Endophytic associations in ethnomedicinal plants of Western Ghats and their antioxidant and antimicrobial potentials

A consolidated progress report submitted to the University Grants Commission (UGC) from the period 30.06.2011 to 31.12.2014 under the UGC-Major Research project Grant No. F. No. 40-307/2011 (SR) dated 30th June 2011

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Introduction

Endophytes of medicinal plants have incited considerable interest and attention for the wide diversity of bioactive metabolites (Caiet al. 2004; Strobel et al. 2004; Newman and Cragg 2007). Medicinal plants afford unique microcosm for the endophytes that reside in the internal organs or cell spaces of healthy plants. Diverse fungal endophytes exist within plant tissues and each plant can host one or more endophytes (Yu et al. 2010). The endophytes apparently stay in a mutualistic association with the host plants and can be a novel source of metabolites of pharmaceutical interest (Huang et al. 2008a). Endophytes are capable of producing similar secondary metabolites as their hosts (Alvin et al. 2014).

Reactive oxygen species (ROS) are continuously generated from the cells because of endogenous (aerobic metabolism) and exogenous (UV irradiation, environmental pollutants, diets etc.) elicits. ROS includes superoxide, hydroxyl ion, hydrogen peroxide, alkoxyl, nitric oxide, sulfhydryl etc. It is well known that excess of ROS is toxic and causes damage to the cell components, including nucleic acids, proteins and lipids. In addition, it causes apoptosis or necrosis (Thannickal and Fanburg, 2000). Oxidative damage to cell components plays a vital role in human diseases *viz.*, diabetes, cardiovascular, cancer, neurodegenerative disorders, Alzheimer's disease, kidney disease etc., (Maldonado et al., 2005). Antioxidants are important compounds with potential therapeutic benefits against these oxidative damages. Antioxidants can inhibit free radicals by binding to the oxidation promoter or by scavenging the free radical chain reaction at early stage, the mechanism is very diverse. Antioxidant compounds can be thiols, ascorbic acids or phenolics.

Bioactive antioxidants are being investigated from various plants since long. Microbes are also gaining importance for the production of various bioactive metabolites as plants. Endophytes are microorganisms which live within the plant tissues without causing any apparent infections (Wilson, 1995). These are not only rich sources of bioactive metabolites as plant itself; exploitation of such organisms also does not harm the biodiversity. The endophytic fungi and actinomycetesproduce a plethora of substances with potential application in modern medicine, agriculture and industry such as antioxidants, novel antibiotics, antimycotics, immunosuppressants and anticancer compounds (Strobel and Daisy, 2003).

India is one of the 17 mega-biodiverse countries in the world as recognized by Conservation International (Mittermeier et al. 1997). The Western Ghats, being a biodiversity hotspot in southern India, is a home for copious medicinal plants used ethnomedicinally. *Zingiber nimmonii* (J. Graham) Dalzell., is an endemic species of the Western Ghats, a biodiversity hotspot of Southern India. The bioactive potential of the plant is well established (Sabulal et al., 2006; Finose and Gopalakrishnan 2014). *Z. nimmonii* is a wild congener of *Z. officinale* (Kavitha et al., 2010). Zingiberaceous species have earlier been subjected to endophytic fungal isolations with the emphasis on the diversity and bioactive potentials of the endophytes (Bussaban et al., 2001; Nongalleima et al., 2013). Despite these findings, attempts are limited on the documentation of antioxidative and antimicrobial potentials from the endophytic fungi and actinomycetes responsible for the bioactivity. Therefore, the primary aim of the present study was to isolate and characterize the fungal endophytes from the plant parts of *Z. nimmonii* and the characterization of secondary metabolites of fungi responsible for the antioxidant activity and antimicrobial activities.

Polygonum chinense L., commonly known as Chinese knotweed belongs to the family Polygonaceae and is common in subtropical and warm temperate regions of Asia (Maharajan et al. 2012). Species of *Polygonum* contain pharmaceutically important bioactive compounds (Ismail *et al.* 2012). The plant itself is used to treat various diseases *viz.*, diarrhea, dyspepsia, hemorrhoid sprains, tonsillitis and poisonous snake bites etc. (Chevallier 1996). In local health traditions, the decoction of stem and roots mixed with jaggery is given to women for post-natal care. Owing to the traditional usage of this plant in providing health benefits, this plant was selected for the isolation of fungal and actinomycete endophytes from the Talacauvery subcluster of Western Ghats. The isolation of endophytic fungi and actinomycetes from *P. chinense* and the characterization of bioactive compounds are limited, although the plant is reported to possess antimicrobial compounds (Ezhilan and Neelamegam 2012). Hence, the primary aim of the study was to isolate and characterize the fungal endophytes from the plant parts of *P. chinense* and characterization of secondary metabolites responsible for the antioxidant and antimicrobial activities.

Justicia wynaadenesis Heyne., is an endemic plant, naturally occurring in the rain forest of Western ghats, East Nilgiris and South Malabar Hills in South India (Gamble, 1928). The plant locally known as ‘Madubanasoppu’ or ‘Maddhuthoppu’, belongs to the family acanthaceae.

The family consists of 250 genera, many of which have ethhnomedicinal properties (Awan et al., 2014). *J. wynaadenesis* also have ethnomedicinal properties. The juice of the stem and leaf of this plant is used locally to cure asthma, boost immunity, as anthelmintic and anti-diabetic (Lingaraju et al., 2013). *J. wynaadenesis* also has cholesterol lowering properties (Subbiah and Norman, 2002). The stem and leaf juice of this plant is extracted in boiling water. A deep purple colored extract obtained through this process is consumed as it is or as sweet dish by the local community. This traditional practice is believed to offer wellness throughout the year. Although, the endemic plant is ethnomedicinal, no reports are available on its endophytic profile. Therefore, the primary aim of the study was to isolate, identify the fungal and actinomycete endophytes morphologically as well as with the help of molecular tools from the ethnomedicinal plant *J. wynaadenesis* and the evaluation of antioxidant and antimicrobial activity of the endophytic strains.

M. frondosa is a rubiaceous shrub with whitish bract leaf and orange flowers. The leaves are used as antiviral treatments for measles, while *E. foetidum* is a herbaceous apiaceae known for its culinary use as alternative to the coriander. The details of the medicinal uses of five plants considered for the study is given in Table 1. Till now, there are no known reports of fungal and actinomycete endophytes from these plant species considered for the study. Therefore, the study has been designed with the following objectives:

Table 1. List of plants employed in the present study

Sl. No.	Plant species	Common name	Family name	Ethno medicinal use
1	*Mussaenda frondosa* L.	Flag bush	Rubiaceae	Antiviral, antioxidant
2	*Polygonum chinense* L.	Chinese knotweed	Polygonaceae	Antioxidant
3	*Justicia wynadensis* Heyne	-	Acanthaceae	Cholesterol-lowering, health wellness
4	*Eryngium foetidum* L.	Spiny coriander	Apiaceae	Anti-oxidant, medicinal
5	*Zingiber nimmonii*	-	Zingiberaceae	Antimicrobial

Objectives of the study

1. **Collection of plants of ethnomedicinal value from natural habitats of Western Ghats and isolation of endophytic fungi &actinomycetes from plant parts**
2. **Characterization of endophytic fungi & actinomycetes by cultural, biochemical and molecular methods**
3. **Microbial fermentation for the production of secondary metabolites**
4. **Screening of extracts for potential antioxidants and antimicrobials**

Methodology

OBJECTIVE 1.Collection of plants of ethnomedicinal value from natural habitats of Western Ghats and isolation of endophytic fungi & actinomycetes from plant parts

Collection of plant materials

The five ethnomedicinal plants viz., *Z. nimmonii*, *P. chinense, J. wynaadensis, M. frondosa* and *E. foetidum* were collected from natural habitats of Talacauvery sub cluster (012°17' to 012°27' N and 075°26' to 075°33'E) of Western Ghats, in Kodagu district, Karnataka state, India (Fig. 1). The roots, rhizomes, leafy stem, leaves and inflorescences of *Z. nimmonii* were collected during November 2011, while the leaves and roots of *E. foetidum* were collected during November 2011. The stem and leaves of *P. chinense, M. frondosa* and *J. wynaadensis* were collected during June and August of 2012 respectively. The plants were identified taxonomically with the help of flora (Gamble, 1928). A herbarium specimen has been preserved and deposited in the Department of Studies in Botany, University of Mysore. The plant parts were collected, kept in zip-lock polythene bags and brought to the laboratory and processed for isolation within 24 h of collection.

Isolation of endophytic fungi and actinomycetes from plant parts

The isolation of endophytic fungi as well as actinomycetes was carried out under aseptic conditions according to Tejesvi et al. (2005). All plant parts were separated and subjected to surface sterilization by soaking in 70% (v/v) ethanol for one min followed by sodium hypochlorite (3.5%) for 3 min and washed 3-4 times in sterile distilled water. The dried plant parts were cut into 1.0 cm × 0.1 cm × 0.1 cm pieces under sterile conditions. Two hundred pieces

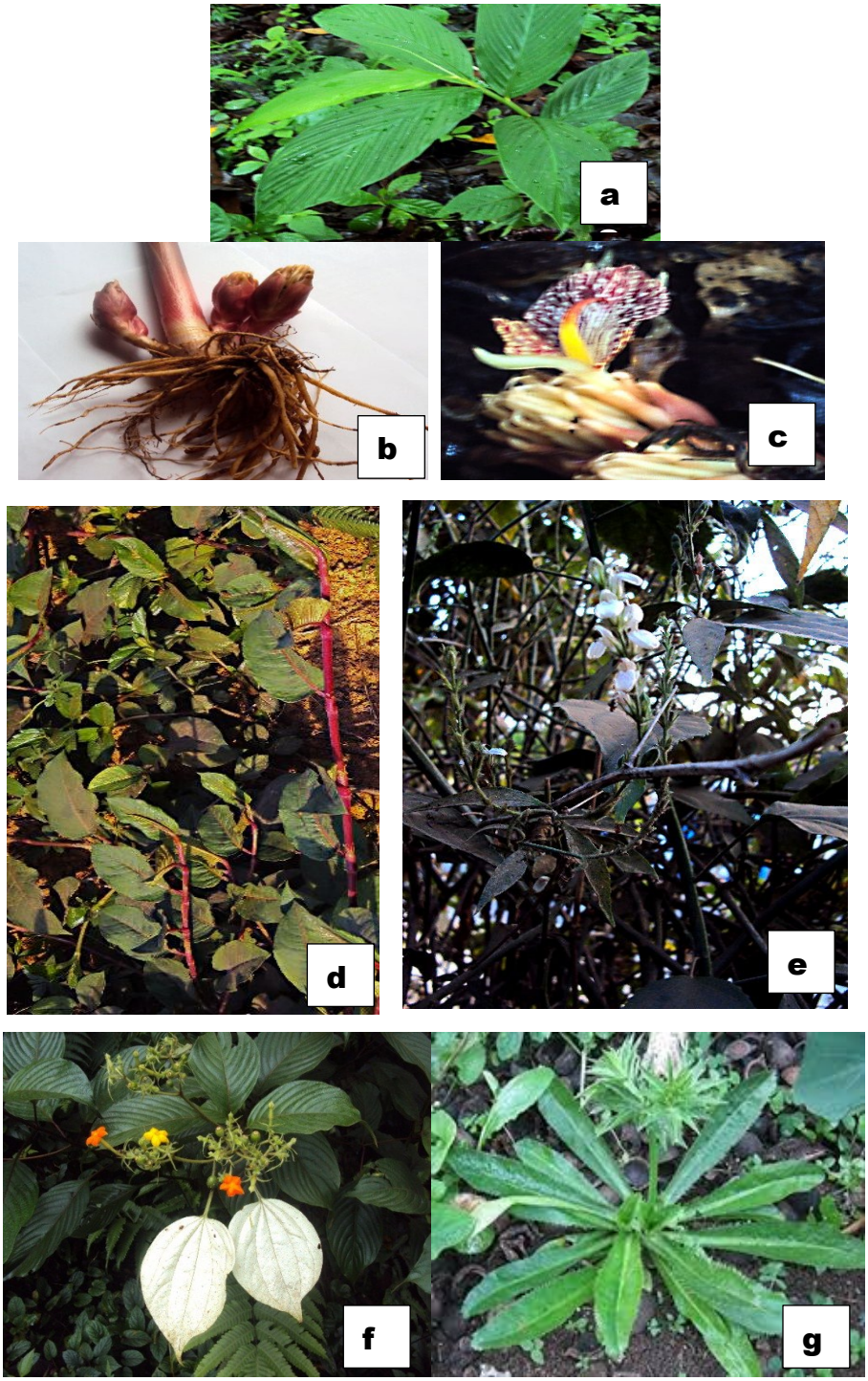

Fig. 1. Medicinal plants collected from the natural habitat of Western Ghats of Southern India.

