Short Communication

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

ABSTRACT

Aims: To isolate and identify the endophytic *Nocardiopsis* sp.5 from Tulsi (*Ocimum sanctum*) leaves 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 – 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 these isolates by primary and secondary streaking methods. Morphological and Molecular Identification of the most bioactive isolate.
Results: Out of 11 endophytes, four isolates (2, 3, 5, and 9) showed antagonistic activity against tea and human pathogens. Among these 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, showing 99% similarity to both *Nocardiopsis synnemataformans* and *Nocardiopsis dassonvillei*.
Conclusion: Endophytic *Nocardiopsis* strain 5 was shown to produce good antimicrobial activity against plant pathogens and human pathogens. Further purification, structure elucidation and characterization of the antimicrobial compound from *Nocardiopsis* 5 strain are recommended. There is definite scope for bioprospecting of antagonistic actinomycetes from Western Ghats once appropriate consistent studies are undertaken.
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Keywords: Endophytic actinomycetes, *Tulsi (Ocimum sanctum),* antimicrobial activity, *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. Even, rare actinomycetes strain like *Nocardiopsis* from marine sediment exhibited antimicrobial activity against multi-resistant bacteria through the production of TP-1161 antibiotic [2] which focusses the researchers' direction towards rare microbes that proved to be the source of potent antibiotic. Soil habitats (96%) have been largely surveyed as compared to other terrestrial resources like endophytic plants (3%) and animal guts (1%) [3]. 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 from these plants.

Therefore, the present research has been targeted on exploring the rare endophytic actinomycetes from the leaves of Tulsi (*Ocimum sanctum)*) and examining their antimicrobial activity against tea pathogens and human pathogens. The isolate that showed maximum bioactivity was identified by both morphological and molecular studies. This is the first work of isolating non-streptomycetes strains from Tulsi leaves.

2. MATERIAL AND METHODS

2.1 Collection of sample medicinal plant

The fresh leaves of common medicinal plant Tulsi (*Ocimum sanctum)* were collected from the medicinal plant garden of Nirmala College for Women, Coimbatore (11.0183° N, 76.9725° E) belonging to Western Ghats region during December 2011 to January 2012. The voucher specimen of *Ocimum sanctum* was preserved in the Herbarium of Botany department of Nirmala College for Women. Surface sterilization of these leaves should be started within four days followed by isolation work. In the meantime, these leaves shall be preserved in refrigerator.

2.2 Isolation of endophytic actinomycetes

The collected Tulsi (*Ocimum sanctum)* leaves were washed by tap water to remove the soil particles and subjected to surface sterilization procedure as per the method of Qin *et al* [4] [5]: washing the leaves in 5% sodium hypochlorite for 4-10 minutes followed by 10-minutes wash in 2.5% sodium sulphite, a5-minutes wash with 75% of ethanol solution, a quick rinse in sterile water followed by final rinse using 10% sodium bicarbonate solution for 10 minutes to disrupt the plant tissues and inhibit the fungal growth. The finally washed solution was inoculated onto Yeast extract Malt extract agar with nalidixic acid (50 mg/lit) and nystatin (100 mg/lit) and observed for microbial growth to check the efficiency of surface sterilization.

Then, the surface sterilized tissues were dried at 100℃ for 15 minutes before inoculation.

High speed centrifugation method for selective isolation of non-streptomycetes has been carried out with slight modifications [5, 6]. Five grams of surface sterilized leaves were centrifuged with 5 ml of sterile tap water for 30 seconds. After allowing it to stand at 27℃ for 60 minutes, the suspension was again centrifuged at 3000 rpm for 10 minutes in order 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 for the growth of rare isolates.

