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# Unveiling the Hidden Allies: Biodiversity, phylogenetic characterisation and antifungal potential of endophytic mycoflora from *Terminalia bellirica* Roxb.

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Endophytes are microorganisms that live and thrive in the tissues of plants without causing any harm to them. *Terminalia bellirica* (Gaertn.) Roxb., belonging to the family Combretaceae, is a widely utilized plant in Indian traditional medicine systems. This research investigates the fungal endophytes of *T.bellirica* to evaluate the diversity and antifungal activity from two different locations in Patna district, Bihar, India. The diversity indices analysis revealed that endophytic mycoflora hosted a huge range of diversity, with the genus *Aspergillus* dominating both the locations. Together, 48 different endophytic mycoflora were isolated from segments of the leaf and bark of *T.bellirica* from both the locations. These isolated fungi were identified by their morphological and cultural characteristics using standard taxonomic manuals as well as by molecular identification using the ITS rRNA gene sequencing method. Out of 48, 21 isolates were from location I and 27 from location II. Antifungal activity of secondary metabolites from fungal endophytes against *Candida albicans* and *Saccharomyces cerevisiae* revealed that *Alternaria* sp., *Aspergillus* sp., *Trichoderma* sp. and *Rhizopus* sp. were the most potent. The leaf tissue segments harbour more endophytic mycoflora than bark tissue. The culture filtrates from all endophytic mycoflora demonstrated antifungal activity against the specific pathogens. The findings suggest that endophytic mycoflora isolated from therapeutic plants might be a source of novel bioactive molecules. This is the first approach to investigate endophytic fungi of *T. bellirica* in the Patna region.

**Keywords :** Antifungal activity, clinical pathogens, diversity, endophytic fungi, *Terminalia bellirica*

## INTRODUCTION

Endophytes are usually fungi and bacteria that dwell within the internal living tissues of healthy plants without the manifestation of any immediate negative effects (Bacon and White, 2000). They have been found in all major groups of plants examined to date including angiosperms (Taylor *et al.,* 1999). Endophytic mycoflora is one of the interesting ubiquitous symbionts of plants that give host defense from natural enemies. They have been recognized as potential sources of novel natural products for exploitation in medicine, agriculture, etc (Strobel *et al*. 2003). A present investigation was carried out to find the diversity of endophytic mycobiota present in one of the important medicinal plants i.e. *Terminalia bellirica.*

The diversity of endophytic fungi within *T. bellirica* presents an intriguing avenue for investigation, offering insights into the microbial biodiversity in this medicinal plant. They are abundant and inhabit the host plants, so one plant may hold over 30 fungal species (Rodriguez *et al.* 2009). The intricate relationship between plants and fungi has long fascinated researchers due to its potential for unlocking novel therapeutic compounds.

Among these, endophytic fungi residing within medicinal plants have emerged as a promising source of bioactive metabolites, offering a treasure trove of pharmacologically relevant compounds (Jha *et al*. 2023).

*Terminalia bellirica* (Gaertn.) Roxb., “Bahera,” stands as one such pharmaceutical plant with a rich reservoir of endophytic fungi yet to be fully

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explored. It has been used as a medicinal plant in Ayurveda and has been extensively studied for its potential therapeutic properties, particularly due to its rich content of bioactive compounds (Gupta *et al.,* 2017). Endophytic fungi offer a vast array of unique organic compounds exhibiting intriguing biological activities and a diverse array of biodiversity. They present an under explored ecological reservoir, with their secondary metabolites displaying significant potency due to their interactions with their host plants (Gupta *et al.* 2023). Recently many researchers worked on the potential of endophytic mycoflora in various ways and they found many beneficial molecules from these microbes (Huang *et al.* 2007). Sampling and characterizing fungal endophytic diversity is an emerging challenge, which leads to the discovery of new species, novel compounds, and a better understanding of their role in the ecosystem (Arnold, 2007). Although many endophytic mycoflora were isolated from various plants, there is a requirement to find new sources and information about their diversity. Variations in the diversity of fungi may be associated with location, climate, and leaf age. Myco endophytes have yielded numerous valuable bioactive compounds that are antimicrobial, insecticidal, cytotoxic, and anticancer in the past two decades (Zhao *et al*. 2011). The present research work focuses on the endophytic mycoflora diversity of *T. bellirica* obtained from two different regions of Patna to characterize the new endophytic fungal isolates and to study their antifungal potential against some clinical pathogens. The study intended to explore the diversity of endophytic fungi and their characterization. It is hypothesised that exploring the diversity of such endophytes in a significant medicinal plant *T.bellirica* will further help utilise them for commercial exploitation. This study aims to isolate and characterize endophytic fungi from two distinct locations, perform molecular identification of the isolated fungi,analyze their diversity, and evaluate their antifungal activity against selected fungal pathogens.

## MATERIALS AND METHODS

### *Study plant and location*

The plant selected for the present study was *Terminalia bellirica* (Gaertn.)Roxb, is a member of the Combretaceae family, commonly known as Bahera. Two different sites were selected from the Patna region (Bihar) India for sample collection. Healthy and asymptomatic plant parts, namely leaves and living tissues of the bark of *T. bellirica* were collected from 2 locations as follows:

Location I- Rajkiya Ayurvedic College & Hospital, Patna-800003

25.608739° N, 85.149541° E

Location II- Sri Krishna Nagar Park No.1, Patna-800001

25.613886° N, 85.128487° E

### *Isolation of endophytic fungi*

Healthy, asymptomatic young and mature tissues of the leaf and bark of *T. bellirica* were collected for isolation and identification of their fungal endophytes. The leaves and living tissues of bark were used as a source of isolated endophytic mycoflora following the methods described by Myovela *et al.* (2024) with minor modification. The collected samples were first cleansed thoroughly under running tap water for about 30 mins to remove dirt and debris from the surface; followed by surface sterilization in laminar airflow. The samples were then submerged in 70% ethanol, treated for one min with a 5% sodium hypochlorite solution, and washed with sterile distilled water and finally dabbed with a sterile paper towel. 1-2 mm portion of the sterilized segmented plant was aseptically seeded on to Petri dishes containing potato dextrose (PDA) medium supplemented with chloramphenicol ( 100 μg/ mL ). The cultures were then observed for at least 7 to 10 days at 25°±2°C. Periodically, the Petri dishes were inspected to monitor the development of the endophytic fungal colonies. The endophytic fungal growth obtained from incubated leaf segments was sub-cultured onto new PDA media and continued sub culturing until a pure culture was achieved. Further, the study was conducted to explore the diversity and characterize the endophytic fungi of *T. bellirica* collected and isolated from the selected region. All experiments were performed in triplicate (Fig 1).

### *Test clinical phytopathogens*

The microorganisms used for the antifungal tests

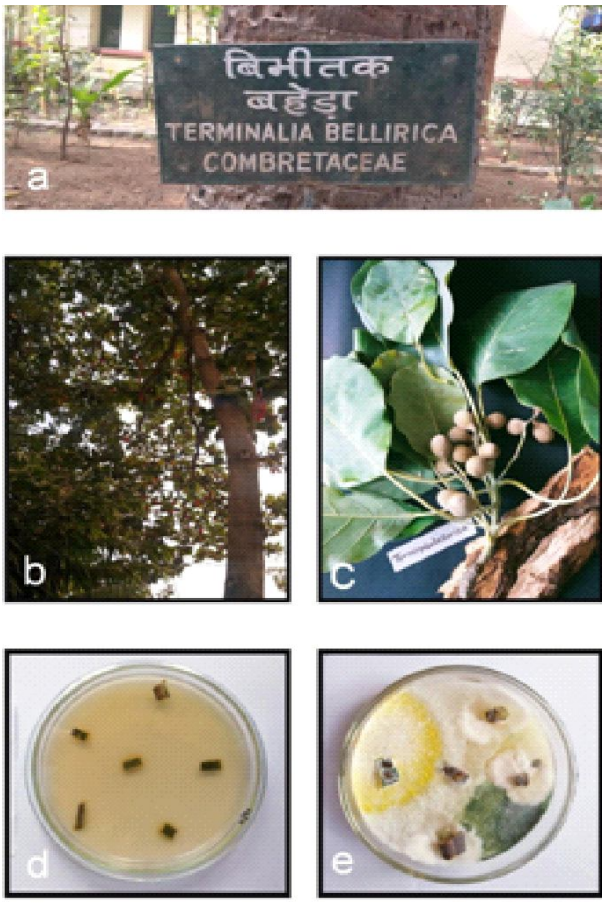



**Fig. 1:** a- Location I, b- Location II ; c- Morphology of the *T.bellirica* tree and d,e- colony morphology of its endophytic fungi

are fungi, clinically pathogenic to humans (*Candida albicans* - MTCC 183 and *Saccharomyces cerevisiae* - MTCC 174 ).

### *Identification of endophytic fungi*

Endophytic fungi were identified in the Plant Pathology & Microbiology Laboratory of the Department of Botany, Patna University, Patna, Bihar, India. The isolates were analyzed morphologically.

### *Morphological identification of endophytic fungi*

An investigation of the fungal morphology was made using both microscopic and macroscopic features. The fungal endophytes were identified by them orphology of the fungal colony media colour, colony and mycelium characters, spore specification, and fruiting structures (front & reverse). Microscopic slides were prepared and examined under a binocular compound microscope. Standard mycological manuals of Barnet and Hunter (1987) and others served as the foundation for both macroscopic and microscopic identification. Identifications were done upto the genus level and specific/ unique codes were assigned for identified fungal endophytes. Unidentified isolates were also allocated codes (UI).

### *Microscopy*

A tiny quantity of endophytic fungal mycelium was added to a drop of Lactophenol cotton blue (LPCB) mounting fluid using a flame-roasted and cooled needle. Every isolate was examined, to determine the mycelium, conidiophores branching, phialides, and conidia.These characteristics were photographed using a digital camera.

### *Molecular identification and phylogenetic analysis of endophytic fungal isolate*

The internal transcribed spacer (ITS) region was used to sequence endophytic fungal culture. Genomic DNA was extracted using the Hi PurA Fungal DNA purification spin column kit (MB543-250PR, Hi Media, India) and checked on 1% agarose gelel ectrophores is. PCR amplification of fungal- specific ITSgene (600bp) was carried out by using primers ITS1 (5'TCCGTAGGTGAACCTGCGG3') and ITS4 (5'TCCTCCGCTTATTGATATGC3')

(White *et al*. 1990). The PCR reaction was performed in a 25 ìL volume containing 10ìL Emerald Amp GT PCR Master Mix, 2x (Takara Bio USA),1 ìL DNA template (50– 100ng), 1.25 ìL (10 ìM) of each primer (forward and reverse) and 11.5ìL of free-nuclease water. PCR amplification was performed using Applied Biosystems Veriti Thermal Cycler as follows: denaturation at 95°C for 5 min followed by 35 cycles of 95°C for 30 s, 55°C for 30s, and 72°C for 1 min and a final cycle at 72°C for 7 min. PCR products were detected by staining with Gel Red Nucleic Acid Gel Stain on 1% agarose electrophoresis gel in (1X) TBE buffer and visualized under UV transilluminator (Protein Simple Red Imager SA-1000). The PCR

product was purified using Exonuclease I and Shrimp Alkaline Phosphatase Purification Kit (NewEngland Biolabs, Inc) and cycle sequenced using the Big DyeTerminatorv.3.1Cycle Sequencing Kit (Applied Biosystems, USA) with conditions as follows: denaturation at 96°C for 1 min followed by 25 cycles of 96°C for 10s, 50°C for 05s, and 60°C for 4 min. Cycle- sequenced amplicons were purified using the sodium acetate ethanol method (Thermo Fisher Scientific) and sequencing reactions were run on a 3500xL Genetic Analyzer (Applied Biosystems, USA). This molecular identification process of selected endophytic fungi was carried out at the CSIR-NCL, PUNE.