a. *Zingiber nimmonii* J. (Graham) Dalzell., aerial part ; b. Roots and spikes arising directly from root stock; c. Flower with yellow lip; d. *Polygonum chinense* L.; e. *Justicia wynaadenesis* Heyne**.;** f. *Mussaenda frondosa* L. with creamy bracts; g. *Eryngium foetidum.* L.

of each plant parts were plated on the actinomycete isolation agar (AIA, 21.7 g/L, Himedia, Mumbai, India) amended with 1% glycerin and cyclohexamide (50 mg/L) for actinomycete isolations. The excised fragments were plated on Water agar (WA, 2% w/v) for the isolation of fungal endophytes supplemented with Streptomycin sulphate (50 mg/L). The plates were sealed and incubated at 28± 2^0C with 12 hours of light and dark cycles for 8-10 weeks. The endophytic actinomycetes that emerged on the fragments were further inoculated onto AIA slants while the fugal hyphae or fruiting bodies that emerged were isolated onto Potato Dextrose Agar medium (PDA) respectively. Both the slants were incubated at 28±2 °C for 10-15 days and maintained as pure cultures at 4°C for further use.

Data analysis

The colonization of actinomycetes was expressed as percentage colonization and calculated by the formula: % colonization = $[F_{col}/F_t] \times 100$, where $F_{col}$is the total number of fragments colonized by the actinomycetes; Ft is the total number of fragments plated (Carroll and Carroll, 1978). The relative frequency (RF) was calculated by the following formula: % RF = $[I_0/I_t] \times 100$, where, I_0 is the number of isolates of one species; I_t is the total number of isolates (Huang *et al*., 2007b).

Data reported as mean ± standard deviation (SD) of three independent replicates. Comparison among means were analysed with one-way ANOVA and Tukey-Kramer multiple comparisons tests using Graph Pad InStat 3.0. Any two data were considered statistically significant at $p < 0.05$ and denoted with different superscripts.

OBJECTIVE 2. Characterization of endophytic fungi & actinomycetes by cultural, biochemical and molecular methods

Identification of endophytic actinomycetes

Mycelia and spore morphology analysis

The morphology of actinomycetes mycelia and spore was studied through the monograph recorded by scanning electron microscope (SEM, S-3400N, Hitachi, Japan). The specimens were prepared by the method of Amano and Gyohbu (2000). The samples were then mounted on a tungsten filament, sputter coated with gold and palladium for 15 min. The specimens were observed and recorded at 10,000X to 20,000X magnification.

Molecular identification of endophytic actinomycetes

The endophytic actinomycetes were identified through the sequencing analysis of 16S rDNA using a set of universal primers 27F and 1492R. The actinomycete isolates were grown in ISP 1 (International Streptomyces Project- 1; 8 g/L, Himedia, Mumbai, India) medium for 21 days at 28±2 ºC. The mycelium was collected for DNA extraction by centrifugation. DNA isolation and purification was done by employing DNA isolation kit RKT 24 (Chromous Biotech Pvt Ltd. Bangalore). The quantification of DNA was done by Nanodrop method (Thermo Scientific Nanodrop 2000/2000c, Bangalore, India). The proper sized DNA was then amplified by PCR amplification using PCR kit PCR 08A (Chromous Biotech Pvt Ltd. Bangalore). The amplification conditions consisted of an initial denaturation step at 95 °C for 2 min, followed by 30 cycles of 94 °C for 1 min, 58 °C for 2 min, 72 °C for 2 min and final extension at 72 °C for 8 min. The amplified actinomycete products were subjected to sequencing at Chromous Biotech Pvt. Ltd., Bangalore, India. The endophyte sequences were aligned with the reference sequences using the BLAST algorithm and submitted to the NCBI GenBank nucleotide collection.

OBJECTIVE 3. Microbial fermentation for the production of secondary metabolites

Fermentation and extraction of metabolites

The pure culture of endophytic actinomycetes was inoculated into 500 ml of ISP-1 medium in duplicates for the secretion of metabolites. The fermentation broth of each endophyte was extracted three times with ethyl acetate at room temperature and further concentrated by a Rotary flash evaporator (Superfit Model, PBU-6D, India). The residue obtained was designated as the crude dry extract and stored in glass vials, until use.

OBJECTIVE 4.Screening of extracts for potential antioxidants and antimicrobials

Antioxidative potential of endophytic fungal and actinomycete strains

a. Determination of total phenolic content

The total phenolic content of the fungal extracts was assessed according to the Folin–Ciocalteau (FC) method of Liu et al. (2007) with some modifications. One ml of FC reagent and 2 ml of sodium carbonate (20%, w/v) was mixed with the crude extracts. The mixture was incubated for 45 min in the dark. The absorbance was read at 765 nm (T- 60, TTL Technology, India). The

total phenolic content of the extracts was expressed as mg of gallic acid equivalence (GAE)/g of the extract.

b. Determination of total flavonoid content

The total flavonoid was determined according to the method of Barros et al. (2007).The fungal extract was mixed with sodium nitrite (5%, 75 µl). After 5 min aluminium chloride (10%, 150 µl) and sodium hydroxide (1 M, 500 µl) were added. The absorbance was measured at 510 nm. The content of flavonoid was calculated using calibration curve of catechin and the results were expressed as mg of catechin equivalent (CE)/g of the extract.

c. Determination of antioxidant capacity by DPPH radical scavenging activity &Reducing power assay

The quenching ability of DPPH was measured according to the procedure of Liu et al. (2007) with some modifications. A methanolic solution of DPPH (0.001 mM) was added to the fungal extract. The absorbance was read at 517 nm after 20 min of incubation. The scavenging activity was expressed as IC_{50} (µg/ml). Ascorbic acid was used as the standard. The scavenging ability of the DPPH radical was calculated by the formula:

% scavenging = $[(A_{control} - A_{sample})/A_{control}]\times 100$.

The reducing power was measured by the method of Oyaizu (1986) with some modifications. The fungal extracts were mixed with phosphate buffer (0.2 M, pH 6.5) and potassium ferricyanide (1%, 0.5 ml). The mixture was then incubated at 50ºC for 20 min. After incubation, TCA (10% w/v, 0.5 ml) was added and centrifuged at 3000 rpm for 10 min. To the supernatant same volume of distilled water and ferric chloride (0.1%, 300 µl) was added and the absorbance was measured at 700 nm. The activity was expressed as mg ascorbic acid (AA)/g dry extract.

Antimicrobial potential of endophytic fungal and actinomycete strains

Detection of antibacterial activity

a. Test organisms*:* Two Gram-positive bacteria *viz*., *Bacillus subtilis* (MTCC 121), *Staphylococcus aureus* (MTCC 7443), and four Gram-negative bacteria *viz*. *Pseudomonas aeruginosa* (MTCC 7093), *Escherichia coli* (MTCC 729), *Enterobacter aerogenes* (MTCC 111), *Klebsiella pneumoniae* (MTCC 661) were used in the study. These test organisms were procured from the Department of Studies in Microbiology, University of Mysore, Karnataka, India.

b. Disk diffusion method: The inhibitory effects of the endophytic fungal and actinomycete strain extracts were tested by disc diffusion method (Bauer *et al*., 1966). The crude extract of endophytic actinomycetes was dissolved in dimethyl sulfoxide (DMSO) and tested on Mueller-Hinton agar medium seeded with the test bacterium at 250 µg per disc (5mm diameter, Whatman no. 1) concentration. Streptomycin (10 µg/ disc) was used as positive control and paper disc loaded with only DMSO was negative control. The test plates were incubated for 24 h at 35 ± 2 ºC and the inhibition zone was measured.

c. Determination of minimal inhibitory and minimum bactericidal concentrations

The minimal inhibitory concentration (MIC) was determined by modified broth dilution method (Xu et al., 2008), using sterile 96-well microplate (Tarsons, Kolkata, India). The wells were filled with reaction mixture containing 90 µl bacterial suspensions (10^6 CFU/ ml) and 10 µl of test sample with different concentrations (2 mg/ml to 0.02 mg/ml). The culture medium with 1% DMSO was used as the negative control and streptomycin sulphate (0.4 mg/ml to 0.01 mg/ml) was the positive control. The microplates were incubated for 24 h at 35 ± 2 ºC. After the incubation, 10 µl of the indicator 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) (0.5 mg/ ml phosphate buffer saline) was added to visualize the microbial growth. The lowest sample concentration at which no blue colour appeared was determined as MIC. Wells containing MIC concentration and above was inoculated onto agar medium to check cell viability. The lowest concentration with no viable cells was determined as minimum bactericidal concentration (MBC).

RESULTS

OBJECTIVE 1.Collection of plants of ethnomedicinal value from natural habitats of Western Ghats and isolation of endophytic fungi & actinomycetes from plant parts

Isolation of endophytic fungi

471 fungal endophytes isolated from 900 plant fragments of *Z. nimmonii* were distributed in 11 different taxa. The strains characterized from *Z. nimmonii* with their genbank accession numbers, colonization and relative frequency (%RF) are depicted in Table 2. *Fusarium equiseti* showed the highest RF (18.5%) followed by *F. chlamydosporum* (17.8%) and *F. solani* (17.4%). The genus *Fusarium* was present in all the plant parts, except rhizome.

Endophytic fungi were isolated from the stem and leaf parts of *P. chinense* L. A total of 264 isolates distributed in 10 endophytic species were recovered from 400 plant fragments. The stem part had number of isolates (141) than leaves (123). The 10 endophytic species belonged to seven genera. Three different species of *Fusarium* and two of *Alternaria* were recovered. *Fusarium solani* showed highest %CF (15.9) followed by *Fusarium chlamydosporum* (15.2). Least %CF was recorded for *Alternaria alternata* (Table 3).

Endophytic fungi were isolated from leaf and stem parts of *J. wynaadensis*. A total of 281 isolates distributed in nine endophytic species were recovered from 400 plant fragments. The isolates were identified with their spore morphology as well as by analyzing the DNA sequence of the ITS region. The identified strains with their genbank accession numbers, isolation number and colonization frequency (%CF) are depicted in Table 4. Results indicated that the stem part had more number of isolates (161) than leaves (120).

Isolation of endophytic actinomycetes

A total of 122 endophytic actinomycetes were isolated from 1,700 plant fragments of three medicinal plants. The percent colonization of actinomycetes on different plant parts of selected host species is given in Table 5. The highest number of isolates were obtained from the rhizome of *Z. nimmonii* (13.5%) followed by stem of *P. chinense* (11.5%) and stem of *J. wynaadensis* (10.5%). From the root of *Z. nimmonii* 2.5% isolates were obtained while, the leafy stem and inflorescence parts were devoid of any isolates. No endophytic actinomycetes were isolated from *M. frondosa* and *E. foetidum*.

