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Antibacterial and Antioxidant Activities of Fungal Endophytes Isolated from Medicinal Plants in Simeulue Island, Aceh

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ABSTRACT

Natural products from endophytic fungi have a wide range of medicinal applications, including antibacterial and antioxidant agents. The research aimed to evaluate the antibacterial and antioxidant activities of compounds derived from endophytic fungi isolated from medicinal plants collected from Simeulue Island by TLC dot-blot and microdilution assays. Eighty-one fungal extracts were screened for antibacterial and antioxidant activities. Antibacterial activity was evaluated against Gram-positive and negative bacteria, i.e., *Staphylococcus aureus* InaCC B-4 and *Escherichia coli* InaCC B-5, respectively. Antioxidant activity was carried out by using free radical scavenging of 1,1-diphenyl-2-picrylhydrazyl (DPPH). The results of the TLC bioassay revealed that the fungal extracts have activities as antibacterial and antioxidant are 65 (against *S. aureus*), 57 (against *E. coli*), and 48 extracts (DPPH free radical scavenger). Two extracts, *Phomopsis* sp. 48BtSi-2.1 and *Xylaria* sp. 55DnSi-1.1, showed strong antibacterial activity against *S. aureus* with MIC value of 32 µg/ml. Furthermore, nine extracts (*Schizophyllum* sp. 20DnSi-1; *Hyphomycetes* 36BhSi-1.1; *Phomopsis* sp. 36DnSi-2.1; *Schizophyllum* sp. 39DnSi-1.1; *Xylaria* sp. 39RpSi-2.1; *Phomopsis* sp. 41BtSi-1.1; *Phomopsis* sp. 48BtSi-2.1; *Lasiodiplodia* sp. 48BtSi-3.1; *Schizophyllum* sp. 50DnSi-3) were strong activity against *E. coli* with MIC value ranges from 32 to 64 µg/ml. One extract, *Xylaria* sp. 04BtSi-2.2 has strong antioxidant activity as DPPH free radical scavenger with an IC_{50} value of 21.66 µg/ml (AAI value of >1).

1. Introduction

Natural products from endophytic fungi have a wide range of uses in medicinal applications, including antibacterial and antioxidant agents (Praptiwi *et al.* 2018). They are also broadly recognized as potential sources of new bioactive compounds of possible agricultural, pharmaceutical, and industrial importance (Qin *et al.* 2009; Schulz *et al.* 1993, 2002). Antimicrobial resistance has become a significant health problem and still causes a problem in the global health care system (Ferri *et al.* 2015). The antibiotic resistance problem has directed the increase of research features, especially bioactive metabolites for antibiotic (Liang *et al.* 2012).

In addition to the problem of increasing antibiotic resistance, there are also growing studies looking for natural antioxidants to treat degenerative diseases. Free radicals from reactive oxygen species (ROS) and reactive nitrogen species (RNS) generated by both endogenous and exogenous sources lead to the oxidative stress process. The exogenous antioxidants from dietary food were needed to prevent damage caused by excessive free radicals (Bharathidasan *et al.* 2012).

In our previous studies, some chemical compounds isolated from endophytic fungi are potential as good antibacterial agents such as (+)-2,2'-epicytoskyrin A (Agusta *et al.* 2015), (+)-bislunatin (Praptiwi *et al.* 2013b), and 3-acetyl-2,5,7-trihydroxy-1,4-naphtalenedione (Praptiwi *et al.* 2013a). The other previous reports have described potent antioxidant compounds of some endophytic fungal metabolites including pestacin, isopestacin, and graphislaetone A (Harper *et al.* 2003; Strobel *et al.* 2002; Song *et al.* 2005). Therefore, this research aimed to evaluate the antibacterial and antioxidant activities of 81 kinds of

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endophytic fungi extracts isolated from 24 medicinal plants collected from Simeulue Island, Aceh, Indonesia.

2. Materials and Methods

2.1. Chemical Reagents

Ethanol 70%, Na-hypochlorite 5.25%, Malt Extract (Bacto), Yeast Extract (Bacto), Corn Meal Agar (Sigma-Aldrich), Potato Dextrose Agar (Difco), Potato Dextrose Broth (Difco), Chloramphenicol (Sigma), (+)-catechin (Sigma), vanillin (Sigma-Aldrich) and Ce(SO4)2 (Merck), iodonitrotetrazolium chloride (INT, Sigma), 2,2-diphenyl-1-picrylhydrazyl (Sigma), Mueller Hinton Broth (Difco), TLC plate (Silica gel GF254, Merck), UV light 254 and 366 nm (Camag).

2.2. Plant Collection

Twenty-four plant materials were collected from Kuala Makmur (2°32'02.2"N, 96°17'22.1"E), Langi (2°49'09.8"N, 95°44'44.6"E), and Amabaan Villages (2°51'00.3"N, 95°50'50.1"E), Simeulue Island, Aceh, Indonesia. Identification of plants was carried out in Bogoriensis Herbarium while identification of endophytic fungi was done in Indonesian Culture Collection (InaCC), Research Center for Biology-LIPI, Bogor, Indonesia.

2.3. Endophytic Fungi Isolation

Parts of the plants were washed, and cut to approximately 1–4 cm, followed by surface sterilization according to Praptiwi *et al.* (2020) as follow: soaking in 70% alcohol for 1 minute, followed by soaking in 5.25% NaOCl for 2.5-3.5 minutes, and then dipped again in 70% alcohol for 30 seconds. Sterile samples were dried aseptically and cut into small pieces with sterile blades, placed on the surface of Corn Meal Malt Agar (CMMA) medium supplementing with 0.05 mg/ml chloramphenicol and incubated for 3-5 days at room temperature. Each fungal colony that was subsequently grown was transferred to PDA medium until a single isolate was obtained. Pure isolates were preserved in 10% glycerol and stored at -80°C until used.

2.4. Endophytic Fungi Identification

Pure endophytic fungi were transferred and grown on Petri dishes containing PDA medium and then incubated at 25-27°C for 5-10 days. Microscopic slides of each selected strain were prepared using 0.01% trypan blue dye in lactophenol as a mounting medium. Fungi isolated identification is carried out based on morphological characteristics. Morphological identification was conducted by observing both macroscopic and microscopic properties. Macroscopic characterizations include observations on color, colony shape, surface, texture, exudate drop, and reverse color. Microscopic characterizations were performed on a light microscope by observing hyphae, hyphae pigmentation, septate, clamp connection, conidia, spores, and other reproductive structures.

2.5. Cultivation and Extraction of Endophytic Fungi

Endophytic fungi were grown on PDB medium and incubated at room temperature in dark conditions for 2-3 weeks. After the incubation period, biomass and media were extracted thricely with ethyl acetate. The extracts were concentrated under reduced pressure by a rotary evaporator. The extracts were stored at -30°C until used.

