

Antimicrobial Activity and Phytochemical Analysis of Endophytic Fungal Extracts Isolated from Ethno-Pharmaceutical Plant *Rauwolfia tetraphylla* L.

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<http://dx.doi.org/10.22207/JPAM.12.1.38>

(Received: 03 December 2017; accepted: 17 January 2018)

Endophytes are the mimicking the plant secondary metabolites as well as vast number chemical synthesizers inside plants and also have been extensively investigated for their endophytic microbial complement. Different parts of *Rauwolfia tetraphylla* L were subjected to the isolation of endophytic fungi. The isolated endophytic fungi were identified morphologically and, isolation, colonization rates and relative frequency of various isolates were calculated. The endophytic fungal isolates were screened for antimicrobial activity against *Staphylococcus aureus*, *Bacillus cereus*, *Pseudomonas aeruginosa*, *Escherichia coli*, *Salmonella typhimurium* and *Candida albicans*. The identification of the prospective endophytic fungi *Curvularia* sp. (RTFs-6) and *Aspergillus* sp. (RTL-6) were selected for the production of secondary metabolites which were grown in rice medium and extracted with ethyl acetate and were screened for their antimicrobial activity by agar well diffusion method and Phytochemical analysis. The extract of *Curvularia* sp. gave effective inhibition to the all the tested organisms whereas extract of *Aspergillus* sp. gave effective inhibition against tested organisms except *P. aeruginosa*. Phytochemical compounds screening of the extracts revealed the presence of alkaloids, terpenoids and tannin compounds.

Keywords: *Rauwolfia tetraphylla* L, Endophytic fungi, Colonization rate, Isolation rate, Antimicrobial, Phytochemicals.

All plants are in natural ecosystem and emerge to be symbiotic with endophytic fungi. Endophytic fungi are endosymbionts residing in plants without causing adverse effects to the host plant and produce a vast range of secondary metabolites with medicinal properties¹. They exhibit mutualistic association with their host and enhance the ability of plants to tolerate abiotic² and biotic³ stresses. It is very important to isolate novel endophytic microorganisms as well as novel bioactive compounds. A specific rationale selection

of each plant for endophyte isolation and bioactive secondary metabolite discovery is used. The reasonable hypotheses are used for plant selection strategy⁴. The number of plant species in the world produce bioactive metabolites, their traditional usage history (use by indigenous people) related to the specific uses or applications and significant technique used to search for endophytes displaying bioactivity⁴. The endophytes of non-grass plants or trees should be screened for bioactive compounds which have rich sources of secondary metabolites⁵.

The demand and need for new antimicrobial agents is growing rapidly as the infectious diseases are still a worldwide problem and the development

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of drug resistance by the pathogens is a growing concern ⁶. The problem extends from the clinical application of antimicrobial drugs and many microorganisms of agricultural concern are also recognized to have acquired resistance to normally used antimicrobial chemicals ⁷, which indicates a growing desire for new bioactive compounds. Historically, a majority of the compounds have been isolated from the normal environment, predominantly plants, and have been used in the treatment of many diseases and illnesses. Many of the drugs obtainable commercially from these natural products and have become potential drug sources ⁸.

The member of *Apocynaceae*, *Rauwolfia tetraphylla* L. a medicinal plant traditionally used for treating various diseases; the seeds and leaves extracts of produces various medicinally important alkaloids ⁹ which are used as ethnopharmaceutical compounds ¹⁰ and in the treatment of snake bite as an antidote ¹¹. It also widely used to encourage uterine expansion in case of difficult delivery and to treat high blood pressure, muscular and rheumatism pain¹⁰.

Recently, more attention is turned towards the endophytic fungi associated with the medicinal plants as they mimic to produce host plant's secondary metabolites ¹²; easy to be grown in laboratory and subjected to large scale production. The crystalline compound mycophenolic acid from *Penicillium glaucoma* was the first anti microbial secondary metabolite, discovered in 1896 by Gosio ¹³. Therefore, it is believed that search for new compounds bound towards endophytic fungi for medicinal purpose and to save the mass utilization of plants to produce secondary metabolites. The bioactive metabolites from endophytic fungi include alkaloids, benzopyranones, benzoquinones, flavonoids, glycosides, steroids, saponins, tannins, and terpenoids, phenol, phenolic compounds, phenylpropanoids, tetralones, xanthenes, and other compounds ¹⁴. The implication of biotechnological tools will enhance the production of fungal secondary metabolites at low cost and environment friendly ¹⁵. The most important quality of secondary metabolites is their unique chemical structures, frequent occurrence and biological activity ¹⁶. Biological activity will be used for interactions between chemicals and molecular targets of living organisms ¹³. Pathogenic microorganisms

like *Staphylococcus aureus*, *Bacillus cereus*, *Salmonella typhimurium*, *Escherichia coli*, *Pseudomonas aeruginosa* and *Candida albicans* cause many infectious diseases which could be fatal. *Staphylococcus aureus*, gram positive coccus bacterium causes cellulitis (inflammation), mastitis in breast feeding women ¹⁷. *Bacillus cereus* is a Gram-positive rod-shaped, beta haemolytic bacterium which causes food borne illness ¹⁸. *Pseudomonas aeruginosa* is a Gram-negative rod-shaped bacteria cause infections of pulmonary tract, urinary tract, burns, wounds, and blood ¹⁹. *Escherichia coli* is a facultative anaerobic gram-negative rod-shaped bacteria and some virulent strains can cause food poisoning and urinary tract infections ²⁰. *Salmonella typhimurium*, Gram-negative rod shaped bacteria predominately found in the intestinal lumen causing typhoid fever ²¹. *Candida albicans* is yeast and grows as filamentous cells causes opportunistic oral, genital infections leading to candidiasis ²².

In the present research an attempt was made to study the diversity of endophytic fungal isolates of *R. tetraphylla*, screen for antimicrobial activity against the pathogenic microorganisms. Also, phytochemical analysis was carried out for the potential endophytic fungal extract exhibiting good antimicrobial activity.

