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ANTIMICROBIAL ACTIVITY OF SECONDARY METABOLITES FROM ENDOPHYTIC FUNGI ISOLATED FROM NERIUM OLEANDER L.

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ABSTRACT

Endophytic fungi found inside the plant tissue are endosymbionts, protecting their host from pests, pathogens, etc. Twenty eight endophytic fungi were isolated from different parts of *Nerium oleander* L., out of which, 54% of isolates were from flower, 39% from stem and 7% from leaf parts. Thirty six percent of the isolates showed antimicrobial activity against tested pathogens. The potential isolates such as *Fusarium semitectum* (Nof-3), *Colletotrichum gloeosporioides* (Nof-7), *Alternaria alternata* (Nof-8) and Mycelia Sterilia sp.1 (Nos-6) were subjected to the production and extraction of secondary metabolites. All the four fungal extracts inhibited *Staphylococcus aureus* and *Bacillus cereus* at 20 µg/mL (MIC). Extracts of *C. gloeosporioides* and Mycelia Sterilia sp.1 showed activity against *Pseudomonas aeruginosa* at MIC of 20 µg/mL. The growth of *Escherichia coli* was suppressed by all the tested extracts at MIC of 20 µg/mL except *F. semitectum*. *A. alternata* & Mycelia Sterilia sp.1 extracts were active against *Salmonella typhimurium* at 20 µg/mL. The growth of *Candida albicans* was inhibited by Mycelia Sterilia sp.1 at 20 µg/mL. The zones of inhibition were statistically significant with respective positive controls.

Keywords: **Nerium oleander L.,Endophytic fungi, Antimicrobial activity, Secondary metabolites, Minimum inhibitory concentration**.

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INTRODUCTION

Endophytic fungi inhabit the host plant, primarily within the aerial tissues, without causing adverse effects. They are associated symbiotically, such that endophytes draw nutrients and protection from the host, but contribute to effective host defence against pathogens, herbivores or abiotic stress [20,2]. Approximately, a million species of endophytic fungi are estimated to be present within the global flora [9]. These endophytic fungi are rich sources of secondary metabolites, which find a wide range of applications in pharmacology as well as in agriculture. They are absolutely needless for the producers and do not involve in their life cycle. The crystalline compound mycophenolic acid isolated from *Penicillium glaucoma* was considered as the first microbial secondary metabolite, discovered in 1896 by Gosio [11]. Most important characteristic features of secondary metabolites are their unique chemical structures, frequent occurrence and biological activity [4]. Biological activity will be used generally for interactions between chemicals and molecular targets of living organisms [11]. Fungal endophytes could also produce metabolites similar to, or with more activity than that of their respective hosts [24]. Therefore, it is believed that search for novel compounds should be directed towards endophytic fungi for medicinal purpose and to save the mass utilization of plants to produce secondary metabolites. Naturally produced bioactive metabolites from endophytic fungi include alkaloids, benzopyranones, benzoquinones, flavonoids, isocoumarin, lignans, phenol and phenolic compounds, phenylpropanoids, steroids, terpenoids, tetralones, xanthones, and other compounds [28]. *Nerium oleander* L. is an evergreen shrub (or small tree) that grows to approximately 6 m. The sticky latex of the stem is highly poisonous to humans, pets, livestock and birds due to the presence of cardiac glycosides, mainly oleandrin. Ingestion causes nausea, vomiting, cardiac arrhythmias, hypotension (low blood pressure) and death. Its sap has been used as rat poison [1,12]. The plant is also proven to have medicinal value like cardiotonic, emetic, antibacterial, anti-inflammatory, anti-nociceptive activity and is used to treat scabies [8,13]. Valuable compounds like cardiac steroid, arabinogalactan and cardenolides are reported to be present in this plant [15,19,22]. Endophytic fungal isolates of *N. oleander* possessing antioxidant, xanthine oxidase inhibitory and antimicrobial activity was reported by Wu-Yang et al [30]. The current investigation aimed at isolation of endophytic fungi from various parts of *N. oleander* L. and to explore their antimicrobial potential against human pathogens.

