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Characterization and Screening of Antagonistic Activity of Endophytic Fungi Isolated from *Muntingia calabura*

Rakshitha Simha [1], Seema J. Patel [1]*, Shruthi Shirur Dakappa [2] and Koushik Bandlu Raghuram [1]

[1] *Department of Studies in Biotechnology, Davangere University, Davangere 577002, India*
[2] *Bioedge Solutions, Bangalore 560058, India*
*(e-mail: ssjp176@gmail.com; Mobile: +91-9448223566)

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ABSTRACT

Endophytic fungi reside within plant tissues and contribute to plant health by producing bioactive secondary metabolites. This study focuses on the isolation, characterization, and antagonistic activity of endophytic fungi from *Muntingia calabura*. Healthy leaf samples were collected from Bangalore, India, and processed under sterile conditions. The fungi were isolated using Potato Dextrose Agar (PDA) and characterized based on macroscopic, microscopic, and molecular techniques using ITS sequencing. Molecular analysis confirmed the isolates as *Aspergillus niger* and *Fusarium thapsinum*. Antibacterial activity was evaluated using the disk diffusion method against Staphylococcus aureus and *Escherichia coli*. The results showed significant inhibition zones, indicating strong antimicrobial activity. This study highlights the potential of endophytic fungi as sources of natural antimicrobial agents for biocontrol and pharmaceutical applications.

Key words: endophytic fungi, *Muntingia calabura*, ITS sequencing, antibacterial activity, *Aspergillus niger*

INTRODUCTION

Endophytic fungi are microorganisms that inhabit the internal tissues of plants without causing apparent harm, often establishing mutualistic relationships with their hosts and providing physiological benefits such as enhanced stress tolerance and disease resistance (Silva et al., 2022; Wen et al., 2022). These fungi are recognized for their ability to produce a wide array of bioactive secondary metabolites, including compounds with antimicrobial, antiviral, antioxidant, and anti-inflammatory activities (Jia et al., 2016; Poveda et al., 2022; Jha et al., 2023; Bhagat et al., 2012). The metabolic versatility of endophytic fungi has attracted interest for applications in agriculture, medicine, and biotechnology, especially as they can synthesize pharmacologically relevant compounds, sometimes mirroring those found in their host plants (Jia et al., 2016; Rajamanikyam et al., 2017).

Muntingia calabura, commonly known as the Jamaican cherry, is a tropical plant valued for its medicinal properties, including traditional uses in treating bacterial infections, inflammation, and digestive disorders (Nirmala et al., 2018). While phytochemical studies have demonstrated the antimicrobial activity of *M. calabura* extracts against pathogens such as *Staphylococcus aureus* and *Escherichia coli*, the diversity and bioactivity of its endophytic fungi remain underexplored, especially in the Indian subcontinent (Toppo et al., 2024).

Recent research has shown that endophytic fungi isolated from *M. calabura* can synthesize secondary metabolites with potent antifungal activity. For example, certain isolates have exhibited significant inhibition zones against *Candida parapsilosis*, attributed to terpenoid and phenolic compounds (Bimantara et al., 2022). These findings highlight the potential of *M. calabura* endophytes as sources of eco-friendly antimicrobial agents.

Despite these promising reports, knowledge gaps remain regarding the identity, diversity, and antagonistic mechanisms of endophytic fungi from *M. calabura*. Addressing these gaps is crucial for harnessing their potential in pharmaceutical and agricultural applications.

MATERIALS AND METHODS

Sample Collection: Healthy leaves of *Muntingia calabura* were collected from five mature plants located in the Peenya region, Bangalore, Karnataka. Ten leaves per plant (total n = 50) were randomly selected, ensuring they were free from visible signs of disease or damage. The samples were placed in sterile bags and immediately transported to the laboratory for further analysis.

Isolation of Endophytic Fungi: Leaves were washed under running tap water and surface

sterilized by immersion in 70% ethanol for 1 min, followed by 4% sodium hypochlorite for 5 min, and rinsed three times in sterile distilled water. To confirm surface sterilization, 100 µL of the final rinse water was plated on Potato Dextrose Agar (PDA); absence of growth after 48 h indicated successful sterilization. Sterilized leaves were aseptically cut into ~0.5 cm^2 segments and placed on PDA plates supplemented with 50 mg/L streptomycin to inhibit bacterial growth. For each leaf, four segments were plated (total n = 200 segments). Plates were incubated at 28 °C for 5–7 days. Emerging fungal colonies were subcultured onto fresh PDA to obtain pure isolates. All experiments were performed in triplicate.

