



Antibacterial Activity of Fungal Endophytes Isolated from Kratom (*Mitragyna speciosa*) in Thailand

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Abstract: Kratom (*Mitragyna speciosa*), which is an indigenous plant in Asia, has been used as traditional medicine for decades. This study focuses on fungal endophytes originally isolated from kratom and their antibacterial activity. Kratom plants were obtained from seven locations in Thailand. In each location, three plant parts, including leaves, stems, and roots, were collected. The plant materials were surface-sterilized and isolated for endophytic fungi on PDA amended with kanamycin. A total of 347 isolates of fungal endophytes were isolated, and 92 isolates were randomly selected for antibacterial activity against *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. They were cultured in 50 ml of potato dextrose broth (PDB) at 25°C for 4 weeks, and the culture broth was tested against 4 bacteria using an agar well diffusion method. 11 isolates (12%) inhibited at least one bacterial species. Eight endophytes inhibited *B. subtilis*, while four, three, and one endophytes inhibited the other three bacteria, respectively. Six isolates of fungal endophytes were chosen for further screening. They were cultured in 2 L of PDB and incubated at 25°C for four weeks. After mycelium was separated, only culture broth was extracted three times with ethyl acetate using rotary evaporation, and the extracts were dissolved in DMSO before testing. Antibacterial activity was determined by disk diffusion, and inhibition zones were recorded. One crude extract (L1-01) partially inhibited *E. coli* (8.28 mm). In the three crude extract tests against *S. aureus*, L5-10 exhibited the highest activity (17.51 mm). Among four crude extracts inhibiting *B. subtilis*, R5-09 had the strongest activity (21.3 mm). These two fungal endophyte isolates (R5-09 and L5-10) were identified based on morphological and molecular characteristics and assigned to *Aspergillus allahabadii* and *Aspergillus terreus*, respectively. This finding emphasized that fungal endophytes from kratom have potential as a source of antibacterial substances and should be further studied for other bioactivities.

Keywords: Antibacterial; *Aspergillus*; fungal endophytes; *Mitragyna speciosa*

1. Introduction

Kratom (*Mitragyna speciosa*), a tropical tree native to Southeast Asia, is found in countries such as Thailand, Malaysia, Indonesia, and Myanmar [1]. In many Asian countries, it has been used in traditional medicine for centuries. It has gained attention for its psychoactive properties. The primary active compound, Mitragynine, is involved in pain relief and mood regulation; therefore, it is used for pain management, mood enhancement, and relief from anxiety or stress [2]. In many Western countries, especially the US, consumption of kratom has been growing substantially since the 2000s. An estimated 1.7

million Americans used kratom in 2021 [3]. In Thailand, kratom was removed from the narcotics list in 2021 and is now legally permitted for cultivation and research purposes. However, certain restrictions remain regarding its sale, advertising, and use among specific groups. Therefore, kratom samples used in this study were collected in accordance with Thai regulations.

Antibiotic resistance is characterized by the ability of pathogenic microbes to resist the effects of medications that once effectively treated infections, and it is a devastating and growing public health concern [4]. The discovery of new antibiotics is one of the most pressing challenges in modern medicine. The pace of discovering new antibiotics has slowed over the past few decades. However, antibiotic resistance has risen dramatically. Given this growing crisis, researchers worldwide are exploring a range of approaches to discover novel antibiotics. Addressing antibiotic resistance is urgent and requires coordinated efforts across multiple sectors, and finding alternative antibacterial substances is needed.

Endophytic fungi are a group of fungi that live asymptotically within the tissues of plants. They can be found in various plant species and often form a mutualistic relationship with their host plant. Various endophytic fungi originally isolated from medicinal plants produce secondary metabolites with antimicrobial, antiviral, or anticancer properties, making them of interest to the pharmaceutical and industrial sectors [5-9]. However, studies on fungal endophytes associated with kratom remain scarce. Currently available information is primarily limited to university-level theses [10, 11], while peer-reviewed publications in scientific journals are still lacking. The objectives of this study were (1) to isolate endophytic fungi from kratom collected in Thailand and (2) to screen for their antibacterial activity against four bacterial species.