The genus *Streptomyces* was isolated from all three host plants. *Arthrobacter* sp. was isolated from two hosts *viz*., Z. *nimmonii* and *J. wynaadensis*. *Curtobacterium* and *Corynebacterium* were isolated from *Z. nimmonii* while, *Nocardiopsis* were recovered from *P. chinense*. Five different species of *Streptomyces* and two different species of *Nocardiopsis* were identified. The %RF of the strains indicated that *S. clavuligerus* showed highest %RF (24.6) followed by *Streptomyces* sp. (18.0). The lowest %RF was recorded for *S. coelicolor* (3.3). The most recurrent genus found was *Streptomyces* which comprised 59% of all the isolates.

OBJECTIVE 2. Characterization of endophytic fungi & actinomycetes by cultural, biochemical and molecular methods

Table 2 Taxonomic identification and percent colonization frequency of the fungal endophytes isolated from *Z. nimmonii*

Endophytic Fungi	Code	Accession No.	Colonization frequency (%) of isolates					Relative frequency of isolation (%)
			Leaf*	Root *	Leafy stem*	Inflorescence†	Rhizome*	
Alternaria tenuissima	ZN-WG-01	KJ547594	28.5	--	--	--	--	12.1
Trichoderma harzianum	ZN-WG-02	KJ547595	--	12	--	--	--	5.1
Fusarium solani	ZN-WG-03	KJ547596	--	7.5	14.5	38	--	17.4
Alternaria consortiale	ZN-WG-04	KM114288	13.5	--	--	--	--	5.7
Bipolaris specifera	ZN-WG-06	KM114290	--	13	--	--	--	5.5
Fusarium chlamydosporum	ZN-WG-07	KM396301	15.5	26.5	--	--	--	17.8
Hypocrea lixi	ZN-WG-08	KM396302	--	5.5	--	--	--	2.3
Aspergillus terreus	ZN-WG-09	KM396303	--	--	--	29	--	6.2
Nectria haematococca	ZN-WG-10	KM396304	--	11.5	--	--	--	4.9
Sarocladium kiliens	ZN-WG-11	KM396305	10.5	--	--	--	--	4.5
Fusarium equiseti	ZN-WG-12	KM396306	14.5	--	29	--	--	18.5
Total			82.5	76	43.5	67	--	100

-- Indicates the absence of the endophytic fungi; * 200 fragments were plated from each part; † 100 fragments were plated from the inflorescence

Table 3 Taxonomic identification and percent colonization frequency (%CF) of the fungal endophytes isolated from *P. chinense*

Endophytic Fungi	Code	Accession No.	Leaf*		Stem*		Total %CF
			I	%CF	*I*	%CF	
Bipolaris sorokinia	PC-WG-01	KY024401	___	___	11	5.5	2.8
Penicillum canescens	PC-WG-02	KY052774	___	___	14	7.0	3.6
Fusarium chlamydosporum	PC-WG-03	KY072925	27	13.5	34	17.0	15.2
Cladosporium tenuissium	PC-WG-04	KY039168	___	___	27	13.5	6.9
Fusarium solani	PC-WG-05	KY039169	25	12.5	39	19.5	15.9
Alternaria alternata	PC-WG-06	KY039171	11	5.5	___	___	2.5
Curvularia geniculata	PC-WG-07	KY052771	26	13.0	___	___	6.2
Fusarium incarnatum	PC-WG-08	KY052774	5	2.5	7	3.5	2.8
Emericella nidulans	PC-WG-10	KY039167	16	8.0	___	___	4.1
Alternaria longipes	PC-WG-11	KY039170	22	11	9	4.5	7.7

- Indicates the absence of the endophytic fungi; *200 fragments were plated from leaf and stem respectively. *I*- Number of isolates.

Table 4 Taxonomic identification and percent colonization frequency (%CF) of the fungal endophytes isolated from *J. wynaadensis*

Endophytic Fungi	Code	Accession No.	Leaf*		Stem*		Total %CF
			I	%CF	*I*	%CF	
Fusarium incarnatum	JW-WG-01	KY052769	5	2.5	19	9.5	6.0
Sarocladium kiliense	JW-WG-02	KY052770	21	10.5	__	__	5.3
Colletotrichum truncatum	JW-WG-03	KY052773	__	__	33	16.5	8.3
Trichoderma harzianum	JW-WG-04	KY072924	13	6.5	__	__	3.3
Colletotrichum lindemuthianum	JW-WG-05	KY484535	27	13.5	31	15.5	13.8
Chaetomium globosum	JW-WG-06	KY484536	18	9.0	25	12.5	10.8
Fusarium oxysporum	JW-WG-07	KY484537	15	7.5	11	5.5	6.5
Fusarium solani	JW-WG-08	KY484538	21	10.5	24	12.0	11.3
Alternaria alternata	JW-WG-09	KY626175	___	__	18	9.0	4.5

*200 fragments were plated from leaf and stem respectively. *I*- Number of isolates.

Identification of fungal endophytes

The isolates were identified morphologically and with the DNA sequence analysis of the ITS region. The strains characterized from *Z. nimmonii* with their genbank accession numbers, colonization and relative frequency (%RF) are depicted in Table 2. The isolates were identified with their spore morphology as well as by analyzing the DNA sequence of the ITS region. The identified strains with their GenBank accession numbers, isolation number and colonization frequency (%CF) is depicted in Table 2. The isolates from *P. chinense* were characterized by analyzing the DNA sequence of the ITS region. The identified strains with their GenBank accession numbers, isolation number and colonization frequency (%CF) is depicted in Table 3. 10 endophytic species belonged to seven genera. The nine endophytic species from *J. wynaadensis* belonged to six genera. The isolates were identified with their spore morphology as well as by analyzing the DNA sequence of the ITS region. The identified strains with their GenBank accession numbers, isolation number and colonization frequency (%CF) is depicted in Table 4. Three different species of *Fusarium* and two of *Colletotrichum* were recovered. *Colletotrichum lindemuthianum* showed highest %CF (13.8) followed by *Fusarium solani* (11.3) and *Chaetomium globosum* (10.8). The least %CF was recorded for *Trichoderma harzianum*.

Identification of actinomycete endophytes

A total of 10 different taxa belonging to five different genera that is, *Streptomyces, Nocardiopsis, Arthrobacter*, *Curtobacterium* and *Corynebacterium* were identified by cultural characteristics and by the scanning electron microscopy (Figs. 4 & 5). The colony characteristics on AIA plates, genebank accession numbers and percent similarity are depicted in Table 5.

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OBJECTIVE 3. Microbial fermentation for the production of secondary metabolites

The fungal and actinobacterial/actinomycetes endophytic strains characterized from the three medicinal plants were subjected to fermentation for the extraction of secreted secondary metabolites.

The pure culture of 10-day old fungal strains were inoculated into PDB medium in duplicates and kept for incubation for 3 weeks at 28 ± 2^{o}C.The pure culture of endophytic actinomycete strains were inoculated into 500 ml of ISP-1 medium in duplicates for the secretion of

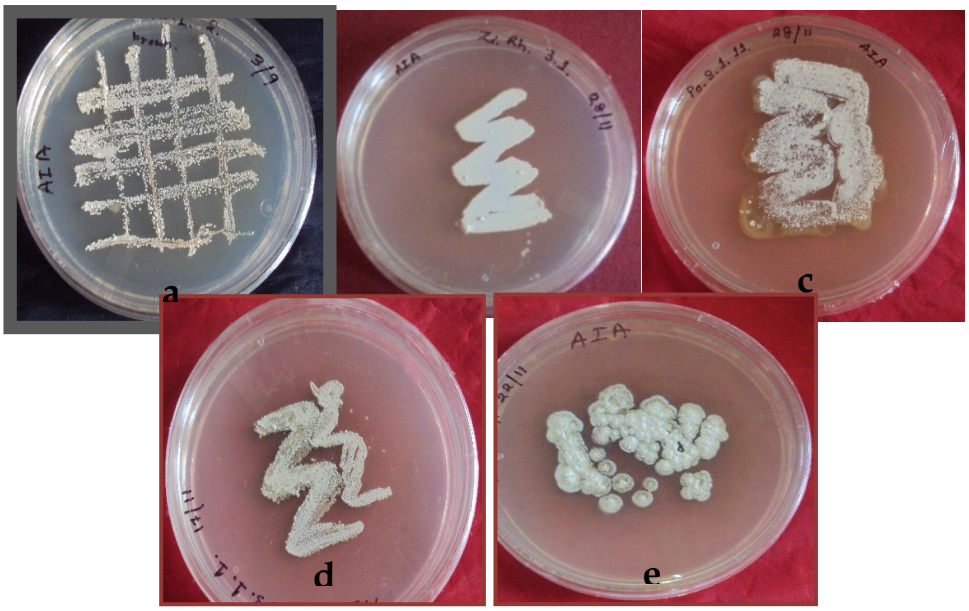

Fig. 4. Pure cultures of five endophytic actinobacterial genera
a.Curtobacterium; b. *Arthrobacter*; c. *Nocardiopsis*; d. *Streptomyces*; e. *Corynebacterium*

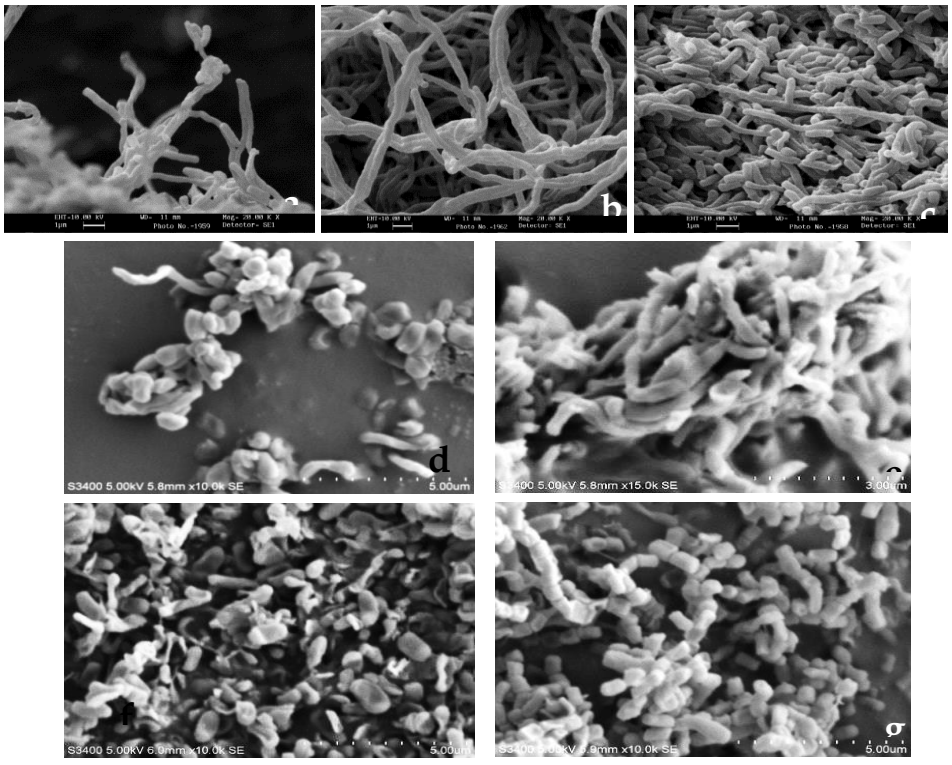

Fig. 5. SEM images of endophytic actinobacterial genera isolated from the medicinal plants
a-c. *Streptomyces* spp. (X20k) d. *Curtobacterium* sp. (X10k); e. *Nocardiopsis* sp. (X15k); f. *Corynebacterium* sp. (X10k); g. *Arthrobacter* sp. (X10k)

