Exploring the Antibacterial Potential of Endophytic Fungi Isolated from Piper betle Leaves

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Abstract: This research aims to investigate the antibacterial activity of endophytic fungi obtained from Piper betel leaves. With the escalating threat of antibiotic resistance, exploring alternative sources of antimicrobial agents becomes imperative. Piper betel, recognized for its medicinal properties, harbors endophytic fungi that could potentially produce

Key Words: Antibacterial activity, Endophytic fungi, Piper Betle Fungal isolates, Natural products, Secondary metabolites, Bioactive compounds, Microbial inhibition, Phytopathogens Medicinal plants, Plant-microbe interactions etc.

INTRODUCTION

Antibiotic resistance poses a global health crisis, necessitating the exploration of unconventional sources for novel antimicrobial agents. Piper betel, a plant with well-documented medicinal properties, hosts endophytic fungi known to play a crucial role in plant health. This study focuses on isolating and assessing the antibacterial potential of endophytic fungi from Piper betel leaves. By doing so, we aim to contribute to the growing body of research aimed at discovering alternative and effective antibacterial compounds. Microorganisms that colonize healthy plant tissue asymptomatically and sustain mutualistic relationship for all or part of their life cycle are referred to as endophytes. The contemporary literature contains several findings on the production of secondary metabolites in a variety of plant species, which is mediated by endophytes. The family Piperaceae includes the genus Piper, which is widely grown in Sri Lanka, India, Thailand, Taiwan, and other Southeast Asian nations. It is also known as "Paan". [1]

Endophytes are bacteria, fungi, and microbes that live in the tissues of healthy plants without showing any signs of illness or injury. They have been demonstrated to generate a wide range of bioactive substances that support the host plant's overall survival and fitness while coexisting in a symbiotic relationship. Numerous of these substances have been discovered to have bioactivities with potential uses in industry, agriculture, and medicine [2,3].

The extensive use of synthetic chemical medications frequently results in dangerous side effects for humans as well as the emergence of drug resistance in clinical microorganisms. Candida albicans is one of those clinical pathogens that should be thoroughly investigated. Candida albicans is a common cause of candidiasis, a fungal infection of the oral cavity. The goal of this study is to identify novel natural chemicals that can be used to combat Candida albicans using the medicinal herb Piper betle [4].

Bioactive substances produced by endophytic fungi have the potential to be employed as promising medications to treat a variety of ailments. To the best of our knowledge, this is the first time that the application of bioactive metabolites from endophytic fungus has been highlighted, especially against multidrug resistant (MDR) microorganisms that live in burn wounds. Thus, the goal of this work is to explore the possibility of using pure benzoic acid (BA), which is obtained from the endophytic fungus Neurospora crassa, which was isolated from Lycium shawii, as a safe and effective alternative antibacterial option for wound healing [5].

The endophytic fungi that grow in plant tissues have a lot of industrial promise since they can frequently produce compounds in vitro that are either exactly the same as or very comparable to those produced by their hosts. The peculiarities of the endophytic lifestyle raise questions about the molecular mechanisms underlying the synthesis of these bioactive compounds in planta as well as the identity of the real producer, which might be either the plant or its occupants. Increasing our understanding of this is crucial to overcoming the current challenges that endophytes face when being used for larger-scale production. The focus of this analysis is on the possible processes by which plant endophytes produce compounds exclusive to their hosts [6].

MATERIALS AND METHODS

Collection of Plant Material Source: Piper betle leaves were collected from a healthy, mature Piper betle plant located in a specified region (e.g., botanical garden, agricultural field).Sterilization:The collected leaves were washed thoroughly under running tap water to remove surface dirt.They were then surface-sterilized using the following sequence:Immersion in 70% ethanol for 1 minute.Immersion in 2% sodium hypochlorite solution for 3 minutes.Rinsing with sterile distilled water three times to remove any residual sterilizing agents.

Isolation of Endophytic FungiProcedure: The sterilized leaves were cut into small segments (approximately 1 cm^2) using sterile scissors. These segments were placed on potato dextrose agar (PDA) plates supplemented with chloramphenicol (to inhibit bacterial growth). The plates were incubated at 25-28°C for 7-14 days, and observed regularly for fungal growth.Emerging fungal colonies were sub-cultured onto fresh PDA plates to obtain pure isolates.

Identification of Endophytic FungiMorphological Identification: Pure fungal isolates were examined microscopically to observe characteristics such as spore shape, septation, and conidiophore structure.Fungal colonies were described based on color, texture, and growth rate.Molecular Identification:Genomic DNA was extracted from the fungal mycelia using a standard protocol (e.g., CTAB method).The internal transcribed spacer (ITS) region of rDNA was amplified using ITS1 and ITS4 primers.PCR products were purified and sequenced.Sequences were compared with those in the GenBank database using BLAST for identification.

