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Antimicrobial Activity of Mangrove Actinomycetes from Soil Sample of Rhizophora Apiculata

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ABSTRACT

Totally 22 actinomycetes were isolated by dry heat (70oC) pre-treatment method on Starch casein agar media, from the soil sample that was collected nearer to the root region of the mangrove Rhizophora apiculata (Blume)-Rhizophoraceae from the back water area, Ariyankuppam, Puducherry (UT). All the 22 actinomycetes were subjected for primary screening against the 10 gram negative bacteria, 2 gram positive bacteria by agar plug method. The total percentage of inhibition by actinomycetes against bacteria in primary screening was noted as E.coli-9%, K. pneumoniae-0%, P.vulgaris-0%, P.aeruginosa-31.8%, S.typhi-13.6%, S.flexneri-13.6%, V.cholera-9%, B.bronchiseptica-68.2%, P.fluorescens-0%, E.faecalis-13.6%, B.subtilis-40.9%, S.aureus-9%. Totally 20 (90.91%) actinomycetes showed antibacterial activity towards any one of the tested bacteria, 2 (9.09%) actinomycetes showed no antagonistic activity. From these, it was noted that the mangrove actinomycetes were strong in their antibacterial activity. Only 4 actinomycetes were selected from R. apiculata and that were subjected for secondary screening. Out of 22 isolates from R.apiculata, 8 (36.36%) were active and 14 (63.64%) were inactive for the Candida albicans. 3 isolates from R.apiculata with strong anticandida activity were selected and subjected to confirmatory screening using cross streak method with 12 bacteria. The 3 active actinomycetes, selected from primary, secondary and cross streak method of antibacterial, anticandida activity were tested against thirteen fungi for antifungal activity by agar plug method and well diffusion method. Broad spectrum antimicrobial activity was confirmed by cross streak method for selected antagonistic actinomycetes.

Keywords: Antimicrobial activity, Screening methods, Mangroves, *Rhizophora apiculata.*

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1. INTRODUCTION

Actinomycetes are group of organisms that share both the characteristics of bacteria and fungi and they have high G+C content. They are the strongest antagonists among microbes. The antibiotic substances produced by them display antibacterial, antifungal, anticancer, antiprotozoic, antiviral, insecticidal properties. The antibiotics produced by the actinomycetes are safer than the antibiotics secreted by the fungi and bacteria. Bio active substances produced by the marine actinomycetes are reported by many researchers [5, ,17, 19, 26]. Few report that mangrove soil is a major source of actinomycetes [21, 23, 4, 7]. Mangrove

ecosystem is the most useful ecosystem diversified with variety of microbes. The search of new and novel antibiotics and other bioactive microbial metabolites is important for the fight against new emerging pathogens [1, 2]. Isolation of actinomycetes from unique unexplored natural habitats is of interest to avoid re-isolation of strains that produce known bioactive metabolites. Neglected habitats are proving to be a good source of novel actinomycetes and bio active compounds [9].

The present investigation aims at finding better antimicrobial compound for controlling the bacterial and fungal human diseases, with the help of mangrove actinomycetes that are selectively isolated from the soil near the root region of *Rhizophora apiculata,* from the Ariyankuppam back water area, Puducherry, India.

2. MATERIALS AND METHODS

2.1. Isolation of Mangrove Actinomycetes

Soil sample was collected near the root region of *Rhizophora apiculata,* from the Ariyankuppam back water area, Puducherry, India. Then the soil sample was air dried for 7-10 days at 40ºC, crushed and sieved to remove the shells and debris and given to Soil testing laboratory, Department of Agriculture, Puducherry, for physico-chemical analysis. The soil sample was subjected to dryheat pretreatment (70^{o}C for 15 min) [6, 7]. One gram soil was mixed and serially diluted in sterile water blanks. 0.1 ml of last dilution (10^{-6}) was inoculated by pour plate method [27 using Starch Casein Agar [11] supplemented with Fluconazole 80µg/ml and Nalidixlic acid 75µg/ml. Plates were incubated at 30 ±ºC for up to 30 days. Plates were regularly examined for actinomycetes colonies. Selected colonies were subcultured in YME agar slants.