Table 5 Taxonomic identification of the endophytic actinobacteria isolated from three medicinal plants

Code	Host	Endophyticactinobacteria	Accession No.	Plant part	Similarity percentage (%)	Colony characteristics	
						Arial	Reverse
A-ZN-01	*Z. nimmonii*	*Corynebacterium* sp.	MF083715	Rhizome	99	White	Gray
A-ZN-02		*Curtobacterium oceansedimenticum*	MF083716	Rhizome	99	Pinkish white	Cream
A-ZN-03		*Arthrobacter*sp.	MF083717	Root	98	White	Cream
A-ZN-04		*Streptomyces griseus*	MF083718	Leaf	100	Pale yellow	light yellowish brown
A-ZN-05		*Streptomyces clavuligerus*	MF083720	Root	100	Grayish green	Grayish yellow
A-ZN-06		*Streptomyces indiaensis*	MF083723	Leaf	99	Gray	Dark violet
A-ZN-07		*Streptomyces coelicolor*	MF083724	Leaf	98	Yellowish	Dark brown
A-PC-01	*P. chinense*	*Nocardiopsis ganjiahuensis*	MF083725	Stem	100	Gray white	Light yellow
A-PC-02		*Streptomyces clavuligerus*	MF083721	Leaf	99	Grayish green	Grayish yellow
A-PC-03		*Streptomyces griseus*	MF083719	Leaf	100	Pale yellow	light yellowish brown
A-PC-04		*Nocardiopsis alba*	MF083726	Stem	100	White	Yellow
A-JW-01	*J. wynaadensis*	*Streptomyces clavuligerus*	MF083722	Leaf	100	Grayish green	Grayish yellow
A-JW-02		*Streptomyces* sp.	MF083728	Stem	100	Brown	Black
A-JW -03		*Arthrobacter*sp.	MF083727	Stem	99	White	Cream

metabolites. The fermentation broth of each endophytic strain was extracted three times with ethyl acetate at room temperature and further concentrated by a Rotary flash evaporator (Superfit Model, PBU-6D, India). The residue obtained was designated as the crude dry extract and stored in glass vials, until further tested for antioxidative and antibacterial properties.

OBJECTIVE 4. Screening of extracts for potential antioxidants and antimicrobials

Antioxidative potentials of endophytic fungi

The fungal endophytes characterized from threemedicinal plants were determined for their antioxidant potentialsby the following methods:

Total phenolic content

TPC of the extracts from *Z. nimmonii* endophytes ranged from 10.17±0.7 to 81.58±6.0 mg GAE/g dry extract. *B. specifera* extracts showed highest total phenolic content (81.58±6.0 mg GAE/g dry extract) followed by *A. terreus*, *A. tenuissima*, *N. haematococca* and *F. chlamydosporum* extracts (52.46±1.3, 47.01±2.6, 28.82±0.7 and 26.64±1.3 mg GAE/g dry extract respectively). TPC of the endophytic extracts from *P. chinense* ranged from 9.9±0.7 to 78.9±2.5 mg GAE/g dry extract. *P. canescens* extract showed the highest total phenolic content (78.9±2.5 mg GAE/g dry extract) followed by *F. chlamydosporum* extracts (50.3±0.5 GAE/g dry extract respectively). *A. longipes* exhibited the least phenolic content (9.9±0.7 5 mg GAE/g dry extract). The total phenolic content (TPC) of the endophytic extracts from *J. wynaadensis* ranged from 4.8±0.08 to 20.74±0.96 mg GAE/g dry extract. *Fusarium incarnatum* extracts showed highest total phenolic content (20.74±0.96 mg GAE/g dry extract) followed by *Trichoderma harzianum* extracts (13.83±0.26 GAE/g dry extract respectively). *F. solani* exhibited least phenolic content (4.8±0.08 mg GAE/g dry extract).

Total flavonoid content

Among the 11 strains of *Z. nimmonii*, flavonoid content was detected in eight strains. TFC ranged from 5.2±0.5 to 24.3±0.9 mg CE/g dry extract. *N. haematococca* exhibited high flavonoid content (24.3±0.9 mg CE/g dry extract). TFC of *F. chlamydosporum* and *A. tenuissima* was recorded as 22.9±1.9 and 19.8±0.5 mg CE/g dry extract respectively. The lowest TFC was recorded for *F. equiseti*. Flavonoid was detected in seven endophytic strains from *P. chinense* (Table 7). The total flavonoid content (TFC) ranged from 5.9±0.4 to 33.6±1.0 mg CE/g dry

Table 7 Total phenolic content (TPC), total flavonoid content (TFC) and antioxidant capacity of fungal endophytes from *P. chinense*

Fungal strains/extracts	TPC (mg GAE/g dry extract)	TFC(mg CE/ g dry extract)	Reducing Power (mg AA/g dry extract)	DPPH radical scavenging capacity (IC_{50} µg/mL)
P. canescens	78.9 ± 2.5^{a}	33.6 ± 1.0^{a}	95.8 ± 2.5^{a}	73.6 ± 3.3^{b}
F. chlamydosporum	50.3 ± 0.5^{b}	12.5 ± 0.5^{d}	84.7 ± 2.1^{b}	126.8 ± 6.7^{c}
A. alternata	24.9 ± 1.1^{c}	14.6 ± 0.2^{c}	48.2 ± 2.0^{c}	319.2 ± 40.2^{d}
B. sorokiniana	22.7 ± 0.8^{d}	17.5 ± 0.5^{b}	44.3 ± 0.8^{d}	366.2 ± 15.8^{d}
F. solani	$21.4 \pm 1.9d^{e}$	10.1 ± 0.1^{e}	40.0 ± 1.1^{e}	440.5 ± 40.8^{e}
C. geniculata	19.3 ± 0.8^{e}	—	37.6 ± 1.1^{e}	472.5 ± 8.7^{e}
C. tenuissimum	17.8 ± 1.6^{f}	—	25.6 ± 0.8^{g}	542.6 ± 44.0^{f}
E. nidulans	16.5 ± 0.9^{f}	7.5 ± 0.3^{f}	30.1 ± 1.1^{f}	581.1 ± 35.2^{f}
F. incarnatum	13.6 ± 0.6^{g}	—	18.8 ± 1.0^{h}	766.9 ± 33.1^{g}
A. longipes	9.9 ± 0.7^{h}	5.9 ± 0.4^{g}	12.8 ± 0.2^{i}	1114.9 ± 71.1^{h}
			Ascorbic acid	7.7 ± 0.02^{a}

Data are reported as mean ± SD of three independent analyses (n=3). Mean with the different superscript within a column are significantly different ($p<0.05$) by one-way ANOVA test.'-' indicates the absence of activity in the respective assays by the endophytic fungal extracts

extract. *P. canescens* exhibited high flavonoid content (33.6±1.0 mg CE/g dry extract). TFC of *B. sorokiniana* and *A. alternata* was documented as 17.5±0.5 and 14.6±0.2 mg CE/g dry extract respectively. The lowest TFC was recorded for *A. longipes*. Flavonoid was not detected in *C. geniculata*, *Cladosporium tenuissimum* and *Fusarium incarnatum* extracts. Flavonoid was detected in four endophytic strains from *J .wynaadensis*. The total flavonoid content (TFC) ranged from 2.1±0.08 to 8.75±0.6 mg CE/g dry extract. *F. incarnatum* exhibited high flavonoid content (8.75±0.6 mg CE/g dry extract), whereas the lowest TFC was recorded for *C. lindemuthianum*.

Antioxidant properties

ABTS$^+$ radical scavenging assay

The total antioxidant capacity of endophytic extracts from *Z. nimmonii* was determined by ABTS$^+$ scavenging capacity. The scavenging capacity of the extracts varied considerably, ranging from 10.93±0.26 to 70.68±0.26 mg TE/g dry extract (Table 6). Among the extracts, *B. specifera*, *A. terreus*, *A. tenuissima*, *N. haematococca* and *F. chlamydosporum* showed high scavenging capacity (70.68±0.26, 68.0±0.26, 67.45±2.24, 62.2±0.17 and 58.18±1.03 mg TE/g dry extract, respectively) in consistence with the TPC. The scavenging capacity of the extracts of endophytes from *J. wynaadensis* varied considerably, ranging from 6.2±0.3 to 25.1±0.1 mg TE/g dry extract (Table 8). Among the extracts, *F. incarnatum* showed high scavenging capacity followed by *T. harzianum* and *S.kiliense* (17.5±0.2 and 15.3±0.3 mg TE/g dry extract, respectively) in consistence with the TPC

DPPH radical scavenging assay

DPPH radical is almost stable and used for antioxidant activity widely. The radical scavenging activity of *Z. nimmonii* fungal endophytes is presented as 50% scavenging capacity (IC_{50}) in Table 6. As depicted, the IC_{50} value of the fungal extracts varied considerably (1337.7±213.6 µg/mL to 96.9±2.4µg/mL). *A. tenuissima* showed highest scavenging capacity with 96.9±2.4 µg/mL, followed by *A. terreus* (123.3±7.6 µg/mL), *N. haematococca* (133.4±5.3 µg/mL) and *F. chlamydosporum* (226.9±23.7 µg/mL). Although, high TPC was exhibited by *B. specifera*, the scavenging capacity of DPPH radical was found to be very less (1057.2±122.3 µg/mL) in the extract.The radical scavenging activity of *P. chinense* endophytic fungal strains is

Table 6 Antioxidant capacity of fungal endophytes isolated from *Z. nimmonii*

Fungal strains	$ABTS^+$ scavenging capacity (mg TE/g dry extract)	Reducing Power (mg AA/g dry extract)	DPPH radical scavenging capacity (IC_{50} µg/mL)	Inhibition of lipid peroxidation capacity (IC_{50} µg/mL)
Bipolaris specifera	70.68 ± 0.26^{a}	110.4 ± 1.1^{a}	1057.2 ± 82.3^{g}	75.3 ± 2.75^{b}
Aspergillus terreus	68.0 ± 0.26^{b}	74.9 ± 2.9^{b}	123.3 ± 7.6^{c}	110.0 ± 3.3^{c}
Alternaria tenuissima	67.45 ± 2.24^{b}	86.9 ± 0.9^{c}	96.9 ± 2.4^{b}	147.0 ± 5.5^{d}
Nectria haematococca	62.20 ± 0.17^{c}	68.0 ± 2.2^{d}	133.4 ± 5.3^{c}	159.8 ± 6.2^{e}
Fusarium chlamydosporum	58.18 ± 1.03^{d}	63.1 ± 0.5^{e}	226.9 ± 23.7^{d}	224.8 ± 5.2^{f}
Sarocladiumkiliens	30.86 ± 2.07^{e}	45.3 ± 1.9^{f}	344.9 ± 21.2^{e}	332.1 ± 11.5^{g}
Fusarium solani	37.39 ± 2.83^{e}	42.7 ± 0.3^{f}	326.2 ± 20.6^{e}	349.4 ± 15.1^{g}
Hypocrea lixi	25.38 ± 1.8^{f}	39.2 ± 0.4^{g}	498.9 ± 40.9^{f}	565.3 ± 17.4^{h}
Fusarium equiseti	20.68 ± 1.47^{g}	28.3 ± 0.2^{h}	1082.3 ± 3.3^{g}	803.9 ± 15.4^{i}
Trichoderma harzianum	14.83 ± 1.29^{h}	23.8 ± 0.5^{i}	546.7 ± 25.7^{f}	897 ± 25.7^{j}
Alternaria consortiale	10.93 ± 0.26^{i}	18.8 ± 0.5^{j}	1337.7 ± 113.6^{h}	1035 ± 30.8^{k}
		Ascorbic acid	7.7 ± 0.02^{a}	30.7 ± 2.1^{a}

Data are reported as mean ± SD of three independent analyses (n=3). Mean with different superscript within a column are significantly different (p<0.05) by one-way ANOVA test.