2.6. Analysis of Secondary Metabolites by TLC Assay

Secondary metabolites analysis were performed by TLC assay according to Praptiwi *et al.* (2020). The extracts were dissolved in acetone to a final concentration of 10 mg/ml. Ten microliters of extracts were transferred on a TLC plate and developed using dichloromethane-methanol (10:1). Separated chemical compounds were monitored under UV light of 254 and 366 nm and chemical substances were detected by spraying with a staining reaction. The samples on TLC were sprayed with the reagents of 0.25% vanillin in 10% sulphuric acid and 1% $Ce(SO_4)_2$ in 10% sulphuric acid. After spraying, the TLC plates were heated at 110°C for optimal color visualization.

2.7. Qualitative Antibacterial Activity by TLC Dot-Blot Assay

Antibacterial activity by TLC Dot-Blot assay according to Praptiwi *et al.* (2020). Ten microliters of 10 mg/ml extract were transferred on TLC plates and evaluated for antibacterial agents against *Staphylococcus aureus* InaCC B-4 and *Escherichia coli* InaCC B-5, by using TLC-DB dot blot assay. After drying, the TLC plate was dipped in a suspension of bacteria and placed in a sterile chamber with sterile wet cotton to keep the moisture. The TLC plates were incubated at 37°C for 18-24 hours. After the incubation period, the plates were sprayed with 4 mg/ml INT. A clear or white zone formation around the extracts against a purple background on the TLC plate indicated the inhibition of bacteria growth.

2.8. Qualitative Antioxidant Activity by TLC-Assay

Antioxidant activity was conducted by TLC- assay according to Praptiwi *et al.* (2020). Ten microliters of 10 mg/ml extract were transferred on a TLC plate and evaluated for antioxidant activity as DPPH free radical scavenger by dot blot assay. After drying, TLC plates were sprayed with 0.2 mg/ml DPPH in methanol and then incubated at room temperature for 5-10 minutes, in dark conditions. A white-yellow zone around the extracts against a purple background on the TLC plate indicated the antioxidant activity of the samples.

2.9. Quantitative Antibacterial Activity by Minimum Inhibitory Concentration (MIC)

Antibacterial activity by microdilution method according to Praptiwi *et al.* (2020). The extracts were dissolved in 2.5% dimethyl sulfoxide (DMSO) sterile and were tested against *S. aureus* and *E. coli*. One hundred microliters of extract were transferred in 1 st well and serially diluted, so that the concentration ranged from 2.0-256 µg/ml at a 96-well microplate then added 100 µl of the bacterial suspension (approximately 10^6 CFU/ml) by using the plate count and incubated at 37°C for 24 hours. The antibacterial test was conducted in triplicate. After incubation, ten microliters of 4 mg/ml INT sterile were added to each well which will result in purple color (the bacterial growth occurred). The lowest concentration that prevents visible growth of a microorganism (or without the color change) was considered as the minimum inhibitory concentration (MIC) value of the sample that inhibits bacteria growth.

2.10. Quantitative Antioxidant Activity by Determining IC_{50} Values of DPPH

Antioxidant activity was performed by determining IC_{50} values of DPPH according to Praptiwi *et al.* (2020). The extracts were dissolved in 1.25% dimethyl sulfoxide (DMSO) in methanol and were tested for antioxidant activity as DPPH free radical scavenging. One hundred microliters of the serial concentration of the extracts range of 1.0-128 µg/ml at a 96-well microplate were added to 100 µl of 61.5 µg/ml DPPH in methanol and were incubated for 90 minutes at room temperature and dark conditions. The antioxidant activity of the extract was conducted in triplicate. The blank was methanol, while the positive control was catechin. The absorbance was observed at 517 nm using Varioskan Flash (Thermo Scientific). Percent inhibition concentration (IC) was calculated as follows:

$$IC\ (\%) = \frac{(A_{control} - A_{sample})}{A_{control}} \times 100\%$$

$A_{control}$ = absorbance of DPPH without samples
A_{sample} = absorbance of DPPH along with different concentrations of extracts

The IC_{50} values of DPPH were obtained from linear calibration between the percentage of inhibition and sample concentration. The antioxidant activity index (AAI) is acquired by the formula below:

$$AAi = \frac{\text{The final concentration of DPPH}}{IC_{50}}$$

2.11. Macroscopic-Microscopic View and Molecular Identification of the Representative Potential Fungal Endophytes: *Xylaria* sp. 04BtSi-2.2 and *Phomopsis* sp. 48BtSi-2.1

The representative potential strains then selected for further identification using the molecular approach. The molecular identification conducted by analyzing the DNA sequence of an internal transcribed spacer (ITS1 and ITS2) of rDNA regions, includes the 5.8S rRNA. The total fungal genomic DNA was isolated using Nucleon PhytoPure, plant and fungal DNA extraction kits (GE Healthcare) according to the manufacturer`s instruction. DNA amplification of the ITS rDNA region was performed by polymerase chain reaction (PCR). PCR amplification was performed in 25-µl reaction mixtures containing 10 µl distilled water, 12.5 µl GoTaq Green Master Mix (Promega), 0.5 µl DMSO, 0.5 µl each primer (10 pmol), and 1 µl (5 to 10 ng) extracted genomic DNA as a template. The primer set of ITS4 (5`-TCCTCCGCTTATTGATATGC-3`) and ITS5 (5`-GGAAGTAAAAGTCGTAACAAGG-3`) was used to amplify approximately 550 nucleotides from ITS1 and ITS 2 including 5.8S rDNA (White *et al.* 1990). Amplification was performed in a TaKaRa PCR Thermal Cycler P650 (TAKARA BIO Inc.), programmed under following conditions: initial denaturation at 95°C for 3 min, 30 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 1 min. The PCR products were then subjected to purification and sequence analysis.

Initial phylogenetic tree construction of selected strains conducted by editing the raw sequence data using ChromasPro (http://www.technelysium.com.

au/ChromasPro.html). The assembled sequences were aligned with those downloaded from National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nlh.gov/) using the Muscle (http://www.ebi.ac.uk/Tools/msa/muscle). The phylogenetic analyses of sequence data were done based on the neighbor-joining (NJ) method (Saitou and Nei 1987) using Molecular Evolutionary Genetics Analysis (MEGA) version 7 program (Kumar *et al.* 2016). The reliability of each branch was evaluated by bootstrapping with 1,000 resampling.

3. Results

3.1. Endophytic Fungi Identification

A total of 81 endophytic fungi were successfully isolated from 24 medicinal plants collected from Simeulue Island (Table 1). These consist of *Colletotrichum* sp. (1 isolate), Dematiaceae (2 isolates), Hyphomycetes (7 isolates); *Lasiodiplodia* sp. (6 isolates), *Phomopsis* sp. (12 isolates); *Schizophyllum* sp. (30 isolates); *Trichoderma* sp. (4 isolates); and *Xylaria* sp. (19 isolates). Isolated endophytic fungi were dominated by the genera *Schizophyllum* sp., followed by *Xylaria* sp. and *Phomopsis* sp, while only one endophytic fungus was isolated from the genera *Colletotrichum* sp.

3.2. Analysis of Secondary Metabolites by TLC Method

The use of the TLC method has advantages such as separating, identifying, and quantifying different substances within the extracts. This method is also an easy, simple, universal, and quick assay to applicate, and cheaper than other chromatography methods. The visualization of chemical components can be done either under UV light or detected by staining spray reagents. The compounds visualized under long-wave UV light 366 nm appeared as bright bands as a fluorescent effect on the dark blue background. While the substances were monitored under short-wave UV light, 254 nm appeared as dark bands on a bright background.

