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Bioactive Compounds of Endophytic *Fungi Lasiodiplodia theobromae* Isolated From The Leaves of Sungkai (*Peronema canescens*)

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Abstract: *Peronema canescens*, known as sungkai, is a plant that is commonly found in Indonesia. Sungkai is used to cure various diseases related to the immune system. Endophytic fungi associated with medicinal plants are a potential natural resource for producing medicinal compounds. In this study, we report on the endophytic fungal species isolated from sungkai leaves and their bioactive compounds. Identification of endophytic fungi was carried out morphologically, and the fungi were analyzed molecularly through the use of phylogenetic trees. The endophytic fungi were cultivated in the potato dextrose broth medium over four weeks in static conditions. Extraction of metabolites was performed using ethyl acetate and then evaporated to gain a dense extract. The 2,2-diphenyl-1-picrylhydrazyl method was used to test antioxidant activity, and the disc diffusion method was used for antibacterial activity. Bioactive compounds were isolated through the chromatographic technique, and the pure compounds were identified spectroscopically to understand the chemical structure. The result of the isolation of endophytic fungi from the sungkai leaves was obtained with the region of difference 4 (RD4) code. Based on morphological and phylogenetic analysis, RD4 was identical to *Lasiodiplodia theobromae*. Two pure compounds were found in the endophytic fungi; namely, 3-methyl-3,4-dihydro-1H-isochromene-1,8(7H)-dione (compound 1) and"3-hydroxy-4(hydroxy(4- hydroxyphenyl)methyl)-γ-butyrolactone (compound 2). Compound 2 had strong antioxidant activity and good antibacterial activity. Based on this research, compound 2 has the potential to be developed as a new source of antioxidant and antibacterial activity following further research.

Keywords: bioactive compound; endophytic fungi; *Lasiodiplodia theobromae*; *Peronema canescens*.

1. Introduction

Various medicinal crops that are found in nature have curative resources for diseases and have been used in contemporary therapies as alternatives to drugs. In developing countries, about 80% of people use herbal medications as their primary source of health care. Many medicinal plants, such as the sungkai plant (*Peronema canescens*), have been tested for their effectiveness against a number of diseases, such as malaria, typhoid, hyperuricemia, and diabetes mellitus. During the COVID-19 pandemic, sungkai leaves were the plant the public trusted the most for increasing the immune system. Chemical compounds in the sungkai plant, namely, peronemin, sitosterol, isopropanol, phytol, diterpenoid, and flavonoid, are effective as antioxidants and antibacterials, enabling the plant to become a natural immunomodulator that can increase immunity [1–3]. The trend toward using medicinal plants is increasing along with the high demand for herbal medicines. However, the horticulture of medicinal crops is faced with many obstacles, such as ecological exaggregation, activities with regard to biological anthropology, and habitats that are damaged by pests, causing plant populations to decrease. As a result, exploring plants' raw materials for medicinal needs is often not adequately undertaken [4], [5].

Endophytic fungi are microorganisms that reside in tissues (all parts of the plant) at certain periods by forming colonies that, rather than crippling the plants, develop a mutualistic relationship with them. Essentially, the plants act as protectors and sources of nutrition for the endophytic fungi, which produce bioactive compounds that help their hosts improve their nutritional statuses to increase growth, resistance to herbivores and disease, and tolerance for physical stress [6, 7]. Endophytic fungi are natural resources that are promising and abundant, containing varied chemical structures, higher biodiversification, and various interesting bioactivities. Penicillin, isocoumarin, and penialidine from the endophytic fungus *Penicillium sp*. isolated from *Melastoma malabatrichum*, *Kandelia candel*, and *Garcinia nibilis* act as antibacterial. It is known that penicillin has been used as an antibiotic. Taxol and brevifoliol derived from *Fusarium redolens* isolated from *Taxus brevifolia* were used as anticancer. These fungi have been identified as repositories of bioactive compounds that can be used in the pharmaceutical and food industries. These compounds include steroids, alkaloids, terpenoids, isocoumarins, lactones, quinones, flavonoids, phenolics, indoles, anthraquinones, xanthones, phenylpropanoids, phenolic acids, and peptides [8, 9].

The natural resources derived from endophytic fungi constitute the most relevant sources for new medicines' invention and molecular diversification. Several studies have shown that most of the bioactive compounds that are acquired from endophytic fungi have uncommon chemical structures. These fungi can produce secondary metabolites that are related to or different from their hosts. This indicates a huge potential for this microbial group to explore new medicines [7, 10].

2. Materials and Methods

2.1. Sample of a plant.

The fresh and healthy leaves of *P. cansescens* were collected in June 2022 from Palembang City, South Sumatra, Indonesia. The plant was identified in the Laboratory of Biosystematic, University of Sriwijaya, with number 302/UN9.1.7/4/EP/2021. Endophytic fungi were isolated from old leaf tissue, the third row from the branch's base.

