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Antibacterial Activity of Endophytic Fungus and Phylogenetic Analysis of *Acinetobacter sp.*

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Abstract: This study investigates the potential of endophytic fungi as sources of antibacterial drugs against *Acinetobacter* species, a group recognized for causing multidrug-resistant nosocomial infections. Soil samples collected from Ulundurpet, Tamil Nadu, India, were used to isolate and characterize *Acinetobacter* species via morphological and 16S rRNA gene sequencing methods. Four endophytic fungal isolates (*Aspergillus welwitschiae*, *Aspergillus indologenus*, *Meyerozyma carpophila*, and *Penicillium citrinum*) previously isolated from the medicinal plant *Abutilon indicum* were tested for antibacterial activity. Using the disk diffusion method, all fungi significantly inhibited *Acinetobacter* growth, with *A. welwitschiae* being the most effective (81.58%). Phylogenetic research revealed genetic variety in *Acinetobacter* strains, shedding light on their evolutionary links and resistance mechanisms. This study demonstrates the potential of endophytic fungi-derived bioactive chemicals as novel antibacterial agents, addressing the important need for alternative treatments for antibiotic-resistant *Acinetobacter* infections. Additional research is needed to isolate and identify the individual bioactive metabolites responsible for the reported antibacterial action.

Keywords: Antibacterial activity, *Acinetobacter*, Endophytic fungal, Phylogenetic analysis

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Introduction

Endophytic fungi are found in numerous plant varieties. A number of species of endophytic fungi may generate a variety of plant-based anti-bacterial compounds. Endophytic fungi produce unique naturally occurring compounds with a wide range of biological functions (Wen *et al.*, 2022). Endophytes are microbial organisms that colonize the inter- and intracellular regions of

plant tissues without producing visible damage and seem to be related to plants of all kinds in natural environments (Nair and Padmavathy, 2014). Fungi, as endophytic microbes, maintain an intimate association with host plants and can create chemicals that increase plant development, productivity, and defense against parasites and diseases (Akram *et al*., 2023). Endophytic fungi have a diverse range of microbial modifications that thrive in unique and unexpected habitats, which makes them an excellent resource for studying and researching novel medications for healthcare, commercial, and agricultural applications (Alam *et al*., 2021).

Natural merchandise researchers are interested in endophytic fungi as a source of anti-bacterial or other active chemicals (Deshmukh *et al*., 2022; Silva *et al.*, 2022). Endophytic bacteria present in medicinal plants have been shown to have an essential role in the generation of pharmaceutically relevant chemicals (Tshikhudo *et al.*, 2023). Investigators discovered that endophytic fungi derived from medicinal vegetation produce high amounts of bactericidal, fungicidal, and cytotoxic compounds (Hashem *et al.*, 2023; Chandra *et al*., 2024).

Acinetobacter is a genus of Gram-negative bacteria classified as Gamma proteobacteria. Bacteria belonging to the genus *Acinetobacter* are abundantly found in the environment (Almasaudi, 2018), and they have garnered greater interest in the past decade because of their ability to inflict significant nosocomial infections (Nocera *et al.*, 2021). *Acinetobacter*, a renowned nosocomial pathogen, can cause significant morbidity and mortality among individuals with chronic underlying illnesses in communities as well as hospitals (Ayoub Moubareck and Hammoudi Halat, 2020). It is typically impervious to all drugs and proliferates most commonly in intubated patients, those with many intravenous lines or monitoring devices, surgical drains, or indwelling urine catheters (Bouhrour *et al*., 2024). The genetic assessment of *Acinetobacter* is truly unpredictable, and typical phenotypic tests are unable to classify species-level strains of *Acinetobacter*, which can be extremely important for clinical applications (Chen *et al.*, 2024). Yet, in recent decades, numerous molecular techniques have proven effective in classifying *Acinetobacter* species.

