sp.</td>
<td>Personal isolate</td>
</tr>
<tr>
<td>Humicola sp.</td>
<td>Personal isolate</td>
</tr>
<tr>
<td>Verticillium sp.</td>
<td>Personal isolate</td>
</tr>
<tr>
<td>Curvularia senegalensis</td>
<td>Personal isolate</td>
</tr>
<tr>
<td>Vibrio cultures</td>
<td>Personal isolate</td>
</tr>
<tr>
<td>Vibrio alginolyticus</td>
<td>Personal isolate</td>
</tr>
<tr>
<td>V. anguillarium</td>
<td>Personal isolate</td>
</tr>
<tr>
<td>V. cholerae</td>
<td>Personal isolate</td>
</tr>
<tr>
<td>V. fluvialis</td>
<td>Personal isolate</td>
</tr>
<tr>
<td>V. mimicus</td>
<td>Personal isolate</td>
</tr>
</tbody>
</table>
3.8. Screening of antibacterial activity

Antibacterial activity was assessed with the radial diffusion assay as described by In Hae Kim et al., (2007). Briefly, an overnight cultures (18 h) of bacteria was grown at 37°C in tripticase soy broth with 1% NaCl and then washed three times by centrifugation at 590 X g for 10 min at 4°C, followed by suspension with cold phosphate–buffered saline (pH 7.0). The bacterial suspension was adjusted to an optical density (OD₅₇₀) of 0.1 (10⁸ CFU/mL). One mL of bacterial suspension was mixed with autoclaved one hundred ml of LB broth containing 1.5% low EEO agarose, 0.5% NaCl, 200 mM phosphate buffer (pH 6.7), and 100mg/mL streptomycin sulphate at 48°C, and poured into sterile Petri dishes. For the radial diffusion assays, 50 µl (5 mg dry weight/mL) of selected eight cyanobacterial crude extracts were transferred into 5-mm diameter wells. Streptomycin sulphate was used as a positive control and solvents were used as a negative control. The plates were incubated at 37°C for 18 h. After incubation, the clearing zone diameters were observed.

3.9. Screening of antifungal activity

Petri dishes with 10 ml of potato dextrose agar were prepared, and mixed with 0.1 ml of 48 h broth cultures of test fungi cultures. The wells were made and filled with 100 µl of cyanobacterial extracts. The inoculated plates were incubated for 48 h at 37°C. After incubation, the diameter of the inhibition zone was measured (Attaie et al., 1987). For the agar well diffusion assays, 50 µl (5 mg dry weight/mL) of algal crude extracts were transferred into 5-mm diameter wells. Kanamycin was used as a positive control and organic solvents were used as a negative control. The plate was incubated at 37°C for 48 h. After incubation the results were observed.

3.10. Screening of antivibrio activity

The sterile petri dishes with 10 ml nutrient agar (pH – 8.5) were prepared, and mixed with 0.1 ml of 18 h broth cultures of Vibrio. The wells were made and filled with 100 µl of nature strains of cyanobacterial extracts. The inoculated plates were incubated for 48 h at 37°C. After incubation, the diameter of the inhibition zone was measured (Attaie et al., 1987). For the agar well diffusion assays, 50 µl (5 mg dry
weight/mL) of algal crude extracts were transferred into 5-mm diameter wells. Ciprofloxacin was used as a positive control and organic solvents were used as a negative control. The plates were incubated at 37°C for 24 h. After incubation the results were observed.

3.11. Thin layer chromatography

TLC plate is a sheet of glass metal or plastic which is coated within a thin layer of solid adsorbent namely silica gel (Volk, 2005). Silica gel was prepared by mixing 0.4g of 0.1N NaOH in 100 ml of distilled water with equal proportions of silica (adsorbent).

