Short Communication

NOCARDIOPSIS SP. 5 ENDOPHYTIC TO TULSI LEAVES – ISOLATION AND ANTIMICROBIAL ACTIVITY

ABSTRACT

Aims: To isolate and identify the rare endophytic *Nocardiopsis* sp.5 from Tulsi (*Ocimum sanctum*) leaves of Western Ghats, India and estimates its antimicrobial activity against various human pathogens and tea pathogens.
Study design: Rare endophytic actinobacteria isolation, identification and estimation of antimicrobial activity.
Place and Duration of Study: Department of Botany and Department of Bioinformatics, Nirmala College for Women, Coimbatore – Western Ghats between December 2011 and December 2012.
Methodology: Pre-sterilization of Ocimum sanctum leaves and isolation of rare endophytic actinomycetes using selective media. Estimation of antimicrobial activity of the isolated rare endophytic actinobacteria by primary and secondary streaking methods. Morphological and Molecular Identification of the most bioactive isolate.
Results: Out of 11 endophytes, 4 showed antagonistic activity against tea and human pathogens. Among the four isolates, isolate no.5 showed maximum activity against all the human pathogenic microorganisms such as *Staphylococcus aureus* (21mm), *Candida albicans* (18 mm), *Enterococcus faecalis* (19mm), *Vibrio cholera* (18mm) and tea pathogens such as *Glomerella cingulata* (14mm) *Hypoxylon serpens* (17mm) and *Pestalopsis theae* (18mm). Hence, morphological and phylogenetic studies show that isolate no.5 belongs to *Nocardiopsis* group.
Conclusion: Further purification, structure elucidation and characterization of the antimicrobial compound from the endophytic rare actinomycete isolate *Nocardiopsis* 5 strain are recommended Hence, there is definite scope for bioprospecting of antagonistic actinomycetes from Western Ghats once appropriate consistent studies are undertaken.
.

Keywords: Western Ghats, endophytic actinomycetes, *Tulsi (Ocimum sanctum), Nocardiopsis* 5, Phylogram analysis.

1. INTRODUCTION

Actinomycetes produce about 70% of total known antibiotics, and remaining 30% are products of filamentous fungi and non-actinomycete bacteria [1]. Hence for the past 2-3 decades, the research has been focused on the antibiotic

producing actinomycetes from varied terrestrial and marine resources due to decreasing rate of discovery of novel antibiotics and the increase in the multi-drug resistant pathogens in recent years. Soil habitats (96%) have been largely surveyed as compared to other terrestrial resources like endophytic plants (3%) and animal guts (1%) [2]. Ancient Indian literatures consider all plants as potential sources of medicinal substances. Hence, medicinal plants have been considered as an important resource of isolating endophytic actinomycetes which can induce secondary metabolites of very important value. However, the work to date is insufficient to understand the actinomycetes diversity of medicinal plants present in Western Ghats. Therefore, the present research has been targeted on exploring the rare endophytic actinomycetes from the leaves of medicinal plants of Western Ghats especially Tulsi (*Ocimum sanctum)*), Tamil Nadu and examining their antimicrobial activity against tea pathogens and human pathogens. This is the first work of isolating rare endophytic actinomycetes from Tulsi leaves.

2. MATERIAL AND METHODS

2.1 Sample Collection

Healthy leaf samples of common medicinal plant Tulsi (*Ocimum sanctum)* with high medicinal value were gathered from the garden of Nirmala College for Women, Coimbatore (11.0183° N, 76.9725° E) which comes under the region of Western Ghats of Southern India during the period of December 2011 to January 2012. The collected plant materials were taken to the laboratory, preserved at 4°C in sealed plastic bags and subjected to isolation work within 96 hours.

2.2 Isolation of endophytic actinobacteria

Healthy leaf samples were cut into small pieces (2*2 cm) and washed by running tap water for 1-2 minutes to remove the soil particles completely. The resultant were subjected to a five-step surface sterilization procedure as per the method of Qin *et al* [3]: a 4 to 10-minutes wash in 5% sodium hypochlorite, followed by a 10-minutes wash in 2.5% sodium sulphite, a 5-minutes wash in 75% ethanol, a wash in sterile water, and a final rinse in 10% sodium bicarbonate for 10 minutes to disrupt the plant tissues and inhibit the fungal growth. At this point, the final washed solution was spread onto Yeast extract Malt extract agar containing nalidixic acid (50 mg/lit) and nystatin (100 mg/lit) and observed for microbial growth to validate the surface sterilized protocol. After the sterility check, the surface sterilized tissues were subjected to continuous drying at 100°C for 15 minutes. Surface-treated tissues were then pretreated by the following method.

