International Journal of Pharmacological Research
ISSN: 2277-3312 (Online)
Journal DOI: https://doi.org/10.7439/ijpr **Research Article**

Antimicrobial Potential of Endophytes from *Datura metel* L.

Madhu Priya M.* and P. Agastian Simiyon Theoder

Plant Biology and Biotechnology, Loyola College, University of Madras, Chennai, India

QR Code

***Correspondence Info:**
Madhu Priya M.
Plant Biology and Biotechnology,
Loyola College, University of Madras,
Chennai, India

***Article History:**
Received: 15/03/2018
Revised: 06/04/2018
Accepted: 06/04/2018
DOI: https://doi.org/10.7439/ijpr.v8i4.4696

Abstract

Endophytes are a suite of microorganisms that grow in the tissues of higher plants. Endophytes constitute a valuable source of secondary metabolites for the discovery of new potential therapeutic drugs. Endophytes can have many effects on their host such as enhancement of stress, insect and disease resistance, and herbicide activities when in association with their host plant. The present study was to examine the antimicrobial activity of ethyl acetate extracts of endophytic fungi (both extra and intracellular) and actinomycete from the stem and root of *Datura metel L.* The crude extract of these endophytic isolates were screened for their antimicrobial potential. Among the endophytes, the extracellular fungal extract showed the highest antimicrobial activity when compared to intracellular fungi and actinomycetes. The studies and isolation of these compounds can be used as a good approach to search of novel products.

Keywords: Endophytes, actinomycete, antimicrobial, extracellular and intracellular.

1. Introduction

Datura metel L. is a medicinal plant which is commonly called as devil trumpet. It belongs to the family solanaceae. It is a short perennial shrub to about 4 – 6 ft. Branches, leaves and stems are tinted purple. The giant flowers can grow to 12". The plant germinates quickly from seed and can bloom in just a few months from planting. All parts of this plant are extremely toxic and should not be ingested in any form. (Figure 1 & 2)

Figure 1: Habit of *Datura metel*

Figure 2: Flower of the plant

Endophytes colonize plant tissues and remain within the tissue, except that fruiting structures may emerge through the surface of the plant tissue. Indeed, leaves may be fully colonized by a variety of fungi within a few weeks of leaf emergence [1]. The colonies remain asymptomatic and some in perennial plant parts may have a very long life.

Endophytic fungi, defined as fungi inhabiting inside healthy plant tissues, are now considered as ubiquitous symbionts of plants from their surprisingly common detection from many plant species [2]. Studies of endophytic fungi in tree leaves have been carried out for many host species since the latter half of the 1970s when their significance as common symbionts and possible mutualists of plants were recognized [3-5].

Endophytic actinomycetes are considered to be potential bio control agents for they can colonize the interior of the host plant avoiding competition by any other microbes in the soil. The actinomycetes are Gram positive, free living, saprophytic bacteria and ubiquitous in nature. Majority of them are found in soil, fresh waters, and surface of water bodies and also in seawater. Odor of freshly turned soil comes from volatile compounds produced by these bacteria. Colonies have pastel colors, soil like odor and are hard and stick into agar. Actinomycetes population has been identified as one of the major group of soil population,

which may vary with the soil type. Soil rich in organic matter is highly suitable for the growth of actinomycete bacteria. The metabolites produced by these organisms are highly effective and biologically active and remain a potent source for antibiotics.

2. Materials and methods

2.1 Isolation and identification of endophytes

Stems and roots of healthy plants of *Datura metel* L. were collected. According to Fisher *et al* [6], different plant parts such as stems and roots of *Datura metel* L. (0.5 - 1.0 cm in diameter) were washed in running tap water several times to remove soil particles. Then they were surface sterilized by sequential immersion in 3 - 5% sodium hypochlorite for 3 min, followed by rinsing with sterile water and further treated with 70% ethanol for 30 seconds. Later the samples were cleaned several times in sterile distilled water. Surface sterilized stem and root samples were split into pieces of 1.0 cm to expose the cortex and vascular bundles. They were then aseptically transferred to petri dishes containing Starch Casein Agar (SCA) for isolation of actinomycetes and Water Agar for isolation of fungi. Nalidixic acid (100 µg/ml) and Actidione (20 µg/ml) were added to Starch Casein Agar to suppress the fungal growth. Streptomycin (250 mg/L) was added to Water Agar to suppress the bacterial growth. Plates were then incubated at 28ºC for a maximum period of three weeks. Actinomycetes and fungi growing on the medium were isolated, subcultured and identified. (Figure 3 & 4)

