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Antimicrobial Properties of Endophytic Fungal Culture Filtrates from *Tinospora crispa*

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Abstract

Endophytic fungi can produce bioactive compounds that are useful as antimicrobials. This study evaluates the antimicrobial potential of culture filtrate extracts derived from endophytic fungi isolated from the medicinal plant *Tinospora crispa*. Isolation was carried out from the roots, leaves, and stems of *T. crispa*, which were then identified based on the ITS gene. The culture filtrate was extracted using ethyl acetate and assessed for antimicrobial activity using the disc diffusion method against *Escherichia coli*, *Staphylococcus aureus*, and *Candida albicans*. A total of 3 endophytic fungal isolates were isolated and identified as *Acrocalymma vagum*, *Diaporthe tulliensis*, and *Colletotrichum truncatum*. The results showed that all culture filtrate extracts of the fungal endophyte isolates exhibited varying antimicrobial activity, with the highest antibacterial activity demonstrated by *C. truncatum* isolates against *Escherichia coli* and *Staphylococcus aureus*. The most significant anticandida activity was by *D. tulliensis* isolates. Endophytic fungi of medicinal plant *T. crispa* can be developed as a source of antimicrobial agents, especially to overcome the increasing antibiotics resistance.

Keywords: Antimicrobial; Culture filtrate extract; Endophytic fungi; *Tinospora crispa*.

INTRODUCTION

Tinospora crispa (L.) Miers ex Hook.fil. & Thomson is one of the most widely recognised medicinal plants in Southeast Asia. This medicinal plant has traditionally been used to treat of various diseases, such as fever, jaundice, malaria, arthritis, diabetes, urinary disorders, high blood pressure, and promoting wellness (Ahmad et al., 2016). The plant is rich in phytochemical compounds such as alkaloids, flavonoids, glycosides, terpenoids, phenolics, as well as other compounds such as lactones, sterols, lignans, and nucleosides, which are known to have diverse biological activities (Shree & Krishnaveni, 2023). In addition to the extensively studied plant parts, microbial components residing within these plant tissues, particularly endophytic fungi, are increasingly recognised as a valuable source of bioactive compounds.

Endophytic fungi are fungi that live symbiotically in plant tissues without causing disease symptoms in the plants they inhabit (Wen et al., 2022). Endophytic fungi in all plants are known to play an important role in supporting plant growth and resistance to various environmental stresses (Li et al., 2025). Endophytic fungi facilitate nutrient absorption, promote plant growth by producing hormones like auxin, and assist plants in coping with stressors such as drought, pathogen attacks, or heavy metal exposure (Baron & Rigobelo, 2022; Fite et al., 2023; Morales-Vargas et al., 2024). Endophytic fungi benefit the host plant and are known for their ability to synthesize valuable bioactive compounds (Hashem et al., 2023). These metabolites hold significant potential in the pharmaceutical industry, particularly as antimicrobial agents and antioxidants. This capability is especially crucial given the rise of microbial resistance to antibiotics.

Endophytic fungi from various medicinal plant species have been reported to produce compounds with antimicrobial activity. *Botryosphaeria mamane* from the medicinal plant *Arrabidaea chica* has potent antibacterial activity against various pathogenic bacteria, including *Staphylococcus aureus* and *Candida parapsilosis* (Gurgel et al., 2023). Additionally, isolates such as *Penicillium sclerotigenum* and *Diaporthe kochmanii* from *Ageratina adenophora* have shown effectiveness against resistant bacteria like MRSA (Wen et al., 2023). Isolates of *Fusarium* sp. and *Acremonium* sp. obtained from *Paederia foetida* produced metabolites that exhibited moderate to strong antibacterial activity against *S. aureus* and *E. coli* (Widjajanti et al., 2021). Endophytic fungi are reported to have great potential to inhibit both gram-positive and negative bacteria (Nguyen et al., 2025).

Previously, it has been reported that the endophytic fungus extract *T. crispa* has antibacterial activity against *E. coli* and *S. aureus* and anticandidal activity (Fathoni et al., 2021, 2022, 2023). However, no studies exclusively utilise liquid culture filtrates, which contain secondary metabolites naturally secreted by fungi and may serve as a more practical source of bioactive compounds. This study aimed to evaluate the antimicrobial and antioxidant activities of the culture filtrate of endophytic fungi from *T. crispa*. This study is expected to provide new insights into the potential of endophytic fungal culture filtrates as promising therapeutic candidates derived from locally sourced biological resources.