The objectives of this work were to extract and recognize *Acinetobacter* sp. from soil samples collected in Ulundurpet, Tamil Nadu, India, and to test the antibacterial efficacy of a previously kept endophytic fungus against these pathogenic bacteria.

Materials and Methods

Sample Collection and isolation:

The soil sample was obtained from the location situated near Ulundurpet, Tamil Nadu, India. The sample underwent dilution using the standard serial dilution technique to promote bacterial growth. Upon concluding the incubation period, the colonies were examined. The morphology of isolated colonies was identified using Gram staining as a method.

Extraction and amplification of DNA:

Genomic DNA was isolated from the bacterial colonies using the NucleoSpin® Tissue Kit (Macherey-Nagel) according to the manufacturer's instructions. To generate pure culture biomass for DNA extraction, isolates were inoculated onto fresh nutrient agar plates and incubated at 25°C for 24 h. The DNA sample was stored at -20°C after confirmation using 1% agarose gel electro-phoresis. DNA extraction was completed, and PCR amplification of the 16S rRNA gene was performed using the NucleoSpin® Plant II Kit (Macherey-Nagel). The forward primer utilized was 16S-RS-F 5'CAGGCCTAACACATGCAAGTC3', while the reverse primer was 16S-RS-R 5'GGGCGGWGTG TACAAGGC3' (Paul, 2023). The PCR amplification was carried out using an ABI 3500 DNA analyzer, a PCR thermal cycler (Gene Amp PCR System 9700, Applied Biosystems), and the Big Dye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA). Finally, the sequences acquired in this work

were uploaded to the GenBank database (http://www.ncbi.nlm.nih. gov/), and BLAST was used to compare their similarity to previously published sequences in the GenBank database. To conduct sequence alignment and alteration, Geneious Pro v5.1 was used. The 16S rRNA gene sequencing data was analyzed using the MEGA5 software program (Alrefaei *et al.*, 2022).

Antibacterial activity:

The current study concentrates on endophytic fungi previously isolated from the root segments of *Abutilon indicum* (Haseena *et al.*, 2024). We sourced four endophytic fungi isolates (*A. welwitschiae*, *A. indologenus*, *M. carpophila*, and *P. citrinum*) from Sri Vinayaga College of Arts and Science, Ulundurpet, Tamil Nadu, India, to assess their antibacterial activity using the disk-diffusion method (Balouiri *et al*., 2016) against the isolated bacteria of *Acinetobacter* sp. Endophytic fungi were grown on PDA media at a temperature of 28 °C for a period of 7 days. Suspensions of the bacteria under investigation were prepared from overnight cultures grown in nutrient broth. The turbidity of the suspension was calibrated to 1.5 $\times 10^{-8}$ CFU/ml using 0.5 culture standards, after which 100 µl of the tested bacterial suspension was added to the Nutrient Agar Medium (NA) plates. A sterile swab was utilized to evenly distribute the bacteria being studied on the NA medium. The seeded plates were subjected to a drying period lasting 15 min. Agar plugs with a diameter of 6 mm, obtained from the growing endophytic fungi culture, were placed onto NA plates that had already been inoculated with the test bacteria. A disc measuring 6 mm in diameter was loaded with 100 µl of ampicillin at a concentration of 0.05 mg/ml, which functioned as the positive control, whereas nutrient broth was employed as the negative control. Plates were incubated at 37°C for 48 h, and the inhibition zones surrounding the agar plugs were measured to assess the antibacterial activity of the fungal isolates.

Statistical analysis:

Data were examined utilizing IBM SPSS statistics 20 by one-way analysis of variance (ANOVA) and Tukey's test to ascertain statistical significance. A p-value of less than 0.05 was deemed statistically significant.

Results

Isolation and Characterization of the 16S rRNA amplicons:

The morphological isolation and identification of gram negative rod like bacteria was done from the paddy field soil. Under microscope specimen of soil bacterial colonies appeared pink rod colonies (Fig. 1A). These studies were carried out for morphological identification of the micro-organisms in soil. *Acinetobacter* species are oxidase-negative, exhibit twitching motility, and occur in pairs under magnification.