2.2. Screening of Actinomycetes for Antimicrobial Activity

2.2.1. Test Organisms Used in This Study

The following test bacteria were procured from Microbial Type Culture Collection-Chandigarh. The gram negative bacteria are *Pseudomonas aeruginosa* (MTCC-424)*, Shigella flexneri* (MTCC-1457)*, Bordetella bronchiseptica* (MTCC-6837)*, salmonella typhi* (MTCC-3220)*, vibrio cholera* (MTCC-3906)*, Proteus vulgaris* (MTCC-744)*, E.coli* (MTCC-1687)*, Klebsiella pneumonia* (MTCC-4031)*, Pseudomonas fluorescens, Enterococcus faecalis* (MTCC-439) and gram positive bacteria are *Staphylococcus aureus* (MTCC-96)*, Bacillus subtilis* (MTCC-441) and One unicellular fungi-*Candida albicans* (MTCC-183).

The multicellular fungi used were *Microsporum gypseum* (MTCC-4494), *Trichophyton mentagrophytes* (MTCC-8476), *Epidermophyton floccosum* (MTCC-7880), *Colletotrichum capsici* (MTCC-3414), *Aspergillus fumigatus* (MTCC-3377), *Aspergillus niger* (MTCC-872), *Fusarium oxysporum* (MTCC-1755) and *Rhizoctonia solani* (MTCC-1236), *Alternaria alternata, Aspergillus flavus, Aspergillus terreus, Curvularia lunata* and *Colletotrichum gloeosporioides.*

2.3. Preparation of Test Organisms

Test bacteria were maintained in nutrient agar broth, pH-7. These were stored in refrigerator at 4^{o}C for future use. 12-24 hours old bacterial liquid cultures and candida culture were used for antimicrobial study.

Test fungi were maintained in potato dextrose broth and in PDA slants, pH-7. These were stored in refrigerator at 4^{o}C for future use. 3-5 days old fungal liquid cultures and plate cultures were used for antifungal study

2.4. Invitro Screening for Antimicrobial Activity

Primary screening by agar plug method was studied by following Mohanraj et al, [14] Secondary screening by agar well diffusion method was done by using Murrey et al, [13] procedure and Cross streak method was studied by Lemos et al, [12].

3. RESULTS AND DISCUSSION

3.1. Isolation and Maintenance of Actinomycetes

Table-1. Physico-chemical characteristics of the soil samples

S. no	Soil sample	Parameters in physico-chemical characteristics										
		ph	Status of		Soil type	Available macro-nutrients (mg/g)			Available micro-nutrients (mg/g)			
			E.C (dsm^{-1})	Lime		N_2	P_2O_5	K_20	Cu	Zn	Mn	Fe
1	*Rhizophora apiculata*	7.5	0.5	N	CL	80 VL	0.77 VL	166 H	0.550 L	0.902 L	1.819 L	4.299 M

N-Normal; **CL**-Clay Loamy; **L**-Low; **VL**-Very Low; **M**-Medium; **H**-High

The abundance of the organic matters, salinity and high degree of moisture content favour the prevalence of actinobacterial population and other life forms in the mangrove ecosystem, same was reported by Nag et al, [15]. The pH of mangrove soil sample collected from *Rhizophora apiculata* was 7.5, usually, the pH of the fresh soil samples was ranging from 7.5 to 8.2 is the optimum range of pH needed for actinomycetes to live and produce the antibiotic substances as secondary metabolites in the soil near to the root region of mangrove plants. The air drying of soil samples for 7-10 days at 50^{o}C helped to eliminate the vegetative bacteria and fungi; it was coincided with the study of Williams et al, [25]. The soil analysis results showed that there were very low available Nitrogen and P_2O_5. Micro-nutrients like Zn, Cu and Mn were low; Fe was medium, K_20 is high in their available form. This may due to the seasonal variations and fluctuating water levels enriching actinomycetes counts. Srivibool, [20] suggested that the abundance of actinomycetes in mangrove soil may actually be due to better quality of the sediments, described in terms of structure, pH and humic substances. Totally 22 actinomycetes were isolated from soil sample of *Rhizophora apiculata* by dry heat (70^{o}C for 15 min) pretreatment method [7]. Dry heat method yielded bioactive actinomycetes for antimicrobial activity. The isolated actinomycetes grew well in yeast malt extract agar (ISP_2) and some of the isolates produce soluble pigments.

