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Research Paper

Antimicrobial activities of marine cyanobacteria isolated from mangrove environment of south east coast of India

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ABSTRACT

Cyanobacteria were isolated from the intertidal zone of three different mangrove environment Pichavaram (11° 27' N 79° 47' E), Parangipettai (11° 29' N 79° 47' E) and Mudasal Odai (11° 28' N 79° 46' E) on southeast coast of India. Seven cyanobacteria were extracted in methanol, and a mixture of chloroform, methanol and water (1:2:0.8), and tested for antimicrobial activity against nine human bacterial pathogens (*Bacillus subtilis*, *Escherichia coli*, *Vibrio parahaemolyticus*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Salmonella typhi*, *Shigella flexneri*, *Staphylococcus aureus* and *Vibrio cholerae*), five plant fungal pathogens (*Colletotrichum falcatum*, *Fusarium solani*, *Helminthosporium oryzae*, *Pyricularia oryzae* and *Rhizoctonia solani*) and four animal fungal pathogens (*Aspergillus flavus*, *A. fumigates*, *Candida albicans* and *Rhizopus* species). Except *Oscillatoria cortiana* methanol extract, the cyanobacterial species exhibited antibacterial activity. Human bacterial pathogens except *Pseudomonas aeruginosa* and *Vibrio cholerae*, were inhibited. Except *Synechocystis salina* methanol extract, the cyanobacterial species showed antifungal activity. Plant fungal pathogens other than *Colletotrichum falcatum* and *Fusarium solani* were inhibited. Human fungal pathogens except *Aspergillus flavus* were inhibited. The maximum antibacterial inhibition zone of 9 mm was observed in *Oscillatoria willei* against *Salmonella typhi*. The maximum antifungal activity with inhibition zone of 5 mm was observed with *Oscillatoria salina* methanol extract against *Fusarium solani*, *Phormidium tenue* methanol extract and *Rhizoctonia solani*.

Keywords: Antimicrobial activity; Fungal, bacterial pathogens; Mangroves; Cyanobacteria.

INTRODUCTION

Nearly 50,000 natural products have been discovered from microorganisms, 10,000 of these are reported to have biological activity and over 100 products are in use today as antibiotics, antitumour agents, and agrochemicals (Carte, 1996). Antibiotics are defined as substances produced by microorganisms that are antagonistic to the growth or life of other microbes (Halstead, 1965). Marine environment covers a wide thermal

range (from below-freezing temperatures in Antarctic waters to about 350°C in deep hydrothermal vents), pressure range (1-1000 atmospheres), nutrient range (oligotrophic to eutrophic) and has both photic and non-photic zones. Marine environment may contain over 80% of world's plant and animal species. With the development of new diving techniques, remote operated machines etc., it is possible to collect marine samples and during the past decade over 5000 novel compounds have been isolated from shallow waters to 900m depths of the sea (McCarthy and Pomponi, 2004). Sea has got plenty of metabolites and other resources in living or dead form. Sponges (37%), Coelenterates (21%) and microorganisms (18%) are the major sources of biomedical compounds followed by algae (9%), echinoderms (6%), tunicates (6%), molluscs (2%) bryozoans (1%) etc., (Blunt, et al., 2004).

Cyanobacteria are one of the most promising groups of organisms for isolation of novel and biochemically active natural products (Patterson, et al., 1993; Burja, et al., 2001). A number of research papers have been published recently about the antimicrobial activities from cyanobacteria (Schaeffer and Krylow, 2000; Jha and Zi-rong, 2004; Prashantkumar, et al., 2006; Biondi, et al., 2008; Zeeshan, et al., 2010; Abed, et al., 2011; Ramamurthy, et al., 2010). The cyanobacterium *Lyngbya majuscula* is responsible for sporadic outbreaks of a contact dermatitis known as 'swimmer itch' (Banner, 1959). The cyanobacteria such as *Nostoc commune* (Bohm, et al., 1995; Jaki, et al., 2000), *Anabaena variabilis* (Ma and Led, 2000), *Nostoc spongiaeforme* (Hirata, et al., 1996), *Microcystis aeruginosa* (Ishida, et al., 1997), *Anabaena flos aquae* (Khairy and El-Kassas, 2010), *Lyngbya majusla* (Ramamuthy and Raveendran, 2009), *Trichodesmium erythraeum* (Thillairajasekar, et al., 2009), *Nodularia harveyana* (Pushparaj, et al., 1999) and *Calothrix brevissima* (Metting and Pyne, 1986) have been popularly reported to produce antimicrobial substances. Heptadecane and tetradecane from *Spirulina platensis* (Ozdemir, et al., 2004), phenolic compounds from *Nostoc muscorum* (El-Sheekh, et al., 2004), peptides, polypeptides, amides and alkaloids from *Fischerella ambigua* (Ghasemi, et al., 2004), lipopeptidases from *Anabaena* spp (Burja, et al., 2001; Fujita, et al., 2002), fatty acids, tetramine, spermine and piperazine derivative from *Anabaena* spp (Mundt, et al., 2003; Shanab, 2007), Tjipanasoles from *Tolypothrix tjipanasensis* (Bonjounklian, et al., 1991), laxaphycins from *Anabaena laxa* (Frankmolle, et al., 1992) and scytophytin from *Scytonema psuedohofmanni* (Ishibashi, et al., 1986) have been reported to possess antimicrobial activity. In order to explore marine cyanobacteria with medical potentials, marine cyanobacteria isolated from mangrove sediment were screened antimicrobial activity against nine human bacterial pathogens, five plant fungal pathogens and four animal fungal pathogens. Most of the cyanobacteria species were new and information about antimicrobial activity very limited.