A 281bp product was obtained by PCR amplification using *Acinetobacter* specific primers from the DNA that was extracted directly from soil samples. These were subjected to 16S rRNA analysis. Well resolved profiles of *Acinetobacter* 16S rRNA gene amplified were obtained as illustrated in Figure 1B. Individual bands were excised, sequenced and identified on the basis of homology with the reference sequences available in the database.

BLAST analysis of isolated 16S rRNA sequence:

In order to confirm the *Acinetobacter* species and its diversity sampled from the soil, the blast search was performed with the isolates in NCBI database as represented in Table 1. For the species 16S rRNA gene sequence matched 100 to 90% to reference species were retrieved from NCBI for phylogenetic analysis.

Phylogenetic analysis of Acinetobacter 16S rRNA sequence:

The isolate that was identified as *Acinetobacter* sp. by using the Maximum Likelihood method was inferred. The optimal tree with the highest log likelihood (-515.4056) is shown. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site. The

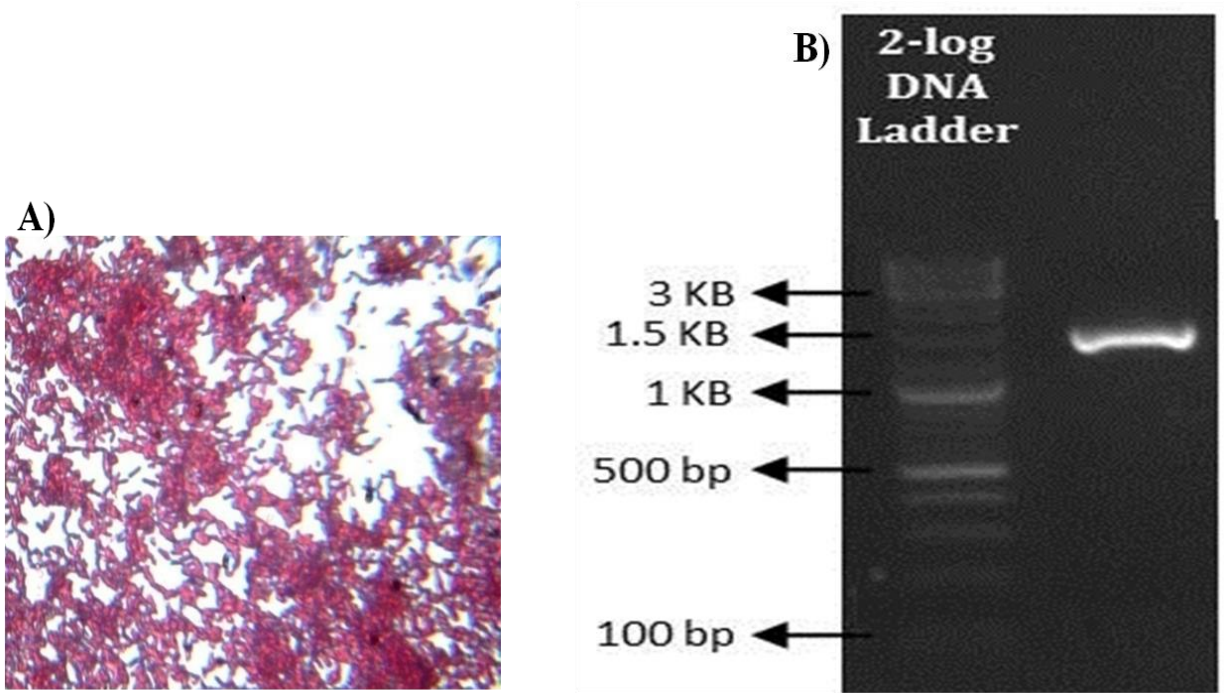

Fig. 1: (A) Morphological structure (Gram's staining); (B) PCR profiles of *Acinetobacter* specific 16S rRNA gene fragments amplified.

