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Research Article

Buchanania lanzan Fungal Endophyte *Penicillium* sp. Therapeutic Properties Attributed to Lapachol in its Extract

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ABSTRACT

Buchanania lanzan, Spr. (Family: Anacardiaceae) with known therapeutic potential is reported as vulnerable and is listed in Red Data Book of IUCN. With immense value for its bark, roots, seeds and gum, exploitation of the host on large scale is reported detrimental. Hence the authors have attempted to study the residing endophytes for metabolites equivalent to the host products. The leaf endophytic fungi were morphotyped based on internal transcribed spacer–deoxyribonucleic acid (ITS–DNA) sequences and *B. lanzan* endophytes identified by molecular typing include *Penicillium* sp, *Fusarium* sp., *Aspergillus* sp and *Fusarium* sp. Molecular typing identified *Penicillium* sp. as *Penicillium gravinicasei* with 98% similarity to the nearest genera and so its extract was screened for therapeutic capacities. Antioxidant activity of the extract exhibited 2,2-diphenyl-1-picrylhydrazyl (DPPH) scavenging capacity with IC_{50} of 55.95 ± 3.29 $\mu g.mL^{-1}$. Antimicrobial activity by disc diffusion in opposition to *Pseudomonas aeruginosa*, *Staphylococcus aureus, Escherichia coli* and *Bacillus subtilis* significantly arrested bacterial growth. Bacterial biofilm inhibition capacity stained by acridine orange and ethidium bromide imaged by confocal laser scanning microscopy revealed bactericidal activity. Lapachol in the endophyte extract detected by TLC could support the therapeutic properties. Thus, studies on unexplored medicinal plant endophytes could pave path to identification of novel secondary metabolites as therapeutic agents and potential drug candidates.

Introduction

Medical practitioners and researchers are in continuous quest for natural medicine with no or less side effects. Ayurveda, a traditional Indian medicine system with about eighty percent population dependent on it utilizes plants as resource. Escalating world-wide research on the effectiveness of herbal medicine for chronic diseases has lead to putting forth scientific evidence for traditional beliefs. *Buchanania lanzan*, Spr. (Family: Anacardiaceae) commonly termed Chironji with its origin from India is reported from deciduous tropical forests in western, northern and central India. A total of seven different species, *B. lanzan*, *B. axillaries*, *B. lanceolata*, *B. platyneura*, *B. lucida*, *B. glabra* and *B. accuminata* are reported. *B. lanceolata* from Kerala's ever green forests is reported as endangered species and *B. platyneura* is reported from forests of Andaman only. *B. lanzan*, *B. platyneura* and *B. axillaries* produce edible fruits. *B. lanzan*, a commercially valuable tree is reported to find application for relieving prickly heat and reducing swelling of glands. Its bark gum extract taken orally is reported to reduce diarrhoea and intercostals pain. However, the negligence and the high demand for various parts of the tree have lead to loss in its numbers leading to its being listed in IUCN Red Data Book.[1,2]

As an alternative to the host, phytotherapists searched for sources and reported endophytes with the capacity to generate related metabolites as the host plant.[3] Plants harbour microorganisms that could be bacteria and fungi collectively known as endophytes.[4] Studies have reported existence of more than one species of these microbes housed in a single host. These microbes could be symbiotic and bordering pathogenic living in plants tissues spending a part or

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their complete life cycle residing either intercellularly and/or intracellularly within the plant causing no overt negative effects. Several studies have indicated the ease with which they can be isolated from any part of the host using commonly available growth medium. They have reviewed on endophytes being pivotal provenance of secondary metabolites with specific biological task.[5-7] Usually, secondary metabolites synthesized by endophytes are highly regulated and modulated at the genetic level; however, when the external environment is tweaked, physiologically is affected resulting in further activation of these genes.[8] Metabolites from endophytes support growth of the host, aid with improvement to tolerate biotic/abiotic stress, increase resistance to pest/insect attack or organic toxins.[9] The products of such interactions, the metabolites exhibit broad spectrum pharmaceutical property. These metabolites are pilots of pharmaceutical revolution leading to discoveries of drug with a range of biological activities are a boon. Secondary metabolites conferring biological activity comprise phenols, alkaloids, quinones, steroids, polyketides, terpenoids, and lignans.[9] These exhibit assorted biological properties such as antioxidant, antibacterial, antidiabetic, cytotoxicity, antimalarial, anti-tubercular and acetylcholinesterase hindrance.[10-12]