MATERIALS AND METHODS

Materials

The materials used in this study included healthy leaves, stems, and roots of Tinospora crispa, PDA, and PDB media with chloramphenicol, and solvents such as ethyl acetate and sterile distilled water. Molecular work involved the Wizard® Genomic DNA Kit, ITS1F and ITS2 primers, GoTaq Green Master Mix, and a PCR ThermoCycler. Antimicrobial assays used E. coli, S. aureus, and C. albicans with chloramphenicol and ketoconazole as positive controls. Paper discs, cotton swabs, and standard microbiological tools were used, and data analysis employed MEGA-X, BioEdit, and NCBI BLAST.

Preparation of Plant Samples

Plant samples were prepared using *Tinospora crispa* plants from the Berbah, Yogyakarta Special Region. Samples collected include healthy leaves, stems, and roots without any spots or defects. After collection, the samples were immediately taken to the Microbiology Laboratory, Universitas Ahmad Dahlan, for the endophytic fungus isolation process.

Isolation of Endophytic Fungi

The isolation process begins with sterilizing the sample surface, where fresh roots, stems, and leaves are washed using running water to remove dirt and soil. Next, the samples were immersed in 70% (v/v) alcohol for one minute, then immersed in 0.5% (v/v) NaClO solution for three minutes, and again immersed in 70% (v/v) alcohol for 30 seconds. After that, the samples were rinsed twice with sterile distilled water and dried in a sterile petri dish. Samples were then aseptically cut using a sterile scalpel into 1-2 cm sizes and inserted into PDA media supplemented with 100 µg/mL chloramphenicol aseptically. Each organ sample was done in two repetitions and coded. To ensure the success of the sterilization process, 0.1 mL of sterile distilled water from the last rinse was poured onto PDA media using the scatter method. The samples were then incubated at room temperature (25-28°C) for seven days. Fungal isolates that successfully grew were then subcultured onto new PDA media. The characteristics of endophytic fungi were observed microscopically by adding lactophenol cotton blue to the mycelial samples and then observing them under a microscope.

Preparation of Culture Filtrate Extract of Endophytic Fungus of *Tinospora crispa* Plant

The endophytic fungal culture filtrate was prepared using the method from Baz et al. (2024). Endophytic fungi that have been rejuvenated for 7 days on PDA media in a petri dish are taken using a scalpel, as many as three snippets in the form of plates. Every three plates of pure fungal hyphae from each strain were put into 2000 mL of PDB media placed in a dark bottle with a capacity of 2500 mL. The culture was then incubated at room temperature (25°C-27°C) for 14 days to allow the production of secondary metabolites. The fermented culture was divided into two components: fungal mycelia and fermentation media. This separation was achieved by filtering the culture through sterile filter paper. The fermentation media, also known as culture filtrate, was then mixed with ethyl acetate solvent in a 1:1 ratio and homogenized. Afterwards, this mixture was evaporated using a vacuum rotary evaporator set at 44°C until a thick extract was obtained.

Antibacterial Activity of Culture Filtrate Extract of Plant Endophytic Fungus *Tinospora crispa*

Determination of the antibacterial activity of the culture filtrate extract of endophytic fungi isolated from *Tinospora crispa* plants was carried out using the disk diffusion method. This test was performed on bacteria *Escherichia coli* and *Staphylococcus aureus.* In this test, sterile distilled water was used as the extract solvent as well as a negative control, while chloramphenicol was used as a positive control with a concentration of 30 µg. Prior to testing, the bacterial suspension was standardised for turbidity using the 0.5 McFarland standard, which represents a bacterial density of 1×10^8 cells/ml. The bacterial suspension was then streaked evenly over Mueller-Hinton Agar (MHA) media using a sterile cotton swab. Paper discs that have been soaked with fungal extracts are placed on the surface of the media. Next, the entire medium was incubated at 30°C for approximately 24 hours to observe the antibacterial activity indicated by the zone of inhibition around the discs.

Anticandidal Activity of Culture Filtrate Extract of Plant Endophytic Fungus *Tinospora crispa*

Anticandida activity testing of filtrate culture extracts against *Candida albicans* was carried out by the paper disc diffusion method. Candida albicans stock that has been rejuvenated for 24 hours at 37°C was taken from Potato Dextrose Agar (PDA) media using an ose needle, then made a suspension in 0.85% NaCl solution and standardized its turbidity using the 0.5 McFarland

standard. The fungal suspension that has met the standard is then taken using a sterile cotton swab and spread evenly on the surface of the PDA media in a Petri dish. Paper discs were dipped for 5 minutes into the filtrate culture extract of endophytic fungi. The plate was then incubated for 24 hours at 37°C. To ensure the reliability of the results, the test was performed three times. After incubation, anticandidal activity was observed by measuring the diameter of the inhibition zone formed around the disk. The inhibition zone results of the extracts were compared with the positive control using ketoconazole (200 mg) and the negative control using sterile distilled water to assess the anticandida effectiveness of the endophytic fungal extracts.