MATERIALS AND METHODS

***Cyanobacterial extraction*:** Seven marine cyanobacteria species (*Synechocystis salina, Spirulina subsalsa, Oscillatoria cortiana, O. salina, O. willei, Phormidium tenue* and *P. fragile*) isolated from three mangrove environment and identified by using the taxonomic publications (Desikachary, 1959; Hum and Wicks, 1980). The cyanobacterial cultures were extracted for antimicrobial substances by two methods. In the first method (Starr, et al., 1962), air-dried cyanobacteria were extracted overnight in methanol and the second method (Bligh and Dyer, 1959), extraction of dried cyanobacteria was carried out in a mixture of chloroform, methanol and water (in a ratio of 1:2:0.8). The extract was centrifuged at 5000 rpm for 20 minutes and the

soluble fraction was evaporated to dryness at 25°C. The residue was extracted two times with a total of 20ml of ether. The ether soluble fraction was evaporated in a water bath at 40°C. The ether soluble residue was dissolved in phosphate buffer at pH 7.2 to a concentration of 100mg/ml.

***Antibacterial and antifungal activities*:** Seven cyanobacterial species were tested using the standard paper disc method. The test microbes used were *Bacillus subtilis, Escherichia coli, Vibrio parahaemolyticus, Proteus vulgaris, Pseudomonas aeruginosa, Salmonella typhi, Shigella flexneri, Staphylococcus aureus* and *Vibrio cholerae,* plant pathogenic fungi such as *Colletotrichum falcatum, Fusarium solani, Helminthosporium oryzae, Pyricularia oryzae,* and *Rhizoctonia solani,* and animal pathogenic fungi such as *Aspergillus flavus, A. fumigates, Candida albicans and Rhizopus* species. The clinical pathogens were obtained from Dr. Rajah Muthiah Medical College Hospital, Annamalai University, and the plant pathogens from the Department of plant pathology, and Department of Agriculture, Annamalai University. The cyanobacteria extract (20µl) were used to saturate the antimicrobial assay paper disks (6mm) with a period of drying between each application. The disks were placed on to the agar surface containing the test microorganisms, and incubated at 37°C for 24h after a diffusion process for 10h at 8°C. The diameter of any inhibition zones formed around the paper disc was then measured.

RESULTS

Data on the antimicrobial activity in terms of inhibition zone exhibited by the cyanobacteria extracted either in methanol or in a mixture of chloroform, methanol and water, are shown in Table 1 for antibacterial and Table 2 for antifungal activities.

***Antibacterial activities*:** The bacterial pathogens such as *Bacillus subtilis, Pseudomonas aeruginosa* and *Vibrio cholerae* did not respond to cyanobacteria extracted in methanol. *Pseudomonas aeruginosa* and *Vibrio cholerae* did not respond to cyanobacteria extracted in a mixture of chloroform, methanol and water. The inhibition zone exceeding 9mm was observed only in one case of methanol extract, *Oscillatoria willei* against *Salmonella typhi* (Tab1e 1). The susceptibility varied with the test bacteria. Species like *Pseudomonas aeruginosa* and *Vibrio cholerae* were resistant to the methanol extracts of all the cyanobacteria tested. *Salmonella typhi* and *Vibrio cholerae* were highly susceptible and inhibited by five cyanobacterial species extracted in methanol. The species like *Vibrio cholerae* and *Pseudomonas aeruginosa* were resistant to the mixture of chloroform, methanol and water extracts of all the cyanobacteria tested. *Staphylococcus aureus* was highly susceptible and inhibited by seven cyanobacterial species extracted in chloroform and methanol. The values among the pathogens were statistically significant at 1% level, however the values were not significant among the cyanobacterial species (Table 3).