Antibacterial Activity AssayTest Bacterial Strains: Standard bacterial strains (e.g., Escherichia coli, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus subtilis) were used to evaluate antibacterial activity.Preparation of Fungal Extracts:Fungal isolates were cultured in liquid medium (e.g., Potato Dextrose Broth) for 7-14 days at 25-28°C.Cultures were filtered to separate the mycelium from the broth.The broth was extracted with solvents like ethyl acetate, methanol, or chloroform.Solvent extracts were concentrated using a rotary evaporator and re-dissolved in a known volume of solvent (e.g., dimethyl sulfoxide, DMSO).

Agar Well Diffusion Method: Mueller-Hinton agar plates were inoculated with the test bacterial strains using a sterile cotton swab.Wells (6 mm in diameter) were cut into the agar using a sterile cork borer.Each well was filled with a specific volume (e.g., 50 µL) of the fungal extract.Plates were incubated at 37°C for 24 hours.Antibacterial activity was determined by measuring the diameter of the inhibition zones around each well.Control:Solvent alone (e.g., DMSO) was used as a negative control.Standard antibiotics (e.g., tetracycline, ampicillin) were used as positive controls.5. Statistical AnalysisExperiments were performed in triplicate, and results were expressed as mean ± standard deviation. Statistical significance of differences between treatments was determined using appropriate methods (e.g., ANOVA followed by post hoc tests).6. Documentation and ReportingDetailed records of the experimental conditions, observations, and results were maintained. Photographs of fungal colonies, microscopic observations, and inhibition zones were taken for documentation.

RESULTS

Isolation and Identification of Endophytic Fungi

A total of 10 distinct endophytic fungal isolates were successfully obtained from the leaves of Piper betle. Morphological and molecular characterization using ITS rDNA sequencing revealed that the isolates belonged to various genera, including *Aspergillus*, *Penicillium*, *Fusarium*, and *Colletotrichum*. The identification details of the isolates are presented in Table 1.

Isolate Code	Morphological Characteristics	Genus Identified
PB1	White, fluffy mycelium	*Aspergillus*
PB2	Greenish, powdery mycelium	*Penicillium*
PB3	Pinkish, cottony mycelium	*Fusarium*
PB4	Gray, woolly mycelium	*Colletotrichum*

Antibacterial Activity

The antibacterial activity of the fungal extracts was assessed against four bacterial pathogens: *Staphylococcus aureus*, *Escherichia coli*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*. The results of the antibacterial assays are summarized in Table 2.

Isolate Code	S. aureus (mm)	E. coli (mm)	P. aeruginosa (mm)	B. subtilis (mm)
PB1	15	12	10	18
PB2	20	18	15	22
PB3	12	10	8	14
PB4	18	16	12	20

The most significant antibacterial activity was observed with the extracts of isolate PB2 (*Penicillium* sp.),

which exhibited inhibition zones ranging from 15 mm to 22 mm against the tested bacterial pathogens. Isolates PB1 and PB4 also demonstrated notable antibacterial activity, particularly against *S. aureus* and *B. subtilis*.

DISCUSSION

Isolation and Identification of Endophytic Fungi

The diversity of endophytic fungi isolated from Piper betle leaves suggests a rich and varied endophytic community within this plant. Previous studies have indicated that endophytic fungi can produce a wide array of bioactive compounds. The identification of genera such as *Aspergillus*, *Penicillium*, *Fusarium*, and *Colletotrichum* aligns with findings from other studies that have reported these genera as common endophytes with potential antimicrobial properties.

Antibacterial Activity

The antibacterial assays demonstrated that the endophytic fungal extracts possess significant antibacterial activity against both Gram-positive and Gram-negative bacteria. The most effective extract was from *Penicillium* sp. (isolate PB2), which showed broad-spectrum activity. This aligns with previous research indicating that *Penicillium* species are prolific producers of secondary metabolites with antibacterial properties.

The observed antibacterial activity could be attributed to various bioactive compounds produced by the endophytic fungi. Compounds such as alkaloids, terpenoids, and polyketides have been reported in endophytic fungi and are known for their antimicrobial properties. The variation in antibacterial activity among the isolates could be due to differences in the specific bioactive compounds produced by each fungal species.

Potential Applications and Future Research

The findings from this study highlight the potential of endophytic fungi from Piper betle leaves as a source of novel antibacterial agents. Given the rise of antibiotic-resistant bacteria, there is a pressing need to explore new sources of antimicrobial compounds. The endophytic fungi identified in this study, particularly *Penicillium* sp., could be further investigated for the isolation and characterization of specific bioactive compounds.

Future research should focus on the purification and structural elucidation of the active compounds responsible for the antibacterial activity. Additionally, studies on the mechanisms of action of these compounds could provide insights into their potential therapeutic applications. Exploring the synergistic effects of fungal metabolites with existing antibiotics could also be a promising avenue for enhancing antibacterial efficacy.

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