MATERIALS AND METHODS

Collection of plant sample and sampling site

The medicinal plant *Rauwolfia tetraphylla* (Fig 1) was collected from Namachilume forest, Tumkur District, Karnataka. Different plant parts were cut from the medicinal plants with knife disinfected with 70% ethanol and was brought to the laboratory. The samples were collected every three months during year 2010- 2011 (July - Sep, Oct – Dec, Jan - Mar, Apr- June) and the collection was repeated.

Authentication of plant samples

The herbarium samples of *R. tetraphylla* was deposited in National Ayurveda Dietetics Research Institute, CCRA&S (Central Council for Research in Ayurveda and Siddha), Department of AYUSH, Ministry of Health and Family Welfare, Govt. of India, Jayanagar, Bangalore with the accession number RRCBI-15390.

Isolation and maintenance of the endophytic fungi

Endophytic fungi were isolated from fresh material of healthy medicinal plant *R. tetraphylla*. The collected samples were washed under running tap water and cut into 0.5cm² segments and were surface disinfected using sodium hypochlorite - ethanol surface sterilization techniques²³. The effectiveness of the sterilization procedure was confirmed by the vitality test²⁴. Ten leaf segments from each individual part were placed in a Petri dish (9cm) containing Potato Dextrose Agar (PDA). Ten surface sterilized segments from each individual part were placed on PDA plates amended with 50mg/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. After incubation at 30°C for 7 to 14 days, purity of the culture was determined by colony morphology.

Morphological identification of endophytic fungi

The endophytic fungi were identified based on the cultural characteristics, the morphology of the fruiting bodies and spores using standard manuals^{25, 26, 27}. The isolates were induced for sporulation by culturing them on different media such as Potato Sucrose Agar (PSA), Potato Carrot Agar (PCA), Malt extract Agar (MEA), Sabouraud Dextrose Agar (SDA), Corn Meal Agar (CMA) and Tap Water Agar (TWA). Non sporulating cultures were distinguished from each other by their cultural characteristics such as colony morphology, hyphal mat characteristics and pigmentation of the colony in the medium and were grouped under Mycelia sterilia²⁸. The fungi were mounted on the clean glass slide with lactophenol, sterile distilled water or lactophenol cotton blue stain and edges were sealed with DPX mountant.

Identification of potential endophytic fungi by molecular methods

In addition to the morphological identification, molecular methods were carried out to confirm the identification of potential endophytic fungi exhibiting antimicrobial activity.

Isolation of the genomic DNA

The potential endophytic fungi were grown on 50mL PDB for 5-6 days at 28±2°C. The mycelia was harvested and washed with

distilled water and ground with liquid nitrogen. Total genomic DNA was extracted using the cetyl trimethyl ammonium bromide (CTAB) method²⁹. The purity of extracted DNA was determined spectrophotometrically and the quality of the DNA was checked by electrophoresis using 1% Agarose gel.

ITS amplification

The ITS region of the rDNA was amplified using primers ITS1 and ITS4²⁹. Amplification reactions were performed in a total volume of 30µL containing 1X PCR buffer, 200µM each dNTPS, 1.4mM MgCl₂, 1µL of each primer (100nmol), 1.0 U *Taq* DNA polymerase, and 20ng of genomic DNA. PCR amplification conditions included an initial denaturation step at 94°C for 4min, cycling conditions were 94°C for 45s, 56°C for 45s, 72°C for 1min (30 cycles), followed by a final extension at 72°C for 5 min. Each set of the experiment included negative controls (without template DNA) to test the presence of contaminating DNA in reagents. The amplicons were checked in 1% agarose gels run parallel to standard DNA molecular weight marker.

Purification and Sequencing of PCR products

The PCR products were purified using GenElute™ PCR clean-up kit according to the manufacturer's instructions and sequencing was carried out in an ABI automated DNA sequencer. The sequencing PCR was set up using ABI-BigDye® Terminatorv3.1 Cycle Sequencing Kit. BLAST analysis was carried out for obtained sequences in the NCBI database and sequences are submitted to NCBI.

Phylogenetic tree construction

The sequence of the highly bioactive isolate was further compared by phylogenetic analysis. Multiple sequence alignments of the obtained sequence and reference sequences retrieved from GenBank were used to generate phylogenetic tree using the online software server phylogeny.fr.³⁰

Data analysis

Isolation rate

Isolation rate (IR) of the endophytic fungi was calculated as number of isolates obtained from tissue segments divided by total number of tissue segments³¹.

Colonization rate

Colonization rate (CR) of endophytic

fungi was expressed as percentage of total number of isolates obtained from different tissue segments divided by total number of isolates obtained from overall tissue segments incubated³².

Relative percentage occurrence of each group of fungi

Relative frequencies (RF) of isolation, used to represent fungal species density was calculated as the number of isolates of each species of the endophytic fungi divided by the total number of isolates and they were expressed as percentage³¹.

Screening of endophytic fungi for antimicrobial activity

Endophytic fungi isolated from medicinal plants of *Rauwolfia tetraphylla*, was screened for the 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 National collection of industrial microorganism (NCIM), NCL, Pune.

Agar plug method

The bacteria were grown in Nutrient Broth (NB) for 24hrs at 37°C and yeast was grown in Sabouraud Dextrose Broth (SDB) for 48hrs at 25°C and the turbidity was matched with 0.5 McFarland standards (10⁶ CFU/mL). The microbial cultures were swabbed on to the respective media (Nutrient agar for bacteria, Sabouraud Dextrose agar for yeast). Five mm cylindrical pieces cut out from well grown culture of the endophytic fungi were placed on Petri dishes swabbed with the test microorganisms, incubated at 2 - 8°C for 12hrs to allow the diffusion of antimicrobial substance. Thereafter, bacterial plates were incubated at 37°C for 24hrs and Yeast plates were incubated at 25°C for 48hrs. The antimicrobial activity was measured as diameter of inhibition zone in mm³³.

