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Antibacterial and antioxidant activity of endophytic fungi extracts isolated from the petiole of sungkai plant (*Peronema canescens*)

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Abstract. *Oktiansyah R, Elfita, Widjajanti H, Salni, Setiawan A. 2023. Antibacterial and antioxidant activity of endophytic fungi extracts isolated from the petiole of sungkai plant (*Peronema canescens*). Biodiversitas 24: 6516-6526.* Indonesia has a diverse number of medicinal plants that are very helpful in preventing infectious diseases. Sungkai (*Peronema canescens* Jack.) is a medicinal plant of the family Verbenaceae that is often used and found in Indonesia. Its leaves possessed antibacterial and antioxidant properties that boost immunity and lessen the signs of infectious disorders. This study explored the types of endophytic fungi found in sungkai petioles and examined their relationship to previously reported sungkai leaf endophytic fungal isolates and spectrum of compounds contained them. Endophytic fungal were isolated from fresh leaf petioles of host plants, and their morphological and molecular characteristics were determined. Endophytic fungal extracts were tested for antibacterial and antioxidant properties. Utilizing the paper disk diffusion technique, the antibacterial characteristics were ascertained, while the DPPH method was used to determine antioxidant activity. Molecular identification was carried out on the fungal isolate with the most potential, and chemical compounds were isolated using column chromatography. The structures of the compounds were determined using spectroscopy, including 1D and 2D NMR. Eight endophytic fungal isolates were obtained from sungkai leaf stalks (RA1-RA8) of various species of the genus *Trichoderma*. Differences in the diversity of endophytic fungi found in leaf stalks and endophytic fungi from leaves were identified. RA1 showed the strongest antibacterial and antioxidant activity and was molecularly identified as *T. harzianum*. Spectroscopic analysis showed that the pure compound contained within was 9-hydroxy-7-methylenebenzo[c]oxepin-3(7H)-one (1), which had never previously been found in sungkai plants and endophytic fungi. The antibacterial activity of Compound 1 is in the strong category (MIC = 64 µg/mL) but is not active as an antioxidant. In terms of producing medicinal ingredients from endophytic fungi, *T. harzianum* in extract form has more potential to be developed.

Keywords: Antibacterial, antioxidant, endophytic fungi, petiole, sungkai

INTRODUCTION

Indonesia is known as a biodiverse country. Plant biodiversity supports the development of traditional community culture and daily life (Rahardjanto et al. 2021; Van de Vuurst and Escobar 2020; Zeng et al. 2022). In particular, the leaves and petioles of sungkai (*Peronema canescens* Jack.) are used by the community to produce traditional medicine because they are believed to reduce symptoms and even cure various diseases, such as infectious diseases.

Infectious diseases are caused by pathogens and adversely affect vulnerable communities and the global economy (Antabe and Ziegler 2020; Seventer and Hochberg 2017). For example, the COVID-19 pandemic caused many deaths around the world, including in Indonesia (Baker et al. 2022; Bloom and Cadarette 2019). Immunity is the most important factor in preventing its rapid spread in the environment. Immunity refers to cells which function to safeguard the body from microbes, viruses, and other foreign substances (Marshall et al. 2018). Innate immunity and adaptive immunity are complementary immune systems. Disorders of the immune system lead to host susceptibility and inappropriate responses to pathogens (García 2020; Gombart et al. 2020). Immunostimulants are needed to improve immunity. These compounds can be used to enhance the defense mechanisms of the body, either specifically or non-specifically. These substances can be synthesized or derived from nature and have antioxidant and antibacterial activity (Alagawany et al. 2021; Alhazmi et al. 2021).

Antioxidants and antibacterials can be agents to protect against various infectious diseases. Antioxidant compounds are needed to increase the ability of immune cells to respond the antigens through the certain mechanism while antibacterials or antibiotics work together with the immune system to respond the antigens (Gombart et al. 2020). In particular, it has been shown that antibiotics (especially macrolide types) can penetrate white blood cells (monocytes) by binding to receptors on the cell membrane to produce cytokines. Cytokines are hormone-like proteins that facilitate communication between immune cells and

are crucial for the development, upkeep, and inhibition of immunological responses. Chemokines (IL-8) are cytokines that can induce leukocyte chemotaxis (immune cells) so that immune cells can approach or respond to antigens (positive chemotaxis) (Zachary 2020). Medicinal plants and cuisines are good source of antioxidant and antibacterial compounds (Farzana et al. 2022; Tomas et al. 2022; Abeyrathne et al. 2022; Llauradó Maury et al. 2020; Lourenço et al. 2019). Sungkai is a plant that is commonly utilized in traditional medicine. Research revealed that sungkai contains many secondary metabolites which have biological effects (Dhalaria et al. 2020; Farzana et al. 2022; Dillasamola et al. 2021; Kusriani et al. 2015). However, existence of sungkai are sometimes not sufficient due to lack of raw materials so that other biotechnology is needed, such as endophytic fungi to overcome these problems.

Endophytic fungi can create distinct or same chemicals from their hosts because they can copy and modify compounds from their host plants through a coevolution process (Alam et al. 2021; Elfita et al. 2014; Sharma et al. 2021; Vigneshwari et al. 2019). Extraction and isolation of endophytic fungal compounds are efficient due to the short cultivation time (Castro et al. 2022; Elfita et al. 2023; Oktiansyah et al. 2023b; Singh et al. 2021). The research results explain that the metabolites of fungal endophyte isolated from medicinal plants show that most of their secondary metabolites have unique chemical structures and can act as antimicrobial and antioxidants (Neamul Kabir Zihad et al. 2022; Wen et al. 2022; Fernando et al. 2022; Tiwari and Bae, 2022; Xu et al. 2021; Gautam 2014; Gautam et al. 2013). Research on endophytic fungi isolated from the sungkai plant has also been reported, such as leaves, stems and roots. Sungkai leaves yielded a total of 12 isolates of endophytic fungus and it was found that the 3-(2,6-dihydroxyphenyl)-2-hydroxyacrylic acid compound isolated from *Penicillium oxalicum* showed strong antibacterial and antioxidant activity (Elfita et al. 2022). Then, 4 isolates of endophytic fungi were obtained from sungkai root bark, where *Penicillium janczewskii* showed strong antioxidant and antibacterial activity (Oktiansyah et al. 2023a). In addition, 20 endophytic fungal isolates were isolated from sungkai stem bark, with *Curvularia intermedia* and *Colletotrichum cliviicola* as the fungi with the most potential antioxidant and antibacterial activity. Both fungi produce two compounds, namely 3-hydroxy-4(hydroxy(4-hydroxyphenyl)methyl)-ɣ-butyrolactone (1) and 5-hydroxy-4-(hydroxymethyl)-2H-pyran-2-one (2), where both compounds are active as antibacterials and antioxidants. Based on this research, endophytic fungi isolated from Sungkai leaf stalks are suggested to have a diversity of endophytic fungi and show strong antioxidant and antibacterial activity. The objective of this study was explored the types of endophytic fungi found in sungkai petioles, examined antioxidant and antibacterial activity of their extracts, and determine the compounds contained them.

