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Research Article

ANTIOXIDATIVE AND ANTIBACTERIAL POTENTIALS OF FUNGAL ENDOPHYTES FROM *JUSTICIA WYNAADENSIS* HEYNE: AN ETHNOMEDICINAL RAIN FOREST SPECIES OF WESTERN GHATS

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ABSTRACT

Objective: Endophytes, living within the medicinal plants, are recognized as an alternative source of bioactive components useful for human health. This study aims to isolate and identify the fungal endophytes from the ethnomedicinal rain forest plant *Justicia wynaadensis* and evaluation of their antioxidant and antimicrobial potentials.

Methods: Endophytic fungi were isolated and identified by sequencing the internal transcribed spacer. The secondary metabolites were extracted with ethyl acetate and evaluated for the total phenolic, flavonoid, antioxidant, and antibacterial potentials. The antioxidative capacities were determined using different experimental models, *viz.,* radical scavenging capacity, reducing power, and inhibition of lipid peroxidation. The antibacterial potential of extracts was determined through disc diffusion method and by evaluating minimum inhibitory concentration through microplate technique.

Results: A total of nine fungal endophytes belonging to six genera were identified. The total phenolic content of the extracts ranged from 4.8±0.08 to 20.74±0.96 mg Gallic acid equivalent/g dry extract. Flavonoid was present in eight extracts in the range of 2.1±0.08 to 8.75±0.6 mg catechin equivalent/g dry extract. *Fusarium incarnatum* was found to have potentially high antioxidant capacity as well as broad spectrum antibacterial activity against Gram-positive and Gram-negative bacteria.

Conclusion: This study reported various endophytic fungi from the plant *J. wynaadensis*. This is the first attempt to explore the treasure of endophytes and their bioactive potentiality from this plant. Hence, our findings encourage the exploration of these fungi and exploit them in search of potential drug discovery.

Keywords: Endophytic fungi, *Justicia wynaadensis,* Western Ghats, Antioxidant, Antibacterial, *Fusarium incarnatum.*

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INTRODUCTION

The importance of medicinal plants as a source of medicine is as old as the civilization. Natural products have been exploited for human use for thousands of years and plants have been the chief source of compounds used for medicine. Around 80% of the world population uses the beneficial effects of the natural compounds of medicinal plants for health care in some form or the other [1]. Medicinal plants have been exploited rigorously to obtain the natural compounds and as a result, the natural reservoir is facing the threats of extinction.

In recent years, microorganisms have been recognized as rich sources of biologically active metabolites that finds wide-ranging exploitation in medicine, agriculture, and industry [2]. Plants may serve as a reservoir of large numbers of microorganisms known as endophytes that live within plant tissues for all or part of their life cycle and cause no apparent infections [3]. Endophytes are chemical synthesizers inside plants [4], in other words, they play a role as a selection system for microbes to produce bioactive substances with low toxicity towards higher organisms [5]. Bioactive natural compounds produced by endophytes have been promising in terms of safety and human health concerns [6].

Microorganisms and humans have developed resistance to the existing antibiotics posing needs of newer therapeutic agents. The fungal endophyte derived bioactive compounds provide us with new choices of novel antibiotic with which can be effectively used against infectious diseases. Endophytes provide a broad variety of bioactive secondary metabolites with a unique structure, including alkaloids, benzopyranones, quinones, flavonoids, phenolic acids, steroids, terpenoids, tetralones, xanthones, and others [7]. Such bioactive metabolites find wide-ranging application as agrochemicals, antibiotics, immune-suppressants, antiparasitics, antioxidants, and anticancer agents [8].

Antioxidant among plethora of substances is an important compound and potential therapeutic agent against the oxidative damage. Oxidative damage to cell component plays vital role in many human diseases, *viz.,* cancer, Alzheimer's disease, and kidney disease. Antioxidant compounds can be thiols, ascorbic acids (AAs), or polyphenols. Phenolic acids and flavonoids are major bioactive components isolated from endophytic fungi [9-11].