2.2. Chemicals and reagents.

The materials used in this study were the endophytic fungal (code: RD4 from sungkai leaves), Potato Dextrose Agar, Potato Dextrose Broth, alcohol 70%, physiological NaCl solution, TLC kiessel gel 60 F254, silica gel G 60 70-230 mesh. The solvents used were n-hexane, ethyl acetate, methanol, H_2O. The test bacteria were *Escherichia coli* (InaCCB5), *Salmonella typhi* (ATCC 1408), *Bacillus subtilis* (InaCCB4) and *Staphylococcus aureus* (InaCCB4). Tetracycline and ascorbic acid were used as positive control.

2.3 Instrumentation.

The characterization of chemical compounds using a NMR spectrum on JEOL JNM-ECZ500R/S1 500 MHz (^{1}H); 125 MHz (^{13}C).

2.4. Isolation of endophytic fungi.

The isolation of the endophytic fungi was done according to the procedure described by Pansanit and Pripdeevech (2018). Fresh leaves were cleaned with distilled water for one minute and then dried. The surfaces were sterilized by dipping the leaves into 70% ethanol and 1% sodium hypochlorite and then rinsing them in sterilized distilled water that had been demineralized. The washed leaves were cut to about 0.5 cm^2 and placed in Petri dishes containing sterile potato dextrose agar (PDA) and 200 g of chloramphenicol. The Petri dishes were incubated at room temperature for approximately 1-to-3 weeks. The mycelium derived from the samples was purified and cultured under the same conditions.

2.5. Molecular identification.

The molecular identification of the region of difference 4 (RD4) involved universal primers, namely, the internal transcribed spacers 1 (primary forward) and 4 (primary reverse). Forward and reverse DNA sequences were determined using the BioEdit program. Also, the DNA sequences in the analysis of intraspecies were similar to the data in the NCBI GenBank using the basic local alignment search tool. The sequences were aligned by using the CLUSTALW method in the MEGA program. Using the neighbor-joining method, a phylogenetic tree was constructed with a 1,000 bootstrap value [11].

2.6. Cultivation and extraction of endophytic fungi.

The endophytic fungal culture on the PDA media was cut ±6 mm in diameter and then transferred to a potato dextrose broth (PDB) medium of 150 mL. After incubation (30 days), the fermented broth was filtered with a vacuum. The PDB culture was then partitioned with ethyl acetate (150 mL × 3). The ethyl acetate extract from the PDB culture was concentrated using a vacuum evaporator at 40°C. The dense crude extract was then stocked at 4°C to test biological activity and the isolation of bioactive compounds [12].

2.7. Antibacterial activity test using the Kirby-Bauer method.

The test of the extract's minimum inhibitory concentration (MIC) was conducted by dissolving the extract in ethanol with concentrations (weight/volume) of 256, 128, 64, 32, 16, 8, and 4 μg/mL. Positive antibacterial activity was determined by diameters of the inhibition zone >9 mm in a series of concentrations. The lowest concentration of positive antibacterial

activity was determined as the MIC value. The MIC value of pure compounds <100 μg/mL is categorized as "good antibacterial" activity [13, 14].

2.8. Antioxidant activity test using the DPPH method.

A solution was prepared to contain 0.05 mM of 2,2-diphenyl-1-picrylhydrazyl in methanol, and this was mixed using 3.8 mL with 0.2 mL of the test sample in methanol at the prepared concentration series of 400, 200, 100, 50, 25, 12.5, 6.75, and 0 g/mL. The blended reaction was thoroughly vortexed and left in the dark for 30 minutes. The mixture absorbance was measured with a spectrophotometer at 517 nm. The absorbance standard used ascorbic acid. The percentage of radical scavenging activity was adjusted by the following formula [15]:

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

A_k = Absorbance of control

A_s = Absorbance of samples

2.9. Isolation and identification of chemical compounds.

The ethyl acetate extract of RD4 isolate was analyzed by thin-layer chromatography (TLC) using eluents, and silica gel as a stationary phase with a ratio of 1:10 was used for column chromatography. Prepared samples using pre-absorption were distributed into the column chromatography and eluted through an increasing polarity of an eluent. The eluate was collected in 10-ml vials, and each was tested using TLC, to be grouped into column fractions. Every column fraction was evaporated using a rotary evaporator and then separated and purified by the column chromatography technique to gain pure compounds.

3. Results and Discussion

3.1. Isolation and morphological identification of endophytic fungi.

In a previous study, 12 endophytic fungi from Sungkai leaves (*P. canescens*) were found with codes RD1–RD12. Screening tests of antioxidant and antibacterial activity showed that RD4 and RD6 had strong activity [16]. This research is a follow-up study by isolating the RD4 fungus from sungkai leaves taken from different leaf positions, namely the 3rd position. Furthermore, RD4 isolates were identified morphologically and molecularly. The macroscopic characteristics of RD4 revealed that the colony's surface was cottony grayish white and pale, while the microscopic characters exhibited hyaline spores, wide ellipsoidal attached to the conidiophores. Colonies become dark gray according to age with slight aerial mycelia (Figure 1).

