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ANTIBACTERIAL ACTIVITY OF MANGROVE-DERIVED MARINE CYANOBACTERIA

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ABSTRACT:

The present study investigated the antibacterial activity of resazurin method by mangrove-derived marine cyanobacteria. Three marine cyanobacteriaS*ynechococcuselangatus* ARKK1, *Gloeocapsa*sp.,ARKK2 and *Phormidium* sp., ARKK3were tested against four clinical pathogenic bacteria *viz,Staphylococcus aureus, Pseudomonas aeruginosa, Klebsiellapneumoniae*and *Proteus vulgaris.* At the beginning of the reaction, all the wells were in blue colour. After 24h of incubation, some of the wells turned into pink colour indicating bacterial growth. *Synechococcus* sp., and *Gloeocapsa* sp., were found to have higher antibacterial activity than *Phormidium* sp., as evident by the colour change. All the bacterial strains showed more sensitivity to both *Synechococcus* sp., and *Gloeocapsa*sp than *Phormidium* sp. The present study revealed that unicellular cyanobacteria such as *Synechococcus* sp., and *Gloeocapsa* sp., were found more potent in antibacterial activity than filamentous *Phormidium* sp. Therefore, it is suggested that the unicellular forms which area promising source of antibacterial can be attempted for developing antibacterial drugs.

Keywords: Mangroves/ Cyanobacteria/ *Synechococcus*sp.,/*Gloeocapsa*sp., Resazurin method.

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INTRODUCTION

Natural products have been isolated from a wide variety of taxa and tested for various biological activities. Among these taxa, cyanobacteria are regarded as potent candidates for drug discovery, especially in pharmaceuticals [7]. Cyanobacteria are known to have antibacterial, antiviral, antifungal, algicide and

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cytotoxic activities [11, 6, 3, 10, 15, 14]. Research on biologically active compounds produced by cyanobacteria has been focused mostly on freshwater species, but not on marine cyanobacterial species [12]. Such a study is important for several reasons: (i) Effective life span of any antibiotic is limited; and (ii)There is an increasing awareness about the problems with the over prescription and misuse of antibiotics. Hence, new sources for novel antibiotics especially marine sources are being tried.

Resazurin is a blue coloured redox dye, generally used as an indicator of chemical cytotoxicity in cultured cells [8]. The assay is based on the ability of viable, metabolically active cells to reduce resazurin to resorufin and finally to colourlessdihydroresorufin. Resorufinthat is formed as a result of resazurinbioreduction is measured colorimetrically. Resazurin is non-toxic to cells and stable in a culture medium, allowing continuous measurement of cell proliferation *in vitro* [2, 5]. In this method, the rate of dye reduction is directly proportional to the number of viable cells present and hence, the toxic effect of drugs on bacterial cells [9]. Therefore, as a direct measure of the metabolic competence of cell cultures, resazurin reduction may provide a convenient index of cell growth. The present study employed the resazurin dye reduction as an indicator of bacterial growth to study the selected cyanobacterial species for their antibacterial activity.

MATERIALS AND METHODS

Preparation of cyanobacterial solution

Three cyanobacterial strains isolated from mangrove environment were identified based on 16s rDNA as *Synechococcuselangatus*ARKK1,*Gloeocapsa* sp., ARKK2 and*Phormidium* sp., ARKK3.The former two strains are unicellular and the last one is filamentous. Six hundred and seventy milligrams of dried powder of each cyanobacterial species were added to 100ml of distilled water and macerated well with help of a pestle and mortar. It was then filtered through a two-layered muslin cloth and the extract was collected. The final volume of extracts yield was calculated for each cyanobacterial species

Preparation of Resazurin Solution

The resazurin solution was purchased from Sigma, India, and 270mg tablet was dissolved in 40 ml of sterile distilled water. A vortex mixer was used to ensure that it was a well homogenous solution.