2.2 Identification of endophytic fungi

The endophytic fungus isolated on Water Agar was subcultured on Potato Dextrose Agar and maintained as pure culture for identification. The fungus was cultured on Synthetic Nutrient Agar (SNA) for 14 days at 25 ºC under near UV-light for sporulation. The cultures were then observed for the morphological characteristics such as the mycelium and the sporulation. The endophytic fungi exhibited grey colour, warty colony texture and non-spore forming on Water Agar. Colonies were found to be fast-growing, reaching 4.5 cm diameter in 4 days at 25ºC, aerial mycelium sparse to abundant and floccose white turning violet or purple on further incubation. The endophytic fungus isolated from the stem of *Datura metel* L. was identified as *Fusarium oxysporum* by staining with lactophenol cotton blue. (Figure 5 & 6)

2.3 Identification of endophytic actinomycetes

Methods and media described by the International Streptomyces Project [7] were used to determine the cultural and physiological features. For morphological characteristics, the presence of aerial hyphae, spore color, distinctive colony color, diffusible pigment synthesis, sporophore and spore chain morphology was noted after 10 days of incubation on ISP-2 medium. The slide culture was identified for microscopic characteristics using Bergey's manual [8]. The culture retained the pink colour and it was confirmed as a gram negative organism (Figure 7). The isolated actinomycete was given for nucleotide sequencing to Lab India Instrument Pvt. Ltd., Haryana. The nucleotide sequence of the isolated actinomycete was subjected to nucleotide blast (BLASTN 2.2.24) for its identification. The endophytic actinomycete isolated from the root of *D. metel* L. was identified as *Stenotrophomonas sp*. by nucleotide blast (BLASTN 2.2.24) (Table 1).

2.4 Mass culture and preparation of crude extracts

The isolated fungus and actinomycete were mass cultured on Modified Nutrient Glucose Broth (MNGB) and Potato Dextrose Broth (Himedia, Mumbai) respectively. The culture flasks inoculated with fungal isolate was maintained as still culture for 10 days at room temperature. The culture flasks inoculated with actinomycete were incubated in rotary shaker at 120 rpm for about 10 days. The broth with full grown culture was centrifuged at 8000 rpm at 4°C for 10 min. The supernatant was collected and dissolved in equal volume of ethyl acetate and the organic layer was separated using the separating funnel. The fungal mat was collected separately for intracellular extraction. Take a small amount of ethyl acetate and homogenise the fungal mat in it. Then filter the extract through the muslin cloth. Measure the filtrate and add equivalent amount of ethyl acetate to it. The organic layer was separated using separating funnel. The crude extract was concentrated on Rota vacuum evaporator and stored at 4°C until further use.

2.5 Antibacterial activity by disc diffusion method

The crude ethyl acetate extracts of endophytic fungus and actinomycete are used to determine the antibacterial activity against a number of bacterial cultures and clinical isolates by disc diffusion method [9]. According to this method, 20 ml of sterile Mueller Hinton Agar (MHA) (Hi-media, Mumbai) was poured into sterile petri plates and solidified. The bacterial strains (100 µl of suspension containing 10^8 CFU/ml bacteria) were swabbed on top of the media and allowed to dry for 10 min. The tests were conducted at three different concentrations of the crude extract, i.e., 5, 2.5 and 1.25 mg/disc. The loaded discs were placed on the surface of the medium and left for 30 min at room temperature for compound diffusion. Streptomycin (10 µg/disc) was used as positive control. The plates were incubated for 24 h at 37°C. Zone of inhibition was recorded in millimeters and the experiment was repeated twice.

2.6 Antifungal assay

The antifungal activity was performed according to the standard reference method (NCCLS, 2002). The extracts were dissolved in water with 2% dimethyl

sulfoxide (DMSO). The initial concentration of the extract was 1 mg/ml and this concentration was serially diluted. Each well was inoculated with 5 µl of suspension containing 10^4 spores/ml. The antifungal agent's fluconazole and ketoconazole are used as positive controls. Minimum Inhibitory Concentration (MIC) was defined as the lowest concentration showing no visible fungal growth after incubation time.

The following fungi were used to determine the minimum inhibitory concentration of crude ethyl acetate extracts: *Aspergillus flavus, Curvularia lunata* 46/01, *Aspergillus niger* MTCC 1344, *Trichophyton rubrum* 57/01, *T. mentagrophytes* 66/01 and *Alternaria solani*. The filamentous fungi were cultured on Sabouraud Dextrose Agar (SDA) slants at 28°C for 10 days and the spores were collected using sterile double distilled water and homogenized.

