MOLECULAR CHARACTERIZATION OF ENDOPHYTIC *ASPERGILLUS FUMIGATUS* FROM *HOUTTUYNIA CORDATA* THUNB. AS AN ANTIBACTERIAL PRODUCER

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ABSTRACT

Endophytic fungi associated with medicinal plants are potential sources of antibacterial metabolites. This study aimed to isolate and molecularly characterize endophytic fungi from Houttuynia cordata Thunb. and evaluate their antibacterial activity against Escherichia coli ATCC 25922 and Staphylococcus aureus ATCC 25923. Endophytic fungi were isolated from healthy roots, stems, and leaves of H. cordata and characterized using morphological examination. Antibacterial activity was evaluated using an agar-block antagonistic assay. Molecular identification was performed through amplification and sequencing of the internal transcribed spacer (ITS) region, followed by BLAST analysis and phylogenetic reconstruction. Four isolates, designated AHC I, BHC II, DHC II, and BHC IV, exhibited antibacterial activity against both test bacteria. BHC IV produced the largest inhibition zone against E. coli (32.24 mm), whereas BHC II exhibited the largest inhibition zone against S. aureus (26.72 mm). ITS sequence analysis revealed 99.19–100% identity with reference sequences of Aspergillus fumigatus, with 100% query coverage. Phylogenetic analysis supported their close genetic relationship with A. fumigatus. These findings demonstrate the antibacterial potential of H. cordata-associated endophytic fungi and support further investigation of their bioactive metabolites.

KEYWORDS

Antibacterial activity; *Aspergillus fumigatus*; Endophytic fungi; *Houttuynia cordata*; ITS sequencing.

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INTRODUCTION

This The emergence and rapid spread of antimicrobial resistance have increased the demand for new sources of antibacterial agents. Natural products derived from microorganisms remain important sources of structurally diverse bioactive compounds. Among these microorganisms, endophytic fungi have attracted considerable attention because they colonize healthy internal plant tissues without causing apparent disease symptoms and are capable of producing a wide range of biologically active secondary metabolites (Singh et al., 2021). Several classes of metabolites produced by fungal endophytes, including alkaloids, polyketides, terpenoids, phenolic compounds, quinones, and cyclic peptides, have demonstrated antibacterial activity against clinically relevant microorganisms (Silva et al., 2022)(Gupta et al., 2020). Consequently, endophytic fungi have increasingly been investigated as potential sources of antibacterial compounds, including metabolites active against Gram-negative bacteria, which exhibit intrinsic resistance to certain antimicrobial agents due to the presence of an outer membrane.

Endophytic fungi establish diverse ecological relationships with their host plants and may contribute to plant growth, stress tolerance, and the production of bioactive metabolites (Elfiati et al., 2022)(Ye et al., 2021). These fungi can produce various biologically active compounds, including phytohormones, siderophores, and hydrolytic enzymes, which may influence plant growth and host–microorganism interactions(Caruso et al., 2023)(Christian et al., 2019)(Gakuubi et al., 2021) (Rana et al., 2020). Furthermore, interactions between endophytic fungi and medicinal plants may influence the biosynthesis and accumulation of secondary metabolites (Christian et al., 2019)(Fanani et al., 2021)(Varghese et al., 2024). These characteristics highlight the potential of endophytic fungi as sources of biologically active compounds for pharmaceutical research(Řebíčková et al., 2020).

Houttuynia cordata Thunb., a medicinal plant belonging to the family Saururaceae, is widely distributed throughout Asia and commonly grows in moist environments (Yang & Jiang, 2009)(Rafiq et al., 2022). This plant has a long history of traditional use for the management of respiratory disorders, inflammation, dysentery, cough, and fever (Chen et al., 2015)(Preethi et al., 2021). Phytochemical investigations have revealed that *H. cordata* contains various secondary metabolites, including flavonoids, alkaloids, terpenoids, and polyphenols. These constituents have been associated with several pharmacological activities, including antibacterial, antiviral, anti-inflammatory, antioxidant, and immunomodulatory effects (Ling et al., 2020)In addition to the bioactive constituents produced by the plant itself, endophytic microorganisms associated with *H. cordata* represent an additional source of biological and chemical diversity (Talukdar et al., 2021). Previous studies have reported the isolation and identification of various endophytic fungi from healthy tissues of *H. cordata*, suggesting their potential as sources of bioactive metabolites (Talukdar et al., 2020)

Although several endophytic fungi have been reported from *H. cordata*, information regarding the occurrence of *Aspergillus fumigatus* as an endophyte of this medicinal plant and the antibacterial activity of its associated isolates remains limited. Furthermore, studies integrating antibacterial screening, molecular identification based on internal transcribed spacer (ITS) sequences, and phylogenetic analysis of endophytic fungi isolated from different tissues of *H. cordata* remain relatively scarce. Molecular characterization is therefore essential for investigating the taxonomic identity and genetic relationships of bioactive fungal isolates and establishing a foundation for further studies of their antibacterial metabolites.