MATERIALS AND METHOD

(I) Sources of endophytic fungi

Different plant parts of *N. oleander* L. were collected (excised using a sterile knife) from Dhanvantri vana located in Mariyappanapalya, Department of forestry, Government of Karnataka, Bangalore, Plant samples were brought to the laboratory in sterile polythene bags. Herbarium of plant sample (with the accession FRLHT Coll. No. 74063) was deposited in Institute of Ayurveda and Integrative Medicine (FRLHT), No. 74/2, Jarakabande kaval, Attur (Post) Via Yelahanka, Bangalore- 560064.

(II) Isolation of endophytic fungi

Different parts of fresh healthy *Nerium oleander* L. plant were cut into small pieces (5 mm × 2 mm) using sterile blade, washed with sterile distilled water. Surface sterilization was done by immersing in 4% sodium hypochlorite solution for 90 Sec followed by 70% ethanol treatment for 5 Sec, thoroughly washed with sterile distilled water [25] and blot dried between sterile paper towels. The surface sterilized samples were placed on potato dextrose agar (PDA) plates amended with 50 mg/L tetracycline to

suppress the bacterial growth and incubated at 28°C to 30°C for 2 to 3 days. The hyphal tip of endophytic fungi growing out from the plant tissue was transferred to fresh PDA plates amended with 50 mg/L tetracycline. After incubation at 30°C for 7 to 14 days, purity of the culture was determined by colony morphology [26]. Colonization rate, expressed in percentage was calculated as the number of fungal isolates from each part of the plant divided by total number of endophytic fungi isolated from the plant.

(III) Identification of endophytic fungi

The endophytic fungi were identified based on the cultural characteristics, morphology of the fruiting bodies and spores, using standard manuals [3,6,27].

(IV) Screening for antimicrobial activity

Endophytic fungi isolated from the medicinal plant *N. oleander* L. were screened for antimicrobial activity against the human pathogenic bacteria (*Staphylococcus aureus* NCIM No. 2079, *Bacillus cereus* NCIM No. 2106, *Pseudomonas aeruginosa* NCIM No. 2200, *Escherichia coli* NCIM No. 2256, *Salmonella typhimurium* NCIM No. 2501) and yeast (*Candida albicans* NCIM No. 3471) procured from NCIM, NCL, Pune. Agar plug method: Cylindrical pieces were cut out from well grown culture of the endophytic fungal strains on PDA. The blocks were placed on the Petri dishes deep inoculated with a fixed amount of test micro-organisms grown in Nutrient agar (NA) medium for bacteria and Sabouraud dextrose agar medium (SDA) for yeast (10^6 cells/mL). The plates were kept for 12 hours at 2–8ºC for the antimicrobial compound diffusion and thereafter they were incubated for the growth of test-micro-organisms at 25ºC for Yeast and 37ºC for bacteria. The antimicrobial activity was measured in the form of diameter zone inhibition after 24 hrs for bacteria (*Staphylococcus aureus, Bacillus cereus, Pseudomonas aeruginosa, Escherichia coli*, *Salmonella typhimurium*) and 48 hrs for yeast (*Candida albicans*) [5] (Fig 3).

(V) Production and extraction of secondary metabolites

Endophytic fungi exhibiting potential antimicrobial activity were subjected to the production of secondary metabolites. The fresh mycelia grown on PDA plates at 28ºC for 3–6 days was inoculated into 500 mL flasks containing 200 mL of potato dextrose broth medium (Dextrose, 20 g; extract of 200 g potato; 1000 mL; autoclaved at 121ºC for 20 min), followed by incubation at 28ºC with 140 rev/min for 15 days. The culture broth of endophytes was filtered using Whatmann No. 1 filter paper to remove mycelium. The secondary metabolites from Nof-3, Nof-7 and Nof-8 were extracted from the culture filtrates using equal volume of ethylacetate. The red pigment produced by Nos-6 isolate was extracted with ethylacetate after acidification with acetic acid at pH-3. The extracts were evaporated under vacuum at 50ºC and dried.

(VI) Antimicrobial activity of ethylacetate extracts of the endophytic fungi by agar well diffusion method

A fixed amount of test micro-organisms (10^6cells/mL) were inoculated in Petri dishes containing NA for bacteria and SDA for yeast. The crude ethylacetate extracts dissolved in DMSO at different concentrations (10µg/mL,20µg/mL, 40 µg/mL, 60 µg/mL, 80 µg/mL, 100 µg/mL) were added into the 5mm diameter well made inoculated Petri dishes. The cultures were kept for 24 hours at 2–8°C for the antimicrobial metabolite diffusion and thereafter they were incubated at the appropriate temperature for the growth of test micro-organisms. The zone of inhibition was measured in mm [29](Fig 4). (VI) Statistical Analysis: The statistical analysis was analyzed by Two way ANNOVA with SPSS 11.5, mean values of the triplicates was compared according to Duncan Multiple Range Test (DMRT) at p = 0.05.