3.2. Antibacterial Activity of Actinomycetes from *R.apiculata*

The great majority of antibiotics that have been isolated in numerous screening programs concerned with the search for new therapeutic agents have been tested primarily for their activity against different bacteria

[24]. Accordingly, ten gram negative bacteria and 2 gram positive bacteria, procured from MTCC, Chandigarh was used for antibacterial study. 22 actinomycetes were isolated from *R.apiculata* and that were subjected for primary screening against human pathogenic bacteria by agar plug method.

Table-2. Primary screening of actinomycetes from *R.apiculata* against human pathogenic bacteria by agar plug method

S.no	Isolate code	Measurement of zone of inhibition in millimeter											
		E.c	**k.p**	**p.v**	**p.a**	**s.t**	**s.f**	**v.c**	**B.b**	**p.f**	**E.f**	**B.s**	**S.a**
1	M46	-	-	-	-	-	-	-	22	-	-	2	-
2	M47	-	-	-	12	-	-	-	-	-	-	8	-
3	M48	-	-	-	-	-	-	8	-	-	-	--	-
4	M49	-	-	-	30	-	10	-	20	-	30	10	-
5	M50	-	-	-	20	8	-	12	20	-	-	20	-
6	M51	-	-	-	14	-	-	-	-	-	-	-	-
7	M52	8	-	-	-	6	-	-	-	-	-	10	6
8	M53	-	-	-	-	-	-	-	-	-	-	-	-
9	M54	-	-	-	-	4	-	-	14	-	-	-	-
10	M55	-	-	-	-	-	-	-	4	-	-	10	-
11	M56	-	-	-	-	-	8	-	-	-	-	-	-
12	M57	-	-	-	-	-	-	-	14	-	-	-	-
13	M58	-	-	-	-	-	-	-	20	-	-	6	6
14	M59	-	-	-	-	-	-	-	-	-	-	-	-
15	M60	-	-	-	10	-	-	-	10	-	-	-	-
16	M61	-	-	-	-	-	-	-	24	-	-	-	-
17	M62	-	-	-	-	-	-	-	26	-	-	6	-
18	M63	-	-	-	20	-	-	-	10	-	-	-	-
19	M64	-	-	-	-	-	-	-	9	-	-	-	-
20	M65	20			15		26		25		20	20	
21	M66	-	-	-	-	-	-	-	4	-	4	-	-
22	M67	-	-	-	-	-	-	-	8	-	-	-	-

Source: From Ph. D unpublished Thesis, T. Janaki- original research work

E.c-*E. coli,* **K.p**-*Klebsiella pneumoniae,* **P.v**-*Proteus vulgaris,* **P.a**-*Pseudomonas aeruginosa,* **S.t-***Salmonella typhi,* **S.f** -*Shigella flexneri,* **V.c**-*Vibrio cholera,* **B.b-***Bordetella bronchiseptica,* **P.f**-*Pseudomonas fluorescens,* **E.f**-*Enterococcus faecalis*, **B.s-***Bacillus subtilis,* **S.a**-*Staphylococcus aureus.*

The total percentage of inhibition by actinomycetes against bacteria in primary screening was noted as *E.coli*-9%, *K. pneumoniae*-0%, *P.vulgaris*-0%, *P.aeruginosa*-31.8%, *S.typhi*-13.6%, *S.flexneri*-13.6%, *V.cholera*-9%, *B.bronchiseptica*-68.2%, *P.fluorescens*-0%, *E.faecalis*-13.6%, *B.subtilis*-40.9%, *S.aureus*-9%. Totally 20 (90.91%) actinomycetes showed antibacterial activity towards any one of the tested bacteria, 2 (9.09%) actinomycetes showed no antagonistic activity. From these, it was noted that the mangrove actinomycetes were strong in their antibacterial activity. Only 4 actinomycetes were selected from *R. apiculata* and that were subjected for secondary screening.