Figure 1. Morphological characteristic of *L. theobromae* isolated from sungkai (*P. canescens*) leaves ((**a**) front view; (**b**) reverse view; (**c**) microscopic characteristic).

3.2. Molecular identification and phylogenetic analysis.

Molecular identification revealed that the endophytic fungus (RD4) was *Lasiodiplodia theobromae* with 99.63% of similarity. The phylogenetic tree can be seen in Figure 2. The sequence of ITS rDNA sequences was as follows:

ATATGCTTTAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCAACCTTGAGAAAA
GTTCAGAAGGTTCGTCCGGCGGGCGACGCCAACCGCTCCAAAGCGAGGTGTATT
CTACTACGCTTGAGGGCTGAACAGCCACCGCCGAGGTCTTTGAGGCGCGTCCGC
AGTGAGGACGGTGCCCAATTCCAAGCAGAGCTTGAGGGTTGTAATGACGCTCGA
ACAGGCATGCCCCCCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGATTCGAT
GATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATC
GATGCCAGAACCAAGAGATCCGTTGTTGAAAGTTTTAGTTTATTAACTTGTTTAT
CAGACGTCTGCGTTTACTGACTGGAGTTTGAAGGTCCTTTGGCGGCCGGAGCCGC
CAAAGCAACAGAGGTACGTTCACAAAGGGTGGGAGAGTCGAGCCGGAGCTCGA
AAACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGAACCCTTGTT.

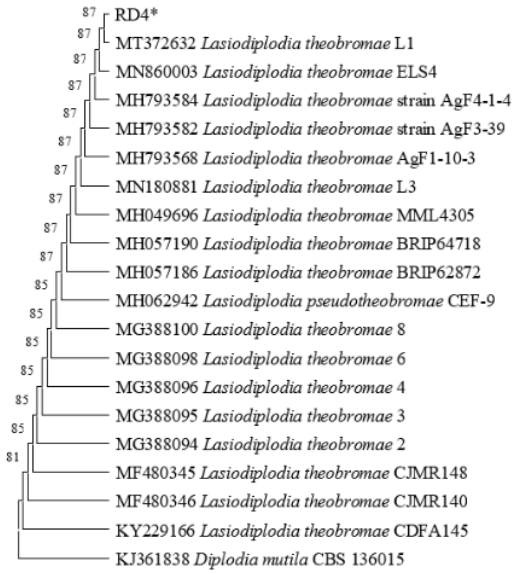


Figure 2. Phylogenetic tree of RD4* (*Lasiodiplodia theobromae*) constructed by Neighbour-Joining method with 1000 bootstrap value.

3.3. Bioactivity of endophytic fungi.

Antibacterial and antioxidant activity of EtOAc extract and pure compound from endophytic fungus *L. theobromae* compared to tetracycline and the ascorbic acid as standard can be seen in Table 1.

Table 1. MIC and IC50 values of EtOAc extract and pure compound from endophytic fungus *L. theobromae* compared with tetracycline and ascorbic acid as standard.

Sample	MIC Values (μg/mL)				IC50 (μg/mL)
	E. coli	*S. aureus*	*S. thypi*	*B. subtilis*	
EtOAc extract	128	128	32	64	12,2 ****
Compound 1	128	128	128	128	> 500 *
Compound 2	32	64	32	32	20,9 ****
Tetracycline[a]	4	4	4	4	
Ascorbic Acid[b]					10,1 ****

[a]Antibacterial positive control; [b]Antioxidan positive control; antioxidant activity IC50 (μg/mL): ****very strong < 20 μg/mL ***strong < 100 μg/mL; **moderat 100-500 μg/mL; * weak > 500 μg/mL

Table 1 reveals the antibacterial and antioxidant activities of the ethyl acetate extract of the endophytic fungus *L. theobromae* and the two compounds isolated from the ethyl acetate extract. The antibacterial test revealed that compound 1 had moderate activity for all test bacteria (MIC = 128 μg/mL), but the antioxidant was in the weak category (inactive) (IC50>500 μg/mL). In contrast to compound 2, it exhibited strong antibacterial activity (MIC 64 μg/mL) and antioxidant (IC50 20.9 μg/mL) with a very strong category.

3.4. Compound isolation and identification.

The EtOAc extract of the endophytic fungus RD4 (2 g) was isolated by a chromatography column using silica gel G60 as a stationary condition.

