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Isolation of Endophytic Fungi from Rui (*Harrisonia perforata* (Blanco) Merr.) and Determining Their Antibacterial, Antioxidant, and Cytotoxic Activity

Syariful Anam[1*], Armini Syamsidi[1], Muh. Fadil Usman Tunreng[1], Helena Fransisca Djaleha[1], Windhy Nur Arisca[1], Gunawan Syaputra[1], Iklima[1], Meri Feki Indriani[1], Agustinus Widodo[1], Ritha Pratiwi[1], Yuliet[1], Abd. Rahman Razak[2]

[1]*Department of Pharmacy, Faculty of Mathematics and Natural Sciences, Tadulako University, Palu 94148, Indonesia*
[2]*Department of Chemistry, Faculty of Mathematics and Natural Sciences. Tadulako University, Palu 94148, Indonesia*

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ABSTRACT

Endophytic fungi produce bioactive metabolites that exhibit antibacterial, antioxidant, and anti-cancer activity. Rui (*Harrisonia perforata* (Blanco) Merr.) is a traditional medicinal plant proven to be an antibacterial and anticancer. Endophytic fungi live in plant tissues without damaging or producing chemicals that infect the host cell. Endophytes produce the same and similar compound as an original plant. This study aimed to isolate the endophytic fungi from Rui and determine their antibacterial, antioxidant, and cytotoxic activity. 13 fungal isolates were successfully isolated using PDA for seven days at 30°C. Molecular identification using the ITS1 and ITS4 DNA sequences only revealed six species: *Diaporthe* sp., *Phomopsis* sp., *Aspergillus tubingensis*, *Aspergillus viridinutans*, *Pseudofusicoccum* sp., and *Daldinia eschscholtz*. Seven isolates showed antibacterial activity based on plate assay. *Aspergillus tubingensis* and RA-1 exhibited antibacterial activity against *Staphylococcus aureus* and *Salmonella typhi*, with the MIC and MBC starting from 24 to 32 mg/ml. The DPPH assay showed the most increased antioxidant activity in *Daldinia eschscholtzii* with an IC_{50} of 98.14±2.39 µg/ml. The Brine Shrimp Lethality Test (BSLT) showed the highest potential cytotoxic activity, as shown by *Aspergillus tubingensis* and *Phomopsis* sp. with LC_{50} of 7.78±7.48 and 30.83±0.39 µg/ml. In conclusion, our study demonstrates that the fungal extract from Rui could be a source of antibacterial, antioxidant, and cytotoxic agents.

1. Introduction

In many decades, plants have become promising sources of natural products, which provide various bioactive compounds, and about 10-15% of plants have been studied (Bisht *et al.* 2006). Indonesia is considered a mega biodiversity country, with about 11% of the world's known plant species, of which around 18,700 are endemic (Fathurahman *et al.* 2016). However, the plant faces limited factors in commercial success as a source of natural products. At this time, many plants needed to provide bioactive components for clinical use. In other cases, bioactive components are mostly from endemic plants, becoming a significant concern regarding biodiversity conservation (Omeje 2017).

The microorganisms living inside the plant tissue can be used to overcome these issues that produce bioactive components as their host plants. Scaling up the fermentation process of these microorganisms enables an increase in the volume of bioactive components and the discovery of new potential bioactive components (Alvin 2014). In our study, we identified Rui (*Harrisonia perforata* (Blanco) Merr.) as an empirical antidiarrheal plant in the Palu and has scientifically proven to have inhibitory activity against *Escherichia coli, Salmonella typhi, Shigella dysentriae*, (Dewi Permatasari 2015; Irna Olvaliani Aimang *et al.* 2015) and cytotoxic activity (Juckmeta *et al.* 2019).

Endophytic fungi are found in plant parts without leading the disease for the plants (Sunitha *et al.* 2013). Several studies have indicated an interaction between endophytic fungi and host plants, with genes and environment as the critical factor. The association of

* Corresponding Author
E-mail Address: syarifulanam1@gmail.com

plant and endophyte may stimulate the secondary metabolites in the host plant (Uzma *et al.* 2018). Endophytic fungi produce metabolites such as steroids, xanthones, quinones, terpenoids, polyketides, alkaloids, peptides, proteins, lipids, and glycosides that possess pharmacological activity that is effective for curing many diseases like cancer, bacterial infections, fungal infections, cholesterolemia, and diabetes (Malik *et al.* 2020).

Studies have shown that the distribution of endophytic fungi is found in most plants, including plants in tropical rainforests, deserts, oceans, and even in the Arctic and Antarctic regions (Jin *et al.* 2021). They have been isolated and cultured from various parts of plants, like roots and above-ground parts (Jin *et al.* 2021; Wen *et al.* 2022). Scientists have identified approximately 69,000 fungal species, and only 11,500 species have been researched. The diversity of fungal species led to many bioactive metabolites (Hawksworth 1991; Wu *et al.* 2019).

The first endophytic strain isolated from ryegrass seeds (*Lolium temulentum* L.) has been proven that over 100 years of endophytic fungi have been well-known (Wen *et al.* 2022). Stierle *et al.* (Stierle *et al.* 1993) discovered *Taxomyces andreanae* from Pacific yew that produce paclitaxel for cancer treatment. Lovastatin is a popular drug for lowering cholesterol as an example of active components isolating from endophytic fungi (Priti *et al.* 2009; Tejesvi and Pirttila 2011). Endophytic fungi can also produce antibiotics; as we know, many microbes are resistant to modern antibiotics, and there is a need to change and discover new antibiotics. Until the 1970s, many new antibacterials were identified and developed, but from 1987 till now, there has been a significant lack of discovery of new antibacterial classes. This necessitates discovering innovative medicinal approaches and antimicrobial therapies (Hutchings *et al.* 2019; Uddin *et al.* 2021).

This discovery generated the attention of pharmacologists on endophytic fungi as a new sustainable source of bioactive components for antibacterial, antioxidant, and cytotoxic activity. Therefore, searching for new potential inhibitors from endophytic fungi should be an invaluable approach. Our study successfully isolated 13 fungal endophytes from *H. perforata* and examined their antibacterial, antioxidant, and cytotoxic activity.

2. Materials and Methods

2.1. Isolation of Endophytic Fungi of *H. perforata*

2.1.1. Sampling and Identification of Rui

The Rui (*H. perforata*) was obtained from Tipo, Ulujadi, Palu, and Central Sulawesi (000°51'60"S and 119°49'47" E). The whole plant was taken in a fresh state and identified by Hasan Ajalia. The voucher specimen was deposited at the Herbarium Celebense, Tadulako University, with number 09/UN.28 UPT-SDHS7LK/2021.