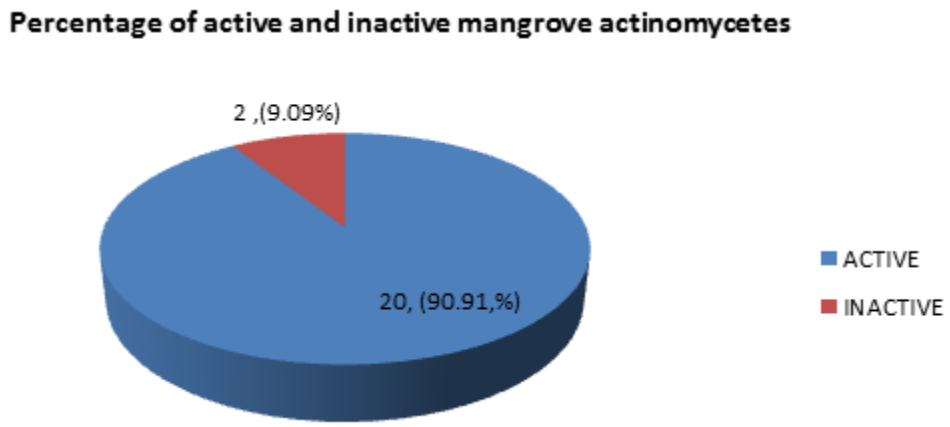

Figure-1. Percentage of active and inactive actinomycetes from *R.apiculata*
Source: From Ph. D unpublished Thesis, T. Janaki- original research work

3.3. Antibacterial Activity of Actinomycetes in Secondary Screening by Agar Well Diffusion Method

The isolates selected from primary screening were subjected for secondary screening by agar well diffusion method. The isolates which produced antibiotic compound in large quantity in liquid media were selected as potent isolates. Well diffusion method helped to study about the antibiosis from liquid media fast. It was noted that the antibiotic production and antibacterial potency of the actinomycetes in liquid media was varying from the antibiotic production and antibacterial potency in solid agar medium [10].

Table-3. Antibacterial activity of active isolates in secondary screening by agar well diffusion method

S.no	Mangrove plant	Isolates code	Zone of inhibition in mm											
			E.c	k.p	p.v	p.a	s.t	s.fl	v.c	B.b	p.f	E.f	B.s	S.a
1	*Rhizophora apiculata*	M49	12	8	10	38	-	16	10	-	--	36	18	8
2		M50	-	-	-	-	-	-	10	-	--	22	12	8
3		M52	8	-	6	12	10	8	-	8	-	32	20	10
4		M65	12	6	12	25	12	20	12	18	-	32	16	10

Source: From Ph. D unpublished Thesis, T. Janaki- original research work

Four active isolates were selected for antibacterial activity in secondary screening by well diffusion method from 22 isolates of *R.apiculata.* The percentage of inhibition of tested bacteria by the actinomycetes were, *E.coli*-75%, *K. pneumoniae*-50%, *P.vulgaris*-75%, *P.aeruginosa*-75%, *S.typhi*-50%, *S.flexneri*-75%, *V.cholera*-75%, *B.bronchiseptica*-50%, *P.fluorescens*-0%, *E.faecalis*-100%, *B.subtilis*-100%, *S.aureus*-100%. It was found that *E.faecalis*, *B.subtilis*, *S.aureus* are totally controlled by all the four active actinomycetes (M49, M50, M52 and M65) in the secondary screening.

It was observed that some actinomycetes grew well in solid medium and had shown antagonistic activity effectively towards the tested bacteria by agar plug method, but same isolates in liquid medium did not show any antagonistic activity towards the tested bacteria by agar well diffusion method in secondary screening. In few cases of actinomycetes, number of inhibition of tested bacteria by the actinomycetes by agar plug method in primary screening coincided with agar well diffusion method in secondary screening. The isolates, strong in

their antibacterial activity both in agar plug method (Solid media) and in agar well diffusion method (Liquid media) were selected for confirmatory test for antibacterial activity.

3.4. Anticandida Activity of the Actinomycetes

It was an effort taken to screen the mangrove actinomycetes for anticandida activity to find better alternative. Out of 22 isolates from *R.apiculata,* 8 (36.36%) isolates were active and 14 (63.64%) were inactive for anticandida activity by agar plug method in the preliminary screening.

Table-4. Anticandida activity of the actinomycetes

S.no	Isolate code	Inhibition in millimeter
1	M46	-
2	M47	-
3	M48	10
4	M49	24
5	M50	-
6	M51	-
7	M52	12
8	M53	-
9	M54	-
10	M55	-
11	M56	12
12	M57	-
13	M58	-
14	M59	10
15	M60	-
16	M61	-
17	M62	8
18	M63	
19	M64	-
20	M65	22
21	M66	-
22	M67	12

Source: From Ph. D unpublished Thesis, T. Janaki- original research work

3.5. Secondary Screening of Actinomycetes for Anticandida Activity

8 isolates from *R.apiculata* with anticandida activity were selected for secondary screening to test their ability to produce the active compounds in liquid medium. It was witnessed that some isolates were not produce active compound for anticandida activity in liquid medium and that type of cultures were omitted after the secondary screening. The isolates that produced active compound both in solid and in liquid medium were taken for the final study as the active isolates for anticandida activity.