The chemical components of fungal endophytic extracts according to the color spots appeared on the TLC plate that was monitored under UV light and after being sprayed with staining reagents. The chemical components appeared yellow (such as A and E, sample No. 39; 62; 73; 77; etc.) and blue fluorescence (such as A and E, sample No. 13; 24; 47; 50; 52; etc.) at a wavelength of 366 nm identified as flavonoids and coumarins, while blue (such as B and F, sample No. 13; 25; 62; 68; etc) and dark spots (such as B and F, sample No. 7; 15; 48; 49; etc) at 254 nm were identified as coumarins and terpenoids. After being

Table 1. Fungal endophytic isolates associated with medicinal plants from Simeulue Island

Fungal taxa	Plant part	Host plant
Hypomycetes-01TdSi-1.1	Petiole	*Pinanga malaiana* (Mart.) Scheff.
Schizophyllum sp. 02DnSi-1.1	Fruit	*Ficus* sp.
Hypomycetes-03BhSi-2.1	Fruit	*Glochidion varians* Miq.
Hypomycetes-03DnSi-2.1	Leaf	*Glochidion varians* Miq.
Schizophyllum sp. 04BhSi-1.1	Fruit	*Poikilospermum suaveolens* (Blume) Merr.
Xylaria sp. 04BtSi-2.1	Stem	*Poikilospermum suaveolens* (Blume) Merr.
Xylaria sp. 04BtSi-2.2	Stem	*Poikilospermum suaveolens* (Blume) Merr.
Schizophyllum sp. 05BgSi-2.1	Flower	*Leucosyke capitellata* Wedd.
Dematiaceae-05BtSi-2.1	Stem	*Leucosyke capitellata* Wedd.
Phomopsis sp. 06DnSi-1.1	Leaf	*Smilax leucophylla* Blume
Xylaria sp.08DnSi-3.1	Leaf	*Ficus septica* Burm.f.
Phomopsis sp. 09BtSi-1.1	Stem	*Gmelina elliptica* Sm.
Lasiodiplodia sp. 09BtSi-1.2	Stem	*Gmelina elliptica* Sm.
Hypomycetes-10DnSi-1.1	Leaf	*Magnolia singapurensis* (Ridl.) H.Keng
Phomopsis sp. 10DnSi-4	Leaf	*Magnolia singapurensis* (Ridl.) H.Keng
Phomopsis sp. 10DnSi-5.1	Leaf	*Magnolia singapurensis* (Ridl.) H.Keng
Phomopsis sp. 10DnSi-5.2	Leaf	*Magnolia singapurensis* (Ridl.) H.Keng
Colletotrichum sp. 12DnSi-2.2	Leaf	*Smilax leucophylla* Blume
Trichoderma sp. 18BtSi-2.1	Stem	*Sonneratia caseolaris* (L.) Engl.
Trichoderma sp. 18BtSi-2.2	Stem	*Sonneratia caseolaris* (L.) Engl.
Dematiaceae-20BtSi-1.1	Stem	*Hibiscus tilliaceus* L.
Schizophyllum sp. 20DnSi-1	Leaf	*Hibiscus tilliaceus* L.
Schizophyllum sp. 30DnSi-1	Leaf	*Melastoma cyanoides* Sm.
Lasiodiplodia sp. 31DnSi-2.1	Leaf	*Ficus septica Burm.* f.
Schizophyllum sp. 33DnSi-1.1	Leaf	*Piper aduncum* L.

Table 1. Continued

Fungal taxa	Plant part	Host plant
Hyphomycetes-36BhSi-1.1	Leaf	*Ficus fulva* Reinw. ex Blume
Phomopsis sp. 36DnSi-2.1	Leaf	*Ficus fulva* Reinw
Phomopsis sp. 36DnSi-2.2	Leaf	*Ficus fulva* Reinw
Schizophyllum sp. 37DnSi-2.1	Leaf	*Rhaphidophora puberula* Engl.
Lasiodiplodia sp. 38DnSi-1.1	Leaf	*Knema laurina* Warb.
Schizophyllum sp. 39DnSi-1.1	Leaf	*Plagiostachys sumatrensis* Ridl.
Xylaria sp. 39RpSi-2.1	Rhizome	*Plagiostachys sumatrensis* Ridl.
Phomopsis sp. 41BtSi-1.1	Stem	*Ficus ribes* Reinw. ex Blume
Schizophyllum sp. 46DnSi-2.1	Leaf	*Smilax leucophylla* Blume
Schizophyllum sp. 46DnSi-2.2	Leaf	*Smilax leucophylla*
Phomopsis sp. 48BtSi-1.1	Stem	*Freycinetia rigidifolia* Hemsl.
Lasiodiplodia sp. 48BtSi-1.2	Stem	*Freycinetia rigidifolia* Hemsl.
Phomopsis sp. 48BtSi-2.1	Stem	*Freycinetia rigidifolia* Hemsl.
Lasiodiplodia sp. 48BtSi-3.1	Stem	*Freycinetia rigidifolia* Hemsl.
Lasiodiplodia sp. 49DnSi-2.1	Leaf	Lauraceae1
Schizophyllum sp. 50DnSi-3	Leaf	Lauraceae2
Xylaria sp. 55DnSi-1.1	Leaf	*Smilax leucophylla* Blume
Phomopsis sp. 56BhSi-1.1	Fruit	*Calamus rotang* L.
Xylaria sp. 56BhSi-1.2	Fruit	*Calamus rotang* L.
Hyphomycetes-01TdSi-1	Petiole	*Calamus rotang* (Mart.) Scheff.
Schizophyllum sp. 02BhSi-1	Fruit	*Ficus* sp.
Xylaria sp. 03BtSi-2	Stem	*Glochidion varians* Miq.
Schizophyllum sp. 04TdSi-1	Petiole	*Poikilospermum suaveolens* (Blume) Merr.
Schizophyllum sp. 05BgSi-1	Petiole	*Poikilospermum suaveolens* (Blume) Merr.
Xylaria sp. 06DnSi-3	Leaf	*Smilax leucophylla* Blume
Schizophyllum sp. 08BtSi-2	Stem	*Ficus septica* Burm.f.
Xylaria sp. 08DnSi-3	Stem	*Ficus septica* Burm.f.
Schizophyllum sp. 09DnSi-1	Leaf	*Gmelina elliptica* Sm.
Xylaria sp. 10BhSi-1	Fruit	*Magnolia singapurensis* (Ridl.) H.Keng
Xylaria sp. 12DnSi-3	Leaf	*Smilax leucophylla* Blume
Xylaria sp. 14BtSi-1	Stem	*Acrostichum aureum* L.
Hypomycetes-14DnSi-4	Stem	*Acrostichum aureum* L.
Schizophyllum sp. 16BtSi-1	Stem	*Acanthus ilicifolius* L.
Schizophyllum sp. 18BhSi-2	Fruit	*Sonneratia caseolaris* (L.) Engl.
Trichoderma sp. 18BtSi-1	Fruit	*Sonneratia caseolaris* (L.) Engl.
Trichoderma sp. 18BtSi-3	Fruit	*Sonneratia caseolaris* (L.) Engl.
Xylaria sp. 19BtSi-1	Stem	*Cerbera manghas* L.
Phomopsis sp. 19BtSi-2	Stem	*Cerbera manghas* L.
Schizophyllum sp. 19DnSi-1	Leaf	*Cerbera manghas* L.
Xylaria sp. 30BtSi-2	Stem	*Melastoma cyanoides* Sm.
Schizophyllum sp. 31BgSi-1	Flower	*Ficus septica* Burm.f.
Xylaria sp. 33BhSi-1	Fruit	*Piper aduncum* L.
Xylaria sp. 33Btsi-2	Stem	*Piper aduncum* L.
Schizophyllum sp. 33DnSi-2	Leaf	*Piper aduncum* L.
Schizophyllum sp. 39BhSi-2	Fruit	*Plagiostachys sumatrensis* Ridl.
Schizophyllum sp. 39BtSi- 1	Stem	*Plagiostachys sumatrensis* Ridl.
Schizophyllum sp. 39BtSi-2	Stem	*Plagiostachys sumatrensis* Ridl.
Xylaria sp. 40Dnsi- 1	Leaf	*Poikilospermum suaveolens* (Blume) Merr.
Schizophyllum sp. 46Dnsi-1	Leaf	*Smilax leucophylla* Blume
Schizophyllum sp. 48DnSi-1	Leaf	*Freycinetia rigidifolia* Hemsl.
Schizophyllum sp. 50BtSi-2	Stem	Lauraceae2
Schizophyllum sp. 50TbSi-1	Petiole	Lauraceae2
Xylaria sp. 55DnSi-2	Leaf	*Smilax leucophylla* Blume
Xylaria sp. 56BhSi-2	Fruit	*Calamus rotang* L.
Schizophyllum sp. 56TbSi-1	Petiole	*Calamus rotang* L.
Schizophyllum sp. 56TbSi-2	Petiole	*Calamus rotang* L.