RESULTS AND DISCUSSION

(1) Isolation and Identification of endophytic fungi

Twenty eight different strains of endophytic fungi were isolated from 300 tissue segments (100 segments from each stem, flower and leaf) of *N. oleander* L. The colonization and isolation rates of endophytic fungi from flower (54%) were higher than that of stem (39%) and least in leaf (7%) parts, whereas Wu-Yang *et al*., [30] described more endophytic fungi in leaves than in stems (Fig1). In the present study, *Curvularia* sp*., Fusarium* spp*.* was found both in stems and flower parts. *Drechslera* sp., *Cladosporium* spp., *Thielavia terricola*, three Mycelia sterilia (non-sporulating strains) and two unidentified sp found in stem segments. To the best of our knowledge, before this there are no reports of *Thielavia terricola* as an endophyte. Wu-Yang *et al*., [30] reported *Chaetomium* sp., *Cladosporium* sp., *Colletotrichum* sp., and *Torula* sp. in the stems of *N. oleander* L. In the present investigation *Torula* sp., *Alternaria alternata*, *Colletotrichum* sp., *Cylindrocephalum* sp., *Aspergillus* sp., *Penicillium* sp., *Cochliobolus* sp., and *Chaetomium* sp. were isolated from flowers. *Bipolaris* sp. and Mycelia sterilia sp.5 were isolated from the leaves of *Nerium oleander* L. Wu-Yang *et al*.,[30] reported that Mycelia sterilia sp. (50% relative frequency) were found predominantly in the leaves along with an *Ascomycete* sp. (Table:1). *Drechslera* sp., *Cladosporium* spp, *Curvularia* sp*., Chaetomium* sp., *Colletotrichum* sp., were commonly found endophytes in medicinal plants [10,18]. *Colletotrichum gloeosporioides* was reported from *Plumeria acutifolia* belonging to *Apocynaceae* [16].

(2) Screening for Antimicrobial activity

Among 28 endophytic fungi screened for antimicrobial activity, 36% of isolates showed activity, 64% of them did not possess any activity against any test pathogens. Six isolates showed potential inhibition against *S. aureus* and seven isolates inhibited the growth of *B. cereus*. Only Mycelia Sterilia sp.1 (Nos-6) suppressed the growth of *C. albicans* and two strains were effective against *P. aeruginosa*. Five isolates exhibited inhibition zone against gram-negative organism *E. coli* and *S. typhi.* (Table.1). Among the tested organisms, four endophytic fungi identified as *Fusarium semitectum* (Nof-3), *Alternaria alternata* (Nof-7), *Colletotrichum gloeosporioides* (Nof-8) and Mycelia sterilia sp.1 (Nos-6) were found to be more promising antimicrobial strains for production and extraction of secondary metabolites. The antimicrobial property of Mycelia sterilia sp.1 was due to production extracellular red pigment. Morphological identification of these four potential endophytic fungi was further confirmed by Agharkar research institute, Pune, India.

(3) Antimicrobial activity of crude extract by agar well diffusion method:

The crude extract of the potential endophytic fungal isolates, exhibited a broad spectrum of antimicrobial activity against the pathogens, when compared with that of standard positive control tetracycline for bacteria and fluconazole for yeast. The zone of inhibition ranged from 1.67 to 28.67 mm at concentrations of 10–100 µg/mL. According to Rios and Recio, [21] extracts of natural origin showing antimicrobial activity above 100 µg/mL concentration should be avoided; hence we used the crude extract at concentrations 10–100 µg/mL. All the four fungal extracts showed MIC at 20 µg/mL against *S. aureus* and *B. cereus*, however, there was no inhibition at concentration below 20 µg/mL. Whereas, Wu-Yang *et al*., [30] reported MIC of endophytic fungal crude extract isolated from *N. oleander* L. at range of 1.25–10 mg/mL for *S. aureus,* 5–25 mg/mL for *B. cereus,* 5–12.5 mg/mL for *E.coli.* This shows that endophytes of *N. oleander* L., from different ecological and geographical conditions exhibit qualitatively and quantitatively varied antimicrobial activity [17]. In this study, *A. alternata* and Mycelia sterilia sp.1 exhibited MIC at 20 µg/mL against *P. aeruginosa*. *A. alternata*, *C. gloeosporioides* and Mycelia sterilia sp.1