These were further analyzed by the Basic Local Alignment Search Tool (BLAST) with the closest culture sequence retrieved from the National Centre for Biotechnology Information (NCBI) database that finds regions of local similarity between sequences (Altschul *et al*. 1990). The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches (Gertz, 2005). The BLAST algorithms are used to infer functional and evolutionary relationships between sequences and help identify members of gene families. (i) Initial search to find potentially closely related type strain sequences using the BLASTN program (Altschul *et al.* 1990), (ii) Pairwise alignment to calculate the sequence similarity values between the query sequence and the sequences identified in step (i) (States *et al*.1991). Therefore, each isolate is reported with the first five-ten hits observed in the said database. Further multiple sequence alignment and phylogenetic analysis were examined using MEGA11 software (Kumar *et al.* 2018) for accurate species prediction and evolutionary relationship (Karlin *et al*. 1990; Myers *et al*. 1988).

### *Extraction of Metabolites*

Each endophytic fungi was picked from actively growing cultures and a 4-5 mm disk was placed into 100-ml potato dextrose broth medium (PDB) in 250 ml Erlenmeyer flasks and maintained at 28°±2°C for 21 days in a shaker incubator at 150 rpm.The cultures were filtered in aseptic condition after the incubation period, using Whatman no.1 filter paper to separate the mycelium.

### *Screening of endophytic fungi for antifungal activity*

An investigation was conducted to determine the antifungal activity of endophytic fungi against clinical pathogens. Antifungal activity was evaluated by the agar well diffusion method. Agar well diffusion assay of secondary metabolites extracts was done.100ìl fungal suspension was poured and spread onto the surface of the plate containing PDA growth medium. All the culture plates were allowed to dry for about 5 mins. Wells were bored in the centre of the agar surface using a cork borer and wells were filled with 100 ìl of endophytic fungal secondary metabolites respectively. For positive control, Ketoconazole, whereas 10% DMSO was used as a negative control. Finally, the inoculated plates were incubated for 24-48 hrs at 30°±2°C. The diameter of inhibition of the zone around the well was measured in mm.

### *Data analysis*

### Colonization frequency (CF)

The colonization frequency of endophytic fungi isolated from the *T. bellirica* trees from two different locations was calculated by Hata and Futai (1995); and Suryanarayana et. al (2003) and expressed in percentage.

$$CF\ \% = \frac{\text{Number of segments colonized by the fungi}}{\text{The total number of segments observed}} \times 100$$

### *Relative frequency (RF)*

To represent the fungal density RF was calculated by using a formula suggested by Huang et al. (2008).

$$RF\ \% = \frac{\text{Number of isolates of a species}}{\text{The total number of isolates}} \times 100$$

### *Species diversity*

Simpson's1949 assessed and quantified endophytic fungal diversity in host plants.

Simpson's index of diversity (D) was calculated using the following formula:1-D

$$D = \frac{\sum n(n-1)}{N(N-1)}$$

Where,
n =The total number of organisms of a particular species
n = The total number of organisms of all species

## RESULTS AND DISCUSSION

### *Identification, Taxonomic Analyses and Characterization*

The taxonomical analysis based on cultural and morphological features was successfully identified at the genus and some at thespecieslevel.Overall, 48 different species of endophytic fungi were isolated from parts of the *T.bellirica* plant from 2 locations in Patna, India. Out of the 48 different species of fungal isolates, 34 fungal colonies are explained in detail with their cultural and microscopic characterization mentioned in Table 1. Different genera and species successfully isolated from *T.bellirica* were- *Aspergillus flavus, A.niger, A.fumigatus, A.oryzae, A. candidus, A. nidulans, A. terreus, A. versicolor, Acremonium strictum, Alternaria* sp.,*Cladosporium* sp., *Curvularia* sp.,*Penicillium notatum, P.chrysogenum, Colletotrichum* sp., *Fusarium* sp., *Neuro spora crassa, N.sitophila, Trichoderma harzianum, T.viride, Phoma* sp., *Sterile mycelium, Bipolaris* sp., *Rhizoctonia* sp., *Mucor, Geotrichum candidum, Rhizopus,* and some unidentified colonies (UI).

In this study, endophytic mycoflora isolates from leaves and the inner tissue of bark were found to belong to phylum Ascomycota and Basidiomycota. Most of the taxa were Ascomycota, as reported in most previous endophytic studies (Crozier *et al*. 2006). Representative culture growths and spore morphology of the identified endophytic fungi are shown in the pictures given in Table 1.