Production and extraction of secondary metabolites

The endophytic fungi exhibiting potent antimicrobial activity was subjected for mass production of secondary metabolites using Rice medium.

The fresh mycelia of endophytic fungi *Curvularia* sp. (RTFs-6) and *Aspergillus* sp. (RTL-

6) were grown on PDA plates at 28 ± 2°C for 3-6 days. They were further inoculated into 1000mL flasks containing 200g of unpolished rice, soaked in 200mL distilled water (autoclaved twice at 121°C for 20 min), followed by incubation for 30 days at 28 ± 2°C³⁴.

Extraction of secondary metabolites from Rice medium culture

The cultures of endophytic fungi grown in rice medium were filled with 300mL of ethyl acetate and allowed to stand for one day, shaken thoroughly and filtered. The following procedure was repeated until most of the metabolites were extracted. Then the ethyl acetate filtrate was extracted with pure distilled water to remove debris and other particles. Finally ethyl acetate extract was treated with anhydrous Sodium sulphate to remove the moisture content and dried using a rotary evaporator³⁵. All the crude extracts were weighed after concentration.

Antimicrobial activity of the crude endophytic fungal extract by agar well diffusion method

The extracted secondary metabolite was dissolved in DMSO at different concentrations of 10µg/mL, 20µg/mL, 40µg/mL, 60µg/mL, 80µg/mL and 100µg/mL; added into the 5mm diameter well bored in Petri dishes (NA for bacteria, SDA for yeast) inoculated with a fixed amount of test microorganisms (10⁶ CFU/mL) so as to obtain a lawn culture. The plates were kept for 12hrs at 2-8°C for the antimicrobial metabolite diffusion and thereafter incubated at optimum temperature for growth. The zone of inhibition was measured in millimeter³⁶.

Statistical analysis

Analysis of variance (ANOVA) was used to determine the significance of difference between treatment groups ($p < 0.05$). Means between treatment groups were compared for significance using Duncan's Multiple Range test.

Phytochemical analysis of ethyl acetate extract of endophytic fungi

The ethyl acetate extract of the potent endophytic fungi were subjected to chemical constituent analysis^{37, 38}. The test details are as follows:

Test for Alkaloids

A drop of extract was spotted on the pre coated TLC plate followed by spraying with Dragendorffs reagent. The orange red or brown

coloration indicated presence of alkaloids.

Test for Steroids and Terpenoids

One mL of extract, 1mL of chloroform, 2-3 mL of acetic anhydride and 1 to 2 drops of concentrated sulfuric acid were added. The dark green coloration of solution indicated the presence of steroids and dark pink or red coloration of solution indicated presence of terpenoids.

Test for Phenols and phenolic compounds

A drop of extract was spotted initially on a filter paper followed by phosphomolybdic acid spot. The blue coloration indicated presence of phenols and phenolic compounds.

Test for Tannins

Two to three mL of extract, 10% alcoholic ferric chloride solution was added. A dark blue or greenish grey coloration of solution indicated the presence of tannins.

Test for Flavonoids

Two to three mL of the extract, a piece of magnesium strip and 1mL of concentrated hydrochloric acid were added. A pink red or red coloration of solution indicated the presence of flavonoids.

Test for the presence of proteins

Ninhydrin test: To the extract, 0.25% Ninhydrin reagent was added and boiled for few a minutes. Formation of blue color indicated the presence of amino acid.

RESULTS

Isolation of endophytic fungi

A total of 26 endophytic fungi were isolated from different parts of *R. tetraphylla* and named as RTS1-4 (*R. tetraphylla* stem isolates), RTFr1-8 (*R. tetraphylla* fruits isolates) and RTL1-14 (*R. tetraphylla* leaf isolates).

Identification of endophytic fungi

The identified endophytic fungi were *Aspergillus* sp., *Cladosporium* sp., *Colletotrichum* sp., *Curvularia* sp., *Cylindrocephalum* sp., *Fusarium* sp., *Paecilomyces* sp., *Penicillium* sp., *Pestalotiopsis* sp., *Thielavia terricola* and *Mycelia sterilia* were found to be associated with *R. tetraphylla* L. The percentage of Relative frequencies of endophytic fungi is shown in Table 1.

All the plant samples were found to be associated with various endophytic fungi with

different isolation rates (IR) and colonization rates (CR). The IR value for endophytic fungi of *R. tetraphylla* L. was found to be highest in the leaf samples (0.7) followed by in fruit samples (0.4), whereas lowest in stem samples (0.2) (Fig 2). The CR of endophytic fungi was maximum in the leaf samples (53.38%) followed by fruit samples (30.76%), whereas lower in stem (15.38%) (Fig 3).

Identification of endophytic fungi by molecular methods

The molecular identification of potential isolates exhibiting antimicrobial activity i.e., RTFs-6 were carried out. Based on the BLAST analysis for sequence of ITS region from 18S rDNA of selected isolates were identified with reference to GenBank NCBI. Five hundred and thirty two (532) bases of RTFs-6 showed the 100% homology with *Curvularia* sp. The ITS rDNA sequence of the strain *Curvularia* sp., (RTFs-6) has been deposited in the NCBI GenBank database with the accession number KF864556.

Screening for antimicrobial activity of endophytic fungi isolated from *R. tetraphylla*

Twenty six (26) endophytic fungi were isolated from *R. tetraphylla* and screened for the antimicrobial activity. Of these, 73% confirmed the antimicrobial activity and rest of the isolates did not exhibit the activity. Out of 19 isolates, 17 isolates showed potential inhibition against *S. aureus* and only 4 isolates inhibited the growth of *B. cereus*. Seven isolates exhibited inhibition against *E. coli* and six isolates showed activity against *S. typhimurium*. Only RTFs-6 isolate suppressed the growth of *P. aeruginosa* and three fungal isolates were effective against *C. albicans* (Table 2). Among the tested isolates, endophytic fungal isolates *Curvularia* sp. (RTFs-6) and *Aspergillus* sp. (RTL-6) revealed better antimicrobial activity was further subjected for production of secondary metabolites (Fig 4).