The endophytes are todays` established expedient supply of natural ad-mixture with therapeutic values.[6] Exploring these as-yet untapped natural legacy increments the chances of unearthing novel entities documented by chemical analysts with use in pharmaceutical sector leading to drug source. However, it remains important to constantly screen for novel entities from nature resources, endophytes and that too from seldom stumbled across medicinal plants.[7]

With this scenario, we attempted to report on the endophytic flora from the leaves of medicinally important Western Ghats tree *B. lanzan* as a lack of reports on endophytes residing in this pharmaceutically important tree was noted. Ethyl acetate extracts of the fungi were prepared, and they were tested for antioxidant and antimicrobial activities. Chemical compound analysis for fungal endophytic extracts was carried out on thin layer chromatography and the presence of lapachol in the endophyte extract is reported to support the therapeutic properties.

Material and Methods

Collection of Leaf Samples

The Kigga region in Western Ghats of Chikmagalore District in Karnataka State was surveyed and *B. lanzan*, Spr. leaves were collected. Voucher specimens (*B. lanzan* # IOE LP0004) were filed at the department. The collected leaf samples were placed in plastic bags and they were processed within 48 hours of collection for fungal endophyte isolation.

Isolation of Fungal Endophytes from Leaves

The leaves were washed several times using running tap water to remove the stuck dust and grime particles. The surface was cleaned under aseptic conditions to avoid contamination by sequential rinse with ethanol (70%; v/v) for 1 minute, followed by 4.5% (v/v) sodium hypochlorite (3% available chlorine) wash for 3 min and finally washed with sterile water (1 minute) to remove traces of surface sterilant.[13] The leaf bits were then dried tapping with a sterile blotting paper to remove excess of moisture. The leaf bits (10–15 bits; 0.5 to 1-cm) were arranged in sterile water agar media (15 g/L) supplemented with 250 ppm chloramphenicol in a sterile petri dish. Incubation was followed for 15 days, at 28°C with alternate dark and light cycles for 16 hours for endophyte growth. The emerging fungal endophytes were carefully harvested and placed in sterile potato dextrose agar (PDA) media to induce sporulation. The fungal endophyte isolated were preserved in cryovials on broth (PDB) containing glycerol (-80°C; 15%, v/v).

Isolation of Genomic DNA of Endophytes, its Amplification by PCR and their Identification by Sequencing of ITS and nBLAST

Fungal endophytes were identified based on their colony characters (Fig. 1b), spore morphology or fruiting bodies (Fig. 1c) visualized by Research Stereo Zoom Microscope (Stereo Discovery V20; Carl Zeiss, Germany) using standard identification manuals.[14,15] Endophytic fungal cultures on PDA media were harvested, their genomic DNA was obtained by CTAB (cetyltrimethyl ammonium bromide) method[16] and was stored in Tris EDTA buffer (100 mL). Quantification and purity of the genomic DNA was checked in Nanospectrophotometer (Thermo 200°C; Thermo Fisher Scientific, USA). Pure DNA (ratio 260–280 nm) with the values (1.6/1.8) devoid of contamination with protein/phenol or RNA was subjected to agarose gel electrophoresis (1%) containing ethidium bromide and was visualized in gel documentation system (Geldoc XRT (BioRad, USA). Amplification by PCR was by using Universal ITS (internal transcribed spacer) primers for fungi, [ITS1:5`-TCC GTAGGTGAACCTGCG G-3`; ITS4: 5`-TCCTCCGCTTATTGATATGC-3`][17] in PCR tubes (0.2 mL) containing reaction mixture (25 mL) with genomic DNA (1-mL; 50 ng/mL) in a cycler (thermal; Master Cycler gradient; Eppendorf, Germany). The program[16] included initial denaturation (95°C for 5 min, 94°C for 3 min), annealing of primer (55°C for 1 min) and extension (2 min and 10 min, 72°C) repeated for 35 cycles. The amplified products (5 mL) were loaded on agarose gel (1%) and DNA bands were visualized along with standard ladder (100–10,000 bp) for molecular weight determination. For sequencing, the PCR amplified products were sent to Chromous biotech, Bangalore and they were analyzed by National Center for Biotechnology Information (NCBI) nucleotide basic local alignment search tool (nBLAST).