A small amount of the methanol extract and ethyl acetate extracts was analyzed and spotted near the bottom of the TLC plate. The TLC plate is then placed in a shallow pool of solvent having a composition of chloroform, methanol, and liquor ammonia in the ratio of 8:2:0.5. The liquid or elute was the mobile phase and it slowly raised upto the TLC plate by capillary action. When the solvent was reached to the top of the plate, the plate was removed from the developing chamber, dried and the separated components of the mixture were visualized under iodine vapour. On visualization, a dark brownish oval spot was observed on the middle of the plate. The compound spotted on the TLC plate was then scrapped along with silica gel further purification process.

3.11.1. Extracts preparation

Alkaloids

One gram of the cyanobacterial samples were extracted with 40% calcium hydroxide solution until the extract was distinctly alkaline to litmus paper and then extract twice with 10ml portions of chloroform then these extracts were used in thin layer plates (Adeloye et al., 2007).

Saponins

About one gram of cyanobacterial samples were extracted with 10ml of 70% ethanol by refluxing for 10 min. Then these extract was filtered by using Whatmann
No.1 filter paper. The filtrate was condensed, enriched with saturated n-butanol, and thoroughly mixed. The butanol was retained, condensed and used for thin layer chromatography (Wagner and Bladt, 1996).

**Phenols**

One gram of cyanobacterial samples were lixiviated in methanol on rotary shaker (180 thaws /min) for 24 h. Then these extract was filtered by using Whatmann No.1 filter paper. The condensed filtrate was used for thin layer chromatography (Harborne, 1998).

**Sterols**

About one gram of selected cyanobacterial samples were extracted with 10ml of methanol in boiling water bath. The condensed filtrate was used for thin layer chromatography (Wagner and Bladt, 1996).

**Flavonoids**

One gram of cyanobacterial samples were extracted with 10ml of ethanol. Then the extract was heated for few minutes and 100µl of extract was applied on the silica gel plates (Ciulei, 1982).

**Amino acid (Sadhasivam and Manickam, 2005)**

Known quantity of sample material (dry / wet) was ground in a mortar and pestle with tenfold volume of 70% ethanol. Shaken the contents were at 55°C for 30 min; centrifuged at 1000 rpm for 10 min and the supernatant was collected and the pellet was at 55°C twice. The supernatants were with equal volume of petroleum ether (40 – 60°C) and shaken vigorously. The petroleum ether layer containing chlorophyll a was discarded. Alcohol fraction was evaporated to dryness under vacuum using either water – pump or rotary evaporators at 40 – 45°C. The residue was dissolved in known volume of absolute ethanol or water.
3.11.2. Sample application

Drew a line lightly with a pencil about 1.5 – 2.0 cm from the bottom. If the thin layer was too soft to draw a pencil line, placed a scale at the bottom and spotted at a distance of 1.5cm and note down the order. The samples were spotted using capillary tubes at 1.5cm distance between them. For preparative TLC, the sample was applied as a band across the layer rather than as a spot.

3.11.3. Solvent preparation

**Alkaloids**

The alkaloids were separated by using n-hexane, ethyl acetate, (3:1) and chloroform, ethyl acetate (9:1) solvent mixture (Adeloye, *et al.*, 2007) or chloroform and methanol (1:2) solvent mixture (Wagner and Bladt, 1996). The presence of alkaloids in the developed chromatograms was detected by spraying the freshly prepared Dragendorff’s reagent. A positive reaction on the chromatogram (indicated by orange spot against a pale yellow background) was confirmatory evidence that the plant extraction contain an alkaloid.

**Saponins**

The saponins were separated by using chloroform, glacial acetic acid, methanol and water in the ratio of 64:34:12:8 solvent mixtures (Wagner and Bladt, 1996). The presence of saponins in the developed chromatograms was detected by iodine vapours. A positive reaction was formation of yellow colour spot.

**Phenols**

The phenols were separated by using chloroform and methanol (27:03) solvent mixture (Harborne, 1998). The presence of phenols in the developed chromatograms was detected by spraying the folin-ciocalteu’s reagent. After at the plates were heated at 80°C for 10 min. A positive reaction was appeared in the formation of blue colour spot (Harborne, 1998).
Sterols

The sterols were separated by using chloroform: glacial acetic acid, methanol, water (64:34:12:8) solvent mixture (Wagner and Bladt, 1996). The presence of sterols in the developed chromatograms was detected by spraying the anisaldehyde sulphuric acid reagent. Then the plates were heated at 100°C for 6 mins. A positive reaction was formation of black colour spot (Wagner and Bladt, 1996).