Production and extraction of secondary metabolites from potent endophytic fungi

The selected potential endophytic fungi were subjected to production and extraction of secondary metabolites. The crude extracts were obtained in rice media from the isolates of *Curvularia* sp. (RTFs-6) and *Aspergillus* sp. (RTL-6) yielded 936mg/100g of rice and 975mg/100g of rice respectively.

Antimicrobial activity of secondary metabolite extract isolated from potential endophytic fungi

The crude extract of the potential endophytic fungi *Curvularia* sp. (RTFs-6) and *Aspergillus* sp. (RTL-6) exhibited a broad spectrum of antimicrobial activity against the test pathogens (Fig 5) when compared with the positive control tetracycline for bacteria and fluconazole for yeast. The zone of inhibition of test pathogens ranged from 6.33mm to 19.67mm at concentrations of 20-100µg/mL of tested crude extracts. The extract of *Curvularia* sp. (RTFs-6) and *Aspergillus* sp. (RTL-6) exhibited antimicrobial activity at the concentration of 20µg/mL, however, there was no inhibition at 10µg/mL. Hence, the MIC was determined to be 20µg/mL. Both the extracts showed inhibition against *S. aureus* and *B. cereus*. The fungal extracts of *Curvularia* sp. (RTFs-6) inhibited *E. coli* and *P. aeruginosa* at different concentrations ranging from 20-100µg/mL. The fungal extract of *Curvularia* sp. (RTFs-6) and *Aspergillus* sp. (RTL-6) exhibited inhibitory activity against *S. typhimurium* at all the tested concentrations. The secondary metabolites of *Curvularia* sp. (RTFs-6) and *Aspergillus* sp. (RTL-6) showed the antifungal activity against *C. albicans* at the concentrations of 20-100µg/mL. These fungal crude extracts exhibited both antibacterial and antifungal activities similar to the

positive controls used; tetracycline for bacteria and fluconazole for fungi (Table 3).

Phytochemical analysis of secondary metabolite extract from endophytic fungi

Phytochemical analysis of the isolated endophytic fungal extracts of *Curvularia* sp. (RTFs-6) and *Aspergillus* sp. (RTL-6) which showed potential antibacterial and antifungal activity was carried out. The phytochemical analysis was performed to determine the presence of chemical components as a prospective source for medicinal and industrial use. Analysis of ethyl acetate extracts of *Curvularia* sp. (RTFs-6) indicated the presence of alkaloids whilst, the secondary metabolites of *Aspergillus* sp. (RTL-6) revealed the presence of alkaloids and tannins (Table 4).

DISCUSSION

Association of endophytic fungi varies from plant to plant, geographical distribution and also different seasons³⁹. The Isolation Rate (IR), Colonization Rate (CR) and Relative Frequency (RF) of the endophytic fungi vary with different medicinal plants³¹. In present study, the IR, CR and RF of endophytic fungi varied with different tissue segments of the medicinal plants studied.

The IR and CR of endophytic fungi of *R. tetraphylla* L. was found to be highest

Table 1. Relative frequency (%) of different endophytic fungal taxa isolated from four medicinal plants of *Apocynaceae*

S. No	Endophytic fungi	<i>Rauwolfhia tetraphylla</i>			Total (%)
		Stem (%)	Leaf (%)	Fruit (%)	
1	<i>Aspergillus</i> sp.	0	11.53846	3.846154	15.3846154
2	<i>Beltrania</i> sp.	3.846154	0	0	3.84615385
3	<i>Cladosporium</i> sp.	0	0	3.846154	3.84615385
4	<i>Colletotrichum</i> sp.	3.846154	7.692308	3.846154	15.3846154
5	<i>Curvularia</i> sp.	0	0	3.846154	3.84615385
6	<i>Cylindrocephalum</i> sp.	0	3.846154	0	3.84615385
7	<i>Fusarium</i> sp.	0	7.692308	3.846154	11.5384615
8	<i>Mycelia sterilia</i> sp.	3.846154	11.53846	0	15.3846154
9	<i>Paecilomyces</i> sp.	0	3.846154	0	3.84615385
10	<i>Penicillium</i> sp.	0	3.846154	3.846154	7.69230769
11	<i>Pestalotiopsis</i> sp.	0	3.846154	3.846154	7.69230769
12	<i>Thielavia terricola</i>	3.846154	0	3.846154	7.69230769
Total	15.38462	53.84615	30.76923	100	

Table 2. Screening for antimicrobial activity of endophytic fungi from *R. tetraphylla* by agar plug method