Table 8 Antioxidant capacity of fungal endophytes isolated from *J. wynaadensis*

Fungal strains	$ABTS^+$ scavenging capacity (mg TE/g dry extract)	Reducing Power (mg AA/g dry extract)	DPPH radical scavenging capacity (IC_{50} µg/mL)	Inhibition of lipid peroxidation capacity (IC_{50} µg/mL)
F. incarnatum	25.1 ± 0.1^a	38.3 ± 0.6^a	379.98 ± 0.8^b	534.69 ± 2.7^b
T. harzianum	17.5 ± 0.2^b	21.15 ± 0.3^b	543.67 ± 16.6^c	931.42 ± 8.2^c
S. kiliense	15.3 ± 0.3^c	16.7 ± 0.6^c	792.15 ± 28.5^d	981.38 ± 16.8^d
C. globosum	12.9 ± 0.1^d	15.24 ± 0.3^d	930.62 ± 10.2^e	105084 ± 3.1^e
F. oxysporum	11.8 ± 0.3^e	14.3 ± 0.1^e	1155.54 ± 5.5^f	1203.45 ± 10.2^f
C. truncatum	10.1 ± 0.5^f	12.3 ± 0.5^f	1172.5 ± 7.2^g	1338.35 ± 22.7^g
A. alternata	8.3 ± 0.1^g	9.5 ± 0.2^g	1328.18 ± 32.4^h	—
C. lindemuthianum	7.6 ± 0.1^h	8.2 ± 0.3^h	1541.74 ± 77.3^i	—
F. solani	6.2 ± 0.3^i	7.8 ± 0.07^h	1920.67 ± 134.0^j	—
		Ascorbic acid	7.7 ± 0.02^a	30.7 ± 2.1^a

Data are reported as mean ± SD of three independent analyses (n=3). Mean with the different superscript within a column are significantly different (p<0.05) by one-way ANOVA test.

presented as 50% scavenging activity (IC_{50}) in Table 7. The results showed that the IC_{50} value of the fungal extracts varied from 73.6±3.3 µg/ml to1114.9±71.1 µg/ml). *P. canescens* showed highest scavenging activity with 73.6±3.3 µg/ml, followed by *F. chlamydosporum* (126.8±6.7 µg/ml). *Bipolaris sorokiniana* and *A. alternata* exhibited the IC_{50} value of 319.2±40.2 and 366.2±15.8 µg/ml respectively. As depicted, the IC_{50} value of the fungal extracts from *J. wynaadensis* varied considerably (379.98±0.8 µg/ml to1920.67±134 µg/ml). *F. incarnatum* showed highest scavenging activity with 379.98±0.8 µg/ml, followed by *T. harzianum* and *S. kiliense* (Table 8).

Reducing power assay

Antioxidant compounds have the reductive ability to transform Fe^{3+} to Fe^{2+} through their functional groups which are susceptible to transfer electron. The reducing power of ferric ion to ferrous ion by the *Z. nimmonii* fungal endophytic extracts is represented in terms of ascorbic acid equivalent (Table 6). The values of reducing power assay ranged from 18.8±0.5 to 110.4±1.1 mg AA/g dry extract. *B. specifera* showed highest activity (110.4±1.1 mg AA/g dry extract) followed by *A. tenuissima* (86.9±0.9 mg AA/g dry extract) and *A. terreus* (74.9±2.9 mg AA/g dry extract). The reducing power activity of *N. haematococca*and *F. chlamydosporum* was 68.0±2.2 and 63.1±0.5 mg AA/g dry extract, respectively.). The values of reducing power assay ranged from 12.8±0.2 to 95.8±2.5 mg AA/g dry extract in endophytic fungal strains from *P. chinense*. *P. canescens* showed highest activity (95.8±2.5 mg AA/g dry extract) followed by *F. chlamydosporum* (84.7±2.1 mg AA/g dry extract) and *A. alternata* (48.2±2.0 mg AA/g dry extract, Table 7).The reducing power of ferric ion to ferrous ion by the fungal extracts from *J. wynaadensis* is represented in terms of ascorbic acid equivalent (Table 8). The values of reducing power assay ranged from 7.8±0.07 to 38.3±0.6 mg AA/g dry extract. *F. incarnatum* showed highest activity (38.3±0.6 mg AA/g dry extract). The reducing power activity of *F. solani* was found to be the lowest.

Inhibition of lipid peroxidation capacity

The peroxidation reaction was induced with Fe^{++}. The inhibition of lipid peroxidation capacity of *Z. nimmonii* fungal endophytic fungal extracts is presented in Table 6. The capacity of the extracts ranged from 75.3±2.75 µg/mL to 1035±30.8 µg/mL. The extract of *B. specifera*

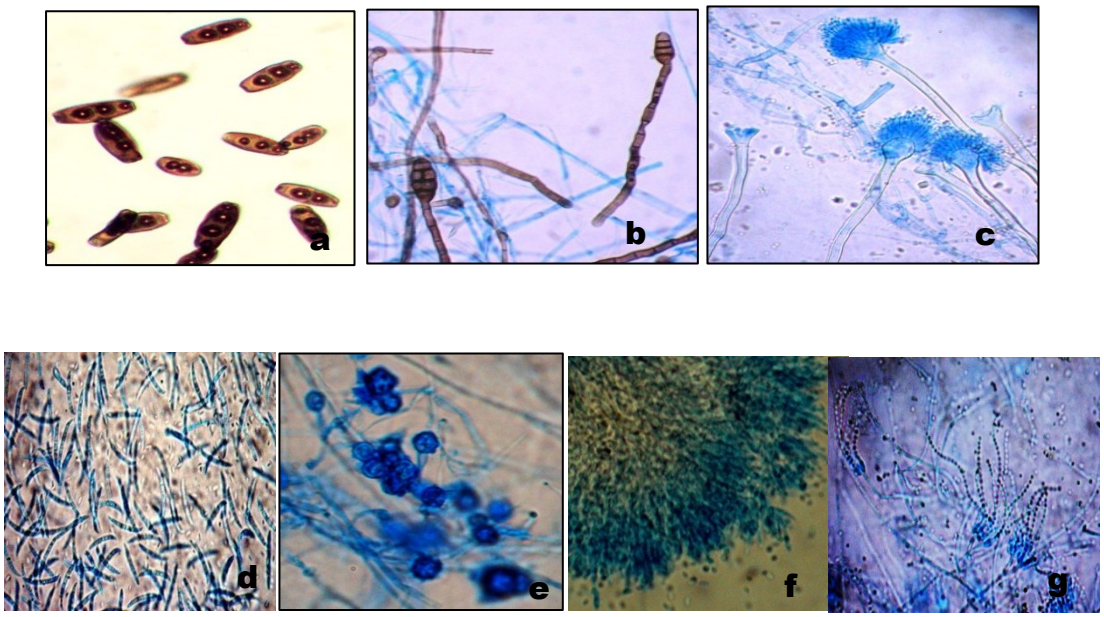

Fig. 2. Light micrography of fungal endophytes isolated from *Z. nimmonii* & *P. chinense*
a. *B. specifera*; b. *A. tenuissima*; c. *A. terreus*; d. *F. chlamydosporum*; e. Chlamydospores of *F. chlamydosporum*; f. *N. haematococca*; g. *P. canescens* from *P. chinense*

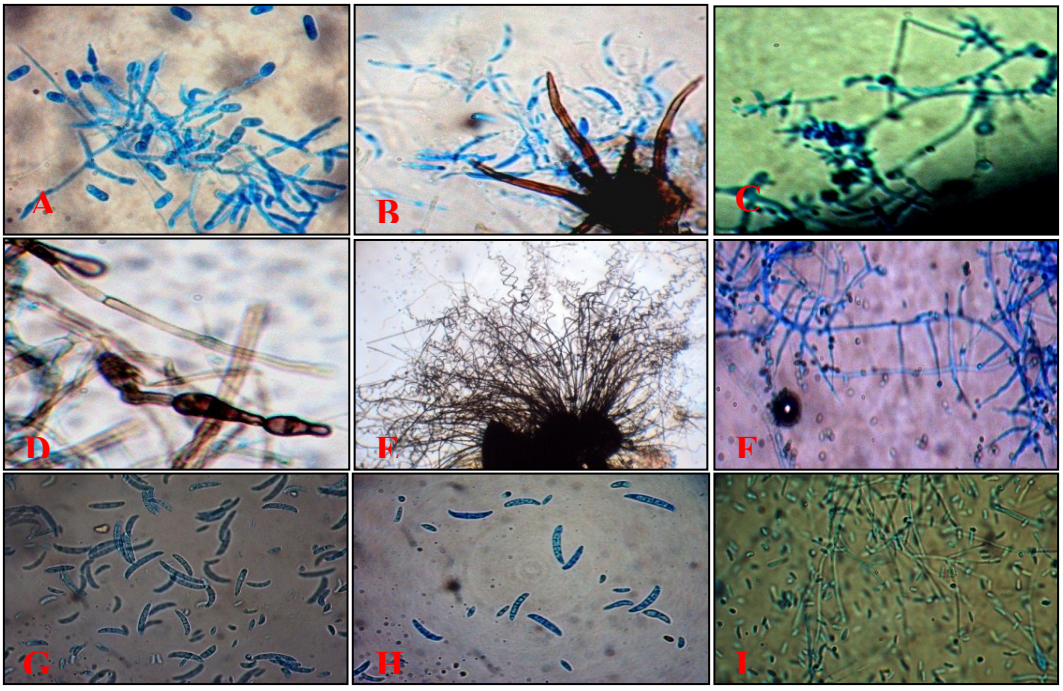

Fig. 3. Spore morphology and light microscopy of fungal endophytes isolated from plant fragments of *J. wynaadensis*
(A) *Colletotrichum lindemuthianum*; (B) *Colletotrichum truncatum*; (C) *Trichoderma harzianum*; (D) *Alternaria alternata*; (E) *Chaetomium globosum*; (F) *Sarocladium kiliense*; (G) *Fusarium incarnatum*; (H) *Fusarium oxysporum*; (I) *Fusarium solani*

showed highest inhibition capacity (75.3±2.75µg/mL) followed by *A. terreus* (110.0±3.3 µg/mL). The inhibition of lipid peroxidation capacity proceeded in the similar pattern as in case of ABTS scavenging capacity.

The extracts of *B. specifera*, *A. terreus*, *A. tenuissima*, *N. haematococca* and *F. chlamydosporum* were found to have high TPC and antioxidant capacity. The light micrograph images of these fungal spore and hyphae is depicted in Fig. 2.

Antioxidative potentials of endophytic actinomycetes

Antioxidant properties

DPPH radical scavenging capacity

The radical scavenging activity is presented as 50% scavenging capacity (IC_{50}) in Table 9. The IC_{50} value of the actinomycete extracts varied from 489.4 ± 2.3 µg/mL to 1968.3±112.0 µg/mL).The results indicated that *Arthrobacter* sp. isolated from *Z. nimmonii* has highest scavenging capacity (489.4±2.3 µg/mL). No significant difference (p<0.05) was recorded between the *Streptomyces clavuligerus* extracts isolated from *Z. nimmonii* and *P. chinense*, whereas *S. clavuligerus* isolated from *J. wynaadensis* contained significant difference (p<0.05) in the scavenging capacity. *Streptomyces griseus* isolated from *P. chinense* exhibited least capacity (1968.3±112.0 µg/mL). The scavenging capacity of *Streptomyces indiaensis* (isolated from *Z. nimmonii*), *Nocardiopsis ganjiahuensis*, and *S. griseus* (isolated from *P. chinense*) was found to be very trivial. No activity was recorded in the extracts of *Curtobacterium oceansedimenticum, Streptomyces coelicolor, S. griseus* (isolated from *Z. nimmonii*) and *N. alba* (isolated from *P. chinense*).