Therefore, this study aimed to isolate and characterize endophytic fungi from healthy root, stem, and leaf tissues of *Houttuynia cordata* Thunb., evaluate their antibacterial activity against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923, and identify selected bioactive isolates through ITS rDNA sequence analysis and phylogenetic reconstruction. By integrating antibacterial screening with molecular characterization, this study seeks to provide further insights into the diversity and antibacterial potential of endophytic fungi associated with *H. cordata*.

RESEARCH METHOD

Materials and Instruments

The materials used in this study included fresh, healthy tissues of *Houttuynia cordata* Thunb., Potato Dextrose Agar (PDA; HiMedia Laboratories, India), Potato Dextrose Broth (PDB; HiMedia Laboratories, India), yeast extract (Merck KGaA, Germany), chloramphenicol (Sigma-Aldrich, USA), Nutrient Agar (NA; HiMedia Laboratories, India), sterile distilled water, 70% ethanol (Merck KGaA, Germany), 5.25% sodium hypochlorite (NaOCl), and ethyl acetate (Merck KGaA, Germany). The bacterial strains used for antibacterial activity testing were *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923.

Genomic DNA was extracted using the FavorPrep™ Fungi/Yeast Genomic DNA Extraction Mini Kit (FAVORGEN Biotech Corp., Taiwan). Molecular analyses were performed using MyTaq™ Red Mix (Meridian Bioscience, USA), agarose (Invitrogen, Thermo Fisher Scientific, USA), a 100 bp DNA ladder (Geneaid Biotech Ltd., Taiwan), and the universal fungal primers ITS1 and ITS4 (Integrated DNA Technologies, USA).

The major instruments used in this study included a Class II biological safety cabinet (Thermo Scientific™ Herasafe™ 2030i, Thermo Fisher Scientific, Germany), an autoclave (Hirayama HVE-50, Hirayama Manufacturing Corp., Japan), an incubator (Memmert IN110, Memmert GmbH + Co. KG, Germany), a shaking incubator (New Brunswick Innova® 44, Eppendorf, USA), an analytical balance (Mettler Toledo ME204, Mettler-Toledo GmbH, Switzerland), a light microscope (Olympus CX23, Olympus Corporation, Japan), and a microcentrifuge (Eppendorf Centrifuge 5424 R, Eppendorf SE, Germany). Genomic DNA concentration and purity were determined using a NanoDrop™ One UV–Vis spectrophotometer (Thermo Fisher Scientific, USA). PCR amplification was performed using a T100™ Thermal Cycler (Bio-Rad Laboratories, USA). The amplified DNA fragments were separated using a Mini-Sub Cell GT electrophoresis system (Bio-Rad Laboratories, USA) and visualized using a Gel Doc™ EZ Imaging System (Bio-Rad Laboratories, USA).

Plant Material Collection and Preparation

Fresh, healthy leaves, stems, and roots of *Houttuynia cordata* Thunb. were collected from Tawangmangu, Karanganyar, Central Java, Indonesia. Plant tissues showing visible signs of disease, microbial infection, or mechanical damage were excluded. The collected plant material was taxonomically identified at Dr. Sardjito General Hospital, Yogyakarta, Indonesia. Botanical identification confirmed that the plant belonged to the family Saururaceae and was identified as *Houttuynia cordata* Thunb., with *Polypara cordata* (Thunb.) H. Buek recorded as a synonym (Identification Report No. TL.02.04/D.XI.6/30138.877/2025, issued on October 28, 2025).

The collected samples were transported to the laboratory and thoroughly washed under running water to remove soil, dust, and other particles adhering to the plant surfaces. The

cleaned samples were subsequently subjected to surface sterilization prior to the isolation of endophytic fungi.