2.1.2. Isolation of Endophytic Fungi

Endophytic fungi were isolated using the modifications described by (Anam *et al.* 2022) and (Julia *et al.* 2022). The leaves, stems, and roots were washed with sterile water, then the leaves were cut into 2 × 2 cm pieces, and the stems and roots were cut to 2 cm lengths. Each organ was surface sterilized separately by soaking in 70% ethanol for 2 minutes, in 1% sodium hypochlorite solution for 5 minutes, and finally rinsed with sterile water three times. All samples were dried on sterile filter paper in a Laminar Airflow. Samples of leaves were cut into 1 × 1 cm, stems and roots were cut into 1 cm lengths, and then three pieces of the organ were placed on PDA (Potato Dextrose Agar) with 1% Chloramphenicol. The samples were incubated for 7 × 24 hours at 30°C. The fungal isolate that grows is sub-cultured until a pure culture is obtained based on the characteristic color of the colony.

2.2. Preliminary Screening for Antibacterial Activity Using Agar Plug Method

2.2.1. Preliminary Screening for Antibacterial Activity Using Agar Plug Method

An antibacterial assay was performed according to Hamzah *et al.* 2018 (Hamzah *et al.* 2018) and S. Silambarasan *et al.* 2012. Nutrient Agar (NA) (Merck) plate was prepared, and 100 µL of bacterial suspension of Bacillus subtilis ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Salmonella typhi* ATCC 14028 was then placed onto the plate separately. A 1 × 1 cm agar plug was cut out from 1 week-old fungal culture, placed onto agar containing bacterial suspension, and then incubated at 37°C for 24 h. The assay was replicated three times for each bacterium combination. Chloramphenicol 0.08% was used as the positive control. The inhibition zone parameter showed

that the fungal isolate had antibacterial activity and thus was continued with the Minimum Inhibitory Concentration (MIC) test of crude fungal extract.

2.2.2. Identification of Endophytic Fungi

Molecular identification of endophytic fungi was performed according to (Malik *et al.* 2020) with slight modification. Genomic DNA extraction from endophytic fungi was performed using the theTIANamp Genomic DNA Kit. Endophytic fungi were mixed into 200 µL GA buffer. Please put it in a 1.5 ml microtube. Add 200 µL proteinase K and mix well with a vortex. Add 200 µL of GB buffer, stir again with Vortex, then incubate at 70°C for 10 minutes to produce a homogeneous solution. Centrifuge briefly on a microcentrifuge to remove droplets inside the lid (12,000 rpm for 30 seconds at 15°C). Add 200 µL of ethanol (96–100%) and stir thoroughly for 15 seconds. Centrifuge briefly on a microcentrifuge to remove any droplets on the sections in the lid (12,000 rpm for 30 seconds at 15°C). Pipette the mixture in step 4 and transfer it to spin column CB3 (in collection 2 ml tube) and centrifuge at 12,000 rpm (13400 x g) for 30 seconds. Discard the flow trough and return the spin column to the collection tube. Add 500 µL of GD buffer to spin column CB3 and centrifuge at 12,000 rpm (13400 x g) for 30 seconds. Flow through Discard, and place the back rotating column into the collecting tube. Add 600 µL of PW buffer to spin column CB3 and centrifuge at 12,000 rpm (13400 x g) for 30 seconds. Flow through Discard, and place the back rotating column into the collecting tube. Centrifuge again at a speed of 12,000 rpm (~13400 x g) for 2 minutes to the drying membrane located at the base of the CB3 spin column. Place the CB3 spin column into a new 1.5 ml microtube, then Pipette 50–200 µL of TE buffer directly into the middle of the membrane. Incubate for 2–5 minutes, then centrifuged for 2 minutes at 12,000 rpm (13400 x g). The supernatant collected in microtubes is the result of ready DNA for analysis. Internal Transcribed Spacer (ITS) of DNA cores was amplified using the MyTaq HS RedMix Polymerase Chain Reaction (PCR) tool (Bioline 25047) (Meridian Bioscience, USA) using primers ITS1 5'–TCCGTAGGTGAACCTGCGG–3' and primes ITS4 5'–TCCTCCGCTTATTGATATGC–3', The final volume of the PCR reaction was 50 µL containing 25 µL of 2X PCR MyTaq Mix Red, 1 µL 10 M ITS1 and ITS4, 200 ng template DNA.T he amplification process consists of initial denaturation at 95°C for 1 min, denaturation at 94°C for the 30s, attachment of primer to DNA template at 58°C for 15s, elongation at 72°C for 30s and final extension at 72°C for 6 min. The amplification processes were repeated 30 times. The PCR product was analyzed with gel electrophoresis using 1% agarose gel with GelRed staining. PCR products were sequenced Bi-directionally using the ABI PRISM 3730 × 1 Genetic Analyzer (Applied Biosystems, USA) at First BASE Laboratory Sdn. Bhd., Selangor, Malaysia. Species names of fungal isolates were assigned based on the NCBI database. The evolutionary history of fungi was analyzed using MEGA X.

2.3. Preparation of Crude Fungal Extracts

The crude fungal extracts were prepared (Wang *et al.* 2015; Hamzah *et al.* 2018). Seven fungal isolates that showed antibacterial activity were inoculated on PDA for seven days. Seven fungal agar plugs were inoculated into 300 mL PDB (Potato Dextrose Broth) (KgaA Darmstadt) in an Erlenmeyer flask incubated at 30°C for 14 days with shaking at 120 rpm. The liquid culture was filtered using a Whatmann paper, and the filtrate was extracted with a 1:1 ratio of ethyl acetate (Brataco Chem.) two times. The concentrated extract was obtained by using a rotary vacuum evaporator.

2.4. Determination of Minimal Inhibitory Concentration (MIC)/Minimum Bactericidal Concentration (MBC)

MIC/MBC of the crude fungal extract was determined using paper disk diffusion (Shafi 1975; Mulyadi *et al.* 2017; Rollando 2019) by placing a disk containing the extract that will diffuse into the agar medium. Nutrient agar (NA) (Merck) plate was prepared, and 100 µL of bacterial suspension of *Bacillus subtilis* ATCC 6633, *Staphylococcus aureus* ATCC 25923, *Escherichia coli* ATCC 25922 and *Salmonella typhi* ATCC 14028 was then placed onto the plate separately. The seven fungal extracts were examined using series concentrations ranging from 50 to 1 mg/ml, dissolving the fungal extract with DMSO (Merck) 10%. A 5 µL extract was dripped in the sterile paper disc and laced onto agar containing bacterial suspension, 37°C for 24 h. Chloramphenicol 2% was used as the positive control. The MIC value was determined based on a concentration series indicating the clear zone using the naked eye observation around the paper disc. MBC value was determined based on the turbidity method in which the MIC concentration was reincubated again on Nutrient Broth (NB) at 37°C for 24 hours. The clarity of the samples indicates the MBC value.