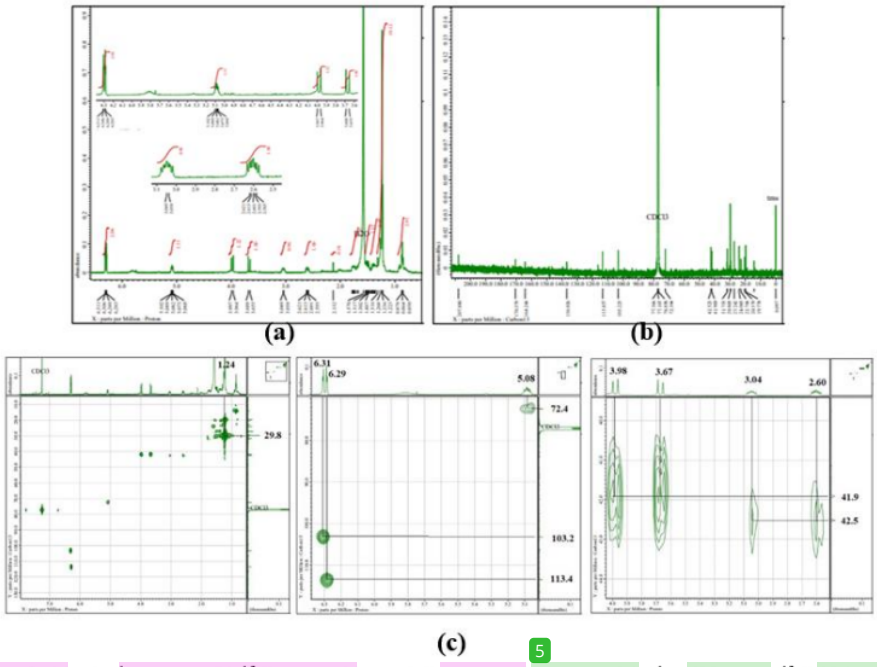


Figure 3. (**a**) The ^{1}H-NMR; (**b**) ^{13}C-NMR; (**c**) HMQC spectra of compound 1 (^{1}H-500 MHz; ^{13}C-125 MHz in $CDCl_3$).

Elution was carried out stepwise with n-hexane:EtOAc (10:0 → 0:10) and EtOAc-MeOH (10:0→8:2) as the mobile phase to produce six subfractions (F1-F6). Subfraction F3 (433 mg) showed purple stains on the TLC plate, so it was purified by column rechromatography and eluted stepwise with n-hexane:EtOAc (10:0→5:5), resulting in four subfractions (F3.1-F3.4). Subfraction F3.2 was precipitated to obtain compound 1 (32 mg). Subfraction F5 (527 mg) showed a potential purple stain on the TLC plate, so it was purified by column rechromatography and eluted stepwise with n-hexane:EtOAc (6:4→0:10), resulting in three subfractions (F5.1-F5.3). Subfraction F5.3 was precipitated until compound 2 (64 mg) was obtained. The results of the antibacterial and antioxidant activity tests of pure compounds are listed in Table 1.

The NMR spectra of compound 1 (Figure 3) showed a slight impurity by long-chain alkanes so that it was identified in the H and C methyl groups but did not interfere with the identification of compound 1. In Figure 1A, there were eight proton signals indicating the presence of 2 vinylic proton signals at δ_H 6.31 (1H, m) and 6.29 ppm (1H, d. J=8.5 Hz), a signal of the proton on oxygenated carbon at δ_H 5.08 ppm (1H, m), four sp3 proton signals with the integration of one at δ_H 3.98 (1H, d. J =16.5 Hz); 3.67 (1H, d. J=16.5 Hz); 3.98 (1H, d. J=16.5 Hz); and 3.67 ppm (1H, d. J=16.5 Hz), and a signal of methyl proton at δ_H 1.24 (3H, d. J=6.5 Hz). The compound 1 spectrum of ^{13}C-NMR (Figure 2B) exhibited the presence of 10 carbon signals consisting of six sp2 carbon signals at δ_C > 100 ppm (δ_C 207.9; 170.6; 136.9; 116.3; 113.4; and 103.2 ppm), a carbon oxygenation signal (δ_C 72.4 ppm), and three other sp3 carbon signals (δ_C 42.5; 41.9; and 29.8 ppm). The HMQC spectrum (Figure 1C) showed five ^{1}H-^{13}C correlations through one bond. There are two vinylic methine signal correlations, a methyl oxygenation signal correlation, two methylene signal correlations, and a methyl signal correlation. Thus, compound 1 was a non-aromatic compound having a ketone carbonyl and an ester carbonyl present in the lowest field, two vinylic groups, an oxygenated methine group, two methylene groups, and a methyl group.