inhibited *E. coli* at the same concentration, whereas, only Mycelia Sterilia sp.1 inhibited *C. albicans* at MIC 20 µg/mL. *C. gloeosporioides*, Mycelia sterilia sp.1 inhibited *S. typhi* at MIC 20 µg/mL. However, none of the other crude extracts tested showed inhibition even at 100 µg/mL. Fernandes [7] reported antimicrobial activity of extract of *A. alternata* isolated from *Coffea arabica* L against *S. aureus* and *E. coli* at the MIC of range 50–100 µg/mL and 400–800 µg/mL respectively; however, it did not show activity in the tested concentrations for *C. albicans.* Reports of Wu-Yang *et al*., [30] showed MIC in the range of 1.25–10 mg/mL for *C. albicans*. Lu *et al*., [14] found that ergosterol derivatives of *Colletotrichum* sp. isolated from *Artemisia annua* inhibited *S. aureus* and *B. subtilis and Pseudomonas* sp. at the range of 25–75 µg/mL and for *C. albicans* 50–100 µg/mL. Endophytic *Fusarium* sp. from plant *Selaginella pallescens* collected in the Guanacaste conservation area of Costa Rica also shows potent activity against *Candida albicans* in agar diffusion assay [23]. According to Wu-Yang *et al*.,[30] most of the tested fungi isolated from *N. oleander* possessed better antibacterial and antifungal activities than the host plant. In the present study, antimicrobial activity of crude extracts of *F. semitectum* and *A. alternata*, at 100 µg/mL concentration**,** were not comparable statistically to the zone produced by the tetracycline (20 µg/mL), whereas, *C. gloeosporioides* is very near to the standard. The activity of Mycelia sterilia sp.1 at 100 µg/mL concentration was statistically significant to the positive control against *S. aureus.* The crude extract of *Fusarium semitectum* at 100 µg/mL was in par with the positive control against *B. cereus. Fusarium semitectum* and *C. gloeosporioides* were inactive against *P. aeruginosa.* However, the zone of inhibition produced by *A. alternata* and Mycelia sterilia sp.1 (20µg/mL) was statistically significant with that of positive control against the same pathogen. *Fusarium semitectum* did not inhibit *E. coli* even at100 µg/mL. However, the antibacterial activity of *A. alternata* and *C. gloeosporioides* against *E. coli* was higher than that of positive control. Both the *F. semitectum* and *A. alternata* showed no inhibition against *S. typhi*, but the crude extract at 60 µg/mL concentration of Mycelia sterilia sp.1 and *C. gloeosporioides* exhibited higher zone than the positive control. Zone of inhibition of Mycelia sterilia sp.1 against *C. albicans* at 80µg/mL was higher than that of fluconazole at 20 µg/mL. (Table 2).

Table 1
Identification and Screening the antimicrobial activity of endophytic fungi isolated from Nerium oleander

Endophytic fungi			**Diameter zone of inhibition in mm**					
Sl. No	Code	Endophytic fungus identified	*S. aureus*	*B. cereus*	*P. aeruginosa*	*E.coli*	*S. typhi*	*C. albicans*
1	Nos -1	*Curvularia* sp.	0^a	0^a	0^a	0^a	2 ± 0^b	0^a
2	Nos-2	*Drechslera* sp.	0^a	0^a	0^a	0^a	0^a	0^a
3	Nos-3	*Fusarium.*sp.	0^a	0^a	0^a	4 ± 0^c	0^a	0^a
4	Nos-4	*Cladosporium* sp.1	0^a	0^a	0^a	0^a	0^a	0^a