MATERIALS AND METHODS

Processing of the sample and isolation of endophytic fungus

The sample was assigned the identifier 302/UN9.1.7/4/EP/2021 at the Plant Systematics Laboratory of the University of Sriwijaya. The petiole was taken from fresh leaves. Before isolating the endophytic fungus, the petiole's surface was first sanitized by being submerged in water for around 5 minutes. The sample was then submerged in 70% alcohol for about three minutes, washed with sterilized distilled water for about one minute, then submerged in a solution of 3% NaOCl for about one minute. The material was initially cut aseptically to a size of 3 x 1 cm before being infected onto a petri dish using PDA (Potato Dextrose Agar). The inoculants were incubated at room temperature for 3 to 14 days. Reinoculating the colonies to new media with petridishes and incubating for 48 hours at room temperature will purify the fungal endophyte (Aini et al. 2022; Hapida et al. 2021).

Morphological identification of endophytic fungi

Endophytic fungi were identified using phenotypic traits. With a 1000X magnification (microscope Hirox 1000), microscopic properties were observed using the slide culture method. The phenotypic traits that emerged were then identified by comparison with a number of sources (Pitt and Hocking 2013; Walsh et al. 2018; Watanabe 2010).

Molecular identification of endophytic fungi

Molecular identification was performed on the endophytic fungal isolates with the highest potential for bioactivity. For identification, the ITS DNA (rDNA) region was used. The primers ITS1 (5'-TCCGTAGGTGAACCT GCGG-3') and ITS 4 (5'-TCCTCCGCTTATTGATATGC-3') were employed in the amplification procedure. BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi) includes the sequences. Additionally, the sequences were aligned using the MEGA11 program's CLUSTAL W technique, and a phylogenetic tree with a bootstrap value of 1000 was created using the Neighbour-joining tree method (Tamura et al. 2021).

Cultivation and extraction

Each isolated endophytic fungal pure culture was grown in 300 mL of potato dextrose broth in six blocks of agar (6 mm in diameter). Each isolate was grown in 15 glass bottles, each with a capacity of one liter. The cultures were then left in the static for 30 days at room temperature (28°C). Using filter paper, the media was removed from the fungal biomass, and then ethyl acetate was added to the growth medium in a 1:1 ratio. A rotary evaporator was used to separate the extracts (Oktiansyah et al. 2023c).

Antioxidant activity test

The DPPH technique, developed by Baliyan et al. (2022), was used to measure antioxidant activity; 0.2 mL of each extract concentration was added to a volume of 3.8 mL of a 0.5 mM DPPH solution. 30 minutes were spent

with the combination in a tube of darkness. Ascorbic acid was employed as a reference and the absorbance was measured using a spectrophotometer UV-Vis (UV-1900) at a maximum of 517 nm. In order to measure antioxidant activity, the percentage of inhibition and IC_{50} values were computed (Abbas et al. 2021).

Antibacterial activity test

The paper disc diffusion technique was employed to assess the antibacterial activity. MHA (Muller Hinton Agar) is the medium that is utilized. *Escherichia coli*, *Salmonella typhi*, *Bacillus subtilis*, and *Staphylococcus aureus* were the test microbes employed. Discs containing 400 µg of endophytic fungus extract were left blank. In order to dilute the extract, DMSO was used. Tetracycline at a standard concentration of 30 µg/disc. Paper discs were put on medium in petri dishes that had been bacterially infected and then administered the extract. After incubating the petri dishes for 24 hours at 37°C, the diameter of the zone of inhibition was determined. Antibacterial activity may be measured using the formula below (Aini et al. 2022):

Strong: $\frac{A}{B}$x 100% > 70 %; Moderate: 50 % <$\frac{A}{B}$x 100% < 70 %;Weak: $\frac{A}{B}$x 100% <50 %

A: Inhibition zone of sample
B: Inhibition zone of antibiotic standard

Isolation and identification of compound

Endophytic fungal concentrated extract (2 g) was completely dissolved and impregnated with silica gel 60 (70-230 mesh) with a ratio of 1:2. Next, the impregnated extract was separated by column chromatography (CC) with silica gel 60 (70-230 mesh) in a ratio of 1:15. The sample that has been impregnated is then inserted into the CC column and then eluted using an eluent with increased polarity. The eluate is gathered in a vial then evaporated using an evaporator and TLC analysis is carried out to see the stain pattern. The eluate with the same stain pattern is merged into one fraction, so that several fractions are obtained (Elfita et al. 2023). RA1 ethyl acetate concentrated extract (2 g) was dissolved in ethyl acetate and impregnation was carried out using silica gel 60 (70-230 mesh)) with a ratio of 1:2 between sample weight and impregnated silica. After that, it is stirred for ± 5 minutes, then evaporated using a rotary evaporator until slightly dry, then dried again by stirring gently at room temperature for ± 30 minutes. The impregnated extract was separated by column chromatography (CC) with 30 g of silica gel 60 dian phase (70-230 mesh) using increasing polarity eluents namely n-hexane:EtOAc (10:0 → 0:10) and EtOAc:methanol (10:0 → 5:5). The eluate was collected in 78 vials of 10 mL each, and the stain patterns were grouped using TLC analysis. After that, the eluates with the same stain pattern were joined to make one fraction, yielding five fractions, namely F1-F5. Fraction F3 was rinsed with a solvent mixture namely hn-hexane: EtOAc (5:5) until compound 1 was obtained in the form of a yellow solid of 41.3 mg.