Surface Sterilization of Plant Tissues

Surface sterilization was performed under aseptic conditions. Healthy leaves, stems, and roots were immersed in 70% ethanol for 2 min, followed by immersion in 5.25% sodium hypochlorite (NaOCl) for 2 min and a second immersion in 70% ethanol for 2 min. The samples were then rinsed twice with sterile distilled water for 1 min to remove residual sterilizing agents. Following surface sterilization, the tissues were dried using sterile tissue paper and aseptically cut into approximately 1-cm segments.

Isolation and Purification of Endophytic Fungi

The surface-sterilized plant segments were aseptically placed onto PDA plates and incubated at 25°C for 5 days. Fungal colonies emerging from the margins of the plant tissues were considered presumptive endophytic fungi. Colonies exhibiting distinct morphological characteristics were individually transferred onto fresh PDA plates and repeatedly subcultured until pure cultures were obtained.

The purified fungal isolates were coded according to their tissue of origin. Four isolates, designated AHC I, BHC II, DHC II, and BHC IV, were selected for further morphological characterization, antibacterial screening, and molecular identification. These isolates were obtained from the root, stem, and leaf tissues of *H. cordata*.

Morphological Characterization of Endophytic Fungi

The purified fungal isolates were characterized based on their macroscopic and microscopic morphological characteristics. Macroscopic characterization included observations of colony color, shape, texture, surface appearance, and growth characteristics on PDA. For microscopic examination, a small portion of fungal mycelium was aseptically transferred onto a clean glass slide and stained with lactophenol cotton blue (LPCB). The preparations were examined under a light microscope to observe the morphological characteristics of the hyphae conidia, conidiophores, and other reproductive structures. These observations were used for preliminary morphological characterization prior to molecular identification.

Antagonistic Activity Screening

The antibacterial potential of the purified endophytic fungal isolates was initially evaluated using an agar-block antagonistic assay against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923. Agar plugs containing pure fungal cultures grown on PDA were aseptically excised using a sterile cork borer. The fungal agar plugs were subsequently placed onto NA plates previously inoculated with the respective test bacteria. The plates were incubated at 37°C for 24 h. Antagonistic activity was assessed based on the formation of clear inhibition zones surrounding the fungal agar plugs. The diameters of the inhibition zones were measured in millimeters (mm). Fungal isolates exhibiting antibacterial activity were selected for further investigation.

Genomic DNA Extraction

Genomic DNA was extracted from the selected endophytic fungal isolates using the FavorPrep™ Fungi/Yeast Genomic DNA Extraction Mini Kit (FAVORGEN) according to the manufacturer's instructions. Approximately 1–5 × 10^6 fungal cells were collected in a 1.5-mL microcentrifuge tube and washed with FA Buffer. Following centrifugation, the supernatant was discarded, and the fungal pellet was subjected to

enzymatic lysis using FB Buffer and lyticase at 37°C for 30 min. RNase A was added when necessary to remove contaminating RNA. The pellet was subsequently resuspended in TG1 Buffer and mechanically disrupted using a bead tube. Proteinase K was added, followed by incubation at 55°C. After centrifugation, the resulting supernatant was mixed with TG2 Buffer and ethanol and transferred to a TG Mini Column to facilitate DNA binding to the silica membrane. The column was sequentially washed with W1 Buffer and Wash Buffer, followed by a final dry-spin step to remove residual ethanol. Genomic DNA was eluted using Elution Buffer or double-distilled water (ddH_2O) and stored at −20°C until PCR amplification.

PCR Amplification of the ITS Region

The internal transcribed spacer (ITS) region of fungal ribosomal DNA (rDNA) was amplified using the universal fungal primer pair ITS1 and ITS4. The primer sequences were as follows:

ITS1 (forward): 5′-TCCGTAGGTGAACCTGCGG-3′
ITS4 (reverse): 5′-TCCTCCGCTTATTGATATGC-3′

PCR amplification was performed using a thermal cycler. The optimized annealing conditions were 50°C for 30 s. Amplification yielded DNA fragments of approximately 500–750 bp. Successful amplification of the ITS region was confirmed by agarose gel electrophoresis prior to DNA sequencing.

Agarose Gel Electrophoresis

The PCR products were subjected to agarose gel electrophoresis to confirm successful amplification of the ITS region. The amplification products were loaded onto an agarose gel alongside a DNA molecular weight marker.
Following electrophoretic separation, DNA bands were visualized under UV illumination using a gel documentation system. The presence of distinct DNA bands at approximately 500–750 bp was considered indicative of successful amplification of the ITS region. PCR products exhibiting the expected band sizes were selected for subsequent DNA sequencing.