Table 1: BLAST matches of GenBank sequences used in the phylogenetic analysis to identify morphotype genus

Strains	Morphotype	Accession No.	Identity (%)	Query coverage (%)	GenBank Sequence Source
SR1773	*Actinobacter sp.*	MN831877	-	-	Present study
NRRL B-41902	*A. lactucae*	NR_152004	98.80	98	Rooney *et al.* (2016)
DR1	*A. oleivorans*	NR_102814	98.80	98	Jung *et al.* (2010)
ATCC 23055	*A. calcoaceticus*	NR_117619	98.80	98	Maslunka (2012)
LMG 1035	*A. pittii*	NR_117930	98.80	98	Nemec *et al.* (2011)
ATCC 17908	*A. junii*	NR_117623	98.20	98	Maslunka (2012)
KNF2022	*A. antiviralis*	NR_115739	98.20	98	Lee *et al.* (2009)
Movanagher 4	*A. movanagherensis*	NR_145841	97.60	98	Moore *et al.* (2014)
DSM 30007	*A. baumannii*	NR_117677	97.60	98	Swiderski (2012)
RUH 2376	*A. nosocomialis*	NR_117931	97.60	98	Nemec *et al.* (2011)
LUH3792	*A. ursingii*	NR_025392	97.60	98	Nemec *et al.* (2001)
LUH 1472	*A. seifertii*	NR_134684	97.60	98	Gundi *et al.* (2009)
AK001	*A. septicus*	NR_116071	97.60	98	Kilic *et al.* (2008)
ATCC 31012	*A. venetianus*	NR_042049	97.01	98	De Baere (2000)
5YN5-8	*A. brisouii*	NR_115871	97.01	98	Anandham *et al.* (2010)
58a	*A. beijerinckii*	NR_042234	97.01	98	De Baere (2004)
ATCC 17909	*A. johnsonii*	NR_164627	96.41	98	Rawat (2019)
ANC 4149	*A. pragensis*	NR_152069	96.41	98	Radolfova-Krizova *et al.* (2016)
JCM 6840	*A. lwoffii*	NR_113346	96.41	98	Kosako *et al.* (2014)
A648	*A. indicus*	NR_117784	96.41	98	Malhotra *et al. (*2012)
DSM 14970	*A. tandoii*	NR_117508	96.41	98	Lee (2010)