S. No	Endophytic fungi	Antimicrobial activity (Diameter zone of inhibition in mm)						
		<i>Staphylococcus aureus</i>	<i>Bacillus cereus</i>	<i>Pseudomonas aeruginosa</i>	<i>Escherichia coli</i>	<i>Salmonella typhimurium</i>	<i>Candida albicans</i>	
1.	RTS-1 <i>Mycelia sterilia</i> sp.1	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
2.	RTS-2 <i>Colletotrichum</i> sp.	9±1 ^g	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
3.	RTS-3 <i>Beltrania</i> sp.	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
4.	RTS-4 <i>Thielavia terricola</i>	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
5.	RTFS-1 <i>Cladosporium</i> sp.	7.33±0.58 ^{cd}	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
6.	RTFS-2 <i>Pestalotiopsis</i> sp.	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
7.	RTFS-3 <i>Fusarium</i> sp.	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
8.	RTFS-4 <i>Penicillium</i> sp.	7.33±0.58 ^{cd}	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
9.	RTFS-5 <i>Aspergillus</i> sp.	9±0 ^g	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
10.	RTFS-6 <i>Curvularia</i> sp.	8.33±0.58 ^{efg}	7.67±0.58 ^b	6.67±0.58 ^b	9±0 ^g	7.67±0.58 ^d	10.33±0.58 ^d	0 ^a
11.	RTFS-7 <i>Thielavia terricola</i>	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
12.	RTFS-8 <i>Colletotrichum</i> sp.	7.33±0.58 ^{cd}	0 ^a	0 ^a	7.33±0.58 ^e	6.33±1.15 ^b	0 ^a	0 ^a
13.	RTL-1 <i>Fusarium</i> sp.	7.33±0.58 ^{cd}	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
14.	RTL-2 <i>Mycelia sterilia</i> sp.2	6±1 ^b	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
15.	RTL-3 <i>Pestalotiopsis</i> sp.	10±0 ^h	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
16.	RTL-4 <i>Colletotrichum</i> sp.	7.33±0.58 ^{cd}	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
17.	RTL-5 <i>Mycelia sterilia</i> sp.3	6±0 ^b	10.33±1.15 ^c	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
18.	RTL-6 <i>Aspergillus</i> sp.	6.67±1.52 ^{bc}	7.33±0.58 ^b	0 ^a	8.33±1.15 ^d	7±0 ^e	8.67±1.15 ^c	0 ^a
19.	RTL-7 <i>Colletotrichum</i> sp.	12±0 ⁱ	0 ^a	0 ^a	7.33±0.58 ^e	0 ^a	0 ^a	0 ^a
20.	RTL-8 <i>Fusarium</i> sp.	7.33±1.15 ^{cd}	7.67±0.58 ^b	0 ^a	0 ^a	8.33±0.58 ^c	0 ^a	0 ^a
21.	RTL-9 <i>Cylindrocephalum</i> sp.	7.67±0.58 ^{de}	0 ^a	0 ^a	6±0 ^b	0 ^a	0 ^a	0 ^a
22.	RTL-10 <i>Penicillium</i> sp.	8.33±1.15 ^{efg}	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
23.	RTL-11 <i>Aspergillus</i> sp.	10.33±0.58 ^b	0 ^a	0 ^a	12±1 ^f	8.33±0.58 ^e	0 ^a	0 ^a
24.	RTL-12 <i>Paecilomyces</i> sp.	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a
25.	RTL-13 <i>Mycelia sterilia</i> sp.4	0 ^a	0 ^a	0 ^a	5.67±0.58 ^h	6±1 ^b	0 ^a	0 ^a
26.	RTL-14 <i>Aspergillus</i> sp.	0 ^a	0 ^a	0 ^a	0 ^a	0 ^a	7.67±0.58 ^b	0 ^a

RTS- *Rauwolfia tetraphylla* stem isolates, RTFS- *Rauwolfia tetraphylla* fruit isolates, RTL- *Rauwolfia tetraphylla* leaf isolates. Values represent mean ± SD of three parallel experiments. In each column, mean values followed by the same letter are not significantly different according to DMRT at p < 0.05.

Table 3. Antimicrobial activity of crude extracts of endophytic fungi isolated from medicinal plants of Apocynaceae.

Endophytic fungi	Concentration of extract in µg/mL	Antimicrobial activity (Diameter of inhibition zone in mm)						
		<i>Staphylococcus aureus</i>	<i>Bacillus cereus</i>	<i>Pseudomonas aeruginosa</i>	<i>Escherichia coli</i>	<i>Salmonella typhimurium</i>	<i>Candida albicans</i>	
<i>Curvularia sp.</i> (RTFs-6)	20	8.33±1.52 ^{ab}	8.33±1.15 ^{bc}	6.67±0.57 ^{bcd}	7±0 ^{bc}	7.67±0.57 ^{bc}	6.33±0.57 ^b	
	40	9.67±0.57 ^{bcde}	10.33±0.57 ^{efghi}	7.67±0.57 ^{ef}	10.33±0.57 ^{gh}	8±1 ^{bc}	9.33±0.57 ^{def}	
	60	10.33±0.57 ^{bcde}	11±0 ^{fghi}	8.67±0.57 ^{gh}	12.33±0.57 ^{ijk}	9±0 ^{cde}	10.33±1.52 ^{ghi}	
	80	11±1 ^{def}	11.33±0.57 ^{ghij}	9.33±0 ⁱ	13.33±0.57 ^{kl}	10.33±0.57 ^{efg}	13.33±0.57 ^{ijk}	
<i>Aspergillus sp.</i> (RTL-6)	100	12.67±0.57 ^{fg}	12.67±0.57 ^{ijkl}	10.67±0.33 ⁱ	15.33±0.57 ^{mm}	12±1 ^{hi}	15.67±1.52 ⁱ	
	20	11.33±0.57 ^{defg}	6.67±0.57 ^a	0 ^a	7±1 ^{bc}	7±0.57 ^b	7.67±0.57 ^c	
	40	13±1 ^{fgh}	7.67±0.57 ^{ab}	0 ^a	9.67±0.57 ^{efg}	9±0 ^{cde}	9.67±1.15 ^{fg}	
	60	15±1 ^{ijk}	9.33±0.57 ^{bcdef}	0 ^a	10.67±0.57 ^{gh}	13±1 ^{hi}	10±0 ^{gh}	
Tetracycline Fluconazole	80	18±1 ^{lm}	11±0 ^{fghi}	0 ^a	12±0 ^{ghi}	15±1 ^{jk}	12.67±0.57 ^{ijk}	
	100	19.67±0.66 ^{mno}	12±1 ^{hijk}	0 ^a	13.33±0.57 ^{kl}	16.67±0.57 ^k	15±1 ^l	
	20	27.33±1.2 ^r	15.33±0.66 ^m	8.66±0.33 ^{gh}	16.33±0.33 ^{no}	11±0.57 ^{gh}	-	
	20	-	-	-	-	-	13.66±0.33 ^k	

Values represent mean ± SD of three parallel experiments. In each column, mean values followed by the same letter are not significantly different according to DMRT at p < 0.05. “-” Not determined.