sprayed with vanillin-sulfuric acid staining reagent, the TLC spots showed the presence of terpenoids (dark green, orange, purple, maroon, or brown color spots such as C, sample No. 1; 44; etc.), saponin (blue zones such as C, sample No. 20; 33; 43; etc.), and phenolic compounds (red spots, such as C, sample no. 21; 23 and 24) (Figure 1). After being sprayed with Cerium (IV) sulfate brown color, or yellowish-brown for alkaloids, and orange (flavonoid)

3.3. Antibacterial Activity: Qualitative and Quantitative Test

Antibacterial activity test by using TLC-DB shows that most extracts were active as antibacterial agents against *S. aureus* (75 extracts), and *E. coli* (68 extracts). On the other hand, 56 kinds of extracts were active against both of the tested bacteria and 11 extracts were not active (Figure 2). Determination of MIC value was carried out for further antibacterial analysis of the active extracts. The lowest concentration that inhibits microorganism growth is considered the MIC value. Criteria of MIC values for extracts: inactive <1000 µg/ml <weak activity < 625µg/ml < moderate < 100 µg/ml < significant. Based on this category, there were two extracts of endophytic fungi that performed strong antibacterial activity against *S. aureus* (*Phomopsis* sp. 48BtSi-2.1 and *Xylaria* sp. 55DnSi-1.1) with MIC value of 32 µg/ml and nine extracts of endophytic fungi that performed strong antibacterial activity against *E. coli* (*Schizophyllum* sp. 20DnSi-1; *Hyphomycetes* 36BhSi-1.1; *Phomopsis* sp. 36DnSi-2.1; *Schizophyllum* sp. 39DnSi-1.1; *Xylaria* sp. 39RpSi-2.1; *Phomopsis* sp. 41BtSi-1.1; *Phomopsis* sp. 48BtSi-2.1; *Lasiodiplodia* sp. 48BtSi-3.1; *Schizophyllum* sp. 50DnSi-3) with MIC value of 32-64 µg/ml (Table 2).

3.4. Antioxidant Activity: Qualitative and Quantitative Test

The test of antioxidant activity shows that several extracts (46 samples) were active as antioxidant agents (Figure 3). The stronger the intensity of the yellowish-white color, the better the antioxidant capacity of the extracts. The antioxidant capacity of the extract was determined according to Antioxidant Activity Index (AAI) values. The criteria of AAI values for the extracts: poor activity < 0.5 < moderate < 1.0 < strong < 2.0 < very strong. There is one extract of endophytic fungus that performed as strong antioxidant activity (*Xylaria* sp. 04BtSi-2.2) with an AAI value of 1.42 and four extracts of endophytic fungi that performed moderate antioxidant activity (*Phomopsis* sp. 10DnSi-4; *Schizophyllum* sp. 20DnSi-1; *Xylaria* sp. 08DnSi-3; *Schizophyllum* sp. 56TbSi- 2) with AAI value of > 0.5 (Table 3).

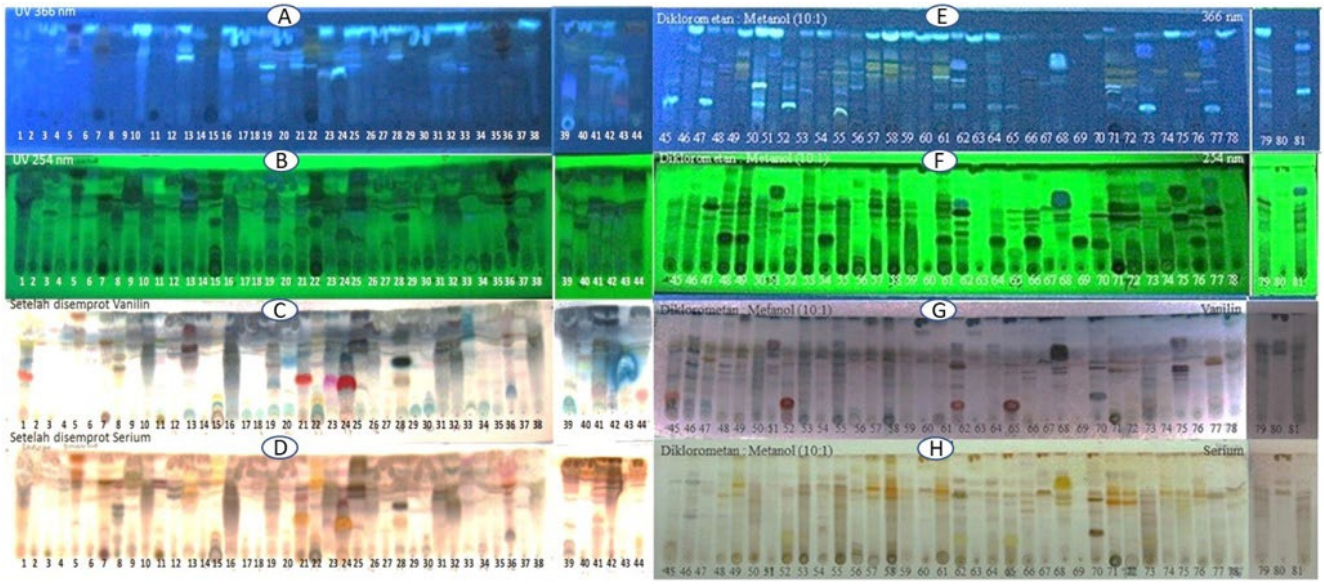

Figure 1. Chromatograms of fungal endophytic extracts developed in dichloromethane-methanol (10:1 v/v), left (No.1-44), right (No. 45-81), viewed under UV-light of 366 nm (A, E), viewed under UV-light of 254 nm (B, F), sprayed with vanillin 0.25% in 10% sulfuric acid-ethanol pa. (C, G), sprayed with 1% $Ce(SO_4)_2$ in 10% sulfuric acid-methanol (D, H)