2.5. Antioxidant Activity

The antioxidant activity of the seven fungal extracts was performed using the 1,1-diphenyl-2picrylhydrazyl (DPPH) (Merck) radical scavenging assay (Hamzah *et al.* 2018) with modifications. The fungal crude extract was diluted in methanol (Merck) to prepare eight series concentrations starting at 500, 250, 125, 62.5, 31.3, 15.6, 7.8, and 3,9 µg/ml as testing solutions. 1.5 ml of the test solution from each concentration and 1.5 ml of 50 ppm DPPH solution putting it in a test tube. Then homogenized and incubated in the dark condition for 30 minutes. Test solutions absorption was measured using a UV-Vis spectrophotometer (Cecile) at 515.5 nm. Inhibition of free radical in percentage (M%) was calculated using the following equation: M% = [(A0-Ai)/A0] × 100, where A0 = absorbance of the control group (without test solutions), and Ai = absorbance of the test solutions. Ascorbic acid was used as the positive control, and all test solutions were measured in triplicates.

2.6. Cytotoxic Activity Using Brine Shrimp Lethality Test

The assay was modified from a previous study (Anam *et al.* 2022). The seven fungal crude extracts were prepared in eight final series concentrations in vials of 500, 250, 125, 62.5, 31.25, 15.62, 7.8, and 3.9 µg/ml with DMSO 10%. The assay was performed by adding a 500 µL test solution in a 10 ml vial, and artificial seawater and 10 Artemia salina larvae were added to reach a volume of 5 ml. Each concentration was repeated three times and compared with the DMSO control. The number of deaths of *Artemia salina* larvae was observed after 24 hours. The cytotoxic of fungal extracts was determined by the value of the LC_{50}, which can kill 50% of the *Artemia salina* population. Toxicity effects were analyzed from observations by calculating the percent mortality (M%) according to the following formula: M% = [(D0-Di)/D0] × 1, where D0 = the number of total Larvae, and Di = the number of dead larvae. The LC_{50} value was obtained by plotting between concentration and percent mortality of larvae using the GraFit version 5.0 program (Erithacus Software Limited, Horley, Surrey, RH69YJ, UK).

3. Results

3.1. Isolation of Endophytic Fungi

Plant Rui was collected from Palu, and leaves stems, and roots were used to isolate the endophytic fungi. Endophytic fungi have been isolated from organs of the plant, represented in Figure 1. Purifying the isolates

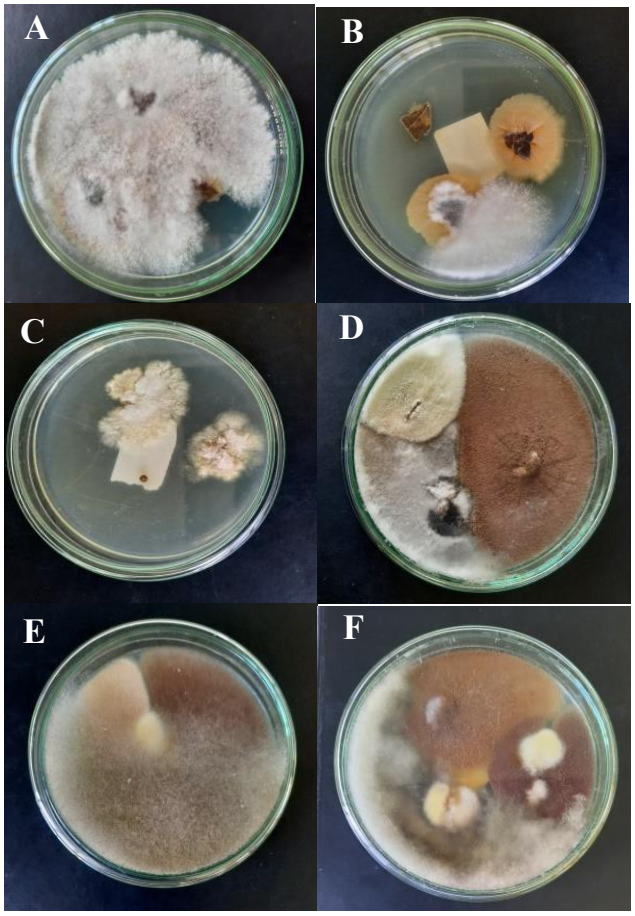

Figure 1. Isolation of endophytic fungi from plant Rui (*H. perforata*) organ on PDA. a-b: leaves, c-d: stems, e-f: roots

was performed by re-culturing in PDA media based on the characteristic color of the colony. In the pure isolate illustrated in Figure 2, there are 13 isolates, including two isolates from the leaves organ, five isolates from the stems organ, and six isolates from the roots organ.

3.2. Preliminary Screening for Antibacterial Activity Using Agar Plug Method

Thirteen fungal isolates were examined for the antibacterial assay with bacteria tested in the preliminary test (Table 1). A preliminary test was carried out in triplicate using an agar plug of endophytic fungi determined based on the inhibition zone around the agar plug (Figure 3-6). The results showed that seven fungal isolates RD-1, RB-1, RB-2, RB-3, RB-4, RB-5, and RA-1 inhibit bacteria.

3.3. Endophytic Identification

Seven fungal isolates were selected for identification based on the preliminary screening antibacterial plate assay. Macromorphology and micromorphology are