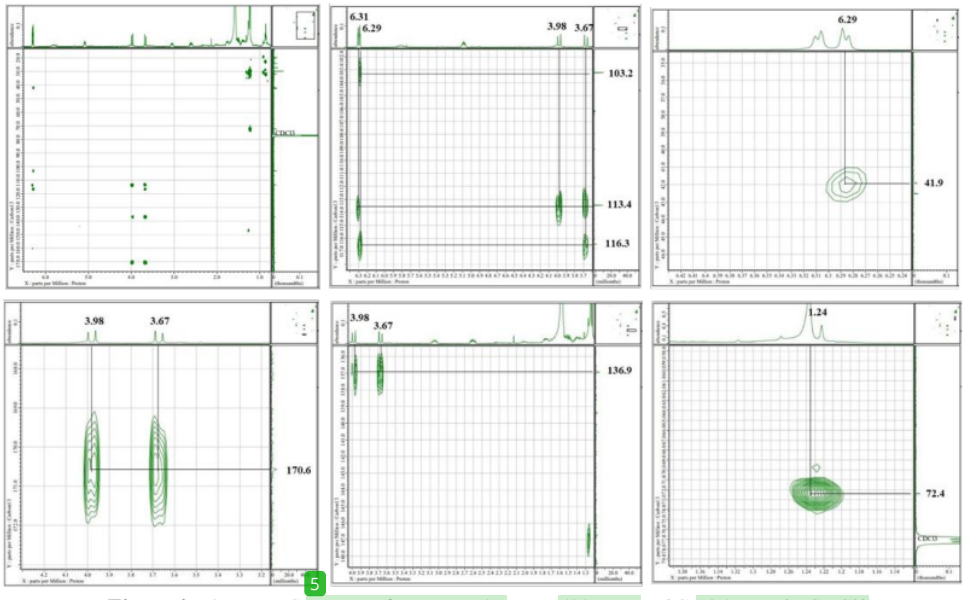


Figure 4. The HMBC spectra of compound 1 (1H-500 MHz; 13C-125 MHz in CDCl3).

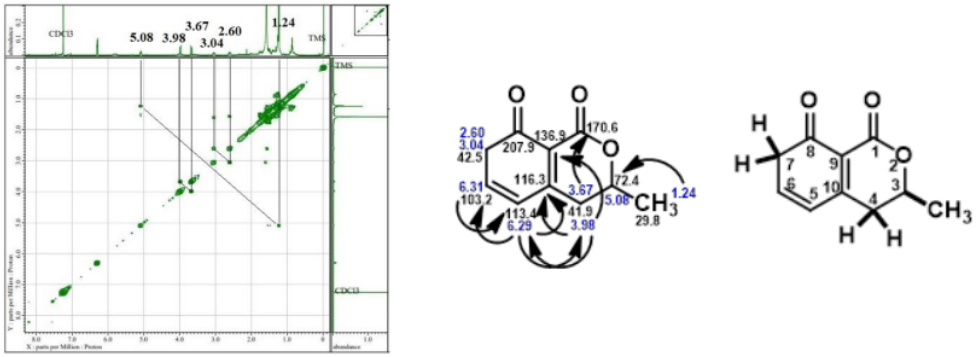
The spectrum of HMBC (Figure 4) exhibited the correlation of ^{1}H-^{13}C throughout two or three bonds. The signal of the vinyl proton at δ_H 6.31 ppm correlated two bonds with another vinyl carbon atom at δ_C 113.4 ppm. The second vinyl proton at δ_H 6.29 ppm correlated two bonds with the carbon atom at δ_C 103.2; 116.3 ppm, and three-bond correlated with the methylene carbon atom at δ_C 41.9 ppm. The two methylene protons with different chemical shifts at H 3.98 and 3.67 ppm correlated to the same four carbon atoms at δ_C 136.9; 170.6; 113.4; 116.3 ppm. The correlation of the four bonds of the methylene proton to the carbonyl ester carbon was due to the long-range coupling by the carbon-carbon double bond.

Furthermore, the correlation between methyl protons at δ_H 1.24 ppm with oxygenated carbon at δ_C 72.4 ppm was also seen. The correlation exhibited that the two vinyl groups were located next to each other in the same ring. The methylene group was in the second ring, which contains a cyclic ester group, evidenced by the presence of an oxygenated methine group attached to a methyl group. The carbonyl carbon of the ketone and another methylene group were placed in the first ring.

Table 2. Data of compound 1 in the NMR, recorded at ^{1}H-500 MHz; ^{13}C-125 MHz in $CDCl_3$.

No	δ_C ppm	Type of C	δ_H ppm (ΣH. Multiplicity (Hz))	HMBC	COSY
1	170.6	C			
3	72.4	CH	5.08 (1H, m)		1.24
3-CH_3	29.8	CH_3	1.24 (3H, d. J=6.5 Hz)	72.4	5.08
4	41.9	CH_2	3.98 (1H, d. J=16.5 Hz) 3.67 (1H, d. J=16.5 Hz)	136.9; 170.6; 113.4; 116.3 136.9; 170.6; 113.4; 116.3	3.67 3.98
5	113.4	CH	6.29 (1H, d. J=8.5 Hz)	41.9; 103.2; 116.3	
6	103.2	CH	6.31 (1H, m)	113.4	
7	42.5	CH_2	3.04 (1H, m) 2.60 (1H, m)		2.60 3.04
8	207.9	C			
9	136.9	C			
10	116.3	C			