Table 4. Phytochemical analysis of ethylacetate extract of endophytic fungi isolated from medicinal plants of *Apocynaceae*

Endophytic fungal extract	Phytochemical analysis						
	Alkaloids	Steroids	Terpenoids	Phenol and phenolic compounds	Tannins	Flavonoids	Proteins and Amino acids
<i>Curvularia sp.</i> (RTFs-6)	+	-	+	-	-	-	-
<i>Aspergillus sp.</i> (RTL-6)	+	-	-	-	+	-	-

Note: + indicates presence and – indicates absence



Rauwolfia tetraphylla L.

Fig. 1. Medicinal plants selected for present study from *Apocynaceae*

in the leaf samples (0.7 and 53.38%) followed by fruit samples (0.4 and 30.76%), and lowest in stem samples (0.2 and 15.38%). Among the total isolates of *R. tetraphylla*, RF value of the endophytic fungi in stem, leaf and fruits of *R. tetraphylla* L. was found to be 15.38%, 53.84% and 30.76% respectively. Endophytic fungi such as *Beltrania sp.*, *Colletotrichum sp.*, *Thielavia terricola*, and *Mycelia sterilia* were found in stem. Likewise, *Aspergillus sp.*, *Colletotrichum sp.*, *Cylindrocephalum sp.*, *Fusarium sp.*, *Paecilomyces sp.*, *Penicillium sp.*, *Pestalotiopsis sp.* and *Mycelia sterilia* were associated with the leaf segments. *Aspergillus sp.*, *Cladosporium sp.*, *Colletotrichum sp.*, *Curvularia sp.*, *Fusarium sp.*, *Penicillium*

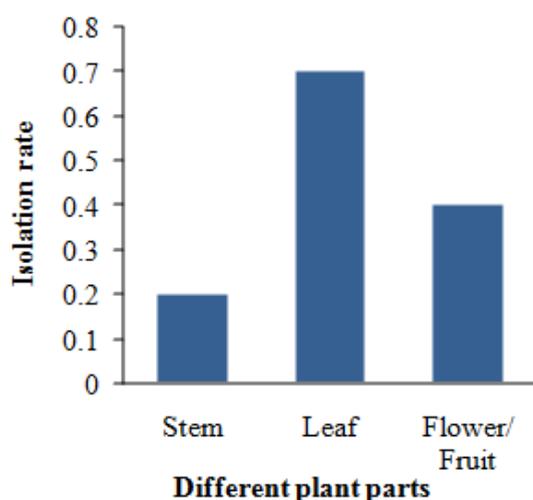


Fig. 2. Isolation rate of endophytic fungi of four medicinal plant parts of *Apocynaceae*

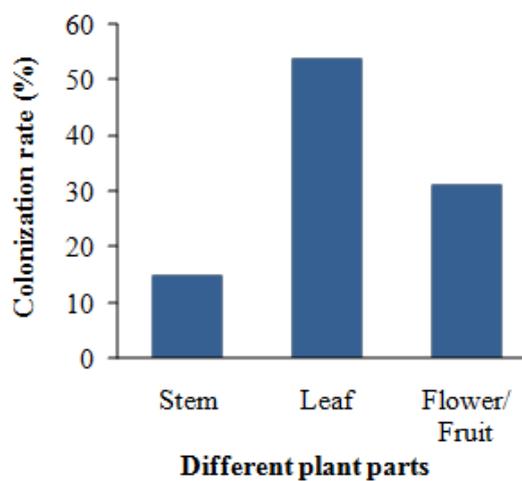


Fig. 3. Colonization rate of endophytic fungi isolated from four medicinal plant parts of *Apocynaceae*

sp., *Pestalotiopsis* sp., and *Thielavia terricola* were found in fruit samples. There are no reports on endophytic fungi from *R. tetraphylla* L. to compare their geographical distribution. Qadri et al.⁴⁰ found *Alternaria brassicae*, *Cladosporium cladosporioides*, *Alternaria alternata*, *Diaporthe helianthi*, *Fusarium proliferatum*, *Alternaria* spp. *Lasiodiplodia theobromae* and *Glomerella* sp. from *Rauwolfia serpentine*.

Host plants of endophytic fungi harbors mutual or commensal symbionts, typically providing nourishment and shelter. A host plant is one that supplies food resources and substrate for certain fungi⁴¹. The tissue specificity is usually defined by the part of the plant that is colonized with specific fungi⁴². Various factors such as environmental conditions, different plant tissues are involved in host and tissue specificity of endophytic fungi^{43,44}. In the present study, endophytic fungal distribution in leaves, stems and fruits were investigated in *R. tetraphylla*. Endophytic fungal composite and abundance varied with the different tissues of *R. tetraphylla*.

The present investigation has not showed any host and tissue specificity for *Aspergillus* sp. which was found in the entire test samples of different part of plants. Many of the previous research also have reported *Aspergillus* sp. as an endophyte from different plants^{31,45,46}. The genus *Beltrania* was found only in stem tissues of *R. tetraphylla* but, Cannon and Simmons⁴⁷ found this genus only in leaf segments of *Carapa guianensis*, *Catostemma fragrans* and *Chlorocardium rodiei*.

In the present study, *Cladosporium* sp. was found in fruit samples of *R. tetraphylla*. This

result agrees with Huang et al.⁴⁸ who reported *Cladosporium* sp. in stem samples of *N. oleander*. Species of *Cladosporium* were also reported from various other plants like *Embelia tsjeriam* and *Ocimum sanctum*^{49,50}. In this study *Curvularia* sp. were associated with stem parts of *R. tetraphylla*. Previous researchers also have reported *Curvularia* sp. associated with root tissues of *C. roseus* and different segments of *Alstonia scholaris*^{32,51}.