Characterization of Endophytic Fungi: Isolates were characterized morphologically by colony color, texture, margin, elevation, and growth rate on PDA. Microscopic features were observed after lactophenol cotton blue staining, including spore shape, size, septation, and hyphal branching. Identification was performed independently by two mycologists.

Molecular Identification: Genomic DNA was extracted from fresh mycelia using the CTAB method. The Internal Transcribed Spacer (ITS) region was amplified using universal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). PCR was performed in 25 µL reactions: 1 µL DNA template, 1 µL each primer (10 µM), 12.5 µL 2X PCR Master Mix, and nuclease-free water. Cycling conditions: 95 °C for 5 min; 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min; final extension at 72 °C for 10 min. PCR products were purified and sequenced (Sanger method). Sequences were compared to NCBI GenBank using BLAST; ≥98% identity was considered species-level identification.

Extraction of Secondary Metabolites: Pure isolates were inoculated into 100 mL Potato Dextrose Broth (PDB) in 250 mL Erlenmeyer flasks and incubated at 28 °C for 14 days under static conditions. Cultures were filtered to separate mycelia, and the broth was extracted three times with equal volumes of ethyl acetate. Combined organic phases were evaporated under reduced pressure at 40 °C. The crude extract was dissolved in DMSO (100 mg/mL) and stored at −20 °C.

Antagonistic activity Assay: Antimicrobial activity was evaluated using the agar well diffusion method. Test organisms included *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), and *Candida albicans* (ATCC 10231). Bacterial suspensions were adjusted to 0.5 McFarland standard and swabbed onto Mueller-Hinton Agar; *C. albicans* was swabbed onto Sabouraud Dextrose Agar. Wells (6 mm) were filled with 50 µL extract (100 mg/mL in DMSO). Positive controls: ampicillin (10 µg/mL) for bacteria, fluconazole (10 µg/mL) for *C. albicans*. Negative control: 50 µL DMSO. Plates were incubated at 37 °C for 24h (bacteria) or 28 °C for 48 h (fungus). Zones of inhibition were measured in mm. All assays were performed in triplicate.

Statistical Analysis: All data are presented as mean ± standard deviation (SD) from three independent experiments. Statistical significance between groups was assessed by one-way ANOVA followed by Tukey's post hoc test, with $p < 0.05$ considered significant.

RESULTS AND DISCUSSION

Isolation and Identification of Endophytic Fungi

Three distinct endophytic fungal isolates were successfully obtained from healthy leaves of *Muntingia calabura* and designated as MCEF-G, MCEF-R, and MCEF-B (Figure 1)). Macroscopic examination revealed clear differences in colony morphology among the isolates (Table 1): MCEF-G formed green, velvety colonies with prominent radial grooves; MCEF-R exhibited a whitish-brown colony with concentric rings; and MCEF-B produced a dark, fluffy colony with irregular margins. Such morphological diversity is consistent with previous reports of endophytic fungi from medicinal plants, where colony color, texture, and growth pattern serve as preliminary indicators of taxonomic diversity. For precise identification, molecular characterization was performed by amplifying and sequencing the ITS region of rDNA from each isolate. BLAST analysis of the ITS sequences revealed high similarity (≥98%) to reference strains in the NCBI database, confirming the identities as *Aspergillus niger* (MCEF-G) and *Fusarium thapsinum* (MCEF-R and MCEF-B). This approach aligns with established methodologies for endophytic fungal identification, where molecular data complement morphological observations to achieve accurate species-level resolution (Jia et al., 2016) (Table 2).

These results align with previous studies, where *Aspergillus* and *Fusarium* species have been frequently isolated as endophytes from medicinal plants and recognized for their bioactive secondary metabolites (Bhagat et al., 2012). The co-existence of these fungi within the same host plant indicates a potential symbiotic relationship that could enhance plant defense mechanisms and produce antimicrobial compounds.

(a) (b) (c)

Fig. 1. Macroscopic observation of isolated fungi. (**a**) MCEF-G (**b**) MCEF-R (**c**) MCEF-B.

Table 1. Macroscopic characterization of different types of fungus.

Isolates	Texture	Surface colour	Reverse colour	Zonation	Spores
MCEF-G	COTTON	GREEN	Dark green	CIRCULAR	Present
MCEF-R	COTTON	BROWN	Brown spherical with black shaded	CIRCULAR	Present
MCEF-B	COTTON	BLACK	Black spherical with brown shaded	CIRCULAR	Present

Microscopic analysis further differentiated the isolates, with each showing unique spore and hyphal structures under lactophenol cotton blue staining. These morphological features provided an initial basis for genus-level identification, as similarly employed in studies of endophytes from other medicinal plants (Figure 2).