RESULTS AND DISCUSSION

Isolation and identification of endophytic fungi

The isolation of fungal endophytes from the petiole of *P. canescens* resulted in eight isolates (RA1-RA8). Macroscopically, the eight isolates showed a variety of shapes and colors, while microscopically, the characteristics of each isolate could be determined (Figures 1 and 2). Endophytic fungi isolates exhibited primarily white and gray hues. The macroscopic and microscopic characteristics of the eight isolates of endophytic fungi can be seen in Tables 1 and 2.

Table 1 and Table 2 revealed each colony of endophytic fungi found on the petiole of sungkai morphologically. This research identified eight isolates of fungal endophytes from the petiole of sungkai, consisting of six species belonging to the genera *Trichoderma*, *Gonatobotrys*, and *Trametes*. The most common species within the *Trichoderma* genus were *T. harzianum*, *T. asperellum*, *T. pseudokoningii*, and *T. aureoviride*. The dominance of the *Trichoderma* genus occurs because the spores of this genus are easily spread. Characteristics that appear based on observations were used as a reference for identification.

Table 1. Endophytic fungi isolated from petiole of *P. canescens* macroscopically

Code	Surface colony	Reverse colony	Structure	Elevation	Pattern	Exudate drops	Radial line	Concentric circle
RA1	White	White	Cottony	umbonate	radiate	-	√	-
RA2	White	White	Cottony	umbonate	radiate	-	√	-
RA3	white Yellowish	white Yellowish	Cottony	umbonate	radiate	-	√	-
RA4	White greentint	White greentint	Cottony	umbonate	radiate	-	√	-
RA5	Yellow	Yellow	Cottony	umbonate	zonate	-	-	√
RA6	White	White	Cottony	rugose	zonate	-	-	-
RA7	White	White	Cottony	umbonate	radiate	-	√	-
RA8	White	White cream	Cottony	umbonate	radiate	-	√	√

Note: (√): detected; (-): not detected

Table 2. Endophytic fungi isolated from petiole of *P. canescens* microscopically

Isolate	Spore	Shape	Hyphae	Characteristic	Identified species
RA1	Sporangia	Globose	Septate	Conidiophores hyaline, phialides short and thick, globose	*Trichoderma harzianum*
RA2	Sporangia	Globose	Septate	Conidiophores dark brown, simple, conidiophores short	*Gonatobotrys simplex*
RA3	Sporangia	Globose	Septate	Conidiophores branched, short and thick, subglobose	*Trichoderma aureoviride rifai*
RA4	Sporangia	Globose	Septate	Conidiophores hyaline, phialides short and thick, globose	*Trichoderma asperellum*
RA5	Conidia	Cylindrical	Septate	Conidiophores hyaline, phialides short and thick, globose	*Trichoderma harzianum*
RA6	Conidia	Globose	Septate	Shelflike, semicircular or fan shaped	*Trametes polyzona*
RA7	Sporangia	Globose	Septate	Conidiophores hyaline, branched, phialides short and thick, subglobose	*Trichoderma pseudokoningii*
RA8	Sporangia	Globose	Septate	Conidiophores hyaline, phialides short and thick, globose	*Trichoderma harzianum*

