Molekul, Vol. 18. No. 3, November 2023: 414 – 425

MOLEKUL
eISSN: 2503-0310

Articles
https://doi.org/10.20884/1.jm.2023.18.3.7881

Potential Endophytic Fungi from the Leaves of *Syzygium zeylanicum* (*L.*) and Their Secondary Metabolite

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Received February 08, 2023; **Accepted** July 15, 2023; **Available online** November 20, 2023

ABSTRACT. Endophytic fungi coexist without harms in the host in some parts or all parts of its lifecycle. Endophytic fungi excrete specialized bioactive compounds beneficial for of its host; the compound itself can be different from what can be found on their host. Endophytic fungi are capable to excrete biologically significant secondary metabolites acting as antidiabetic, antioxidant, antimicrobial, and cytostatic agents. Endophytic fungi existence are common across different plants; including *Syzygium zeylanicum* that can be found in Indonesia. This study aims to isolate endophytic fungi found in leaves of *S. zeylanicum*, L., to elucidate their characterized morphologically, and to examine its antimicrobial and antioxidant properties in addition to their chemical structures. Characterization of endophytic fungi was conducted by their macroscopic and microscopic features, followed by molecular characterization of highly bioactive metabolites. Antimicrobial activities were measured by disc diffusion method. Antioxidant properties were measured with DPPH. Secondary metabolites were chromatographically isolated and identified with spectroscopy techniques (NMR ID and 2D). Four endophytic fungi isolates were obtained: *Penicillium citrinum* (SZ1), *Colletotrichum lindemuthianum* (SZ2), *Aspergillus nidulan* (SZ3), *Scopulariopsis asperula* (SZ4). *P. citrinum* (SZ1) showed antimicrobial activities against four different bacteria (71.3% against *E. coli*; 74.1% against *S. aureus*; 76.2% against *S. typhi*; and dan76.9% against *B. subtilis*). Antioxidant activity ini all ekstrak of endophytic fungi showed very activity (IC_{50} SZ3 extract = 3.85 μg/mL). Potential endophytic fungi SZ1 was molecularly identified as *P. citrinum*. Extracts from SZ1 fungi contains bioactive 4-hydroxy-2-(4-hydroxyphenyl)-ɣ-butyrolactone-3-yl) methyl acetate. The newly obtained substance could be developed into antimicrobial and antioxidant agents in further studies.

Keywords: Antibacterial, antioxidant, endophytic fungi, secondary metabolite, *Syzygium zeylanicum*

INTRODUCTION

Endophytic fungi colonizing plant without exerting significant adverse effect in its host (Jia et al., 2016). Endophytic fungi are capable to colonize various tissues (i.e., leaves, fruit, seeds, stems, and roots) without harming their hosts; some plant-endophytic fungi symbioses are even known to be mutually beneficial. Plants provides cover and nutrients for endophytic fungi (Mbilu et al., 2018). On the other hand, some endophytic fungi are able to improve efficiency of syntheses of bioactive substrates in the plant. The discovery of endophytic fungi may provide new methods to discover bioactive substrates, including drugs, in plant. The discovery of substances in mutually beneficial symbioses between endophytic fungi and their hosts may help resolving the scarcity of natural resources and extinctions of highly-sought plants (Zheng, 2021).

Ogan community in South Sumatra utilized different parts from *Syzigium zeylanicum* for traditional remedies. *S. zeylanicum* leaves has been traditionally used against hypertension and diabetes mellitus (Nguyen et al., 2019), while its bark and pistils are commonly used for diarrhea. Numerous literatures have reported the use of spicate eugenia around the world for traditional remedies against bacterial infection. In addition, essential oil of spicate eugenia has been extracted and used for natural mosquito repellent (Govindarajan & Benelli, 2016).

Deepika et al. (2014) discovered spicate eugenia extract activities against *Escherechia coli, Pseudomonas aeruginosa, Klebsiella pneumoniae, Proteus vulgaris, Staphylococcus aureus*, and *Bacillus subtilis*. Dominant phenolic and flavonoids compounds found in spicate eugenia lends to strong antimicrobial activity of spicate eugenia (Deepika et

al., 2014 ; Gouda et al., 2016; Palanisamy et al., 2011;Shilpa & Krishnakumar, 2015; Elfita et al., 2022; Elfita et al., 2023). Shilpa and Krishnakumar (2015) reported *S. zeylanicum* contains 18.11 mg/g of phenolic compound when extracted with water. The phenolic extraction efficiency can be increased by methanol (to 28.18 ± 2.7 mg/g). Phenolic compounds have direct, important role in antioxidant activity through the scavenge of free radicals (Akter et al., 2022). In one example, leaf stalks of endophyte *Beltrania rhombica* contains antioxidant 3-(hydroxyl(4-hydroxyphenyl) methyl)-3-4-dihydro-2H-pyran-4,5,6-triol (Habisukan et al., 2021). *Fusarium verticillioides*, isolated from the stem bark of *S. jambos,* contains 3-hydroxy-4-(hydroxyl (4-hydroxyphenyl)methyl)dihydrofuran-2-on as its active antioxidant compound (Aini *et al*., 2022). *Tritirachium oryzae* found in fruit of *S. malaccense* contains antioxidant 2-(4-Hydroxy-phenyl)-4-methoxytetrahydrofuran-3-ol (Hapida et al., 2021).

Discovery of new antioxidant and antimicrobial compounds exist as continuous and urgent priorities around the world. Secondary metabolites of plants are known to be one of the potential sources for antioxidant and antimicrobial compounds. Some secondary metabolites are known to prevent endophytic fungal and bacterial infection in plants (Ikram et al., 2019). Unfortunately, studies on secondary metabolites of endophytic fungi are still limited. In this study, we explore potential antimicrobial and antioxidant compounds from secondary metabolites of endophytic fungi found in spicate eugenia. We aim to explore prospective secondary metabolites extracted from endophytic fungi of *Spicate eugenia* and discuss its potential use in medical field.