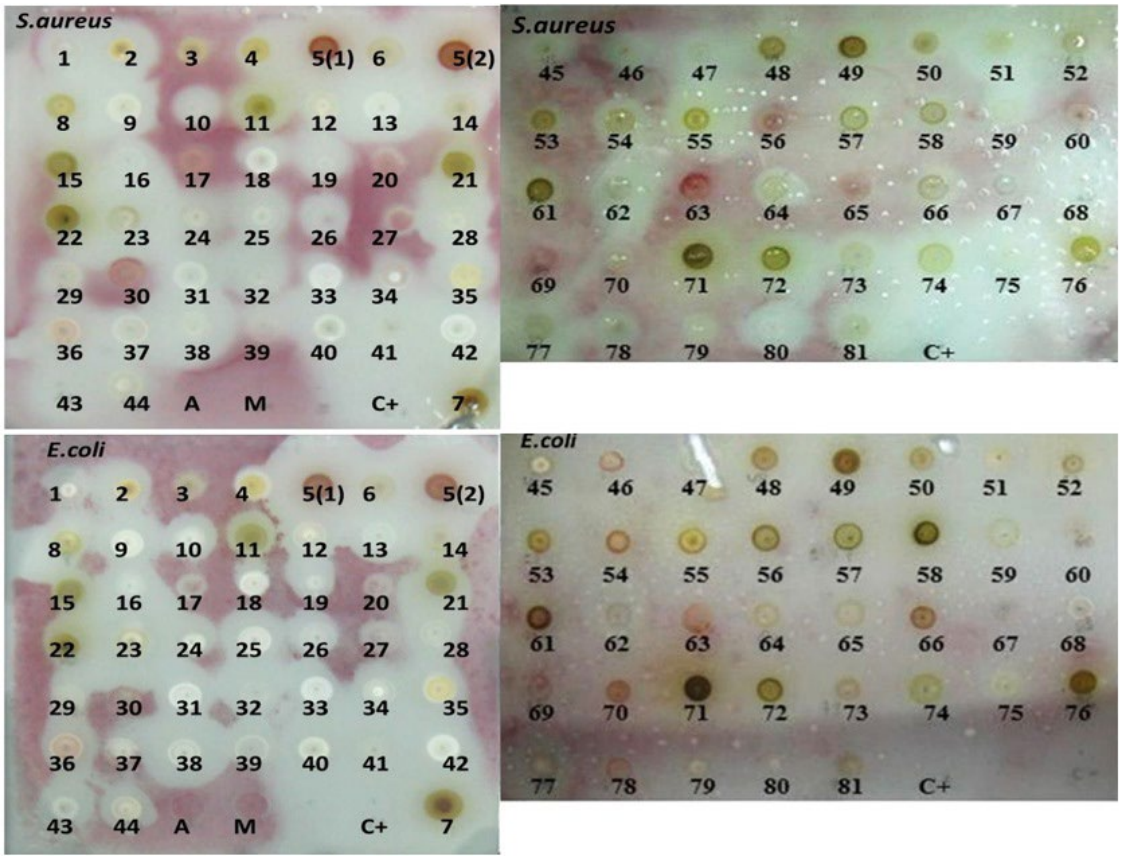

Figure 2. TLC dot-blot assay for antibacterial activity of the fungal endophytes extracts against *S. aureus* and *E. coli*

Table 2. MIC values of the fungal endophytic extracts against *S. aures* and *E. coli*

Fungal extract	MIC against *S. aureus* (µg/ml)	Category of MIC against *S. aureus*	Fungal extract	MIC against *E. coli* (µg/ml)	Category of MIC against *E. coli*
Hypomycetes 03DnSi-2.1	256	Moderate	*Xylaria* sp. 04BtSi-2.2	256	Moderate
Xylaria sp. 04BtSi-2.2	128	Moderate	*Dematiaceae* 05BtSi-2.1	256	Moderate
Xylaria sp. 08DnSi-3.1	256	Moderate	*Phomopsis* sp. 06DnSi-1.1	256	Moderate
Phomopsis sp. 10DnSi-4	256	Moderate	*Phomopsis* sp. 10DnSi-4	256	Moderate
Phomopsis sp. 10DnSi-5.1	256	Moderate	*Phomopsis* sp. 10DnSi-5.1	128	Moderate
Schizophyllum sp. 20DnSi-1	256	Moderate	Trichoderma sp. 18BtSi-2.1	128	Moderate
Schizophyllum sp. 30DnSi-1	256	Moderate	*Schizophyllum* sp. 20DnSi-1	64	Strong
Hyphomycetes 36BhSi-1.1	256	Moderate	*Schizophyllum* sp. 30DnSi-1	256	Moderate
Phomopsis sp. 36DnSi-2.2	128	Moderate	Hyphomycetes 36BhSi-1.1	32	Strong
Schizophyllum sp. 39DnSi-1.1	256	Moderate	*Phomopsis* sp. 36DnSi-2.1	32	Strong
Xylaria sp. 39RpSi-2.1	256	Moderate	*Phomopsis* sp. 36DnSi-2.2	256	Moderate
Phomopsis sp. 41BtSi-1.1	128	Moderate	*Schizophyllum* sp. 39DnSi-1.1	32	Strong
Phomopsis sp. 48BtSi-2.1	32	Strong	Xylaria sp. 39RpSi-2.1	32	Strong
Lasiodiplodia sp. 48BtSi-3.1	128	Moderate	*Phomopsis* sp. 41BtSi-1.1	64	Strong
Schizophyllum sp. 50DnSi-3	128	Moderate	*Schizophyllum* sp. 46DnSi-2.1	256	Moderate
Xylaria sp. 55DnSi-1.1	32	Strong	*Phomopsis* sp. 48BtSi-1.1	256	Moderate
Schizophyllum sp. 08BtSi- 2	256	Moderate	Lasiodiplodia sp. 48BtSi-1.2	256	Moderate
Xylaria sp. 33BtSi- 2	256	Moderate	*Phomopsis* sp. 48BtSi-2.1	32	Strong
Schizophyllum sp. 48DnSi- 1	128	Moderate	Lasiodiplodia sp. 48BtSi-3.1	64	Strong
Schizophyllum sp. 56TbSi- 1	128	Moderate	Lasiodiplodia sp. 49DnSi-2.1	256	Moderate
			Schizophyllum sp. 50DnSi-3	32	Strong
			Phomopsis sp. 56BhSi-1.1	128	Moderate