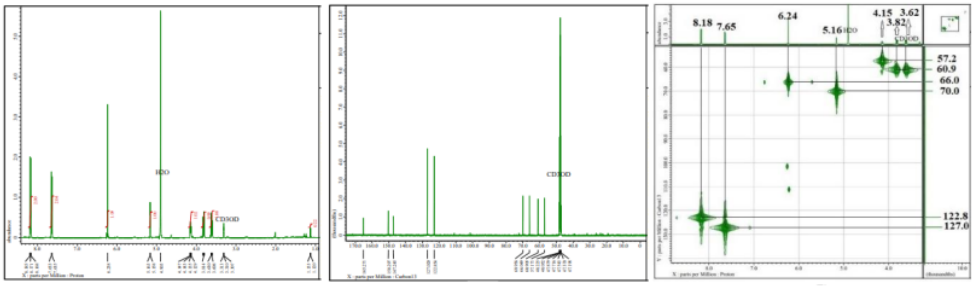
The COSY spectrum (Figure 5A) revealed a ^{1}H-^{1}H correlation through two or three bonds. There were three ^{1}H-^{1}H correlations through two bonds, namely the correlation between methylene protons bonded to the same carbon but with different chemical shifts. In addition, there was also the correlation of methyl protons with oxygenated methylene protons through three bonds. The spectral data of 1D and 2D NMR for compound 1 are shown in Table 1.



(a) **(b)** **(c)**

Figure 5. (a) The COSY spectra; (b) the HMBC correlation; (c) the COSY correlation of compound 1 as 3-methyl-3,4-dihydro-1H-isochromene-1,8(7H)-dione

According to the spectral analysis of ^{1}H-NMR, ^{13}C-NMR, HMQC, HMBC, and COSY, it could be explained that compound 1 had two rings as a cyclic ketone ring and a cyclic ester with two vinyl methine groups in the first ring and a methyl group in the second ring. Both rings contained a methylene group. In addition, compound 1 had two sp2 quaternary carbons. The molecular formula of compound 1 was $C_{10}H_9O_3$, with the equivalent double bond is 5. Hence, the chemical structure of compound 1 was proposed as 3-methyl-3,4-dihydro-1H-isochromene-1,8(7H)-dione, shown in Figure 5C.



(a) (b) (c)

Figure 6. (**a**) The ^{1}H-NMR; (**b**) ^{13}C-NMR; (**c**) HMQC spectrum of compound 2 (^{1}H-500 MHz; ^{13}C-125 MHz in CD_3OD).

The NMR spectrum of compound 2 (Figure 6A) exhibited the existence of seven proton signals. There were two proton signals in the H aromatic region at δ_H 7.50-8.20 ppm, each of which had two integrations, doublet cleavage with a constant coupling of 8.5 (ortho). These two signals indicated that compound 2 is an aromatic compound substituted at the para position. Thus, the ortho and meta proton positions are equivalent. Five other signals appeared at δ_H 3.50-6.30 ppm, indicating the presence of methine protons and protons on oxygenated carbon. The ^{13}C-NMR spectra of compound 2 in (Figure 6B) revealed the existence of nine carbon signals. Five sp^2 carbon signals appeared at C > 100 ppm consisting of two carbon signals in the low field, namely at δ_C 165.3 ppm as carbonyl ester carbon and δ_C 150.3 as oxyaryl carbon. Subsequently, three oxygenated sp^3 carbon signals appeared at δ_C 60.0-70.0 ppm and a methine sp^3 carbon signal at δ_C 57.2 ppm. The HMQC spectrum (Figure 1C) showed seven ^{1}H-^{13}C correlations through one bond. There were two aromatic proton signal correlations, four proton signal correlations on oxygenated carbon, two methine proton signal correlations, and two methylene proton correlations with different chemical shifts to the same carbon atom. In addition, there was a correlation between an sp^3 methine proton to a tertiary carbon atom. This correlation indicated that the aromatic ring substituent of compound 2 consists of a hydroxyl group and a cyclic ester group that binds to a hydroxyl group.

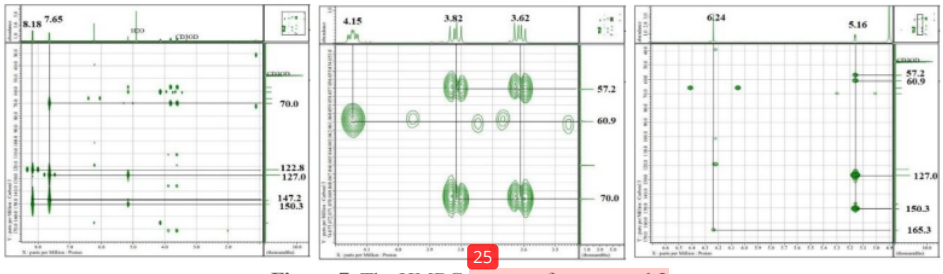


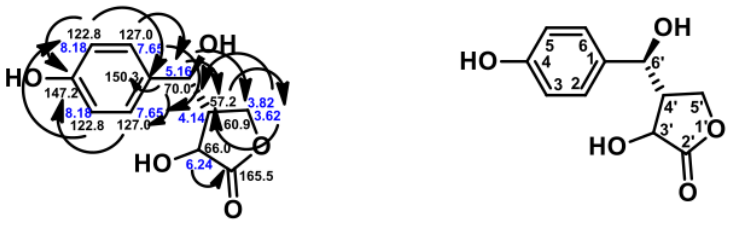
Figure 7. The HMBC spectra of compound 2.