3. Results and discussion

In the present study, the antimicrobial activities of crude extracts of endophytic fungi (both extracellular and intracellular) and actinomycete were tested against selected reference cultures.

The extracellular fungal extract was found to exhibit significant antibacterial activity compared with intracellular which shows higher activity of about (27 mm) against *Enterobacter aerogens* when compared to positive control, followed by 26 mm of zone of inhibition against *E. faecalis.* Minimum activity of about 20 mm was found against *Staphylococcus aureus* (Figure 8). Comparatively, the intracellular fungal extract showed only mild activity of about 13 mm against *Enterobacter aerogens,* followed by 11 mm of zone of inhibition against *Enterobacter faecalis* and not showing any activity against other reference cultures (Table 2).

Based on the antibacterial activity in reference cultures, they were screened for the antibacterial activity against clinical isolates as well as drug resistance microbes (Table 3). Extracellular fungal extract shows higher activity of about 29 mm of clear zone against ATCC – 29213 [MSSA], followed by 25 mm against ESBL *Klebsiella* (ICMR-6), and MRSA (clinical pathogens). Moderate activity of 20 mm was found against MRSA [Methicillin Resistant *S. aureus*] ICMR – 5 and trace activity of 12 mm was found against *Staphylococcus aureus* [Methicillin Sensitive *S. aureus*] (Figure 8). Intracellular fungal extracts showed lesser activity of about 12mm of inhibition zone against ICMR-24 [*E. coli*] Cipro R, followed by minimal activity of about 10 mm against ESBL *Klebsiella* (ICMR-6), *Klebsiella pneumonia* (clinical pathogens), *MRSA* (Methicillin Resistant *S. aureus*) ICMR-5 and ATCC – 29213 [*MSSA*] and no activity was recorded against other clinical isolates.

In the case of the ethyl acetate extracts of endophytic actinomycete, this shows lesser activity of about 10 mm of inhibition zone against *Yersinia enterocolitica* and *E. faecalis* and showed no activity against other reference cultures (Figure 8) (Table 4). Endophytic actinomycete showed activity of about 11 mm of inhibition zone against MRSA [Methicillin Resistant *S. aureus*] ICMR – 5 and ESBL, *E.coli* (clinical pathogens), tracer activity of 10 mm against ATCC – 29213 [*MSSA*] and *MRSA* (clinical pathogens) and showed no activity against some of the clinical isolates (Figure. 8) (Table 5).

Totally six fungal pathogens were used to study the antifungal activity of the crude ethyl acetate extracts of endophytic fungus and actinomycete. Based on the results obtained from the antibacterial activity, the intracellular fungal extracts have shown only trace activity or no activity against bacterial cultures. Extracellular fungal extract inhibited the growth of *T. rubrum* (25 µg/ml) and in the case of actinomycete, the minimum inhibitory concentration required to inhibit the activity of *Alternaria solani* was found to be 50µg/ml (Table 6).

Table 1: Molecular Characterization of Endophytic Actinomycete

Query ID	lcl|12475	Database Name	nr
Description	None	Description	All GenBank+EMBL+DDBJ+PDB sequences (but no Query EST, STS, GSS, Environmental samples or phase 0, 1 or 2 HTGS sequences)
Length	342		
Molecule type	nucleic acid		
		Program	BLASTN 2.2.24+
gb|HM582659.1| Stenotrophomonas sp. 4D-W-1 16S ribosomal RNA gene, partial sequence Length=708			
Score = 551 bits (298), Expect = 1e-153 Identities = 319/340 (93%), Gaps = 0/340 (0%) Strand=Plus/Plus			

```
Query  3     GGTGGCGAGTGGCGGACGGGTGAGGAATRCATCGGAATSTACTTTTTMGTGGGGGATAAC  62
             ||||||||||||||||||||||||||||  |||||||||  ||||||||  ||||||||||||
Subject  43  GGTGGCGAGTGGCGGACGGGTGAGGAATACATCGGAATCTACTTTTTCGTGGGGGATAAC  102
Query  63    GTAGGGAAACYTWMGCTAATACCGCATACGMCCTACGGGKGAAAGCAGGGGATCTTMGGA  122
             ||||||||||  |   ||||||||||||||||  ||||||||  ||||||||||||||||  |||
Subject  103 GTAGGGAAACTTACGCTAATACCGCATACGACCTACGGGTGAAAGCAGGGGATCTTCGGA  162
Query  123   CCTTGCRCGATTRAATSRGCCGATGTCGGATTAGCTAGTTGGYGGGGTAAAGGCCCACCA  182
             ||||||  |||||  |||   ||||||||||||||||||||||  ||||||||||||||||
Subject  163 CCTTGCGCGATTGAATGAGCCGATGTCGGATTAGCTAGTTGGCGGGGTAAAGGCCCACCA  222
Query  183   AGGCGACGATCCGTAGCTGGTYTGAGAGGATGAYCAGCCACACTGGRACTGAGACACGGY  242
             ||||||||||||||||||||  ||||||||||  ||||||||||  ||||||||||||
Subject  223 AGGCGACGATCCGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGT  282
Query  243   CCAGACTCCTACGGGAGGCAGCAGTGGGGAATWTTGGACAATGGGSGCAAGCCTGATCCA  302
             ||||||||||||||||||||||||||||||  ||||||||||  ||||||||||||||
Subject  283 CCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCA  342
Query  303   GCCATMCCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAG                     342
             |||||  ||||||||||||||||||||||||||||||||
Subject  343 GCCATACCGCGTGGGTGAAGAAGGCCTTCGGGTTGTAAAG                     382
```