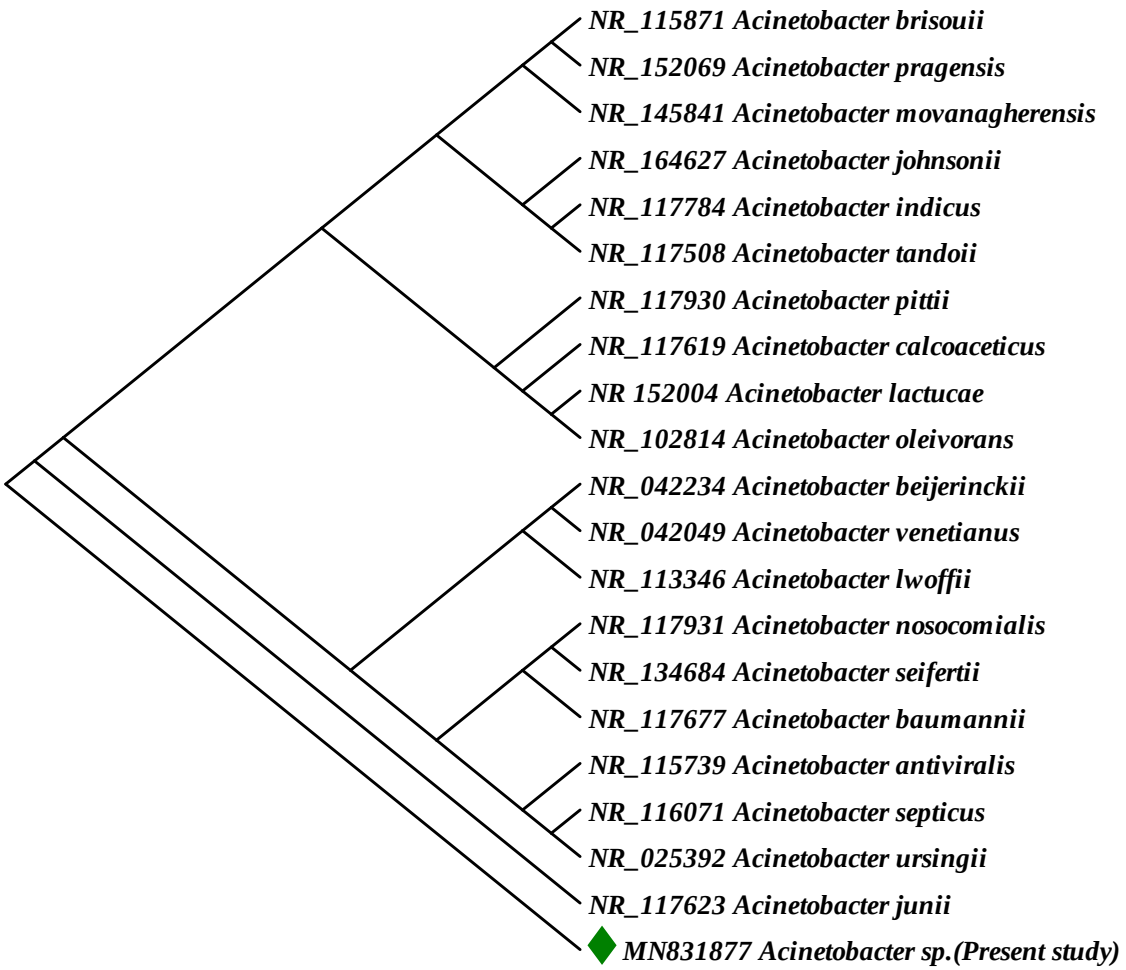

Fig. 2: Molecular Phylogenetic analysis of *Acinetobacter sp*. with similarity sequences of BLAST in NCBI database by Maximum Likelihood method.

analysis involved 21 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 245 positions in the final dataset.

The phylogenetic analysis revealed that most of the *Acinetobacter* strains was obtained from the studied isolates. The phylogenetic analysis of strain of *Acinetobacter* revealed that there was great diversity within the strains of *A. junii* (96.76 % similarity). Whereas the present study using *Acinetobacter* sp. shows different clades diversity within same genus are *A. ursingii*, *A. baumannii*, *A. johnsonii*, *A. septicus*, *A. brisouii*, *A. beijerinckii*, *A. venetianus* and *A. lwoffii*. Although, *Acinetobacter* sp. 16S rRNA gene showed similarity within the group genus of species as represented in Figure 2.

The 16S rRNA gene substitution matrix was estimated within the similarity sequences. From that the *Acinetobacter* sp., the rates of different transitional substitutions were shown in nucleotide and those of transversional substitutions having the relative values of instantaneous nucleotide frequency was evaluated (Table 2). Substitution pattern and rates were estimated under the Tamura-Nei model. Relative values of instantaneous r should be considered when evaluating them. For simplicity, sum of r values is made equal to 100, the nucleotide frequencies are A = 25.83%, T/U = 19.24%, C = 21.57%, and G =

Table 2: Maximum Likelihood Estimate of Substitution Matrix of *Acinetobacter sp*. 16S rRNA sequences

	A	T/U	C	G
A	-	2.28	2.56	17.66
T/U	3.06	-	23.76	3.95
C	3.06	21.20	-	3.95
G	13.68	2.28	2.56	-

Table 3: Antibacterial activity of the four endophytic fungi compared to a standard antibiotic (ampicillin)