EXPERIMENTAL SECTION

Plant Material

Fresh leaves of *S. zeylanicum* were collected from Penukal Abab Lematang Ilir (PALI), South Sumatra, Indonesia. This plant was identified in the Laboratory of Biosystematics, Department of Biology, University of Sriwijaya, Indonesia, as accession number 331/UN-9.1.7/4/EP/2020. Sampling was carried out in February 2020The leaves used are the leaves in the third position of the test.

Isolation of Endophytic Fungi

The surface of fresh leaves was sterilized. Wash with tap water until clear for ± 5 minutes then wet with 70% alcohol for ± 3 minutes, then rinse with sterile distilled water for ± 1 minute and wet with sodium hypochlorite (NaOCl) 3% (w/v) for 1 minute. Aseptically sterilized leaves were cut to measure ±3 x 0.5 cm. Samples were placed on potato dextrose agar (PDA) medium in Petri dishes and incubated at room temperature for 3–14 days. Fungal growth is observed daily until fungal colonies appear. Then, fungal colonies with different morphological characteristics (shape, color and size) were cleaned. Purification was performed by transferring the colonies to fresh PDA medium using the single spore isolation method and then incubating at room temperature for 2 x 24 hour. Purified fungal colonies were grown in PDA medium as working cultures (in petri dishes) and stock cultures (in test tubes) (Fitriarni & Kasiamdari 2018; Hanin & Fitriasari 2019; Habisukan et al. 2021; Oktiansyah et al. 2023a).

Identification of Endophytic Fungi

Identification of endophytic fungi is based on their macroscopically and microscopically characters. Observations of colony characteristics include: (i) colony color and background color, (ii) colony surface: granular, dusty, hilly, smooth. (iii) presence or absence of exudate dots, (iv) presence or absence of radial lines (radial furrows) from the center of the colony circumference, (v) presence or absence of concentric circles. Microscopic observations are the shape of the hyphae or mycelium, the shape of the spore, the color of the spores, the presence or absence of a septum in the hyphae and other microscopic characteristics. Comparison of phenotypic identification data with key identification literature was performed using Illustrated Soil Atlas and Fungal Seed Morphology of Cultivated Fungi and Species Guides (Watanabe 2010), Laron's Medicinally Important Fungi (Walsh et al. 2018) and Fungi and Food Spores. (Pitt and Hawking, 2009).

Cultivation and Extraction

All fungal species grown on PDA (±6 mm diameter) were cultivated by placing 6 blocks of pure culture in 300 mL of potato dextrose broth (PDB). Each isolate was inoculated with a volume of 300 mL of PDB in 5 Erlenmeyer flasks 1000 mL. Cultures were then grown for 4 weeks under static conditions at room temperature. After the incubation, use filter paper to separate the mycelia from the broth culture. Then ethyl acetate solvent was added to the culture medium (1:1). The ethyl acetate extract was isolated from the fungal culture medium and used in a rotary evaporator (Deepika et al. 2014; Habisukan et al., 2021; Oktiansyah et al., 2023b). The product is stored in the oven at a temperature of 40 °C. Accumulated draft and biomass were weighed using an analytical balance.

Antibacterial Activity Test

The antibacterial activity was performed by the Kirby-Bauer method using NA medium (Nutrition agar) against four bacterial isolates, two Gram-negative bacteria (*E. coli* InaCCB5 and *S. thypi* ATCC1048) and two Gram-positive bacteria (*Staphylococcus aureus* InaCCB4 and *B. subtilis* InaCCBI1204). The endophytic fungal extract was dissolved with dimethyl sulfoxide (DMSO). The blank paper disc was dripped with 20 μL of endophytic fungal extract at a concentration of 400 μg/disc and left until all solvent was completely evaporated. The positive control was tetracycline 30 μg/disc. The disc

containing the test solution was inoculated with the test bacteria in NA medium. It is then incubated at 37 °C for 1x24 hours. The measurement of antibacterial activity of the sample and the diameter parameters of the zones were determined according to the following formula (Elfita et al., 2019; Oktiansyah et al., 2023c):

$$\text{Weak} = \frac{A}{B} \times 100\% < 50\%$$

$$\text{Middle} = 50\% < \frac{A}{B} \times 100\% < 70\%$$

$$\text{Strong} = \frac{A}{B} \times 100\% > 70\%$$

$$\%Inhibition = \frac{A_k - A_s}{A_s}$$

Where:
A: Inhibition zone (mm) of the test sample
B: Inhibition zone (mm) of standard antibiotics

Antioxidant Activity Test

Antioxidant activity was determined using the DPPH (1,1-diphenyl-2-picrilhydazyl) method. The fractions obtained from the extraction procedure were dissolved into concentrations of 1000, 500, 250, 125, 62.5, 31.25, 15.625 μg/mL. 0.2 mL of each concentration was added 3.8 mL of 0.5 mM DPPH. As a standard antioxidant ascorbic acid was used and at least three replicates of each concentration were considered (Goswami & Ray, 2017). The mixture was homogenized and left in a dark tube for 30 minutes. Absorption was measured using a UVVis spectrophotometer at max 517 nm. In this test, ascorbic acid was used as a standard positive control and methanol as a negative control. Antioxidant activity can be represented by the value of DPPH absorption inhibition, which is calculated by the percentage inhibition of DPPH absorption and the IC_{50} value.

$$\%\ \text{Inhibition} = \frac{A_k - A_s}{A_s}$$

A_k = Absorbance of control
A_s = Absorbance of samples

Molecular Analysis of ITS rDNA

Identification of isolates based on the internal transcribed spacer (ITS) region of DNA (rDNA). Amplification was performed using the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') (Diongue et al., 2019). The DNA sequences of the forward and reverse primers were assembled using the Bioedit program. Sequence results were then identified to the species level of taxa using the online bioinformatics method of the Basic Local Extent Search Tool (BLAST) at the website http://blast.ncbi.nlm.nih.gov/Blast .cgi. Then, multiple alignments were performed using the Mega 6 program (Tamura et al., 2013) using the CLUSTAL W method, and the phylogenetic tree was constructed using the Neighbor-joining tree method with a bootstrap value of 1000 (Katoch & Pull, 2017; (Kuswytasari *et al*., 2019; Potshangbam *et al*., 2017).