### *Data analysis of endophytic fungal diversity*

To characterize the diversity of isolated endophytic fungi we calculated the colonization frequency, relative frequency, and Simpson's diversity of index. The colonization frequency of endophytic fungi was recorded higher in leaves as compared to bark. Whereas, 48 different species were identified and among them 21 species were isolated from location I & 27 from location II (Fig. 2). Relative frequencies was calculated and- 20.0% of isolates of genus *Aspergillus* showed its dominance in endophytic fungal diversity in both the locations followed by- *Penicillium* 11.2%, *Curvularia* 6.4%, *Alternaria* 4.8%, *Rhizopus* 5.6%, *Colletotrichum* 3.2%, and *Cladosporium* 2.4% (Fig 3). All the isolates were also calculated in terms of their order (Fig5). The Simpson's Diversity Index values were higher in leaves followed by bark of the plant .Loc1 (1-D=0.92) leaves and (1-D=0.94) bark, Loc2(1-D=0.95)leaves and(1-D=0.93) bark (Table 2). Conclusively, 4 *Fusarium* spp., 8 *Aspergillus* spp., 3 *Alternaria* spp., 4 *Penicillium* spp., 2 *Bipolaris* spp., 2 *Colletotrichum* spp, 2 *Neurospora* spp, 3 *Trichoderma* spp., one species of each of *Acremonium strictum, Cladosporium, Curvularia, Phoma sp, Sterile mycelium, Rhizoctonia, Mucor, Geotrichum candidum,* and *Rhizopus,* were successfully identified. Eleven fungal taxa were designated as unclassified fungi.

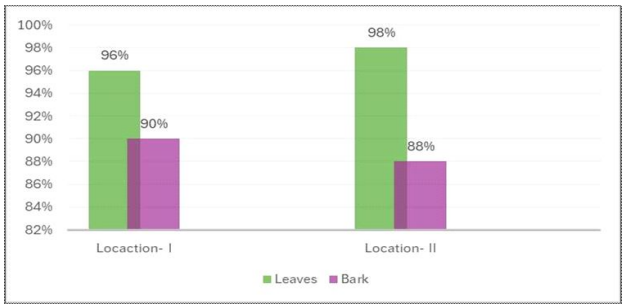



**Fig. 2:** Colonization frequency of endophytic fungi isolated from the *Terminalia bellirica*

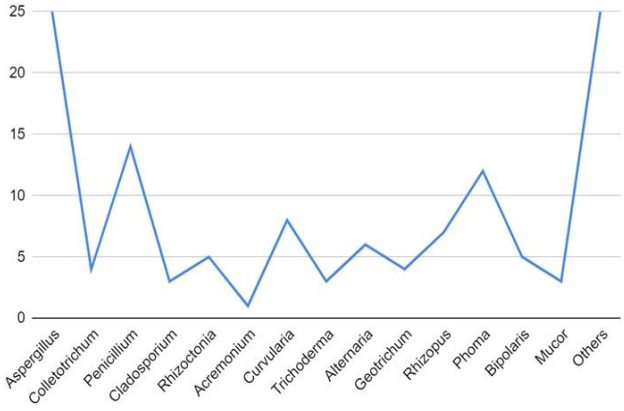
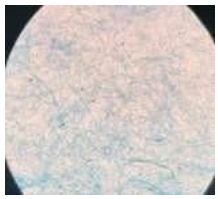
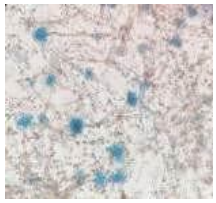
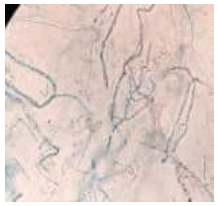
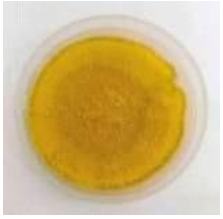
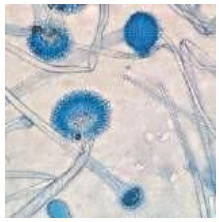
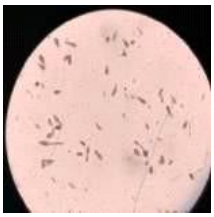
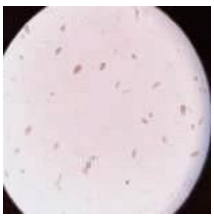
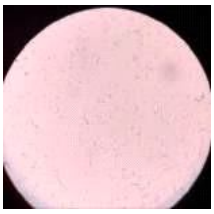
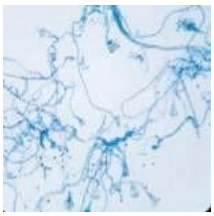
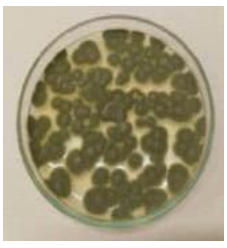
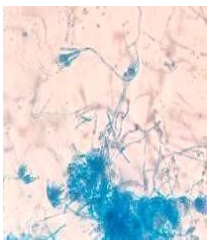


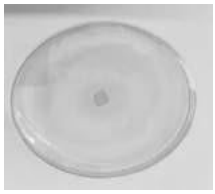
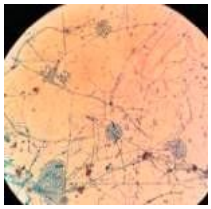

**Fig. 3:** Relative frequency of endophytic fungi isolated from the *Terminalia bellirica*

**Table 1:** Endophytic fungi isolated from leaves and bark tissues of *T.bellirica* with their front, reverse, microscopic images and characteristics

**A. Location I**

| Isolates code/name | Front view | Reverse view | Microscopic view | Characteristics |
|---|---|---|---|---|
| LEAF MATbL1 *Diaporthe pterocarpicola* |  |  |  | The Colonies were cottony white, and fast -growing, and gradually they formed a lacy structure. Mycelium was tangled, aerial, cottony, and non-septate. |
| LEAF MATbL2 *Rhizoctonia* sp. |  |  |  | The colonies were dark grey in colour with a cottony texture. Reverse it had black colour. Hyphae appeared hyaline & thick-walled with darkly pigmented. |
| LEAF MATbL3 *Aspergillus oryzae* |  |  |  | The colony was green and granular in colour & texture. Conidial heads were biseriate, septate metulae, and globose swelling. Reverse was also green in colour. |
| LEAF MATbL4 *Aspergillus terreus* |  |  |  | The colour of the colonies was light brown to sand -brown with yellow-brown in reverse. Conidial heads were compact, columnar, and also biseriate. Conidia was globose shaped. |
| LEAF MATbL5 UI |  |  |  | White colour circular colony with cottony texture whereas, the reverse was light peach colour pigmentations with a darker center. Hyphae may septate and are thread like structures that make up the mycelium. |
| LEAF MATbL6 *Aspergillus flavus* |  |  |  | The colonies were bright yellow and fast -growing. Granular, flat with radical grooves. The Heads of the conidia were radiate ,biseriate and some were uniseriate.Pale yellow colour in reverse. |