Antibacterial Activity of Culture Filtrate Extract of Plant Endophytic Fungus *Tinospora crispa*

The isolation and genetic identification of endophytic fungi begins with harvesting of four-day-old mycelium from Potato Dextrose Broth (PDB) media. The mycelium was filtered, then frozen in a freezer until it froze. After that, the frozen mycelium was ground to destroy the cell tissue, and 40 mg was used for genomic DNA isolation using the Wizard® Genomic Kit method (Promega Corp). Amplification of the ITS-1 rDNA gene was performed using the Polymerase Chain Reaction (PCR) method. The primer pairs were ITS1F (5'-CTTGGTCATTTAGAGGAAGTAA-3') and ITS2 (5'-GCTGCGTTCTTCATCGATGC-3'). PCR reactions were prepared in a total volume of 50 µL, consisting of 6 µL of isolated DNA, 25 µL of GoTaq Green Master Mix, 2 µL of each primer, and 15 µL of Nuclease-Free Water. Amplification was performed in a PCR ThermoCycler (Bio-Rad) with 35 cycles, including pre-denaturation at 95°C for 3 minutes, denaturation at 95°C for 10 seconds, annealing at 54°C for 30 seconds, elongation at 72°C for 45 seconds, and final elongation at 72°C for 8 minutes. PCR products were analyzed using 1.5% agarose gel electrophoresis with 0.5x TAE buffer. Electrophoresis was run for 60 minutes with a voltage of 50 V, and the gel results were observed using a UV transilluminator. The PCR amplification products were then sent for DNA sequencing. After sequencing, the chromatogram images were analyzed to obtain the nucleotide base sequence. Furthermore, the DNA sequences were analyzed using NCBI's BLAST program. Finally, based on the BLAST results and sequence analysis, a phylogenetic tree was constructed to determine the evolutionary relationship between the fungal isolates. Phylogenetic tree construction was performed using the Neighbor-Joining method with 1000 bootstraps on MEGA-X software.

Data analysis

The test was conducted three times to ensure accurate results. Data on the percentage of inhibition was calculated by measuring the diameter of the inhibition zone of each culture filtrate extract of *Tinospora crispa* endophytic fungus. Statistical tests were conducted using Analysis of Variance (ANOVA). If significantly different results were found, the Duncan test was performed with a confidence level of $\alpha = 0.05$.

RESULTS AND DISCUSSION

Fungal Endophyte Isolation and Identification

Endophytic fungi were isolated using *Tinospora crispa* plants in perfect, healthy, and non-deformed conditions. The isolation process uses PDA media added with chloramphenicol, which inhibits the growth of contaminant and endophytic bacteria. Isolation was carried out using the direct planting method with three repetitions. From the isolation results, three endophytic fungal isolates were obtained, each from the root (isolate A), stem (isolate B), and leaf organs (isolate D). Endophytic fungal isolates obtained are observed based on the shape and colour of the colonies as an initial step in morphological characterisation to obtain pure isolates. Based on ITS gene nucleotide similarity, it shows that Isolate A is 98.09% similar to *Acrocalymma vagum* (MH141292.1), Isolate B is 99.65% similar to *Diaporthe tulliensis* (KX688170.1), and Isolate D shows 100% similarity to *Colletotrichum truncatum* (KX621963.1). Figures 1, 2, and 3 show the observation results for each isolate, including the top and bottom views of fungal colonies grown on PDA medium, microscopic observations of hyphae, and the phylogenetic tree based on ITS gene sequences.

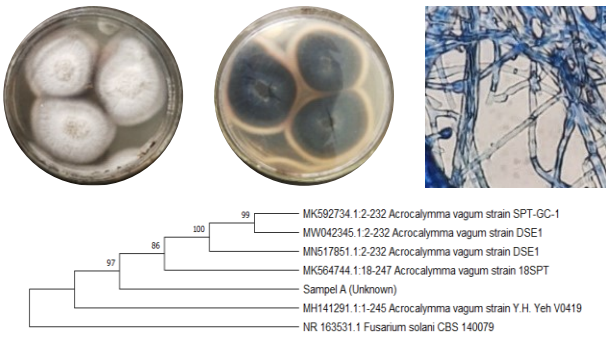

Figure 1. Endophytic fungal isolate A (*Acrocalymma vagum*).