Justicia wynaadensis Heyne is an endemic plant, naturally occurring in the rain forest of Western Ghats, East Nilgiris, and South Malabar Hills in South India [12]. The plant is locally known as "Madubana soppu" or "Maddhu thoppu," belongs to the family Acanthaceae. The family Acanthaceae consists of 250 genera, many of which have ethnomedicinal properties [13]. *J. wynaadensis* also has ethnomedicinal properties. The juice of the stem and leaf of this plant is used locally to cure asthma, boost immunity, as anthelmintic, and antidiabetic [14]. *J. wynaadensis* also has cholesterol lowering properties [15]. 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 a 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 endophytes morphologically as well as with the help of molecular tools from the ethnomedicinal plant *J. wynaadensis* and the evaluation of antioxidant and antimicrobial activity of the endophytic fungal isolates.

METHODS

Chemicals

Gallic acid, trolox, AA, 2, 2'-azino-bis(3- ethylbenzthiazoline-6-sulphonic acid) (ABTS), and 1, 1-diphenyl-2- picrylhydrazyl (DPPH) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Folin–Ciocalteu's reagent was purchased from SRL Pvt. Ltd. (Mumbai, India). Sodium hypochlorite, potassium ferricyanide, trichloroacetic acid (TCA), ferric chloride, sodium nitrite, aluminum chloride, potassium persulfate, thiobarbituric acid (TBA), and sodium dodecyl sulfate (SDS) and all other general chemicals and solvents were of analytical grade. Triple distilled water was used wherever necessary. Antibiotic discs and Mueller-Hinton medium were purchased from Hi Media (Mumbai, India).

Collection of the plant material

J. wynaadensis was collected from the Talacauvery sub-cluster (012°17' to 012°27' N and 075°26' to 075°33'E) of the Western Ghats, in Kodagu district, Karnataka state, India, during August 2012 (Fig. 1). The plant parts such as stems and leaves were collected in zip lock polyethylene bags and brought to the laboratory and processed for the isolation within 24 hrs of collection. A herbarium specimen has been preserved in the Department of Studies in Botany, University of Mysore.

Isolation of endophytic fungi from the plant parts

Endophytic fungal isolation was carried out under aseptic conditions, according to Tejesvi *et al*. [16]. The plant parts were surface sterilized with ethanol (70%, 1 minute), sodium hypochlorite (3.5%, 3 minutes) and then 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 and 400 plant fragments were placed on water agar media (2%, w/v) for the isolation of endophytic fungi, supplemented with the antibiotic streptomycin (50 mg/L) to inhibit bacterial growth. The effectiveness of the surface sterilization was confirmed [17]. The plates were sealed with Clingwrap™ and incubated at 28±2°C with 12 hrs of light and dark cycles for 4-6 weeks. The fungal hyphae that emerged on the fragments (Fig. 2) were further inoculated onto potato dextrose agar slants and incubated at 28±2°C for 10-15 days and maintained as pure cultures at 4°C for further use.

Identification of the fungal endophytes

Microscopic slides of each endophyte were prepared using lactophenol cotton blue stain (Hi Media, Mumbai, India). The slides were examined under the light microscope (Labovision, India) for morphological analysis. Based on the cultural characteristics and spore structure, nine endophytic fungi were selected for the molecular identification and further analysis of their metabolites.

Molecular identification of fungal endophytes

Actively grown mycelial plugs from nine morphologically different endophytic fungi were inoculated into potato dextrose broth (PDB, Hi Media, Mumbai, India). The isolates were grown in still culture at 28±2°C for 7-10 days. The genomic DNA was extracted from the freeze-dried fungal mats by cetyltrimethylammonium bromide method with slight modifications [18]. The DNA concentration was estimated by measuring the absorbance at 260 and 280 nm (Thermo Scientific Nanodrop 2000/2000c, Bengaluru, India). Target regions of the rDNA internal transcribed spacer (ITS) 1 and 2 regions and 5.8 rRNA genes were amplified using primers ITS 1 and ITS 4. The amplification was performed in a total reaction volume of 25 µl containing 200 µmol/L deoxynucleotide triphosphates, 10 pmol/µl of each primer and 50 ng template DNA. The amplification conditions consisted of an initial denaturation step at 95°C for 3 minutes, followed by 35 cycles of 92°C for 1 minute, 50°C for 1 minute, 72°C for 2 minutes, and a final extension at 72°C for 10 minutes. The amplified product was subjected to sequencing at Chromous Biotech Pvt. Ltd. Bengaluru, India. The endophyte sequences were aligned with the reference sequences using the BLAST algorithm and submitted to the NCBI GenBank nucleotide collection.