Preparation of bacterial cultures

Four species of clinical strains of bacteria obtained from Muthaiah medical college, Annamalai University (*Staphylococcus aureus,*

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*Pseudomonas aeruginosa, Klebsiellapneumoniae*and *Proteus vulgaris*) were used. A single colony of each bacterial species was transferred into 10ml of nutrient broth; it was placed in an incubator at 37°C for overnight. The overnight cultures of bacterial species were spun down using a centrifuge set at 4000 rpm for 5min. The supernatant was discarded and the pellet was resuspended using 20 ml of normal saline. Thus the test bacterial cultures were prepared for antibacterial assay.

Antibacterial assay using Resazurin method

The antibacterial activity of cyanobacterial species was estimated in a microtitre plate method by using Resazurin assay [13]. A sterile 96 well plate (2 sets) were labeled (Fig.1). A volume of 5-120µl (5,10,20,40, 60, 80, 100 and 120) of each cyanobacterial extracts were pipetted into the first three row of the plate, and it was labeled as C1(Control 1),C2 (Control 2) and C3(Control 3). To all other wells, 50µl of nutrient broth or normal saline was added. To each well, 10µl of resazurin indicator solution was added. Using a pipette 30µl of 3.3× strength broths was added to each well to ensure that the final volume was single strength of the nutrient broth. Finally, 10µl of bacterial suspension was added to each well to achieve a concentration of 5×10^{5} CFU.ml^{-1}. Each plate had a set of controls: a column with a broad-spectrum antibiotic as a positive control withciproflaxicin in serial dilution and labeled as C5a,C5b,C5c and C5d. A column with all solutions except for the cyanobacterial extracts were labeled as C4a,C4b,C4c and C4d, and a column with all solutions without bacterial solution adding 10µl of nutrient broth instead were labeled as C1,C2 and C3.The test pathogens along with the cyanobacteria were taken on microtitre plate separately and labeled as P1,P2,P3,P4,S1,S2,S3,S4,G1,G2,G3,G4. The plates were prepared in triplicate and incubated at 37°C for 24h. The colour change was then assessed visually. The colour retaining property of the sample recorded as positive. The lowest concentration of cyanobacteria extract in which colour was not changed was taken as the Minimum Inhibitory Concentration (MIC) value. The average of the three values was calculated.

RESULTS

At the beginning of the reaction, all the wells were in blue. After 24hof incubation, some of the wells turned into pink colour indicating bacterial growth(Figs.1, 2). *Synechococcus* sp.,and*Gloeocapsa* sp., were found to have higher antibacterial activity than *Phormidium* sp. as evident by the colour change(Table 1). All the bacterial strains showed more sensitivity to both

Synechococcus sp., and *Gloeocapsa* sp., than *Phormidium* sp. The results are shown in Table 1.

Table 1. Antibacterial potential of cyanobacterial extracts against four clinical bacterial pathogens.

	***Phormidium*sp.**				***Synechococcus*sp.**				***Gloeocapsa* sp.**			
Conc .of cyano-bacteria (µl)	*S. aur*	*P. aer*	*K. pne*	*P. vul*	*S. aur*	*P. aer*	*K. pne*	*P. vul*	*S. aur*	*P. aer*	*K. pne*	*P. vul*
5	-	-	-	-	-	-	-	-	-	-	-	-
10	++	-	-	-	-	+	-	+++	-	-	+++	+++
20	+	-	-	+	-	+	++	++	-	-	++	+++
40	++	-	-	+	+	+	+	+	+	+	+	++
60	++	++	++	++	+	+	+	+	+	+	+	++
80	+++	++	++	++	++	++	+	+	+	+	+	+++
100	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++
120	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++	+++

(- No activity, + activity, ++ moderate activity, +++ good activity) (*S.aur*= *Staphylococcus aureus, P.aer =Pseudomonas aeruginosa, K.pne = Klebsiellapneumoniae, and P.vul = Proteus vulgaris*).