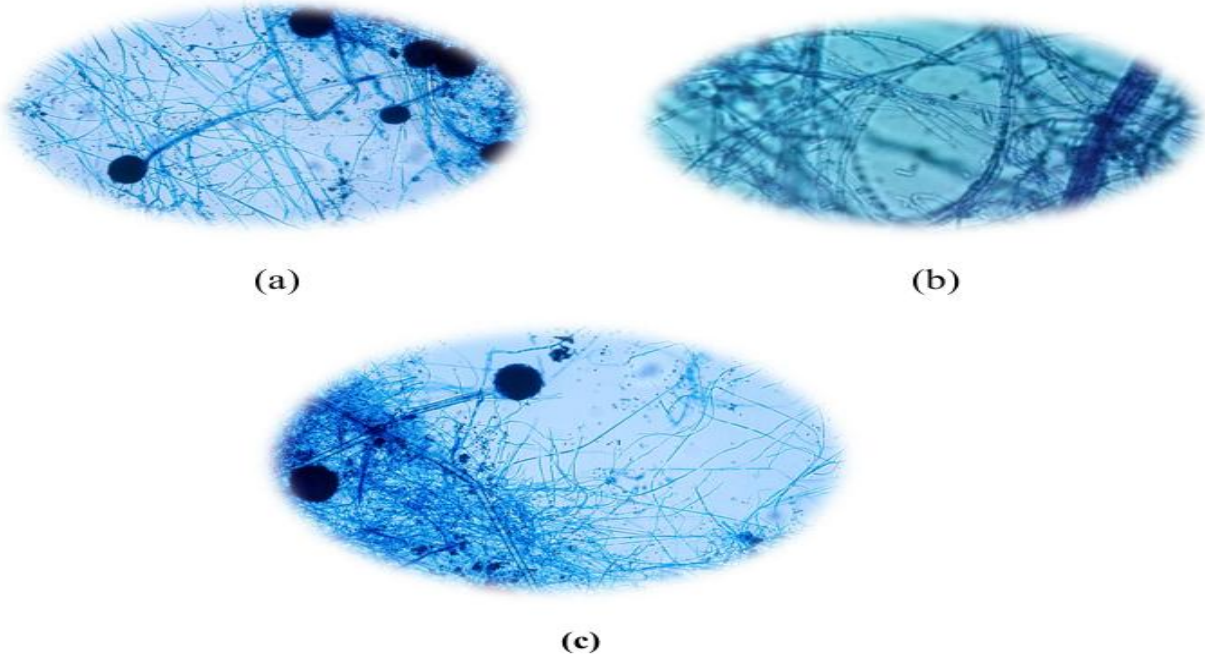

Fig. 2. Microscopic images of isolated fungi. (**a**) MCEF-G (**b**) MCER-R (**C**) MCEF-B.

Table 2. Data analysis of obtained FASTA sequences with the NCBI.

SL No.	Code	HIT	Percentage	Query	E-value	Accession No.
1	MCEF-G-ITS	*Aspergillus niger* isolate IDSGBT-01-22 small subunit ribosomal RNA gene.	100%	100%	0.0	OP001987.1
2	MCEF-R-ITS	*Fusarium thapsinum* isolate IDSG-BT03-22 internal transcribed spacer 1.	96.84%	100%	0.0	OP002008.1
3	MCEF-B-ITS	*Aspergillus niger* isolate IDSGBT-01-22 small subunit ribosomal RNA gene	95.54%	94%	0.0	OP001987.1

Molecular Characterization

Isolation of Genomic DNA

Three different types of fungus are collected in tubes to isolate the DNA.
Three different fungal isolates are taken and crushed with sand particles using pestle and mortar. That sample are taken in tubes for isolation of genomic DNA.

Amplified ITS gene for fungus (550 bp)

The isolated 3 samples are loaded in polymerase chain reaction machine and then set the PCR program to get many copies of DNA. Then Visualization of PCR amplification on 1% agarose gel. LaneM, 1-kb Marker, Lane 1 to 3, isolates of DNA from *Muntingia calabura* contains 550 bp (Figures 3 and 4).