The present study revealed tissue specificity of *Cylindrocephalum* sp. which varied with different plants. *Cylindrocephalum* sp. was found in the leaf segments of *R. tetraphylla*, where as Sunitha et al.⁵² reported this genus in *Alpinia calcarata*. The current study revealed no tissue specificity for *Fusarium* spp., which were found fruit samples of *R. tetraphylla*. Zhang et al.⁵³ also has isolated *Fusarium oxysporum* from the phloem (inner bark) of *C. roseus*. Huang et al.³¹ also reported *Fusarium* sp. from different Chinese medicinal plants. This study revealed the occurrence of *Paecilomyces* sp. in leaf segments of *R. tetraphylla*. Garcia et al.⁵⁴ has reported *Paecilomyces* sp. in leaf of *Sapindus saponaria* L. and Tunali et al.⁵⁵ reported *Paecilomyces* sp. from Turkish Grass seeds.

In the present investigation, *Penicillium* sp. did not exhibit host specificity, this agrees with many previous research reports^{56,57,58,59}. *Pestalotiopsis* sp. was reported in *Catharanthus roseus*, *Terminalia morobensis*, *Mangifera indica*^{60,61,62}. There are no reports on *Thielavia terricola* as an endophyte but, the present study revealed its presence in stem and fruit samples of *R. tetraphylla*. In the current study there was no observation for

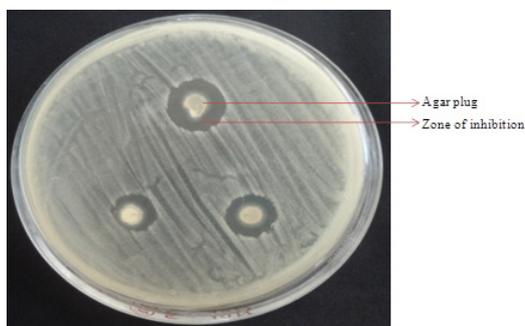


Fig. 4. Antimicrobial activity of endophytic fungi was determined by agar plug method

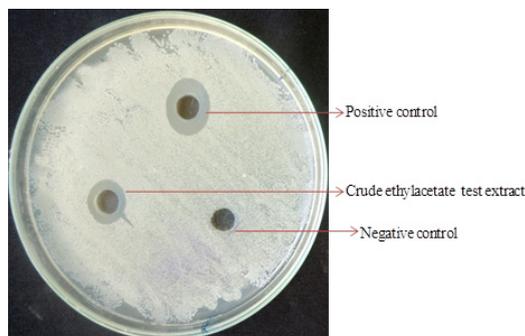


Fig. 5. Antibacterial activity of crude extract of endophytic fungi was determined by agar well diffusion method

tissue and host specificity for *Mycelia sterilia* spp. whereas, Huang et al.⁴⁸ reported 50% of *Mycelia sterilia* spp. in *N. oleander*.

The broad spectrum novel antibiotics are frequently required for treatment due to increase in the existence of naturally resistant bacteria, progression of new diseases caused by pathogenic microorganisms and toxicity of some of the current synthetic or natural compounds⁶³. Even though there are many antibiotics that have been effectively produced, new screening techniques are extensively required to isolate fresh and novel bioactive secondary metabolites from different sources in nature. The endophytic fungi are rich sources of secondary metabolites which kill wide variety of harmful disease causing agents⁵. Therefore, the utilization of endophytic fungi as sources of antimicrobial agents leads up to new areas for biotechnological exploitations, which open the isolation and cultivation of these organisms. In previous studies many endophytic fungi have been identified from different plants species belonging to *Apocynaceae* which have shown potential antimicrobial properties^{48, 64}. In the present study, the endophytic fungi isolated from *R. tetraphylla* exhibited broad spectrum of antimicrobial activity against the test microorganisms.

Twenty six endophytic fungi of *R. tetraphylla* were tested for antimicrobial activity, among them 19 isolates produced inhibition zone of 6mm to 10.33mm and *Curvularia* sp. (RTFs-6) exhibited antimicrobial activity against all the tested pathogens. The endophytic fungi *Curvularia* sp. (RTFs-6) and *Aspergillus* sp. (RTL-6) also exhibited potential antimicrobial activity. *Curvularia* sp. (RTFs-6) showed antimicrobial activity against *S. aureus*, *B. cereus*, *P. aeruginosa*, *E. coli*, *S. typhimurium* and *C. albicans* with inhibition zone of 8.33mm, 7.67mm, 6.67mm, 9mm, 7.67mm and 10.33mm respectively. Whereas, *Aspergillus* sp. (RTL-6) suppressed the growth of *S. aureus*, *B. cereus*, *E. coli*, *S. typhimurium* and *C. albicans* with inhibition zone of 6.67mm 7.33mm, 8.33mm, 7mm and 8.67mm respectively. To the best of our knowledge, there are no previous reports on antimicrobial activity of endophytic fungi isolated from *R. tetraphylla* but, endophytic fungi *F. proliferatum*, *Cladosporium cladosporioides* and *Glomerella acutata* isolated

from *R. serpentina* exhibited activity against *E. coli* and *S. aureus*; *Alternaria* spp. showed activity only for *S. aureus*⁴⁰.

The selected endophytic fungi, *Curvularia* sp. (RTFs-6) and *Aspergillus* sp. (RTL-6) in rice media it gave yield of 936mg/L and 975mg/L respectively. The secondary metabolite biosynthesis in microbes is sometimes controlled by regulatory mechanisms to avoid over production and these regulatory mechanisms process to undesirably low levels⁶⁵.