2. Materials and Methods

2.1 Isolation of fungal endophytes

Samples of kratom were collected from seven locations in Chachoengsao Province, located in the eastern part of Thailand. The kratom plants used in this study were cultivated by residents and collected from residential areas and household surroundings, which were predominantly surrounded by rice fields and agricultural land. Three plant parts of kratom, including leaves, stems, and roots, were collected, and the samples were brought back to the lab for isolation within 48 hours. Healthy and symptomless plant materials were used in this study. Plant materials were rinsed with tap water to remove dirt and then dried at room temperature for 1 hour in a biosafety cabinet. Leaves were cut into 10 pieces (1x1 cm²) while stems and roots were cut into 10 pieces (1 cm). They were surface-sterilized using a method modified from that described by Singwongwatana [10]. The plant materials were submerged in 95% ethanol for 1 minute, then in 5% sodium hypochlorite for 5 minutes, then in 95% ethanol for 30 seconds, and finally rinsed twice in sterile distilled water for 1 minute. The effectiveness of surface sterilization was verified by plating the final sterile rinse water onto PDA to confirm the absence of epiphytic microbial contamination. Sterile plant samples were placed on potato dextrose agar (PDA) amended with kanamycin (50 mg/L) (5 pieces/plate). PDA plates were incubated at 25°C for 7 days. After fungal hyphae were observed in plant materials, they were transferred to new PDA without antibiotics, and pure cultures of endophytes were maintained in PDA slants at 4°C throughout the study.

2.2 Determination of antibacterial activity

2.2.1 Agar well diffusion with culture broth

Endophytes were cultured in 250-ml flasks containing 50 ml of potato dextrose broth (PDB). The flasks were incubated at 25°C for four weeks. Their antibacterial activity was tested against four bacterial species: *Bacillus subtilis*, *Staphylococcus aureus*, *Escherichia coli*, and *Pseudomonas aeruginosa*. Culture broths of the fungal endophytes were screened using an agar well diffusion method. Inocula were adjusted to 0.5 McFarland Standard (equivalent to 1.5×10^8 CFU/ml) using sterile normal saline solution (NSS). The inoculum was swapped onto Mueller-Hinton agar (MHA) using a sterile cotton swab. A seeded agar plate was punctured with a 3 (6 mm) cork borer, and 30 µl of 4-week culture broth of fungal endophytes was added to each well. The experiment was done in triplicate. After incubation at 37°C for 18 hours, the inhibition zones (diameter in millimeters) were observed, measured, and recorded. The spectrum of antimicrobial activity was the primary criterion for selecting endophytic fungal isolates, while the size of the inhibition zone was a secondary criterion.

2.2.2 Disk diffusion with crude extracts

Potential endophytes were cultured in 500 flasks containing 250 ml of PDB, with 8 replicates (8 flasks/isolate). The flasks were incubated at 25°C under stationary conditions, as static cultivation may promote the production of specific secondary metabolite profiles. After 4 weeks, the culture broth from each isolate was pooled for chemical extraction. To separate the culture broth from the mycelia, the broth was filtered through a sterile Whatman filter paper (No. 1, 90 mm in diameter). The culture broths were extracted three times with ethyl acetate and evaporated to dryness using a rotary evaporator at 45°C. All extracts were kept at room temperature. Extracts were dissolved in DMSO (1% v/v) at a concentration of 1 g/ml and stored as a stock solution. A similar method was used for inoculum preparation as previously described in an agar well diffusion method. A paper disk (Whatman No. 1, 6 mm in diameter) was placed on the surface of the MHA seeded with the test bacteria. A total of 10 µl of the extract was pipetted onto the disk, resulting in 10 mg/disk, and 10 µl of DMSO (1% v/v) was used as a control. The plates were incubated using the same conditions mentioned earlier. Inhibition zones were observed and recorded.

2.3 Identification of potential fungal endophytes

2.3.1 Morphological identification

Fungal endophytes that exhibited antibacterial activity were identified based on their morphological characteristics. They were cultured on PDA and incubated at 25°C for two weeks. Their colony appearances were observed and photographed. Colors of fungal colonies were measured and rated using the Online Auction Color Chart (OAC), and a slide culture technique was used to induce sporulation and observe some fungal reproductive structures. The fungi were stained with lactophenol-cotton blue and studied under a microscope.