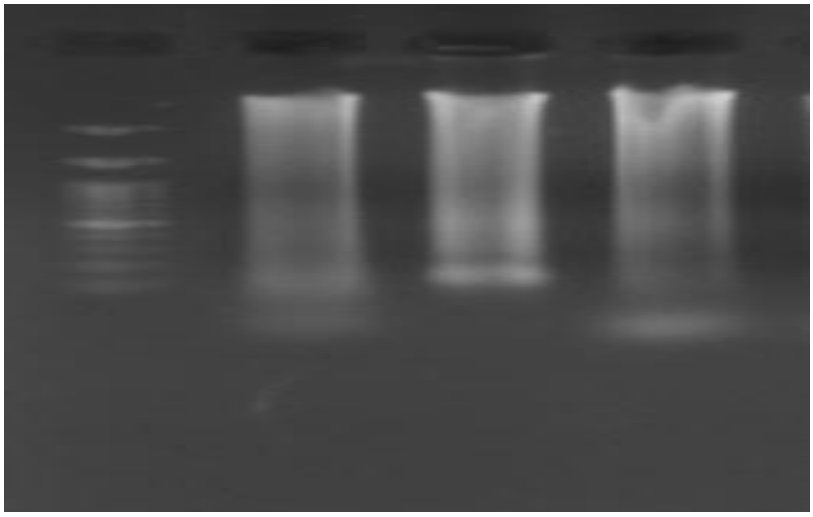

Fig. 3. Genomic DNA compared to 1000 bp ladder.

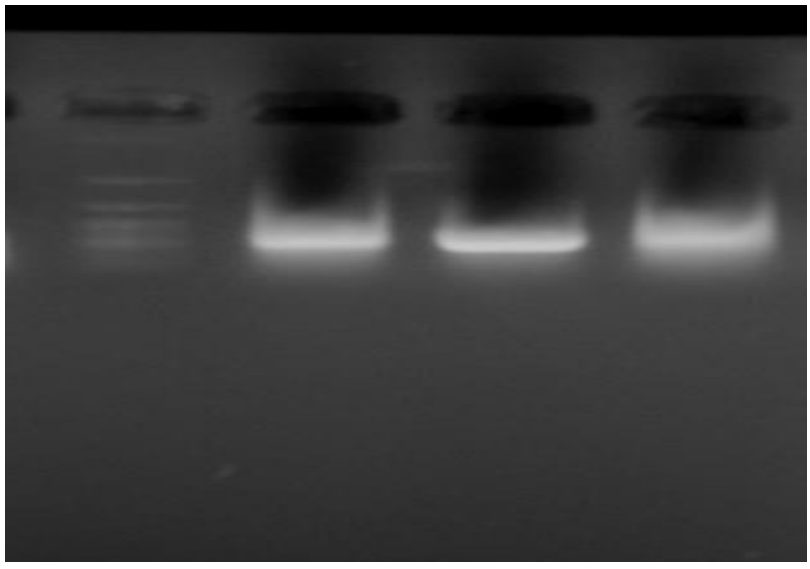

Fig. 4. PCR product of ITS gene (550 bp) compared to 1000 bp ladder.

Sanger Sequence

In this technique we can add only single primer either Forward primer or Reverse primer. For 2 mL of template DNA, 1 mL of ITS2 primer is added then 0.5 mL of BDT (Big dye terminator) solution and 2 mL of buffer and 4.5 mL of water added and then, kept for PCR which includes Denaturation at 950 °C for 5 min, Annealing takes place at 550 °C for 30 min, then termination occurs at 600 °C for 1 min. Then 2nd, 3rd and 4th steps are repeated for about 30 cycles and 40 °C held forever. Then run for capillary electrophoresis. Here 3130× L genetic analyzer is used.
Electropherogram: Picture of Electropherogram of 3 verities of fungus showing the Sequences of Data produced by an Automated DNA Sequencing Machine (Figure 5).