5	Nos-5	*Cladosporium* sp.2	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
6	Nos-6	Mycelia sterilia sp.1	4±1[e]	5.33±0.58[e]	8±1[b]	8.33±0.58[e]	7±0[d]	5.67±0.57[b]
7	Nos-7	Un identified	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
8	Nos-8	Un identified	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
9	Nos-9	*Thielavia terricola*	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
10	Nos-10	Mycelia sterilia sp.2	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
11	Nos-11	Mycelia sterilia sp.3	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
12	Nof-1	*Aspergillus* sp.	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
13	Nof-2	*Cochliobolus* sp.	0[a]	4±0[d]	0[a]	5.33±0.58[d]	2±0[b]	0[a]
14	Nof-3	*Fusarium semitectum*	3.67±0.58[de]	13.33±1.53[f]	0[a]	0[a]	0[a]	0[a]
15	Nof-4	*Curvularia brachyspora*	2±0[c]	2±0[b]	0[a]	0[a]	0[a]	0[a]
16	Nof-5	*Alternaria brassicola*	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
17	Nof-6	*Chaetomium* sp.	1±0[b]	0[a]	0[a]	0[a]	2.67±0.58[c]	0[a]
18	Nof-7	*Alternaria alternata*	3.33±0.58[d]	3.67±0.58[d]	9±0[c]	3±0[b]	0[a]	0[a]
19	Nof-8	*Colletotrichum gloeosporioides*	10.33±0.58[f]	4±0[d]	0[a]	4±0[c]	6.67±1.53[d]	0[a]
20	Nof-9	*Cochliobolus* sp.	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
21	Nof-10	*Cylindrocephalum* sp.	0[a]	3±0[c]	0[a]	0[a]	0[a]	0[a]
22	Nof-11	*Colletotrichum* sp.	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
23	Nof-12	Mycelia sterilia sp.4	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]

24	Nof-13	*Torula* sp.	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
25	Nof-14	Un identified	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
26	Nof-15	*Penicillium* Sp.	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
27	Nol-1	Mycelia sterilia sp.5	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]
28	Nol-2	*Bipolaris* Sp.	0[a]	0[a]	0[a]	0[a]	0[a]	0[a]

Nos- Nerium oleander stem host isolate, Nof- Nerium oleander flower host isolate, Nol- Nerium oleander leaf host isolate.
In each column, mean values followed by the same letter are not significantly different according to DMRT at p = 0.05(SPSS ver 11.5).

Table 2
Antimicrobial activity of ethylacetate by agar well diffusion method

Endophytic fungi	Concentration of extract µg/mL	Antimicrobial activity (Diameter of inhibition zone in mm)					
		S. aureus	*B. cereus*	*P.aeruginosa*	*E. coli*	*S. typhi*	*C. albicans*
Nof3	20	3.67±0.58[a]	10.33±2.89[d]	0[a]	0[a]	0[a]	0[a]
	40	6.33±0.58[c]	12.67±1.15[e]	0[a]	0[a]	0[a]	0[a]
	60	8.33±0.58[d]	16.33±0.58[f]	0[a]	0[a]	0[a]	0[a]
	80	12.67±1.15[f]	19.67±2.51[hi]	0[a]	0[a]	0[a]	0[a]
	100	15.0±2.64[g]	21.00±1.00[i]	0[a]	0[a]	0[a]	0[a]
Nof7	20	4.00±1.00[a]	4.67±0.58[ab]	5.67±0.58[b]	1.67±0.58[b]	0[a]	0[a]
	40	4.33±1.15[ab]	8.00±0.00[c]	8.33±0.58[c]	4.33±0.58[cd]	0[a]	0[a]
	60	8.33±0.58[d]	10.33±1.15[d]	11.67±0.58[de]	5.67±0.58[d]	0[a]	0[a]
	80	11.67±0.58[ef]	14.33±0.58[e]	12.33±1.15[e]	8.33±0.58[e]	0[a]	0[a]
	100	15.33±0.58[g]	18.33±0.58[h]	21.00±1.73[h]	13.0±0.00[gh]	0[a]	0[a]
Nof8	20	6.67±1.15[cd]	3.30±0.58[a]	0[a]	2.00±0.00[b]	5.00±0.00[c]	0[a]
	40	10.67±0.58[e]	4.67±0.58[ab]	0[a]	3.00±0.00[bc]	14.0±1.00[e]	0[a]
	60	15.33±0.58[g]	5.67±0.58[b]	0[a]	5.3±0.58[d]	17.33±1.15[g]	0[a]
	80	16.33±1.52[gh]	8.67±0.58[cd]	0[a]	8.33±0.58[e]	20.33±0.58[h]	0[a]
	100	22.0±0.00[j]	13.0±1.00[e]	0[a]	12.67±2.08[gh]	24.0±1.00[i]	0[a]
Nos6	20	6.00±1.00[bc]	4.00±0.00[ab]	5.67±0.58[b]	10.67±1.15[f]	3.67±0.58[b]	3.0±0.00[b]
	40	10.67±1.15[e]	8.67±1.15[cd]	10.67±1.15[d]	16.0±0.00[i]	12.67±1.15[d]	5.33±0.58[c]
	60	14.67±1.15[g]	13.0±1.00[e]	12.33±0.58[e]	20.67±1.15[j]	21.33±1.15[h]	7.67±0.58[d]
	80	18.0±2.0[h]	16.67±1.15[fg]	13.67±0.58[f]	24.67±1.15[k]	24.67±1.15[i]	12.00±1.00[f]
	100	20.0±0.00[i]	19.33±1.15[hi]	15.33±0.58[g]	28.67±1.15[l]	27.33±1.15[j]	14.67±0.58[g]
Tetracycline	20	22.33±0.8[j]	10.33±0.58[d]	5.33±0.58[b]	11.33±1.15[fg]	15.33±0.58[f]	-
Fluconozole	20	-	-	-	-	-	8.67±0.58[e]