Table-5. Anticandida activity of actinomycetes by agar well diffusion method in secondary screening

S.no	Mangrove plants	Isolates code	Zone of inhibition in mm
1	*Rhizophora apiculata*	M49	22
2		M52	14
3		M65	20

Source: From Ph. D unpublished Thesis, T. Janaki- original research work

Based on the secondary screening, 3 isolates from *R.apiculata* with strong activity were selected and subjected to confirmatory screening using cross streak method. The output of our study related to anticandida activity is better than the study of Susithra et al, [22]. Our study report that the actinomycete isolates efficiently controlled the *Candida albicans* under in-vitro conditions. This output confirmed that the mangrove sources are precious sources for anticandida drug delivary from actinomycetes.

3.6. Antifungal Activity of Selected Isolates

The 3 active actinomycetes, selected from antibacterial, anticandida activity were tested against thirteen fungi for antifungal activity by agar plug method and well diffusion method. Results indicated that the mangrove actinomycetes are very active in controlling the growth of both phytofungal pathogens and zoophilic fungal pathogens [9]. Actinomycete-fungus antagonism has been demonstrated for a wide variety of plant pathogens such as *Alternaria sp.* Chattopadhyay and Nandi, [3] *Rhizoctonia sp*. [18] and *Cuvvularia sp*. [16].

Table-6. Antifungal activity of selected isolates by agar plug method

Isolate code	Fungi used in antifungal activity, Inhibition in mm												
	M.g	T.m	E.f	C.l	A.a	A.fu	A.n	A.f	A.t	R.s	C.ca	C.g	F.o
M49	8	4	6	12	8	-	-	-	-	-	10	8	6
M52	-	-	-	-	-	-	6	4	8	-	-	-	-
M65	4	6	4	-	-	-	12	-	-	12	8	14	12

M.g- Microsporum gypseum, **T.M**- Trichophyton mentagrophytes, **E.f**- Epidermophyton floccossum, **C.l**- Curvularia lunata, **A.a**- Alternaria alternata, **A.fu**-Aspergillus fumigatus, **A.n**- Aspergillus niger, **A.f**- Aspergillus flavus, **A.t**-Aspergillus terrus, **R.s**-Rhizoctonia solani, **C.ca**-Colletotrichum capsici, **C.g**-Colletotrichum gleosporioides, **F.o**-Fusarium oxysporum

3.7. Cross Streak Method to Confirm the Antimicrobial Activity of Selected Isolates

Cross streak method was used for confirmation of antimicrobial activity for most active isolates. The antibacterial and anticandida activity of the isolates was studied better in nutrient agar plates and antifungal activity was studied with PDA plates for cross streak method. It was observed that inhibition of bacteria by isolates in cross streak method was better than the agar plug method, because the antibiotic compound in the cross streak plates was not disturbed, the whole production of antibiotic compound was in the same plate but in the agar plug method only 8mm radial agar plugs were cut and tested for antibacterial activity [10]. The antimicrobial activity of actinomycetes was varying from one method to other in antimicrobial screening.

4. CONCLUSION

Mangrove actinomycetes from *Rhizophora mucronata* inhibited both gram positive as well as gram negative bacteria efficiently and able to lyse and destroy the cell wall types of both gram +ve and gram –ve bacteria. The gram–ve bacteria; *Pseudomonas aeruginosa, Bordetella bronchiseptica* and gram positive bacteria; *Bacillus subtilis* were more sensitive to the mangrove actinomycetes from *Rhizophora mucronata*. The overall sensitivity of bacteria to isolates added valuable information that the mangrove soil is the efficient source for isolating potent antinomycete isolates for the bacteria those involve in causing nosocomial infections in human beings. Anticandida study revealed information that the actinomycete isolates from

mangrove sources are very effective and it can be used in the pharmaceutical field for anticandida drug delivary. Antifungal activity added valuable information about mangrove actinomycetes and its application in the field plant protection and pharmaceutical field for human welfare.

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