Fig. 1: *Justicia wynaadensis* Heyne., collected from the natural habitat of the Western Ghats

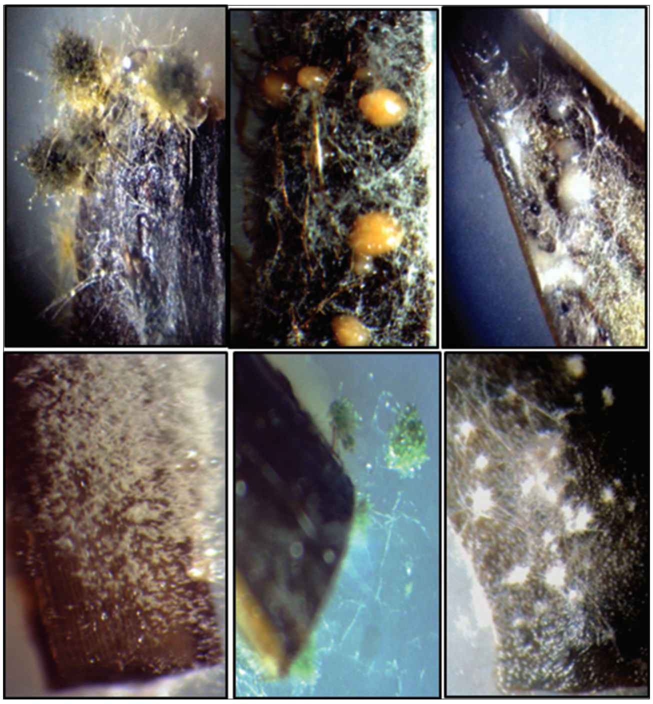

Fig. 2: Emergence of fungal hyphae from plant fragments plated on agar media

Fermentation and extraction of metabolites

The pure cultures of 10-day-old isolates were inoculated into 500 ml of PDB contained in Erlenmeyer flasks in duplicates and kept for incubation for 3 weeks at 28±2°C. The fermentation broth of each endophyte was extracted with ethyl acetate thrice 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.

Determination of the total phenolic content (TPC)

The TPC was assessed according to the Folin–Ciocalteau (FC) method of Liu *et al*. with some modifications [19]. 1 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 minutes in the dark. The absorbance was read at 765 nm (T-60, TTL Technology, India). The TPC of the extracts was expressed as mg of Gallic acid equivalent (GAE)/g of the extract.

Determination of total flavonoid content (TFC)

The total flavonoid was determined according to the method of Barros *et al*. [20]. The fungal extract was mixed with sodium nitrite (5%, 75 µl). After 5 minutes aluminum 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.

Determination of antioxidant capacity

$ABTS^+$ radical scavenging assay

The $ABTS^+$ scavenging capacity was determined by the method of Re *et al*. [21]. $ABTS^+$ was generated by the mixing of ABTS (7 mM) and potassium persulfate (2.45 mM). The working solution was prepared by diluting with methanol to obtain an absorbance of 0.70 at 734 nm. The activity was expressed as Trolox equivalent antioxidant capacity (mg TE/g dry extract).

DPPH radical scavenging assay

The quenching ability of DPPH was measured according to the procedure of Liu *et al*. with modifications [19]. A methanolic solution of DPPH (0.001 mM) was added to the fungal extract. The absorbance was read at 517 nm after 20 minutes of incubation. The scavenging activity was expressed as inhibitory concentration 50% (IC_{50}) (µg/ml). AA was used as the standard. The scavenging ability of the DPPH radical was calculated by the formula:

$$\% \text{ Scavenging} = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Reducing power assay

The reducing power was measured by the method of Oyaizu with modifications [22]. 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 50°C for 20 minutes. After incubation, TCA (10% w/v, 0.5 ml) was added and centrifuged at 3000 rpm for 10 minutes. To the supernatant, the 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 AA/g dry extract.