Isolation of Bioactive Compound

Isolation of bioactive compound was carried out on selected ethyl acetate extracts with the highest antibacterial and antioxidant activity, namely isolate ZL6. Concentrated ethyl acetate extract (2.0 g) was separated by gravity column chromatography (CC) method with a gradient eluent system, namely 100% *n*-hexane (100 mL) eluent, a mixture of *n*-hexane and ethyl acetate with increasing polarity, (n- hexane:ethyl acetate 9:1 (100 mL), 8:2 (100 mL), 7:3 (100 mL), 5:5 (100 mL); 2;8 (100 mL), 100% ethyl acetate (100 mL) mL), and ethyl acetate and methanol (9:1 30 mL), 8:2 (30 mL), 7:3 (30 mL)). The stationary phase used was silica gel 60 G (70-230 mesh). The separation results were collected using vials (10 mL) and obtained as many as 80 vials. The eluate was then analyzed using thin layer chromatography (TLC) with a mixed eluent of *n*-hexane and ethyl acetate (5:5). TLC with similar chromatogram patterns were combined into one fraction. Based on the results of the chromatogram pattern obtained 4 fractions (F1-F4). The F2 fraction was rinsed with *n*-hexane-ethyl acetate (6:4) to obtain compound 1 in the form of white crystals (37 mg).

RESULTS AND DISCUSSION

Four isolates were found and labelled as SZ1 through 4 (**Figure 1**). Identification of macroscopic features encompassed following parameters: color, texture, topography, pattern, exudate drop, radial line and concentric circle. Identification of microscopic features encompassed type of spores, hyphae, and other microscopically-defining characteristics. Comparison of macroscopic and microscopic features were made to literatures in order to identify the species in question (Watanabe, 2010).

Endophyte SZ1: dark green colony on both sides, velvet-like texture, flat surface, with zonate growths. Microscopic examination revealed conidial, rounded spores with septate hyphae and conidial head chains on different branches. Comparison with known morphological features rendered SZ1 as *Penicillium citrinum* (Cole, 1974; Watanabe, 2010). Endophyte SZ2: dark green colony with light green colorization on opposite side, cotton-like texture, flat surface, with zonate growths. Microscopic examination revealed conidiophores and conidia, thick-walled macroconidia, 2-3 layer of septae, with pointed peak. Identified as *Colletotrichum linemuthianum* (Watanabe, 2010). Endophyte SZ3: dark green colony with orange colorization on the opposite site, velvety texture, flat surface, with zonate growths. Microscopic examination revealed septate hyphae with straight conidiophores and hyaline with short columnar conidial head. Identified as *Aspergillus nidulans* (Akmalasari *et al.*, 2013). Endophyte SZ4: pale brown colony with light orange colorization on the opposite side, cotton-like texture, flat surface covering all surface of medium, with zonate growths. Microscopic examination revealed philaid conidial spores and

septate hyphae, basipetally catenulate conidia. Identified as *Scopulariopsis asperula* (Watanabe, 2010). Phenotypic analysis showed that these 4 isolated were grouped into four classes Eurotiomycetes (SZ1), Ascomycetes (SZ2), Deuteromycetes (SZ3) and Sardariomycetes (SZ4).

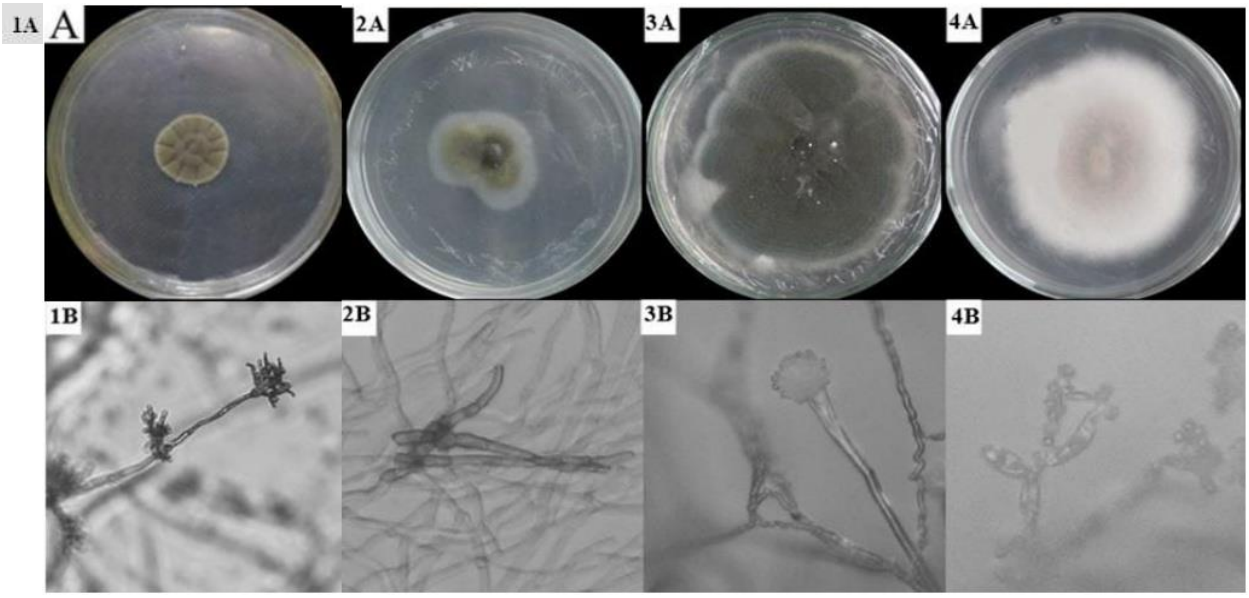


Figure 1. Colony and microscopic morphology of endophytic fungal species; SZ1**(1)**; SZ2**(2)**; SZ3**(3);** SZ4 (**4); A.** macroskopic ; **B.** microskopic

Table 1. Macroscopic characteristics of endophytic fungi isolated from jambu nasi-nasi leaves