High speed centrifugation method for selective isolation of actinomycetes has been carried out with slight modifications [4]. Five grams of the sterility checked and surface-treated leaf tissues were placed in a 50 ml volume centrifugation tube, containing 5 ml of sterile tap water and stirred with a mixer for 30 seconds. After allowing it to stand at 27°C for 60 minutes, the suspension was centrifuged at 3000 rpm for 10 minutes to remove soil particles. The supernatant was then centrifuged at 10000 rpm for 10 minutes and the resulting supernatant was further centrifuged at 20000 rpm for 20 minutes. The resulting supernatant was filtered through 0.22 µm pore size membrane filter and the condensed supernatant was streaked onto Humic acid Vitamin agar supplemented with nalidixic acid (50 mg/lit) and nystatin (100 mg/lit). The media was then incubated at room temperature (28 ± 2ºC) for 2 to 8 weeks.

2.3 Identification of actinomycetes

The isolates picked up from Humic acid Vitamin agar plates were purified on Yeast extract Malt extract media and incubated at room temperature (28 ± 2ºC) for 30 days. The isolates were identified according to morphological criteria, including characteristics of colonies on plate, morphology of substrate and aerial hyphae, morphology of spores and pigment production [5,6]. The isolates were preserved in 20% (v/v) glycerol for subsequent investigation.

2.4 Microbes used

ATCC cultures like *Escherichia coli* (25922), *Staphylococcus aureus* (25923), *Enterococcus faecalis* (29212) and *Pseudomonas aeruginosa* (27853) were obtained from Piramal Diagnostics, Mumbai. Tea plant pathogens like *Macrophoma* sp, *Hypoxylon serpens, Pestalopsis theae, Glomerella cingulate* and *Botryiplodia theobromae* were collected from UPASI, Valparai. Other lab isolates like *Klebsiella pneumoniae, Acinetobacter* sp., *Bacillus* sp., *Vibrio cholera, Shigella dysenteriae, Aspergillus niger* and *Candida albicans* were collected from PSG Hospital and Microlab, Coimbatore. All these isolates were used for this study.

2.5 Primary screening

Primary streaking was performed by cross streak method as described by Sivakumar *et al* [7]. A loop full of actinobacterial inoculum was streaked in the middle of the petri dish containing MHA agar medium. After inoculation, petri dishes were incubated at 28±2°C for 5 days for actinomycetes. Same way, a fresh colony of bacterial strain was streaked and incubated at 30°C for 24 hours to allow the isolates to secrete antibiotics into medium as described by Ahmed *et al* [8]. 24hrs old pathogenic strains were cross streaked to the growth line of antimicrobial metabolite producing actinomycetes and bacteria. Each streaking was started near the edge of the plates and streaked toward the central growth line and incubated at 37°C for 24-48 hrs. The inhibition zone produced between the bioactive strains and the pathogenic bacteria were measured.

2.6 Secondary screening

Isolates that showed broad spectrum against test pathogens in primary screening were further subjected to secondary screening by Kirby Bauer paper disc method. Bioactive isolates were inoculated into 50 ml of Yeast extract Malt extract broth and incubated at 28±2°C for 5 days at 180 rpm. The culture broth was centrifuged and the activity of the supernatant was determined against test organisms by adding 0.1 ml of the culture filtrate into 6mm sterile paper disc. The discs were then placed on fresh lawn culture of test organisms, kept at 4 ºC for 30 min for the diffusion of the culture broth [9] and then incubated at their respective optimum temperature. Each test was repeated three times and the antibacterial activity was expressed as the mean of diameter of the inhibition zones (mm) produced by the secondary metabolite when compared to controls, which was recorded after 18 - 24 hours and for yeasts after 2 -3 days.