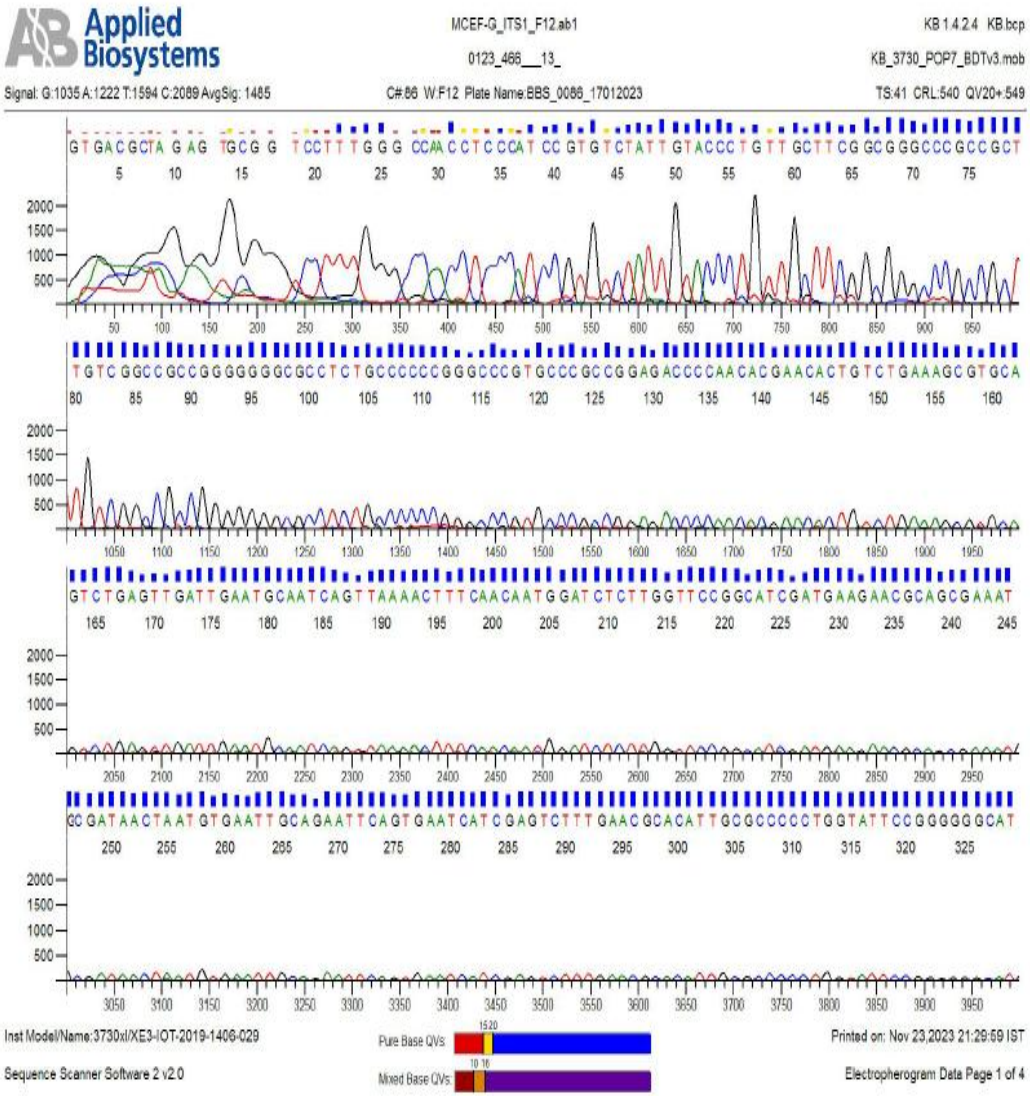

Fig. 5. Electropherogram in pdf format.

FASTA Sequences, Phylogenetic Trees and the DNA Barcode of All Three Isolates (Figures 6–11)

>MCEF-G_ITS
TCCGTAGGTGAACCTGCGGAAGGATCATTACCG
AGTGCGGGTCTTTATGGCCCAACCTCCCACCCG
TGACACGATGCATGCATCTATTGTACCTTGTTG
CTTCGGCGGGCCCGCCAGCGTTGCTGGCCGCCG
GGGGGCGACTCGCCCCCGGGCCCGTGCCCGCCG
GAGACCCCAACATGAACCCTGTTCTGAAAGCTT
GCAGTCTGAGTGTGATTCTTTGCAATACCAGTT
AAAACTTTCAACAATGGATCTCTTGGTTCCGGC
ATCGATGAAGAACGCAGCGAAATGCGATAACT
AATGTGAATTGCAGAATTCAGTGAATCATCGA
GTCTTTGAACGCACATTGCGCCCCCTGGTATTC
CGGGGACGATGGGCATGCCTGTCCGAGCGTCAT
TGCTGCCCTCAAGCCCGGCTTGTGTGTTGGGCCC
TCGTCCCCCGGCTCCCGGGGGACGGGCCCGAAAG
GCAGCGGCGGCACCGCGTCCGGTCCTCGAGCGTA
TGGGGCTTCGTCTTCC
GCTCCGTAGGCCCGGCCGGCGCCCGCCGACGCAT
TTATTTGCAACTTGTTTTTTTCCAGGTTGACCTC
GGACGATCGATCATCAGGTAGGGATACCCGCTGA
ACTTAAGCATATCAATAAGCGGAGGAAAAGAAA
CCAACCGGGATTGCCACAGCTTC

Hit: *Aspergillus niger* isolate IDSG-BT-01-22 small subunit ribosomal RNA gene,
Percentage identity: 100%
Total score: 1101
Query coverage: 100%
E value: 0.0
Accession No.: OP001987.1
Phylogenetic tree:

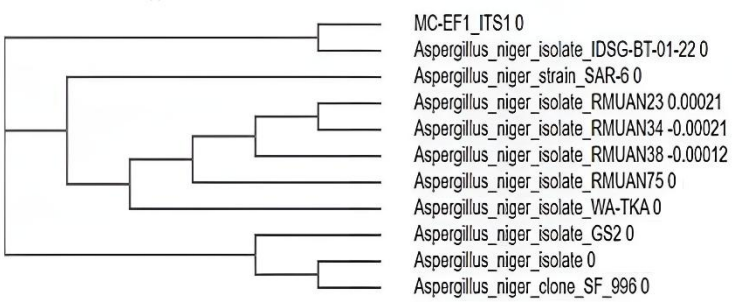

Fig. 6. Phylogenetic tree of isolate MCEF-G.