Table 2: The ethyl acetate extracts of endophytic fungus isolated from the stem of *D. metel* L. were tested against reference cultures to prove their antibacterial activity (zone of inhibition in mm)

Test organisms	Ethyl acetate extract						
	EF (mg/disc)			IF (mg/disc)			S (µg/disc)
	1.25	2.5	5.0	1.25	2.5	5.0	10
S. aureus (ATCC 25923)	12	15	20	-	-	-	13
Y. enterocolitica (MTCC 840)	15	21	23	-	-	-	24
P. aeruginosa (ATCC 15380)	-	-	-	-	-	-	10
V. fischeri (ATCC 1738)	13	19	21	-	-	10	13
E. aerogens (MTCC 111)	17	22	27	-	10	13	19
E. faecalis (ATCC 29212)	17	23	26	-	-	11	10
Xanthomonas oryzae pv. oryzae (MTCC 2760)	14	20	24	-	-	10	11

EF – Extracellular filtrate; **IF** – Intracellular filtrate; **S** – Streptomycin

Table 3: Antibacterial test done with the extracts of ethyl acetate of fungus isolated from the stem of *D. metel* L. against clinical isolates (zone of inhibition in mm)

Code no	Test organisms	Ethyl acetate extract					
		EF (mg/disc)			IF (mg/disc)		
	Clinical isolates	1.25	2.5	5.0	1.25	2.5	5.0
17	ESBL *Klebsiella* (ICMR-6)	15	19	25	-	-	10
40	*Klebsiella pneumonia* (clinical pathogens)	10	13	15	-	-	10
28	ESBL, *E. coli* (clinical pathogens)	-	-	-	-	-	-
41	*Proteus*	-	10	11	-	-	-
21	*Staphylococcus aureus* (Methicillin sensitive *S. aureus*)	-	10	12	-	-	-
15	*MRSA* (Methicillin Resistant *S. aureus)* ICMR - 5	11	14	20	-	-	10
24	*MRSA* (clinical pathogens)	17	20	25	-	-	-
19	ICMR-24 *[E. coli]* Cipro R	10	16	19	10	10	12
7	ATCC – 29213 *[MSSA]*	14	21	29	-	-	10
20	ICMR-19 *Acetobacter baumanii* (Carbapenem R)	-	-	10	-	-	-
45	*Salmonella paratyphi*	-	-	10	-	-	-

EF – Extracellular filtrate; **IF** – Intracellular filtrate

Table 4: Antibacterial action of extracts of ethyl acetate from endophytic actinomycete isolated from the root of *D. metel* L. against reference cultures (zone of inhibition in mm)

Test organisms	Ethyl acetate extract			
	EPA (mg/disc)		S (µg/disc)	
	1.25	2.5	5.0	10
S. aureus(ATCC 25923)	-	-	-	13
Y. enterocolitica(MTCC 840)	-	-	10	24
P. aeruginosa(ATCC 15380)	-	-	-	10
V. fischeri(ATCC 1738)	-	-	-	13
E. aerogens(MTCC 111)	-	-	-	19
E. faecalis(ATCC 29212)	-	-	10	10
Xanthomonas oryzae pv. oryzae (MTCC 2760)	-	-	-	11