DNA Sequencing and Sequence Analysis

PCR products showing successful amplification of the ITS region were subjected to Sanger sequencing using the ITS1 and ITS4 primers. The nucleotide sequences obtained from the fungal isolates were processed and compared with reference sequences available in the National Center for Biotechnology Information (NCBI) GenBank database using the Basic Local Alignment Search Tool (BLAST).

Sequence similarity was evaluated based on query coverage, E-value, and percentage identity between the obtained sequences and closely related reference sequences deposited in GenBank. Reference sequences exhibiting high similarity to the fungal isolates were selected for subsequent phylogenetic analysis. Molecular identification was performed by considering the overall BLAST results in conjunction with phylogenetic relationships rather than relying solely on a fixed sequence identity threshold for species identification.

Multiple Sequence Alignment and Phylogenetic Analysis

The ITS sequences obtained from the endophytic fungal isolates and closely related reference sequences retrieved from GenBank were subjected to multiple sequence alignment using Clustal X version 2.1.

Phylogenetic relationships were reconstructed using MEGA version 12 based on the Neighbor-Joining (NJ) method. The resulting phylogenetic tree was used to evaluate the genetic relationships between the endophytic fungal isolates obtained from *H. cordata* and closely related fungal taxa represented in GenBank.

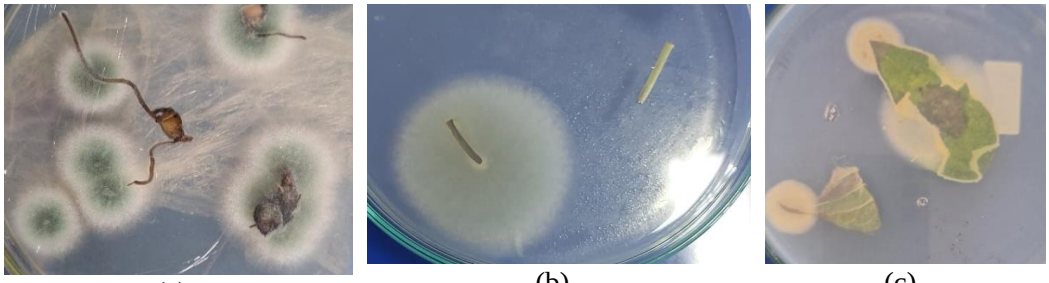
RESULT AND DISCUSSION

Isolation and Identification of Endophytic Fungi

The isolates produced in this study were named strains AHC I, BHC II, BHC IV and DHC II which were isolated from the roots, stems, and leaves of *H. cordata* plants and then grown on PDA media for 10 days (Figure 1), thus obtaining pure isolates from H. cordata plant tissue (Figure 2). Based on colony morphology and microscopic reproductive structures. The isolates were grown on PDA agar for identification. BHC II colonies on PDA were round, white, with white to grayish aerial mycelium, like cotton without visible conidial masses. Growth on PDA was slow, showing a diameter of approximately 55 mm in 5 days at 25°C (Figure 2a). AHC I colonies on PDA were round, green, with dark green aerial mycelium, like powder. Growth on PDA was fast and spreading (Figure 2b). DHC II colonies on PDA were round, green, with green aerial mycelium, like powder. Growth on PDA was fast and spreading (Figure 2c). Conidia measuring 20–22 × 5–7 µm and septate, smooth-walled, hyaline, and cylindrical with rounded tips, often with conspicuous basal papilla scars, dripping, and slightly narrowed in the center (Figure 2(c)). Growth on PDA is fast, colonies are white in 7 days at 25°C (Figure 2d). Endophytic fungi isolated from healthy leaves, stems, and leaves of H. cordata have similar characteristics both in terms of colony shape and colony color on the parts of the *H. cordata* plant. *Aspergillus sp*. isolates (Figure 3) have microscopic characteristics of spherical, transparent, and smooth-surfaced conidia and 1-cell conidia. The observed microscopic characteristics, including septate hyphae, conidiophores, terminal vesicles, phialides, and conidial structures, were consistent with the general morphological characteristics of the genus *Aspergillus*. However, morphological characteristics alone may not provide sufficient resolution for distinguishing closely related species within this genus. Therefore, molecular identification based on ITS rDNA sequencing was performed to support the preliminary morphological identification (Samson et al., 2014). The macroscopic and microscopic morphological characteristics of isolated endophytic fungi can be shown in Table 1 and Figures 2 & 3.