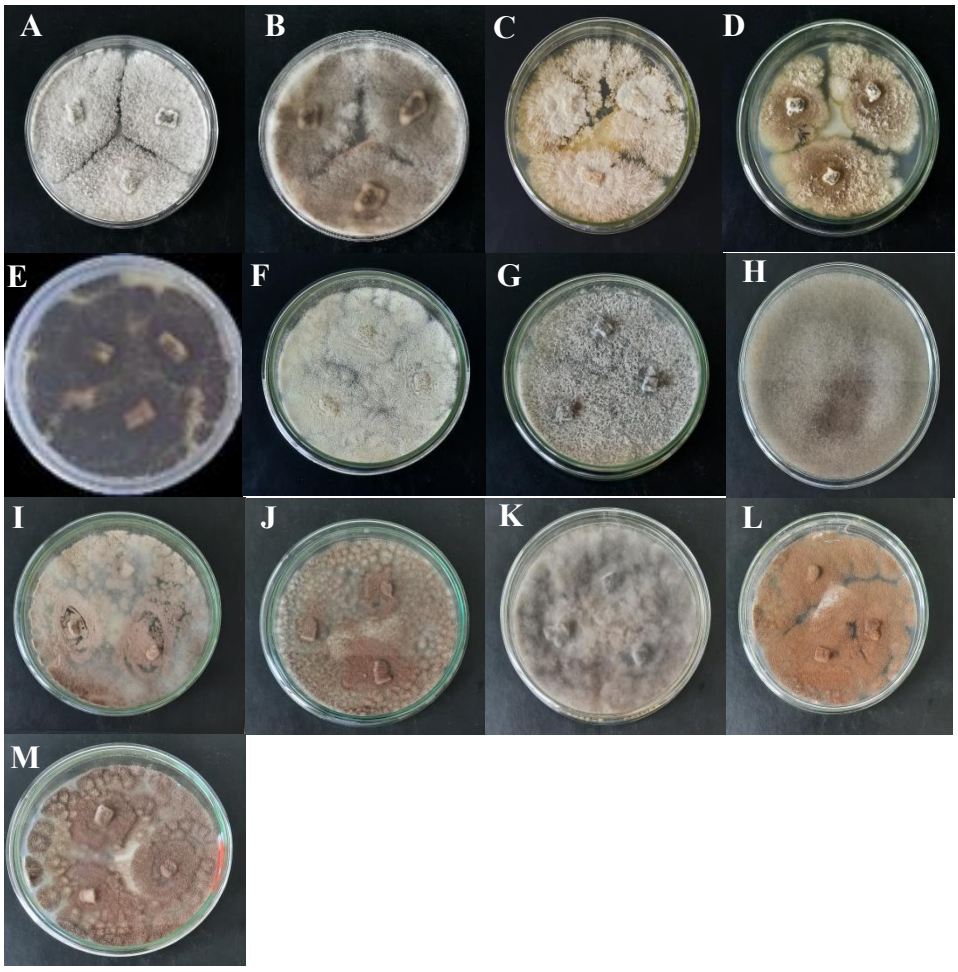

Figure 2. The pure culture of 13 endophytic fungi from plant Rui (*H. perforata*) on PDA. (A) RD-1, (B) RD-2, (C) RB-1, (D) RB-2, (E) RB-3, (F) RB-4, (G) RB-5, (H) RA-1, (I) RA-2, (J) RA-3, (K) RA-4, (L) RA-5 dan (M) RA-6

Table 1. The preliminary test of agar plugs fungal isolates against bacteria test using agar plug

Fungal isolate	*B. subtilis*			*S. aureus*			*E. coli*			*S. typhi*		
	i	ii	iii	i	ii	iii	i	ii	iii	i	ii	iii
RD-1	+	+	+	+	+	+	+	+	+	+	+	-
RD-2	+	-	-	+	+	+	-	+	-	-	-	-
RB-1	+	+	+	+	+	+	+	+	+	+	-	+
RB-2	+	+	+	-	+	+	+	+	+	-	-	-
RB-3	+	+	-	+	+	+	+	+	-	-	-	+
RB-4	+	+	+	+	+	+	+	+	+	+	+	-
RB-5	+	+	+	+	+	+	+	+	+	+	-	-
RA-1	+	+	+	-	+	+	+	+	+	-	+	+
RA-2	+	-	+	-	-	+	+	-	+	-	+	+
RA-3	-	-	+	+	-	+	-	-	-	-	-	-
RA-4	+	+	+	+	+	+	+	+	+	+	-	-
RA-5	-	+	-	-	+	+	-	-	-	-	-	+
RA-6	-	-	-	-	-	-	-	+	+	+	-	-
Chloramphenicol	+	+	+	+	+	+	+	+	+	+	+	+

"+" the inhibition of fungal isolates, and "–" no inhibition. Chloramphenicol was used as the positive control

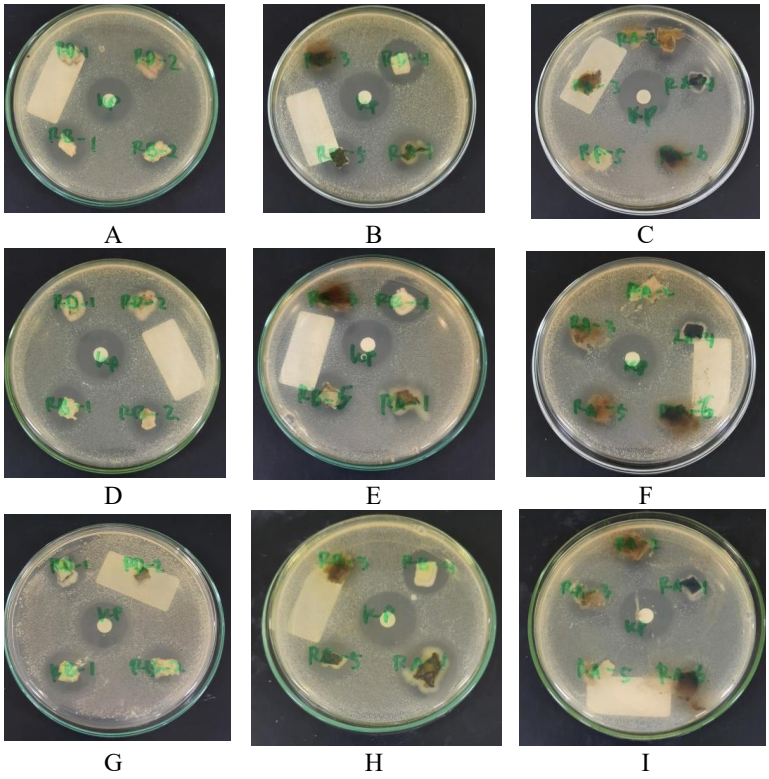

Figure 3. Preliminary screening of fungal isolates against *Bacillus subtilis* using agar plug method. A-C: replicate 1, D-F: replicate 2, G-I: replicate 3

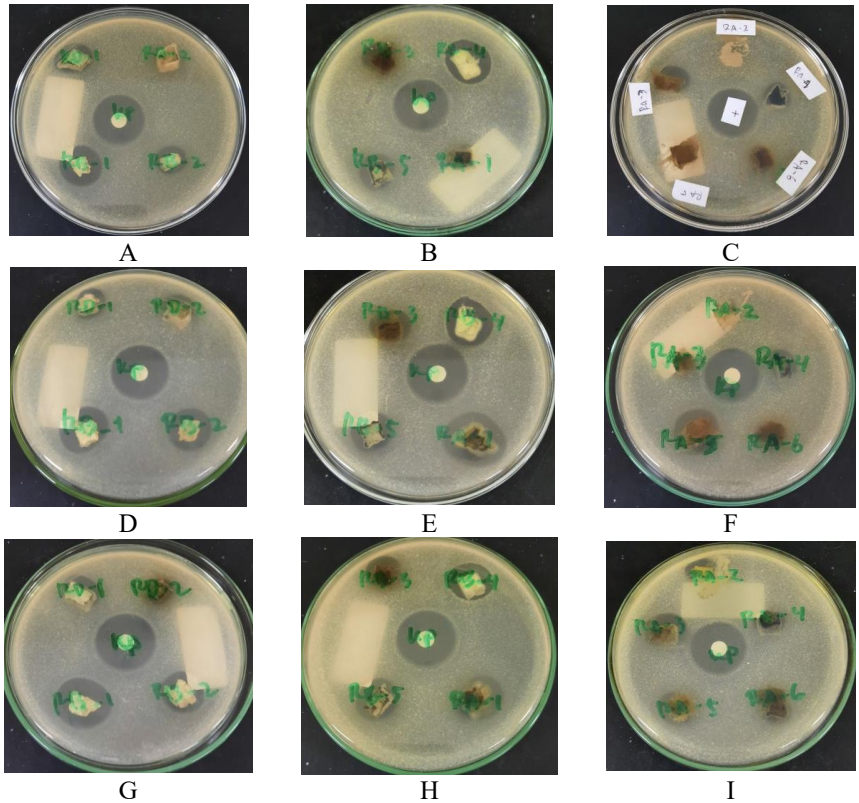

Figure 4. Preliminary screening of fungal isolates against *Staphylococcus aureus* using agar plug method. A-C: replicate 1, D-F: replicate 2, G-I: replicate 3

