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Molecular Identification and Antibacterial Activity of *Colletotrichum siamense* Isolated from *Hardwickia binata* Leaves

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Abstract: Plant-associated endophytes and epiphytes are a sustainable, eco-friendly reservoir of novel bioactive secondary metabolites, offering a viable alternative to the destructive harvesting of wild plant species. This study isolated fungal strains from the tissues of *Hardwickia binata* (Anjana tree), determined their taxonomic identity, and evaluated the antibacterial potential of their crude secondary-metabolite extracts. Morphological characterization, paired with rDNA-ITS sequence analysis and phylogenetic tree construction, confirmed the isolated fungal strain as *Colletotrichum siamense.* Antimicrobial assays against key human pathogens revealed variable antibacterial efficacy. Among the tested Gram-positive strains, *Staphylococcus aureus* exhibited the highest susceptibility, with a maximum zone of inhibition of 3.24 ± 1.06 mm, whereas *Bacillus subtilis* showed the least sensitivity (1.24 ± 0.42 mm). Against Gram-negative bacteria, *Escherichia coli* showed moderate sensitivity (2.48 ± 0.56 mm), followed by *Pseudomonas aeruginosa* (1.72 ± 0.16 mm). These findings highlight the potential of *Hardwickia binata*-derived *Colletotrichum siamense* as a valuable bioprospecting source for developing novel antibacterial agents. These findings provide an essential empirical baseline that directly informs future research into targeted epidemiological management, novel therapeutic interventions, and sustainable control protocols for this emerging biological threat.

I. INTRODUCTION

Plants naturally carry many harmless microscopic organisms. These include fungi that live on the surfaces of their leaves and stems (epiphytes) and those that live within their healthy tissues (endophytes) without causing disease (World Health Organization, 2021). Plants naturally carry many harmless microscopic organisms. These include fungi that live on the surfaces of their leaves and stems (epiphytes) and those that live within their healthy tissues (endophytes) without causing disease (Jia et al., 2016). These fungi and their host plants have grown together over time, helping each other out through chemical signals. Because of this close relationship, fungi taken from medicinal or special plants can produce powerful natural compounds—such as alkaloids, terpenoids, and flavonoids. These compounds can be used to make medicines, especially to fight harmful bacteria and fungi. (Strobel, 2018; Manganyi & Ateba, 2020). Studying fungi isolated from plants is a smart and eco-friendly way to discover new drug compounds without harming wild plants or overharvesting them. Identifying fungi by appearance alone is often unreliable because their features vary with the environment. Instead, sequencing target genes such as the ITS region or -tubulin has become the standard method for precise, species-level identification (Schoch et al., 2012; Raja et al., 2017). Systematically isolating fungi from target plant tissues, identifying them through precise DNA sequencing, and screening their crude metabolic extracts against clinically relevant bacterial strains are essential steps toward identifying promising natural antibacterial sources.

In the present study, we selected leaves of the *Hardwickia binata* (Anjana) plant from Pal, Dist. Jalgaon, Maharashtra, and screened them for endophytic fungi. This plant is found in the Satpura of India; the Satpura is a 900-kilometer-long range of hills in central India that runs east-to-west across Gujarat, Maharashtra, Madhya Pradesh, and Chhattisgarh. *Hardwickia binata* is important for nutritious animal fodder, strong natural fibers, and traditional medicinal treatments, and plays an important role in protecting the biodiversity of this region (ICRA 2013 and Seetharaam and Haleshi, 1998).

This study aims to:

1. Isolate fungal strains associated with the tissues of *Hardwickia binata* (Anjana tree).
2. Determine their molecular identities through rDNA-ITS sequence analysis and phylogenetic tree construction.
3. Evaluate the antibacterial activity of their crude secondary-metabolite extracts against key Gram-positive and Gram-negative bacterial pathogens.

II. Materials and Methods

1. Collection of Plant Material

The present study was conducted at the Postgraduate Laboratory, Department of Botany, at Maratha Vidya Prasarak Samaj's Nutan Maratha College, Jalgaon, Jalgaon District, Maharashtra, India. Diseased leaves were collected in September 2025 from the Pal Satpura mountain range in Jalgaon District, India. Dense forests surround Pal and are part of the Yawal Wildlife Sanctuary, which is home to diverse flora and fauna. The sanctuary provides a safe habitat for a variety of wildlife species. The entire planting area was about 176 square kilometers, and samples were taken from within 15 square kilometres. We collected symptomatic leaves of *Hardwickia binata* (Indian name "Anjana").