In each column, mean values followed by the same letter are not significantly different according to DMRT at p = 0.05.
'-' Not determined.

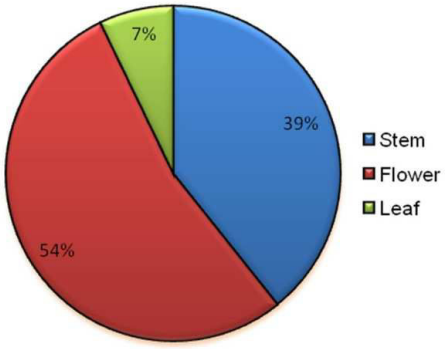

Figure 1.
Endophytic fungi isolates of different parts of Nerium oleander L.

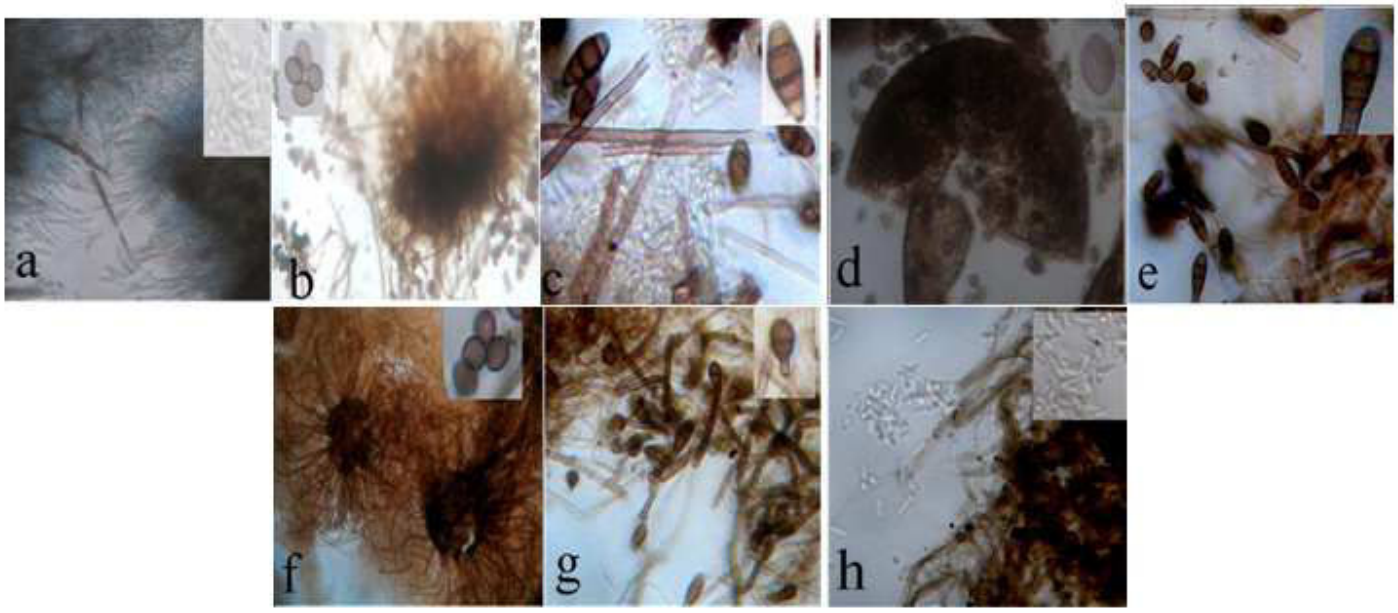

Figure 2.
Microscopic pictures of endophytic fungi with conidia from Nerium oleander L. a. Colletotrichum sp., b. Chaetomium sp., c. Curvularia sp., d. Thielavia terricola, e. Alternaria sp., f. Chaetomium sp., g. Bipolaris sp., h. Cylindrocephalum sp.

Figure 3.
Antimicrobial activity of endophytic fungi by agar plugs method.

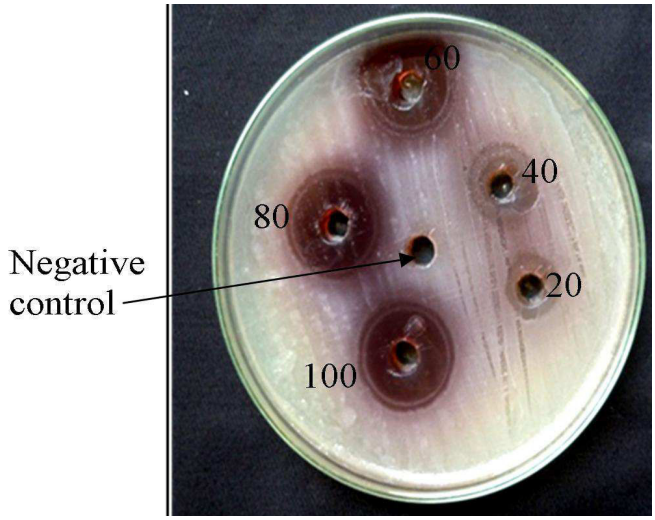

Figure 4
Antimicrobial activity of ethyl acetate extract by agar well diffusion method

CONCLUSION

The present findings indicate that antimicrobial properties of endophytic fungi vary with the geographical distribution of the host plant. *F. semitectum* (Nof-3), *A. alternata* (Nof-7), *C. gloeosporioides* (Nof-8) and Mycelia sterilia sp.1 from *Nerium oleander* are potential sources of new antibiotic and further research is required to determine the chemical nature and efficacy of the compounds.

