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Molecular characterization and antibacterial properties of endophytic fungi *Lasidiplodia theobromae* in *Lobelia nicotianifolia* Roth ex Schult. of central Western Ghats of Karnataka, India

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ABSTRACT

Endophytic fungi are the great symbiotic microorganisms present in the plants. The fungal endophytes are recognized as valuable sources for novel secondary metabolites with various activities such as antimicrobial, antioxidant, antiviral, and anticancer. The present investigation focused on isolation, molecular identification, and phylogenetic studies of the endophytic fungi *Lasidiplodia theobromae* in the leaves of *Lobelia nicotianifolia* of Chikkamagaluru. The endophytic fungi *L. theobromae* was isolated, molecularly characterized by ITS (ITS1 and ITS4), and LSU (LROR and LR5) gene sequencing, and the antibacterial activity of the ethyl acetate fungal extract by agar well diffusion method was evaluated. *Lasidiplodia theobromae* expressed high antibacterial activity against all the tested clinical pathogenic bacteria and greater inhibition in *Escherichia coli* (MTCC-1599) compared to *Xanthomonas campestris* (MTCC-228), *Klebsiella pneumoniae* (MTCC-7028), and *Staphylococcus aureus* (MTCC-4734).

1. INTRODUCTION

The word endophyte is derived from the Greek word "endo" meaning within and "phyte" meaning plants [1]. Endophytes are the microorganisms [2,3] that colonize the living internal tissues of healthy plants, either intercellularly or intracellularly [4], without causing any negative effects or disease symptoms [5,6], and extraordinarily unstudied as potential assets of novel natural merchandise for exploitation in remedy, agriculture, and industries [7,8]. Endophytes are isolated from almost every organ of every plant [9]. The plants are associated with endophytic fungi [10], and are also recognized for their ecological roles influencing host population, plant communities, biosynthesis, biotransformation, and biodegradation [11], and act as biological control agents, enhance plant growth, and promote plants initiation in adverse conditions [12,13]. An intensive search for more recent antimicrobial agents is diagnosing the use of endophytic fungi as novel potential sources of bioactive secondary metabolites, over 25% of prescribed drugs used in human medicine are obtained from fungal endophytes [14].

Fungal endophytes provide a broad variety of bioactive secondary metabolites such as alkaloids, benzapyaranones, flavonoids, phenolic acids, quinones, steroids, terpenoids, tetralones, xanthones [15,16], isocoumarin derivatives, phenols, and peptides [17]. Many fungal endophytes act as the defense systems of the host-plants [18]. These secondary metabolites are known to possess a wide variety of biological activities such as antibiotic, anticancer, antioxidant, anti-inflammatory agents [19,20], antimycotics, immunosuppressants [21], antiviral, and antimicrobial properties [22].

Lobelia nicotianifolia Roth ex Schult. belongs to the family Campanulaceae, commonly known as wild tobacco. The plant has the presence of lobeline, a pyridine alkaloid mainly used for antiepileptic treatment [23]. It is used in numerous ethnobotanical treatments with reported antibiotic, antiseptic [24], anti-inflammatory activities [25], snakebite [26], wounds healing [27], scorpion bite, epilepsy, and the number of respiratory diseases such