Isolates	Colony color	Reverse colony color	Texture	Topography	Pattern	Radial line	Concentric circle
SZ1	Dark green	Dark green	Velvety	Rugose	Zonate	√	-
SZ2	Dark green	Light green	Velvety	Flat	Zonate	-	-
SZ3	Dark green	White and orange in center	Velvety	Flat	Zonate	-	√
SZ4	Pale Brown	Light orange	Cottony	flat	Zonate	√	-

Note: (-) = characteristic doesn't appear; (√) = characteristic appear

Table 2. Microscopic characteristics of endophytic fungi isolated from jambu nasi-nasi leaves

Isolates	Type of spore	Shape of spore	Shape of spore	Hyphae	Specific characteristic	Species
SZ1	Conidia	Dark green	Subglobose	Septate	Bearing catenulate in each branch	*P. citrinum*
SZ2	Conidia	Light green	Phialosporous	Septate	Conidia simple or branched, erect	*C. lindemuthianum*
SZ3	Conidia	White and orange in center	Phialosporous	Septate	Conidial head have short columnarce	*A. nidulan*
SZ4	Conidia	Light orange and light	Phialosporous	Septate	Catenulete conidia basipetally	*S. asperula*

Table 3. Antibacterial and antioxidant activities of endophytic fungi from *S. zeylanicum*. leaves

Fungi code	Genus/Species of Identification	Ethyl acetate extract weight (gram)	% Antibacterial activity				Antioxidant activity IC50 (μg/mL)
			E. coli	*S. aureus*	*S. thypi*	*B. subtilis*	
SZ1	*P. citrinum*	5.8	71.3 ± 1.55 ***	74.1 ± 1.00 ***	76.2 ± 0.30 ***	76.9 ± 0.70 ***	7.29 ****
SZ2	*C lindemuthianum*	5.2	84.1 ± 0.41 ***	59.5 ± 0.74 **	82.9 ± 0.62 ***	76.5 ± 0.41 ***	3.99 ****
SZ3	*A. nidulan*	5.5	78.5 ± 1.64 ***	62.9 ± 0.98 **	71.4 ± 0.37 ***	60.5 ± 0.43 **	3.85 ****
SZ4	*S. asperula*	5.3	87.7 ± 0.39 ***	63.4 ± 0.65 **	67.6 ± 0.37 **	66.6 ± 0.44 **	7.69 ****
Positive control			Tetracycline 30μg/disc				Ascorbic Acid 30μg / disc
			100 ****	100 ****	100 ****	100 ****	2.73 ****

Note : * = Low antibacterial or weak antioxidant; ** = moderate antibacterial or antioxidant; *** = strong antibacterial or antioxidant ; **** = very strong antibacterial or antioxidant.

Antimicrobial activity of endophytic fungi ethyl acetate extract from *S. zeylanicum* leaves was tested using standard disc diffusion method (at 400 μg/disc). Endophytic fungi extract was tested on *S. aureus, B. subtilis, E. coli, and S. typhi* (**Table 3**). Extracts with strongest antibacterial activity were subjected to continuous isolation to obtain the active compound.

Extract from SZ1 showed widest antibacterial spectrum and strongest antibacterial activity against gram-positive and gram-negative bacteria. Meanwhile, SZ4 extract showed strong antibacterial activity against *E. coli* and moderate activity against other three bacteria tested. Activity of SZ2 and SZ3 extracts were strong against gram-negative bacteria and moderately active against gram-positive bacteria. Endophytic fungi are believed to be able to mimic, duplicate, and modify secondary metabolites of their hosts (Gouda *et al*., 2016). Antibacterial activity of endophytic fungi found in spicate eugenia correlates with its host's antibacterial activity. Deepika *et al*. (2014) reported antimicrobial activity of spicate eugenia against *E. coli, P. aeruginosa, Klebsiella pneumoniae, Proteus vulgaris, Staphylococcus aureus, dan B. subtilis*. Spicate eugenia's antibacterial activity mainly comes from phenolic and flavonoid compounds (Deepika *et al*., 2014; Microbiol *et al*., 2016; Palanisamy *et al*., 2011; Shilpa & Krishnakumar, 2015).

The ethyl acetate extracts of endophytic fungi found in spicate eugenia were tested for antioxidant activity using DPPH method (**Table 3**). Based on its IC_{50} value, antioxidant properties of extracts were classified into following categories: very high (IC_{50}<50 μg/mL), high (IC_{50} 50-100 μg/mL), moderate (IC_{50} 100-500 μg/mL), and weak (IC_{50}<500 μg/mL) (Mbekou *et al*., 2021; Metasari et al., 2020). All samples of endophytic fungi showed very high antioxidant activities; pursuing further studies as antioxidant candidates are feasible. The highest antioxidant activity of endophytic fungi extracts was shown by isolate SZ3(IC_{50} = 3.85 μg/mL). It is known that antioxidants are necessary to scavenge free radicals in the cell. Antioxidants are able to scavenge free radicals by donating one hydrogen atom to act as reductor in redox reaction against free radicals. Stabilizing free radicals also prevents further free radicals formation (Barreca, 2021; Setiawan *et al*., 2018).

The identified SZ1 isolate of *P. citrinum* was also found in the medicinal plant *Stephania kwangsiensis* with potential to control phytopathogens that contained citrinin and emodin compounds (Luo *et al*., 2019). The SZ2 isolate identified by Colletotrichum was also found in *Buxus sinica* plant with antibacterial potential against *S. aureus, P. aeruginosa, E. coli and B. subtilis* bacteria due to contained chermisione B and colletoreichones (Wang *et al*., 2016). The SZ4 isolate identified by Scopulariopsis was also found in soybean (*Glycine max* L. Merr.) and the SZ3 Aspergillus isolate was found in corn (*Zea mays* L.) with potential as pest control (Russo *et al*., 2016). The SZ4 isolate identified by Scopulariopsis was also found in cotton (*Gossypium hirsutum*), which had a synergistic effect on the pathogenicity of *Verticillium dahlia* (Li *et al*., 2017).