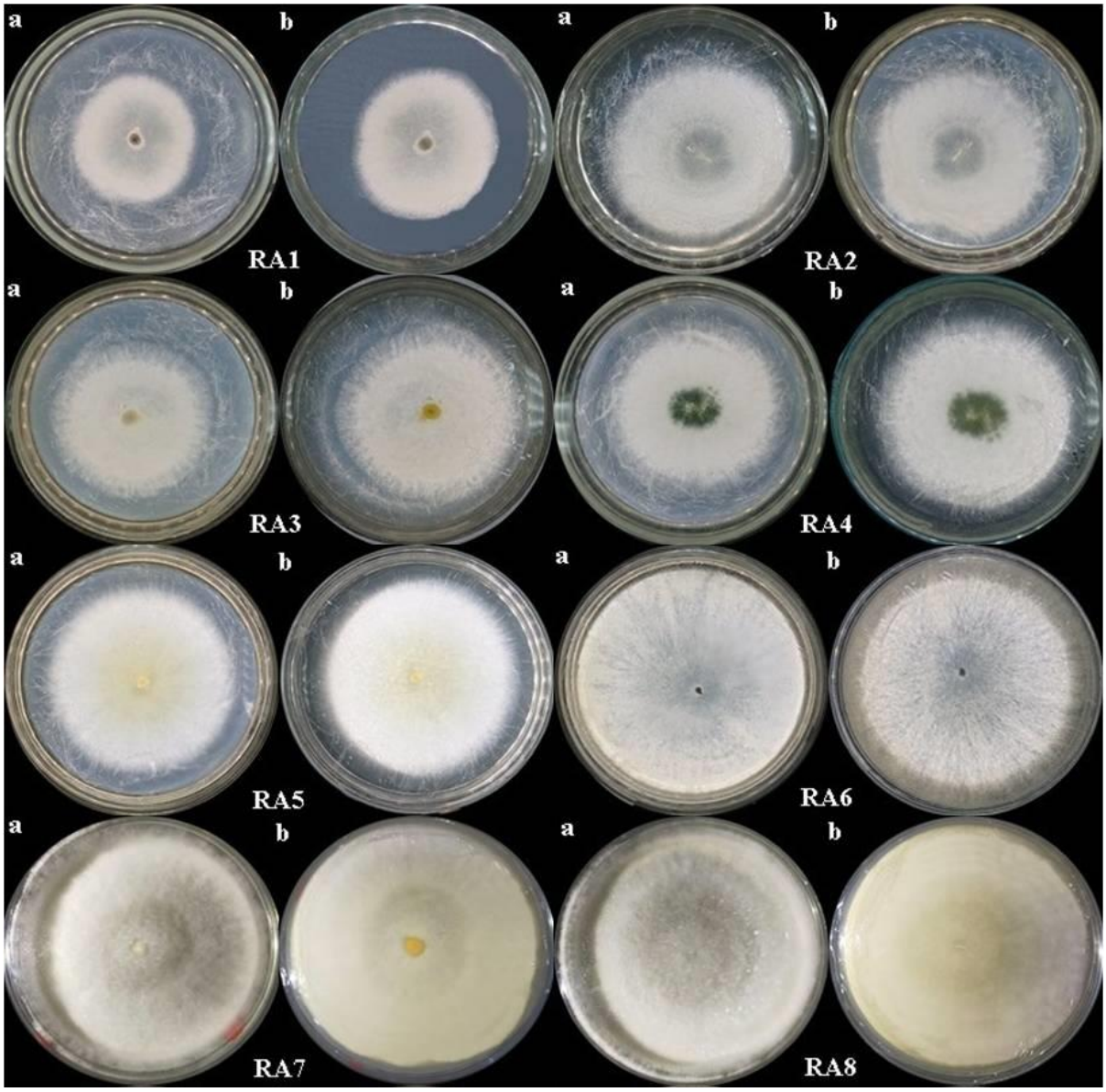

Figure 1. Characteristics of endophytic fungi colonies found on the petiole of sungkai morphologically (RA: isolate code (1-8); a: front view; b: reverse view)

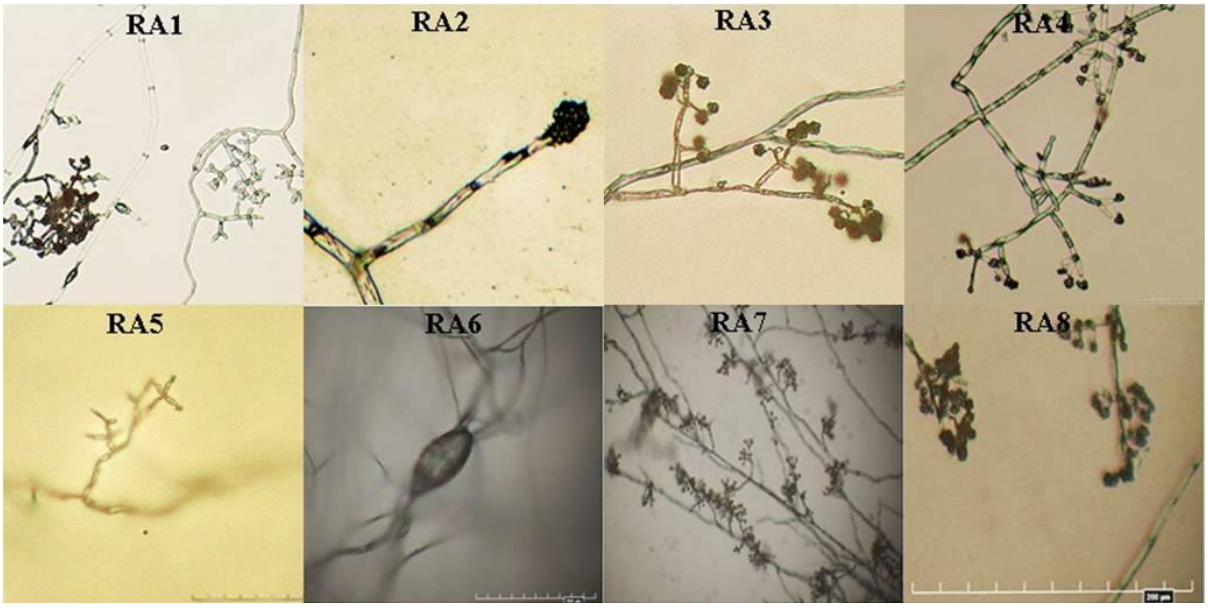

Figure 2. The microscopic characteristic of fungal endophytes found from the petiole of sungkai (RA: isolate code (1-8))

Table 3. Antibacterial activity percentation and IC_{50} endophytic fungus extract obtained from sungkai petiole vs positive controls

Code isolate	Genus/identified species	Weight of extract (g)	% Antibacterial activity				Antioxidant activity IC_{50} (µg/mL)
			E. coli	*S. aureus*	*S. thypi*	*B. subtilis*	
Methanol of sungkai's petiole			70.3 ± 0.74 ***	71.2 ± 1.16 ***	80.4 ± 0.93 ***	80.6 ± 0.82 ***	13,75 ***
RA1	*Trichoderma harzianum*	2	71.7 ± 2.17 ***	81.0 ± 0.27 ***	74.8 ± 0.61 ***	88,4 ± 2,60 ***	21,02 ***
RA2	*Gonatobotrys simplex*	3.2	45.7 ± 2.02 *	80.8 ± 0.75 ***	79.4 ± 0.40 ***	86,1 ± 0,35 ***	32,40 ***
RA3	*Trichoderma aureoviride rifai*	3.1	55.4 ± 0.28 **	69.4 ± 0.96 **	72.4 ± 0.65 ***	82,1 ± 0,05 ***	135,27 **
RA4	*Trichoderma asperellum*	2.9	70.8 ± 1.05 ***	70.1 ± 0.63 ***	74.6 ± 0.95 ***	82,9 ± 0,77 ***	23,81 ***
RA5	*Trichoderma harzianum*	2.5	57.5 ± 0.27 **	75.7 ± 0.91 ***	87.6 ± 0.99 ***	78,6 ± 1,90 ***	157,17 **
RA6	*Trametes polyzona*	3.2	70.8 ± 2.89 ***	81.8 ± 0.51 ***	74.6 ± 1.79 ***	70,3 ± 0,80 ***	131,77 **
RA7	*Trichoderma pseudokoningii*	3.4	69.4 ± 0.33 **	75.5 ± 1.82 ***	82.2 ± 0.95 ***	70,7 ± 2,50 ***	36,29 ***
RA8	*Trichoderma harzianum*	3.6	60.2 ± 1.15 **	80.2 ± 0.97 ***	80.6 ± 0.40 ***	83,7 ± 0,92 ***	129,52 **
Positive control			Tetracyclin 100 ***	Tetracyclin 100 ***	Tetracyclin 100 ***	Tetracyclin 100 ***	Ascorbic acid 10.08 ****