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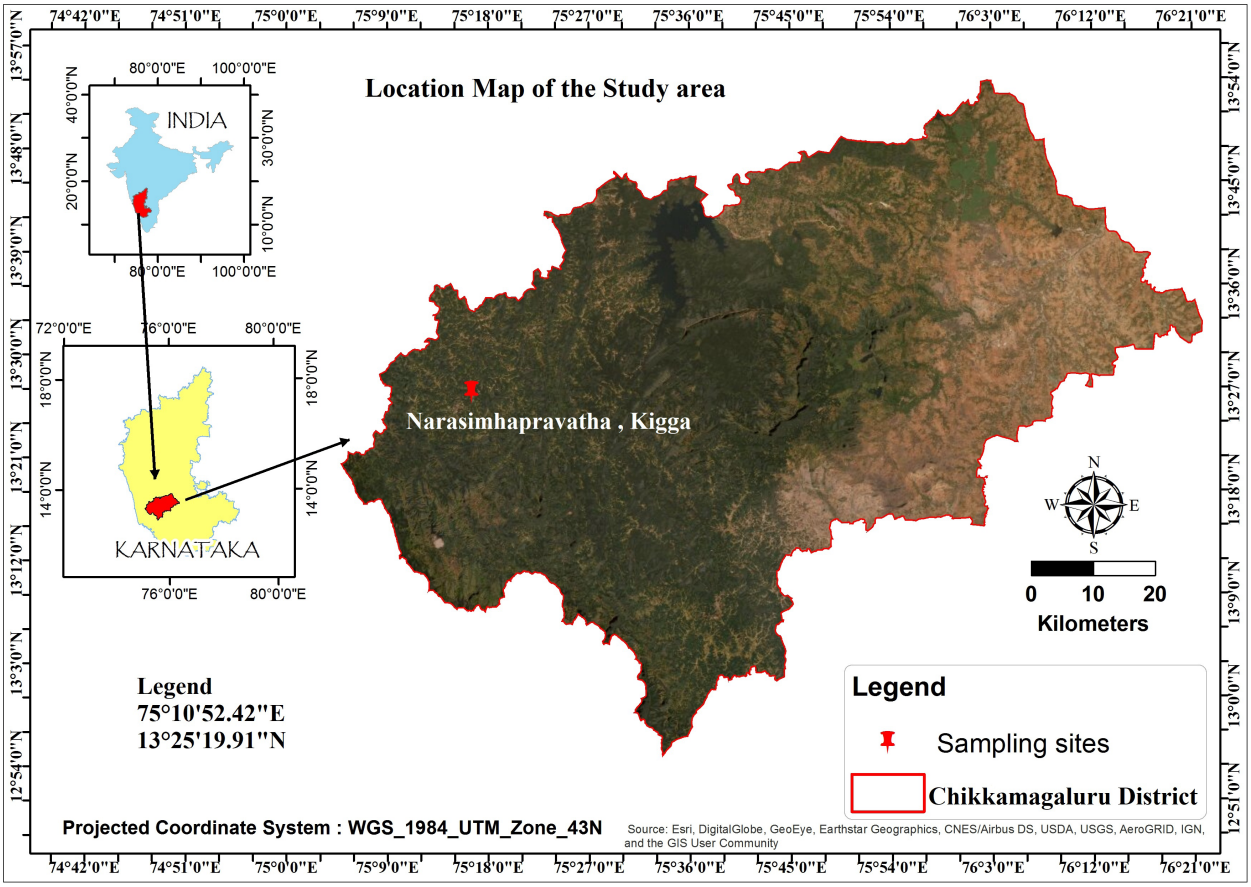

Figure 1: Map showing the study area of plant collection site, Narashima Parvatha, Kigga, Chikkamagaluru, Karnataka, India.

as asthma, bronchitis, and pneumonia [28]. The study investigates the isolation, identification phylogenetic study, and antibacterial activity of the endophytic fungi *Lasidiplodia theobromae* on the leaves of *L. nicotianifolia* from the central Western Ghats region of Chikkamagaluru, Karnataka.

2. MATERIALS AND METHODS

2.1. Study Area and Collection of Samples

Healthy matured leaves of *L. nicotianifolia* (Fig. 2A) were collected in sterilized polythene bags from the central Western Ghats area of Chikkamagaluru district during December 2020, situated at 13°25′19″N; 75°10′52″E (Fig. 1). The samples were brought to the lab and cultured within 24 hours of collection [29].

2.2. Chemicals and Reagents

The molecular biology chemicals and potato dextrose agar (PDA), from HiMedia Laboratories, dimethyl sulfoxide (DMSO), from Merck Life Science Private Limited, and ethyl acetate, from SDFCL Sd Fine Limited.

2.3. Isolation of Endophytic Fungi

Surface sterilization was carried out by using the method described by Ghimire *et al*. [30] with slight modifications (95% ethanol for the 30 seconds, 70% ethanol for 5 minutes, and 3% sodium hypochlorite). The surface-sterilized tissues were rinsed three times with sterile water, blot dried, cut into 1 cm^2 pieces in aseptic condition, then placed on PDA amended with Amoxicillin and incubation at 25°C ± 2°C, and examined regularly for emerging fungal colonies. The pure cultures were stored on PDA slants at 4°C for later use and 20% glycerol at −20°C for long-term preservation.