Inhibition of lipid peroxidation capacity

Inhibition of lipid peroxidation in the egg yolk of the hen was determined according to the procedure of Ohkawa *et al*. with modifications [23]. The egg yolk of hen (0.5 g) was emulsified with phosphate buffer (0.1 M, pH 7.4) to achieve the final volume of 25 g/L. Then, 0.5 ml of the egg homogenate was mixed with 0.1 ml of each sample. The volume was made up to 1 ml with distilled water. In this mixture 0.05 ml ferrous sulfate (0.07 M) was added and incubated for 30 minutes at room temperature to induce lipid peroxidation. After incubation, 1.5 ml of acetic acid (20%), 2 ml of TBA (1% w/v) in 1.1% SDS, and 0.05 ml TCA (20%) was added and vortexed. This mixture was again incubated for 60 minutes in a boiling water bath. After cooling, 5.0 ml of butanol was added and centrifuged at 3000 rpm for 10 minutes. The organic upper layer was taken in a separate tube, and the absorbance was measured at 532 nm. The inhibition of lipid peroxidation capacity was expressed as IC_{50} (µg/ml). Catechin was used as the standard. The ability to inhibit lipid peroxidation was calculated by the following formula:

$$\text{Inhibition of lipid peroxidation capacity } (\%) = \frac{A_{control} - A_{sample}}{A_{control}} \times 100$$

Detection of antibacterial activity

Test organism

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

Antibacterial activity

The inhibitory effect of the endophytic fungal extract was tested by paper disc diffusion method [24]. The crude extract of endophytic fungi was dissolved in dimethyl sulfoxide (DMSO) and tested on the Mueller-Hinton agar medium seeded with the test bacterium at 250 µg per disc (5 mm diameter, Whatman No. 1) concentration. Streptomycin (10 µg/disc) was used as positive control and the paper disc loaded with only DMSO was negative control. The test plates were incubated for 24 hrs at 35±2°C, and the inhibition zone was measured. The test was done in three replicates.

Determination of minimal inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

The MIC was determined by the modified broth dilution method [25]. MIC test was done using sterile 96 well microplate (Tarsons, Kolkata, India). The wells were filled with the reaction mixture containing 90 µl bacterial suspensions (10^6 CFU/ml) and 10 µl of the test sample with different concentrations (2 mg/ml to 0.02 mg/ml). Culture medium with 1% DMSO was the negative control, and streptomycin sulfate (0.4-0.01 mg/ml) was the positive control. The microplates were incubated for 24 hrs at 35±2°C. After incubation, 10 µl of indicator 3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyltetrazolium bromide (0.5 mg/ml phosphate buffer saline) was added to visualize the microbial growth. The lowest sample concentration at which no blue color appeared was determined as MIC. Wells containing MIC concentration and above were inoculated onto agar medium to check cell viability. The lowest concentration with no viable cells was determined as MBC. The test was done in three replicates.

Data and statistical analysis

The colonization frequency (CF) was calculated by the following formula:

$$\% \text{ CF} = \frac{N_{col}}{N_t} \times 100$$

Where, N_{col} is number of tissue segments colonized by a fungus; N_t is the total number of tissue segments plated [26].

Data reported as mean±standard deviation of three independent replicates. Comparison among means was analyzed with one-way ANOVA and Tukey-Kramer multiple comparison tests using GraphPad InStat 3.0. Any two data were considered statistically significant at $p<0.05$ and denoted with different superscripts.

RESULTS

Isolation and identification of fungal endophytes

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 (Fig. 3) as well as by analyzing the DNA sequence of the ITS region. The identified strains with their Genbank accession numbers, isolation number and %CF are depicted in Table 1. Results indicated that the stem part had more number of isolates (161) than leaves (120). The nine endophytic species belonged to six genera. Three different species of *Fusarium* and two