2.7 Genomic DNA Isolation

The total genomic DNA from actinomycetes was isolated by following standard methods [10]. Briefly, the strain was grown in tryptic soy broth (TSB) for five days and the mycelium was separated by centrifugation and washed thrice with distilled water. Approximately 200 mg of mycelium was resuspended in 800 pl of lysis solution (100 mM Tris HC1, pH 7.5, 20 mM EDTA, 250 mM NaC1, 2% SDS, 1 mg/ml lysozyme); to the cleared lysate, *5* pl of RNase (50 mglml) was added and incubated at 37°C for 3 hr. Then. 10 p1 of proteinase K solution (20 mglml) was added and incubated at 37°C for 1 hr. The lysate mas extracted with an equal volume of pheno1: chloroform (24:l), then centrifuged to obtain the aqueous phase. DNA was precipitated by adding 2 volumes of 95% ice cold ethanol to the aqueous phase. After centrifugation, the DNA pellet was washed twice with 70% ethanol and resuspended in 50 p1 of TE buffer (10 mM Tris HCl pH 7.4 and 1 mM EDTA pH 8). The DNA was tested for purity and quantity by spectrophotometer at 260 and 280 nm.

2.8 PCR amplification

16S rRNA gene of the actinomycetes strain was amplified between the positions 8 to 1492 using forward and reverse primers mentioned below.

Forward primer: 5'- AGAGTTTGATCMTGGCTCAG -3'

Reverse primer: 5'- TACGGYTACCTTGTTACGACTT -3'

94°C	94°C	55°C	72°C	72°C
5 min	30 sec	30 sec	1 min	5 min
Initial Denaturation	Denaturation	Annealing	Extension	Final Extension
	35 cycles			

A positive control (*E.coli* genomic DNA) and a negative control in the PCR were also included.
Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were resolved on an Applied Biosystems model 3500XL automated DNA sequencing system (Applied BioSystems, USA).

2.9 Sequence similarities and Phylogeny analysis

Nucleotide sequence of 16S rRNA of actinomycetes strain was determined and compared for similarity level with the reference species of present in genomic database bank. The NCBI BLAST program was employed in order to assess the degree of DNA similarity. Multiple sequence alignment and the phylogenetic tree was constructed using MEGA software [11].

3. RESULTS AND DISCUSSION

The isolation procedure is a critically important step in studies of endophytic actinobacteria. It is necessary to improve traditional selective isolation methods in order to recover the untapped majority of rare endophytic actinobacteria [3]. The surface sterilization was proved effective due to absence of microbial growth by spreading the final washed solution onto Yeast extract Malt extract agar. Hence, the isolated microbes from the surface sterilized leaves when inoculated on another media will be considered as endophytes.

By inoculating the surface sterilized Tulsi leaves in Humic acid Vitamin agar, eleven actinomycetes strains were isolated. Of these, four actinomycetes strains showed antagonistic activity against tea pathogens and human pathogens. Crude extract obtained from their bioactive strains were utilized for secondary screening and the results were obtained in the Table 1.

Among the four actinobacterial strains, Isolate no. 5 showed maximum activity against all the human pathogenic microorganisms such as *Staphylococcus aureus* (21mm), *Candida albicans* (18 mm), *Enterococcus faecalis* (19mm), *Vibrio cholera* (18mm) and tea pathogens such as *Glomerella cingulata* (14mm) *Hypoxylon serpens* (17mm) and *Pestalopsis theae* (18mm) (Table 1).

Table 1. Antimicrobial screening of the given isolates against different plant and human pathogens (Secondary screening)

S.No.	Test organisms	Inhibition zones (mm)			
		2	**3**	**5**	**9**
1	*Macrophoma* sp.	8	10	12	9
2	*Hypoxylon serpens*	-	9	17	11
3	*Pestalopsis theae*	9	10	18	8
4	*Glomerella cingulata*	11	-	14	-
5	*Botryiplodia thebromae*	10	9	13	-
6	*Escherichia coli*	-	5	-	-
7	*Klebsiella pneumoniae*	9	-	14	12
8	*Pseudomonas aeruginosa*	-	10	13	-
9	*Acinetobacter* sp.	10	11	13	10
10	*Bacillus* sp.	11	12	15	7
11	*Vibrio cholerae*	-	10	18	14
12	*Staphylococcus aureus*	15	12	21	20
13	*Shigella dysenteriae*	10	10	12	11
14	*Enterococcus faecalis*	7	13	19	10
15	*Candida albicans*	11	12	18	14

Isolate no. 5 produced irregular, white powdery colonies with reddish brown color on reverse side of Yeast extract Malt extract agar surface. Dark brown melanin pigment was produced on Tyrosine agar. By viewing under 1000X magnification, gram positive rods with rounded ends; branched hyphae with non-motile spores were found. Therefore, based on actinomycetes identification key [5,6], the isolate was assigned to the genus *Nocardiopsis.*

The amplified products were visualized on 2% ethidium bromide (10mg/ml) stained agarose gel using UV transilluminator. Unincorporated PCR primers and dNTPs from PCR products were removed by using Montage PCR Clean up kit (Millipore). The purified PCR products of approximately 1,400 bp were sequenced by using 2 primers as described above.