2.4. Identification of Endophytic Fungi

2.4.1. Morphological and molecular identification

The pure culture of fungal endophyte was identified based on morphological characteristics [31]**.** The genomic DNA was extracted from the freshly cultured mycelia using the cetyltrimethylammonium bromide (CTAB) method [32] with modifications. The 5–7–day-old fresh fungal cultures were grown in both liquid broth and culture plates. The fungal mass from the culture plate was scraped out of the mycelium with the help of a spatula. Approximately 300 mg of fungal mycelium was homogenized with 500 µl of 2X CTAB extraction buffer pre-warmed to 65°C in a 1.5 ml microcentrifuge tube with the help of a micropestle, vortexing and incubated in a water bath at 65°C for 1 hour. The centrifugation was for 13,000 rpm for 20 minutes and 1 µl of RNase A added and incubated for 10 minutes at 37°C, and

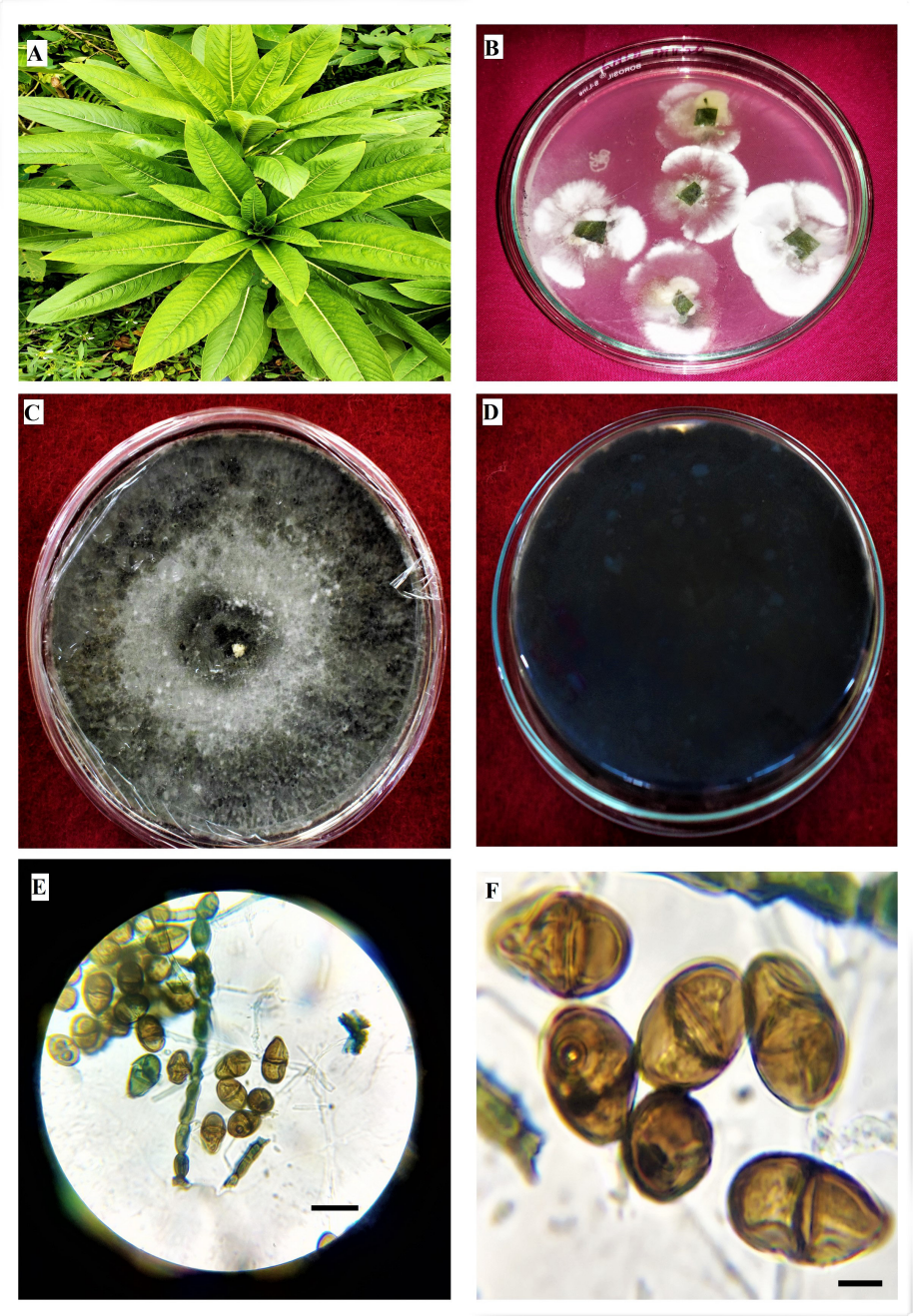

Figure 2: Isolation, culture, and identification of *L. theobromae.* A. *Lobelia nicotianifolia* Roth ex Schult. B. Isolation of fungal endophytes C. Pure culture of *L. theobromae*, D. Reverse side of the colony. E and F. Conidia. Bars = 10 µm.