2.3 Identification of actinomycetes

The endophytic actinomycetes isolated from Humic acid Vitamin agar plates were purified on Yeast extract Malt extract (ISP2) media and incubated at 28 ± 2ºC for 30 days. The pure colonies were identified morphologically including both microscopic and macroscopic characteristics [7, 8]. 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* [9]. 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℃ for 5 days for actinomycetes. S ame way, a fresh colony of bacterial strain was streaked and incubated at 30℃ for 24 hours to allow the isolate s to secrete antibiotics into medium as described by Ahmed *et al* [10]. 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℃ for 24-48 hrs. The inhibition zon e produced between the bioactive strains and the pathogenic bacteria were measured. For fungal cultures, the same cross streak methodology has been followed on Sabourauds Dextrose agar and incubated at 24℃ for five days.

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℃ 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 [11] 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. Same way, spot inoculation method was followed in order to detect antifungal activity against tea pathogens and the width of zone of inhibition was measured after incubation at 24℃ for 5 days.

2.7 Genomic DNA Isolation

The total genomic DNA from actinomycetes was isolated by following standard methods [12]. 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℃ for 3 hr. Then. 10 p1 of proteina se K solution (20 mglml) was added and incubated at 37℃ 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 to run PCR. Sequencing was performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA). Sequencing products were determined on an Applied Biosystems model 3500XL automated DNA sequencing system (Applied BioSystems, USA) [5, 13].

2.9 Phylogenetic identification

The 16S rRNA sequence that was retrieved from actinomycetes strain was proceeded with sequence similarity search using NCBI BLAST program. Multiple sequence alignment was carried out followed by phylogenetic tree construction using MEGA6 software [5, 14].

3. RESULTS AND DISCUSSION

Since actinomycetes produce about 70% of total known antibiotics and remaining 30% are only the products of filamentous fungi and non actinomycetes bacteria [1], the focus is directed towards actinomycetes studies in plants. The isolation procedure seems to be the significant initial step for endophytic actinomycetes studies. Selective isolation strategies need to be developed further to recover the unexploited rare endophytic actinobacteria [4]. 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 (Isolate 1-11) were isolated. Of these, four actinomycetes strains (Isolate 2, 3, 5, and 9) 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 and Fig 1,2). Hence, isolate no.5 was selected for further identification studies since its zone of inhibition was more comparatively.

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

S.No.	Test organisms	Inhibition zones of actinomycetes isolates (mm)			
		Isolate 2	Isolate 3	Isolate 5	Isolate 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

Morphologically, Isolate no. 5 produced irregular, white powdery colonies with reddish brown color on reverse side of Yeast extract Malt extract (ISP2) agar surface (Fig. 3). 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 (Fig. 4). Therefore, based on actinomycetes identification key [7, 8], the isolate was assigned to the genus *Nocardiopsis.*