Flavonoids

The flavonoids were separated by using n-butanol, glacial acetic acid, water (4:1:5; V/V/V) mixture (Ciulei, 1982). The presence of flavonoids was detected by the formation of color in the plate. A positive reaction was formation of yellow colour spot.

Amino acid

The amino acids were separated by using ethanol, water (70/30) or butanol, acetic acid, water (80/20/20) solvent mixture. The plates were sprayed with 0.1 % ninhydrin in acetone and heated the plates for 5 min. at 100 – 110° C. After incubation the formation of pink or purple colour spot was considered as a positive result (Sadhasivam and Manickam, 2005).

3.11.4. Plate development

The chromatographic tank was filled with developing solvent to depth of 1.5cm and equilibrated for about 5 h. The thin layer plate was placed gently in the tank and allowed to stand for about 60 min. Make sure the spots do not touch the solvent directly; capillary action caused the solvent to ascend as in paper chromatography and the separation of compounds takes place. As the solvent front reaches about 1.2cm from the top of the plate, the plate was removed, solvent front was marked with a pencil immediately and allowed to air dry by placing the plate upside down.

Retention factor value (Rf) is a variable used to characterize the portion of a substance in a chromatogram. It is an index of the degree of retention of a compound.
It is defined as the distance between the start line and the solvent front line divided by the distance between start and solute front line. Thus, the Rf of solute (a component of sample mixture) is ratio of distance migrated by solvent (eluent) to the distance migrated by the solute, in a chromatogram.

\[
R_f = \frac{\text{Distance migrated by solute}}{\text{Distance migrated by solvent}}
\]

3.11.5. Purification

In purification process, the scrapped portion of the compound was then transferred to a glass bottle with stopper and shaken vigorously for 15 min. in solvent system chloroform and methanol in the ratio of 1:1 was added. It was left undisturbed until the silica gel was settled at the bottom of the glass bottom. By using Whatmann No.1 filter paper the compound was recorded from silica gel. The compound was further studied by using spectral analysis.

3.12. Fourier Transform - Infrared spectroscopy (FT - IR)

To obtain IR spectra, the purified red pigment was dissolved in chloroform and 1-2 µl of sample was mixed with KBr and pellet was formed. Pellet was applied to the sample injector and inserted to the FT-IR Spectrophotometer (Perkin Elmer) and the data was recorded.

3.13. \( ^1H \)- Nuclear Magnetic Resonance (\(^1H\) - NMR) (Volk, 2005)

Thirty five milligrams of purified phytocompounds were dissolved in 500µl of CDCl\(_3\). An NMR spectrum was recorded on a Bruker Advance 300 Spectrophotometry (300 mHz), using chloroform solvent peak as a reference.


The GC – MS analysis was carried out using a Clarus 500 Perkin – elmer (Auto system XL) Gas Chromatograph equipped and coupled to a mass detector Turbo mas gold – Perkin Elmer Turbomass 5.1 spectrometer with an Elite – 1 (100%
Dimethyl poly siloxane), 30m x 0.25 mm ID x 1µm of capillary column. The instrument was set to an initial temperature of 110°C, and maintained at this temperature for 2 min. At the end of this period the oven temperature was rose up to 280°C, at the rate of an increase of 5°C/min, and maintained for 9 min. Injection port temperature was ensured as 250°C and Helium flow rate as one ml/min. The ionization voltage was 70eV. The samples were injected in split mode as 10:1. Mass spectral scan range was set at 45-450 (m/z).

Using computer searches on a NIST Ver.2.1 MS data library and comparing the spectrum obtained through GC – MS compounds present in the crude sample were identified.