The 16S rRNA sequence of isolate 5 (around 1088 bp) was determined and deposited in Genebank under the accession number **KF909127**. Using BLAST search in the NCBI data bank, sequence homologous to our isolate was collected. The DNA sequences were aligned and phylogenetic tree was constructed using MEGA4 software (bootstrap method) [11]. (Fig.1).The evolutionary history was inferred using the Neighbor joining method. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) was shown next to the branches. Only values greater than 50% were shown.

Fig. 1. Phylogram depicting the taxonomic position of *Nocardiopsis* sp.5

Comparison of the 16S rRNA nucleotide gene sequence of *Nocardiopsis* strain A5 with corresponding *Nocardiopsis* sequences clearly showed that the organism form a distinct phyletic line in the *Nocardiopsis* spp. (Fig. 1). The isolate was related to the type strain of *Nocardiopsis synnemataformans* and *Nocardiopsis dassonvillei* sharing a 16S rRNA gene sequence similarity of 99% each. Still, DNA-DNA hybridizations and phenotypic comparisons need to be performed to confirm its novelty.

4. CONCLUSION

Further purification, structure elucidation and characterization of the antimicrobial compound from the endophytic rare actinomycete isolate *Nocardiopsis* 5 strain are recommended to know the quality and novelty which can be applied for the treatment of different pathogenic infections since the test isolate shows prominent antimicrobial activity. Hence, there is definite scope for bioprospecting of antagonistic actinomycetes from Western Ghats once appropriate consistent studies are undertaken.

REFERENCES

1. Akshatha M.D., Manjunatha B.K., Pooja.R., Umesh.T.M., Sreevijeth.R. Screening for Novel Antibiotic Producing Actinomycetes from Western Ghats of Karnataka State, India. Indian journal of Research. 2013; 2(2).
2. Sonashia Velho-Pereira & Nandkumar M. Kamat. Actinobacteriological research in India. Indian Journal of Experimental Biology. 2013; 51: 573-596.
3. Qin, S., Li, J., Chen, H.-H., Zhao, G.-Z., Zhu, W.-Y., Jiang, C.-L.*, et al.* Isolation, diversity, and antimicrobial activity of rare actinobacteria from medicinal plants of tropical rain forests in Xishuangbanna China. Appl. Environ. Microbiol. 2009; 75: 6176–6186.
4. Kizuka M, Enokita R, Takahashi K, Okazaki T. Distribution of the actinomycetes in the Republic of South Africa investigated using a newly developed isolation method. Actinomycetologica 1997; 11:54–58.
5. Shirling, E.B. and Gottlieb. D. Methods for characterization of *Nocardiopsis* species. Int J Syst Bacteriol. 1966; 16:313–340.
6. Williams S.T, Sharpe M.E. and Holt J.G. Bergey's Manual of Systematic Bacteriology. Vol 4. Williams and Wilkins, Baltimore, 1989.
7. Siva kumar J, Santhanam P and Masilamani Selvam M. Antimicrobial activity of actinomycetes isolated from the Western Ghats of Tamilnadu. International Journal of Pharma and Bio Sciences 2011; 2(1).

8. Ahmed.N, Uzair.B, Ayaz.S, Ahmed.V. Antibacterial activity of marine bacteria from Arabian Sea of Pakistan. The Internet Journal of Microbiology. 2007; 4(2).
9. Deepika Sharma, Talwinder Kaur, Chadha BS and Rajesh Kumari Manhas. Antimicrobial activity of actinomycetes against Multidrug Resistant *Staphylococcus aureus*, *E. coli* and various other pathogens. Tropical Journal of Pharmaceutical Research 2011; 10 (6): 801-808.
10. Boudjella H., Bouti K, Zitouni A., Mathieu F., Lebrihi A. and Sabaou N. Taxonomy and chemical characterization of antibiotics of *Streptosporangium* Sg 10 isolated from a Saharan soil. Microbiol. Res. 2006; 161:288-298.
11. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, and Kumar S. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance and Maximum Parsimony Methods. Molecular Biology and Evolution 2011; 28: 2731-2739.