2.3.2 Molecular identification

Molecular identification of the potential fungal endophytes was based on sequence analyses of the internal transcribed spacer region (ITS). The method follows our previous study with some modifications [12]. Their genomic DNA was extracted using a CTAB method. PCR master mix (50 µl reaction volume) contains 10X buffer, 2.5 mM MgCl₂, 0.2 mM dNTPs, 0.2 µM of each primer (ITS5 and ITS4), and 1 U Taq DNA polymerase (Vivantis Technologies). Their ITS region was amplified using primers ITS5/ITS4 using following PCR condition (1) preheat 96°C (2 min) (2) denature 96°C (1 min) (3) annealing 53°C (1 min) (4) extension 72°C (1 min 30 s), (5) repeat of (2)-(4) for 35 cycles (6) final extension 72°C (10 min) (7) final hold 15°C [13]. PCR products were purified and sequenced in both directions by Macrogen Inc., Korea. The DNA sequences of endophytes were compared with sequences deposited in the GenBank database, and the sequences with the highest similarity values were downloaded. The dataset was aligned using BioEdit v.7.2.5 and MEGA v.11.0.13. Phylogenetic analyses were performed using the Maximum Likelihood (ML) and Maximum Parsimony (MP) methods in PAUP v4.0a169, and two statistical supports, ML bootstrap (MLBS) and MP bootstrap (MPBS), were calculated. The phylogenetic tree was constructed and edited using TreeView v0.5.0. The sequences of these fungal endophytes were deposited in the GenBank Database, where they were assigned accession numbers PX715484 and PX715485.

2.4 Statistical analysis

The results for antibacterial activity of culture broth and crude extracts were expressed as the mean ± standard deviation ($\bar{X} \pm SD$) of 3 replicates (3 Petri plates).

3. Results and Discussion

3.1 Sources and diversity of endophytic fungi from kratom

From seven locations, three plant parts (leaves, stems, and roots), and ten plant materials from each plant part, there were 210 pieces of plant material. A total of 347 isolates of fungal endophytes were recovered from this study. The recovery rate was 1.6 isolates per plant piece. Considering plant parts, there were 170, 95, and 82 isolates originally from leaves, stems, and roots, respectively. Therefore, 51, 21, and 20 endophyte isolates were randomly selected based on morphotype for further study. Therefore, 92 endophyte isolates were screened for antibacterial activity. Some of the different morphotypes of endophytes are presented in Figure 1.

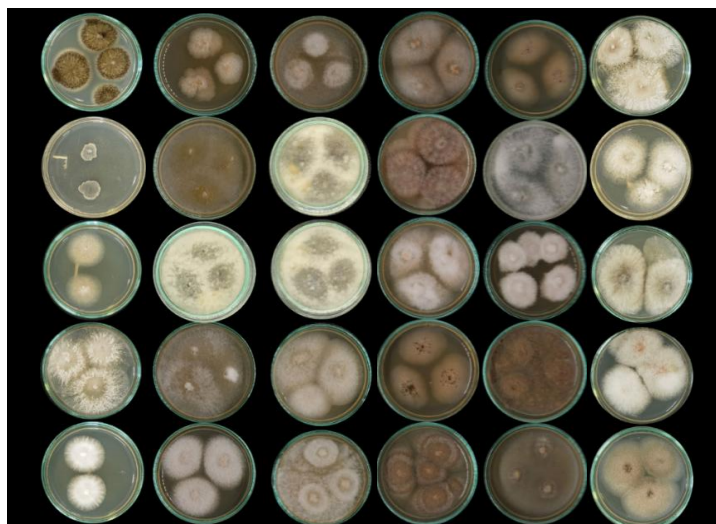


Figure 1. A sample of fungal endophytes originally isolated from kratom (*Mitragyna speciosa*) in Thailand. A diverse and wide range of colony appearances of endophytes were obtained. The endophytes were cultured on PDA and incubated at 25°C for 7 days.

Although kratom has been extensively studied on its pharmaceutical and medicinal properties, there is little information on fungal endophytes originally isolated from this herbal plant. In a previous study conducted in Indonesia, 43 fungal endophyte isolates from kratom twigs and leaves showed higher diversity than those from leaves [11]. In this study, however, leaves exhibit greater endophyte diversity than stems and roots. A total of 170 isolates were obtained from leaves, while 95 and 82 isolates were obtained from stems and roots, respectively. Our results emphasize that leaves are a major source of endophytes and that fungal endophytes have great diversity awaiting further discovery. The majority of research related to kratom mostly focuses on the chemical properties of natural products extracted directly from this herbal plant. Despite being recognized as an important source of bioactive compounds, fungal endophytes of kratom have been overlooked and underestimated. This finding also expands the knowledge of kratom and its fungal diversity. Other plant parts of kratom, for example, the flower, bark, and trunk, should be studied for fungal endophytes, which might lead to further discoveries of greater diversity.