Preparation of Endophyte Extracts

Agar pieces (0.5 cm^2) with actively growing fungal endophytic colony were inoculated onto sterile potato dextrose broth (PDB; 1L) and the flasks were incubated with alternate light (8 hours) and dark cycles (16 hours) in stationary phase (25 ± 2°C) for 15 days. The mycelial mat in the broth was sonicated and the whole mass was filtered through double layer muslin cloth. For extraction of metabolites, the filtrate was mixed with equal volumes of ethyl acetate in a separating funnel and the blend was strongly agitated. The upper solvent layer was separated, concentrated to solid sticky mass (10 mg) in Heidolph rotary evaporator set at 42°C[18] and was stored in a colored glass container (4°C) for analysis.

Estimation of Total Phenol Content in the Extract

The phenol content in endophyte ethyl acetate extracts was determined by Folin-Ciocalteu calorimetric method.[1,2] Test sample (100 µL) was reacted with Folin-Ciocalteu reagent (0.75 mL; diluted 10 fold) and allowed to stand for 5 minutes. After neutralization with saturated sodium carbonate (60 g/L) it was incubated in the dark for 1.5 hours at 22°C and the absorbance was measured at 725 nm using UV/visible spectrophotometer (U-3900;Hitachi). Gallic acid standard (25–250 µg/mL) was prepared under similar conditions. Total phenol content was quantified using this gallic acid standard graph and phenol content was expressed as gallic acid equivalence (GAE; µg/mg of extract).

Free Radical Scavenging Ability of the Extracts was Tested by DPPH Radical Scavenging Assay

DPPH (1, 1-Diphenyl-2-picryl hydrazyl) method for determining the antioxidant potential was followed as described by Brand-Williams *et al.*[19] at 37°C in dark for 30 min and the absorbance was recorded at 517 nm (Spectra max 340, multimode plate reader, Molecular devices). The reaction mixture included extract (5 µL; 1-mg/mL) mixed with DPPH solution (95 µL; 300 µM). Scavenging activity of DPPH radical was estimated in comparison with a methanol (negative control). Ascorbic acid and quercetin (25–250 $\mu g.mL^{-1}$; Sigma-Aldrich, St. Louis, MO, USA) were used as positive control. The values (IC_{50}) representing the concentration of endophyte extract required to scavenge DPPH (50%) radicals was reported.

Anti-microbial Activity

a. Agar Disc Diffusion Method

Gram (–) bacteria *Pseudomonas aeruginosa* (ATCC 27853); *Escherichia coli* (MTCC 724) and Gram (+) bacteria, *Bacillus subtilis* (MTCC 441) and *Staphylococcus aureus* (MTCC 96) were procured from the Institute of Microbial Technology, Chandigarh, India. The extract (1:10 diluted; 1, 2.5, 5.0, and 10.0 µg) was loaded on two 10 mm discs placed on these plates and incubated (15 to 18 hours at 37°C). The diameter of inhibition zones was reported comparing against chloramphenicol (positive standard) under similar experimental conditions.[20]

b. Assay of Loss of Bacterial Biofilm Analyzed by Confocal Laser Scanning Microscopy

To observe the biofilm structure, CLSM (LSM 710 Carl Zeiss, Germany) was used. The samples were prepared [21] from the overnight cultures of Gram (-) bacteria, *P. aeruginosa* nd *E. coli* with the absorbance set at 1.5 ± 2.0 at 600 nm. The assay was conducted in a 6-well plate containing cover slips and growth media (2 mL) in all the wells. To the test wells, overnight bacterial suspensions (750 µL) were introduced and after 24 hours incubation at 37°C the extracts (200 µL, 750 µg) were added. The biofilm formed on the cover slips in wells were removed carefully and were washed with PBS. Ethidium bromide and acridine orange (20 µL; 1 µg in 400 µL; HiMedia, India) were used to stain the biofilm (5 minutes) at room temperature. After three washes with PBS, the cover slips with stained biofilm on them were placed on glass slide in an inverted position and the CLSM images of the samples were imaged. The results presented are images of three independent experiments.