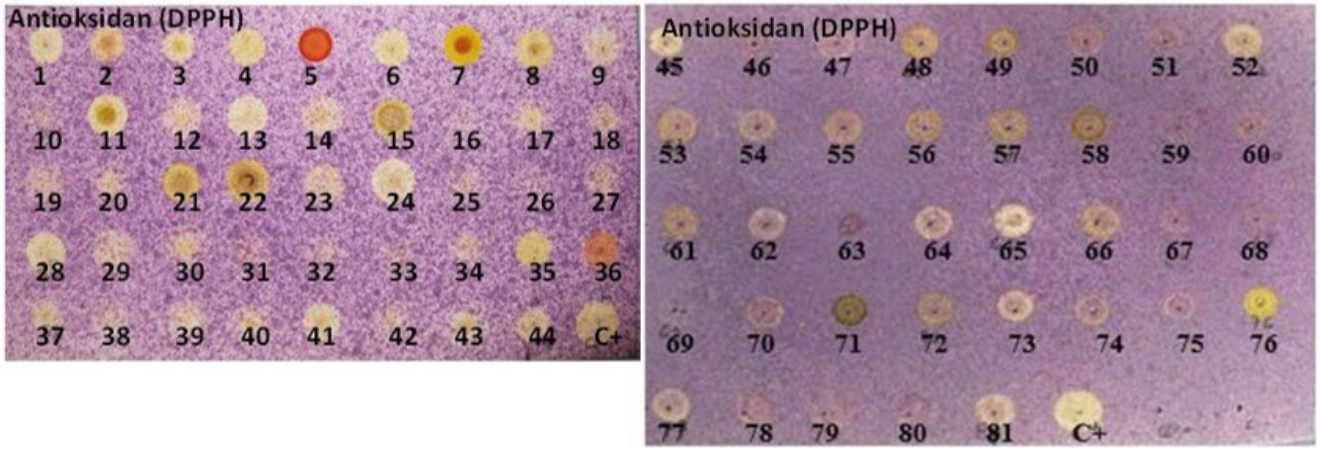

Figure 3. TLC assay for antioxidant activity of the fungal endophytes extracts

Table 3. IC_{50} and AAI values of the fungal endophytes extracts

Sample	IC_{50} value (ug/ml)	AAI value	Category of AAI
Xylaria sp. 04BtSi-2.2	21.66	1.420	Strong
Phomopsis sp. 10DnSi-4	53.45	0.575	Moderate
Schizophyllum sp. 20DnSi-1	53.20	0.578	Moderate
Schizophyllum sp. 56TbSi- 2	38.59	0.797	Moderate

3.5. Macroscopic-Microscopic View and Molecular Identification of the Representative Potential Fungal Endophytes: *Xylaria* sp. 04BtSi-2.2 and *Phomopsis* sp. 48BtSi-2.1

The macroscopic and microscopic appearance data (Figure 4) are consistent with the molecular identification data. Based on the neighbor-joining tree of fungal endophytes strain 04BtSi-2.2 based on ITS rDNA sequence revealed that endophytes strain 04BtSi-2.2 has closest taxa with Xylaria sp. strain LCM 895.01 (100%) and *Xylaria* sp. ICMP 20641 (99.15%) (Table 4 and Figure 5). While, the fungal endophytes strain 04BtSi-2.2 based on ITS rDNA sequence revealed that endophytes strain 48BtSi-2.1 has closest taxa with *Phomopsis* sp. strain TW25 (99.14%) and *Phomopsis* sp. strain PB-42 (98.62%) (Table 4 and Figure 6).

4. Discussion

This study revealed that *Schizophyllum* sp. was the most dominant fungi in this study, followed by *Xylaria* sp. and *Phomopsis* sp. Genera of *Schizophyllum* generally causes tree decay and was considered as wound parasitic fungi as the plant pathogen or the saprobic or even mutualistic fungi (Vulinovic *et al.* 2018; Zhai *et al.* 2017). *Xylariaceous* fungi may be considered the mutualistic fungi in their host plants (Davis *et al.* 2003) and show inhibitory effects against the fungal pathogens *Fusarium oxysporum*, *Ceratocystis pirifera*, and *Botrytis cinerea* (Gonzalez-Teuber 2016). *Phomopsis* spp. or teleomorph *Diaporthe* is the most common endophytic fungi found in tropical plants (Murali *et al.* 2006; Udayanga *et al.* 2011). Some species of *Phomopsis* are plant diseases, while others are mutualistic fungi (Hu *et al.* 2018; Urbes-Torres *et al.* 2013).

Endophytic fungi from plants produced numerous substances that exhibited antimicrobial activity (Sheeba *et al.* 2019), plant growth promoter (Das and Varma 2009), and insecticide (Vega *et al.* 2008), yield promoter (Lugtenberg *et al.* 2016). The difference in susceptibility between Gram-negative and Gram-positive bacteria may be due to the differences in the structure of their cell walls (Epand *et al.* 2016). In this study, two extracts (*Phomopsis* sp. 48BtSi-2.1 and *Xylaria* sp. 55DnSi-1.1) performed strong antibacterial against Gram-positive, and nine extracts (*Schizophyllum* sp. 20DnSi-1; *Hyphomycetes* 36BhSi-1.1; *Phomopsis* sp. 36DnSi-2.1; *Schizophyllum* sp. 39DnSi-1.1; *Xylaria* sp. 39RpSi-2.1; *Phomopsis* sp. 41BtSi-1.1; *Phomopsis* sp. 48BtSi-2.1; *Lasiodiplodia*