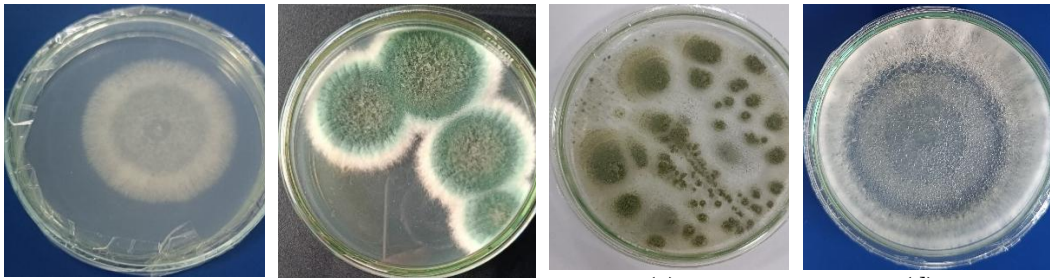
Table 1. Macroscopic and microscopic morphology of endophytic fungi from *H. cordata* plant tissue

Macroscopic characterization of fungal colonies	**AHC I**	**BHCII**	**DHC II**	**BHC IV**
Colony Color	Green	White	Green	White
Conidia Shape	Oval	Oval	Oval	Oval
Spore Color and Shape	Odorless Green	Yellowish White	Odorless Green	Yellowish White
Conidiophore Surface and Color	Smooth Transparent	Smooth Transparent	Smooth Transparent	Smooth Transparent
Hyphae	Septate and Branched	Septate and Branched	Septate and Branched	Septate and Branched



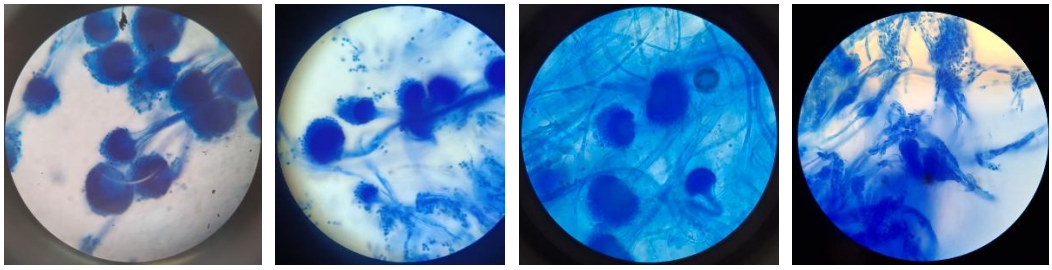
(a) (b) (c)

Figure 1. Isolation of *H.cordata* tissue endophytic fungi (a) Roots (AHC 1); (b) Stems (BHC II& IV); (c) Leaf (AHC II)



(a) (b) (c) (d)

Figure 2. Macroscopic morphology of endophytic fungal colonies in *H.cordata* tissue on PDA media: (a) BHC II; (b) DHC II; (c) AHC I; (d) BHC IV



(a) (b) (c) (d)

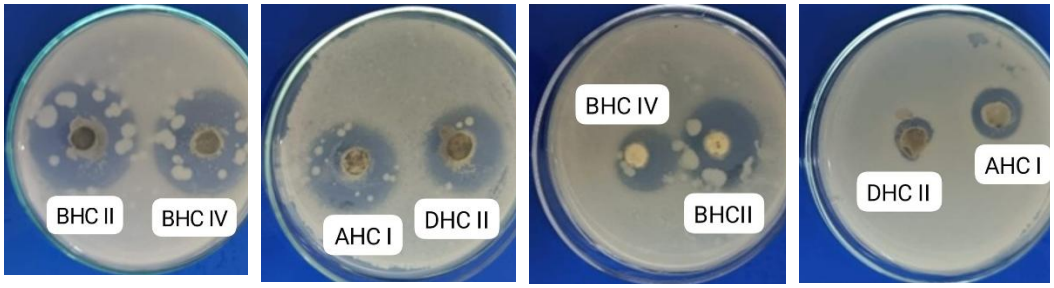
Figure 3. Microscopic morphology of endophytic fungi in *H.cordata* plant tissue

Antagonistic Activity Screening

Based on the results of the research conducted from the antagonistic test results between endophytic fungi from H. cordata tissue on *Staphylococcus aureus* and *Escherichia coli*, the diameter of the inhibition zone (in mm) was measured using a caliper with the agar-block antagonistic assay. The average diameter of the inhibition zone obtained can be seen in table 2 and figure 4 below.