Isolate	**Endophytic fungus with Ampicillin (Control) sample**				
	A. welwitschiae	*A. indologenus*	*M. carpophila*	*P. citrinum*	Ampicillin
Acinetobacter sp.	14.32±1.31	13.01±1.06	12.05±0.64	11.63±0.25	14.85±1.20
% of inhibition	81.58%	72.76%	66.29%	63.46%	100%

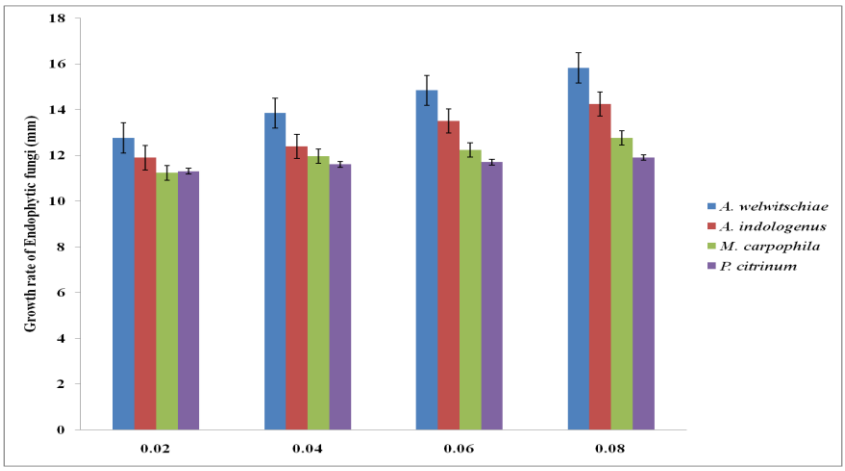

Fig. 3: Antibacterial activity of four endophytic fungi against isolated *Acinetobacter sp*.

33.35% as shown in Table 2. For estimating ML values, a user-specified toplogy was used. The maximum Log likelihood for this computation was -515.406, which was in a total of 245 positions.

Antibacterial activity of Endophytic fungi:

The extract of endophytic fungi was evaluated on isolated *Acinetobacter* sp. Testing with antibiotics revealed that all four endophytic fungi effectively inhibited the growth of *Acinetobacter sp*. Nonetheless, the endophytic fungus *A. welwitschiae* demonstrated the highest growth rate inhibition at 81.58%, with an average mean of 14.32±1.31. This was followed by *A. indologenus* at 72.76% (13.01±1.06), *M. carpophila* at 66.29% (12.05±0.64), and *P. citrinum* at 63.46% (11.63±0.25), as illustrated in Table 3. However, observed significant variances ($p > 0.05$) between the four endophytes and growth rate were found to be highest at all the concentrations of *A. welwitschiae* those tested against *Acinetobacter* sp. (Fig. 3).

Discussion

The present study indicates that the endophytic fungus extracted from the root segments of *Abutilon indicum* has significant antibacterial properties against *Acinetobacter* sp. The inhibitory zone noted in the agar diffusion assay indicates that the secondary metabolites generated by the fungus have significant antibiotic efficacy. The metabolites may damage the cell membrane

integrity of *Acinetobacter* or inhibit certain bacterial enzymes involved in cell wall formation (Vaou *et al*., 2021). However, further bioassay-guided fractionation is required to identify the active chemicals (Parmanik *et al.*, 2022). Many earlier studies have shown that endophytic fungi, such as *Aspergillus*, *Penicillium*, and *Trichoderma*, can kill different types of bacteria, including Gram-negative and Gram-positive bacteria like *Acinetobacter* (Fadiji and Babalola, 2020; Yadav and Meena, 2021; Singh and Kumar, 2023). It is believed that endophytes' antibacterial properties originate from their symbiotic relationship with the host plant, which could exert selection pressure on the production of bioactive chemicals (Wu *et al.*, 2021). The development of new and effective drugs from endophytic fungi is particularly intriguing for *Acinetobacter*, recognized for its multi-drug resistance, as it provides an alternative to traditional antibiotics (Silva *et al*., 2022).