DNA Barcode
>MCEF-G_ITS

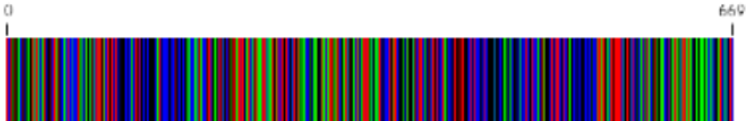

Fig. 7. DNA Barcode > MCEF-G_ITS isolate.

>MCEF-R_ITS
TACTGTGCGGGACTCTGGGTCAACCTCCCATCC
GTGTCTCTTGTACCCTGTTGCTTCGGCGGGCCC
GCCTTCATGGCCGCCGGGGGGCTTCTGCCCCCG
GGCCCGCGCCCGCCGGAGACTCCAACATTGAAC
ACTGTCTGAAGGTTGCAGTCTGAGTTTTCTATA
AGAAAAATCGTTAAAACTTTCAACAACGGATC
TCTTGGTTCCGGCATCGATGAAGAACGCAGCGA
AATGCGATACGTAATGTGAATTGCAGAATTCA
GTGAATCATCGAGTCTTTGAACGCACATTGCGC
CCTCTGGTATTCCGGGGGGCATGCCTGTCCGAG
CGTCATTGCTGCCCTCAAGCACGGCTTGTGTGT
TGGGCCCCCGTCCCGGTTCTCCAAGCCGGGACG
GGCCCGAAAGGCAGCGGCGGCACCGTGTCCGGT
CCTCGAGTGTATGGGGCTCTGTCACTCACTCGT
GTAGGTCCGGCCGGCGGCCAGCCTCTTCAACCAT
TTTTTCACCAGGTTGACCTCGGATCAGGTAGGGA
TACCCGCTGAACTTAAGCATATCAATAAGAGCA
AGCACTCGCGGAGGGGGACCCGGGGGACAAAAA
CCCCGGGGGCCCTTTGCGTGCTTTTTCGGCGCCC
CCATATTGTGCTGGA

Hit: *Fusarium thapsinum* isolate IDSG-BT-03-22 internal transcribed spacer 1
Percentage identity: 96.84%
Total score: 832
Query coverage: 100%
E value: 0.0
Accession No.: OP002008.1
Phylogenetic tree:

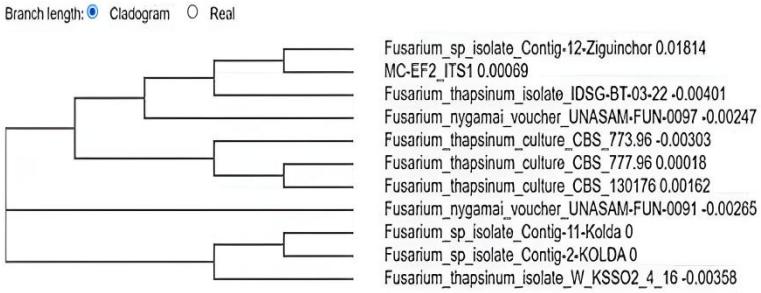

Fig. 8. Phylogenetic tree of isolate MCEF-R.

DNA Barcode.
>MCEF-R_ITS

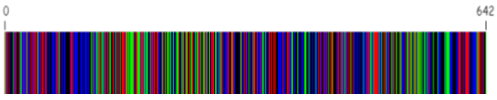

Fig. 9. DNA Barcode > MCEF-R_ITS isolate.

>MCEF-B_ITS
AAGGATCATTAAATAATCAATAATCTTGGCTT
GTCCATTATTATCTATTTACTGTGAACTGTATT
ATTATACGTACGATCGATTGACATTTGAGGGA
TGTTCCAATGTTATAAGGATAGACATTGGAAA
TGTTAACCGAGTCATAATCAGGTTTAGGCCTGG
TATCCTATTATTATTTACCAAATGAATTCAGAA
TTAATATTGTAACATAGACCTAAAAAAACAGC
TAGCATCTCTATAAAACAACTTTTAACAACGGA
TCTCTTGGTTCTCGCATCGATGAAGAACGTAGC
AAAGTGCGATAACTAGTGTGAATTGCATATTC
AGTGAATCATCGAGTCTTTGAACGCAACTTGCG
CTCATTGGTATTCCAATGAGCACGCCTGTTTCA
GTATCAAAACAAACCCTCTATCCAACTTTTGTT
GTATAGGATTATTGGGGGCCTCTCGATCTGTAT
AGATCTTGAAATCCCTGAAATTTACTAAGGCCTG
AACTTGTTTAAATGCCTGAACTAATATAAAGGA
AAGCTCTTGTAATTGACTTTGATGGGGCCTCCCA
AATAAATCTCTTTTAAATTAGCAGGCATCGATC
TGATCTGAAATCAGGCGGGATTACCCGCTGAACT
TAA