EPA- Endophytic actinomycete extract; **S** – Streptomycin

Table 5: Antibacterial activity done with the extracts of actinomycete isolated from the root of *D. metel* L. against clinical isolates (zone of inhibition in mm)

Code no	Test organisms	Ethyl acetate extracts		
		EPA (µg/disc)		
	Clinical isolates	1.25	2.5	5.0
17	ESBL *Klebsiella*(ICMR-6)	-	-	-
40	*Klebsiella pneumonia* (clinical pathogens)	-	-	-
28	ESBL, *E. coli* (clinical pathogens)	11	11	11
41	*Proteus*	-	-	-
21	*Staphylococcus aureus* (Methicillin Sensitive *S. aureus*)	-	-	-
15	*MRSA* (Methicillin Resistant *S. aureus*) ICMR - 5	-	-	11
24	*MRSA* (clinical pathogens)	-	-	10
19	ICMR-24 *[E.coli]* Cipro R	-	-	-
7	ATCC – 29213 *[MSSA]*	-	-	10
20	ICMR-19 *Acetobacter baumanii* (Carbapenem R)	-	-	-
45	*Salmonella paratyphi*	-	-	-

EPA - Endophytic actinomycete extract

Table 6: Antifungal activity of EPF and EPA isolated from *D. metel* L. using broth micro dilution method (MIC)

Tested fungi	EPF (µg/ml)	EPA (µg/ml)	Fluconazole (µg/ml)	Ketoconazole (µg/ml)
Aspergillus flavus	50	100	50	<12.5
Alternaria solani	100	50	25	<12.5
Curvularia lunata 46/01	50	>200	<12.5	<12.5
Aspergillus niger MTCC 1344	200	200	100	<12.5
T. rubrum 57/01	25	100	25	<12.5
*T. mentagrophytes*66/01	50	>200	25	<12.5

EPF - Endophytic fungal extract; **EPA -** Endophytic actinomycete extract

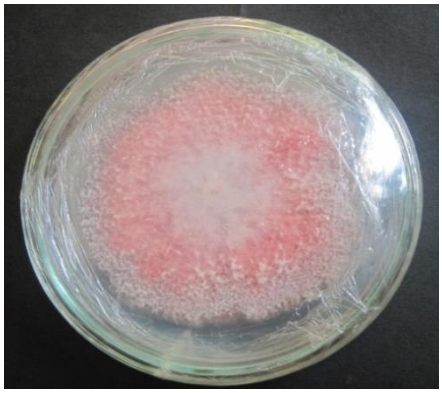
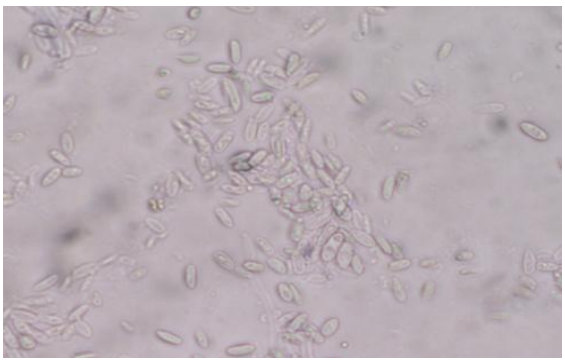


Figure 3: Isolation of endophytic fungus

Figure 4: Isolation of endophytic actinomycete



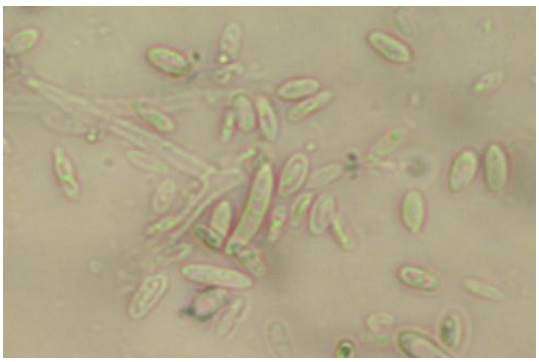


Figure 5 & 6: *Fusarium oxysporum* isolated from *D. metel* showing micro and macro conidia