Table 2. Inhibition Zone of the endophytic fungus *H. cordata* against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923

Sample	Inhibition Zone *E. coli* ATCC 25922 (mm)	Inhibition Zone *S. aureus* ATCC 25923 (mm)
BHC II	31.09	26.72
BHC IV	32.24	19.05
AHC I	25.65	10.96
DHC II	26.72	15.60



(a) (b)

Figure 4. (a) Endophytic fungi antagonist test against *Echerchia coli* ATCC 25922, (b) Endophytic fungi antagonist test against *Staphylococcus aureus* ATCC 25923

The results of the antibacterial activity test showed that all samples (BHC II, BHC IV, AHC I, and DHC II) were able to inhibit the growth of *E. coli herichia coli* ATCC 25922 and *S. aureus* ATCC 25923, as indicated by the formation of inhibition zones around the test discs. However, there was significant variation in the diameter of the inhibition zones between samples and between bacterial types. In general, all samples demonstrated stronger antibacterial activity against Gram-negative bacteria (*E. coli*) than Gram-positive bacteria (*S. aureus*). This was evident from the larger inhibition zone diameter in *E. coli*, particularly in BHC IV (32.24 mm) and BHC II (31.09 mm). This difference may be attributed to the structure of the bacterial cell wall. Gram-negative bacteria have an outer membrane containing lipopolysaccharides that can affect the penetration of active compounds, but in some cases are more susceptible to certain metabolites such as lipophilic compounds or antimicrobial peptides. BHC IV demonstrated the highest activity against *E. coli* (32.24 mm), but decreased activity against *S. aureus* (19.05 mm). Conversely, BHC II demonstrated relatively high and more balanced activity against both bacteria (31.09 mm against *E. coli* and 26.72 mm against *S. aureus*). This indicates that BHC II likely contains bioactive compounds with a broader antibacterial spectrum. Sample AHC I demonstrated the lowest antibacterial activity against *S. aureus* (10.96 mm), although it was still relatively active against *E. coli* (25.65 mm). Meanwhile, DHC II demonstrated moderate activity against both bacteria, with inhibition zones of 26.72 mm against *E. coli* and 15.60 mm against *S. aureus*. This variation suggests that the composition of secondary metabolites produced by each isolate likely differs, both qualitatively and quantitatively. Based on the general classification of antibacterial activity (zone of inhibition diameter >20 mm = very strong, 10–20 mm = strong, 5–10 mm = moderate), BHC II and BHC IV are categorized as having very strong activity against *E. coli*. Against *S. aureus*, only BHC II is categorized as very strong, while the other samples are in the strong to moderate category. This difference in antibacterial activity is likely influenced by the type and concentration of secondary metabolites produced, such as alkaloids, flavonoids, terpenoids, or polyketides, which are known to have mechanisms of action such as damaging cell membranes, inhibiting protein synthesis, or interfering with bacterial DNA replication. Overall, these results indicate that sample BHC II is the most potential candidate as a broad-spectrum antibacterial agent, while BHC IV has higher specific potency against Gram-negative bacteria. The differences in inhibition zone diameters between *E. coli* and *S. aureus* may reflect differences in bacterial susceptibility to diffusible antifungal metabolites, as well as variations in the production and diffusion of bioactive compounds among fungal isolates. Endophytic fungi are known to produce diverse secondary metabolites with antibacterial properties, including compounds active against Gram-negative bacteria(Silva et al., 2022)(Gupta et al., 2020). However, the specific metabolites

responsible for the observed antibacterial activity in the present study remain to be identified.

DNA Isolation, Electrophoresis and Sequencing.

Genomic DNA was successfully extracted from four endophytic fungal isolates obtained from *Houttuynia cordata* Thunb. The concentration and purity of the extracted DNA were evaluated using UV–Vis spectrophotometry prior to molecular identification. As presented in Table 3, the DNA concentrations ranged from 12.8 to 15.2 ng/µL, with the highest concentration observed in isolate DHC II (15.2 ng/µL) and the lowest in isolate BHC IV (12.8 ng/µL). The A260/A280 ratios ranged from 1.37 to 1.56, whereas the A260/A230 ratios ranged from 0.20 to 0.59. These values were below the commonly accepted purity ranges for DNA, suggesting the possible presence of residual contaminants from the extraction process

The relatively low A260/A280 ratios may indicate contamination by proteins or other UV-absorbing substances, whereas the low A260/A230 ratios may reflect the presence of residual extraction reagents or other compounds that absorb at approximately 230 nm (Desjardins & Conklin, 2010). However, spectrophotometric purity ratios alone cannot establish the identity or concentration of these contaminants. Furthermore, the presence of UV-absorbing substances may affect the accuracy of DNA concentration measurements. Therefore, DNA concentration and purity should be interpreted as complementary indicators of DNA quality rather than as definitive measures of its suitability for downstream molecular applications.