Note: Activity of antibacterial percentage: *** strong (≥70%), **moderate (50-70%), and *weak (<50%). Antioxidant activity IC50 (µg/mL): ****very strong <10 µg/mL ***strong < 100 µg/mL; **moderate 100-500 µg/mL; * weak >500 µg/mL (Elfita et al. 2022)

Antibacterial and antioxidant activity of endophytic fungi

Fungal endophytes isolated from the petiole of sungkai have potential as antioxidants and antibacterials in ethyl acetate extracts. The results revealed that the endophytic fungi extract had antibacterial properties against the four tested bacteria. Three isolates showed strong capabilities against the four tested bacteria. All extracts showed strong antibacterial activity against *Streptococcus aureus*, *Salmonella thypi*, and *Bacillus subtilis* bacteria, while only three extracts demonstrated significant antibacterial action against *Escherichia coli*. Regarding antioxidant activity, endophytic fungi extracts isolated from petiole showed strong and moderate levels of IC_{50}.

Experimental results described the extract of fungal endophytes from petiole of sungkai which inhibited the growth of the bacteria and the IC_{50} were categorized as moderate and strong. Additionally, the host plant's

methanol extract shown excellent antioxidant and antibacterial activity categories. The isolates RA1, RA4, and RA6 had strong antibacterial activity against the four test bacteria, but only RA1 isolates had antioxidant activity close to the IC_{50} value of ascorbic acid (positive control), namely 21.02 µg/mL. Based on these data, RA1 isolates were carried out for molecular identification.

Molecular identification of endophytic fungi

Compared to the other isolates, RA1 demonstrated the most antibacterial and antioxidant activity, making it the most promising isolate. The results of the molecular identification of RA1 are shown in Figure 3. The order of the sequences was as follows: AACCTGCGGAGGCATCATTACCGAGTTTACAACTC CCAAACCCAATGTGAACGTTACCAAACTGTTGCCT CGGCGGGATCTCTGCCCCGGGTGCGTCGCAGCCCC GGACCAAGGCGCCCGCCGGAGGACCAACCAAAAA CTCTTATTGTATACCCCCTCGCGGGTTTTTTTATAA TCTGAGCCTTCTCGGCGCCTCTCGTAGGCGTTTCG AAAATGAATCAAAACTTTCAACAACGGATCTCTTG GTTCTGGCATCGATGAAGAACGCAGCGAAATGCG ATAAGTAATGTGAATTGCAGAATTCAGTGAATCAT CGAATCTTTGAACGCACATTGCGCCCGCCAGTATT CTGGCGGGCATGCCTGTCCGAGCGTCATTTCAACC CTCGAACCCCTCCGGGGGGGTCGGCGTTGGGGATC GGCCCTGCCTCTTGGCGGTGGCCGTCTCCGAAATA CAGTGGCGGTCTCGCCGCAGCCTCTCCTGCGCAGT AGTTTGCACACTCGCATCGGGAGCGCGGCGCGTCC ACAGCCGTTAAACACCCAACTTCTGAAATGTTGAC CTCGGATCAGGTAGGAATACCCGCTGAACTTAAG CATATCAATAAGCCGGAGGAA. RA1 showed 99.84% similarity up to 1 clade with *Trichoderma harzianum*.

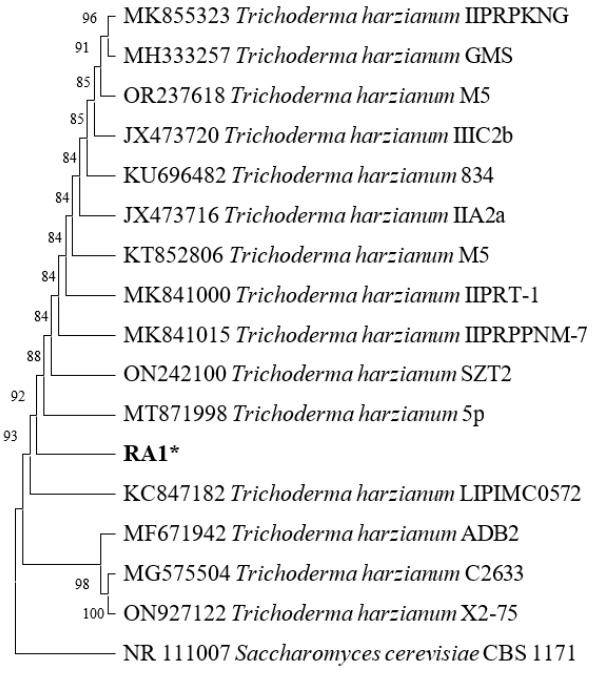

Figure 3. Phylogenetic tree of RA1 reconstructed by using the Neighbour-Joining bootstrap value of 1000

Isolation and identification of pure compound

The ^{1}H-NMR spectrum (500 MHz, CD3OD) (Figure 4) displays the presence of five proton signals, each appearing at δ_H 8.84 (1H, d, J = 8.0), 8.56 (1H, dd, J = 8.0 and 1.5), 8.38 (1H, dd, J = 8.5 and 1.5), 8.36 (1H, dd, J = 8.5 and 1.5), 8.11 (1H, s), 8.09 (1H, m), and 8.06 (1H, m) ppm. These signals indicate that Compound **1** consists of five sp2 protons attached to unprotected carbon atoms. The coupling constant value of 7.2 Hz for protons at δ_H 8.84 and 8.56 ppm indicates that the two protons are neighbors. The protons at δ_H 8.38 and 8.36 ppm were identified as being bound to one carbon atom, and from the cap constant values (8.5 and 1.5 Hz), it was found that the two protons were correlated with each other and correlated to four bonds, each with the protons at δ_H 8.09 (1H, m) and 8.06 (1H, m). Apart from that, a proton at δ_H 8.11 ppm had a singlet multiplicity and was in close chemical shift to the other two vinyl protons. This vinyl proton was identified as having no neighboring protons and was bound to an oxygenated carbon atom.