3.2 Antibacterial activity

3.2.1 Antibacterial activity from culture broth

Endophytic fungal isolates were incubated for 4 weeks due to variations in growth rate. While some fungi grew rapidly, others grew more slowly. The extended cultivation period was therefore intended to ensure complete fungal development and to facilitate the production of diverse secondary metabolites. When a 4-week-old culture broth of endophytes was screened for antibacterial activity, three samples from three isolates (L1-01, L1-04, and S1-01) inhibited *E. coli*, with inhibition zones of 7.53, 8.63, and 7.56 mm, respectively (Table 1 and Figure 2). While only one sample (S1-01) also inhibited *P. aeruginosa*, with a weaker inhibition zone of 6.74 mm. Four samples demonstrated antibacterial activity against *S. aureus*. They include L5-10, S5-10, R5-09, and L6-20, and the inhibition zones were 11.13, 17.13, 15.63, and 15.12 mm, respectively. Eight samples inhibited *B. subtilis*, with inhibition zones ranging from 8.78 to 18.42 mm; sample R5-09 showed the strongest activity.

3.2.2 Antibacterial activity from chemical extracts

Only six isolates of fungal endophytes were chosen for further screening. They were cultured on PDB, and the culture broth was extracted with ethyl acetate. Six crude extracts were obtained from the culture broth of these endophytes (Table 2). Antibacterial activity against *P. aeruginosa* was not determined due to low activity. Only one extract from Isolate L1-01 showed partial inhibitory activity against *E. coli* (8.28 mm in diameter). Four extracts showed antibacterial activity against *B. subtilis*, with one extract from Isolate R5-09

exhibiting the highest activity (21.3 mm in diameter; Figure 3). Three crude extracts inhibited *S. aureus*, while one extract from Isolate L5-10 produced the highest activity toward *S. aureus* (17.51 mm in diameter).

Table 1. Antibacterial activity of culture broth of fungal endophytes isolated from kratom

Isolate	Inhibition zone (diameter in mm±SD)			
	<i>B. subtilis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
L1-01	–	–	7.53 ± 0.00	–
L1-04	–	–	8.63 ± 0.00	–
S1-01	–	–	7.56 ± 0.00	6.74 ± 0.09
R3-14	8.78 ± 0.98	–	–	–
S4-12	12.53 ± 0.00	–	–	–
L5-10	14.99 ± 0.75	11.13 ± 0.41	–	–
L5-13	11.07 ± 0.82	–	–	–
S5-10	16.87 ± 0.12	17.13 ± 0.19	–	–
R5-09	18.42 ± 0.45	15.63 ± 0.29	–	–
L6-20	16.60 ± 0.82	–	–	–
R6-04	17.06 ± 0.12	15.12 ± 0.76	–	–
PDB (Control)	0.00	0.00	0.00	0.00

Note –, No activity

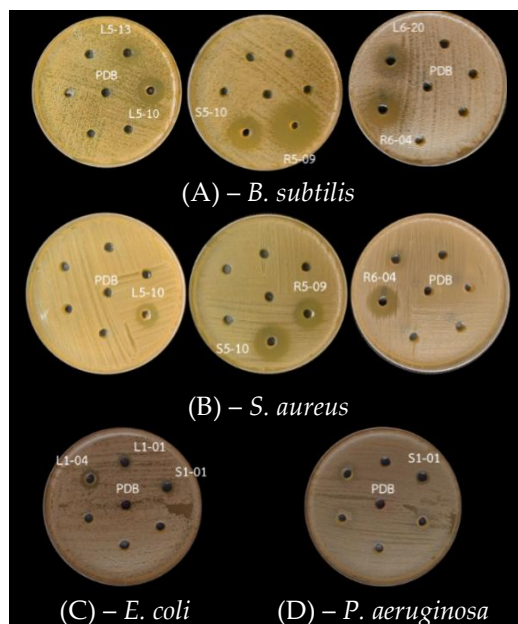


Figure 2. Antibacterial activity against four bacteria using the agar well diffusion method. (A) – *Bacillus subtilis*, (B) – *Staphylococcus aureus*, (C) – *Escherichia coli* and (D): *Pseudomonas aeruginosa*. The test bacteria were cultured on MHA and incubated at 37°C for 18 hours.