finally Phenol:Chloroform:Isoamyl alcohol (25:24:1) was added with invert mixing. Then it was centrifuged at 10,000 rpm for 10 minutes at room temperature and the supernatant was extracted. To precipitate the DNA, added 500 µl of ice-cold isopropanol and incubated overnight at 4°C, followed by centrifugation at 10,000 rpm for 10 minutes at 4°C to pellet the DNA and washed two times with 70% ethanol, dried and dissolved in 50 µl of 1X TE buffer. 2 µl of genomic DNA was subjected to 0.8% agarose gel electrophoresis and observe under gel doc (BIO-RAD).

PCR reactions were carried out in 0.2 ml PCR tubes with 50 µl reaction mixture containing, 25 µl double distilled water, 8 µl 10X PCR buffer A. 2.5 µl of each primer, 0.5 µl of Taq DNA polymerase (3 U/µl), 1.5 µl deoxynucleoside triphosphate mixture, and 10 µl of DNA template. The primer pairs ITS 1 and ITS 4 for the ITS region and LROR and LR5 for the LSU region were used. The thermal cycling for ITS amplification was as follows: 4′ 94°C, 32 cycles of 30″ 94°C, 1′ 52°C, 1′ 72°C and a final extension step of 7′ 72°C, for LSU 5′ 94°C, 30 cycles of 30″ 94°C, 1′ 47°C, 1′ 72°C and a final extension step of 7′ 72°C. The PCR products were observed on 1% agarose gel stained with ethidium bromide, under

Table 1: List of *L. theobrame* ITS species, origin, and GenBank accession numbers of the ITS sequences used in phylogenetic analysis. A newly generated sequence is in bold.

Sl No	Species	Zss GenBank accession number	Strain	Origin and year
1	*L. theobromae*	MW858234	LS3	India, 2021
2	*L. theobromae*	MT644474	ZW 50-1	China, 2020
3	*L. theobromae*	MN634046	IRNKB244	Spain, 2019
4	*L. theobromae*	MK792507	COUFAL0103	Brazil, 2019
5	*L. parva*	KX227558	LTY9	Ghana, 2016
6	*L. parva*	KX227552	LTY3	Ghana, 2016
7	*L. marypalmiae*	NR 147341	CMM 2275	Brazil, 2013
8	*L. marypalme*	KC484841	CMM2274	Brazil, 2013
9	*L. iranensis*	MF580781	EB5	Malaysia, 2017
10	*L. iranensis*	MH057172	BRIP63318	Australia, 2018
11	*L. citricola*	MT849766	XGWY44	China,2020
12	*L. citricola*	MT849764	XGWY43	China, 2020
13	*L. pseudotheobromae*	MN341226	KoRLI047143	South Korea,2019
14	*L. pseudotheobromae*	MN341225	KoRLI047140	South Korea,2019
15	*Botryosphaeria dothidea*	MK178544	LTNM2	USA, 2018

gel image documentation system (Bio-Rad) followed by PCR product purification and sequencing. Both forward and reverse sequences were trimmed using molecular evolutionary genetics analysis (MEGA X) and generated the consensus sequences using Bio Edit and the Basic Local Alignment Search Tool search in the GenBank nucleotide database and both ITS and LSU sequences were deposited to GenBank [33].

2.5. Phylogenetic Analysis Maximum Likelihood Method

The molecular phylogenetic examination was conducted by using ITS and LSU sequences independently. The datasets of 15 ITS sequences (Table1) and 16 LSU sequences (Table 2) were recovered from the National Center for Biotechnology Information (NCBI) GenBank. The multiple sequence alignment was carried out by using the Claustal W and the bootstrap study was examined up to 1,000 replicates. The evolutionary history was inferred by using the Maximum Likelihood method Tamura three-parameter model [34]. Evolutionary analysis was conducted in MEGA X [35].