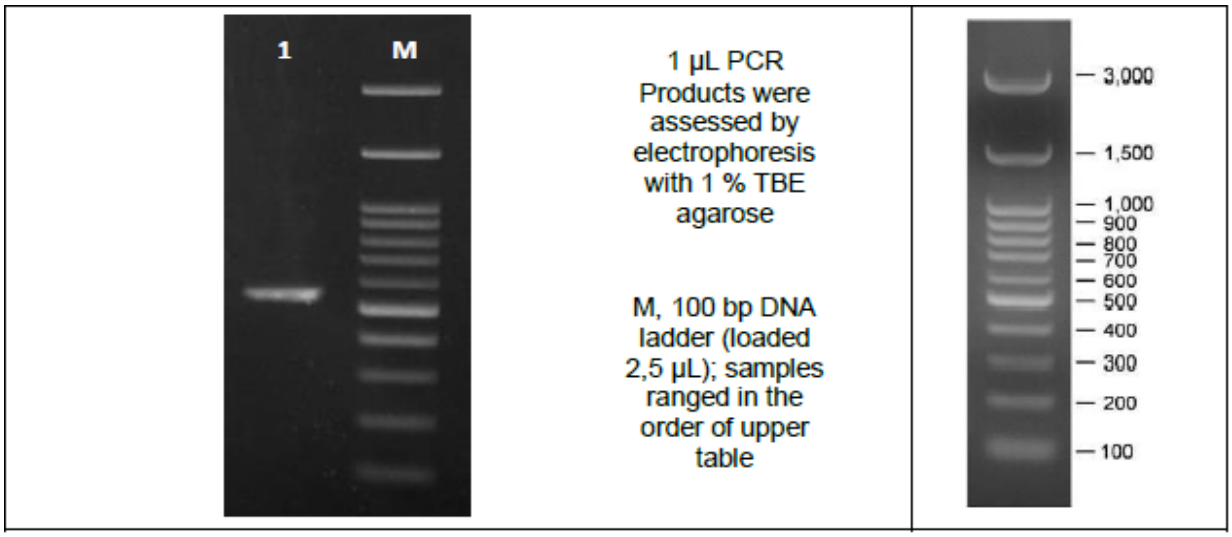


Figure 2. Electrophoresis results of ITS rDNA sequences of endophytic fungi of Isolate SZ1

>OK639011.1 Pestalotiopsis uvicola isolate SZT2 small subunit ribosomal RNA gene, partial sequence; internal transcribed spacer 1, 5.8S ribosomal RNA gene, and internal transcribed spacer 2, complete sequence; and large subunit ribosomal RNA gene, partial sequence
CGTAGGGTGAACCTGCGGAGGGATCATTATAGAGTTTTCTAAACTCCCAACCCATGTGAACTTACCATTG
TTGCCTCGGCAGAAGCTGCTCGGTGCACCTTACCTTGGAATGGCCTACCCTGTAGCGCCTTACCCTGGAA
CGGCTTACCCTGTAGCGGCTGCCGGTGGACTACCAAACTCTTGTTATTTTATTGTAATCTGAGCGTCTTA
TTTTAATAAGTCAAAACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGAAGAACGCAGCGAAATGC
GATAAGTAATGTGAATTGCAGAATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCCCATTAGTAT
TCTAGTGGGCATGCCTGTTCGAGCGTCATTTCAACCCTTAAGCCTAGCTTAGTGTTGGGAGCCTACTGCT
TTTGCTAGCTGTAGCTCCTGAAATACAACGGCGGATCTGCGATATCCTCTGAGCGTAGTAATTTTTATCT
CGCTTTTGACTGGAGTTGCAGCGTCTTTAGCCGCTAAACCCCCCAATTTTTAATGGTTGACCTCGGATCA
GGTAGGAATACCCGCTGAACTTAAGCATATCAATAAGCGG

Figure 3. Nucleotide base sequence of endophytic fungi Isolates SZ1

The endophytic fungi isolates selected for the molecular identification were SZ1 fungi isolated from leaves. Extracts SZ1 fungi produce more yield than other extracts Fungi. The potential for endophytic fungi to be developed as a new source of medicinal raw materials, apart from the ability to biological activity, can also be viewed from the yield of the resulting extract. Molecular test results are presented in the form of a phylogenetic tree (**Figure 4**) to assist in the identification process.

Isolate SZ1 has similarity of 100% is in the name with clade with *P. citrinum*. The phylogenetic tree construction of theendophytic fungi isolates SZ1 in **Figure 3** uses the Neighbor-Joining method. Endophytic isolates of *S. zeylanicum* indicated by an asterisk [*] were subjected to phylogenetic analysis with related species using neighboring phylogenetic trees (bootstrap value= 1000). Sequences were obtained from BLAST results. The value on the branch shows the bootstrap value (percentage of 1000X replication). According to Deepika et al., (2014) the evolutionary level of a species is indicated by different line lengths in the phylogenetic tree. The eluate from column chromatography was monitored using thin-layer chromatography (TLC) with a mobile phase of *n*-hexane and ethyl acetate (5:5). TLC profile with a similar chromatogram pattern was combine into one fraction. Based on the results of the chromatogram pattern, 4 fraction were obtained, namely F1-F4. The form of the pure compound indicated by the F2 fraction is white crystals weighing 37 mg.

The ^{1}H-NMR spectrum of compound 1 showed 8 proton signals; two of them are doublet aromatics with 2 proton integration: δ_H 7.70 (1H, d, J= 8.5 Hz) and δ_H 8.17 ppm (1H, d, J= 8.5 Hz) with ortho coupling constant. The result indicated that Compound 1 is a para-substituted aromatic compound, with two equivalent proton pairs. Further, six signals on chemical shift δ_H < 6.5 ppm were found: at δ_H 1.96 (3H, s); 4.21 (1H, m); 4.32 (1H, m); 4.42 (1H, m); 5.23 (1H, s); and 6.32 ppm (1H, d, J= 1.0 Hz). The chemical shift signals originated from protons of methyl, methine, and oxygenated methine groups. Based on these defining characteristics, Compound 1 was identified as para-substituted aromatic compound containing hydroxyl, methyl, methine, and oxygenated methine groups.