The minimum inhibitory concentration (MIC) of *Phormidium*sp.,was 67.5x10^{-3} mg.ml^{-1} against *S.aureus,* 405x10^{-3} mg.ml^{-1} against *P.aeruginosa,* 405x10^{-3} mg.ml^{-1} against *K.pnuemoniae,* 270x10^{-3} mg.ml^{-1} against *P.vulgaris.*The MIC of *Synechococcus*sp., was 270x10^{-3} mg.ml^{-1} against *S.aureus,* 135x10^{-3} mg.ml^{-1} against *P.aeruginosa,* 135x10^{-3} mg.ml^{-1} against *K.pnuemoniae,* 67.5x10^{-3}mg.ml^{-1} against *P.vulgaris.*The MIC of *Gloeocapsa*sp.,was 270x10^{-3} mg.ml^{-1} against *S. aureus*, 270x10^{-3} mg.ml^{-1} against *P. aeruginosa*, 67.5x10^{-3} mg.ml^{-1} against*K. pnuemoniae*, 67.5x10^{-3}mg.ml^{-1} against *P. vulgaris.*The Gram positive bacterium *Staphylococcus aureus* was found to be more sensitive to all the three cyanobacterial extracts tested. The inhibitory effect of cyanobacterial extracts was comparable with the standard antibiotic (Ciproflaxicin). The results of MIC are tabulated in Table 2.

Table 2. Minimum Inhibitory concentration (MIC) of cyanobacterial extracts against clinical bacterial pathogens.

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Species	Minimum Inhibitory Concentration (MIC) ($x10^{-3}mg.ml^{-1}$)			
	S.aureus	*P .aeruginosa*	*K. pnuemoniae*	*P. vulgaris*
***Phormidium*sp.**	67.5	405	405	270
***Synechococcus*sp.**	270	135	135	67.5
***Gloeocapsa*sp.**	270	270	67.5	67.5

DISCUSSION

Resazurin technique is generally used in determining the bacterial load in food quality control. However, a few reports are available on the use of this technique to test antibacterial activity [4]. The present study is the first work to use the resazurin technique to assess the antibacterial potential of marine cyanobacteria for quick screening. Results were obtained in a short period of time (24h.) and with very good sensitivity. It is an inexpensive and easy method to perform. The application of this technique will not only reduce the experimental cost but also the time, compared to conventional agar plate methods (such as well diffusion and disc diffusion).

Inhibitory activity of any toxic substance is concentration dependent. This fact was also substantiated in the present work that the inhibition increased with increasing concentration of the cyanobacterial extracts used. It seems likely that biosynthesis of such active substances often confers an important selective advantage for cyanobacteria in nature. A noticeabledecrease of gram-positive bacteria in lakes during the occurrence of cyanobacterial waterblooms has been reported by Chrost [1] and the production of antibacterial substances may be one of the reasons for this phenomenon.

Today infectious diseases are the main cause of death in developing countries and worldwide they hold the second position after heart diseases. Because of the growing bacterial resistance against commercialantibiotics the search for new active substances with antibacterial activity against hospital-based multi-resistant *Staphylococcus aureus*(MRSA-strains) is of increasing importance [16,17,18].Among the clinical pathogens tested in the present study, the *Staphylococcus aureus* was found more sensitive to all the three cyanobacterial extracts tested. *Staphylococcus aureus* can cause a range of illnesses from minor skin infections, to life-threatening diseases such as pneumonia and meningitis.

The present study revealed that unicellular cyanobacteria such as *Synechococcus* sp., and *Gloeocapsa* sp., were found more potent in

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antibacterial activity against clinical pathogens than filamentous form (*Phormidium* sp.) (Table 1) Therefore, it is suggested that the unicellular forms such as *Synechococcus* sp., and *Gloeocapsa* sp., which are a promising source of antibacterials can be attempted for developing antibacterial drugs.

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Figure.1. Microtitre plates showing the colouring by Resazurin with clinical bacterial pathogens with or without the treatment of three cyanobacterial at the beginning of the reaction.