Hit: *Aspergillus niger* isolate IDSG-BT-01-22 small subunit ribosomal RNA gene,
Percentage identity: 95.54%
Total score: 839
Query coverage: 94%
E value: 0.0
Accession No.: OP001987.1
Phylogenetic tree:

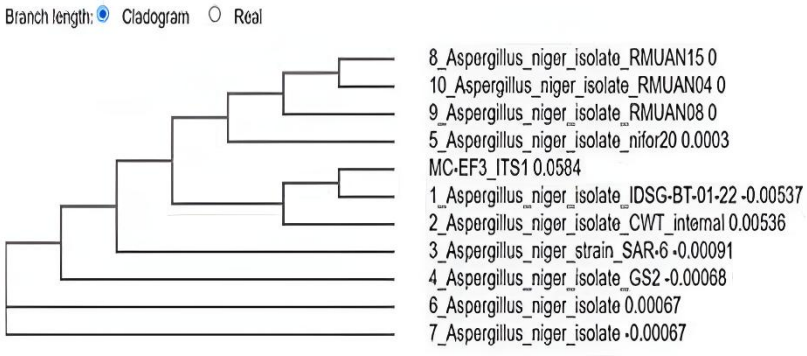

Fig. 10. Phylogenetic tree of isolate MCEF-B.

DNA Barcode
>MCEF-B_ITS

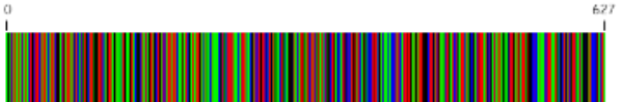

Fig. 11. DNA Barcode>MCEF-B_ITS isolate.

Antagonistic Activity

The fungal extracts exhibited significant antagonistic activity, as indicated by the zones of inhibition against two bacterial pathogens, Staphylococcus aureus and *Escherichia coli*. (Table 3) (Figure 12).

To contextualize the antibacterial activity observed in this study, the inhibition zones produced by the endophytic fungal extracts were compared with values reported in the literature. The *Aspergillus niger* isolate (MCEF-G) exhibited inhibition zones of 24 mm against Staphylococcus aureus and 25 mm against *Escherichia coli*, which are at the higher end or slightly above the typical ranges reported for Aspergillus niger extracts (usually 12–22 mm for *S. aureus* and 10–20 mm for *E. coli*) in previous studies (Jia et al., 2016; Bhagat et al., 2012). Similarly, the *Fusarium thapsinum* isolate (MCEF-R) showed inhibition zones of 20 mm (*S. aureus*) and 17 mm (*E. coli*), also aligning with or exceeding the upper values reported for *Fusarium* spp. from medicinal plants (10–18 mm for *S. aureus* and 8–16 mm for *E. coli*). These findings indicate that the endophytic fungi isolated from *Muntingia calabura* possess strong antibacterial properties, comparable to or greater than those documented in earlier research, and highlight their potential as promising sources of natural antimicrobial agents.

The diversity observed in *Muntingia calabura* endophytes is comparable to findings from other medicinal plants, where multiple genera-including *Fusarium, Aspergillus*, and Colletotrichum-are commonly reported. Notably, previous research has documented a higher number of isolates from *M. calabura* (up to 15 in some studies), reflecting both the rich endophytic community associated with this host and the influence of sampling and isolation

protocols. In this study, the focus on healthy leaf tissues and stringent surface sterilization ensured that only true endophytes were recovered.

The identification of both *Aspergillus niger* and *Fusarium thapsinum* as endophytes of *M. calabura* is significant, as these genera are well-known for their ability to produce a wide spectrum of bioactive secondary metabolites, including compounds with antimicrobial and antifungal properties. This sets the stage for subsequent evaluation of their antagonistic activity and potential application as sources of natural antimicrobial agents.