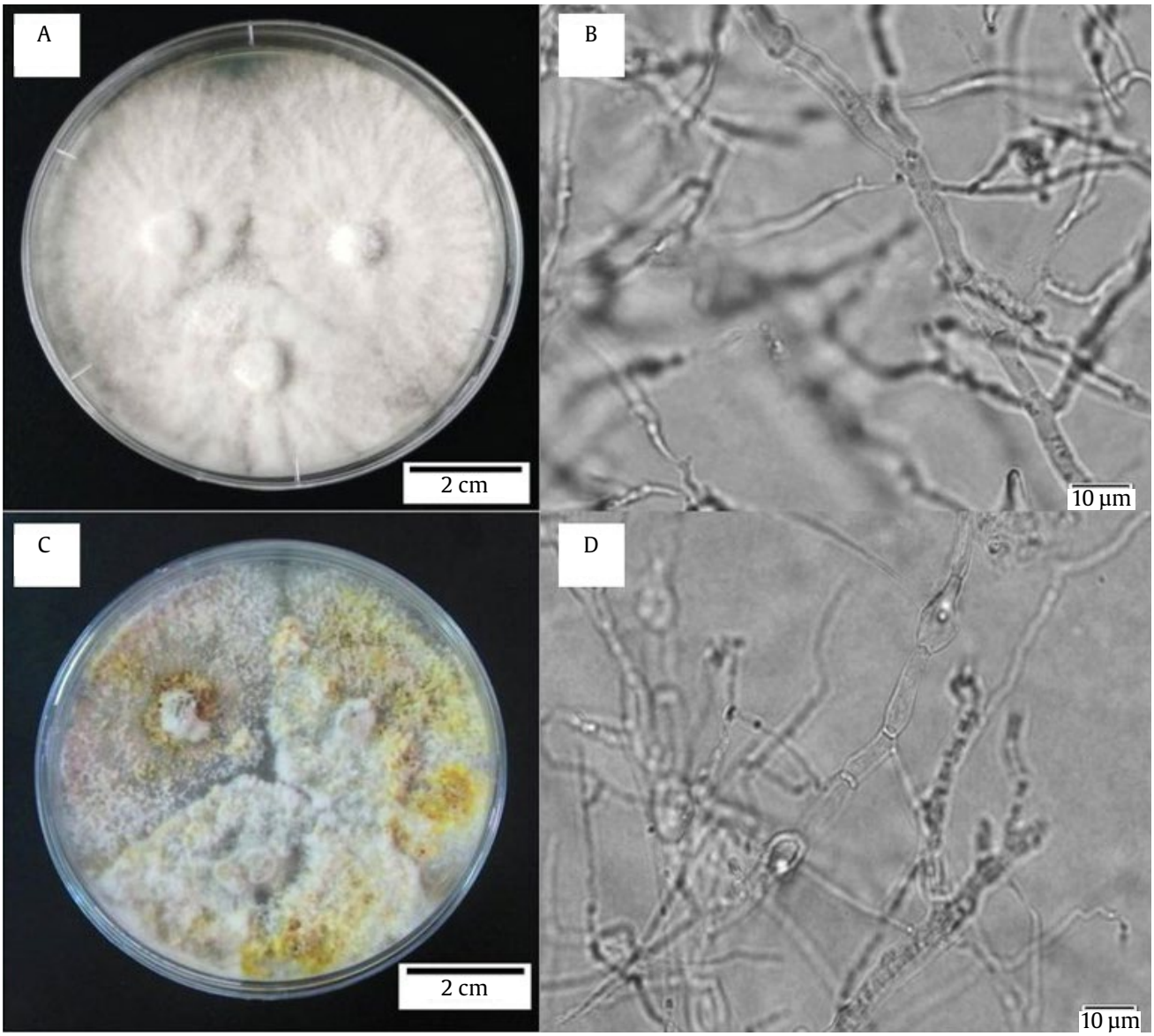

Figure 4. The macroscopic and microscopic view of the representative potential fungal endophytes *Xylaria* sp. 04BtSi-2.2 (A, B) and *Phomopsis* sp. 48BtSi-2.1 (C, D) were grown in PDA media, 7 days incubation at 27°C

Table 4. The BLAST result of the potential fungal endophytes strain 04BtSi-2.2 and 48BtSi-2.1 based on ITS rDNA sequence according to NCBI BLAST (https://blast.ncbi.nlm.nih.gov/)

Fungal endophytes strain	1st and 2nd closest taxa on NCBI BLAST (https://blast.ncbi.nlm.nih.gov/)
04BtSi-2.2	*Xylaria* sp. strain LCM 895.01 (Accession no: MF495427) [similarity: 100.00%; max score: 1094; total score: 1094; query coverage: 99%; e-value: 0.0; max identities: 592/592 (100%); gaps: 0/592 (0%)]
	Xylaria sp. ICMP 20641 (Accession no: KP689112) [similarity: 99.15%; max score: 1064; total score: 1064; query coverage: 99%; e-value: 0.0; max identities: 586/591 (99%); gaps: 0/591 (0%)]
48BtSi-2.1	*Phomopsis* sp. isolate TW25 (Accession no: MH930426) [similarity: 99.14%; max score: 1040; total score: 1040; query coverage: 100%; e-value: 0.0; max identities: 574/579 (99%); gaps: 2/579 (0%)]
	Phomopsis sp. strain PB-42 (Accession no: MK333962) [similarity: 98.62%; max score: 1024; total score: 1024; query coverage: 100%; e-value: 0.0; max identities: 571/579 (99%); gaps: 1/579 (0%)]

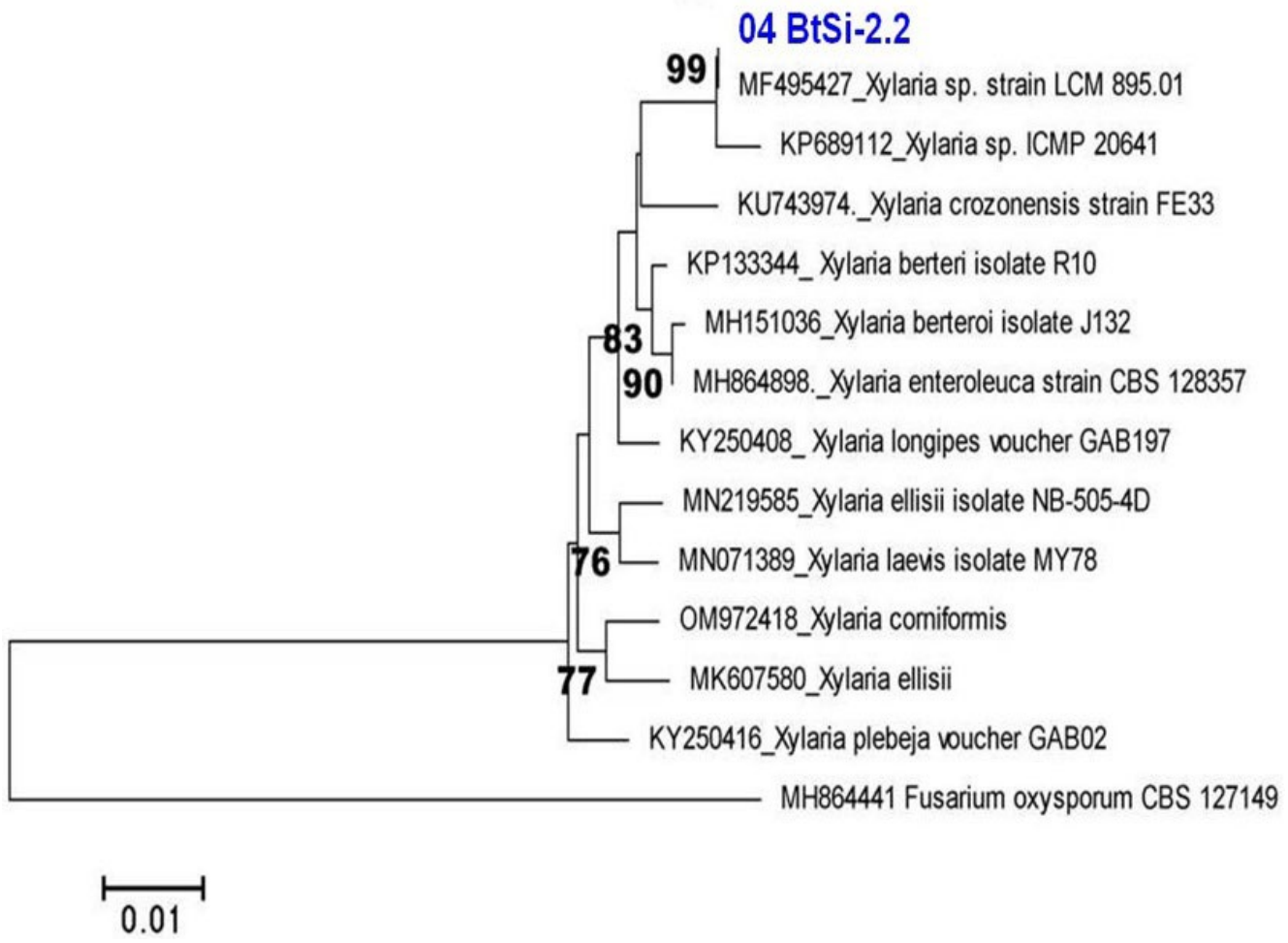

Figure 5. Neighbor-joining tree of fungal endophytes strain 04BtSi-2.2 based on ITS rDNA sequence and *Fusarium oxysporum* as outgroup. Only bootstrap values above 70 are shown