| | | | | |
|---|---|---|---|---|
| LEAF<br>MATbL7<br>UI |  |  |  | The grey colony with white colour margin. Reverse it had a dark grey colour. Conidiophores were dark brown elongated and simply single obpyriform conidia. |
| LEAF<br>MATbL8<br>UI |  |  |  | The colonies were grey cottony whereas in reverse it had black colour. Conidia were obclavate. Conidiophores were erect, un branched, and septate. |
| LEAF<br>MATbL9<br>*Colletotrichum* sp. |  |  |  | Colonies were slow-growing cottony white with an orange rust colour in the centre and reverse it appeared white to paleorange. Hyaline,septate mycelia; falcate as eptate conidia. |
| LEAF<br>MATbL10<br>*Penicillium* sp. |  |  |  | Colonies showed concentric ring-like growth in green colour whereas, pale brown from the reverse.It has septate hyphae and brush -like conidiophores, with chains of round conidia borne on phialides. |
| BARK<br>MATbB1<br>*Penicillium chrysogenum* |  |  |  | The colonies were in itially green in colour and gradually became dark green, textured was velvety and even. Reverse it had yellowish -brown pigmentations. Conidiophores were branched with round -shaped conidia in chains. |
| BARK<br>MATbB2<br>*Cladosporium* sp. |  |  |  | Greenish brown velvety nap colonies whereas the reverse side had the darkest green which gives a black colour in appearance, Hyphae & conidiophores are dark, separate, and branched. |

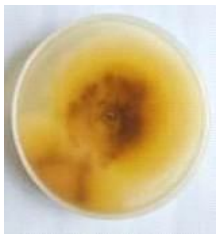
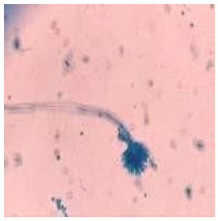
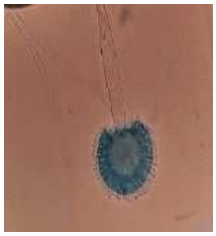
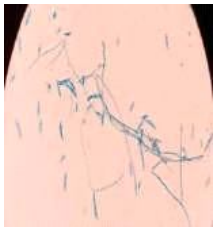
| | | | | |
|---|---|---|---|---|
| BARK<br>MATbB3<br>*Aspergillus* sp. |  |  |  | Granular colonies were brown in colour, with sulcation parallel grooves. The Reverse was light brown with a dark centre. Erect unbranched conidiophores. Phialides are long. Conidia ellipsoidal & hyaline, biseriate brown rough walled. |
| BARK<br>MATbB4<br>*Aspergillus niger* |  |  |  | The colonies were granular and black in colour. Conidial heads were biseriate with phialides which were brown. Conidia were globose shapes. |
| BARK<br>MATbB5<br>*Neurospora crassa* |  |  |  | Colonies were bright orange in colour and the reverse was orange pigmentation. Fast growing. The mycelial pigmentation of mutant strains buff, ropy, and conidial bands closely resembled the orange colour. |
| BARK<br>MATbB6<br>UI |  |  |  | Pure white colour furry velvety fast growing colony. The Reverse had a white colour. Mycelium was aerial, and non - septate. |
| BARK<br>MATbB7<br>*Acremonium* sp. |  |  |  | Colonies were cream to ivory in colour. The Reverse had peach colour pigmentation. Slow - growing, suede-like, or floccose with age. Hyphae are fine and hyaline with mostly simple awl - shaped erect phialides with inconspicuous collarettes. |

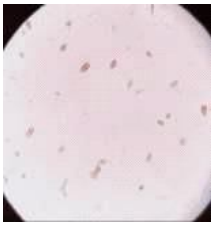
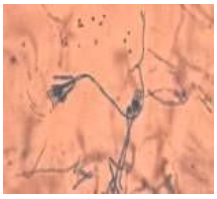
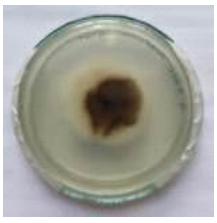
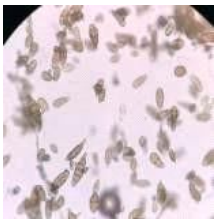
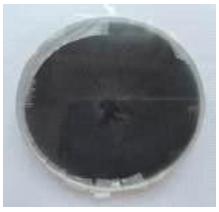
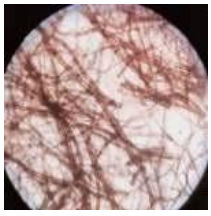
Among the fungal genera isolated, *Aspergillus* spp. was the most prevalent in both of the studied *Terminalia* species.According to Wang *et al*. (2018), *Aspergillus* strains exhibit significant chemical diversity, producing metabolites such as alkaloids, sterols, terpenoids, and butenolides. These compounds have demonstrated various bioactivities, including antibacterial, antifungal, anticancer, and cytotoxic effects. Additionally, Zhang *et al.(*2016) reported that an extract from the endophytic strain *A. carneus* inhibited the potato pathogen *Fusarium oxysporum*.