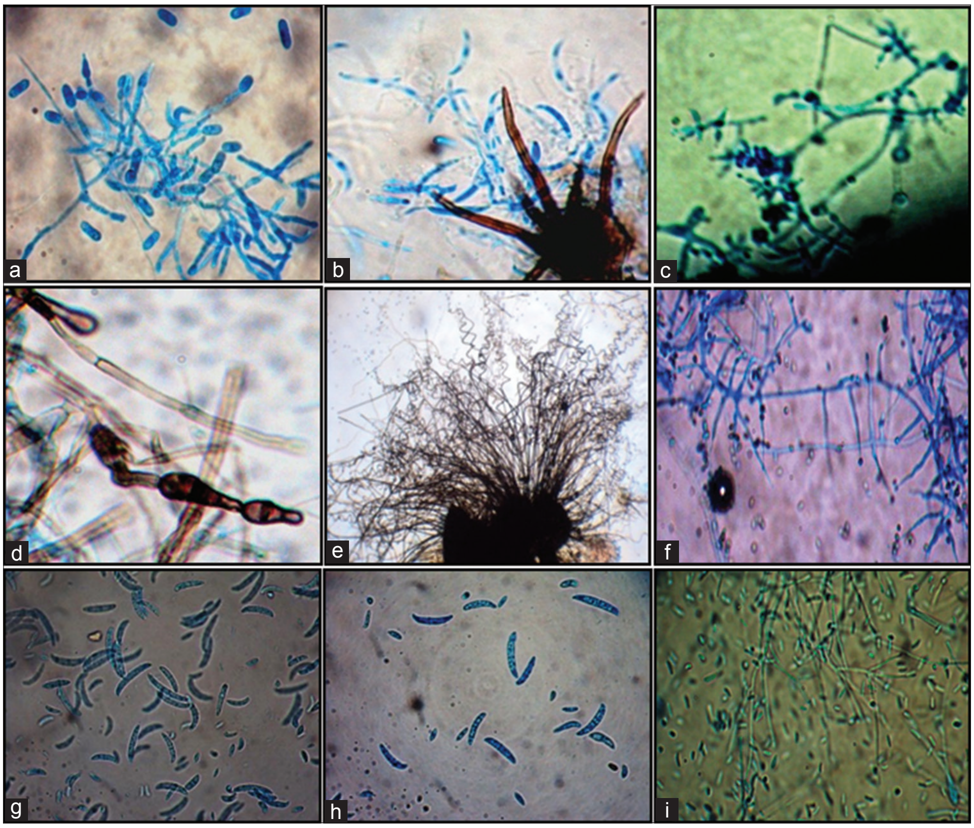

Fig. 3: Spore morphology of fungal endophytes isolated from plant fragments. (a) *Colletotrichum lindemuthianum*, (b) *Colletotrichum truncatum*, (c) *Trichoderma harzianum*, (d) *Alternaria alternata*, (e) *Chaetomium globosum*, (f) *Sarocladium kiliense*, (g) *Fusarium incarnatum*, (h) *Fusarium oxysporum*, and (i) *Fusarium solani*

Table 1: Taxonomic identification and percent %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, CF: Colonization frequency

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*.

Determination of TPC and flavonoid content

The TPC of the extracts is represented in Fig. 4. TPC of the extracts ranged from 4.8±0.08 to 20.74±0.96 mg GAE/g dry extract. *Fusarium incarnatum* extracts showed highest TPC (20.74±0.96 mg GAE/g dry extract) followed by *T. 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).

Flavonoid was detected in four endophytic strains (Fig. 4). The 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 capacity

$ABTS^+$ radical scavenging assay

The total antioxidant capacity of endophytic extracts was determined by $ABTS^+$ scavenging capacity. The scavenging capacity of the extracts varied considerably, ranging from 6.2±0.3 to 25.1±0.1 mg TE/g dry extract (Table 2). Among the extracts, *F. incarnatum* showed high scavenging capacity followed by *T. harzianum* and *Sarocladium 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

The DPPH radical is almost stable and used for antioxidant activity widely. The radical scavenging activity is presented as 50% scavenging activity (IC_{50}) in Table 2. As depicted, the IC_{50} value of the fungal extracts varied considerably (379.98±0.8 to 1920.67±134 µg/ml). *F. incarnatum* showed highest scavenging activity with 379.98±0.8 µg/ml, followed by *T. harzianum* and *S. kiliense*.

Reducing power assay

Antioxidant compounds have the reductive ability to transform Fe^{3+} to Fe^{2+} through their functional groups which are susceptible to electron transfer. The reducing power of ferric ion to ferrous ion by the fungal extracts is represented in terms of AA equivalent (Table 2). 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 the 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 fungal extracts is presented in Table 2. The capacity of the extracts ranged from 534.69±2.7 µg/ml to 1338.3±22.7 µg/ml. The extract of *F. incarnatum* showed highest inhibition capacity (534.69±2.7 µg/ml) followed by *T. harzianum* (931.42±8.2 µg/ml). *Alternaria alternata*, *C. lindemuthianum,* and *F. solani* did not show any activity, whereas *Colletotrichum truncatum* and *Fusarium oxysporum* showed very little activity.