REFERENCES

1. Al-Yahya MA, Preliminary toxicity study on the individual and combined effects of *Citrullus colocynthis* and *Nerium oleander* in rats. Fitoterapia, 71: 385-391, (2000).
2. Arnold AE, Mejia LC, Kyllo D, Rojas EI, Maynard Z, Robbins N, Herre EA, Fungal endophytes limit pathogen damage in a tropical tree. Proc Natl Acad Sci USA 100:15649-15654, (2003).
3. Barnett HL, Hunter BB, Illustrated genera of imperfect fungi. APS Press: St. Paul, Minnesota. ISBN: 0-89054-192-2, (1998).
4. Demain AL, Fang A, The natural functions of secondary metabolites. In: Scheper T (ed) Advances in Biochemical Engineering/ Biotechnology, Springer Verlag, Berlin 69:1–39, (2000).
5. Denitsa N, Mariana N, Screening the antimicrobial activity of Actinomycetes strains isolated from Antarctica. Journal of Culture Collections, 4: 29-35 (2004-2005).
6. Ellis MB, Dematiaceous Hyphomycetes. Commonwealth Mycological Institute, Kew, Surrey, England (1971).
7. Fernandes MDRV, Silva TACe, Ludwig HP *et al*., Biological activities of the fermentation extract of the endophytic fungus *Alternaria alternata* isolated from *Coffea arabica* L. Braz J of Pharm Sci 45(4): 677-686, (2009).
8. Fu L, Zhang S, Li N, Wang J, Zhao M, Sakai J, Hasegawa T, Mitsui T, Kataoka T, Oka S, Kiuchi M, Hirose K, Ando M, Three new triterpenes from *Nerium oleander* and biological activity of the isolated compounds. J Nat Prod 68:198–206, (2005).
9. Ganley RJ, Brunsfeld SJ, Newcombe G, A community of unknown endophytic fungi in western white pine. Proc Nat Acad Sci USA 101:10107–10112, (2004).
10. Huang WY, Cai YZ, Hyde KD, Corke H and Sun M, Biodiversity of endophytic fungi associated with 29 traditional Chinese medicinal plants. Fungal Divers 33:61-75, (2008).
11. Janos B, Bioactive Microbial Metabolites. J. Antibiot 58(1): 1–26, (2005).
12. Langford SD, Boor PJ, Oleander toxicity: An examination of exposures human and animal toxic. Toxicology 109: 1-13, (1996).
13. Li P, Antony JM, Leeuwenberg, David JM, *Apocynaceae*. Flora of China 16: 143–188, (1995).
14. Lu H, Wen Xin Zou, Jun Cai Meng, Jun Hu, Tan RX, New bioactive metabolites produced by *Colletotrichum* sp., an endophytic fungus in *Artemisia annua*. Plant Science 151 (2000) 67–73, (2000).
15. Mostaqul HM, Jabbar A, Rashid MA, Hasan CM, A novel antibacterial and cardiac steroid from the roots of *Nerium oleander*. Fitoterapia 70: 5-9, (1999).