The HMBC spectra of compound 2 (Figure 7) exhibited the correlation of seven protons to the carbon atom through two or three bonds. The two pairs of equivalent aromatic protons appeared to be correlated with each other to their equivalent carbon atoms. In addition, a pair of aromatic protons equivalent to δ_H 7.65 ppm was seen to be correlated with the oxygenated carbon atom at δ_C 70.0 ppm, which was part of the para substituent. This indicated that the oxygenated-methine carbon atom was precisely bonded to the benzene ring. Furthermore, it can be seen that the oxygenated methine carbon atom (δ_C 70.0 ppm) also correlates with two aromatic carbons δ_C 127.0; 150.3 ppm and also correlates with a methine carbon at δ_C 57.2, an oxygenated methylene carbon at δ_C 60.9 ppm, and vinyl proton signal at δ_H 6.31 ppm. In addition, the correlation of an oxygenated methine proton (δ_C 66.0 ppm) with the carbonyl ester carbon indicated the existence of a hydroxyl group adjoining the ester carbonyl group. The spectrum of 1D and 2D NMR data for compound 2 are demonstrated in Table 3. The HMBC correlation of compound 2 is shown in Figure 8.

Tabel 3. The NMR data of compound 2, recorded at ^{1}H-500 MHz; ^{13}C-125 MHz in $CDCl_3$.

No	δ_C ppm	Type of C	δ_H ppm (ΣH. Multiplicity, Hz)	HMBC
1	150.3	C		
2	127.0	CH	7.65 (1H, d, J= 9)	70.0; 127.0; 147.2
3	122.8	CH	8.18 (1H, d, J= 9)	122.8; 147.2; 150.3
4	147.2	C		
5	122.8	CH	8.18 (1H, d, J= 9)	122.8; 147.2; 150.3
6	127.0	CH	7.65 (1H, d, J= 9)	70.0; 127.0; 147.2
2'	165.5	C		
3'	66.0	CH	6.24 (1H, s)	165.5
4'	57.2	CH	4.15 (1H, m)	60.9
5'	60.9	CH_2	3.82 (1H, m) 3.62 (1H, m)	57.2; 70.0 57.2; 70.0
6'	70.0	CH	5.16 (1H, d, J= 2.5)	57.2; 60.9; 127.0; 150.3

Based on 1D and 2D NMR spectrum analysis, compound 2 was a phenolic compound with a para-substituted aromatic ring skeleton with 3-hydroxy-4-(hydroxymethyl)-ɣ-butyrolactone substituent. Thus, the chemical structure of compound 2 was proposed as 3-hydroxy-4(hydroxy(4-hydroxyphenyl)methyl)-ɣ-butyrolactone shown in Figure 4.



(a) **(b)**

Figure 8. (a) The HMBC correlation of compound 2; (b) the structure of compound 2 as 3-hydroxy-4(hydroxy(4-hydroxyphenyl)methyl)-ɣ-butyrolactone.

3.5. Discussion.

The endophytic fungi *Lasiodiplodia theobromae* was first isolated on sungkai leaves. The results of the literature study revealed that the endophytic fungus *L. theobromae* has been found to be from the Botryosphaeriaceae family, which is known as a fungal pathogen in tropical plants [17, 19]. However, research has revealed that this fungus can spread as plant-associated endophytic microorganisms, and there is no reported evidence of host specificity for *L. theobromae* [20,21]. Most of the endophytic fungi isolated from plants, especially medicinal

plants, are known to have good bioactivity. Endophytic fungi are able to synthesize the bioactive compounds used by host plants for defense against pathogens, and some of these compounds have been shown to be useful as new medicinal resources [10, 22, 23]. Several studies reveal that *L. theobromae*, isolated from medicinal plants, can synthesize large numbers of bioactive compounds that act as antimicrobials, antioxidants, and anti-inflammatories [18], [24 – 26]. *L. theobromae* contained cyclohexenes, cyclohexenones, indoles, lactones, jasmonates, mellein, and phenol compounds. These secondary metabolites, especially lactone, and phenol have antibacterial and antioxidant activities [25], [27 – 29].