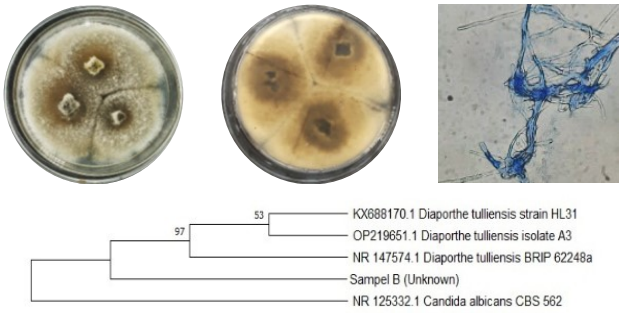

Figure 2. Endophytic fungal isolate B (*Dilaporthe tulliensis*).

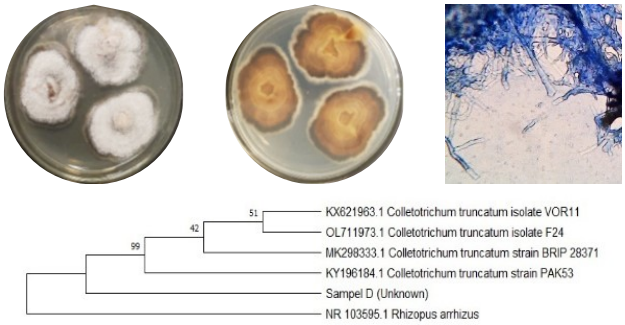

Figure 3. Endophytic fungal isolat D (*Colletotrichum truncatum*).

Antibacterial Activity of Culture Filtrate Extract of Plant Endophytic Fungus *Tinospora crispa*

Culture filtrate extracts from endophytic fungi demonstrated antibacterial activity against *Escherichia coli* and *Staphylococcus aureus*, as observed in Table 1 and Figure 4. The culture filtrate extract from the three isolates (A, B, and D) demonstrated inhibition zones against *E. coli* and *S. aureus*. Statistical analysis using one-way ANOVA revealed significant differences between the treatment groups. The culture filtrate extract of isolate D showed the highest antibacterial activity compared to the other isolates against both bacteria.

Table 1. Antibacterial activity of culture filtrate extract of fungal endophyte.

Categories	Inhibition Zone (mm)	
	E. coli	***S. aureus***
Negative control	$00{,}00 \pm 0{,}00^{a}$	$00{,}00 \pm 0{,}00^{a}$
Positive control	$27{,}33 \pm 0{,}21^{e}$	$30{,}00 \pm 0.96^{d}$
A	$10{,}00 \pm 0{,}20^{b}$	$11{,}33 \pm 0.76^{b}$
B	$10{,}66 \pm 0{,}24^{c}$	$13{,}33 \pm 1.16^{c}$
D	$11{,}00 \pm 0{,}14^{d}$	$13{,}66 \pm 0.41^{c}$

Note: Data represent the average inhibition zone diameter (± standard deviation). Statistical analysis using one-way ANOVA showed significant difference among treatments ($p > 0.05$).

Anticandidal Activity of Culture Filtrate Extract of Plant Endophytic Fungus *Tinospora crispa*

The results of the anticandidal activity test of the fungal endophytic culture filtrate extract, as shown in Table 2 and Figure 4, indicate that the culture filtrate extract can produce an inhibition zone against *Candida albicans*. The highest activity was shown by the culture filtrate extract of endophytic fungal isolate B. Statistical analysis using one-way ANOVA showed significant differences between treatments ($p > 0.05$).

Table 2. Anticandidal activity of culture filtrate extract of fungal endophyte.

Categories	Inhibition Zone (mm)
	Candida albicans
Negative control	$00{,}00 \pm 0{,}00^{a}$
Positive control	$28{,}03 \pm 0{,}24^{d}$
A	$6{,}50 \pm 0{,}59^{b}$
B	$9{,}05 \pm 0{,}81^{c}$
D	$8{,}21 \pm 0{,}43^{c}$

Note: Data represent the average inhibition zone diameter (± standard deviation). Statistical analysis using one-way ANOVA showed significant difference among treatments for *Candida albicans* ($p > 0.05$).