sp. 48BtSi-3.1; and *Schizophyllum* sp. 50DnSi-3) performed strong antibacterial against Gram-negative bacteria. So, one fungal extract of *Phomopsis* sp. 48BtSi-2.1 showed strong antibacterial against both Gram-positive and Gram-negative bacteria. In another study, the endophytic fungus *Phomopsis* sp. isolated from *Notobasis syriaca* exhibited good antibacterial activity and is known as a source of new compounds of antibacterial agents such as phomosine K, pyrenocines J-M (Hussain *et al.* 2011, 2012), phomosine I (Krohn *et al.* 2011).

The results of the antioxidant activity test of the fungal extracts showed that several extracts could scavenge DPPH free radicals. In this study, one extract (*Xylaria* sp. 04BtSi-2.2) performed strong antioxidant activity as free radical scavenging of DPPH. In a previous study, *Xylaria* sp. DAP KRI-5 isolated from *Albertisia papuana* had produced antibacterial and antioxidant metabolites, such as a phenolic compound of phloroglucinol (Delfanian *et al.* 2021; Fathoni *et al.* 2013). The presence of diverse secondary metabolites such as flavonoids and phenolic compounds in fungal extracts may contribute to antioxidant activity. phenolic and flavonoid components are associated with their ability as antioxidant agents by donating hydrogen to deactivate free radicals (Aryal *et al.* 2019). The study by Shrestha and Dhillion (2006) indicated a strong correlation between total phenolic and flavonoid contents with antioxidant capacity. The bioactive compounds within the extract may have a synergistic action that causes vigorous activity for free radical scavenging as antioxidant agents (Praptiwi *et al.* 2018). Our study demonstrated that the fungal endophytic isolates associated with medicinal plants collected from Simeulue Island had a significant range of biological activities as antioxidant and antibacterial agents.

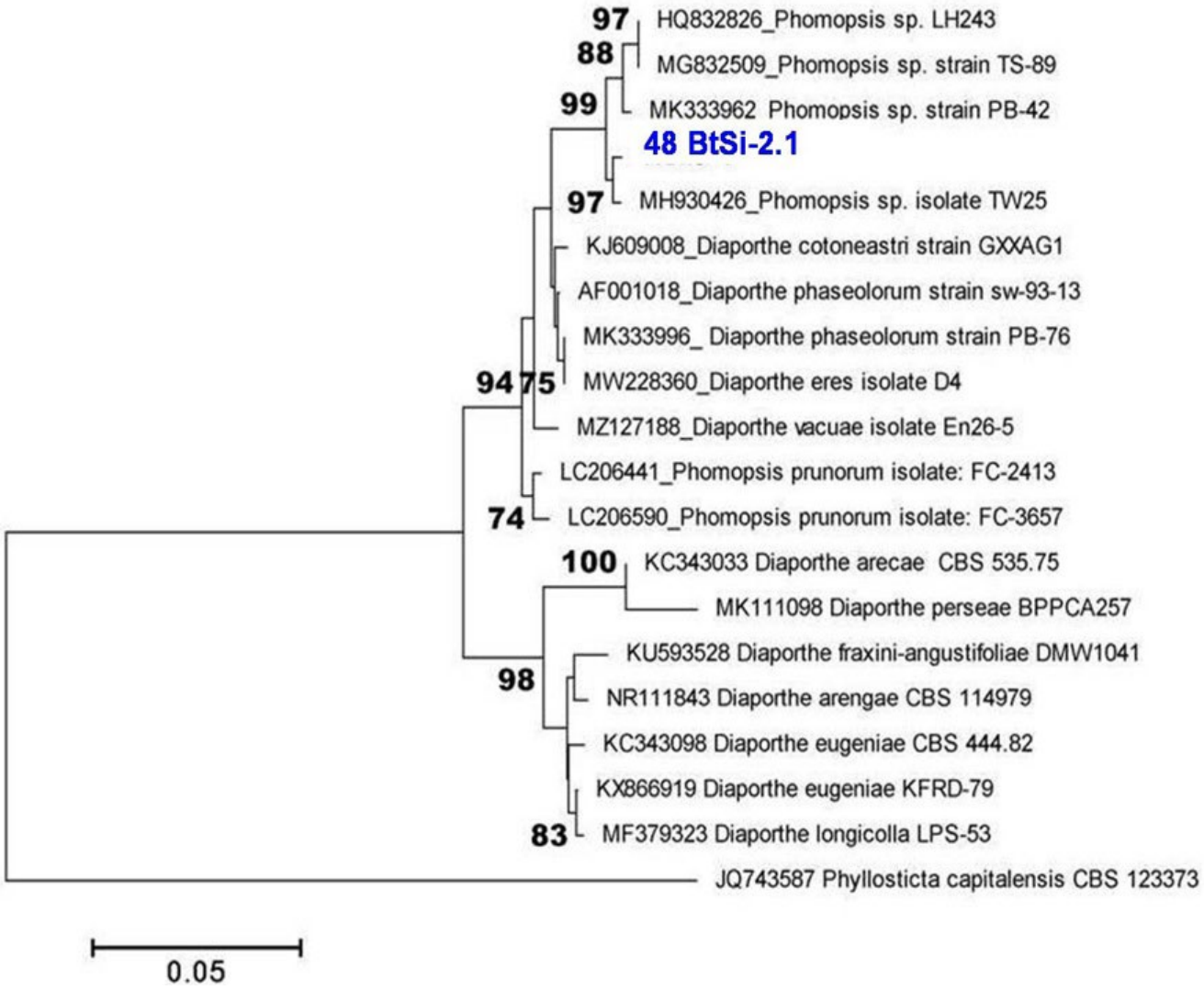

Figure 6. Neighbor-joining tree of fungal endophytes strain 48BtSi-2.1 based on ITS rDNA sequence and *Phyllosticta capitalensis* as outgroup. Only bootstrap values above 70 are shown

This study concluded that the extracts of the fungal endophytic isolated from medicinal plants collected from Simeulue Island showed antibacterial and antioxidant activity. Purification of the active compounds, determination of chemical structure, and evaluation of their biological activities need to be done for further study. These reports indicated that endophytic fungi of medicinal plants collected from Simeulue Island could be potential sources of future drug discovery from natural products.

Conflict of Interest

The authors declare no conflict of interest.

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