3.15. Screening of antioxidant activity

Measurement of DPPH (2, 2-Diphenyl-1picrylhydrazyl) radical scavenging activity (RSA)

The scavenging activity of the algal extracts on the DPPH (radical was determined by a photometry assay based on procedure described by Kuda et al., 2005a. The algal extract solution (0.2 ml) was mixed with equal volume of ethanol 0.001 M DPPH (Fluka)/methanol solution (0.025ml) was added to the solution and absorbance at 550 nm was measured after 30 min. The RSA was calculated as a percentage of DPPH discoloration using the equation

$$\text{RSA} \% = 1 - \frac{A_T}{A_O} \times 100$$

Where $A_T$ is the absorbance of the test solution at 30 min. time and $A_O$ is the absorbance at zero time (initial absorbance).

3.15.1. Statistical analysis

Analytical values represent means of three independent experiments each with duplicate measurements. The AOA results were compared by one - way analysis of variance (one – way ANOVA) to test for significant differences (Lapin, 1997). Paired
t test when needed, was used to assess significant difference between AOA of WE and EE in terms of DPPH scavenging activity (Vining, 1998). Difference among (or between) sample means were reported to be significant when P < 0.05.

3.15.2. Determination of total phenolic content

Total phenolic content was determined according to the method of Taga et al., (1984) and Yuan et al., (2005a) briefly, 0.2 ml of the sample solution was mixed with 1.0 ml of 10% Folin – Ciocalteu solution and 0.8 ml of 7.5% sodium carbonate solution. The mixture was incubated for 1 h at room temperature. The absorbance at 760 nm was measured and converted to phenolic contents according to the calibration curve of gallic acid.

3.16. Protein profile (SDS – PAGE) (Maniatis et al., 1990)

3.16.1. Preparation of separating gel solution

The separation gel mixture was prepared by mixing 30% acrylamide, 0.8% bisacrylamide and 4X tris HCl in distilled water and the pH was adjusted to 8.8. The gel mixture was poured in side – arm flask and degassed in vacuum for 10 to 15 min. into which 10% ammonium persulphate and tetraethylene methylenediamide (TEMED) was added and mixed gently.

3.16.2. Preparation of stacking gel solution

The separating gel mixture was prepared by mixing of 30% acrylamide, 0.8 % bisacrylamide and 4X tris HCl in distilled water and the pH was adjusted to 6.8. The gel mixture was poured in side-arm flask and degassed in vacuum for 10 to 15 min. And 10% ammonium per sulphate and tetraethylene methylenediamide (TEMED) was added and mixed gently.

3.16.3. Gel matrix preparation

The glass plates were assembled for casting a slab gel, into which the separating gel mixture was poured between the plates upto the mark without air bubbles. The separating gel mixture was carefully overlayed with butanol saturated with water or separating gel overlay solution and the gel was left undisturbed for
polymerization for about 15 – 20 min. After polymerization, poured off the overlay solution and the separating gel was washed for several times with distilled water and drained. The appropriate comb was inserted and the stacking gel mixture was poured without air bubbles and allowed for polymerization for about 15 – 30 min. After polymerization, the comb was removed without distorting the shapes of the wells and wells were washed with distilled water to remove unpolymerized acrylamide by flushing with a syringe or a pipette and placed in a appropriate vertical gel electrophoresis system with running buffer at the bottom reservoir.

3.16.4. Sample preparation

Equal volumes of protein sample (5µl) with the 2X sample buffer was mixed and kept in a boiling water bath for 90 seconds and the sample was placed in ice till loading. Approximately 10 µl of sample standard marker protein was loaded to each well using a micropipette. Equal volume of 1X sample buffer was also added to any empty well to prevent spreading of adjoining lanes. The top chamber was slowly filled up with the tank buffer and the wells were also filled with buffer gently using a syringe.

3.16.5. Gel running

The tank was connected to the power supply and approximately 10 – 15 mA current was applied and run continuously (90 min) till the marker dye (bromothymol blue) reached the bottom of the separating gel.