Table 2. Antibacterial activity of ethyl acetate crude extracts from fungal endophytes isolated from kratom

Extract	Inhibition zone (diameter in mm±SD)			
	<i>B. subtilis</i>	<i>S. aureus</i>	<i>E. coli</i>	<i>P. aeruginosa</i>
L1-01	ND	ND	8.28 ± 0.15	ND
R3-14	13.5 ± 0.55	ND	ND	ND
L5-10	13.85 ± 0.25	17.51 ± 0.75	ND	ND
R5-09	21.3 ± 0.91	14.1 ± 0.10	ND	ND
R6-04	8.14 ± 0.74	8.23 ± 0.91	ND	ND
DMSO (Control)	0.00	0.00	0.00	ND

ND = Not determined

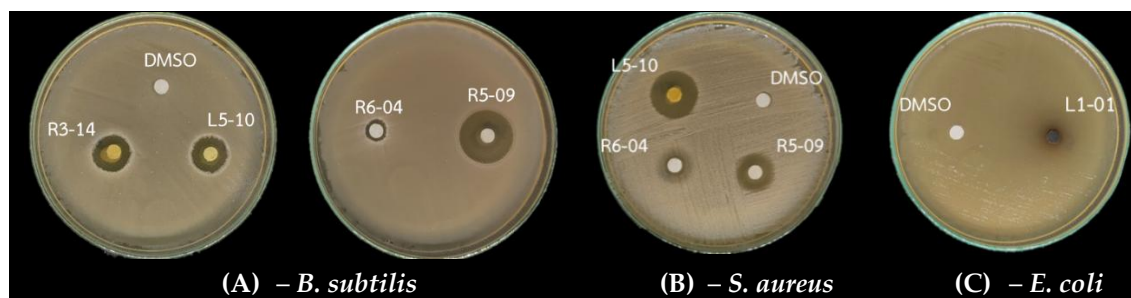


Figure 3. Antibacterial activity against four bacteria using the disk diffusion method: (A) – *Bacillus subtilis*, (B) – *Staphylococcus aureus*, and (C) – *Escherichia coli*. The test bacteria were cultured on MHA and incubated at 37°C for 18 hours.

Most of the fungal endophytes tested in this study failed to inhibit *E. coli* and *P. aeruginosa*, two representatives of Gram-negative bacteria. However, numerous fungal endophyte isolates inhibited *B. subtilis* and *S. aureus*, with wide inhibition zones. Antibacterial activity of 65 endophyte isolates from *Indigofera suffruticosa* leaves was tested against five bacteria, including *B. subtilis*, *S. aureus*, *E. coli*, *K. pneumoniae*, and *P. aeruginosa* [14]. Only one fungal endophyte inhibited *P. aeruginosa*, while the majority of endophytes inhibited the other four bacteria. It clearly demonstrates that Gram-negative bacteria are more resistant to antibiotics than Gram-positive bacteria. Gram-negative bacteria possess an outer membrane (OM) that serves as an effective permeability barrier, contributing to their greater antibiotic resistance compared with Gram-positive bacteria [15]. For antimicrobial agents to exert their activity, they must penetrate this outer membrane to reach intracellular targets. Gram-negative bacteria can further enhance resistance through several mechanisms, including porin modification, alterations in membrane hydrophobicity and lipopolysaccharide (LPS) composition, efflux pumps, and enzymatic inactivation of antimicrobial compounds. In contrast, Gram-positive bacteria lack this additional outer membrane, making them generally more susceptible to antibacterial agents.

Antibacterial activities of fungal endophytes against Gram-positive bacteria, including *B. subtilis* and *S. aureus*, have been widely reported. Among numerous fungal endophyte species isolated from *Dendrobium devonianum* and *D. thyrsiflorum*, the endophytic fungus *Phoma* sp. demonstrated the strongest activity against *B. subtilis* [16]. Three compounds extracted from the culture broth of *Phomopsis* sp., an endophytic fungus of *Garcinia kola*, inhibited *S. aureus* ATCC 25923 [17]. An extract obtained from *Penicillium brevicompactum*, an endophytic fungus isolated from *Abies numidica*, exhibited inhibitory effect against various strains of *S. aureus*, including *S. aureus* ATCC25923 and five other strains of multidrug-resistant *S. aureus* [18]. Metabolites from *Xylaria grammica*, an endophyte isolated from *Urtica fissa*, also inhibited *S. aureus* by inducing morphological deformations in the cell surface and enhancing cell membrane permeability, leading to cytoplasmic leakage [19]. Our findings are consistent with these previous reports and thus confirm that fungal endophytes are a good source of antibacterial compounds. Ethyl acetate was chosen as a solvent for chemical extraction due to its many advantages. It has moderate polarity, with good solubility properties for a wide range of natural products, particularly secondary metabolites. Ethyl acetate is considered less toxic and safer than other solvents, and it also has a lower boiling point; therefore, it allows the extraction of compounds without excessive heat that might degrade sensitive natural products. Among ten publications related to crude extracts of endophytes, ethyl acetate was the most used solvent, with eight publications [20]. Meanwhile, other solvents such as butanol, methanol, and ethanol should be used for further studies, as they may lead to the discovery of wider classes of natural products.