Reducing power assay

The reducing power of endophytes is represented in terms of ascorbic acid equivalent in Table 9. The values of reducing power assay ranged from 9.3±0.3 to 27.3±0.2 mg AAE/g dry extract. *Arthrobacter* sp., isolated from *Z. nimmonii* displayed highest activity (27.3±0.2 mg AAE/g dry extract). *N. alba* from *P. chinense* and *S. coelicolor* from *Z. nimmonii* exhibited lowest reducing capacity.

Table 9 Antioxidant capacity of actinobacterial endophytes isolated from three medicinal plants

Host plant	Actinobacterial strains	DPPH radical scavenging capacity (IC_{50} µg/mL)	Reducing Power (mg AAE/g dry extract)
Z. nimmonii	*Arthrobacter* sp.	489.4 ± 2.3^{b}	27.3 ± 0.2^{a}
	Corynebacterium sp.	913.6 ± 11.6^{d}	20.4 ± 0.2^{c}
	S. clavuligerus	1185 ± 21.5^{e}	17.5 ± 0.06^{d}
	S. indiaensis	1554.5 ± 15.2^{g}	14.9 ± 0.1^{f}
	C. oceansedimenticum	—	12.5 ± 0.2^{h}
	S. griseus	—	11.9 ± 0.4^{i}
	S. coelicolor	—	9.5 ± 0.2^{j}
P. chinense	*S. clavuligerus*	934.1 ± 17.3^{d}	17.7 ± 0.2^{d}
	N. ganjiahuensis	1890.7 ± 124.0^{h}	13.6 ± 0.1^{g}
	S. griseus	1968.3 ± 112.0^{i}	12.3 ± 0.5^{h}
	N. alba	—	9.3 ± 0.3^{j}
J. wynaadensis	*Arthrobacter* sp.	554.7 ± 27.3^{c}	24.0 ± 0.2^{b}
	S. clavuligerus	1210 ± 38.0^{ef}	16 ± 0.2^{e}
	Streptomyces sp.	1313 ± 87.2^{f}	15.2 ± 0.3^{f}
	Ascorbic acid	7.7 ± 0.02^{a}	

Data are reported as mean ± SD of three independent analyses (n=3). Mean with the different superscript within a column are significantly different (p<0.05) by one-way ANOVA test.AAE- Ascorbic acid equivalent.

Total phenolic and flavonoid content

The total phenolic content (TPC) of the extracts ranged from 5.2±0.1 to 24.4±0.08 mg GAE/g dry extract. *Arthrobacter* sp. extracts isolated from *Z. nimmonii*, showed high TPC (24.4±0.08 mg GAE/g dry extract) followed by *Arthrobacter* sp. from *J. wynaadensis* (21.5±0.09 mg GAE/g dry extract).*Nocardiopsis alba* from *P. chinense* and *Streptomyces coelicolor* from *Z. nimmonii* exhibited lower phenolic content (5.2±0.1 mg GAE/g dry extract and 5.3±0.2 mg GAE/g dry extract, respectively).Flavonoid was detected only in three endophytic strains. The total flavonoid content (TFC) ranged from 3.2±0.1 to 5.6±0.3 mg CE/g dry extract.

Antimicrobial potentials

Antibacterial activity of fungal endophytes

Nine endophytic fungal extracts from *Z. nimmonii*, showed inhibitory activities against at least two of the six test bacterial strains. *Bipolaris specifera* (KM114290) exhibited the highest inhibition zones ranging from 15.1±0.3 to 26.7±1.1 mm (diameter), against all six test bacteria in the agar disk diffusion assay, and with Minimum Inhibitory Concentrations (MIC's) of 0.04 to 0.14 mg/mL, followed by *Aspergillus terreus* (Tables 10 & 11; Fig. 6). The antibacterial activity of endophytic fungal strains from *P. chinense* was tested against six pathogenic bacteria and the appeared area of inhibition zone is presented in Table 12. The strains were further tested for MIC and MBC. The results are depicted in Table 13. *Penicillium canescens* (Fig. 2g) exhibited highest inhibition zone against all the pathogens except *B. subtilis* followed by *F. chlamydosporum*. The inhibition zone formed by *P. canescens* is represented in Fig. 7. *B. subtilis* was found to be resistant against all the extracts. *Alternaria longipes* did not show any activity. *Curvularia geniculata* did not depict any inhibition zone at 250 µg but showed inhibition at higher concentration against a Gram-positive (*P. aeruginosa*) and Gram-negative (*E. coli*) bacterium. All the endophytic fungal extracts showed inhibitory activity against *E. coli,* the concentration ranged from 2 mg/ml to 0.06 mg/ml. *Emericella nidulans* showed an inhibition zone only against *E. coli*. The antibacterial activity of fungal strains from *J. wynaadensis* was tested against six pathogenic bacteria and the area of inhibition zone is presented in Table 14. The strains were further tested for MIC and MBC. The results are depicted in Table 15. *F. incarnatum* exhibited highest inhibition zone against all the pathogens except *B. subtilis* followed by *C. lindemuthianum* and *C. truncatum*. The inhibition zone formed by *F. incarnatum*

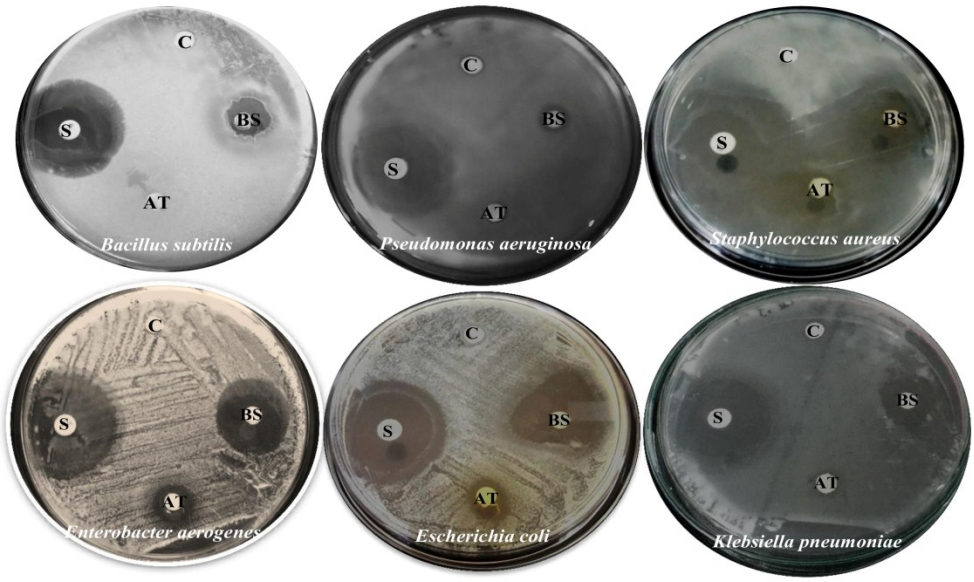

Fig. 6 Antibacterial inhibition zone formed by fungal endophytic extracts from Z. *nimmonii* (S) Streptomycin disc (10 µg/ disk), (C) Negative control, (BS) *B. specifera*, (AT) *A. terreus* (250 µg/ disk).

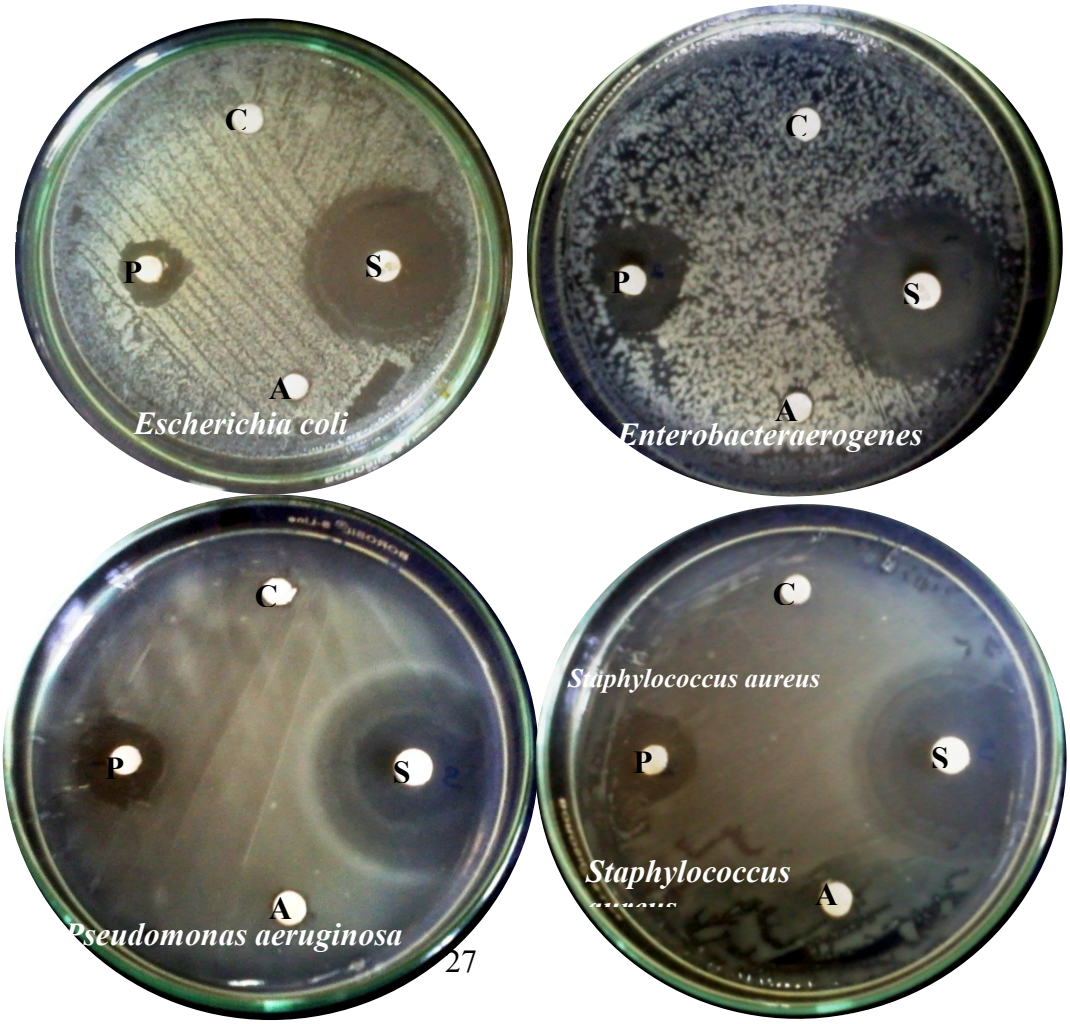

Fig. 7

Antibacterial inhibition zone formed by *P. canescens* extract
S- Streptomycin disc; C- Negative control; P- *P. canescens* extract; A- *A. longipes* extract