The ethyl acetate extract of the endophytic fungi *L. theobromae* that is associated with sungkai leaves showed good antibacterial activity for the test bacteria *S. thypi* and *B. subtilis* (MIC <100 μg/mL) and moderate activity against the test bacteria *E. coli* and *S. aureus* (MIC = 128 μg/mL). The antioxidant activity of the ethyl acetate extract was in a very strong category, with an inhibitory concentration value of 50% (IC50), which was almost the same as ascorbic acid as a standard antioxidant. The good bioactivities of this extract were related to the secondary metabolites that it contained. Two secondary metabolites were found in this study, namely, a new compound, 3-methyl-3,4-dihydro-1H-isochromene-1,8(7H)-dione (compound 1), and a known compound, 3-hydroxy-4(hydroxy (4-hydroxyphenyl) methyl)-γ-butyrolactone (compound 2) that has been previously identified [30].

Compound 1 revealed moderate antibacterial activity, but its antioxidant activity was in the weak/inactive category (IC50 > 500 μg/mL). The antibacterial activity of compound 1 was caused by the presence of the lactone and carbonyl groups. The lactone group with the oleyl chain was important for increasing antibacterial activity [31]. Lactone containing this side chain is able to show significant antibacterial activity. The carbonyl group also shows activity as an antibacterial. Monoterpenoids belong to a carbonyl group that has antimicrobial activity, and the enantiomeric form and camphor monoterpenoids greatly affect the antimicrobial activity [32 – 34]. The inactivity of compound 1 as an antioxidant resulted from the absence of a hydroxyl group, so there were no protons that free radicals could abstract. Studies explain that the removal of hydroxyl groups can reduce coplanarity, which can reduce the capability of compounds to free radical scavenging [35, 36]. Substituting the hydroxyl group at its position (C3) with a glyxosyl or methyl group can eliminate the quercetin activity as an antioxidant [37 – 40]. Also, phenolic acids that contain the number of hydroxyl groups devoted to the aromatic ring are not significantly different in their antioxidant agents. The 4-hydroxy-3-methoxy benzoic acid has greater antioxidant properties than the 3-hydroxy-4-methoxy benzoic acid [37], [41], [42]. This indicates that the presence and position of a hydroxyl group significantly affect the antioxidant properties of a compound.

Compound 2 exhibited good antibacterial activity against all test bacteria and strong antioxidant activity (IC50 = 20.9 μg/mL). Studies have found that the hydroxyl group at a particular position on the aromatic ring enhances the antibacterial effect. One hydroxyl group on ring A (especially at C7) is essential for the antibacterial activity of flavonoids [43 – 45]. Nevertheless, studies reveal that the number of hydroxyl groups on the two aromatic rings can reduce the antibacterial effect [46 – 50]. The findings of this study indicate that the position of the hydroxyl group can affect the antibacterial activity. The strong antioxidant activity of compound 2 was caused by the presence of several hydroxyl groups, so the proton abstraction of compound 2 by free radicals produced new stable free radicals through the distribution and delocalization of free radicals in the molecule. The antioxidant activity of compound 2 was included in the strong category but was still less than the antioxidant activity of the ethyl acetate

extract. This might be caused by the synergy between the compounds in the extract, thereby increasing its antioxidant properties. Another possibility is that other antioxidant compounds had not been isolated in the extract. Compound 1 is not acting as an antioxidant because it does not have an OH group. The antioxidant activity of compound 1 can be increased by modifying the structure, namely the addition of the OH group. As an illustration, the easiest position for the entry of the OH group is at the C-5 and C-6 alkene groups through the pi-bond oxidation reaction without breaking the sigma bond. The reagent used is potassium permanganate under alkaline and cold condition. Compound 2 has had strong antioxidant activity. However, if the structure is modified by adding OH groups at positions C-3 and C5, the antioxidant activity of compound 2 will be comparable to the positive control of ascorbic acid. The reaction begins with the substitution of electrophilic nitration (HNO_3/H_2SO_4) followed by reducing the nitro group using Fe/HCl. The reaction is continued with the addition of $NaNO_2$/HCl to form a diazonium salt which readily forms an OH group by reaction with a hot (in water) acid solution. The description of the modified structure of compounds 1 and 2 with side-by-side OH groups will have high antioxidant activity when free radicals abstract hydroxyl protons. Free radicals on the O atom can be stabilized through electron distribution and delocalization which can ultimately produce neutral compounds with the formation of a diketo group. Thus, compounds 1 and 2 can be starting compounds as candidates for new drug ingredients [51].

4. Conclusions

The endophytic fungi *Lasiodiplodia theobromae* that is found on sungkai leaves produced 3-methyl-3,4-dihydro-1H-isochromene-1,8(7H)-dione (compound 1) and 3-hydroxy-4(hydroxy(4-hydroxyphenyl) methyl)-γ-butyrolactone (compound 2). The antibacterial and antioxidant activities of compound 1 were categorized as moderate and inactive, respectively. The antioxidant activity of compound 2 was included in the strong category but is still below the antioxidant activity of the ethyl acetate extract. For future development of the endophytic fungi *L. theobromae*, sungkai leaves as medicinal resources can be used in the form of extracts whose bioactive compounds have been identified.

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Conflicts of Interest

The authors declare no conflict of interest.

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