To further understand the basis of the observed antimicrobial activity, it would be valuable to analyze the secondary metabolites produced by the endophytic fungi isolated from *Muntingia calabura.* Previous studies have shown that endophytic *Aspergillus niger* and *Fusarium species* are capable of synthesizing a wide range of bioactive compounds, including alkaloids, terpenoids, phenolics, and polyketides, which are often responsible for their antimicrobial effects (Jia et al., 2016; Bhagat et al., 2012). Specifically, compounds such as nigericin, gliotoxin, and fumonisin from *Aspergillus* and *Fusarium* have been reported to exhibit potent antibacterial activity. Although the present study did not include chemical profiling, future work involving chromatographic and spectrometric analyses (such as GC-MS or LC-MS) is recommended to identify and characterize the active constituents in the crude extracts. Such detailed investigation would help pinpoint the specific metabolites responsible for the inhibition of *Staphylococcus aureus* and *Escherichia coli*, and could facilitate the development of novel antimicrobial agents from these endophytic fungi.

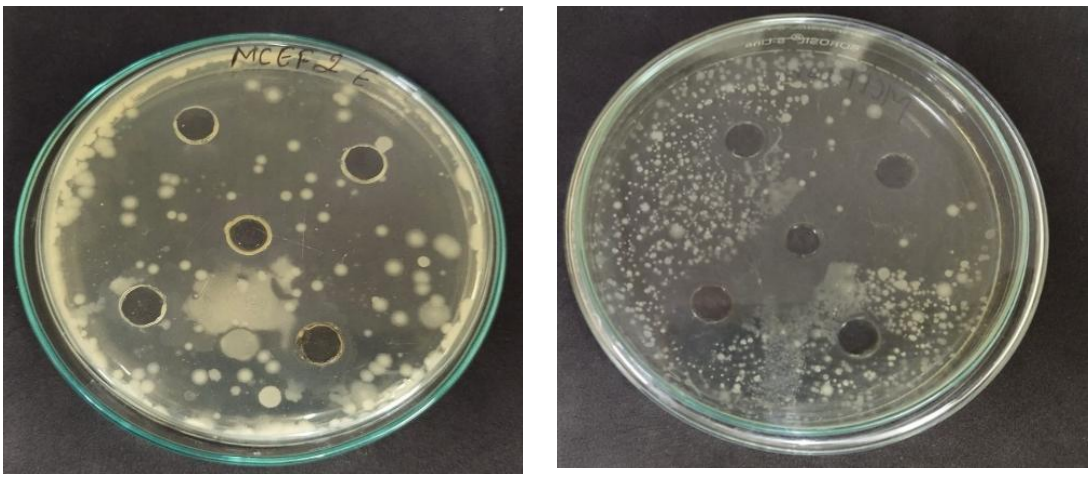

Fig. 12. Antagonistic activity of fungal extracts against *Escherichia coli* (**left**) and *Staphylococcus aureus* (**right**) respectively.

Table 3. Antagonistic activity of selected fungus.

Sl No.	Sample	Inhibition zone	-ve Control(8mm) (Inhibition zone negative control)
	S. aureus		
	MCEF-G	24	16
1	MCEF-R	20	12
	MCEF-B	12	4
	(+ve) Ampicillin	30	22
	E. coli		
	MCEF-G	25	17
2	MCEF-R	17	9
	MCEF-B	15	7
	(+ve) Ampicillin	32	24

MCEF-G, MCEF-R, and MCEF-B show the zone of inhibition against the test pathogen *Staphylococcus aureus* and *E. coli.*

CONCLUSIONS

This study provides the first report of *Aspergillus niger* and *Fusarium thapsinum* as endophytic fungi isolated from *Muntingia calabura* in India, demonstrating their significant antibacterial activity against *Staphylococcus aureus* (inhibition zones: 18.2 ± 1.3 mm) and *Escherichia coli* (15.6 ± 0.9 mm). The results validate our hypothesis that endophytes from this plant produce bioactive metabolites with antimicrobial potential, aligning with previous reports on medicinal plant-associated fungi (Jha et al., 2023;

Rajamanikyam et al., 2017). These findings highlight the value of *M. calabura* as an underexplored reservoir of natural antimicrobial agents, particularly in combating antibiotic-resistant pathogens.

Study limitations include the small sample size (n = 50 leaves) and the lack of toxicity evaluation for the crude extracts. Future research should prioritize:

1. Purification and structural elucidation of active metabolites from *A. niger* and *F. thapsinum*.
2. Toxicity profiling of extracts using mammalian cell lines to assess safety for therapeutic use.
3. Field trials to evaluate the efficacy of these endophytes as biocontrol agents in agricultural settings.

By addressing these gaps, researchers can advance the development of eco-friendly alternatives to synthetic antibiotics and sustainable solutions for crop protection. The symbiotic relationship between *M. calabura* and its endophytes underscores the untapped potential of plant-microbe interactions in addressing global health and agricultural challenges.

CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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