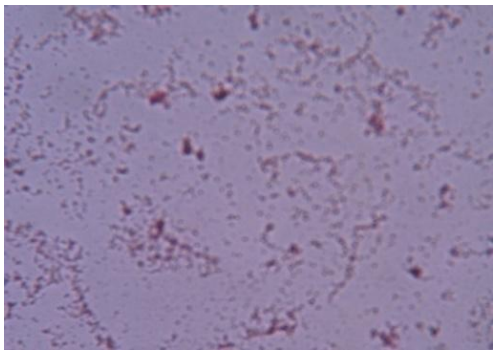
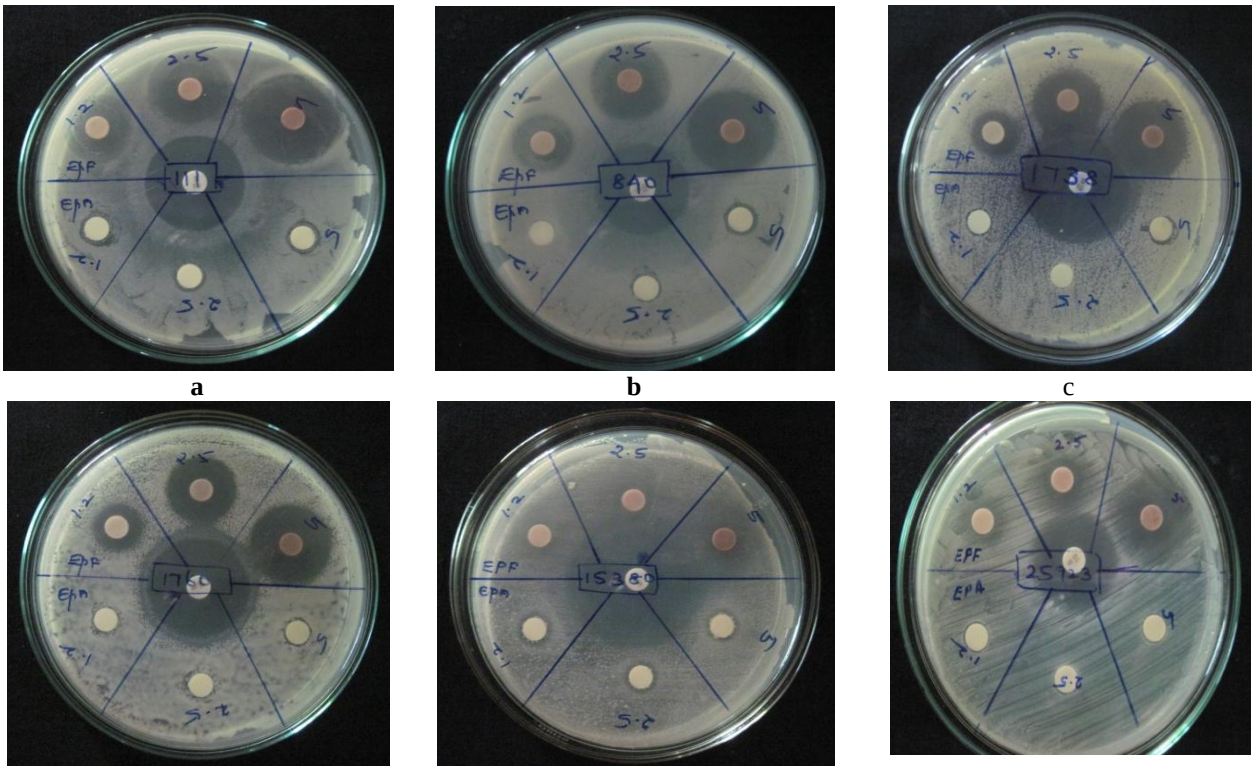


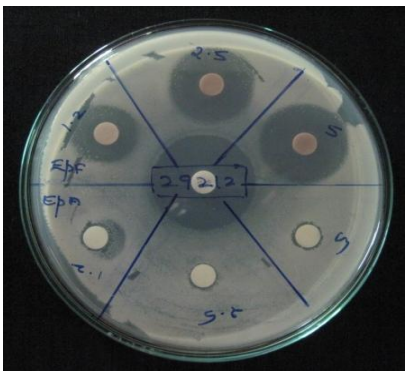
Figure 7: Gram staining of endophytic actinomycete

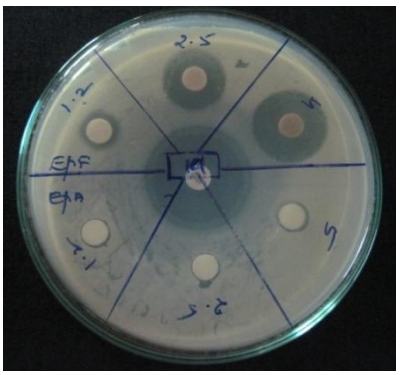
Figure 8: Antibacterial activities of ethyl acetate extract of endophytic fungus and actinomycete on different bacterial strains