The crude extract of the potential endophytic fungal isolates, exhibited a broad spectrum of antimicrobial activity against the pathogens, when compared with that of positive control tetracycline for bacteria and fluconazole for yeast. The zone of inhibition ranged from 6.33 mm to 19.67 mm at concentrations of 20-100µg/mL. According to Rios and Recio³⁶ extracts of natural origin showing antimicrobial activity above 100µg/mL concentration should be avoided, hence in the present study, the concentrations of crude extract were limited to 10-100µg/mL. Both the fungal extracts of *R. tetraphylla* showed no inhibition at concentrations less than 20µg/mL and hence, MIC was determined to be 20µg/mL against test pathogens. Whereas, Huang et al.⁴⁸ reported the MIC to be 1.25-10mg/mL for *S. aureus*, 5-25mg/mL for *B. cereus*, 5-12.5mg/mL for *E. coli* when tested with the crude extracts of endophytic fungi isolated from *N. oleander* L. These results proved that endophytes from different host conditions exhibited varied antimicrobial activity⁶⁶.

In this study, extracts of *Curvularia* sp. (RTFs-6) exhibited MIC at 20µg/mL against *P. aeruginosa*. The extracts of *Curvularia* sp. (RTFs-6) and *Aspergillus* sp. (RTL-6) inhibited *S. typhimurium* at MIC 20µg/mL. The activity of crude extracts was found to be similar to that of reference positive control - tetracycline for bacteria and fluconazole for *C. albicans*. These findings strongly suggest that the metabolites screened for antimicrobial activity have broad spectrum activity and can be successfully used as potent antimicrobial agents⁶⁷.

In previous report, the antimicrobial activity of crude extract of *A. alternata* isolated from *Coffea arabica* L against *S. aureus* and *E. coli* showed the MIC at a range of 50-100µg/mL and 400-800µg/mL respectively. However, it did

not show any activity against *C. albicans* at all the tested concentrations⁶⁸. The extracts of endophytic fungi isolated from *N. oleander* showed MIC in the range of 1.25-10mg/mL for *C. albicans*⁴⁸. Lu et al.⁶⁹ found that ergosterol derivatives of *Colletotrichum* sp. isolated from *Artemisia annua* inhibited *S. aureus* and *B. subtilis* and *Pseudomonas* sp. at the range of 25-75µg/mL and 50-100µg/mL for *C. albicans*. Endophytic *Fusarium* sp. isolated from plant *Selaginella pollescens* collected in the Guanacaste conservation area of Costa Rica also showed potent activity against *Candida albicans* in agar diffusion assay⁴. The extract of endophytic fungus *Cochliobolus intermedius* isolated from *Sapindus saponaria* showed antibacterial activity against *Escherichia coli*, *Staphylococcus aureus*, *Salmonella typhimurium*, *Micrococcus luteus* and *Enterococcus hirae*⁵⁴. According to Huang et al.⁴⁸ most of the extracts of endophytic fungi from *N. oleander* possessed better antibacterial and antifungal activities when compared to the extracts of host plant. The secondary metabolite of endophytic fungus *Curvularia lunata* isolated from *Catharanthus roseus* exhibited antimicrobial activity against *B. subtilis*, *S. paratyphi*, *P. vulgaris*, *V. cholerae*, *S. aureus* and *E. coli*⁷⁰. The ethyl acetate extract of *Aspergillus* sp. isolated from *Ficus carica* showed significant antimicrobial activity against *Pseudomonas aeruginosa*⁷¹.

In the present study, antimicrobial activity of crude extracts of *Curvularia* sp. (RTFs-6) and *Aspergillus* sp. (RTL-6) varied statistically with the zone of inhibition produced by the standard drug tetracycline (20µg/mL) and fluconazole (20µg/mL). The crude extract of *Curvularia* sp. (RTFs-6) and *Aspergillus* sp. (RTL-6) at 100µg/mL was in par with the positive control against *B. cereus*. Ethyl acetate extract of *Aspergillus* sp. (RTL-6) was inactive against *P. aeruginosa*. However, the antibacterial activity of *Curvularia* sp. (RTFs-6) and *Aspergillus* sp. (RTL-6) against *E. coli* was almost similar and higher than that of positive control. Inhibition zone of crude extracts of *Curvularia* sp. (RTFs-6) and *Aspergillus* sp. (RTL-6) against *C. albicans* of 100µg/mL was either near or higher than that of fluconazole. The endophytic fungi thus screened for antimicrobial activity exhibited potent activity against the test pathogens and proved to be promising as a drug

in near future. The present results correlated with the previous findings of endophytes as potent antimicrobial agents⁷².

Phytochemical analysis was carried out for endophytic fungal extracts to find the presence of chemical components as a prospective source for medicinal and industrial use. Their presence is an indicator so that, can be exploited as precursors in the development and advancement of synthetic drugs⁷³. Phytochemical analysis has been carried out in several plant species but very few reports are available on endophytes⁴⁸.

The active metabolites of fungal extracts contain chemical groups such as phenols, steroids, flavonoids, quinines, terpenoids, xanones, peptides, cytocatalasins, alkaloids, aliphatic compounds, and phenyl propanoids⁷⁴. In the current study, phytochemical analysis of extract *Curvularia* sp. (RTFs-6) revealed the presence of alkaloids and terpenoids. Similarly, the terpenoid was identified from extract of endophytic fungus *C. lunata* isolated from *Catharanthus roseus*⁷⁰. Extract of *Aspergillus* sp. (RTL-6) showed presence of alkaloids and tannins. Our results are well in accordance with previous reports of Lai et al.⁷⁵ who have reported the presence of different phytochemicals viz alkaloids, steroids, tannins⁷⁶; phenolic compounds and flavonoids⁷⁷ and are also known to possess strong antimicrobial activities.

CONCLUSION

The present study concludes that, the 26 endophytic fungi associated with *R. tetraphylla*, *Curvularia* sp. (RTFs-6) and *Aspergillus* sp. (RTL-6) are effective alternative sources of antimicrobial drugs and can be further commercially exploited.

ACKNOWLEDGEMENTS

The authors are thankful to Dr. Siddamallaya for authenticating the plant sample and maintaining the herbarium in National Ayurveda Dietetics Research Institute. (Central Council for Research in Ayurveda and Siddha, Department of AYUSH, Ministry of Health and Family Welfare. Govt. of India, New Delhi) Jayanagar, Bangalore.

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