16. Nithya K and Muthumary J, Growth studies of *Colletotrichum gloeosporioides* (Penz.) Sacc. A taxol producing fungus from *Plumeiria acutifolia.* Ind. J. Sci. Technol. **2** (11):14-19, (2009).
17. Petrini O, In: Fokkema NJ, Van den Hueval J (ed) Microbiology of the Phyllosphere. Cambridge, UK, Cambridge University Press, 175-187, (1986).
18. Photita W, Taylor PWJ, Ford R, Lumyong P, McKenzie EHC, Hyde KD, and Lumyong S, Morphological and molecular characterization of Colletotrichum species from herbaceous plant in Thailand, Fungal Divers 18: 117-133, (2005).
19. Qun D, Ji-nian F, Structural elucidation of a new arabinogalactan from the leaves of *Nerium indicum.* Carbohyd Res 332: 109-114, (2001).
20. Redman RS, Sheehan KB, Stout RG, Rodriguez RJ, Henson JM, Thermotolerance generated by plant/fungal symbiosis. Science 298:1581, (2002).
21. Rios JL, Recio MC, Medicinal plants and antimicrobial activity. J. Ethnopharmacol 100:80-84, (2005).

22. Sabira B, Bina SS. Razia S, Atiya Z, Amin S, Bioactive cardenolides from the leaves of *Nerium oleander*. Phytochemistry 50: 435-438, (1999).
23. Strobel G, Daisy, Bioprospecting for Microbial Endophytes and Their Natural Products Microbial. Mol biol Rev 67:491-502, (2003).
24. Strobel GA, Rainforest endophytes and bioactive products. Crit Rev Biotechnl 22: 31-33 (2002).
25. Suryanarayan TS, Thennarasan S, Temporal variation in endophyte assemblages of *Plumeria rubra* leaves. Fungal Divers 15:197-204, (2004).
26. Suthep W, Nongluksna S, Wattana P, Nutawan T, Kannawat DK, Nijisiri R, Vithaya M, Endophytic fungi with antimicrobial, anticancer and antimalarial activities isolated from Thai medicinal plants. World J Microbiol Biotecnol 20: 265-272, (2004).
27. Sutton BC, the *Coelomycetes*: *Fungal imperfecti with pycnidia, acervuli and stoma*. Commonwealth Mycological Institute, Surrey, England, (1971).
28. Tan RX, Zou WX, Endophytes: a rich source of functional metabolites. Nat Prod Rep 18:448–459, (2001).
29. Visalakchi S, Muthumary J, Antimicrobial activity of the new endophytic *Monodictys castaneae* SVJM139 pigment and its optimization. Afr J of Microbiol Res 3(9):550-556, (2009).
30. Wu-Yang H, Yi-Zhong C, Hyde KD, Harold C, Mei S, Endophytic fungi from *Nerium oleander* L. (*Apocynaceae*): main constituents and antioxidant activity. World J Microbiol Biotechnol 23:1253–1263,(2007).