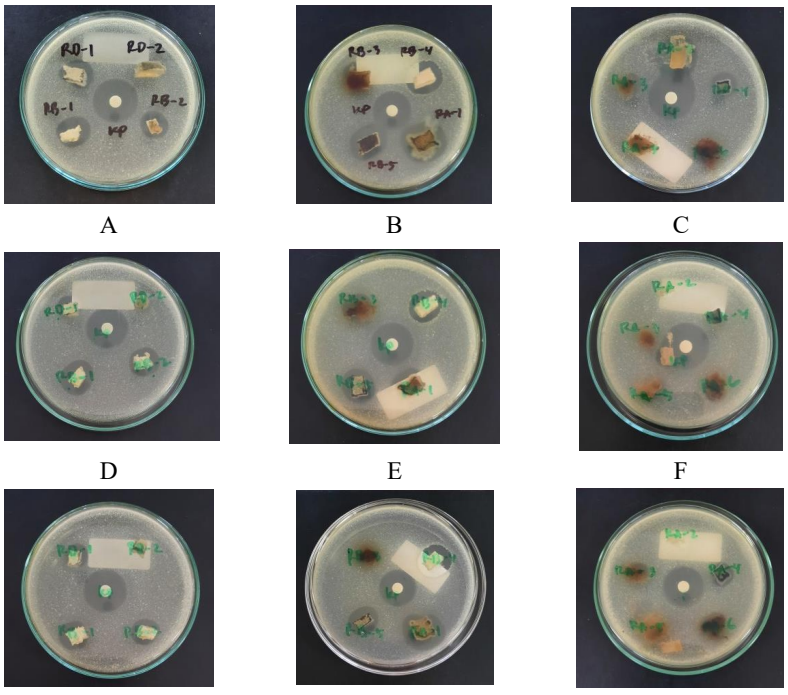

Figure 5. Preliminary screening of fungal isolates against *Escherichia coli* using agar plug method. A-C: replicate 1, D-F: replicate 2, Ga-I: replicate 3

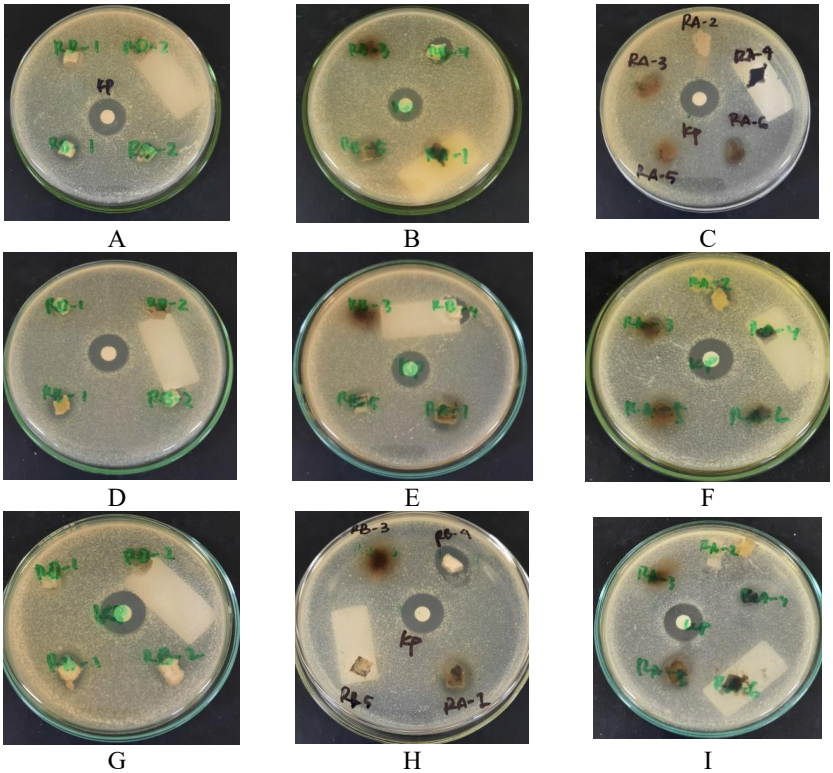

Figure 6. Preliminary screening of fungal isolates against *Salmonella typhi* using agar plug method. A-C: replicate 1, D-F: replicate 2, G-I: replicate 3

represented in Figures 7 and 8. Molecular identification using primers ITS1 and ITS4. BLAST searches revealed their identities as six genera represented in Table 2. The phylogenetic tree of seven genera is illustrated in Figures 9-14.

3.4. Determination of Minimal Inhibitory Concentration (MIC)/Minimum Bactericidal Concentration (MBC)

Seven crude fungal extracts were selected to investigate the minimum concentration that inhibits the

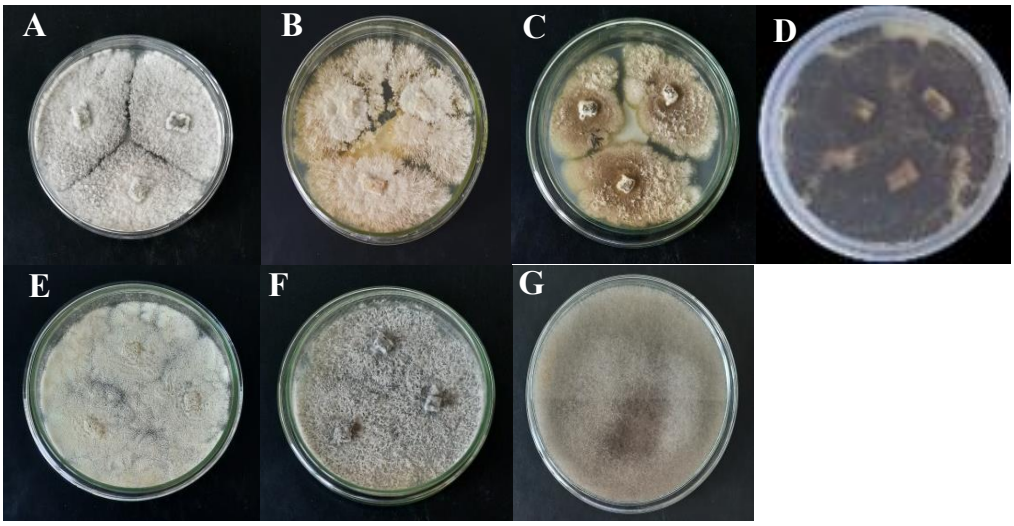

Figure 7. Macro-morphology of seven selected endophytic fungi on PDA. A: RD1, B: RB1, C: RB2, D: RB3 E: RB4, F: RB5, G: RA1