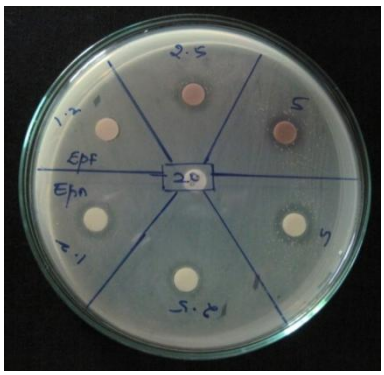


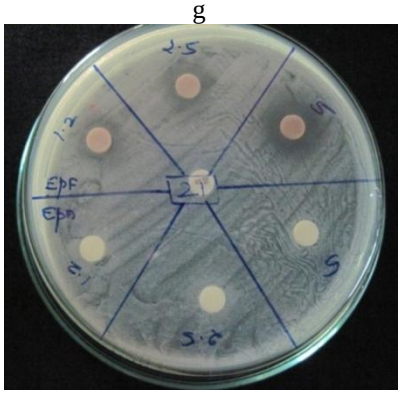
a **b** c

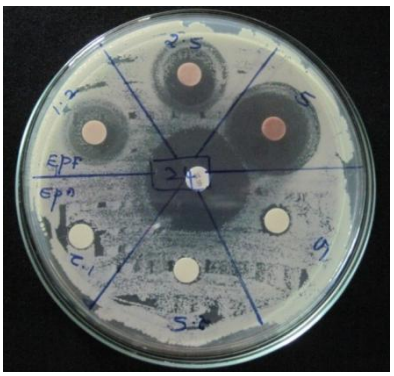
d e f

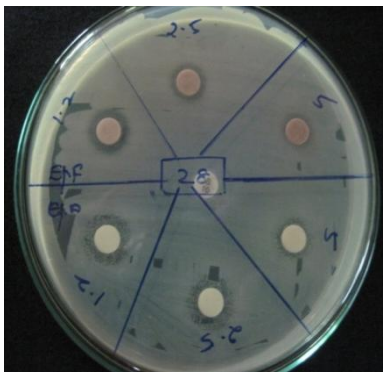

g

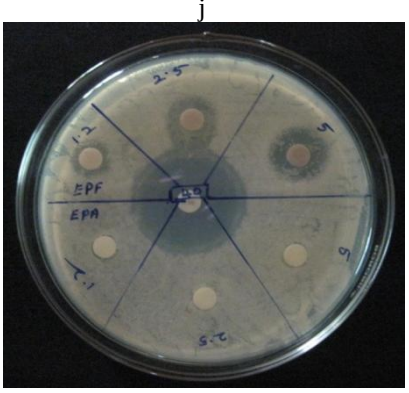

h

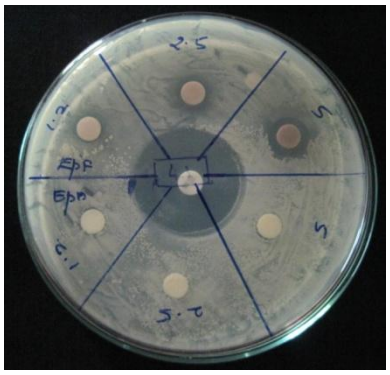

i

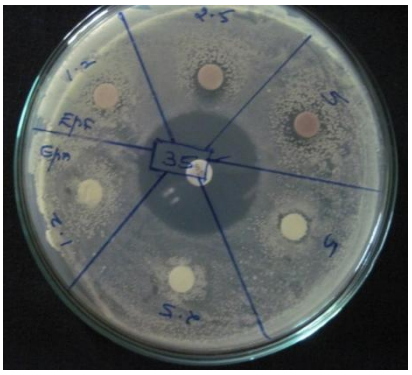

j

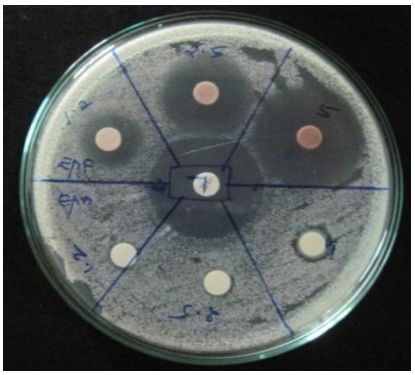

k

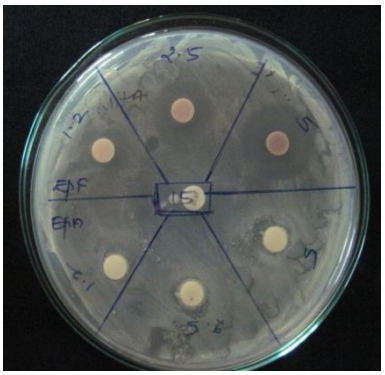

l

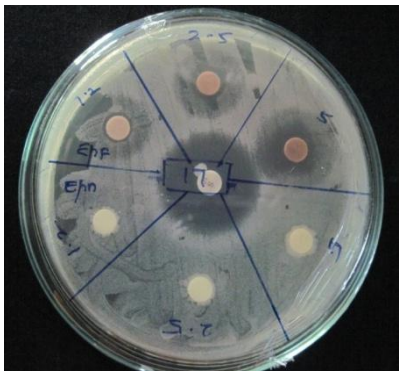

m


n


o


p


q


r

a) *Enterobacter aerogens*
b) *Yersinia enterocolitica*
c) *Vibrio fischeri*
d) *Xanthomonas aeupest. Pv, oryzae*
e) *Pseudomonas aeruginosa*
f) *Staphylococcus aureus*
g) *Enterococcus faecalis*
h) [*E.coli*] Cipro R
i) *Acetobacter baumanii* (Carbapenem R)
j) *Staphylococcus aureus* [Methicillin Sensitive *S. aureus*]
k) *MRSA* (clinical pathogens)
l) ESBL, *E.coli* (clinical pathogens)
m) *Klebsiella pneumoniae* (clinical pathogens)
n) *Proteus*
o) *Salmonella paratyphi*
p) [*MSSA*]
q) *MRSA* [Methicillin Resistant *S. aureus*]
r) *ESBL Klebsiella*

4. Conclusion

An endophyte is a bacterial or a fungal microorganism, which spends the whole or part of its life cycle colonizing inter and /or intra cellular healthy tissues of the host plant, typically causing no apparent symptoms of disease. The endophytic population of a given species varies from several to a few hundreds of bacterial and fungal strains.

Endophytic organisms can be secluded from meekly surface sterilized tissues of plants and can be cultivated on nutrient agar. The relationship between the endophyte and its host plant may range from latent phytopathogenesis to mutualistic symbiosis.

Hence, the plan of the current study was to isolate the endophytes from *Datura metel L*. and to investigate their pharmacological properties. During the path of the study, the endophytic fungus was identified as *Fusarium oxysporum* and the endophytic actinomycete was identified as *Stenotrophomonas sp.*