Antibacterial activity

The antibacterial activity of strains was tested against six pathogenic bacteria, and the area of inhibition zone is presented in Table 3. The strains were further tested for MIC and MBC. The results depicted in Table 4. *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* is represented in Fig. 5. *B. subtilis* found to be resistant against all the extracts *F. oxysporum* did not show any activity. All endophytic fungal extracts

Table 2: 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)
Fusarium incarnatum	25.1±0.1^{a}	38.3±0.6^{a}	379.98±0.8^{b}	534.69±2.7^{b}
Trichoderma harzianum	17.5±0.2^{b}	21.15±0.3^{b}	543.67±16.6^{c}	931.42±8.2^{c}
Sarocladium kiliense	15.3±0.3^{c}	16.7±0.6^{c}	792.15±28.5^{d}	981.38±16.8^{d}
Chaetomium globosum	12.9±0.1^{d}	15.24±0.3^{d}	930.62±10.2^{e}	105084±3.1^{e}
Fusarium oxysporum	11.8±0.3^{e}	14.3±0.1^{e}	1155.54±5.5^{f}	1203.45±10.2^{f}
Colletotrichum truncatum	10.1±0.5^{f}	12.3±0.5^{f}	1172.5±7.2^{g}	1338.35±22.7^{g}
Alternaria alternata	8.3±0.1^{g}	9.5±0.2^{g}	1328.18±32.4^{h}	-
Colletotrichum lindemuthianum	7.6±0.1^{h}	8.2±0.3^{h}	1541.74±77.3^{i}	-
Fusarium solani	6.2±0.3^{i}	7.8±0.07^{h}	1920.67±134.0^{j}	-
		AA	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. AA: Ascorbic acid, ABTS^{+}: 2, 2'-azino-bis (3- ethylbenzthiazoline-6-sulphonic acid), TE: Trolox equivalent, DPPH: 1, 1-diphenyl-2- picrylhydrazyl, IC_{50}: Inhibitory concentration 50%, *J. wynaadensis: Justicia wynaadensis*

Table 3: Antibacterial activity of fungal endophytes isolated from *J. wynaadensis* against six pathogenic bacteria

Fungal strains extract	*Pseudomonas aeruginosa*	*Bacillus subtilis*	*Staphylococcus aureus*	*Escherichia coli*	*Enterobacter aerogenes*	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*
Fusarium incarnatum	15.0±0.9	-	12.5±0.5	16.9±0.2	15.6±0.7	10.0±0.1	15.0±0.9
Trichoderma harzianum	9.2±0.2	-	-	10.0±0.5	11.5±0.1	-	9.2±0.2
Sarocladium kiliense	9.0±0.6	-	9.0±0.1	10.8±0.3	8.0±0.2	-	9.0±0.6
Chaetomium globosum	-	-	-	-	8.0±0.5	-	
Fusarium oxysporum	-	-	-	-	-	-	
Colletotrichum truncatum	9.0±0.1	-	9.3±0.1	10.3±0.2	8.0±0.4	7.5±0.4	9.0±0.1
Alternaria alternata	8.2±0.2	-	-	9.5±0.1	-	8.5±0.2	8.2±0.2
Colletotrichum lindemuthianum	10.5±0.5	-	7.0±0.5	11.0±0.1	9.2±0.2	8.5±0.2	10.5±0.5
Fusarium 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	33±0.3

Data are reported as mean±SD (in mm) of three independent analyses (n=3). *Streptomycin 10 µg disc used. SD: Standard deviation, *J. wynaadensis: Justicia wynaadensis*