Despite the suboptimal spectrophotometric purity values, successful amplification of the fungal internal transcribed spacer (ITS) region was observed in all four isolates. Agarose gel electrophoresis revealed distinct PCR products within the expected size range of approximately 500–750 bp (Figure 6). These findings indicate that the extracted DNA contained sufficient amplifiable template for the PCR assay and that the residual contaminants, if present, did not completely inhibit the amplification reaction under the conditions employed. Thus, the extracted DNA was suitable for ITS amplification in the present study, although its spectrophotometric purity was below the commonly accepted ranges.

The ITS region was amplified using the universal fungal primers ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGCTTATTGATATGC-3′), with an annealing temperature of 50°C for 30 s. The fungal ITS region comprises ITS1, the 5.8S rRNA gene, and ITS2, which are located between the 18S and 28S rRNA genes. The relatively high sequence variability of the ITS regions makes them useful molecular markers for fungal identification. The amplified products were subsequently subjected to Sanger sequencing to obtain nucleotide sequence information for molecular characterization.

Table 3. Concentration and spectrophotometric purity of genomic DNA extracted from endophytic fungal isolates of Houttuynia cordata Thunb

Sample code	Concentration (ng/µL)	A260/280	A260/230
AHC I	14,1	1,37	0,28
BHC II	14,7	1,56	0,37
DHC II	15,2	1,43	0,59
BHC IV	12,8	1,44	0,20

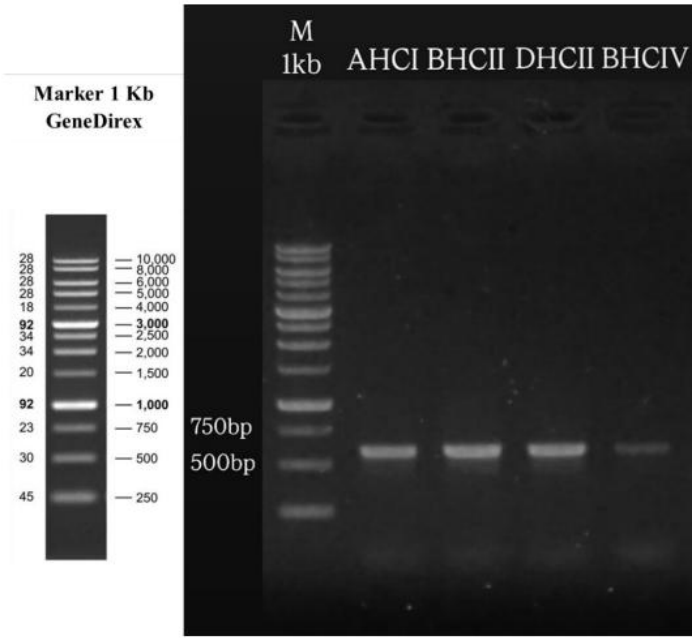


Figure 6. Agarose gel electrophoresis of PCR-amplified internal transcribed spacer (ITS) regions from four endophytic fungal isolates of Houttuynia cordata Thunb. M: 1 kb DNA ladder; AHC I, BHC II, BHC IV, and DHC II: PCR products obtained from the respective fungal isolates. The amplified DNA fragments were observed within the expected size range of approximately 500–750 bp

The resulting ITS sequences were compared with reference sequences available in the NCBI GenBank database using BLAST. Sequence similarity was evaluated based on percentage identity, query coverage, and E-value. The BLAST results were subsequently interpreted in conjunction with phylogenetic analysis to assess the genetic relationships between the fungal isolates and closely related reference taxa. The high ITS sequence similarity and phylogenetic clustering of the four isolates with *Aspergillus fumigatus* reference sequences support their close taxonomic relationship with this species. However, ITS sequence analysis alone may not provide sufficient resolution to distinguish closely related species within *Aspergillus*. Therefore, a polyphasic approach integrating morphological characteristics and additional molecular markers, such as calmodulin and β-tubulin, may be required to confirm species-level identification (Schoch et al., 2012).