The ^{13}C NMR spectrum (Figure 5.A) shows that the isolated compound has 11 carbon atoms, namely at δ_C 128.0-167.5 ppm, Siverstein et al. (1991) classified the chemical shift of carbon in an organic compound. Alkene sp2 carbons are at δC 100-140, aromatic sp2 carbons at δC 125-160 ppm, ester carbonyl carbons at δC 160-180 ppm. Thus, all the carbons in compound 1 are sp2 carbons. The proton at δ_C 167.5 ppm was identified as ester carbonyl and the carbon at δ_C 144.0 ppm was identified as oxyvinyl carbon. The HMQC spectrum (Figure 5.B) shows the relationship of seven proton signals that are directly bound to the carbon atom. The spectrum shows a correlation between seven vinyl protons with a δ_H value of 8.84; 8.56; 8.38; 8.36; 8.11; 8.09; and 8.06 ppm which binds directly to sp2 carbon respectively at δ_C 136.2; 134.5; 128.0; 130.2; 133.1; and 131.9 ppm. Two vinyl protons at δ_H 8.38 and 8.36 ppm bond directly with δ_C 128.0 ppm which indicates compound 1 has a methylene group.

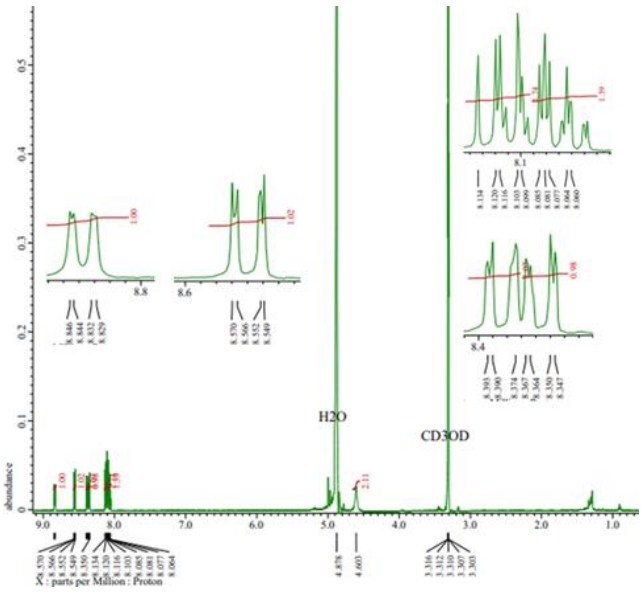

Figure 4. The ^{1}H-NMR spectral of compound 1

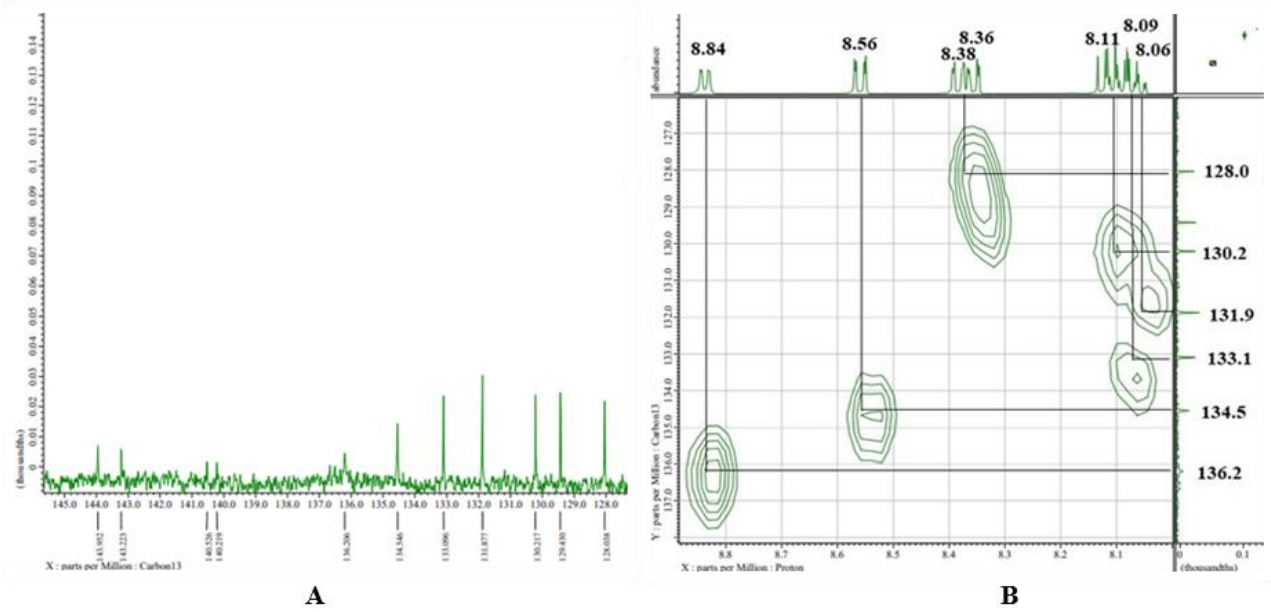

Figure 5. The ^{13}C-NMR (A) and HMQC (B) spectral of compound 1

Table 4. The compound 1 NMR data (1H-500 MHz, 13C-125 MHz in CD_3OD)

No. C	δ_C ppm	δ_H ppm (ΣH. Multiplicity, Hz)	HMBC
	1	1	1
1	130.2	8.11 (1H, s)	143.2
3	167.5		
4	136.2	8.84 (1H, d, J=8.0)	134.5; 140.2; 167.5
5	134.5	8.56 (1H, dd, J=8.0 & 1.5)	136.2; 140.2
6	133.1	8.09 (1H, m)	129.4; 140.2
7	129.4		
8	131.9	8.06 (1H, m)	144.0; 128.0
9	144.0		
10	143.2		
11	140.2		
12	128.0	A. 8.38 (1H, dd, J=8.5 & 1.5)	131.9; 144.0
		B. 8.36 (1H, dd, J=8.5 & 1.5)	133.1