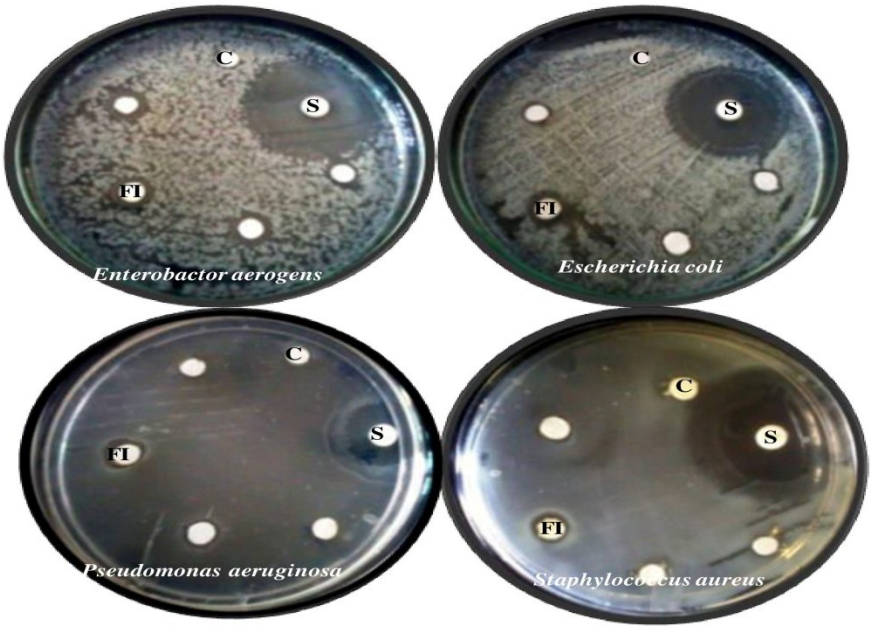

Fig. 8 Antibacterial zone formed by the extract of *F. incarnatum* isolated from *J. wynaadensis*
(S) Streptomycin disc (10 µg/ disk), (C) Negative control, (FI) *F. incarnatum* extract (250 µg/ disk).

is represented in Fig. 8. *B. subtilis* found to be resistant against all the extracts. *F. oxysporum* did not show any activity. All endophytic fungal extracts showed inhibitory activity against *E. coli* between concentrations ranged from 2 mg/ml to 0.16 mg/ml except *F. oxysporum*. *C. globosum* showed inhibition zone only against *E. aerogenes*.

Antibacterial activity of actinomycete endophytes

The antibacterial activity of strains was tested against six pathogenic bacteria and the appeared area of inhibition zone is presented in Table 16. The strains were further tested for MIC and MBC.The results depicted in Table 17. *Streptomyces indiaensis* isolated from *Z. nimmonii* exhibited highest inhibition zone against all the pathogens followed by *Streptomyces clavuligerus* strain isolated from *J. wynaadensis*. The inhibition zone formed by *S. indiaensis*is represented in Fig. 9. *Staphylococcus aureus* was found to be susceptible only to *S. indiaensis*. All actinomycete extracts assayed showed inhibitory activity against *E. coli* and *E.aerogene*

*s*within the concentration range of 1.6 to 0.02 mg/mL and 2 to 0.02 mg/mL respectively. *S. indiaensis* showed good antibacterial activity against all the pathogens.

Table 10 Antibacterial activity of fungal endophytes isolated from *Z. nimmonii* against six pathogenic bacteria

Extracts of endophytic strains		Gram-positive		Gram-negative			
	Gen bank accession No.	***Bacillus subtilis***	***Staphylococcus aureus***	***Pseudomonas aeruginosa***	***Escherichia coli***	***Enterobacter aerogenes***	***Klebsiella pneumoniae***
Bipolaris specifera	KM114290	17.8±0.7	18.9±1.0	15.1±0.3	26.7±1.1	25.4±0.3	16.0±0.6
Aspergillus terreus	KM396303	-	13.9±0.2	7.2±0.1	14.8±0.4	16.5±0.5	7.5±0.2
Alternaria tenuissima	KJ547594	-	9.9±0.3	-	13.0±0.3	9.2±0.2	10.2±0.4
Alternaria consortiale	KM114288	-	-	-	-	-	-
Nectria haematococca	KM396304	-	12.5±0.5	11.1±0.1	14.2±1.0	17.2±0.5	11.7±0.1
Fusarium chlamydosporum	KM396301	-	10.6±0.4	9.2±0.2	11.5±0.5	16.8±0.3	7.0±0.5
Fusarium solani	KJ547596	-	8.6±0.4	10.5±0.2	11.2±0.1	7.5±0.2	9.7±0.5
Fusarium equiseti	KM396306	-	-	-	9.3±0.1	8.2±0.1	-
Hypocrealixi	KM396302	-	-	-	-	-	-
Sarocladium kiliens	KM396305	-	8.1±0.1	9.5±0.3	10.2±0.2	8.5±0.1	-
Trichoderma harzianum	KJ547595	-	-	11.2±0.2	12.7±0.5	11.5±0.1	-
Streptomycin*	-	32±0.1	31.5±0.5	33±0.3	20±0.1	22±0.1	30±0.2

Data are reported as mean ± SD (in mm) of three independent analyses (n=3). * Streptomycin 10 µg disc used; '-' indicates the absence of inhibition zones in the disk diffusion assay with the endophytic fungal extracts as tested against the test bacterial pathogenic strains.

Table 11 Minimal inhibitory (MIC mg/mL) concentrations and minimum bactericidal concentrations (MBC mg/mL) of fungal extracts from *Z. nimmonii* endophytic strains

Endophytic fungal strains/ extracts	Test bacterial strains											
	S. aureus		***B. subtilis***		***P. aeruginosa***		***E. coli***		***E. aerogenes***		***K. pneumoniae***	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
B. specifera	0.1	0.12	0.1	0.12	0.14	0.16	0.04	0.06	0.04	0.06	0.14	0.16
A. terreus	0.16	0.18	-	-	1.4	1.6	0.16	0.16	0.12	0.14	1.2	1.4
A. tenuissima	1.0	1.0	-	-	1.8	2.0	0.18	0.2	1.0	1.2	0.8	1.0
A. consortiale	-	-	-	-	-	-	-	-	-	-	-	-
N. haematococca	0.6	0.8	-	-	0.8	1.0	0.16	0.18	0.10	0.12	0.6	0.8
F. chlamydosporum	0.8	1.0	-	-	1.0	1.2	0.6	0.8	0.12	0.14	1.4	1.6
F. solani	1.2	1.4	-	-	0.8	1.0	0.8	1.0	1.2	1.4	1.0	1.2
F. equiseti	-	-	-	-	1.8	2	1.0	1.2	1.2	1.4	-	-
H. lixi	-	-	-	-	-	-	-	-	-	-	-	-
S. kiliense	1.2	1.4	-	-	1.0	1.2	0.8	1.0	1.2	1.4	2.0	-
T. harzianum	-	-	-	-	0.8	1.0	0.6	0.6	0.6	0.8	2.0	-

Table 12 Antibacterial activity of fungal endophytes isolated from *P. chinense* against six pathogenic bacteria

Fungal strains extract	*P. aeruginosa*	*B. subtilis*	*S. aureus*	*E. coli*	*E. aerogenes*	*K. pneumoniae*
P. canescens	19.5±0.9	-	18.6±1.0	21.9±2.1	23.4±0.7	15±1.1
F. chlamydosporum	12.8±1.1	-	14.0±0.5	16.8±0.6	21.2±0.1	13.5±0.9
A. alternata	11.3±0.2	-	9.9±0.3	13.8±0.3	8.9±0.2	11.2±0.1
B. sorokinia	14.4±1.3	-	10.5±0.4	12.2±1.4	9.2±0.1	10.2±0.5
F. solani	14.6±0.2	-	10.6±0.4	11.6±1.0	-	11.7±0.5
C. geniculata	-	-	-	-	-	-
C. tenuissium	11.7±0.9	-	9.5±0.7	-	-	10.5±0.2
E. nidulans	-	-	-	7.3±0.1	-	-
F. incarnatum	-	-	-	9.0±0.1	8.5	8.3±0.3
A. longipes	-	-	-	-	-	-
Streptomycin*	33±0.3	32±0.1	31.5±0.5	20±0.1	22±0.1	30±0.2

Data are reported as mean ± SD of three independent analyses (n=3). * Streptomycin 10µg and Chloramphenical 30µg disc used

Table 13 Minimal inhibitory (MIC mg/ml) concentration and minimum bactericidal concentration (MBC mg/ml) of fungal endophytes from *P. chinense*

Endophytic fungal strain extract	*P. aeruginosa*		*S. aureus*		*E. coli*		*E. aerogenes*		*K. pneumoniae*	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
P. canescens	0.1	0.12	0.1	0.12	0.08	0.1	0.06	0.08	0.14	0.16
F. chlamydosporum	0.14	0.16	0.18	0.2	0.12	0.14	0.08	0.1	0.18	0.2
A. alternata	0.18	0.2	0.2	0.4	0.14	0.16	0.4	0.6	0.18	0.2
B. sorokinia	0.12	0.14	0.18	0.2	0.18	0.2	0.4	0.6	0.2	0.2
F. solani	0.18	0.2	0.18	0.18	0.18	0.2	-	-	0.16	0.18
C. geniculata	1.8	2	-	-	1.6	1.6	-	-	-	-
C. tenuissium	0.16	0.18	0.4	0.6	1.4	1.6	-	-	0.2	0.4
E. nidulans	-	-	-	-	1.2	1.4	-	-	2.0	-
F. incarnatum	2.0	-	-	-	0.4	0.6	0.6	0.8	0.6	0.8
A. longipes	-	-	-	-	2	-	-	-	-	-

Table 14 Antibacterial activity of fungal endophytes isolated from *J. wynaadensis* against six pathogenic bacteria

Fungal strains extract	*P. aeruginosa*	*B. subtilis*	*S. aureus*	*E. coli*	*E. aerogenes*	*K. pneumoniae*
F. incarnatum	15.0±0.9	-	12.5±0.5	16.9±0.2	15.6±0.7	10.0±0.1
T. harzianum	9.2±0.2	-	-	10.0±0.5	11.5±0.1	-
S. kiliense	9.0±0.6	-	9.0±0.1	10.8±0.3	8.0±0.2	-
C. globosum	-	-	-	-	8.0±0.5	-
F. oxysporum	-	-	-	-	-	-
C. truncatum	9.0±0.1	-	9.3±0.1	10.3±0.2	8.0±0.4	7.5±0.4
A. alternata	8.2±0.2	-	-	9.5±0.1	-	8.5±0.2
C. lindemuthianum	10.5±0.5	-	7.0±0.5	11.0±0.1	9.2±0.2	8.5±0.2
F. solani	-	-	-	8.0±0.1	10.5	-
Streptomycin*	33±0.3	32±0.1	31.5±0.5	20±0.1	22±0.1	30±0.2

Data are reported as mean ± SD (in mm) of three independent analyses (n=3). * Streptomycin 10µg disc used

Table 15 Minimal inhibitory (MIC mg/ml) concentration and minimum bactericidal concentration (MBC mg/ml) of fungal endophytes from *J. wynaadensis*

Endophytic fungal strain extract	*P. aeruginosa*		*S. aureus*		*E. coli*		*E. aerogenes*		*K. pneumoniae*	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
F. incarnatum	0.18	0.2	0.2	0.4	0.16	0.18	0.18	0.2	0.6	0.8
T. harzianum	1.0	1.2	-	-	0.8	1.0	0.8	1.0	1.8	2.0
S. kiliense	1.2	1.2	1.2	1.4	1.0	1.2	1.6	1.8	0.18	0.2
C. globosum	2	-	-	-	1.8	2	1.6	1.8	-	-
F.oxysporum	-	-	-	-	-	-	-	-	-	-
C. truncatum	1.4	1.6	1.4	1.6	1.0	1.2	1.4	1.6	1.8	2.0
A. alternata	1.4	1.4	-	-	0.8	1.0	1.6	1.8	1.2	1.4
C. lindemuthianum	0.6	0.8	1.8	2.0	0.8	1.0	0.8	1.0	1.2	1.4
F. solani	2.0	-	-	-	1.2	1.4	1.0	1.2	-	-