Using 16S rRNA gene sequencing to look into the phylogeny of *Acinetobacter* strains found several groups of related species, proving that this genus has a lot of genetic diversity (Sachdev *et al.*, 2010). The phylogenetic tree revealed a close relationship between *A. baumannii* strains and other *Acinetobacter* species, such as *Acinetobacter pittii* and *Acinetobacter nosocomialis*, both of which are known for their opportunistic infections (Adjei *et al*., 2021; Yaikhan *et al*., 2024). This finding fits with earlier research which showed that there is a large group of genetically related *Acinetobacter* species. Some of these species are very contagious and are known for causing illnesses that people get in hospitals. The study showed that some *Acinetobacter* strains have a lot of genetic differences, which could affect how pathogenic they are, how long they last in different environments, and how resistant they are (Nocera *et al*., 2021). Some strains in the same evolutionary group may share similar virulence factors, antibiotic resistance genes, or environmental adaptation processes (Larsson and Flach, 2022). This could help explain why their clinical effects and antimicrobial resistance are different. The genetic variety among *Acinetobacter* species presents considerable obstacles to infection treatment, as the establishment of resistant variants can hinder therapeutic approaches (Vázquez-López *et al*., 2020).

This study highlights the significance of investigating endophytic fungus as a potential source of innovative antimicrobial drugs, especially given the increasing menace of antibiotic-resistant bacteria like *Acinetobacter*. Numerous bioactive metabolites generated by endophytic fungi remain underexplored for their potential as antibacterial agents (Deshmukh *et al*., 2022). Because current antibiotics do not work well enough against *Acinetobacter* species, especially multidrug-resistant variants, we need to find new antibiotics with different ways of working right away (Kyriakidis *et al.*, 2021). The extraction and identification of bioactive substances from endophytes may serve as an effective approach to mitigate this deficiency.

The phylogenetic research of *Acinetobacter* not only identifies possible treatment medicines but also offers significant insights on the evolution of this species, its ecological distribution, and its pathogenic mechanisms (Singh *et al.*, 2024). Knowing the genetic connections and resistance patterns of different *Acinetobacter* strains can help doctors make better treatments and make medicines that are more effective against the specific strains that cause infections (Moosavian *et al.*, 2020). Future research must address some limitations as this study provides significant insights into the antibacterial properties of endophytic fungi and the genetic variety of *Acinetobacter* strains (Kandasamy and Kathirvel, 2023; Hyde *et al.*, 2024). We initially assessed the antibacterial activity using a crude extract of fungal metabolites, which requires subsequent research to separate and identify the individual bioactive components responsible for the observed antimicrobial activities. Second, the phylogenetic analysis only used a small number of *Acinetobacter* strains. Adding more clinical isolates to the dataset might give us a better

picture of genetic diversity and possible toxicity. This study adds to the evidence that endophytic fungi can be a source of new antibiotics. It also gives us important information about the genetic makeup of *Acinetobacter* species, which are clinically important because they are resistant to many drugs.

Conclusion

This study underscores the promise of endophytic fungus as a source of novel antimicrobial drugs targeting *Acinetobacter* species, which are progressively resistant to traditional antibiotics. The phylogenetic investigation of *Acinetobacter* strains demonstrates significant genetic diversity and resistance patterns that affect their clinical management. Further research is required to extract and identify the bioactive chemicals from endophytic fungus, and to explore their potential as a component of a comprehensive strategy to address antibiotic resistance in *Acinetobacter* and other pathogenic bacteria.

Ethical Statement

Not applicable for this study.

Author Contributions

M.H. and *A.S*: Conceptualization; methodology; investigation; formal analysis; visualization; writing - original draft; writing - review and editing; *G.S.*: Investigation; formal analysis; Writing - review and editing; *A.F.M.*: Visualization; writing - review and editing. All authors have read and agreed to the published version of the manuscript. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no conflicts of interest.

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