Overall, the successful amplification and subsequent sequence analysis of the ITS region demonstrate that the extracted DNA was adequate for the molecular characterization workflow employed in this study, despite its suboptimal spectrophotometric purity. These findings also highlight the importance of evaluating DNA quality in relation to the requirements of the intended downstream application rather than relying exclusively on spectrophotometric purity ratios(Yunita et al., 2016).

Table 4. Blast data sequencing results

Isolate	Homologous fungal species	*Max score*	*Query coverage* (%)	E value	*Identities*	Accession
AHC I	*Aspergillus fumigatus* isolate R6	1081	100%	0.0	99.83%	KM111293.1
	Aspergillus fumigatus strain TD10	1079	100%	0.0	99.83%	OR939714.1
BHC II	*Aspergillus fumigatus* isolate AA233	1066	100%	0.0	100.00%	MN006406.1
	Aspergillus fumigatus starin TD10	1066	100%	0.0	100.00%	OR939714.1
	Aspergillus fumigatus starin YTF5	1066	100%	0.0	100.00%	OM841708.1
DHC II	*Aspergillus fumigatus* isolate FOAOA-3	896	100%	0.0	99.19%	MN994279.1

Isolate	Homologous fungal species	*Max score*	*Query coverage* (%)	E value	*Identities*	Accession
	Aspergillus fumigatus starin NR41	896	100%	0.0	99.19%	MH270569.1
	Aspergillus fumigatus starin ND102	896	100%	0.0	99.19%	MH270608.1
BHC IV	*Aspergillus fumigatus* isolate AA233	1066	100%	0.0	100.00%	MN006406.1
	Aspergillus fumigatus starin TD10	1066	100%	0.0	100.00%	OR939714.1
	Aspergillus fumigatus starin YTF5	1066	100%	0.0	100.00%	OM841708.1



Figure 7. Phylogenetic tree of endophytic fungal isolates obtained from *Houttuynia cordata* Thunb. based on ITS rDNA sequences. The phylogenetic tree was constructed using the Neighbor-Joining method in MEGA 12. Bootstrap values are shown at the nodes. The sequences obtained in this study (AHC I, BHC II, BHC IV, and DHC II) are indicated in bold. GenBank accession numbers are provided for reference sequences.

Fungal isolate extracts from culture filtrates showed significant antimicrobial activity against several human clinical trial pathogens. Accurate molecular identification is crucial to ensure the taxonomic clarity of fungal isolates. The Internal Transcribed Spacer (ITS4 and ITS5) regions of ribosomal DNA have been widely accepted as universal barcode markers for fungal identification due to their high interspecies variability and ease of amplification. Molecular characterization allows for precise identification and supports the reproducibility and scientific validity of the study. Figure 7 shows the results of a phylogenetic tree using 1000 bootstrapping replicates using the Neighbor Joining (NJ) method. The identification results in the roots, stems, and leaves of the *H. cordata* plant were Aspergillus fumigatus. In this study, the endophytic fungus identified as *Aspergillus fumigatus* was isolated from healthy plant tissue of Houttuynia cordata Thunb., a potential ethnomedicinal plant used for the treatment of(Wu et al., 2021). Identification of endophytic fungi using molecular identification is highly sensitive, fast, and accurate. The identity of endophytic fungi can be determined down to the species level based on BLAST analysis of DNA sequencing results. With the advancement of molecular biology, this method has become the preferred method for identification, especially when morphological identification is difficult.

CONCLUSION

This study demonstrated the antibacterial potential of endophytic fungal isolates obtained from the roots, stems, and leaves of *Houttuynia cordata* Thunb. Four isolates, designated AHC I, BHC II, DHC II, and BHC IV, exhibited antibacterial activity against *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923. Among the isolates, BHC IV exhibited the largest inhibition zone against *E. coli* (32.24 mm), whereas BHC II showed the largest inhibition zone against *S. aureus* (26.72 mm). Molecular characterization based on ITS rDNA sequencing revealed 99.19–100% sequence identity with reference sequences of *Aspergillus fumigatus*, with 100% query coverage. Phylogenetic analysis further supported the close genetic relationships between the four isolates and *A. fumigatus* reference sequences. These findings indicate that endophytic fungi associated with *H. cordata* represent potential sources of antibacterial metabolites. Further studies involving additional molecular markers, bioactive compound isolation, chemical characterization, and safety evaluation are required to confirm species identity and identify the compounds responsible for the observed antibacterial activity.

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