3.3 Identification of potential fungal endophytes

3.3.1 Morphological identification (Figure 4)

The two isolates of potential fungal endophytes (L5-10 and R5-09) were identified based on morphological features and molecular data. Based on morphological studies, they grew rapidly on PDA and showed considerable differences in colony appearance and mycelial pigmentation on the medium. Under the microscope, they all produced conidiophores. Their conidiophores were unbranched stipes with a swollen apex (vesicle) and phialides borne directly on the vesicle. Conidia were globose and formed in dry chains

attached to phialides. These are common features for the genus *Aspergillus*. Therefore, these two isolates were identified at the generic level as *Aspergillus* species.

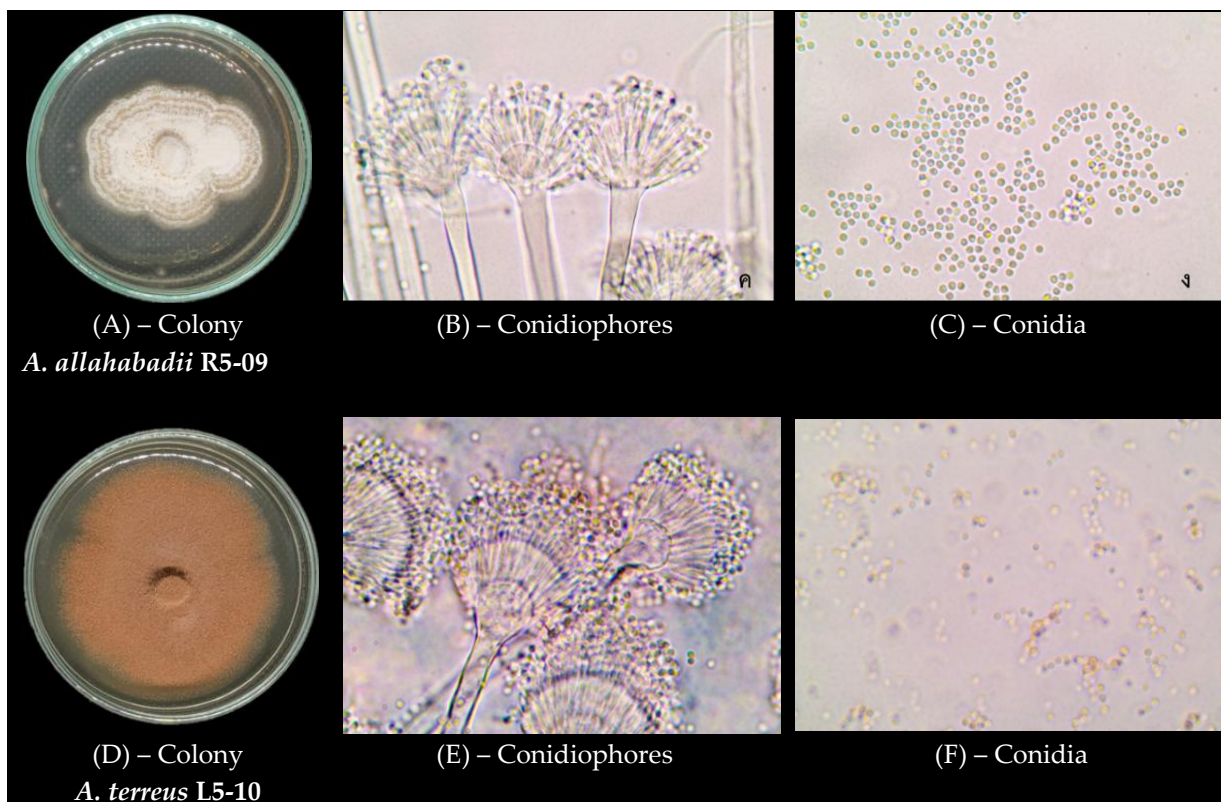


Figure 4. Morphological characteristics of two potential fungal endophytes from kratom. They were cultured on PDA incubated at 25°C for 7 days. *Aspergillus allahabadii* R5-09 (upper row). (A) – Colony on PDA, (B) – Conidiophores and (C) – Conidia.; *Aspergillus terreus* L5-10 (lower row), (D) – Colony on PDA, (E) – Conidiophores and (F) – Conidia.