Chromatography by Thin Layer Chromatography

Chemical compound analysis for the extract was carried out on thin layer chromatography plates (silica gel GF_{254}, Merck). Extract (10 µL; 10 mg/mL) was applied onto TLC plates and was developed in the solvent combination (CH_2Cl_2:MeOH; 10:1). The separated compounds on the TLC plates were visualized under 366 nm and also 254 nm, ultraviolet (UV) light.

Statistical Analysis

All experiments and measurements were made in triplicate and the values are reported as the mean ± standard deviation (SD). The results were subjected to variance analysis followed by Tukeys` test to analyse differences between the endophyte ethyl acetate extract and controls. Statistically significant differences ($p < 0.001$) were reported.

Results

The present study gives an insight into the endophytic fungal community associated with the leaves of *B. lanzan*, Spr. (Family: Anacardiaceae) as this could be the first report on the endophytes from *B. lanzan* leaves.

Isolation and Identification of Fungal Endophytes by Morphotyping and Molecular Typing

A total of 33 isolates were recovered from a total of 150 leaf segments. Endophytic DNA was extracted as reported in materials and methods and the amplified ITS-DNA presented a clear band on agarose gel (1%) of molecular weight 560 bp (Fig. 1).

Lanes MW: 100–1000 bp DNA ladder; lane 2: *Penicillium* sp; lane 3: *Fusarium* sp; lane 4: *Aspergillus* sp; lane 5: *Fusarium* sp

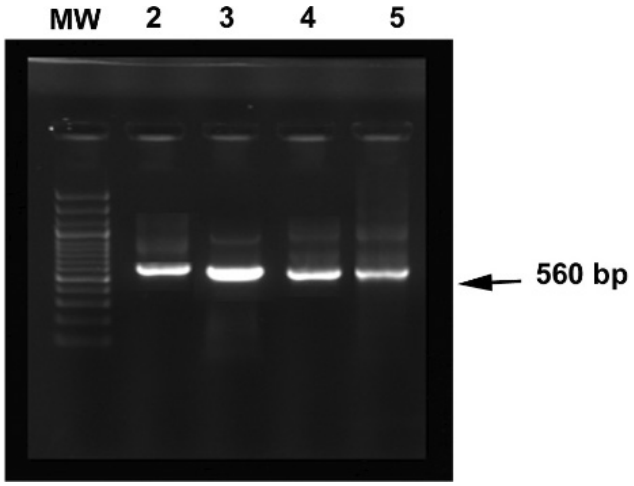

Fig. 1: PCR amplifications of ITS regions of selected endophytes on agarose gel (1% w/v)

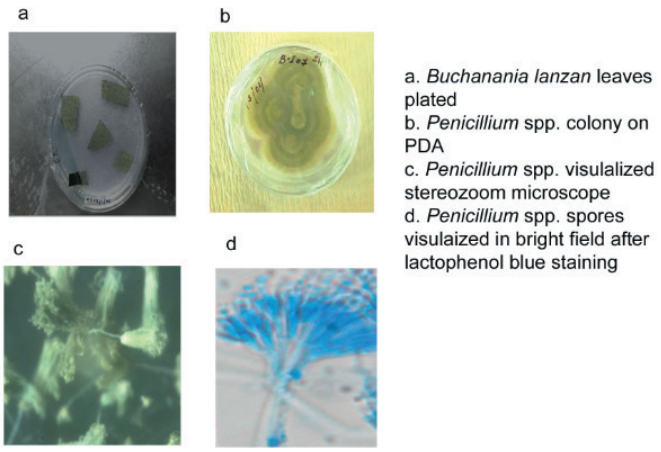

Fig. 2: Main approach of the study with stages involved in isolation and identification of endophytic fungi *P. gravinicasei* from leaf bits of host *Buchanania lanzan* leaves.