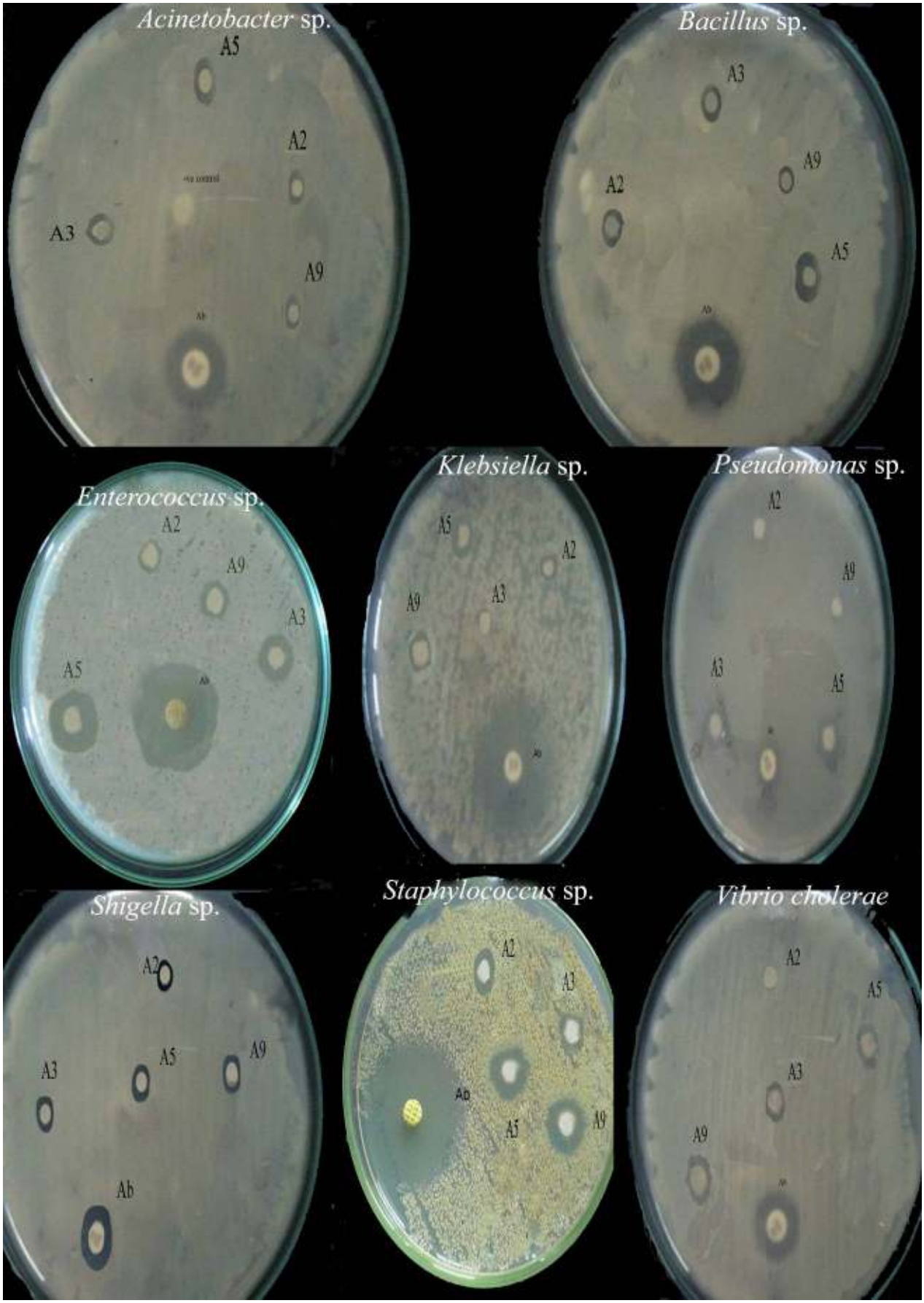

Acinetobacter sp.
A5
A2
A3
A9
Bacillus sp.
A3
A9
A2
A5
Enterococcus sp.
A2
A9
A3
A5
Klebsiella sp.
A5
A2
A9
A3
Pseudomonas sp.
A2
A9
A3
A5
Shigella sp.
A2
A3
A5
A9
Ab
Staphylococcus sp.
A2
A3
A5
A9
Vibrio cholerae
A2
A5
A3
A9