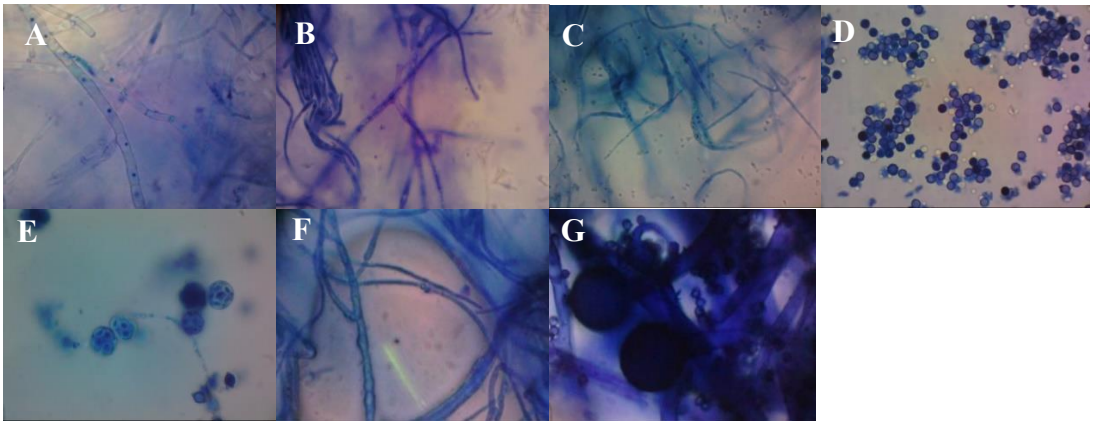

Figure 8. Micro-morphology of seven endophytic fungi. (A) isolat RD1, (B) isolat RB1, (C) isolat RB2, (D) isolat RB3, (E) isolat RB4, (F) isolat RB5, (G) isolat RA

Table 2. Match identity for six fungal isolates

Fungal ID	Percent Identity (%)	E-value	Max score	Total score	Query cover (%)	Species	GenBank accession
RB-1	98.55	0.0	966	966	99	Diap*orthe* sp	MW220855
RB-2	99.07	0.0	968	968	98	*Phomopsis* sp.	HQ315847
RB-3	99.65	0.0	1037	1037	98	*Aspergillus tubingensis*	MT729937
RB-4	99.46	0.0	1014	1014	97	*Aspergilus viridinutans*	HM473077
RB-5	99.28	0.0	998	998	98	*Pseudofusicoccum* sp.	MZ854244
RD-1	99.26	0.0	981	981	98	*Daldinia eschscholtzii*	MT597438
RA-1	-	-	-	-	-	-	-

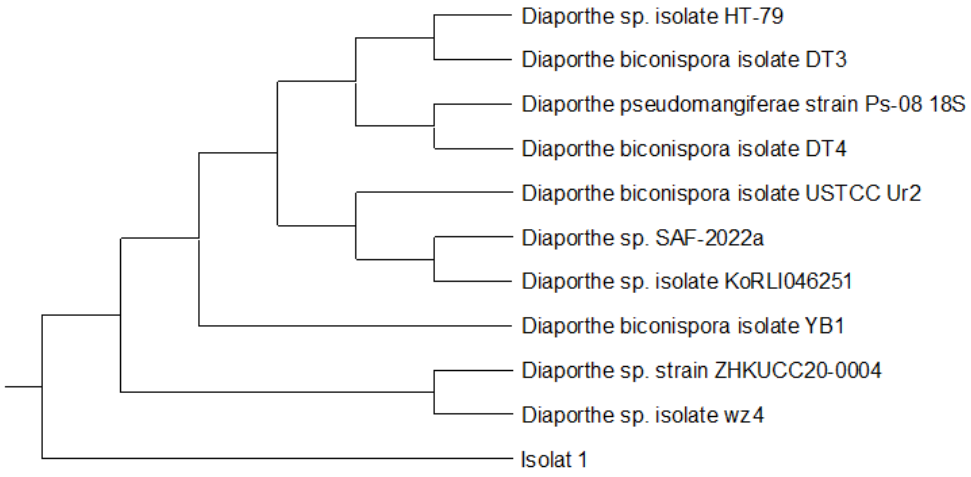

Figure 9. Phylogenetic tree of endophytic fungi RB1 (*Diaporthe* sp.)

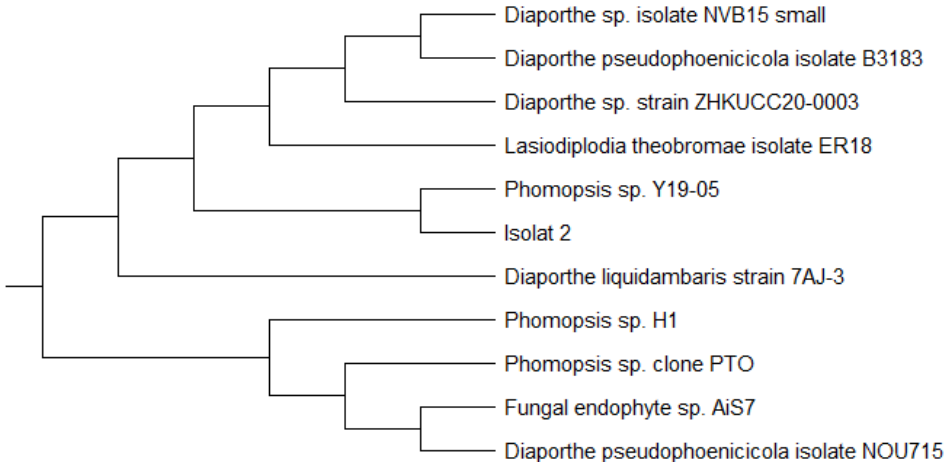

Figure 10. Phylogenetic tree of endophytic fungi RB2 (*Phomopsis* sp.)

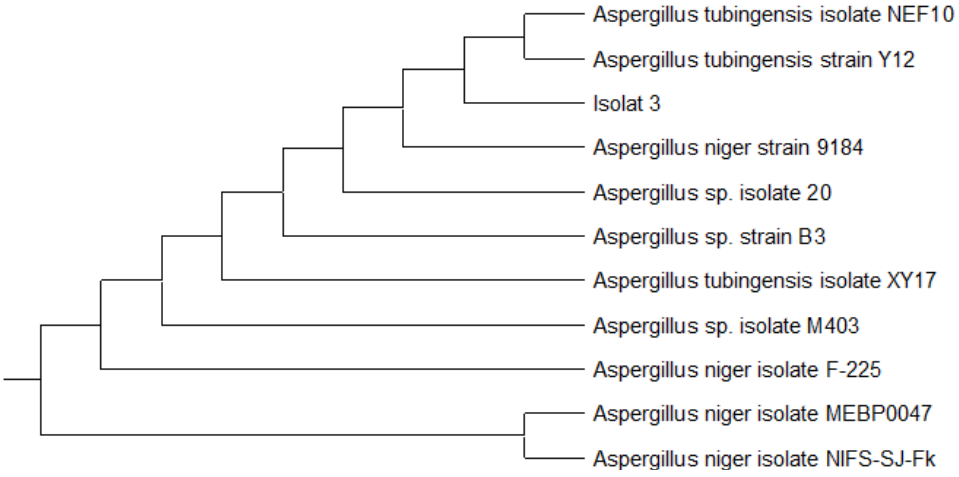

Figure 11. Phylogenetic tree of endophytic fungi RB3 (*Aspergillus tubingensis*)