Table 1: Concentration and purity of the DNA extracted from fungal endophytes and identification of endophytes based on the ITS sequences

Endophyte code	*Endophyte identified*	*DNA (ng/100 mL)*	*Total DNA*	*Percentage similarity to the nearest genera*	*GenBank accession number*
BL-1	*Penicillium* sp	196	1.83	*Penicillium gravinicasei* (98%)	MG600581.1
BL-6	*Fusarium* sp	182	1.81	*Fusarium solani* (92%)	MH109274
BL-23	*Aspergillus* sp	193	1.76	*Aspergillus* sp (91%)	MH594213
BL-31	*Fusarium* sp	195	1.71	*Fusarium proliferatum* (94%)	MH109276

Data expressed are mean (n = 3) ± SD (Standard deviation)
BL – *Buchanania lanzan*

The purified band was sequenced, and its analysis was done by NCBI, n BLAST. *B. lanzan* endophytes identified by molecular typing include *Penicillium* sp, *Fusarium* sp, *Aspergillus* sp and *Fusarium* sp. (Table 1).

Penicillium sp. exhibited 98% similarity to its nearest genera was identified as *P. gravinicasei* by its colony, spore morphology (Fig. 2b, c) and by the analysis of ITS regions of the DNA (Table 1). Ethyl acetate extract of this microbe was prepared and it was subjected to evaluation of biological activities in the present studies.

Total Phenol Content and DPPH Radical Scavenging Assay of *P. gravinicasei* Extract

The total phenol content was expressed as equivalents of Gallic acid in microgram/mg of the sample. The *P. gravinicasei* extract exhibited a phenol concentration of 42.80 ± 0.28 mg GAE/mg for the extract. *P. gravinicasei* endophyte extract exhibited scavenging of DPPH with the IC50 value of 55.95 ± 3.29 µg/mL^{-1}. The reference compounds, ascorbic acid and quercetin exhibited IC50 values of 28 ± 3.12 and 21 ± 1.32 µg.mL-1 (Table 2).

Antibacterial Activity

a. Agar Disc Diffusion Assay

Inhibition zone studies by agar-disc diffusion method exhibited a clear antimicrobial effect of the extract. A statistically significant inhibition of both gram negative and gram positive bacteria were recorded (Fig. 3). *P. gravinicasei* extract were screened using four microbes, Gram (–) bacteria *E. coli* (MTCC 724), *P. aeruginosa* (ATCC 27853) and Gram (+) bacteria, *S. aureus* (MTCC 96) and *B. subtilis* (MTCC 441) by disc diffusion. Extract (ethyl acetate; 10 mg) significantly arrested *P. aeruginosa* (1.1 cm) and *S. aureus* (0.5 cm) followed by bacteria *E. coli* and *B. subtilis* (0.2 cm).

b. Assay of Loss of Bacterial Biofilm Visualized by Confocal Laser Scanning Microscopy

Gram (-) strains of *P. aeruginosa* (ATCC 27853) and *E. coli* (MTCC 724) commonly reported as biofilm forming strains were included in the present study. From sample observations, biofilm formation on the cover slip support was observed for the controls, *P. aeruginosa* (Fig. 4a) and *E. coli* (Fig. 4c). They were live cells visualized green stained by acridine orange. The addition of the extract (200 µL, 750 µg) resulted in a loss in the biofilm and this observation was visualized by CLSM studies. Patches and broken down biofilm architecture and with decreased micro-colonies containing dead cells of *P. aeruginosa (Fig. 4b)* and *E. coli* (Fig. 4d) was observed red stained by ethidium bromide.

Lapachol Detection by TLC in the Endophyte Extract

Secondary metabolite profiling by TLC identified several bands under UV light and in white light.

Table 2: Antioxidant and total phenol concentration of *P. gravinicasei* endophyte extract isolated from *B. lanzan*

Endophyte extracts/ positive controls	*DPPH (IC_{50}) (µg/mL)*[a]	*Total Phenol content (mgGAE/mg)*[a]
P. gravinicasei extract	*55.95 ± 3.29*	*42.80 ± 0.28*
Ascorbic acid	*28 ± 3.12*	-
Quercetin	*21 ± 1.32*	-

*Total phenol concentration was measured and reported as gallic acid equivalent (GAE; µg/mg) of the fungal extract
[a]mean ± SD, – represents no activity from endophytic extracts