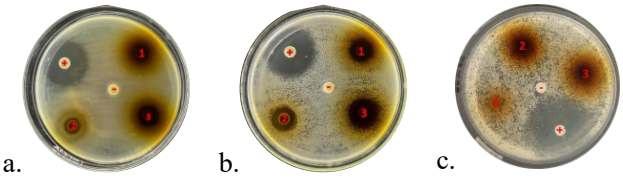

Figure 4. The antibacterial and anticandidal activity of culture filtrate extracts from endophytic fungi. (a) *Escherichia coli*, (b) *Staphylococcus aureus*, and (c) *Candida albicans*. Each plate shows: negative control, positive control, and extracts from three fungal isolates (1: Isolate A, 2: Isolate B, and 3: Isolate C).

Discussion

This study showed that culture filtrate extract of endophytic fungi from *Tinospora crispa* had varying antimicrobial activity against gram-negative *Escherichia coli*, gram-positive *Staphylococcus aureus*, and pathogenic *Candida albicans*. The three isolates obtained, *Acrocalymma vagum* (isolate A), *Diaporthe tulliensis* (isolate B), and *Colletotrichum truncatum* (isolate C), demonstrated the ability to produce inhibition zones against all test microbes, although at varying intensities. The highest antibacterial activity against *E. coli* and *S. aureus* was shown by isolate C. This study is in line with several previous studies that also reported the antimicrobial potential of this species. *C. truncatum* has been successfully isolated as an endophytic fungus from various plants, including *Chloranthus japonicus* (An et al., 2020), *Gynura japonica* (Riga et al., 2025), and *Musa acuminata* (Yansombat et al., 2023). Riga et al. (2025) reported that sydonic acid compounds isolated from endophytic *C. truncatum* showed antibacterial activity against *Staphylococcus aureus*, *Escherichia coli*, and *Streptococcus pyogenes*.

Endophytic fungi are an important source of novel antimicrobial compounds with diverse chemical structures and the ability to inhibit human, plant and marine pathogens, thus contributing significantly to the global challenge of antimicrobial resistance (Caruso et al., 2022). The difference in antibacterial potential between endophytic isolates could be due to variations in the secondary metabolites produced by each fungal species. The culture filtrate used in this study contains metabolite compounds secreted naturally during fermentation in liquid media. Extraction with ethyl acetate solvent allows good separation of these active compounds. This suggests that using culture filtrate, without involving the fungal biomass directly, is potentially effective as a source of antimicrobial compounds. The higher level of inhibition against *S. aureus* than *E. coli* in most isolates was due to the difference in cell wall structure between Gram-positive and Gram-negative bacteria. Gram-negative bacteria, such as *E. coli,* have a complex lipopolysaccharide outer membrane, which can inhibit the penetration of antimicrobial compounds (Choi & Lee, 2019).

Anticandidal activity testing revealed that the culture filtrate extract from isolate B (*Diaporthe tulliensis*)

showed the largest zone of inhibition against *Candida albicans*. Genus *Diaporthe* have been successfully isolated from various plants, such as *Orthosiphon stamienus* (Tong et al., 2017), *Prunus domestica* (Abramczyk et al., 2022), *etc*. The anticandidiasis activity observed in this study is in line with the findings of Yedukondalu et al. (2017), who reported that xylarolide compounds isolated from the endophytic fungus *Diaporthe terebinthifolii* showed antifungal activity against *Candida albicans*. In addition, *Diaporthe* sp. ED2 isolate from *O. stamienus* produced a new ketone compound, 3-hydroxy-5-methoxyhex-5-ene-2,4-dione, which showed fungicidal activity against *Candida albicans* (Tong et al., 2017). Endophytic fungi of the genus *Diaporthe* are also reported to produce various active compounds that have the potential to be used as biofertilizers and biopesticides (Hilário & Gonçalves, 2022). This finding align with previous reports on the antimicrobial activity of endophytic fungi from various medicinal plants. In addition, using culture filtrate extracts can be an alternative in exploring microbial resources as potential therapeutic agents. The results of this study indicate the potential utilisation of endophytic fungi from *T. crispa*. Future research should focus on the purification and structural elucidation of the active metabolites from these endophytic fungi, as well as in vivo testing to assess their therapeutic efficacy and safety.

CONCLUSIONS

The culture filtrate extract of endophytic fungal isolated from *Tinospora crispa* has antimicrobial activity against *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*. The results showed that secondary metabolites secreted into the culture medium were able to inhibit microbial growth, with *Colletotrichum truncatum* isolate showing the highest antibacterial effect against *Escherichia coli* and *Staphylococcus aureus* while *Diaporthe tulliensis* showed the highest anticandida activity. These findings support the potential application of fungal culture filtrates as an alternative source of bioactive compounds for antimicrobial therapy.

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