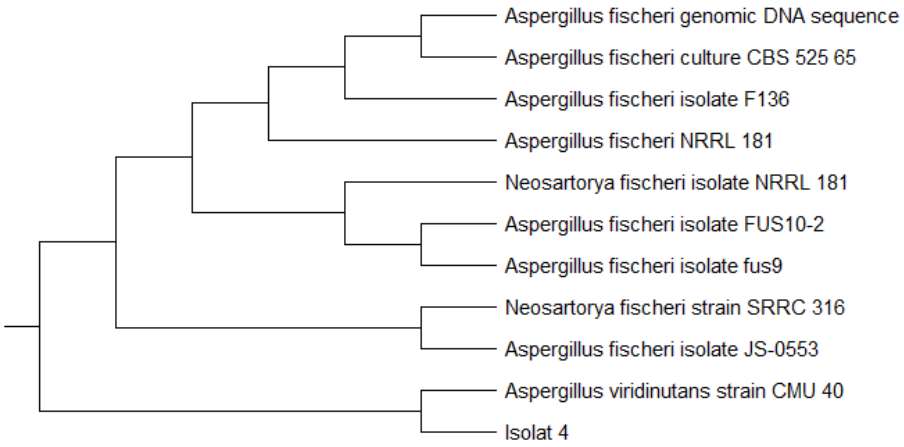

Figure 12. Phylogenetic tree of endophytic fungi RB4 (*Aspergillus viridinutans*)

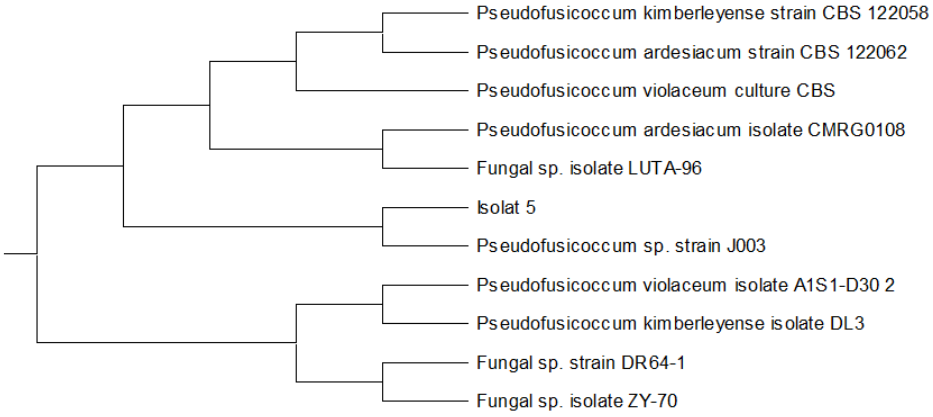

Figure 13. Phylogenetic tree of endophytic fungi RB5 (*Pseudofusicoccum* sp.)

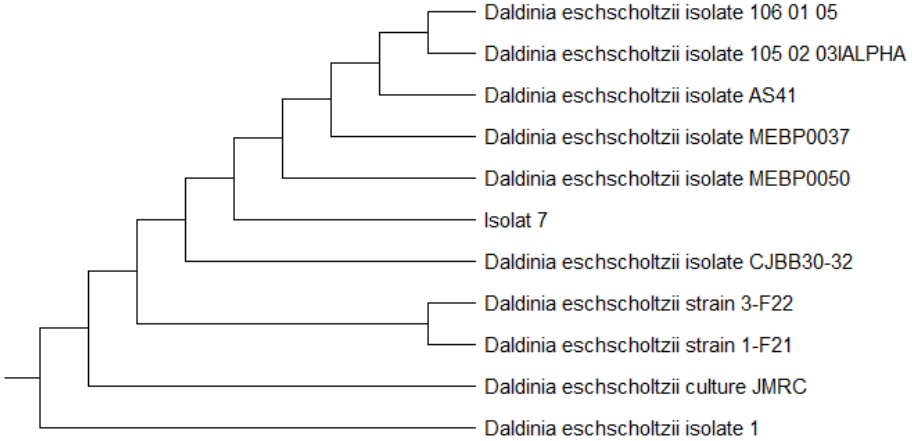

Figure 14. Phylogenetic tree of endophytic fungi RD1 (*Daldinia eschscholtzii*)

bacteria. The result showed that Aspergillus tubingensis and RA-1 inhibited the bacteria, as the MIC/MBC value indicated. Crude extract of *Aspergillus tubingensis* inhibit adequately *S. aureus* at 28 mg/ml; at 31 mg/ml, it was fair to deter *S. typhi*; at 45 and 50 mg/ml, it inhibits *E. coli* and *B. subtilis*, respectively. Fungal extract of RA-1 inhibits *S. aureus, E. coli*, and *S. typhi* with MIC/MBC value at 24/25 mg/ml (Table 3).

Table 3. MIC and MBC value of seven fungal extracts using the diffusion agar method

Isolate	MIC/MBC (mg/ml)			
	B. subtilis	*S. aureus*	*E. coli*	*S. typhi*
Diaporthe sp.	-	50/-	50/-	50/-
Phomopsis sp.	-	50/-	-	50/-
Aspergillus tubingensis	50/-	28/29	45/-	31/32
Aspergilus viridinutans	-	-	-	-
Pseudofusicoccum sp.	-	-	-	-
Daldinia eschscholtzii	50/-	-	50/-	50/-
RA-1	-	24/25	24/25	24/25

3.4. Antioxidant Activity

Seven fungal extracts were evaluated for their capacity to inhibit the DPPH. The fungal extract of *Daldinia eschscholtzii* reduced the absorbance of DPPH free radical with an IC_{50} value of 98.14 µg/ml. Meanwhile, other fungal extracts required a high concentration of more than 200 µg/ml to inhibit the DPPH free radical (Table 4).

3.5. Cytotoxic Activity

The Brine ShrimpLethality Test (BSLT) examined seven fungal extracts for cytotoxic activity. The fungal extract of *Aspergillus tubingensis* showed the highest inhibition with an LC_{50} value of 7.78 µg/ml (Table 5).

4. Discussion

4.1. Endophytic Diversity

This study is the first to isolate endophytic fungi from the plant Rui, representing 13 endophytic fungi successfully isolated. We used the plant Rui's three organs: leaves, stems, and roots. The isolation results illustrated in Figure 1 show that more than one fungal emerges from one organ. Further isolation was carried out to obtain the pure fungal culture by sub-culturing them based on the characteristic color of the colony. Finally, we got 13 fungal isolates. Figure 2 illustrates the macro-morphology of the 13 fungal isolates, showing various colony colors that represent the diversity of endophytic fungi from plant Rui. Molecular identification of selected endophytic fungi using ITS sequences identified six fungal isolates, including *Diaporthe* sp., *Phomopsis* sp., *Aspergillus tubingensis, Aspergillus viridinutans, Pseudofusicoccum* sp., *Daldinia eschscholtzii* representing in Table 2 and the phylogenetic tree. The six genera are parts of the phylum Ascomycota. These results showed similar findings to the previous study, in which Ascomycota was the most frequently endophytic fungi encountered by plants (He *et al.* 2012; Koukol *et al.* 2012).