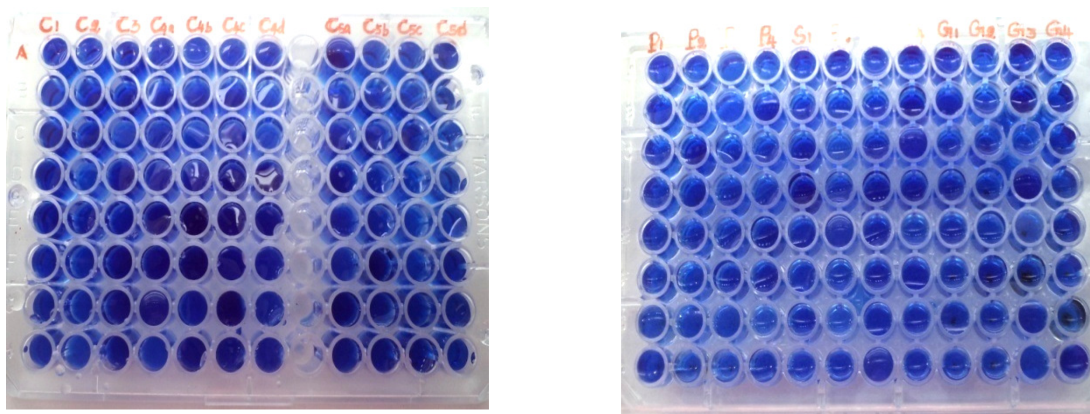

Figure.2. Plates after 24 h in resazurin assay [pink colour indicating the growth and blue means inhibition of growth] C1= *Phormidium*sp. alone,C2= *Synechococcus*sp. alone,C3=*Gloeocapsa*sp. alone, C4a =*Staphylococcus aureus*alone, C4b= *Pseudomonas aeruginosa*alone,C4c=*Klebsiellapneumoniae*alone, C4d= *Proteus vulgaris* alone; C5a =*Staphylococcus aureus*+ Ciproflaxicin in dilution, C5b= *Pseudomonas aeruginosa* + Ciproflaxicin at diff.,C5c*Klebsiellapneumoniae*+ Ciproflaxicin in dilution, C5d = *Proteus vulgaris* + Ciproflaxicin in dilution; P1-G4=The Cyanobacterial extracts in different concentrations viz.120 (5,10,20,40,60,80,100,120 µl). P1 =*Staphylococcus aureus*+ *Phormidium*sp., in dilution, P2= *Pseudomonas aeruginosa* + *Phormidium*sp., P3*Klebsiellapneumoniae*+ *Phormidium sp.*,P4 = *Proteus vulgaris* + *Phormidium*sp., S1

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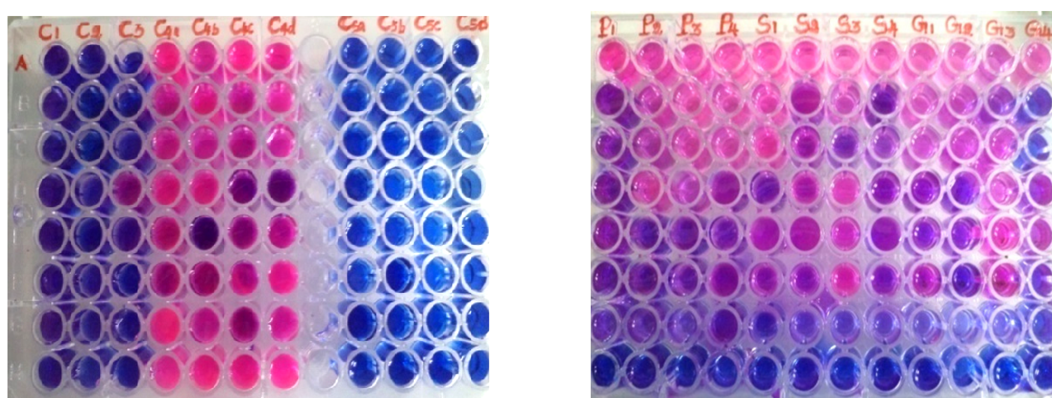
=*Staphylococcus aureus*+ *Synechococcus*sp., S2= *Pseudomonas aeruginosa* + *Synechococcus*sp.,S3*Klebsiellapneumoniae*+ *Synechococcus*sp., S4 = *Proteus vulgaris* + *Synechococcus*sp.; G1 =*Staphylococcus aureus*+ *Gloeocapsa*sp. , G2= *Pseudomonas aeruginosa* + *Gloeocapsa*sp.,G3*Klebsiellapneumoniae*+ *Gloeocapsa*sp., G4 = *Proteus vulgaris* + *Gloeocapsa*sp.



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