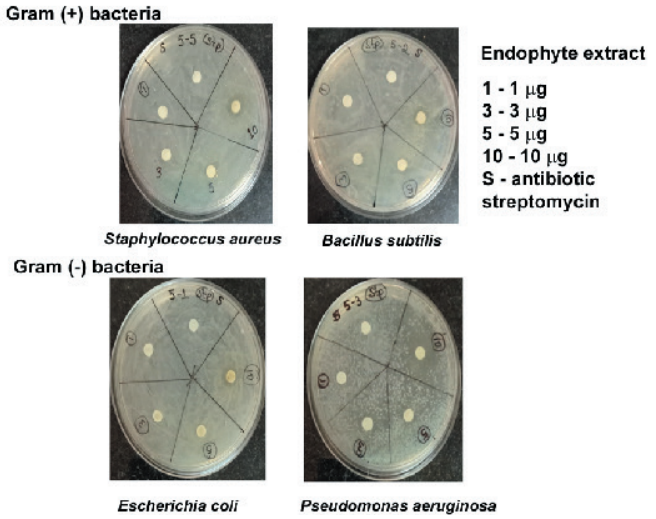

Fig. 3: Antibacterial assay of *P. gravinicasei* extract by disc diffusion method

From the earlier reports, presence of lapachol in *Penicillium* sp was known. Hence in the current study standard lapachol was run in TLC mode under similar conditions. The presence of lapachol in the endophyte extract by TLC analysis in comparison with standard lapachol (Fig. 5) is reported. Further research could be directed to characterize other metabolites in the extract.

Discussion

Treatment of bacterial induced infections involves antibiotics, and its prolonged application has resulted in antibiotic resistance exhibited by them leading to hindering the ability to treat even commonly reported bacterial infections. These infections could be pneumonia, gonorrhea, tuberculosis, or blood poisoning resulting it impossible to cure with antibiotics becoming less useful. A rise in research interest related to endophytic microbes in medicinal plants for their ability to generate broad spectrum metabolites with therapeutic potential is observed.[3-13] Bioprospecting has been applied as a tool to commercialize medicinal plants and the diverse endophytes residing in its tissues. Endophytes are reported paragon of metabolites like flavonoids, phenols, chinones, alkaloids etc., exhibiting several biological activities, including antioxidant,[22] antibacterial[23] and anti-cancer[24]

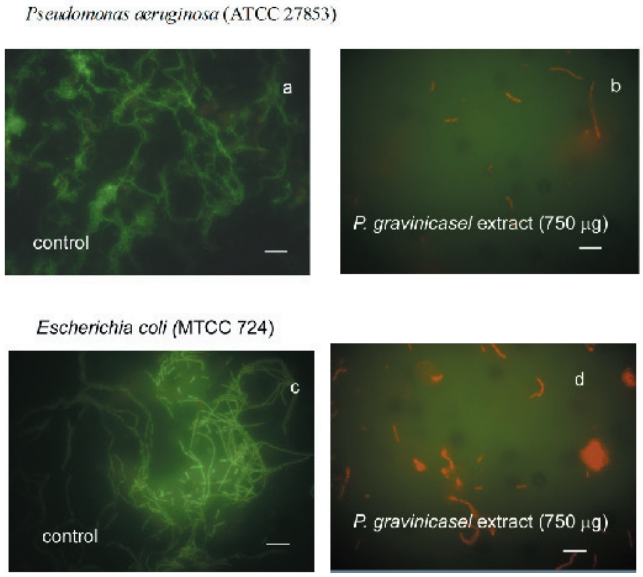

Fig. 4: Biofilm inhibition assay of *P. gravinicasei* ethyl acetate extract. Controls, *P. aeruginosa* (a); *E. coli* (c). *P. gravinicasei* extract (ethyl acetate) + *P. aeruginosa (Fig. b); P. gravinicasei* extract (ethyl acetate) + *E. coli* (d).