2.6. Preparation of Fungal Extract

The pure culture of *L. theobromae* was cultivated in 1,000 ml of potato dextrose broth liquid medium and incubated at 28°C on a shaker at 160 rpm for 4 weeks. The culture filtrate was filtered by vacuum filtration. Afterward, the culture filtrate was extracted with ethyl acetate using a separatory funnel (solvent–solvent extraction). The rotary evaporator with the extract was dried and the crude extract was dissolved in DMSO, and then evaluated for antibacterial activity [36].

2.7. Antibacterial Activity

The antibacterial activity was determined using the agar well diffusion method against three human bacterial pathogens (*Escherichia coli*, MTCC-1599; *Klebsiella pneumoniae*, MTCC-7028; and *Staphylococcus aureus*, MTCC-4734), and one plant pathogen (*Xanthomonas campestris*, MTCC-228). The crude fungal extract was prepared in DMSO. The 6 mm wells are made by using a sterile borer and loaded with 40 µl of the test sample. The antimicrobial drugs amoxicillin and DMSO were used as positive and negative controls distinctly. The inoculated plates were incubated at 37°C overnight and the zone of inhibition (mm) was recorded [37].

3. RESULTS

3.1. Morphological and Molecular Identification

The colony morphology of the endophytic fungi *L. theobromae* showed dark gray mycelia, septate, conidia elliptic in shape, curved, and broad septate cells (20.5 × 10.5 µm) (Fig. 2). Molecular identification was carried out by ITS and LSU gene sequencing. The sequences were edited using MEGA X and BIO edit 7.2 software and the consensus sequences were submitted to NCBI GenBank with accession numbers MW858234 and MW876233.

3.1.1. Taxonomy

3.1.1.1. *Lasiodiplodia theobromae* (Pat.) Griffon & Maubl., Bull. trimest. Soc. Mycol. Fr. 25: 57 (1909). Mycobank 188476.

Table 2: List of *L. theobrame* LSU species, origin, and GenBank accession numbers of the ITS sequences used in phylogenetic analysis. A newly generated sequence is in bold.

Sl No	Species	GenBank accession number	Strain	Origin and year
1	L. theobromae	MW876233	LS3	India, 2021
2	*L. theobromae*	MN181372	L3	China, 2019
3	*L. theobromae*	MK348011	C443B	Thailand, 2018
4	*L. theobromae*	MH876839	CBS 129758	The Netherland, 2017
5	*L. theobromae*	MH876835	CBS 129754	The Netherland, 2017
6	*L. parva*	LN907364	UTHSC: DI16-221	Spain, 2015
7	*L. parva*	KF766362	CBS 456.78	South Africa , 2013
8	*L. hyalina*	NG 069536	CGMCC 3.17975	China, 2017
9	*L. hyalina*	MG321677	CGMCC 3.17975	China, 2017
10	*L. iranensis*	NG 069920	CBS 124710	The Netherland, 2019
11	*L. iranensis*	MH874918	CBS 124710	The Netherland,2017
12	*Lasiodiplodia margaritacea*	NG 069371	CBS 122519	The Netherland, 2017
13	*L. margaritacea*	KX464354	CBS:122519	The Netherland, 2017
14	*L. venezuelensis*	MH876836	CBS 129755	The Netherland, 2019
15	*L. venezuelensis*	KF766363	CMW 13512	South Africa, 2013
16	*Botryosphaeria dothidea*	MW981605	QOC 20	China, 2021

The colony was 4–4.2 cm diameter within 7 days (Fig. 2C), mycelium initially white and become dark, immersed, branched, and septate. Paraphyses hyaline, cylindrical, thin and septate. Conidiomata pycnidial, and the pycnidia was superficial, globose, dark brownish and uninoculated. The conidiophores were absent and conidiogenous cells cylindrical, hyaline, smooth and holoblastic. Conidia (Fig. 2E and F) hyaline with medium septum, dark brown, longitudinal striate and oval shaped measuring 22.5 × 14.5 µm two celled (100X Magnification) using Binocular microscope Lawrence and Mayo.

3.1.1.2. Substrate. Healthy matured leaves of *L. nicotianifolia* Roth ex Schult.

3.1.1.3. Material examined. India, Karnataka, Kuvempu University, Department of Applied Botany, *L. nicotianifolia* Roth ex Schult., January 24, 2020, Vinu. K, LS3.