The HMBC spectrum (Figure 6) displays the correlation between seven vinylic protons at δ_H 8.84, 8.56, 8.38, 8.36, 8.11, 8.09, and 8.06 ppm, which were correlated with carbon through two and three bonds. Protons at δ_H 8.84 ppm (1H, d, J = 8.0) were correlated with carbon at δ_C 134.5, 140.2, and 167.5 ppm, and protons at δ_H 8.56 ppm (1H, dd, J = 8.0 and 1.5) were correlated with carbon at δ_C 136.2 and 140.2 ppm. This correlation implies that the two protons are neighbors. The proton at δ_H 8.84 ppm was correlated with three bonds with two quaternary carbon atoms, one of which was ester carbonyl carbon. The two methylene protons at 8.38 δ_H (1H, dd, J = 8.5 and 1.5) and 8.36 (1H, dd, J = 8.5 and 1.5) were correlated with each other, and each was also correlated via three bonds with the proton at δ_H 131.9 and 144.0 ppm and δ_H 133.1 ppm. This indicates that the methylene proton was bound by three bonds with both vinyl carbons. This indication is supported by the correlation of vinylic protons at δ_H 8.09 ppm with carbon at δc 129.4 and 140.2 ppm and vinylic protons at δ_H 8.06 ppm with carbon at δ_C 144.0 and 128.0 ppm. Furthermore, the results showed a correlation of vinylic protons at δ_H 8.11 ppm through two bonds with carbon at δ_C 143.2 ppm. This indicates that the vinyl proton was bound to the oxygenated carbon atom, namely, the ester group. Table 4 summarizes the 1D and 2D NMR spectrum data for Compound 1.

Compound 1 was found using 1D and 2D NMR spectroscopy data as a bicyclic ring containing a 7-membered unsaturated lactone ring (ε-caprolactone). Compound 1 has five vinylic methylene protons, two protons from a vinylic methylene group and a hydroxyl group. The ε-caprolactone ring has three vinylic protons. Thus, the molecular structure of compound 1 was proposed, namely 9-hydroxy-7-methylenebenzo[c]oxepin-3(7H)-one. The molecular structure of compound 1, complete with carbon atom numbering, is shown in Figure 7.

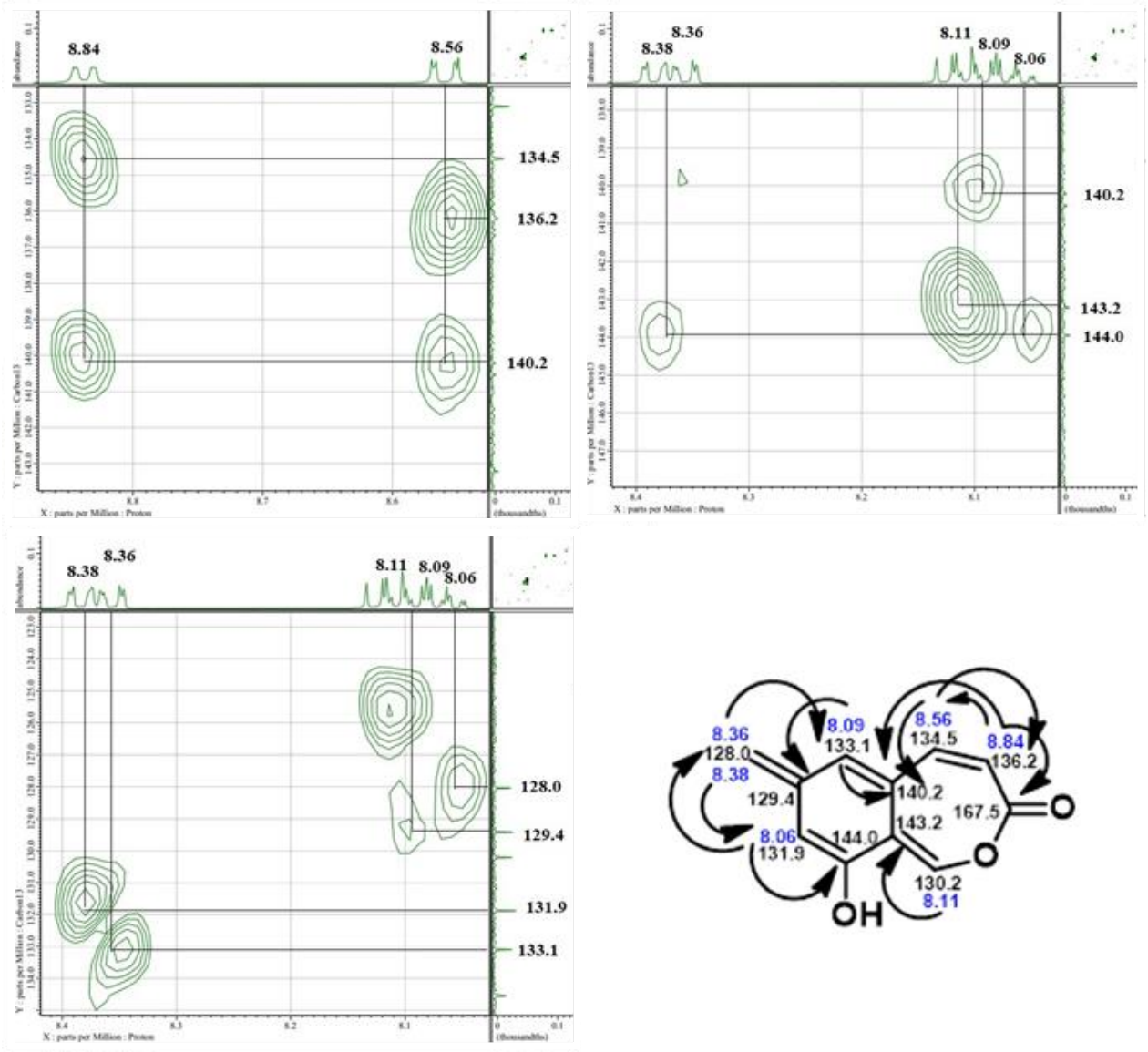

Figure 6. The HMBC spectral and HMBC correlation of compound 1

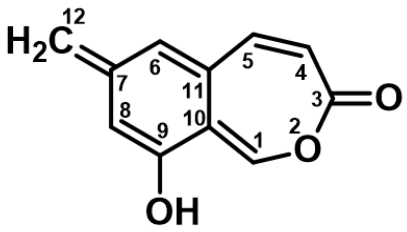

Figure 7. Chemical structure of compound 1 as 9-hydroxy-7-methylenebenzo[c]oxepin-3(7H)-one