The crude extracts of both the endophytic organisms were tested for antimicrobial potential. Extracellular endophytic extracts seemed to have better activity where the zone of inhibition is almost equal to that of the antibiotics used. So the extracts of these fungal extract can be equally considered to an effective antibiotic.

References

[1]. Verma VC, Gange AC, editors. Advances in endophytic research. Springer *Science & Business Media*; 2013 Nov 12

[2]. Petrini O. (1991) Fungal Endophytes of Tree Leaves. In: Andrews J.H., Hirano S.S. (eds) Microbial Ecology of Leaves. Brock/Springer Series in Contemporary Bioscience. Springer, New York, NY.

[3]. Carroll, F.E., Müller, E. & Sutton, B.C. Preliminary studies on the incidence of needle endophytes in some European conifers. *Sydowia*. 1977; 29: 87-103.

[4]. Bernstein ME, Carroll GC. Internal fungi in old-growth Douglas fir foliage. *Canadian Journal of Botany*. 1977 Mar 15; 55(6):644-53.

[5]. Carroll JS. The effect of imagining an event on expectations for the event: An interpretation in terms of the availability heuristic. *Journal of experimental social psychology*. 1978 Jan 1; 14(1): 88-96.

[6]. Fisher PJ, Petrini O, Scott HL. The distribution of some fungal and bacterial endophytes in maize (*Zea mays* L.). *New Phytologist.* 1992 Oct 1; 122(2):299-305.

[7]. Shirling ET, Gottlieb D. Methods for characterization of *Streptomyces species*. *International Journal of Systematic and Evolutionary Microbiology*. 1966 Jul 1; 16(3):313-40.

[8]. Holt RA, Lawton JH. The ecological consequences of shared natural enemies. *Annual review of Ecology and Systematics*. 1994 Nov; 25(1): 495-520.

[9]. Murray N, Chiang J. Active Galactic Nuclei Disk Winds, Absorption Lines, and Warm Absorbers. *The Astrophysical Journal Letters.* 1995 Dec 1; 454(2): L105.