Table 16 Antibacterial activity of actinobacterial endophytes against six pathogenic bacteria

Host plant	Actinobacterial strains extract	Gram - positive		Gram - negative			
		B. subtilis	***S. aureus***	***P. aeruginosa***	***E. coli***	***E. aerogenes***	***K. pneumoniae***
Z. nimmonii	*Arthrobacter* sp.	-	-	9.2±0.2	11.1±0.5	10.3±0.4	8.1±0.1
	Corynebacterium sp.	-	-	-	9.9±0.3	9.2±0.2	-
	S. clavuligerus	10.0±0.2	-	7.2±0.1	12.1±0.3	10.5±0.5	-
	S. indiaensis	17.0±0.2	17.5±0.5	14.2±0.1	28.8±0.2	29.4±0.1	16.3±0.3
	C. oceansedimenticum	-	-	8.2±0.2	10.5±0.5	-	-
	S. griseus	11.1±0.1	-	9.5	13.3±0.1	12.9±0.1	7.2±0.4
	S. coelicolor	11.5±0.5	-	10.1±0.2	12.0±0.2	7.8±0.2	-
P. chinense	*S. clavuligerus*	9.2±0.1	-	7.5±0.3	10.2±0.3	9.7±0.2	-
	N. ganjiahuensis	-	-	8.0±0.1	-	-	-
	S. griseus	8.8±0.4	-	8.5±0.5	10.2±0.4	9.0±0.1	-
	N. alba	12.1±0.2	-	12.5±0.8	13.3±0.3	10.1±0.5	11.2±0.2
J. wynaadensis	*Arthrobacter* sp.	8.5±0.2	-	-	9.2±0.1	10.5±0.3	-
	S. clavuligerus	14.2±0.3	-	12.4±0.1	18.5±0.5	17.3±0.5	14.3±0.2
	Streptomyces sp.	12.3±0.2	-	13.1±0.1	12.9±0.1	9.2±0.2	-
	Streptomycin*	32±0.1	22.0±0.1	31.5±0.5	33±0.3	30±0.2	20±0.1

Data are reported as mean ± SD (in mm) of three independent analyses (n=3). * Streptomycin 10µg disc used. Actinomycete strains extract of 250 µg /disk used.

Table 17 Minimal inhibitory (MIC mg/mL) concentration and minimum bactericidal concentration (MBC mg/mL) of actinobacterial endophytes

Host plant	Endophytic actinobacterial strain extract	Test bacterial strains											
		P. aeruginosa		***B. subtilis***		***S. aureus***		***E. coli***		***E. aerogenes***		***K. pneumoniae***	
		MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Z. nimmonii	*Arthrobacter* sp.	1.0	1.2	-	-	-	-	0.6	0.8	0.8	0.8	1.4	1.6
	Corynebacterium sp.	2.0	-	1.8	2.0	-	-	0.8	1.0	1.0	1.2	-	-
	S. clavuligerus	1.6	1.8	0.8	1.0	2.0	-	0.4	0.6	0.6	0.8	1.6	1.8
	S. indiaensis	0.16	0.18	0.1	0.12	0.1	0.12	0.02	0.04	0.02	0.04	0.14	0.16
	C. oceansedimenticum	1.4	1.6	-	-	-	-	0.8	1.0	2.0	-	-	-
	S. griseus	1.0	1.2	0.4	0.6	-	-	0.2	0.4	0.2	0.4	1.8	1.8
	S. coelicolor	0.8	1.0	0.6	0.8	-	-	0.4	0.6	1.4	1.6	2.0	-
P. chinense	*S. clavuligerus*	1.4	1.6	1.0	1.2	2.0	-	0.8	1.0	1.0	1.0	1.8	2.0
	N. ganjiahuensis	1.4	1.6	-	-	-	-	1.6	1.8	2.0	-	-	-
	S. griseus	1.2	1.2	1.0	1.2	-	-	0.8	0.8	1.0	1.2	-	-
	N. alba	0.2	0.4	0.2	0.4	-	-	0.18	0.2	0.8	1.0	0.6	0.8
J. wynaadensis	*Arthrobacter* sp.	1.2	1.4	1.8	2.0	-	-	0.6	0.8	1.0	1.2	-	-
	S. clavuligerus	0.2	0.4	0.16	0.18	1.6	1.8	0.08	0.1	0.1	0.12	0.16	0.16
	Streptomyces sp.	0.18	0.2	0.2	0.4	-	-	0.18	0.2	1.0	1.2	1.8	2.0
	Streptomycin sulphate	0.02	0.02	0.02	0.04	0.02	0.04	0.04	0.06	0.04	0.06	0.04	0.04

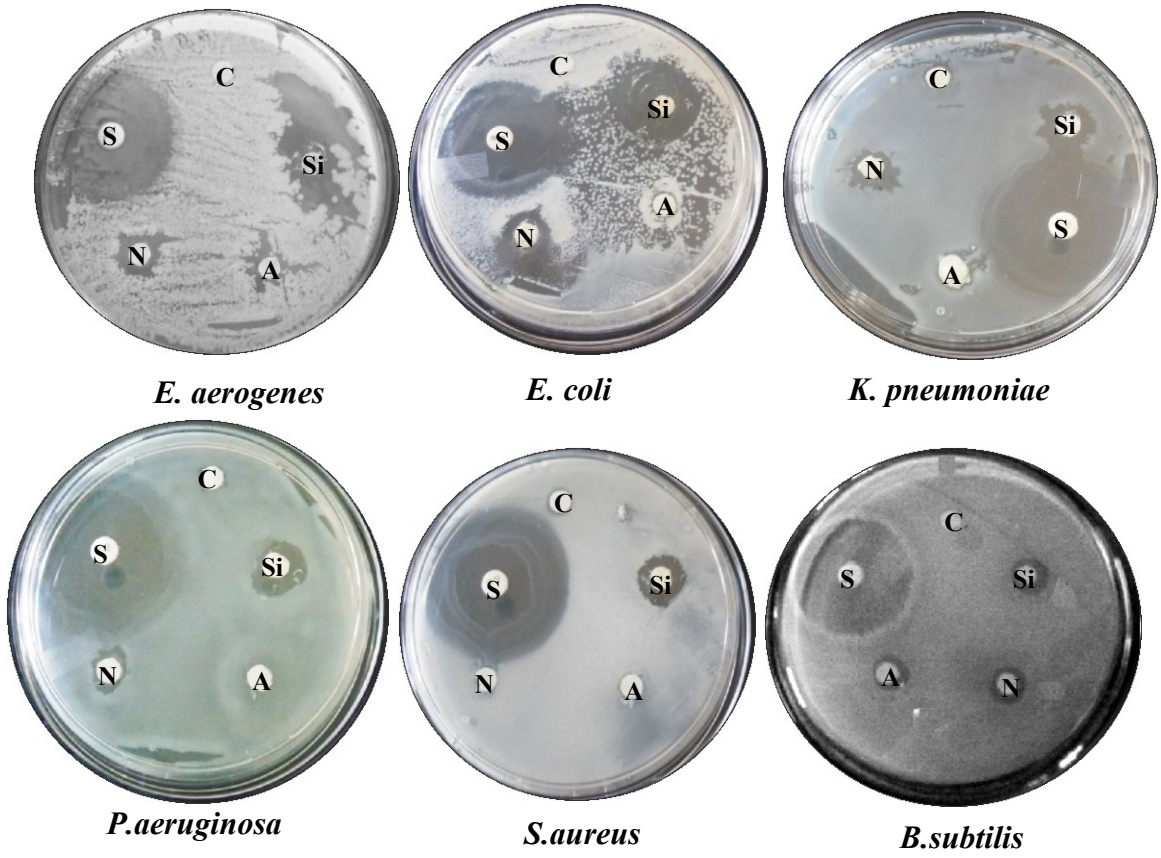

Fig. 9 Antibacterial inhibition zones formed by endophytic actinobacterial extracts (250 µg extract/ disk)

S- Streptomycin disk; C- Negative control; Si- *S. indiaensis* extract; N- *N. alba* extract; A- *Arthrobacter* sp. extract isolated from *J. wynaadensis*

OUTCOMES OF THE PROJECT

1. **PUBLICATIONS (International and indexed journals)**

a. **Das, M., Prakash, H.S. and Nalini, M.S. 2017a.**Antioxidative properties of phenolic compounds isolated from the fungal endophytes of *Zingiber nimmonii* (J. Graham) Dalzell. **Frontiers in Biology 12(2):151–162, DOI 10.1007/s11515-016-1441-z** (Springer Business Media + Higher Education Press China) (Copy enclosed).

b. **Das, M., Prakash, H.S. and Nalini, M.S. 2017b.** Antioxidative and antibacterial potentials of fungal endophytes from *Justicia wynaadensis* Heyne., an ethnomedicinal rainforest species of Western Ghats. **Asian Journal of Pharmaceutical & Clinical Research 10(6):203-209.** (Innovare Academic Sciences, Bhopal, India), ISSN: 2455–3891 (Copy enclosed).

c. **Das, M., Prakash, H.S. and Nalini, M.S. 2018**.Bioactive sesquiterpene, plasticizer, and phenols from the fungal endophytes of *Polygonum chinense* L. **Annals of Microbiology** 68:595-609. **(Springer Nature Impact Factor = 1.57)** (Copy enclosed).

d. **Das, M., Prakash, H.S. and Nalini, M.S.** Antibacterial metabolites from *Bipolaris specifera*, an endophytic fungus from the endemic medicinal plant, *Zingiber nimmonii* (J. Graham) Dalzell. **3 Biotech 10:310-317 (Springer Nature;2019 IF = 1.798)** (Copy enclosed).

2. **PAPERS/ POSTER PRESENTED IN CONFERENCES**

a. **International Conference by Omics Group;** Poster presented on **"Endophytic fungi from *Zingiber nimmonii* (J. Graham) Dalzell. (Zingiberaceae): An endemic medicinal plant of Western Ghats, southern India** – Das, M., Nalini, M.S**.** and Prakash, H.S., Organized by Omics International, **12th to 14th September, 2012 at Hyderabad, India.**

b. International Symposium on Role of Fungi and Microbes in the 21st century-A Global Scenario, Poster presented on **"Antioxidative potentials of fungal endophytes from the endemic medicinal plant of Western Ghats –*Zingiber nimmonii* (J. Graham) Dalzell**. – Das, M., Nalini, M.S**.** and Prakash, H.S., Organized by Indian Mycological Society, in collaboration with Department of Botany, University of Calcutta, **20th to 22nd February, 2014.**

c. Participated and presented a poster on "**Bioactive antibacterial compounds from *Streptomyces indiaensis*, an endophyte of the endemic medicinal plant, *Zingiber nimmonii***

(J. Graham) Dalzell., in two days "**International Workshop on Biology & Applications of Actinomycetes"-** Nalini M.S., Das, M. and Prakash, H.S., held on 31st October to 1st November 2019 organized byUniversity of Mysore, Mysore, in association with Helmholtz Center for Infection Research & Technical University of Braunschweig, Germany.

3. THESIS SUBMITTED BY PROJECT FELLOW

A PhD thesis on **"Isolation of endophytic fungi and actinomycetes from selected medicinal species of the Western Ghats and evaluation of their bioactive potential"** was submitted by the Project Fellow Mrs. Madhuchhanda Das on 3rd July, 2017 to the University of Mysore for the award of the Degree of Doctor of Philosophy in Botany (Scanned copy of the front and back cover page, Copies of the PhD notification and the Degree certificate enclosed).

Signature of the Principal Investigator

Signature of the Registrar with the Institution seal