Table 4: MIC (mg/ml) and (MBC mg/ml) of fungal endophytes

Endophytic fungal strain extract	*Staphylococcus aureus*		*Escherichia coli*		*Enterobacter aerogenes*		*Klebsiella pneumoniae*		*Klebsiella pneumoniae*		*Pseudomonas aeruginosa*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
Fusarium incarnatum	0.2	0.4	0.16	0.18	0.18	0.2	0.18	0.2	0.6	0.8	0.6	0.8
Trichoderma harzianum	-	-	0.8	1.0	1.0	1.2	0.8	1.0	1.8	2.0	1.8	2.0
Sarocladium kiliense	1.2	1.4	1.0	1.2	1.2	1.2	1.6	1.8	0.18	0.2	0.18	0.2
Chaetomium globosum	-	-	1.8	2	2	-	1.6	1.8	-	-	-	-
Fusarium oxysporum	-	-	-	-	-	-	-	-	-	-	-	-
Colletotrichum truncatum	1.4	1.6	1.0	1.2	1.4	1.6	1.4	1.6	1.8	2.0	1.8	2.0
Alternaria alternata	-	-	0.8	1.0	1.4	1.4	1.6	1.8	1.2	1.4	1.2	1.4
Colletotrichum lindemuthianum	1.8	2.0	0.8	1.0	0.6	0.8	0.8	1.0	1.2	1.4	1.2	1.4
Fusarium solani	-	-	1.2	1.4	2.0	-	1.0	1.2	-	-	-	-

MIC: Minimal inhibitory concentration, MBC: Minimum bactericidal concentration

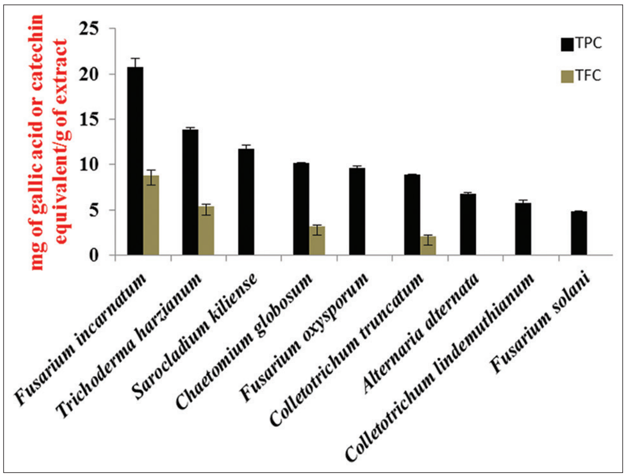

Fig. 4: Total phenolic content and total flavonoid content of fungal endophytes. Data are reported as mean±standard deviation of three independent analyses (n=3)

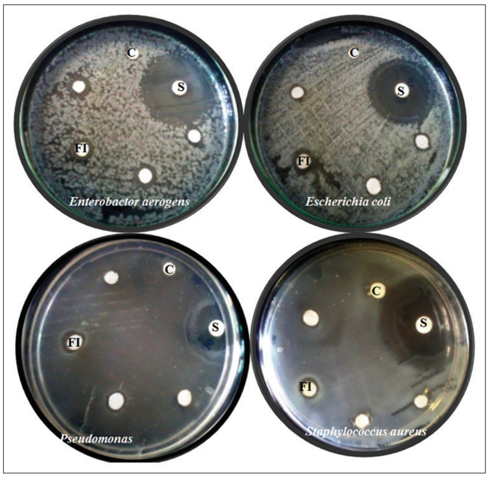

Fig. 5: Antibacterial zone formed by *Fusarium incarnatum*. S - Streptomycin disc (10 µg/disc), C - Negative control, FI - *Fusarium incarnatum* extract (250 µg/disc)

showed inhibitory activity against *E. coli* between concentrations ranged from 2 to 0.16 mg/ml except *F. oxysporum*. *C. globosum* showed inhibition zone only against *E. aerogenes*.

DISCUSSION

Two regions in India have been recognized as the "biodiversity hotspot" *viz*., the Eastern Himalayas and the Western Ghats. Exceptional levels of plant endemism are observed in these regions which in turn attract researchers to unfold the treasure house of Mother Nature, causing irreparable damage to biodiversity. Therefore, interest is building on the study of endophytes as well as its secondary metabolites for bioprospecting. Many reports are present on the isolation of endophytes from the medicinal plants of the Western Ghats [11,26-28]. *J. wynaadensis* is one of the endemic and ethnomedicinally important plants of this region. To our knowledge, there are no reports on endophytic study of this plant. This study deals with the isolation of endophytic fungi and their bioactive potentials from *J. wynaadensis*. A total of nine endophytic fungal strains belonging to six genera, *viz*., *Fusarium*, *Trichoderma*, *Sarocladium*, *Chaetomium*, *Colletotrichum,* and *Alternaria* were identified. *C. lindemuthianum* showed highest %CF (13.8). The genus *Colletotrichum* was previously isolated from different species of genus *Justicia* [29]. The isolates were identified morphologically (mycelial characters, spore morphology) as well as with the help of DNA sequence analysis of the ITS region. The isolates showed 95-100% similarity to their assigned taxa.