3.16.6. Staining proteins in gels

The location of protein in a gel can be determined by Coomassie blue staining. After the marker dye has reached the bottom of the gel, the gel plates were removed and the gel was placed in Coomassie staining solution for 3- 4h. Followed by this, the gel was transferred to destaining solution, shacked intermittently and changed the destain solution several times until a clean background was obtained. The protein bands were examined in a white light transilluminator.

3.17.1. Principle

MTT, (3-(4, 5-Dimethyl thiazol-2yl)-2, 5-diphenyl tetrazolium bromide, is cleaved by mitochondrial dehydrogenase of viable cells, yielding a measurable purple formazan product. This formazan production is proportionate to the viable cell number and inversely proportional to the degree of cytotoxicity.

3.17.2. Requirements

- Dimethyl sulphoxide was obtained from Sigma Aldrich Chemicals, USA.
- MTT stock solution (5mg/ml)
  
  It was prepared as follows:
  
  MTT - 50 mg
  PBS - 10 ml

  After vortexing for 20 min it was filtered through 0.45µ filter. The bottle was wrapped with aluminium foil or paper to block light, as MTT is light-sensitive. The preparation was stored at 4°C.

3.17.3. Analysis

Cytotoxicity was determined by means of a colorimetric micro culture assay (MTT assay, MTT=3-(4,5-dimethyl − 2 thiazolyl) − 2, 5 − diphenyl − 2H tetrazolium bromide). For this purpose HBL − 100 (Breast cancer cell line) were harvested from cultures flasks by trypsinization and seeded in 200µl aliquots in RPMI supplemented with 15% heat an activated fetal bovine serum, 1mM sodium pyruvate, 4mM L − glutamine and 1% non essential amino acids (100%) (all purchased from sigma − Aldrich) in 96 well micro culture plates in all derivatives of 5 X 10^3 cells per well, respectively, in order to ensure exponential growth of untreated controls throughout drug exposure. For 24 hr cells were allowed to settle and resume exponential growth, followed by the addition of dilution of the test compounds in aliquots if 200µl well⁻¹ in the same medium. Only for the less water soluble compounds (alkaloids, amino acids, sterol and phenol), stock solution had to the
prepared in (DMSO) dimethyl sulfoxide. The DMSO content in the actually tested dilution did not exceed 0.5%. After continuous exposure for 24 to 48 hrs drug solution were replaced by a 130μl well – 1 RPMI 1640 culture medium (supplemented with 15% heat inactivated fetal bovine serum and 4mML – glutamine plus 20μl well – 1 MTT solution in phosphate – buffered saline (5mg mL⁻¹). After incubation for 4h, the medium / MTT mixture were removed, and the formazen crystals formed by viable cells were dissolved in 100μl of DMSO per well. Optional derivatives at 550nm were measured with a micro plate reader (Bio – Red), using a reference wavelength of 690nm to corrected for unspecific adsorption. The quantity of vital was expressed in terms of (Test/Control) T/C values by comparison to untreated control micro cultures, and 50% inhibitory concentration (ID 50) were calculated from concentration effect curves by interpolation. Evaluation is bared on means format least three independent experiments, each comprising three replicates per concentration level.

3.17.4. Cell culture and drug treatment

HBL – 100 cell line was maintained in RPMI 1640 medium supplemented with 0.2% penicillin, streptomycin and 15% fetal bovine serum and grown at 37ºC in a 5% CO₂ humified environment. Cells were plated in 6 well tissue culture plates at a cell density of 2.5 X 10⁵ cells per well. The medium was replaced with medium containing compounds after 24h and inoculated for 24 and 48 hrs. The cells were treated with compounds with indicated times (easy assay) and the cells were harvested and extent of apoptosis was assessed by light micropil observation, ethidium bromide fluorescent staining.

Analysis

A graph of absorbance (Y- axis) against the concentration of the drug (X-axis) was plotted and the IC₅₀ concentration was determined as the dye concentration that was required to reduce the absorbance to half that of control. The data was then converted to percentage inhibition curve, to normalize a series of curves.
3.17.5. Assessment of cell morphology

Preparation of Acridine orange & Ethidium bromide staining

One part of 100µg/ml acridine orange in PBS and one part of 100µg/ml ethidium bromide in PBS.