***Antifungal activities*:** None of the cyanobacteria extracted in methanol showed antifungal activity against plant pathogens like *Collectorichum falcatum, Pyricularia oryzae* or animal pathogenic fungi like *Aspergillus flavus, Candida albicans* and *Rhizopus* species. Similarly, the cyanobacteria extract with mixture solvents also did not exhibit antifungal activity against plant pathogens like *Collectotricum falcatum* and *Helminthosporium oryzae,* or animal pathogenic fungi like *Aspergillus flavus* and *A. fumigatus.* The inhibition zone exceeding 6mm was observed only in one case: *Oscillatoria salina* extracted in methanol against *Fusarium solani,* and *Phormidium tenue* extracted with mixture of solvents against *Rhizoctonia solani* (Table 2). The susceptibility varied with the test fungi. The species like *Aspergillus flavus, Candida*

albicans, *Colletotrichum falcatum* and *Pyricularia oryzae* were resistant to the methanol extracts of all the cyanobacteria tested. *Rhizoctonia solani* was highly susceptible and was inhibited by six cyanobacterial species extracted in methanol. The species like *Aspergillus flavus, A. fumigates*, *Colletotrichum falcatum* and *Helminthosporium oryzae* were resistant to the mixture of chloroform, methanol and water extracts of all the cyanobacteria tested. *Rhizoctonia solani* was highly susceptible and was inhibited by five cyanobacterial species extracted in chloroform and methanol. The values among the pathogens were statistically significant; however the values were not significant among the cyanobacterial species (Table 3).

DISCUSSION

In the present study, the bacterial pathogen *Staphylococcus aureus* was found be inhibited by majority of the cyanobacterial strains with inhibition zones of 4-7mm followed by *Shigella flexneri* by six cyanobacterial strains with inhibition zone of 4-6mm and *Salmonella typhi* by five cyanobacteria strains with highest range of inhibition zone of 2-9mm. Among nine human bacterial pathogens, two of them (*Vibrio cholerae* and *Pseudomonas aeruginosa*) were very sensitive and there was no inhibition observed by any cyanobacterial species except *Phormidium tenue* which was inhibited (4mm) by only *Phormidium tenue* methanol extract. The plant fungal pathogens like *Rhizoctonia solani* were highly inhibited (2-6mm) by all cyanobacteria species. It was followed by *Fusarium solani* with inhibition of 2-6mm by five cyanobacteria species. Plant fungal pathogen *Colletotrichum falcatum*, and animal fungal pathogen *Aspergillus flavus* were very sensitive to cyanobacterial extracts (Tables 1, 2).

Extracts from *Oscillatoria princeps* were active against *Bacillus subtilis, Staphylococcus aureus, Escherichia coli* and *Brucella bronchiseptica* (Gupta and Shrivastava, 1965). Partially purified compounds from marine cyanobacterium *Oscillatoria willei* enhanced immunoreactive cells of Swiss albino male mice (Thirunalasundari, et al., 2003). Unicellular marine cyanobacteria such as *Synechocystis* and *Synechococcus* reportedly cause inhibition of Gram positive bacteria (Martins, et al., 2008). Ether and water-soluble fractions obtained from *Lyngbya majuscula* have been reported to have antibacterial action against *Pseudomonas fluorescens, Micrococcus pyogenes* and *Mycobacterium smegmatis* (Starr, et al., 1962). In the present study, chloroform and methanol extracts have some effect against *Pseudomonas* species (Table 1). Extract from *Phormidium fragile* has antagonised effect against *Bacillus subtilis* (Palaniselvam, 1998) which is confirmed in the present study.