Discussions

This research identified eight isolates of fungal endophytes from the petiole of sungkai with *Trichoderma* as genus the most found. Several studies have also found *Trichoderma* on sungkai leaves—namely, *Trichoderma* sp. and *T. asperellum*— (Elfita et al. 2022; Oktiansyah et al. 2023c) but the number of species in this genus did not vary, as was found in the petioles. The variation in the species of *Trichoderma* on leaf stalks is caused by the function of these leaf stalks as a perch for organisms, such as birds, and a place for soil insects to pass so that spores from fungi that stick to the bodies of these animals can move to the petioles of sungkai leaves. Soil is the main habitat of *Trichoderma*. However, the presence of wind and other organisms, such as birds and insects, makes it very easy to find fungi from *Trichoderma* on petioles. Studies have reported that fungi from *Trichoderma* do not have a specific host and are easily found on other plant organs (Kim et al. 2020; Tyśkiewicz et al. 2022). Additionally, fungi of the genus *Trichoderma* spp. can adapt to changing environmental conditions and produce many conidia and chlamydospores. Research indicated that this group of

fungi has a very wide temperature tolerance range for its growth and development, so it is very often found on plant parts, especially the petioles of host plants (Contreras-Cornejo et al. 2020; Filizola et al. 2019; Peng et al. 2021).

The methanol extract of sungkai petiole showed strong antibacterial and antioxidant properties (Table 3). This bioactivity is suspected to be related to the content in the sungkai petiole. The chemical content found in the sungkai petiole is similar to that found in the leaves and stem bark, namely phenolics, saponins, alkaloids, tannins, and steroids it's just differ in the total of the content (Dillasamola et al. 2021; Kusriani et al. 2015). These metabolites have been proven to be antibacterial and antioxidant (Almanaa et al. 2022; Hmamou et al. 2022; Karimi et al. 2011; Konappa et al. 2021).

The extract of fungal endophytes that found in the petiole of the sungkai also exhibited strong antioxidant and antibacterial activity. Isolates RA1, RA4, and RA6 inhibited the growth of the four test bacteria and based on the IC_{50} value for determining antioxidant activity, they were included in the strong category. Table 3 shows that from a total of 8 isolates of fungal endophytes residing in sungkai petiole, isolate RA1 had antibacterial and antioxidant properties equivalent to its host. The position of the leaf and the petiole are not too far apart so that the secondary metabolite content is suggested similar. In this study, RA1 contained lactone compound where several scientific references explain that this compound has antibacterial and antioxidant activity. Molecular Identification showed that RA1 was *Trichoderma harzianum*.

The extract produced by the *T. harzianum* fungus has strong antibacterial properties (MIC = 64 µg/mL) because it is able to inhibit the four tested bacteria but is not active as an antioxidant. After this compound was isolated, this endophytic fungus produced the compound 9-hydroxy-7-methylenebenzo[c]oxepin-3(7H)-one (1). This compound belongs to the lactone group which has never been found in other parts of the sungkai plant or its endophytic fungi. This discovery indicates that this compound is a different compound from its host plant and other endophytic fungi. The same agro-climatic conditions and stresses on plants cause similar compounds to be found in different endophytic fungi from different plant parts and different plant species (Divekar et al. 2022; García-Mier et al. 2013; Li et al. 2022). The long coevolution of the endophyte with its host plant causes the endophyte to adapt to its microenvironment so that it can molecularly absorb some plant DNA into its genome. This event is thought to be the cause of the endophytes being able to synthesize chemical components related to the host plant, including secondary metabolites. Certain environmental conditions and pressures can activate biosynthesis gene clusters, in the form of "silent genes", so that fungal endophyte can produce different compounds to help their host plants survive dangerous circumstances of environmental stress (Fontana et al. 2021; Pfannenstiel et al. 2019; Rashmi and Venkateswara Sarma 2018; Tiwari and Bae 2020; Zhao et al. 2010). This means that plant species in the same environment can produce endophytic fungi with different secondary metabolites.

Studies have shown that *T. harzianum* contains lactone (Lakhdari et al. 2023; Xiao et al. 2023). Lactone substances can stop bacteria from growing and lessen oxidative stress. This substance can prevent bacteria from synthesizing proteins and DNA by inhibiting ribonucleic acid reductase activity and decreasing the surface permeability of bacterial cell walls. These properties are provided by the ester cyclic in the chemical structure (El Khatib et al. 2021; Hechaichi et al. 2023; Li et al. 2022). According to the literature, the secondary metabolites of the host plant are identical to those detected in endophytic fungal extract. This suggests that because of their function in mutualistic relationships, endophytic fungi can duplicate secondary metabolites of their host plants. The opportunity to obtain the same compounds as the host plant is very large considering that raw materials for drugs to overcome antibiotic resistance are increasingly difficult to obtain. The new compounds produced by endophytic fungi can also be an alternative to treat various infectious diseases. Endophytic fungi isolated from sungkai petiole obtained 8 isolates. RA1 isolate (*Trichoderma harzianum*) was the isolate with the most potential bioactivity. The pure compound isolated from *T. harzianum*, namely 9-hidroksi-7-methylenebenzo[c]oxepin-3(7H)-one, was a lactone compound. This compound had strong antibacterial activity and inactive antoxidant. In this study, we found 9-hydroxy-7-methylenebenzo[c]oxepin-3(7H)-one. This compound belongs to the lactone group which has never been found in other parts of the sungkai plant or its endophytic fungi. This discovery indicates that this compound is a different compound from its host plant and other endophytic fungi. According to studies, this chemical can be modified in a number of ways to be employed as a novel medicinal material.

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