Working solution

Five µl of the stock solution was taken and the volume was made up to 500µl using distilled water.

Method

Cells were grown in 6 well micro titre plates (5x10³ cells/well) for 24 hr. The cells were then incubated with the IC50 dose of all compounds for 24 h. The medium was discarded and the cells were washed with PBS. The cells were then trypsinized and placed on a glass slide and stained with acridine orange and ethidium bromide stain. The cells were then viewed in an epifluorescent microscope (Olympus, Japan).

3.18. Biosynthesis of silver nanoparticles (Govindaraju, et al., 2008)

The selected marine cyanobacterial samples were collected from our laboratory. Before experimentation, the biomass was washed thrice in deionized water to remove the unwanted materials, for the synthesis of silver nanoparticles, silver nitrate (AgNO₃) (qualigens) was used as received. Double - distilled deionized water was used for all the experiments. Silver and biometallic nanoparticles formations were carried out by taking 500mg of dry blue green algae in a 250ml Erlenmeyer flask with 10⁻³ M aqueous AgNO₃, with different molar concentrations and incubated at room temperature. The pH was checked during the course of reaction and it was found to be 5.6. The bioreduction of pure AgNO₃, was monitored using UV – Vis spectroscopy at regular intervals. UV-Vis spectra was recorded as a function of time of reaction on a UV-Vis 1601 Schimadzu spectrophotometer operated at a resolution of 1nm. The silver nanoparticles synthesized using selected cyanobacteria were subjected to Fourier transform infrared (FT-IR) spectrum analysis to identify (if possible) whether the biomolecules are stabilizing and reducing agents. The complete reduction of Ag⁺ ions by blue green algae was monitored using UV-Vis spectrum. The metal
nanoparticles were centrifuged at 6000rpm for 15 min. separately to isolate the silver nanoparticles from free proteins. The silver nanoparticle pellets obtained after centrifugation were redispersed in water prior to FT-IR analysis. For FT-IR data spectroscopy measurements were done on a Thermo Nicolet Arator 300 instrument in the diffuse reflectance mode at a resolution of 4cm⁻¹ in KBr pellets. Samples for high-resolution scanning electron microscopy (HR-SEM) analysis were prepared by drop-coating Ag, nanoparticle solutions on a carbon-coated copper SEM grids. The films on the SEM grids were allowed to stand for 2 min. following which, the extra solutions were removed using a floating paper and the grid was allowed to dry prior to measurement. HR-SEM analysis was performed using a JEOL 3010 instrument operated at an accelerating voltage of 120 kV.

3.19. Isolation and extraction of chromosomal DNA

Isolation of cyanobacteria were grown up to the late exponential phase in ASN II medium at 28 ± 2°C, washed twice with Tris EDTA buffer or 10.3% sucrose prior to DNA preparation. Chromosomal DNA was isolated by resuspending 0.5 – 1.0g of cells with 5ml lysis buffer (25mM Tris; 25mM EDTA; pH 8.0; 10 – 15 µg lysozyme; 50µg/ml RNase), and incubated for 20 – 80 min. at 37°C, followed by the addition of 500 µl of 5 M NaCl solutions. The suspension was agitated on a vortex mixer until the cell suspension became translucent. Cells were lysed by the addition of 1.2 ml of 10% SDS. The lysate were incubated for 15 -30 min at 65°C. After addition of 2.4 ml of 5 M potassium acetate, the solution was mixed and left on ice box for 20 min. The precipitate was removed by centrifugation for 30 min. at 6000 rpm and the volume of the supernatant was adjusted to 8 ml. The DNA was recovered by precipitation with isopropanol. The precipitate was dissolved in 700µl/g 50mM Tris/10mM EDTA (pH 8.0). Any insoluble substances were spun off and the aqueous phase was transferred to a 1.5ml microfuge tube. Subsequently, 75µl 3M sodium acetate and 500µl isopropanol were added and the solution was centrifuged for 30 seconds to 2 min. The precipitate was washed with cold 70% ethanol, dried and dissolved in 100µl TE (10mM tris/1mM EDTA, pH 8.0).
3.20. PCR amplification of 16S rDNA (Weisburg et al., 1991)