Fig. 1. Antibacterial activity of actinobacterial isolates

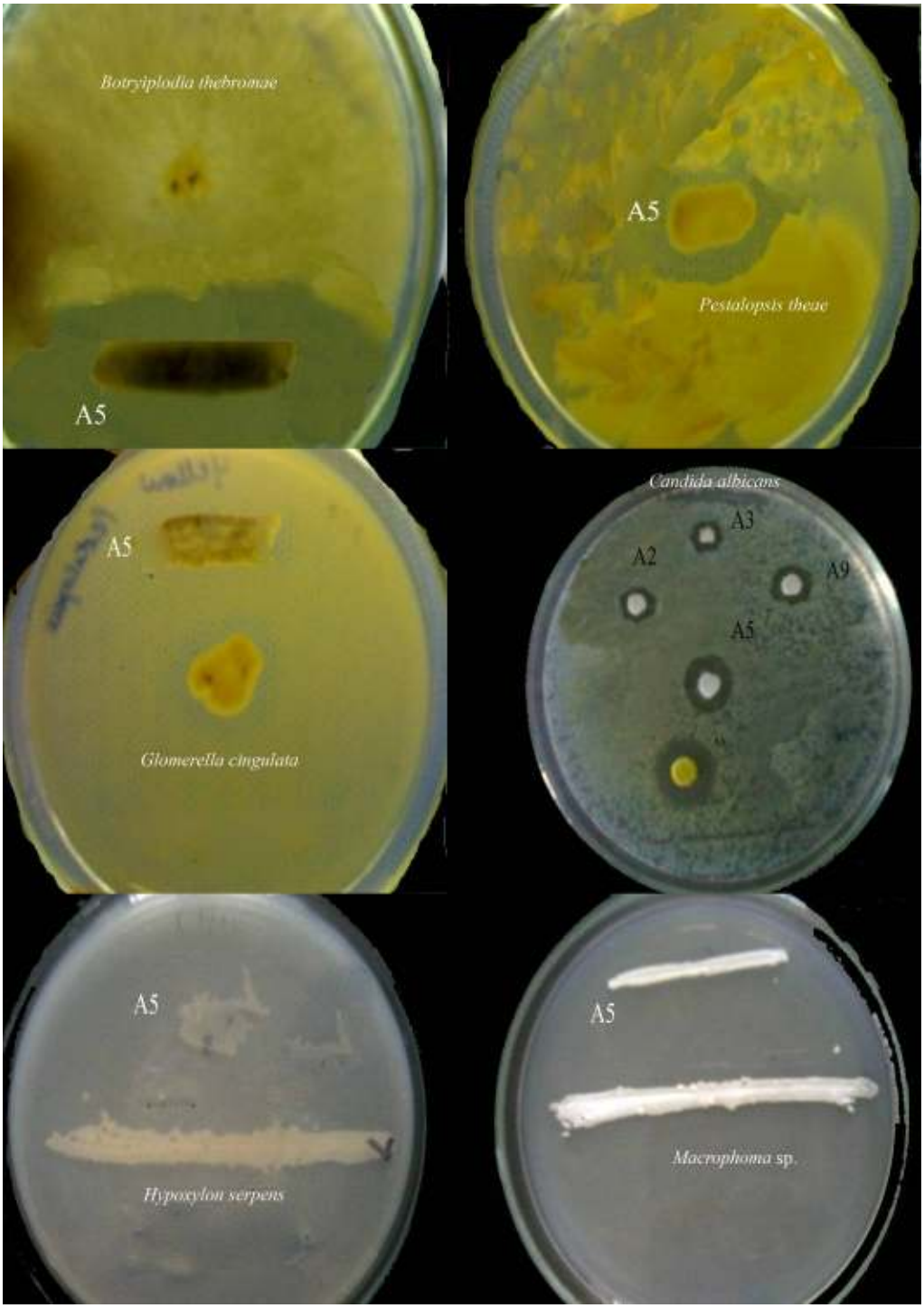


Fig. 2. Antifungal activity of actinobacterial isolates

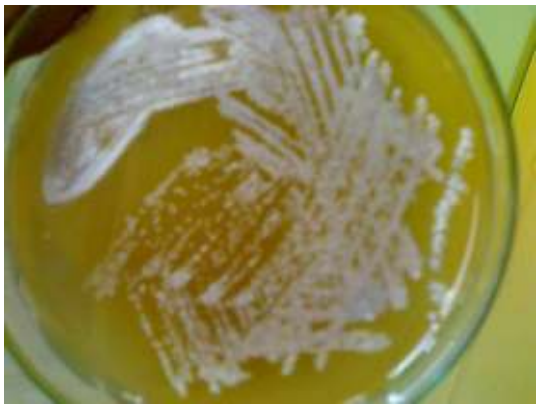


Fig. 3. *Nocardiopsis* sp. 5 growth in ISP2 agar

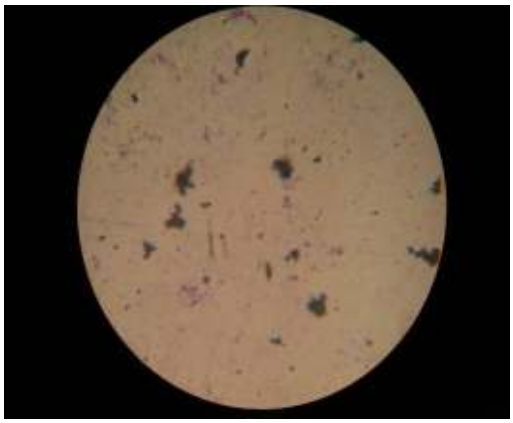
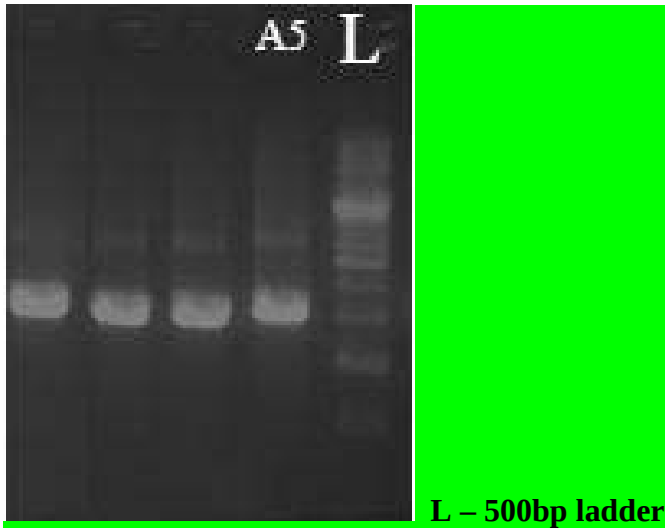


Fig. 4. Microscopic structure of *Nocardiopsis* sp.5

Fig.5. PCR Amplification of 16S rRNA gene of *Nocardiopsis* sp.5



Under molecular characterization, the PCR amplified product of 16S rRNA gene from Isolate 5 were visualized on 2% ethidium bromide (10mg/ml) stained agarose gel using UV transilluminator (Fig 5). 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 two primers as described above [15].

The 16S rRNA sequence of isolate 5 (around 1088 bp) was determined and deposited in Genbank under the accession number **KF909127**. Using BLAST search in the NCBI data bank, the corresponding sequences homologous to our test isolate were collected and the phylogenetic tree was constructed by employing MEGA6 software (bootstrap method) [14]. (Fig.6).The evolutionary history was predicted by using Neighbor joining method. The percentage of replicate trees where the related taxa grouped together in bootstrapping test based on thousand replicates was shown clearly next to their branches [5].