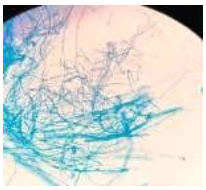
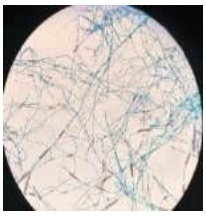
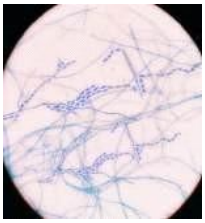
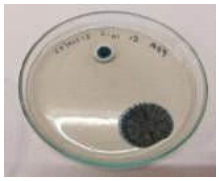
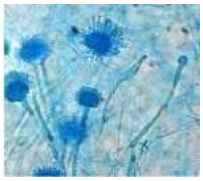
*Colletotrichum* spp.is commonly known as a plant pathogen ; however, it has also been found to produce secondary metabolites with diverse bioactivities. Notably, *Colletotrichum* spp. has been identified as a novel endophytic fungus capable of producing taxol,acompound with potentcy totoxic activity (Zhao *et al*. 2013).

The present study successfully isolated *Colletotrichum* from *Terminalia bellirica* at both study locations (I and II).

**Location II**

| Isolates code/name | Front view | Reverse view | Microscopic view | Characteristics |
|---|---|---|---|---|
| LEAF MKTbL1 *Colletotrichum* sp. |  |  |  | Colonies were fluffy white cottony at an early stage. The Reverse had pale yellow and brown colour pigmentation going on. Septate hyphae and curved crescent-shaped conidia are produced in acervuli. |
| LEAF MKTbL2 *Aspergillus flavus* |  |  |  | Colonies were yellow initially but quickly became yellow- green with ageing. Reverse it had a pale yellow colour. Granular, flat radial grooves. Conidiophores were hyaline and conidia were globose. Conidial heads were radiate, biseriate. |
| LEAF MKTbL3 *Aspergillus niger* |  |  |  | The colonies were black granular and fast - growing. Conidiophores simply upright arise from a septate mycelium and terminate in the globus head. Conidia were in chains developed at the end of sterigma. |
| LEAF MKTbL4 *Aspergillus candidus* |  |  |  | The colonies were white in colour with parallel grooves on the colony. The reverse colony had an ivory colour. Phialides are attached directly to the vesicles . Conidiophores are long, unbranched & hyaline. Fast-growing colony. |
| LEAF MKTbL5 UI |  |  |  | White pink cottony colonies with abrownish reverse centre. Septatehyphaes lightly curved conidia. Conidia are fusiform or cylindrical in shape. |
| LEAF MKTbL6 *Curvularia* sp. |  |  |  | Colonies were velvety brownish in colour where as the reverse was black with brown pigmentation on the sides. Multi -septate ,crescent shaped conidia with bent, swollen central cells. Hyphae septate and often pale brown. |

| | | | | |
|---|---|---|---|---|
| LEAF<br>MKTbL7<br>*Trichoderma viride* |  |  |  | Colonies were cottony white then changed to dark green colour with white margins. Reverse it was green. Branched conidiophores, septate, smooth mycelium, and conidia were colourless and globose. |
| LEAF<br>MKTbL8<br>UI |  |  |  | The Woolly colony surface with olive -green to greyish brown colour. Reverse was dark in colour. Hyphae are septate and dark, and Conidia arelargeandusuallycontain4 cells. Conidiophores were simple branched. |
| LEAF<br>MKTbL9<br>*Penicillium* sp. |  |  |  | The colonies were dark green in colour. The filaments (hyphae) are there along with some conidia(a sexual spores). Colourless, tapering, tubular branched septate hyphae |
| LEAF<br>MKTbL10<br>*Alternaria* sp. |  |  |  | The colonies are black to olivaceous-black or greyish and aresuede -like to floccose. Branchedacropetalchains and multicelled, obclavate to obpyriform conidia with short conical beaks. |
| BARK<br>MKTbB1<br>*Lasiodiplodia* sp. |  |  |  | Colonies were cottony velvet dark grey colour. Reverse it was black in colour. Hyphae are branched and hyaline. Conidia are produced in chains and are dark brown to black. The growth of the colony was fast and rapid. |
| BARK<br>MKTbB2<br>*Trichoderma harzianum* |  |  |  | Colonies were in the form of concentric rings in the centre with bright green colour and surrounded by light green & white fluffy culture. Reverse it was all white. The rapid growth of culture-branched conidiophores, slightly ovoid conidia, paired branches, and ampulli form phialides. |

| | | | | |
|---|---|---|---|---|
| BARK<br>MKTbB3<br>*Fusarium graminearum* |  |  |  | Circular shape growth of the colony with distinct margin. Red pigmentation with white cottony texture overlapping throughout. The reverse side shows a darker and more intense red color. Hyphae are septate. Conidia are present in clutter. |
| BARK<br>MKTbB4<br>UI |  |  |  | Colonies were initially white but with age, it turned into grey. Reverse it had a white and dark grey colour pigmentation. Mycelium was hyaline, septate hyphae with conidiophores and conidia. Long branched structures. |
| BARK<br>MKTbB5<br>*Aspergillus fumigatus* |  |  |  | The colonies showed green concentric ring -like growth both on the front and reverse sides with a white margin around the colony. Conidia had pigmented chains attached to the phialide with an obovoid vesicle. |
| BARK<br>MKTbB6<br>*Geotrichum candidum* |  |  |  | Colonies were flat white and the reverse was dull white & with no pigmentation. Fast-growing finely velvety to suede-like and floccose with no reverse pigment. Hyphae are hyaline, septate, and branched,<br>producing arthro conidia that are terminal, hyaline, and suborder to subglobose or cylindrical. |
| BARK<br>MKTbB7<br>*Aspergillus versicolor* |  |  |  | Colonies were usually velvety green, Biseriate phialides Loosely radiating conidial heads, typically blue-greenish spores. Reverse colonies were green-brown. |

### ***Molecular identification and phylogenetic analysis of endophytic fungal isolate***

The identification of endophytic fungal isolate was done by ITS. The phylogenetic tree was constructed using the neighbor-joining cluster method through MEGA11 software (Saitou *et al*.1987). A 1000 replicates were used to infer the bootstrap tree (Felsenstein 1985). The BLAST analysis showed a 100% similarity with *Lasiodiplodia mahajangana*, the sequence is available in the NCBI database. The assessed nucleotide sequence(s) NCBI genebank accession number is: PQ720995 (Fig .5).