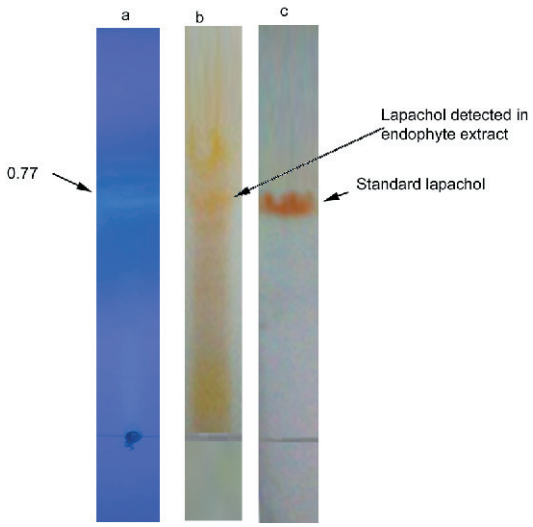

Fig. 5: Thin layer chromatography detecting presence of lapachol in *P. gravinicasei* extract as visualized under UV (a) and white light (b). TLC of standard lapachol (c) was executed under similar conditions. The Rf value (0.77) for lapachol is presented in the figure. The results are indicative of 3 independent experiments.

revolutionizing pharmaceutical industry. It is reported that of the 35% microbes isolated from medicinal plants several of them (about 80%) are reported to provide molecular entities with biological activity contributing 82% of the natural drugs to the commercial establishments. This has led to discoveries of several unique secondary metabolites that have been used as drug.[25,26]

This edged the authors in the present study to initiate scientific screening of endophytes from a valuable western Ghats medicinal tree, *B. lanzan*. It is reported that plant-based drug is generally not produced to the

required quantities as its production is dependent on specific developmental stage, environmental conditions, stress and/or availability of specific nutrient. As a plant and/or tree takes several years to grow, the production and accumulation of specific secondary metabolite may take several years. With these limitations associated with plants as sources of secondary metabolites, it was visualized that microbes (endophytes) residing in their tissues could be screened for potential sources of novel natural production that could find applications in agriculture, medicine, and drug industries.

In this direction, our work could be the first study of fungal endophytes from *B. lanzan* tree leaves. The fungal endophytes were cultivated, and four fungal isolates were morphotyped with their molecular identity revealed by ITS–DNA sequences analysis as *Penicillium* sp, *Fusarium* sp., *Aspergillus* sp and *Fusarium* sp. One of the isolates, screened for therapeutic capacities was the *Penicillium* sp. identified as *P. gravinicasei* by molecular typing exhibiting 98% similarity to the nearest genera. Antioxidant capacity of ethyl acetate extract exhibited DPPH scavenging capacity with IC50 of 55.95 ± 3.29 $\mu g.mL^{-1}$ is reported.

Further, we provide here the anti-bacterial capacity of *P. gravinicasei* extract screened against four microbes (Fig. 3) which significantly arrested the growth of *P. aeruginosa* and *S. aureus* followed by *E. coli* and *B. subtilis*. Bacterial biofilm inhibition capacity stained by acridine orange and ethidium bromide imaged by confocal laser scanning microscopy revealed the loss of microcolonies. In the present study, secondary metabolite from *P. gravinicasei* extract was characterized by TLC and the presence of lapachol was reported as observed in earlier report,[27] with Rf value of 0.77 both under UV and white light. The existence of bioactive, lapachol (a naphthoquinones) detected via TLC could support these anti-microbial properties. Several reports involving, naphthoquinones like lapachol exhibiting therapeutic properties are available. It was observed that these compounds exhibited pharmacological capacity including antineoplastic, anti-fomenting, anti-trypanosome, antimalarial and microbicidal activity.[28,29] Their therapeutic activity may be related to molecular characteristics of these molecules. Their mechanism of action could include generation of reactive oxygen species (ROS), bioreduction of quinone regions by NAD (P) H: quinone oxirreductase-1 (NQO1) and specific interaction with topoisomerase.[30]

The present report on endophytic fungi *P. gravinicasei* screened from consortia of endophytes from *B. lanzan* tree leaves, its characterization via morphotyping and molecular typing by ITS-DNA sequence analysis adds to the current trend of knowledge with respect to several studies indicating *Penicillium* spp. as a rich provenance of compounds with biological activity.[27] Although, lapachol from the extract was reported by TLC analysis in comparison to use of standard lapachol, further investigations are required to determine other secondary metabolites from *P. gravinicasei*. This scientific activity could lead to identification of many more new molecules with potential in future drug development. Our laboratory is continuing to pursue this research. Data collection is a valuable exercise for understanding the extent of the potential of endophytic *Penicillium* species reported to be a true fungal factory for exploitation directed towards wide variety of applications.

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