A mixture of sterile distilled deionized water 40 µl upstream primer (100 pmols) 10µl, (8F:5’ GGGG AATYTTCC GCAATGG 3’) (1492R:5’ GACTAC WGGGGTATCTAAT CC WTT 3’) downstream primer (100 pmols), 10µl, 10x PCR buffer 10µl, 25mM MgCl2 8µl, dNTP mix 6µl, cyanobacteria template DNA (50ng), 5µl and Taq DNA polymerase (30/µl), 2µl in a 0.5ml micro centrifuge tube was taken. The total 100µl mixture in a tube was gently spun for 10 seconds and allowed to settle the contents. The samples were kept in Eppendorf PCR Thermal cycler. The amplification was carried out in the following manner of 35 cycles, denaturation for 60 seconds at 92°C primer annealing for 60 seconds at 72°C. Finally the tubes were ensured complete polymerization at 72°C for 15 min. 10µl of PCR products with 2µl of loading dye was mixed and loaded on a 1.2% agarose gel analysed electrophoretically at 60 volts for 45 minutes. The gel was observed on UV transillumination and compared with 1 kb DNA ladder.

3.21. 16s rDNA Sequencing (Weisburg et al., 1991)

The full length 16S rDNA fragment was amplified by PCR from the above isolated genomic DNA. A single discrete PCR amplicon band of 1.5 Kb was observed when resolved on agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reaction of PCR amplicon was carried out using BDT 3.1 cycle sequencing kit on ABI 3730xl genetic analyser. Consensus sequence of 1434bp of 16S rDNA gene was generated from forward and reverse sequence data using aligner software. The 16S rDNA sequence was used to carry out BLAST with the NR data base of NCBI genebank data base.

3.22. Nucleotide sequence accession


3.23. Phylogenetic tree

The reference sequences required for comparison were down loaded from the EMBL database using the site [http://www.ncbi.nlm.nih.gov/genebank](http://www.ncbi.nlm.nih.gov/genebank). All the
sequences were aligned using the multiple sequence alignment program CLUSTAL W development by (Higgins *et al.*, 1992). The evolutionary history was inferred using the Neighbour-joining method (Saitou and Nei, 1987). The bootstrap consensus tree from 500 replicates (Felsenstein, 1993) was taken to represent the evolutionary history of the taxa analysed. Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicates tree in which the associated taxa clustered together in the bootstrap test (500 replicates) is shown next to the branches. The evolutionary distances were computed using the Kimura2-parameter method (Kimura, 1980) and are in the units of the number of base substitutions per site. Codon position include were 1st + 2nd + 3rd + non coding. All positions containing gaps and missing data were eliminated from the dataset (complete deletion option). There were a total of 1372 positions in the final dataset. Phylogenetic analyses were conducted in MEGA 4. (Tamura, *et al.*, 2007).

**3.24. 16S rDNA secondary structure prediction of cyanobacterial species**

The secondary structure of cyanobacterial species NCBI were predicted using the Bioinformatics tools available in online [www.genebee.msu.su/services/rna2-reduced-html](http://www.genebee.msu.su/services/rna2-reduced-html).

**3.25. Restriction site analysis of 16S rDNA of cyanobacterial isolates**

The restriction sites in 16S rDNA of *Phormidium angustissimum* (SGBRA 01), *Phormidium* sp. (SGBRA 02), *Phormidium tenue* (SGBRA 03), *Oscillatoria subbrevis* (SGBRA 04), *Oscillatoria* sp. (SGBRA 05), *Oscillatoria amphibia* (SGBRA 06), *O. chlorina* (SGBRA 07) and *Microcoleus* sp. (SGBRA 08) were analysed using NEB cutter programme version 2.0 tools in online [www.neb.com/NEB Cutter 2/](http://www.neb.com/NEB Cutter 2/) index.php.