>NR_147325.1*Lasiodiplodia mahajangana*

GGAAGGATCATTACCGAGTTTTCGAGCTTC
GGCTCGACTCTCCCACCCTTTGTGAA
CGTACCTCTGTTGCTTTGGCGGCTT
CGGCCGCCAAAGGACCTTCAAA CTCCAG
TCAGTAAACGCAGACGTCTGATAAACAAGTT
AATAAACTAAAACTTTCAACAACGGATCTCTT

**Table 2:** Diversity indices of endophytic mycoflora isolated from *Terminalia bellirica*

| *Terminalia bellirica* | LocI (Leaf) | LocI (Bark) | LocII (Leaf) | LocII (Bark) |
|---|---|---|---|---|
| Simpson'sIndex(D) | 0.08 | 0.06 | 0.05 | 0.07 |
| Simpson's Diversity Index(1-D) | 0.92 | 0.94 | 0.95 | 0.93 |

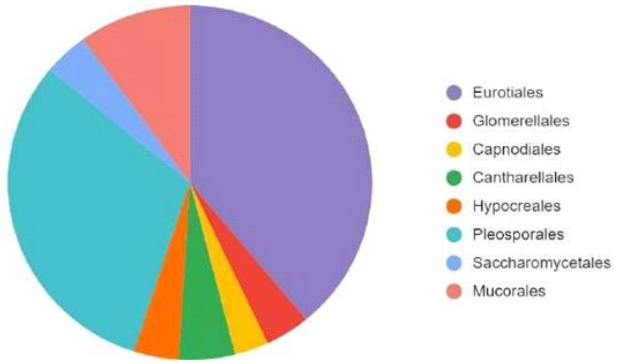



**Fig . 4:** Number of isolates in Order

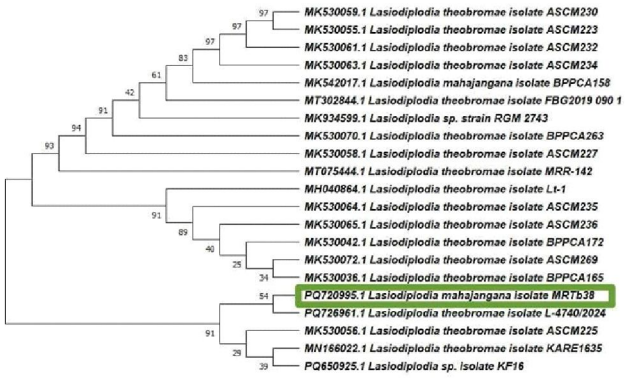



**Fig. 5:**Phylogenetic tree of endophytic fungal isolate MKTbB1(*Lasiodiplodia mahajangana*)

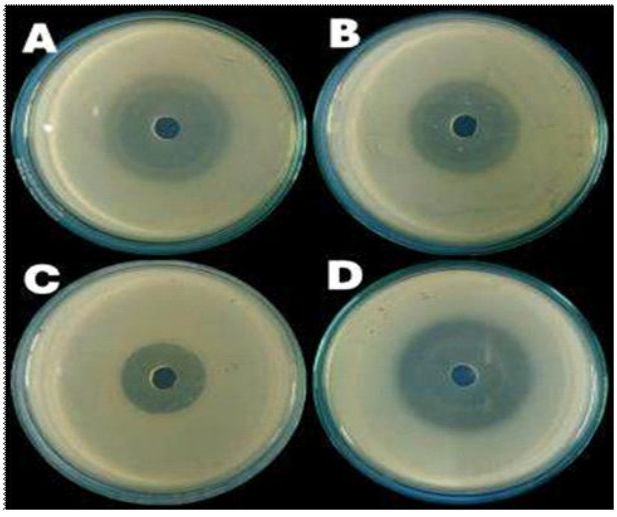



**Fig 6:** Zone of inhibition of some endophytic fungal extracts against *Candida albicans* (A),(C) *and* against *Saccharomyces cerevisiae* (B),(D)

GGTTC TGGCAT CGATGA AGAACG CAGCG AAATGCGATAAGTAATGT GAATT GCAGAATTCA G T G A A T C A T C G A A T C T T T G A A C G C A C A T T G C G C C C C T T G G T A T T C C G G G G G G C A T G C C T G T T C G A G C G T C A T T A C A A C C C T C A A G C T C T G C T T G G A A T T G G G C A C C G T C C T C A C T G C G G A C G C G C C T C A A A G A C C T C G G C G G T G G C T G T T C A G C C C T C A A G C G T A G T A G A A T A C A C C T C G C T T T G G A G C G G T T G G C G T C G C C C G C C G G A C G A A C C T T C T G A A C T T T T C

### *Screening of endophytic fungi for antifungal activities against some clinical pathogens*

The antifungal potential of all endophytic fungi isolated from *T.bellirica* was evaluated against two clinical pathogens *Candida albicans* (CA) and *Saccharomyces cerevisiae* (SC) on PDA media plates in triplicates. The results revealed a broad spectrum fanti fungal activity among the endophytic fungi ,with variability ineffectiveness. Out of the 22 isolates, several displayed significant antifungal activity against both the pathogens (Table 3). The clear zones of inhibition observed around the wells demonstrated the antifungal potency of the selected isolates (Fig. 6).