Description of Aspergillus allahabadii R5-09

Macroscopic feature: Colony diameter in 32.3 mm on PDA for 7 days at 25°C (Figure 4 A-C). Surface: Concentric, slightly raised at center, velvety texture. Colony form: Irregular margin with concentric rings. Colony color: White to pale. Microscopic feature: Mycelium: Hyaline hypha, unbranched conidiophore, Conidia: Globose, hyaline to light green, smooth.

Description of Aspergillus terreus L5-10

Macroscopic feature: Colony diameter in 43.4 mm on PDA for 7 days at 25°C (Figure 4 D-F). Surface: Smooth and flat. Colony form: Circular with a margin. Colony color: Dark orange to light brown. Microscopic feature: Mycelium: Hyaline hypha, hyaline conidiospore or slightly coloured. Conidia: Globose to ellipsoidal, smooth, translucent, and orange pigment, hyaline to light yellow.

3.3.2 Molecular identification

The two most promising endophytes (R5-09 and L5-10) were identified using sequence analyses of the internal transcribed spacer (ITS) region. *Penicillium digitatum* NR176692 was used as the outgroup (Figure 5). Eleven species of *Aspergillus* were included in this analysis. The first endophyte R5-09 is closely related to five sequences of *Aspergillus allahabadii*. This species also forms a sister clade with *A. niveus* and *A. neoniveus* with high statistical support (93 MLBS and 80 MPBS). The second endophyte, L5-10, is closely related to six *A. terreus* sequences, with high statistical support (100 MLBS, 100 MPBS). In comparison, other *Aspergillus* species, including *A. flavus*, *A. iranicus*, *A. nidulans*, *A. niger*, *A. oryzae*, and *A. parasiticus*, are placed in separate clades with strong statistical support. This clearly demonstrates that the two candidate fungal endophytes are identified as *A. allahabadii* and *A. terreus*.

Aspergillus is one of the most common filamentous fungi in the Ascomycota, and it is commonly found across various substrates and habitats. The genus *Aspergillus* is among the most studied endophytes and has been reported to produce a wide range of bioactive compounds, particularly antibacterial substances [21, 22]. *Aspergillus terreus* is commonly found worldwide, especially in terrestrial and aquatic habitats. An ethyl acetate extract from an endophytic fungus, *A. terreus*, originally isolated from a leaf of *Psidium guajava*, was reported to have antibacterial activity against numerous bacterial species, including *Bacillus sphaericus*, *Enterobacter aerogenes*, *E. coli*, and *S. aureus* [23]. This fungus is regarded as one of the most promising sources of bioactive compounds because it can produce diverse secondary metabolites with various applications in medical, industrial, and biotechnological contexts [24]. *Aspergillus allahabadii* is also commonly found in various substrates, ranging from decomposer organisms in soil to stone, because it can adapt to hostile environments. Our finding is consistent with a previous study, which reported that *A. allahabadii*, an endophytic fungus originally isolated from the root of *Cinnamomum subavenium*, exhibited bacterial activity against *Bacillus cereus* [25]. *A. allahabadii* has been reported to possess a wide range of biological activities, including antimicrobial, antioxidant, antidiabetic, and mosquito larvicidal properties, while exhibiting no detectable cytotoxicity [26]. In this study, *A. allahabadii* exhibited antibacterial activity against *B. subtilis* and *S. aureus*, expanding knowledge of this fungus's bioactivity. These findings emphasize that endophytic fungi are among the most important sources of bioactive compounds with potential therapeutic and biomedical applications.

4. Conclusions

Kratom (*Mitragyna speciosa*) is a medicinal plant used in traditional medicine, and a diverse group of fungal endophytes was isolated from kratom. The fungal endophytes were cultured in potato dextrose broth, and the culture broth and chemical extracts were tested for antibacterial activity using agar well diffusion and disk diffusion methods, respectively. Two fungal endophyte isolates exhibited strong antibacterial activity against *Bacillus subtilis* and *Staphylococcus aureus*. The two most promising fungi were identified based on morphological and molecular studies and were identified as *Aspergillus allahabadii* and *Aspergillus terreus*. Additionally, *A. allahabadii* is a relatively understudied endophytic fungus, and this study highlights its potential as a source of antibacterial activity.