3.2. Phylogenetic Analysis

The phylogenetic evolutionary study was conducted separately for ITS and LSU sequences by NCBI-Blastn, MEGA-X, and Fig Tree software program. The phylogenetic investigation was using the Maximum Likelihood method and Tamura three-parameter model. The trees with the highest log-likelihood for ITS (−1,742.99) and LSU (−955.41) are shown. The difference in Gamma distribution rate between ITS [Type 5 (+G, parameter = 0.4515) and LSU Type 5 (+G, parameter=0.0575)] sites. There are 849 positions in ITS and 556 positions in the final dataset in LSU. In the evolutionary analysis of ITS (Fig. 3) and LSU (Fig. 4), *Botryosphaeria dothidea* was selected as the out group with GenBank accession numbers MK178544 and MW981605, respectively.

3.3. Antibacterial Activity

The present investigation revealed that that ethyl acetate extract of *L. theobromae* showed strong antibacterial activity against *E. coli,* S. *aureus*, *K. pneumoniae*, *X. campestris* compared to Amoxicillin (Fig. 5). *Escherichia coli* showed a high zone of inhibition among the tested bacterial strains (Table 3).

4. DISCUSSION

The present study showed that a naturally growing medicinal plant *Lobelia nicotianifolia*, which is grown in the mountain ranges is a natural niche for various fungal endophytes. The need to look for bioactive compounds with antimicrobial values arises from the evolutionary reaction of microbes to existing antibiotics with the resulting increase in resistance rates [38]. To combine economic value with biodiversity, bioprospecting studies have shown the biotechnological potential of endophytes against a variety of pathogens [39]. Most of the existing studies on the biological activity of *L. theobromae* metabolites have evaluated their potential phytotoxicity, cytotoxicity, and antibacterial activity [40]. The present study reveals the isolation, molecular characterization (ITS & LSU), phylogenetic analysis, and the production of secondary metabolites from the novel endophytic

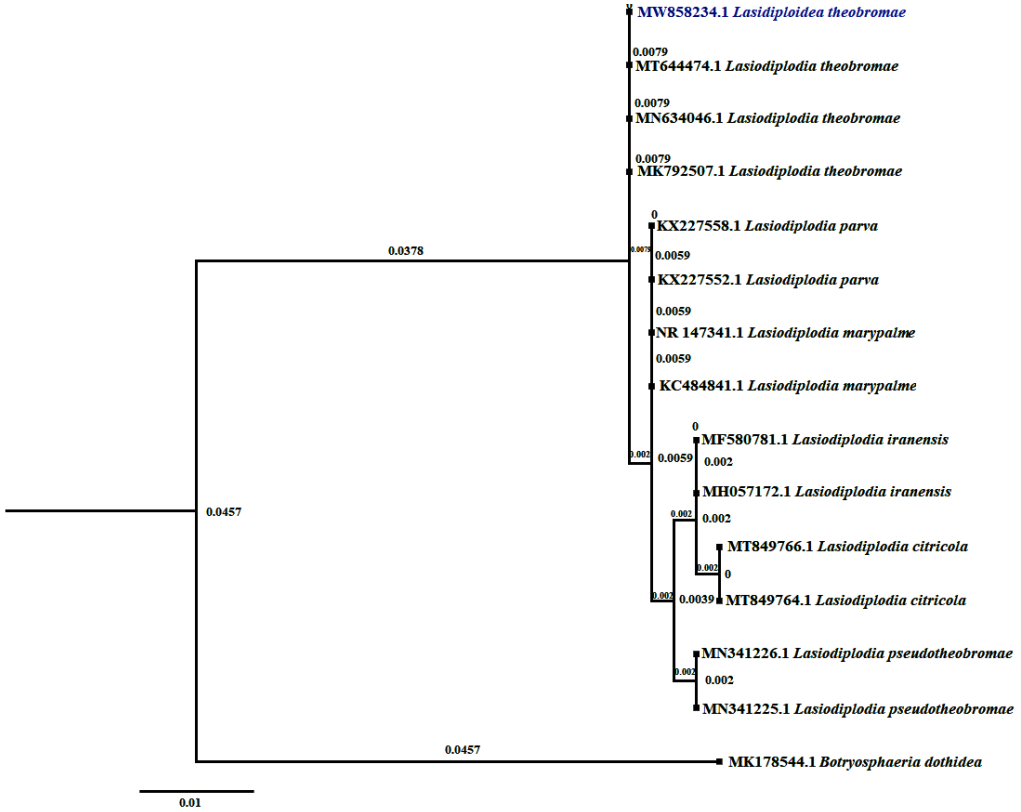

Figure 3: Maximum likelihood tree of ITS sequence data. It illustrates the relationships between *L. theobromae* (MW858234) with other *Lasidiplodia* species and *Botryosphaeria dothidea* selected as an out group.