2. Isolation of Endophytic Fungi

Leaf samples were surface-sterilized by sequential immersion in 70% ethanol for 30 seconds, 1% sodium hypochlorite (NaCl) for 3 minutes, and sterile distilled water three times. Small segments (approximately 5 mm × 5 mm) from the lesion margins were aseptically excised from the sterilized leaves with a sterile scalpel.

Potato Dextrose Agar (PDA) was prepared according to standard protocols, with 200 g of potato infusion, 20 g of dextrose, and 15 g of agar per liter of distilled water. The medium was sterilized by autoclaving at 121°C and 15 psi for 15 minutes. PDA plates were allowed to cool to room temperature before inoculation. Following the methodology documented in previous isolation work (as referenced in Isolation of Colletrichum siamense fungi from leaf of terrestrial plant), we used PDA as the primary culture medium because it effectively supports fungal growth. Sterilized leaf segments (3-5 per plate) were placed at the centre of PDA plates and incubated at 25 ± 2°C under a 12-hour light/dark photoperiod for 7 days. Daily observations were recorded to monitor fungal growth and colony morphology (Bhalerao V A and Chavan A M , 2020)

3. Morphological And Molecular Identification

Fungi were isolated from a *Hardwickia binata*-infected leaf sample. Preliminary identification was based on colony characteristics and morphological features observed under a microscope. Identification and confirmation were made by preparing slides of fungal cultures and observing them under a compound microscope. Fungi were identified using authentic literature, manuals, and books (Barnett, 1998; Ellis, 1993; Gilman, 2001; Raper and Fennel, 1965; Thom and Raper, 1941; Subramanian, 1971). Pure cultures of these fungi were prepared and maintained on potato dextrose agar (PDA) slants and used for further studies. Morphological methods using differential media are among the most reliable and sensitive assays for identifying fungal species; microscopy and culture remain essential tools for identification.

4. DNA Extraction And Sequencing

The researcher provided the fungal culture samples. DNA was extracted using the ProGenome Life Science Fungal DNA Extraction Kit (In-House Kit) and quality-checked by 1% agarose gel electrophoresis. The gel was visualized using a Gel Documentation System - UV Transilluminator (Himedia). The ITS gene was amplified using the ITS1 and ITS4 primers. A single discrete PCR amplicon band was observed when resolved on a 1.3% agarose gel. The PCR amplicon was purified to remove contaminants. Forward and reverse DNA sequencing reactions of the PCR amplicon were carried out with the forward and reverse primers using the BDT v3.1 Cycle sequencing kit on an ABI 3730xl Genetic Analyzer. The ITS gene sequence was used to perform BLAST against the NCBI GenBank database. Based on the maximum identity score and alignments generated with the multiple alignment software Clustal W, a distance matrix was generated, and the phylogenetic tree was constructed using MEGA 11.

5. Species Confirmation Using ITS Marker

The ITS region was amplified using Hi-PCR® REDy Master Mix. Amplified PCR products were visualized on a 1.3% agarose gel.

Table 1. Details of ITS polymerase chain reaction composition

Component	Component Volume
REDy MasterMix	12.5 µL
ITS1 primer	1.5 µL
ITS4 Primer	1.5 µL
Template DNA	3.0 µL
Nuclease-Free Water	6.5 µL
Total reaction volume	25 µL