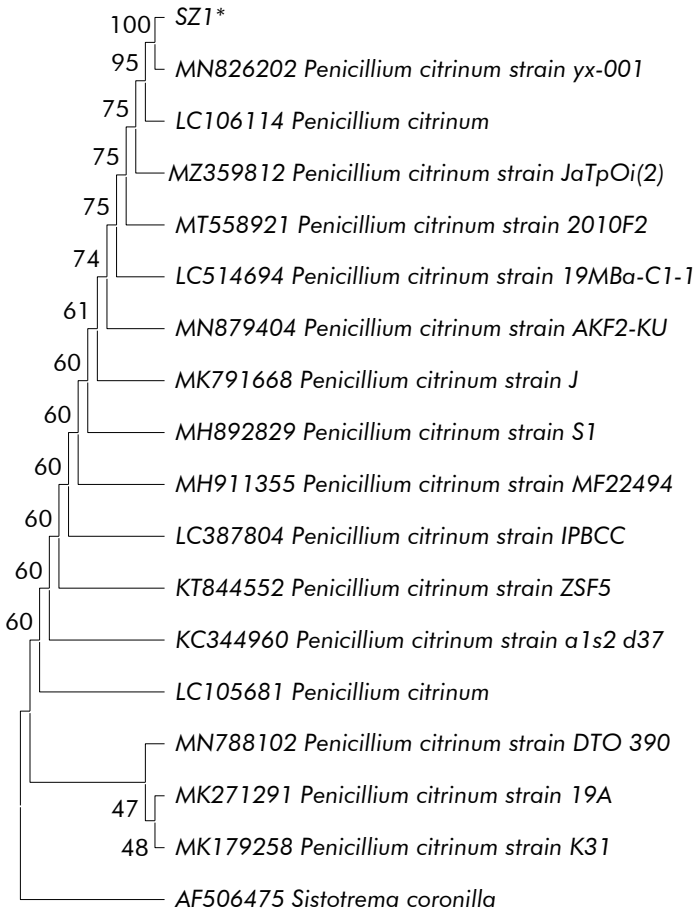
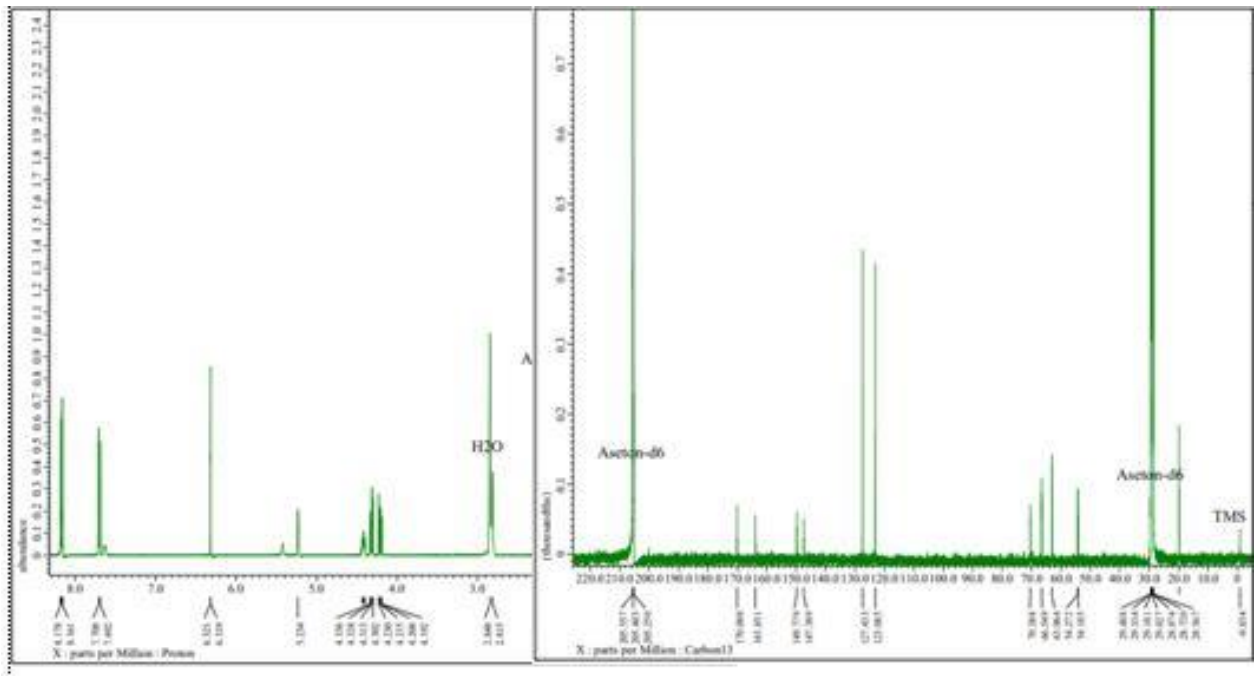


Figure 4. SZ1 Phylogenetic Tree Construction using the Neighbor-Joining method (Saitou & Nei, 1987) with bootstrap values (1000 replicates) displayed next to branches (Felsenstein, 1985). This analysis involved 18 nucleotide sequences. There are a total of 540 positions in the final dataset. Evolutionary analysis carried out in MEGA11 (Tamura et al., 2021).



A B

Figure 5. ^{1}H-NMR (A) and 13C-NMR (B) spectra of substrate A (1H-500 MHz in acetone)

The ^{13}C-NMR spectrum of compound 1 showed 11 carbon signals. Two high-intensity carbon signals were observed, indicating two pairs of equivalent aromatic carbons. Two other aromatic carbons exist as quaternary carbons and showed low intensity signals at δ_C 147.4 and 149.8 ppm. Two other carbon signals were found at lowest fields at δ_C 163.8 and 170.1 ppm; providing evidence for the existence of ester carbonyl carbons. Three carbon signals at δ_C 60.0 – 71.0 ppm (63.1; 66.5; and 70.4 ppm) and one carbon signal at δ_C 54.3 ppm are methine carbon signals; three of them are oxygenated methine signals. Results of ^{13}C-NMR were further confirmed on HMQC spectra. HMQC spectra showed eight correlations of ^{1}H-^{13}C exist through single bond. Proton signals at δ_H 4.21 (1H, m) and 4.32 (1H, m) correlated to one single carbon atom at δ_C 63.1 ppm; indicating existence of methylene group in the ring. Thus, analysis of Compound 1 spectra showed substituted benzene ring and lactone ring substituted with methyl acetate (**Figure 5**).