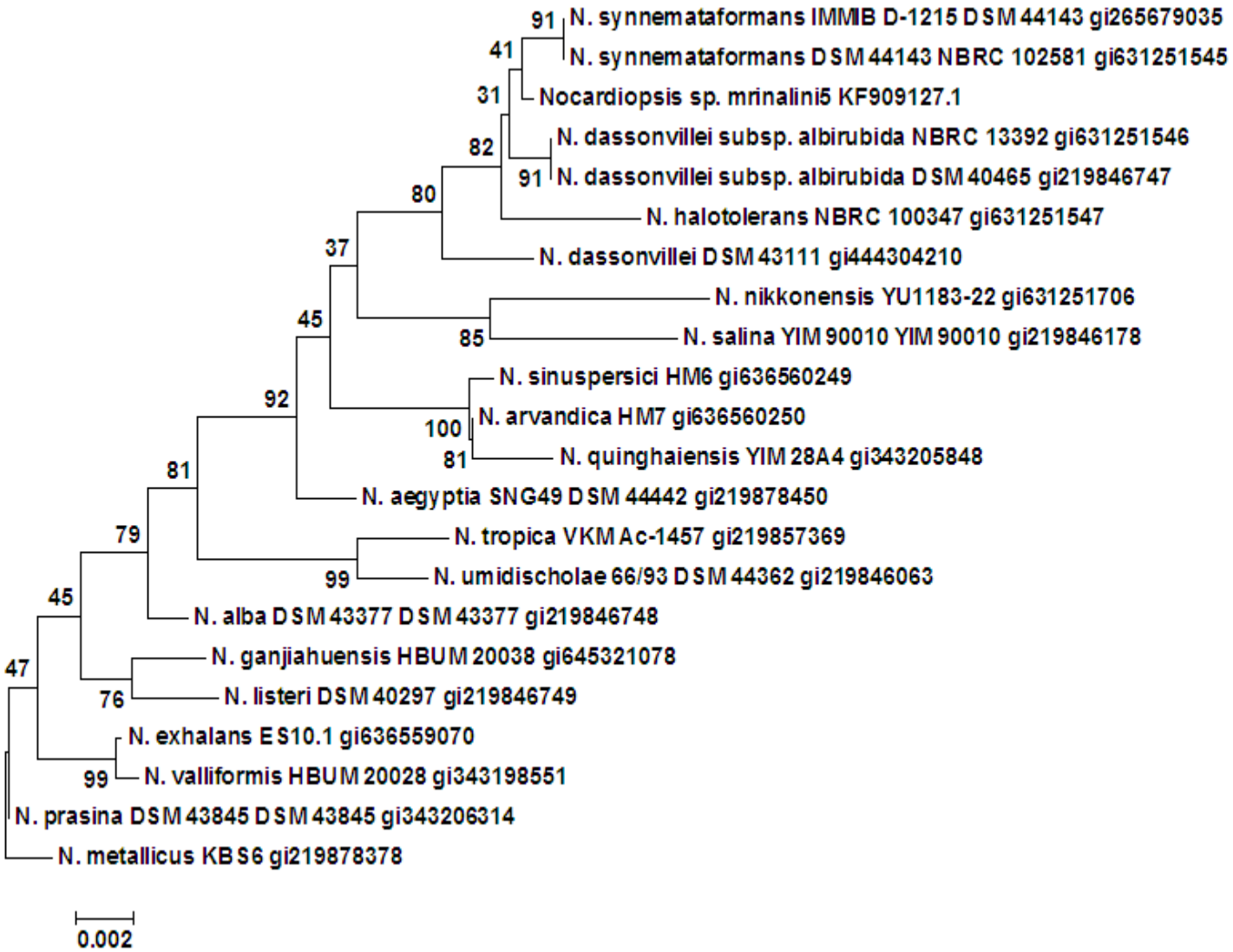


Fig. 6. Phylogram depicting the taxonomic position of *Nocardiopsis* sp.5

The comparison of 16S rRNA sequence of *Nocardiopsis* strain A5 with corresponding homologous DNA sequences clearly revealed that the test organism form a distinct phyletic line in the *Nocardiopsis* spp. (Fig. 6) [5]. The phylogeny tree clearly depicted that the isolate A5 was related to both *Nocardiopsis synnemataformans* and *Nocardiopsis dassonvillei* by sharing a 16S rRNA gene sequence similarity of 99% each. *Nocardiopsis metallicus* was selected as outgroup. Gene cloning of 16S rRNA gene can also be considered further to obtain its nearly full length of around 1400 bp for taxonomic characterization. Still, DNA-DNA hybridizations and phenotypic comparisons need to be performed to confirm its novelty.

4. CONCLUSION

Isolate no. 5 which was shown to belong to *Nocardiopsis* genera of rare endophytic actinomycetes was shown to produce good antimicrobial activity against both plant pathogens and human pathogens. Further purification, structure elucidation and characterization of the antimicrobial compound from the 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 other medicinal plants once appropriate consistent studies are undertaken.

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