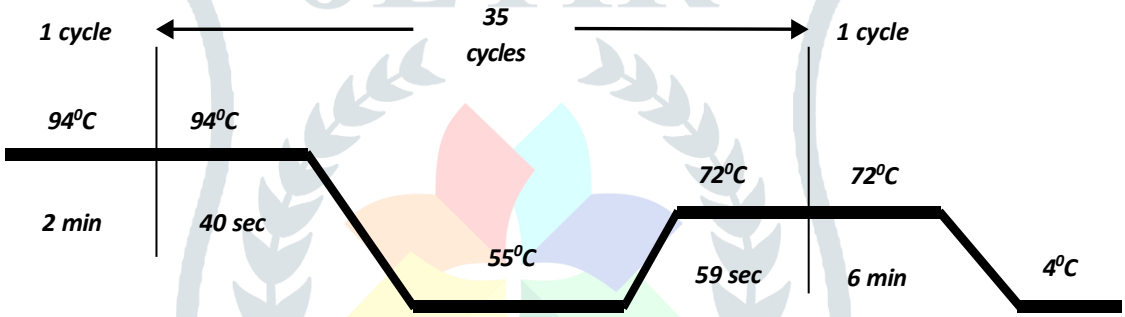

Figure 1. ITS thermal cycling conditions used for amplification

6. Sequencing

PCR products were cleaned up to remove unincorporated nucleotides and residual primers using Exonuclease I and Shrimp Alkaline Phosphatase (1 unit/µL), followed by a cycle sequencing reaction using the BigDye® Terminator v.3.1 Cycle Sequencing Kit (Applied Biosystems, Inc.). For ITS amplicon sequencing, the same PCR primers were used. Thermal cycler conditions included an initial denaturation of 2 min at 96°C, followed by 35 cycles of 30 sec at 96°C, 15 sec at 55°C, and 4 min at 60°C. Cycle sequencing was followed by ethanol precipitation cleanup, then the template was dissolved in HiDi formamide and bidirectionally sequenced on an ABI 3730 Genetic Analyzer.

7. Antibacterial Assay

The antibacterial activity of the fungal extract was evaluated using the standard agar well diffusion method. Clinically pure isolates of eight major pathogens, including *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*, were used for analysis. Nutrient agar was prepared using Beef extract-3gm, Glucose-2.5gm, Peptone-5gm, Agar-15gm, distilled water-1000 mL, and adjusted to pH 6.8. A 0.9% NaCl solution was used to prepare the bacterial suspension. The agar well diffusion method was used to assess antibacterial activity. After inoculating the media, the Petri plates were left to dry for 15 minutes under controlled aseptic conditions, and wells were punched with sterile cork borers. The wells were then filled with 10 µL of Colletrichum siamense extract. The prepared Petri plates were incubated at 37 °C for about 24 hours. The diameter of the clear inhibition zone around each well was measured using a ruler. All antibacterial assays were performed in triplicate, and results were expressed as mean ± SD. Statistical analysis was carried out using an unpaired two-tailed Student's t-test, with $P < 0.05$ considered statistically significant. (Atikya Farjana et al., 2014).

III. Results And Discussion

Isolation of Endophytic Fungi from *Hardwickia binata* Plant

Infected leaf samples were collected from *Hardwickia binata* plants and subjected to surface sterilization and culturing on Potato Dextrose Agar (PDA). Fungal growth was observed on the samples (80% colonization frequency), indicating a high prevalence of endophytic fungi within *Hardwickia binata* tissues. The affected area measures 1 mm to 5 mm and is brown in color. Multiple fungal colonies with varied morphologies were isolated and cultured, yielding 3 fungal isolates from plant tissue.

Most isolates were identified as *Colletotrichum* species. Therefore, we selected *Colletotrichum* species for further molecular studies. The cultures were evaluated for colony morphology, and spores and mycelia were examined microscopically (Figure 2). Based on morphological and microscopic features, the culture (S4A) was identified as *Colletotrichum siamense*.