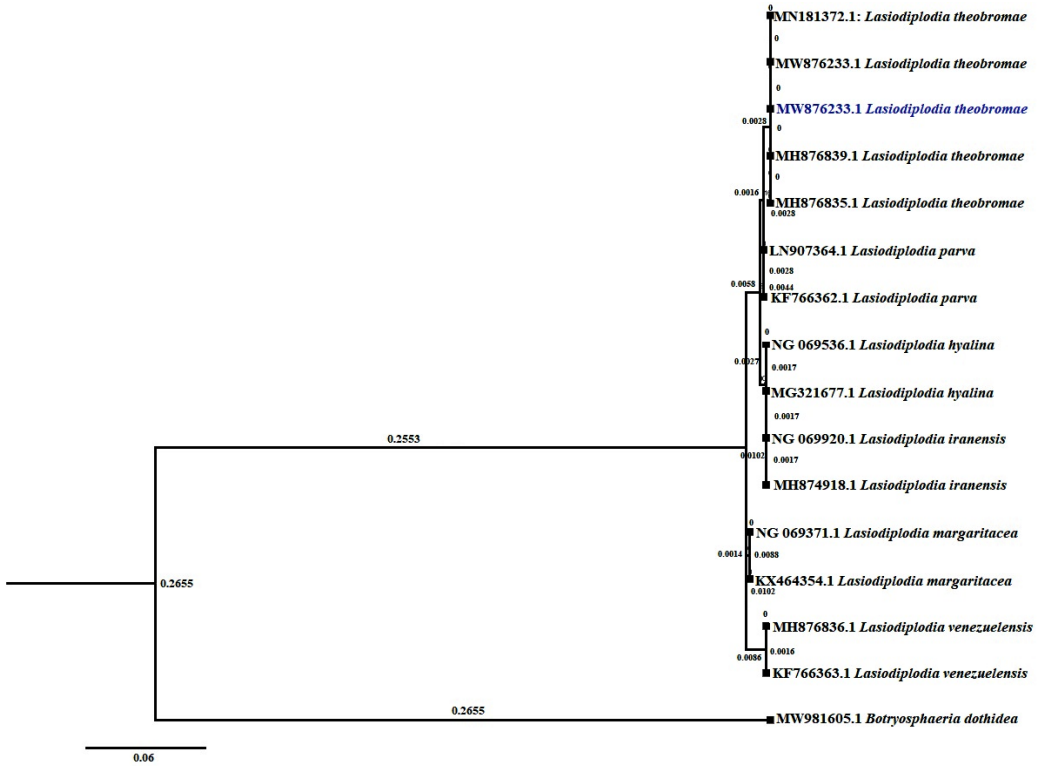

Figure 4: Maximum likelihood tree of LSU sequence data. It illustrates the relationships between *L. theobromae* (MW876233) with other *Lasidiplodia* species and *Botryosphaeria dothidea* selected as an out group.