Extracts from *Phormidium corium* an exhibited inhibition zone (46mm) against *Staphylococcus aureus* (Madhumathi, et al., 2011), it was inhibited by all the cyanobacterial extracts with a smaller inhibition zone (4-7mm) during the present study (Table 1). Extracts from *Microchaete tenera* is shown to have activity (14mm) against human pathogenic bacterium *Proteus vulgaris* (Prashantkumar, et al., 2006), and in the present study it was inhibited (6 mm) by extracts collected from *Spirulina subsala* and *Oscillatoria willei*. Extract from *Tolypothrix* sp. is reported to have inhibition zone (17 and 21mm) against *Bacillus cereus* and *Staphylococcus epidermidis* (Zeeshan, et al., 2010). Extracts of *Plectonema boryanum* and *Anabaena variabilis* are reported to exhibit inhibition zones (17, 12mm) against *Stayphylococcus epidermidis* (Suhai, et al., 2011). The extract from *Nostoc paludosum* shows the inhibition zone (23mm) against *Bacillus subtilis* and *Candida albicans* (Ramachandra

Rao, 1994). In the present study, *Bacillus subtilis* extract exhibited maximum inhibition (7mm) against bacterial pathogens and four fungal pathogens, and *Bacillus subtilis* were inhibited only by the *Phormidium tenue* extracts (Tables 1, 2). The antimicrobial activity of the extract could be due to the presence of different chemicals that may include flavonoids and triterpenoids besides phenolic compounds and free hydroxyl group, (Yu, et al., 2009) amides and alkaloids (Ghasemi, et al. 2004), metabolities such as tannin, alkaloids, protein, and flavonoids (Zeeshan, et al., 2010), heptadecane and tetradecane (Ozdemir, et al., 2004), polypeptides, lipopeptidases (Burja, et al., 2001; Fujita, et al., 2002), fatty acids, tetramine, spermine and piperazine (Prashantkumar, et al., 2006; Shanab, 2007).

Acetone extract of *Phormidium corium*, methanol extract of *Lyngbya martensiana* and diethyl ether extract of *Microcystis aeruginosa* gave the largest inhibition zone tested against fungal pathogens (Madhumathi, et al., 2011). Acetone extract of cyanobacteria shows maximum inhibition zone against *Echerichia coli*, *Bacillus subtilis* and *Pseudomonas aeruginosa* (De Mule, et al., 1991; Ishida, et al., 1997). Methanol extracts of cyanobacteria was recorded maximum antimicrobial activity against *Proteus vulgaris*, *Bacillus cereus*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Aspergillus niger* and *Aspergillus flavus* (Prashantkumar, et al., 2006). Ethanol extracts of cyanobacteria mat gave maximum inhibition zone against *Staphylococcus aureus*, *Streptococcus pyrogenes* and *Pseudomonas aeruginosa* (Abed, et al., 2011). Acetone and methanol extracts from *Spirulina platensis* showed more or less similar inhibition zones against *Staphylococcus aureus* and *Staphylococcus typhimurium* (Kumar, et al., 2011). Pyridine and n-Butanol extracts of *Oscillatoria subbrevis*, *Oscillatoria amphibia* and *Oscillatoria chlorina* gave maximum activity against *Bacillus cereus*, *Enterobacter* sp., *Enterococcus faecalis*, *Staphylococcus aureus* and *Salmonella typhi*, *Aspergillus wentii*, *Candida albicans*, *Vibrio alginolytics*, *Vibrio fluvialis*, *Vibrio mimicus*, *Vibrio anguillarum* and *Vibrio cholerae* (Prabakaran, 2011). Ethanol extract of *Anabaena oryzae*, diethl ether and acetone extract of *Spirulina platensis* had highest antibacterial and antifungal activity (Abedin and Taha, 2008; Ozdemir, et al., 2004). A variety of solvents (water, methanol, ethanol, acetone, petroleum ether, and hexane) used for *Arthrospira platensis* and methanol gave the highest inhibition zone (75.75%) than other solvents (Challouf, et al., 2011). In the present study also methanol extracts of *Oscillatoria salina* and *Oscillatoria willei* showed maximum inhibition zone against *Salmonella typhi*.

CONCLUSION

It is concluded from this study that extracts of some cyanobacterial strain showed antimicrobial activity against the pathogens used in the present investigation. Further researches should be made to identify and purify natural product from these cyanobacteria against antibacterial and antifungal activity. Improvement knowledge of the composition, analysis, and the properties of these cyanobacteria with respect to antimicrobial compounds would assist in efforts for the pharmaceutical application.

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Table- 1: Antibacterial activity in terms of Inhibition Zone (in mm) expressed against nine human pathogenic bacteria by seven cyanobacterial species extracted in methanol, and mixture of chloroform, methanol and water (1:2:0.8).