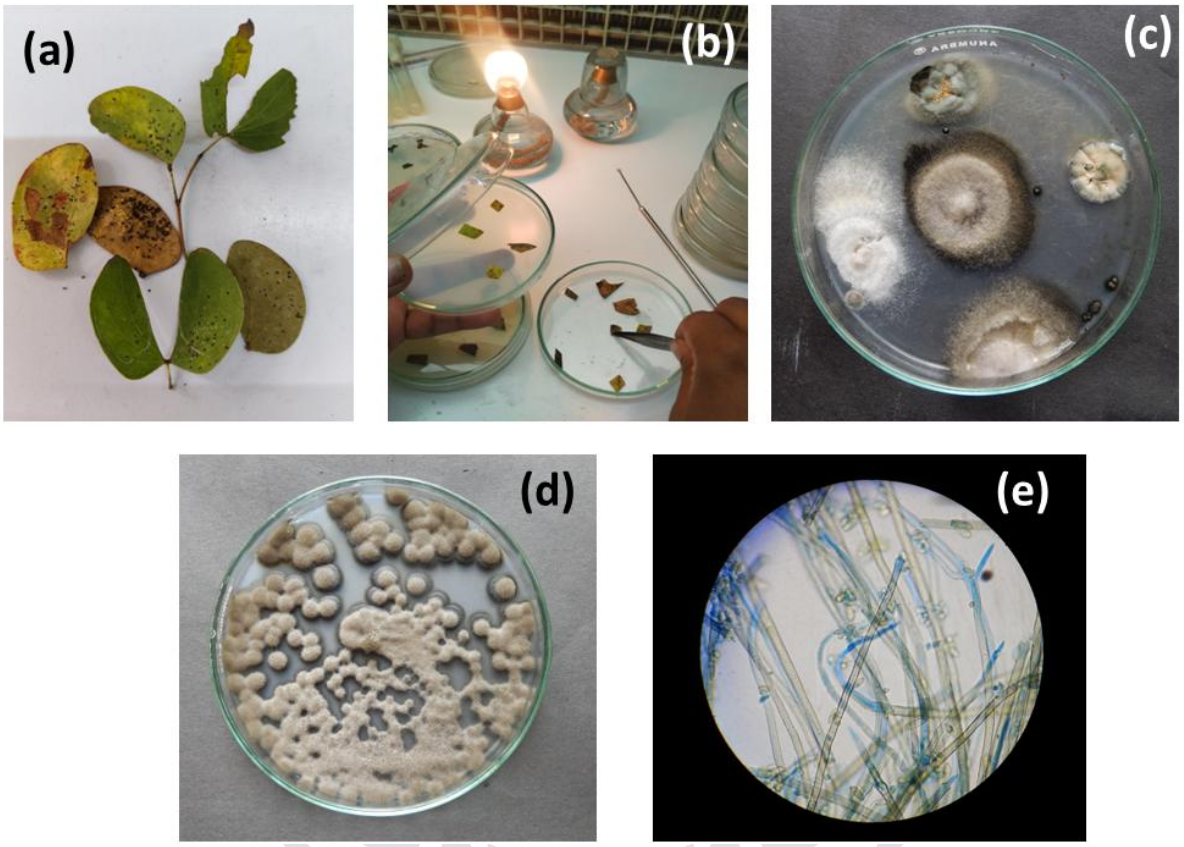

Figure 2. Morphological characteristics of endophytic fungi isolated from *Hardwickia binata*. (a) Infected leaf sample of *Hardwickia binata;* (b) Leaf sample used for fungal isolation; (c) Different mycoflora; (d) Colony morphology of *Colletrichum siamense* (S4A) on culture medium; (e) Microscopic view of conidia of *Colletrichum siamense*

Morphological Characterization and Molecular Identification

ITS Sequencing Results

Isolates from the S4A strain group were selected for molecular identification. The sequenced ITS region contained the 5.8S ribosomal RNA gene. DNA extraction from pure cultures yielded high-quality genomic DNA with 260/280 nm absorbance ratios of 1.85-1.95. PCR amplification of the ITS region with universal primers produced amplicons of approximately 620-650 bp. Bidirectional sequencing of the purified PCR products was successful for the isolates.

BLAST Analysis

BLAST analysis of the obtained ITS sequences against the GenBank nucleotide database revealed 99-100% sequence identity to previously published Colletrotrichum siamense sequences. Comparison with reference strains showed the highest similarity to C. siamense, accession number PZ112912, for S4A (Table 2). The isolates from plant leaves displayed identical or nearly identical ITS sequences, confirming their assignment to the species Colletrotrichum siamense. Table 3 three shows brief Sample Information Sample ID and Accession Number from NCBI.

Table 2. Sample IDs showing Similarity Searches in Sequence Alignment and Phylogenetic Analysis

Sample ID	Description	Max Score	Total Score	Query Cover	E-value	Per Ident
PZ112912 (S4A)	*Colletotrichum siamense* isolate YH 2-3 internal transcribed spacer 1, partial sequence	791	791	100%	0.0	99.54%

Table 3. Sample Information Sample ID and Accession Number by NCBI:

Sr. No.	Sample ID	Name	Accession Number
1	S4A	*Colletrichum siamense*	PZ112912

Sequence Alignment and Assembly

PCR products were then processed for direct bidirectional sequencing on an ABI PRISM 3730 × 1 Genetic Analyzer (Applied Biosystems, USA). The resulting DNA sequences were aligned using CLUSTALW in MEGA 11 (Tamura, Stecher, and Kumar 2021), then manually trimmed and edited to obtain complete sequences. Species confirmation was based on sequence similarity scores. Homology searches were conducted using the BLASTn program against the NCBI GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi). An NJ tree was constructed in MEGA 11, and all positions with gaps or missing data were included in the analysis. Clade support was calculated from 1,000 bootstrap replicates.