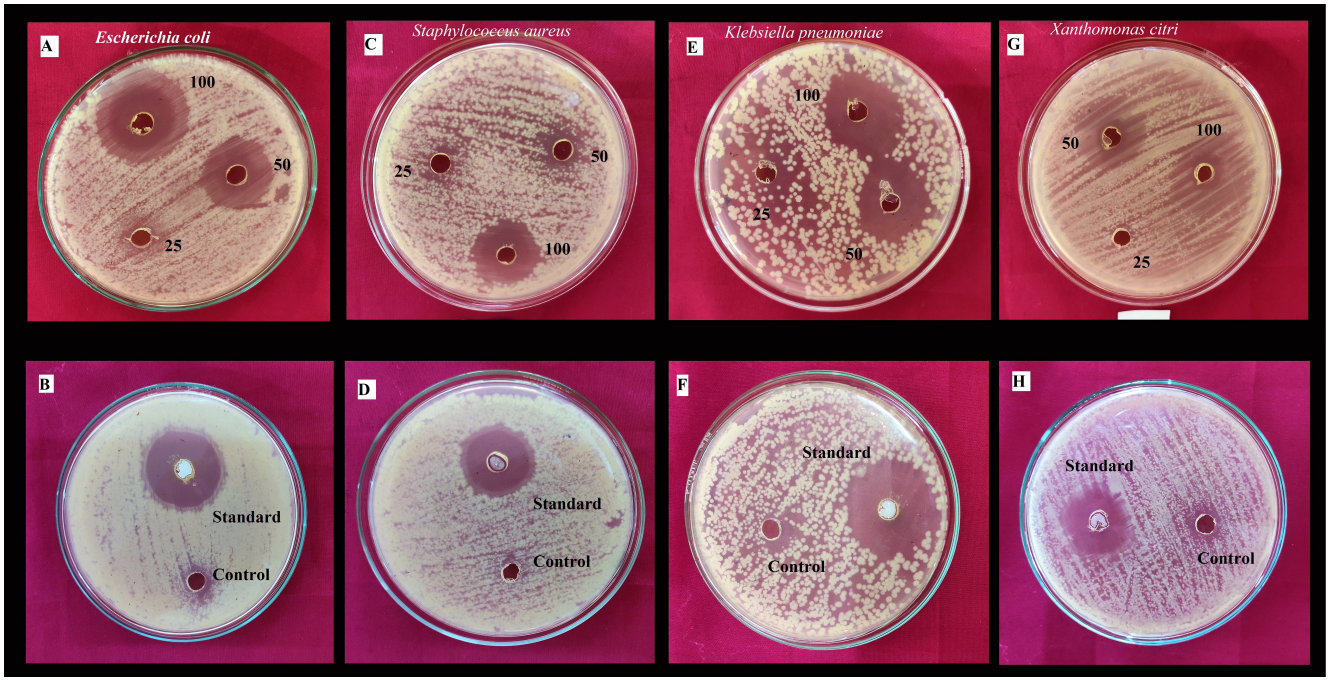

Figure 5: The antibacterial activity of the ethyl acetate extract of *L. theobromae* against pathogenic bacterial strains. A and B. *Escherichia coli*. C and D. *Staphylococcus aureus*. E and F. *Klebsiella pneumoniae*. G and H. *Xanthomonas citri.*

Table 3: Antibacterial activity of the ethyl acetate extract of *L. theobromae* against the selected test strains.

Sl No	Name of bacterial strains	Inhibition Zone (In mm)				
		Concentration (mg/ml)			Standard (amoxicillin)	Control (DMSO)
		100%	50%	25%		
1	*Escherichia coli*	12.33 ± 0.33	7.33 ± 0.33	2.33 ± 0.33	11.66 ± 0.33	0
2	*Staphylococcus aureus*	8.66 ± 0.57	5.33 ± 0.33	2.66 ± 0.33	10.66 ± 0.33	0
3	*Klebsiella pneumoniae*	11.33 ± 0.33	10.66 ± 0.33	6 ± 0.57	11.33 ± 0.33	0
4	*Xanthomonas campestris*	11.66 ± 0.33	8.6 ± 0.33q	1.3 ± 0.03	9.33 ± 0.33	0

fungi *L. theobromae* in *L. nicotianifolia*. The ethyl acetate extract of the endophytic fungi observed significant antibacterial activity than the standard drug, by showing more inhibition zone against X. campestris when compared to the other selected pathogenic bacteria at 100% concentration level.

5. CONCLUSION

Endophytes are a novel and significant microbial resource for showing a wide range of biological activities. The biologically active secondary metabolites produced by endophytes have shown promising potential and benefits for human health and safety. The present study showed that the isolation of endophytic fungi *L. theobromae* and molecular characterized by ITS and LSU gene sequencing. The consensus sequences were submitted in NCBI GenBank and with accession numbers MK178544 and MW981605. The antibacterial activity of the ethyl acetate extract of *L. theobromae* showed the effective antibacterial activity against the clinical tested bacteria (*E. coli*, MTCC-1599), (*K. pneumoniae*, MTCC-7028), (*S. aureus*, MTCC-4734), and (*X. campestris*, MTCC-228).

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7. CONFLICT OF INTEREST

The authors declare no conflicts of interest.

8. AUTHORS' CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current

journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the International Committee of Medical Journal Editors (ICMJE) requirements/guidelines.

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ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

DATA AVAILABILITY

All data generated and analyzed are included within this research article.

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