HMBC spectra (**Figure 6**) showed correlation of ^{1}H-^{13}C through two or three bonds. Proton aromatic signal at δ_H 8.17 ppm showed correlations to three aromatic carbons at δ_C 123.1; 147.4; and 149.8 ppm; showed involvements of their equivalent carbon atoms. Aromatic proton at δ_H 7.70 ppm correlates with two aromatic carbon atoms at δ_C 127.4 and 147.4 ppm and oxygenated carbon at aromatic substituent (at δ_C 70.4 ppm). Oxygenated methine proton at δ_H 5.23 ppm correlates with two aromatic carbons at δ_C 127.4 and 149.8 ppm. Proton at δ_H 5.23 ppm appear singlet, possibly because a proton that is three bonds away is in the farthest geometric position. Those correlations showed direct bind of oxygenated methine group to aromatic ring and para-substituted at hydroxyl group. Further analysis showed correlations of two methylene protons at δ_H 4.21 (1H, m) and 4.32 (1H, m) to single carbon atom at δ_C 54.3; 70.4; and 170.1 ppm. Correlations of two methine protons at δ_H 4.42 ppm to δ_C 63.1 ppm carbon indicates the methylene proton binding to methine carbon and ester carbonyl carbon on open chain. On spectral analysis, correlation of methyl proton at δ_H 1.96 ppm to ester carbonyl carbon atom at δ_C 170.1 ppm were found; strongly suggesting existence of carbonyl ester group on side chain. HMBC spectra analysis indicates binding of lactone ring to aromatic ring and methyl acetate, in addition of binding to hydroxyl group. NMR 1D and 2D spectra data of Compound 1 can be found at **Table 4**.

Combined analysis of ^{1}H-NMR, ^{13}C-NMR, HMQC, and HMBC spectra lend evidence to structure of compound 1, contains para-substituted benzene ring between hydroxyl and ɣ-butyrolactone group. The ɣ-butyrolactone ring further binds to hydroxyl and methyl acetate groups. Thus, proposed chemical structure of Substrate A is (4-hydroxy-2-(4-hydroxyphenyl)-ɣ-butyrolactone-3-yl) methyl acetate **Figure 7**.

The chemical structure of compound 1, (4-hydroxy-2-(4-hydroxyphenyl)-ɣ-butyrolactone-3-yl) methyl acetate, contains hydroxyl (-OH) group and aromatic (phenolic) ring. Phenolic compounds are acidic; they easily dissociate H^+ ion from hydroxyl (-OH) group bound to aromatic ring (Kusumaningrum *et al*., 2021). Compound 1 (4-hydroxy-2-(4-hydroxyphenyl)-ɣ-butyrolactone-3-yl) methyl acetate) contains 1 aromatic ring substituted with one hydroxyl (-OH), and at the para- position of binds five esters (R-COO-R′) with one hydroxyl (-OH) substituent and one other ester substituent at ring five. Compound 1 to act ab antioxidant by donating hydrogen atoms to free radicals are defined as atoms or molecules that have one or more unpaired electrons. The odd electrons possesses by free radicals us able to attact electrons from other molecules to make them stable (Alugojo, et al, 2014). Compound 1 another group at the para (p) position attached to the benzene ring so that it can stabilize the radicals formed in the compound by donating free electron from the group to the aromatic ring, resulting in resonance and producing a more stable lowering the electron density at one position (Bendary et al, 2003).

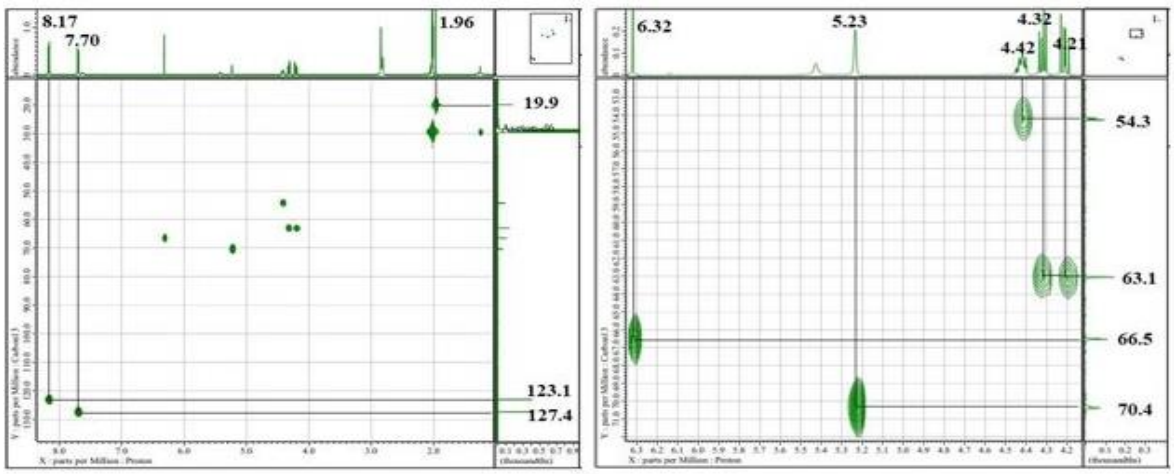


Figure 6. Spectrum HMQC of compound 1 (1H-500 MHz; 13C-125 MHz in aceton)