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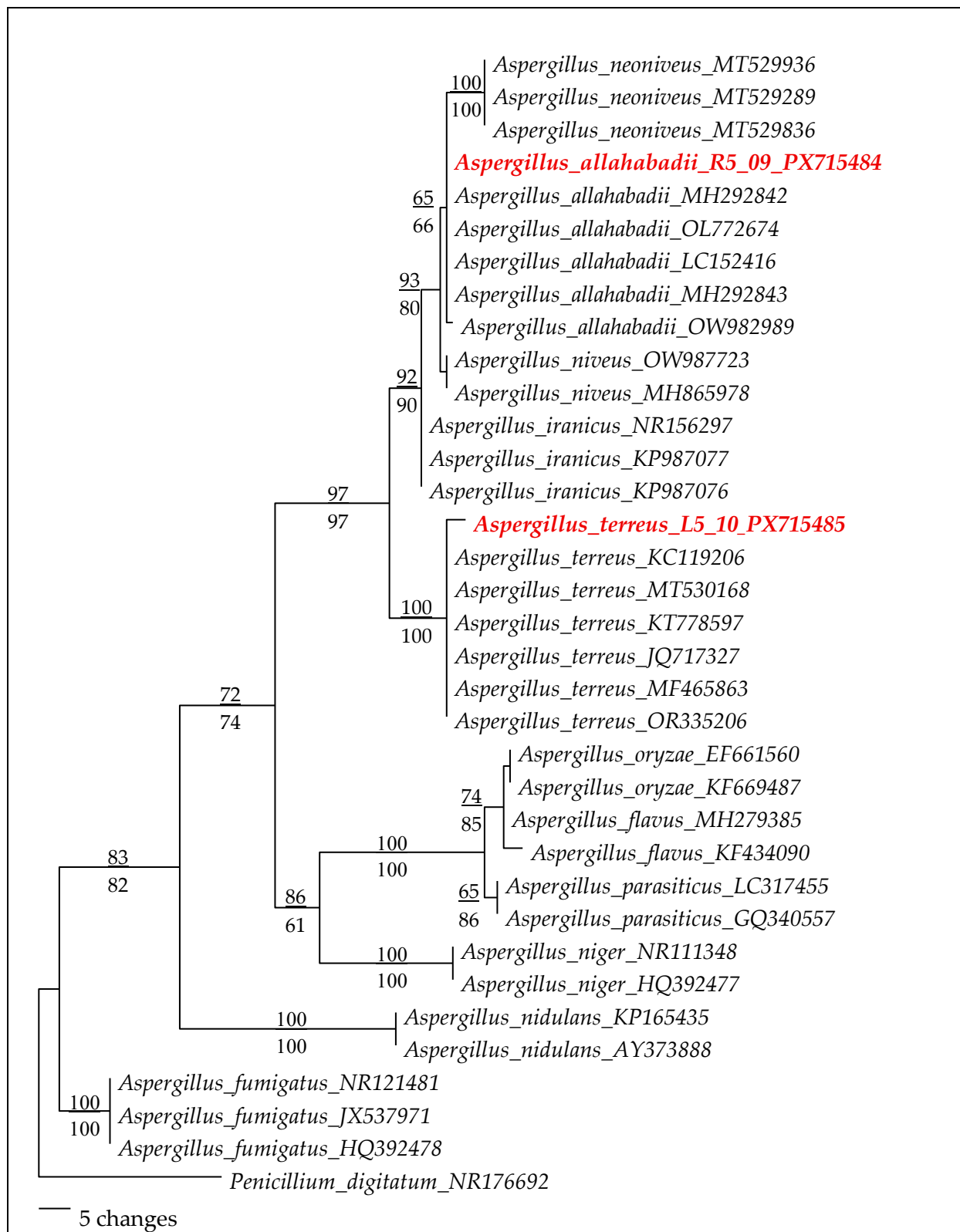


Figure 5. A phylogenetic tree of *Aspergillus* spp. based on molecular analyses of the internal transcribed spacer (ITS) region. Bootstrap support for Maximum Likelihood (MLBS) (above) and Maximum Parsimony (MPBS) (below) is shown on branches of the tree. Two fungal endophyte isolates, R5-09 and L5-10, are highlighted in red and identified as *Aspergillus allahabadii* and *Aspergillus terreus*, respectively.

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