The endophytic *Alternaria* species, exhibited maximum inhibition zones of 29 mm and 32mm against CA and SC, respectively which was surpassing ketoconazole, showing inhibition zones of 28 mm and 23 mm against CA and SC, respectively. Several other isolates, including isolate numbers 22, 20, 8, 5, and 21, also demonstrated high antifungal activity, with inhibition zone diameters reaching 29 mm against CA. Similarly, significant antifungal activity was noted against SC, though some isolates like isolate 17 (*Phoma*) showed lower inhibition zones of 10mm and 12mm against CA and SC,respectively. Isolates 15 and 19, however, exhibited more notable inhibition zones of 14 mm and 13 mm against CA.

**Table 3:** Antifungal activity of the endophytic fungal secondary metabolites in agar well diffusion method

| Isolates codes | Endophytic fungi | Inhibition Diameter Zone(mm) | |
|---|---|---|---|
| | | *Candida albicans* | *Saccharomyces cerevisiae* |
| MKTbL10 | *Alternaria* sp. | 29 ± 0.2 | 32 ± 0.3 |
| MATbB7 | *Acremonium strictum* | 16 ± 0.2 | 18 ± 0.1 |
| MKTbL2 | *Aspergillus flavus* | 19 ± 0.4 | 23 ± 0.2 |
| MATbL4 | *Aspergillus terreus* | 20 ± 0.3 | 24 ± 0.1 |
| MKTbB5 | *Aspergillus fumigatus* | 21 ± 0.2 | 23 ± 0.1 |
| MATbB4 | *Aspergillus niger* | 19 ± 0.1 | 24 ± 0.1 |
| MATbL3 | *Aspergillus oryzae* | 18 ± 0.2 | 16 ± 0.1 |
| MATbB9 | *Aspergillus nidulans* | 22 ± 0.3 | 21 ± 0.2 |
| MKTbL4 | *Aspergillus candidus* | 16 ± 0.2 | 18 ± 0.4 |
| MATbB12 | *Bipolaris* sp. | 18 ± 0.2 | 19 ± 0.1 |
| MATbB2 | *Cladosporium* sp. | 19 ± 1.1 | 23 ± 0.1 |
| MATbL9 | *Colletotrichum* sp. | 18 ± 0.2 | 16 ± 0.5 |
| MKTbL6 | *Curvularia* sp. | 18 ± 0.2 | 20 ± 0.1 |
| MKTbB6 | *Geotrichum Candidum* | 15 ± 0.4 | 19 ± 0.2 |

| | | | |
|---|---|---|---|
| MATbB1 | *Penicillium chrysogenum* | 14 ± 0.2 | 23 ± 0.1 |
| MKTbL14 | *Penicillium notatum* | 18 ± 0.7 | 22 ± 0.1 |
| MKTbL16 | *Phoma* sp. | 10 ± 0.2 | 12 ± 1.0 |
| MKTbL20 | *Rhizopus* sp. | 19 ± 0.5 | 21 ± 0.1 |
| MATbL2 | *Rhizoctonia* sp. | 13 ± 0.1 | 19 ± 0.3 |
| MKTbB2 | *Trichoderma harzianum* | 24 ± 0.0 | 19 ± 1.2 |
| MKTbL7 | *Trichoderma viride* | 21 ± 0.1 | 20 ± 0.2 |
| MATbL1 | UI | 29 ± 0.4 | 21 ± 0.3 |
| Positive Control | Ketoconazole | 28 ± 0.2 | 23 ± 0.2 |
| NegativeControl | DMSO | 0 | 0 |

Overall, 11 out of the 22 isolates showed strong antifungal activity against CA, with the remaining isolates displaying moderate to low activity. The high antifungal activity of isolate FEB1 (*Rhizopus* sp.) against *Fusarium oxysporum* observed by Nuraihi et al. (2017) is consistent with the current findings. Previous studies have also highlighted the antifungal potential of *Aspergillus* species, including *Aspergillus capensis* CanS-34 A, isolated from *Brassica napus* (Qin *et al*.2019). Besides, pharmacological activity of endophytes have also been documented earlier ( Thangavel *et al*. 2022; Upadhyaya *et al*. 2014; Vini *et al*. 2017).In this study, *Aspergillus* spp. from *T.bellirica* ranked second among the most potent isolates (Table 3), show casing excellent antifungal potency.

## CONCLUSION

Endophytic fungi ,known for colonizing host plants asymptomatically ,are valuable sources of bioactive compounds. In this study, 48 endophytic fungi were successfully isolated from *Terminalia bellirica*, a medicinal plant with rich phytochemical diversity. These fungi, particularly from the genera *Aspergillus* and *Penicillium*, exhibited significant antifungal activity against clinical pathogens. Notably , leaf tissues hosted a greater diversity of endophytic fungi compared to bark tissues.The culture filtrate so fall isolates demonstrated abroad spectrum of antifungal potential, highlighting the role of endophytes as key producers of microbial metabolites—contributing to 45% of known bioactive compounds.The

discovery of bioactive compounds from these endophytic fungi opens new avenues for exploring their potential as natural antifungal agents, with promising applications in both pharmaceutical and agricultural industries. Given the increasing threat of drug-resistant pathogens,these endophytes offer anovel and sustainable source of antifungal compounds that could help combat infectious diseases. This study expands our understanding of the diversity of endophytic fungi in *T. bellirica* and underscores their untapped potential as producers of novel bioactive metabolites. Future research should focus on the isolation, purification, and characterization of these compounds to unlock their full therapeutic potential.

To the best of our knowledge, this is the first study investigating the endophytic fungi of *T. bellirica* in the Patna region, setting the stage for further exploration in this promising field.

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