All nine strains were tested for the TPC, TFC, antioxidant, and antimicrobial capacities. The presence of phenolic and flavonoid phytochemicals in leaf and stem extracts of *J. wynaadensis* were reported by researchers [30,31]. The TPC of leaf and stem extracts (0.65±0.08 mg of GAE/g and 0.16±0.01 mg of GAE/g, respectively) as was previously reported by Medapa *et al*. is found to be significantly lower than that of the endophytes isolated from this plant in this investigation (4.8±0.08 to 20.74±0.96 mg GAE/g dry extract) [29]. The TFC is also found to be of significantly higher concentration than plant extract as reported by Medapa *et al*. [30]. However, our results of TPC and TFC of endophytes were significantly lower than the methanolic extract of *J. wynaadensis* leaf as reported by Abhishek *et al*. [31].

Among all the isolated strains, *F. incarnatum* was found to have potentially high antioxidant capacity. The DPPH radical scavenging capacity of leaf extract (IC_{50} 540.2485 µg/ml) of the plant was found to be lesser than the endophyte *F. incarnatum* (IC_{50} 379.98±0.8 µg/ml) [31]. *F. incarnatum* also displayed significant $ABTS^+$ radical scavenging (25.1±0.1 mg TE/g dry extract) and ferric reducing (38.3±0.6 mg AA/g dry extract) capacities. Hence, these findings encourage the exploration and bioprospecting of endophytic fungi.

In stressed condition, highly reactive OH^- radical reacts with polyunsaturated fatty acids. Due to lipid peroxidation, various products such as malondialdeyde, 4-hydroxyl 2-nonenal, hydrocarbons, volatile ketones, and lipid polymers are produced which react with the cell macromolecules and effect cellular functions and biochemistry [32,33]. Therefore, the inhibition of lipid peroxidation is necessary for cell viability in stressed conditions. The extracts of *F. incarnatum* exhibited the highest activity (534.69±2.7 µg/ml) whereas, *C. globosum*, *F. oxysporum,* and *C. truncatum* showed significantly lower activity and *A. alternata*, *C. lindemuthianum* had no activity.

In this study, we have shown that the endophytes have broad spectrum antimicrobial activity among which *F. incarnatum* demonstrated the maximum activity. Although *Colletotrichum* spp. did not show antioxidative potentiality, they were found to show broad spectrum antimicrobial activity. Endophytic *Colletotrichum* sp. isolated from *Prosopis juliflora* also showed significant activity with a zone of inhibition against *E. coli* (24.6±0.33) and *S. aureus* (23.8±0.88) [34]. In consistent with this study *Alternaria* sp. isolated from medicinal plant *Rauvolfia serpentina* showed a zone of inhibition against *K. pneumoniae* (17.6±0.58 mm) and *E. coli* (05.7±00.43 mm) [35]. All the isolated strains except *F. oxysporum* formed inhibition zone against *E. coli* at 250 µg concentrations. The endophytic *Aspergillus* sp. isolated from *Justicia adhatoda* has been reported to produce a stronger zone of inhibition against *E. coli*, *Pseudomonas,* and *Klebsiella* which corroborates our findings [36].

CONCLUSION

The Western Ghats of India is a biodiversity hotspot and a tropical rain forest. This place has a wealth of many medicinal plants which are not fully explored, for the presence of fungal endophytes. This study reported the occurrence of endophytic fungi from *J. wynaadensis*. To the best of our knowledge this is the first attempt to explore the treasure of endophytes and their bioactive potentiality from this plant. The strain *F. incarnatum* displayed broad spectrum antibacterial properties against Gram-positive and Gram-negative pathogens as well as significant antioxidative capacities. The strain *T. harzianum* displayed significant antiradical and ferric reducing capacities, and both species of *Colletotrichum* proved as potential antimicrobial agents. Hence, our findings encourage the exploration of these fungi and exploit them in search of potential drug discovery.

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