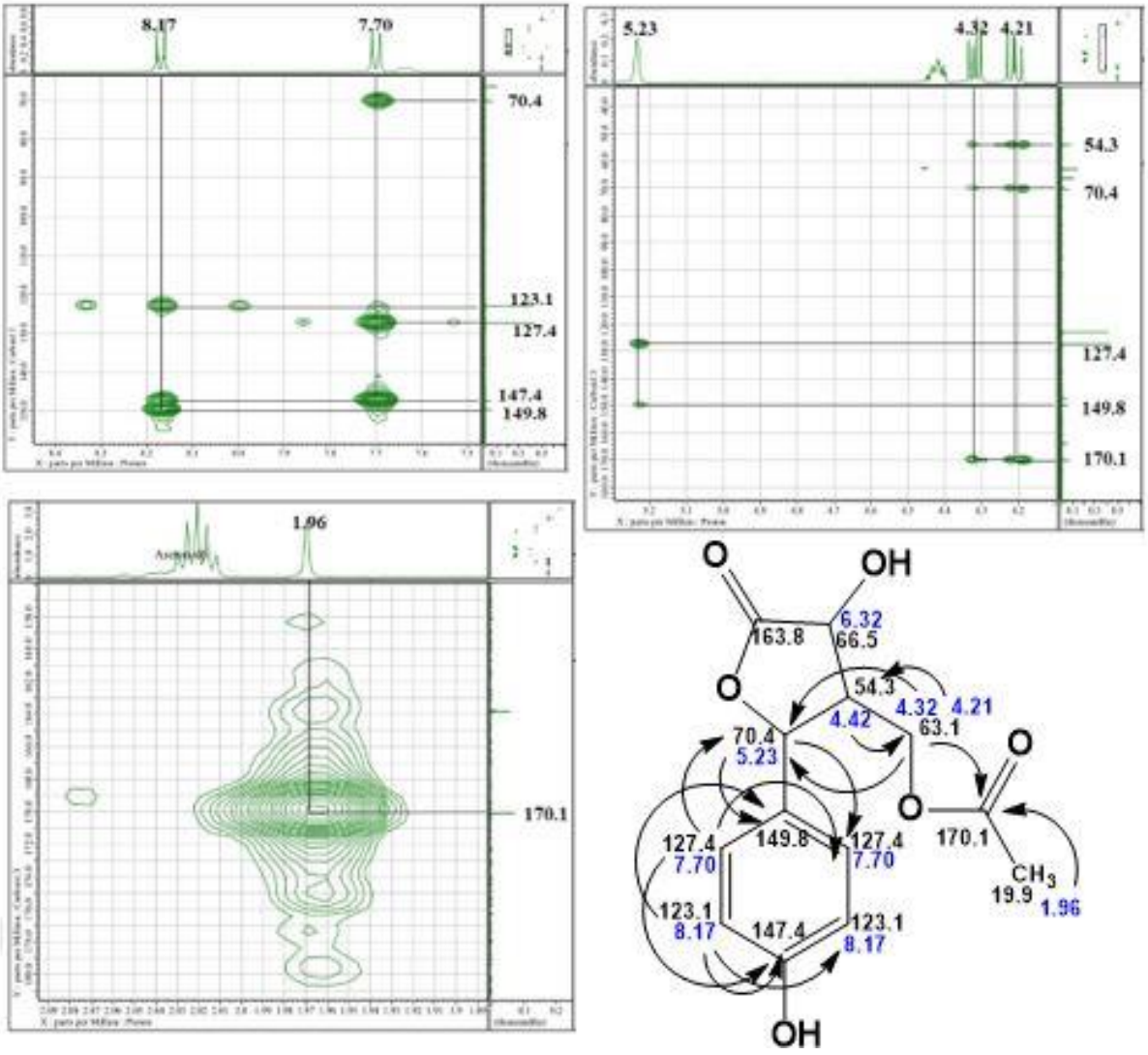


Figure 7. Spectrum HMBC of compound 1 (1H-500 MHz; 13C-125 MHz in Aceton)

Table 4. Data of NMR from compound 1, in 1H-500 MHz; 13C-125 MHz in CDCl3

No C	δ_C ppm	Type of Carbon binding	δ_H ppm (ΣH. Multiplicity (Hz))	HMBC
2	70.4	CH	5.23 (1H, s)	2′ (6′); 1′
3	54.3	CH	4.42 (1H, m)	
4	66.5	CH	6.32 (1H, d, J= 1.0 Hz)	
5	163.8	C		
6	63.1	CH_2	A. 4.32 (1H, m) B. 4.21 (1H, m)	2; 3; 7
7	170.1	C		
8	19.9	CH_3	1.96 (3H, s)	7
1′	149.8	C		
2′	127.4	CH	7.70 (1H, d, J= 8.5 Hz)	2; 2′ (6′); 4′
3′	123.1	CH	8.17 (1H, d, J= 8.5 Hz)	3′ (5′); 4′; 1′
4′	147.4	C		
5′	123.1	CH	8.17 (1H, d, J= 8.5 Hz)	3′ (5′); 4′; 1′
6′	127.4	CH	7.70 (1H, d, J= 8.5 Hz)	2; 2′ (6′); 4′

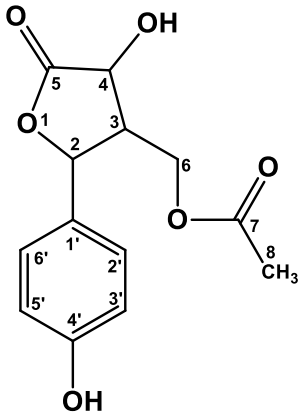


Figure 8. Structure of compound 1: (4-hydroxy-2-(4-hydroxyphenyl)-ɣ-butyrolactone-3-yl)methyl acetate

CONCLUSIONS

Endophytic fungi have been successfully cultured and 4 isolates of fungi were obtained from leaves (coded SZ1, SZ2, SZ3, SZ4). All endophytic fungi have potential antibacterial and antioxidant activity. The molecular results of the isolate with the highest antibacterial showed that the isolate SZ1 was identified as *P. citrinum* with a compound structure (4-hydroxy-2-(4-hydroxyphenyl)-ɣ-butyrolactone-3-yl)methyl acetate). Thus isolate can be used as potential candidates for producing antibacterial and antioxidant compounds in the pharmaceutical in the future.

ACKNOWLEDGEMENTS

The authors thank to the SP DIPA Kemenristek Republic of Indonesia, which provided research funding through Hibah Doctoral Dissertation 2022, with contract no. 0064.03/UN9.3.1/PL/2022

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