Table 4. Inhibition concentration (IC_{50}) of seven fungal extracts against DPPH

Fungal extracts	IC_{50} value (µg/ml)[a]
Diaporthe sp.	747.02±39.32
Phomopsis sp.	651.76±43.04
Aspergillus tubingensis	218.75±1.87
Aspergilus viridinutans	312.02±26.20
Pseudofusicoccum sp.	585.26±8.39
Daldinia eschscholtzii	98.14±2.39
RA-1	623.26±31.55
Ascorbic acid	2.55±0.94

[a]triplicates

Table 5. Lethal concentration (LC_{50}) of seven fungal extracts using BSLT

Fungal extracts	IC_{50} value (µg/ml)[b]
Diaporthe sp.	39.20±9.07
Phomopsis sp.	30.83±0.39
Aspergillus tubingensis	7.78±7.48
Aspergilus viridinutans	443.41±251.78
Pseudofusicoccum sp.	211.10±993.91
Daldinia eschscholtzii	81.42±4.94
RA-1	175.67±35.52

[b]triplicates

Diaporthe (incl. its Phomopsis state) has been reported as one of the most frequently encountered genera of endophytic fungi in several plant hosts (Murali *et al.* 2006; Botella and Diez 2011; Gomes *et al.* 2013). The genus has also often been recognized as a producer of exciting enzymes and secondary metabolites (Dai *et al.* 2005; Isaka *et al.* 2001) with antibiotics (Dettrakul *et al.* 2003) or anticancer activity (Kumaran and Hur 2009). Furthermore, species of Diaporthe have been applied as bioherbicides (Ash *et al.* 2010). Among the numerous existing endophytic fungi, *Aspergillus* strains constitute one of the most prolific sources of secondary metabolites with diverse chemical classes and interesting biological activities. The isolated metabolites were chemically varied and exhibited various biological activities such as antibacterial, anti-cancer, anti-plasmodial, anti-inflammatory,

antioxidant, immunosuppressive, and antifungal activities (Hagag *et al.* 2022). *Pseudofusicoccum* species have been reported to produce secondary metabolites and exhibit cytotoxic activity (Sobreira *et al.* 2018). Daldinia is accommodated in Hypoxylaceae (Wendt *et al.* 2018). It is a ubiquitous wood-inhabiting ascomycete with a wider geographic area and host range (U'Ren *et al.* 2016). Daldinia demonstrated broad biological activity in various areas of human welfare, such as antimicrobials (Narmani *et al.* 2019).

One fungal isolate, RA1, could not be identified. The sequence obtained is not in the database. The Blast results for RA1 in NCBI show that the sequences are somewhat different. Therefore, in this study, we mention the RA1 isolate.

4.2. Antibacterial Properties

In a previous study, the leaf extracts of *H. perforata* showed significant inhibition on the growth of *Escherichia coli* and *Salmonella typhi* (Irna Olvaliani Aimang *et al.* 2015). In our effort to pursue new antibacterial compounds, it could be suggested that the endophytic fungi isolated from *H. perforata* exhibit antibacterial activity as their host plant.

In our study, 13 fungal isolates were isolated from *H. perforata*, and preliminary antibacterial activity screening was conducted using the agar plug method. The potential fungal isolates were selected based on preliminary screening results for antibacterial activity. Seven fungal isolates that inhibit the four tested bacterial pathogens were selected as potent strains and were continued with the MIC/MBC test of crude fungal extract.

Fungal extract of *Aspergillus tubingensis* and RA-1-inhibited bacteria were tested, as the MIC/MBC values indicated that the remaining other fungal extract showed no inhibition. Our findings have shown results similar to those of Mohamed *et al.* 2022, who reported that compounds of *A. tubingensis* extract showed antibacterial activities against *Pseudomonas aeruginosa* and *Escherichia coli*. In addition, endophytic *Aspergillus* species are prominent sources of antimicrobial metabolites (Mohamed *et al.* 2022; Padhi *et al.* 2017).

4.3. Antioxidant Activity

All fungal extracts have performed antioxidant activity using free radical scavenging DPPH. The result showed the seven fungal extracts exhibited antioxidant activity represented by the IC_{50} value. *Diaporthe* sp., *Phomopsis* sp., *Pseudofusicoccum* sp., and RA-1 have IC_{50} values above 500 µg/ml, while two *Aspergillus* species have IC_{50} around 200-300 µg/ml. The fungal extract of *Daldinia eschscholtzii* had the highest antioxidant activity with an IC_{50} value of about 98.14±2.39 (Table 4). Chutulo 2020 reported that the isolate of *D. eschscholtzii* from *Psidium guajava* showed antioxidant activity (Chutulo 2020). In addition, isolate D. eschscholtzii from Musa paradisiaca showed a percent inhibition of 65.9% at a 500 µg/ml concentration in the DPPH assay. High-pressure chromatography identifies the extract/fraction containing aureonitol, pestalotiolactone A, cyclopenol, and palitantin (Chigozie *et al.* 2020).

4.4. Cytotoxic Activity

Larvae *A. salina* was used as a bioindicator in this study because this method is easy and straightforward, requires relatively few samples, and has a reasonably high sensitivity (Elsy Puspitasari and Hendri 2018). Therefore, toxicity tests using these larvae were used as an initial screening that can provide a positive correlation with the cytotoxicity of the material test on cancer cells (Carballo *et al.* 2002; Elsy Puspitasari and Hendri 2018; Anam *et al.* 2022), so it is hoped that this study will provide recommendations for isolates and endophytic fungal isolate extract, which has activity against *A. salina* to be continued on testing using cancer cells. The cytotoxic assay showed that *A. tubingensis* had the highest activity, followed by *Phomopsis* sp. with IC_{50} of 7.78±7.48 and 30.83±0.39 µg/ml, respectively (Table 5). In a previous study, bioactive compounds produced by *A. tubingensis* (Asperazine, Pyranonigrin A, Fonsecin, TMC 256 A1) showed inhibition to the L5178Y cell line (Mohamed *et al.* 2022).

Aspergillus tubingensis is the fungal isolate that showed potential antibacterial and cytotoxic activity. The fungal extract of *A. tubingensis* showed the best value in MIC/MBC and LC_{50} compared to other fungal extracts. These results indicate a relationship between cell toxicity and the ability to kill bacterial cells. In contrast, *Daldinia eschscholtzii* showed the highest antioxidant but did not show adequate antibacterial and cytotoxic activity. In conclusion, our study demonstrates that the fungal extract of endophytic fungi isolated from *H. perforata* could be a source of antibacterial, antioxidant, and cytotoxic agents.

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