Antimicrobial Activity of Fungal Endophytes:

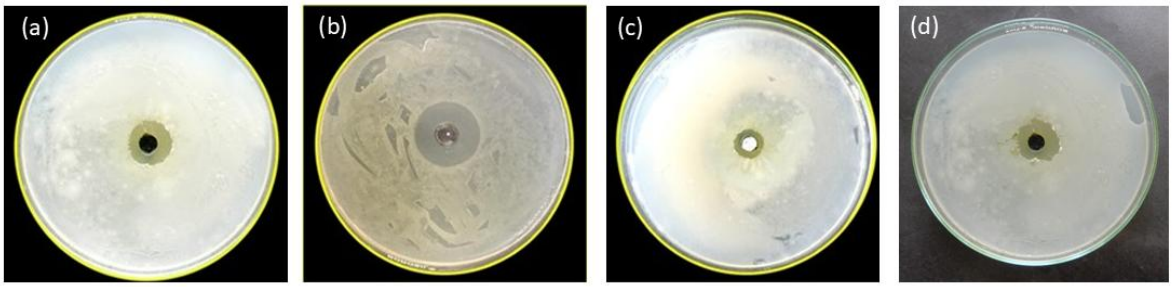

Figure 3. Antibacterial activity of the fungus *Colletotrichum siamense* isolated from *Hardwickia binata*, assessed by dual-culture inhibition zone diameter (mm) for (a) *Escherichia coli*, (b) *Staphylococcus aureus*, (c) *Pseudomonas aeruginosa*, and (d) *Bacillus subtilis*

The antibacterial activity of the endophytic fungal isolate *Colletotrichum siamense* (S4A). The dual-culture assay revealed that the endophytic fungus *Colletotrichum siamense* (strain accession number: PZ112912) (Table 3), isolated from *Hardwickia binata*, exhibits differential antibacterial activity against both Gram-positive and Gram-negative bacterial pathogens. Broad-spectrum antibacterial efficacy was evaluated by measuring the zone of inhibition (mm) against four reference bacterial strains: *Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, and Bacillus subtilis*. The fungal isolate demonstrated varying levels of antibacterial potency across the tested pathogens. The highest susceptibility was observed in the Gram-positive pathogen *Staphylococcus* aureus, which exhibited the largest clear zone of inhibition at 3.24 ± 1.06 mm.

Among the Gram-negative test organisms, *Escherichia coli* displayed moderate sensitivity to the fungal secondary metabolites, with an inhibition zone of 2.48 ± 0.56 mm, followed by *Pseudomonas aeruginosa* at 1.72 ± 0.16 mm. Conversely, *Bacillus subtilis* was the least susceptible bacterium, yielding the smallest recorded inhibition zone of 1.24 ± 0.42 mm (Table 4) and shown in Figure 3. Our results, when compared with the literature, show good agreement with each other (Kundekar et al. 2026; Li et al. 2023; and Ruth et al. 2020)

Table 4: Antibacterial activity of the fungus Colletrichum siamense isolated from *Hardwickia binata*, assessed by a dual culture assay

Isolated Fungi	**Inhibition Diameter Zones (Mm)**			
	Escherichia coli	***Staphylococcus aureus***	***Pseudomonas aeruginosa***	***Bacillus subtilis***
***Colletotrichum siamense* (PZ112912)**	**2.48 ± 0.56mm**	**3.24±1.06 mm**	**1.72±0.16 mm**	**1.24±0.42 mm**

*Values are expressed as Mean ± Standard Deviation (SD) of triplicate determinations.

IV. Conclusion:

Colletotrichum siamense can be successfully isolated from the foliage of *Hardwickia binata*. Our findings illustrate that endophytic fungal communities play a comprehensive role in ecosystem functioning by simultaneously governing host plant growth dynamics and potent antimicrobial properties. The distinct biological activities demonstrated by *Colletotrichum siamense* underscore the vast, largely unexplored reservoir of functional secondary metabolites within the endophytic microflora of native plants such as *Hardwickia binata*. These findings establish a critical empirical baseline that will inform subsequent research into targeted epidemiological management, therapeutic interventions, and sustainable control protocols for this emerging biological threat.

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