Pathogens	I		II		III		IV		V		VI		VII		VII
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	
Bacillus subtilis	-	-	-	-	-	-	-	-	-	-	-	-	4	-	1
Escherichia coli	-	-	3	6	-	-	-	3	-	-	-	-	6	5	3
Vibrio parahaemolyticus	-	-	3	4	-	-	-	-	-	-	-	4	3	4	3
Proteus vulgaris	-	3	6	4	-	-	4	-	-	6	-	-	3	2	5
Pseudomonas aeruginosa	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Salmonella typhi	-	-	6	4	-	-	8	2	9	5	8	7	6	5	5
Shigella flexneri	-	-	-	4	-	4	3	4	3	4	4	4	4	5	6
Staphylococcus aureus	6	5	7	7	-	4	5	7	-	4	4	7	6	4	7
Vibrio cholerae	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
No. of bacteria inhibited	1	2	5	6	0	2	4	4	2	4	4	4	6	7	

- I – *Synechocystis salina*; II – *Spirulina subsalsa*; III – *Oscillatoria cortiana*; IV – *Oscillatoria salina*; V – *Oscillatoria willei*; VI – *Phormidium fragile*; VII – *Phormidium tenue*; VII – Number of potential cyanobacteria.
- A – Methanol extract; B – Chloroform, Methanol and Water (1:2:0.8)
- – no activity

Table- 2: Antifungal activity in terms of Inhibition Zone (in mm) expressed against five plant fungal pathogen and four animal fungal pathogen by seven cyanobacterial species extracted in methanol, and the mixture of chloroform, methanol and water (1:2:0.8).

Pathogens	I		II		III		IV		V		VI		VII		VII
	A	B	A	B	A	B	A	B	A	B	A	B	A	B	
Colletotrichum falcatum	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Fusarium solani	-	-	4	4	-	-	6	4	-	3	2	-	3	3	5
Helminthosporium oryzae	-	-	-	-	-	-	3	-	-	-	-	-	-	-	1
Pyricularia oryzae	-	-	-	-	-	-	-	3	-	-	-	3	-	-	2
Rhizoctonia solani	-	2	3	4	2	2	4	3	2	-	3	-	6	3	7
Aspergillus flavus	-	-	-	-	-	-	-	-	-	-	-	-	-	-	0
Aspergillus fumicatus	-	-	-	-	-	-	-	3	-	-	-	-	-	-	2
Candida albicans	-	-	-	-	-	-	-	3	-	-	-	-	-	-	1
Rhisopus sp.	-	-	-	3	-	-	2	-	-	-	-	-	-	3	3
No. of bacteria inhibited	0	1	2	3	1	2	3	3	1	1	2	1	3	4	

- I – *Synechocystis salina*; II – *Spirulina subsalsa*; III – *Oscillatoria cortiana*; IV – *Oscillatoria salina*; V – *Oscillatoria willei*; VI – *Phormidium fragile*; VII – *Phormidium tenue*; VII – Number of potential cyanobacteria
- A – Methanol extract; B – Chloroform, Methanol and Water (1:2:0.8)
- – no activity

Table- 3: ANOVO for the seven cyanobacterial extracts tested against nine human bacterial pathogens, five plant fungal pathogens and four animal fungal pathogens.

No.	Pathogens	Level of significance between pathogens	Level of significance between cyanobacterial species
Antibacterial activity of methanol extract			
1	*Escherichia coli*	165.00 **	1.81 NS
2	*Vibrio parahaemolyticus*	18.10 **	0.17 NS
3	*Proteus vulgaris*	68.20 **	1.00 NS
4	*Shigella flexneri*	29.00 **	0000 NS
5	*Staphylococcus aureus*	21.27 **	1.81 NS
Antibacterial activity of chloroform, methanol and water extract			
1	*Bacillus subtilis*	16.00 **	1.00 NS
2	*Escherichia coli*	12.42 **	0.35 NS
3	*Vibrio parahaemolyticus*	54.00 **	0000 NS
4	*Proteus vulgaris*	21.00 **	1.00 NS
5	*Shigella flexneri*	112.00 **	2.50 NS
6	*Staphylococcus aureus*	10.50 *	2.50 NS
Antifungal activity of methanol extract			
1	*Aspergillus fumicatus*	6553 NS	6553 NS
2	*Fusarium solani*	33.40 **	0.29 NS
3	*Helminthosporium oryzae*	655 **	6550 **
4	*Rhizoctonia solani*	6.07 *	0.12 NS
Antifungal activity of chloroform, methanol and water extract			
1	*Candida albicans*	25.00 **	1.00 NS
2	*Fusarium solani*	45.00 **	2.50 NS
3	*Pyricularia oryzae*	25.00 **	2.50 NS
4	*Rhizoctonia solani*	25.38 **	2.14 NS
5	*Rhizopus* spp.	17.50 **	2.50 NS

- **Significant at 1% ; *Significant at 5% ; NS - Not Significant