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Secondary metabolites of endophytic fungii isolated from the stem bark of Sungkai (*Peronema canescens* Jack.)

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ABSTRACT: Infectious diseases are a global challenge today. One preventive measure is to maintain and improve humans' immunity. Many people consume sungkai, a medicinal plant that is believed to cut down the exposure of severe covid-19 because it is related to antioxidant and antibacterial activity. This research investigated the antioxidant and antimicrobe agents of fungal endophyte extracts from the stem bark of sungkai. Endophytic fungal was isolated from the fresh tissue of the stem bark of sungkai and identified morphologically. The antioxidant of the endophytic fungi extract was tested with the DPPH, and antibacterial agent was tested by using the paper disc diffusion method. Molecular identification of endophytic fungi was done to extract that showed the most potential antioxidant and antibacterial activity. The pure compounds were isolated by chromatographic techniques. Structural determinations of the compounds were accomplished using a spectroscopic method, comprising 1D and 2D NMR. Twenty isolates of fungal endophyte were found residing in the stem bark of sungkai, specifically RB1-RB20. Isolates RB4 and RB6 showed the most potential antioxidant and antibacterial activity and were identified molecularly. The results of molecular identification showed that RB4 and RB6 were *Curvularia intermedia* and *Colletotrichum cliviicola*. Based on the spectroscopic analysis, the compounds identified from the two fungi were the different compound, specifically 3-hydroxy-4(hydroxy(4-hydroxyphenyl)methyl)-γ-butyrolactone (1) and 5-hydroxy-4-(hydroxymethyl)-2H-pyran-2-on (2). Compound 1 and 2 showed antibacterial and antioxidant in strong to moderate. These compounds and the endophytic fungi extract can be used as a new ingredient for medicine because it has antioxidant and antibacterial activity with further research.

KEYWORDS: Antibacterial; antioxidant; endophytic fungi; secondary metabolites; sungkai (*Peronema canescens*).

1. INTRODUCTION

Covid-19, the infectious disease caused by the novel coronavirus, is a global challenge today. Since being announced a pandemic, covid-19 has surged in many countries around the world [1–3]. Because of the current covid-19 resulting from ineffective prevention and the appearance of new variants of the virus, immunity is the most important way to prevent its spread. This can be achieved by consuming substances containing antioxidants and antibacterials [4].

Antioxidants and antibacterials can increase immunity to inhibit or diminish the risk of numerous 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 to antigens. 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 enable immune cells to communicate, and play an important role in the

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initiation, maintenance, and suppression of immune responses. Chemokines (IL-8) are cytokines that can indice leukocyte chemotaxis (immune cells) sof that immune cells can approach or respond to antigens (positive chemotaxis) [5–10]. A healthy immune system causes the body to be better prepared to respond to antigens [11,12]. Almost all Covid-19 sufferers in Indonesia consume Sungkai plants to strengthen their immunity during this disease crisis. There are limitations in the cultivation of sungkai plants in Indonesia, so in this study the potential sources of immunostimulants from endophytic fungi were explored.

Immunostimulant is a compound that can be used to enhance the body's defense mechanism either specifically or non-specifically [13,14]. Immunostimulants can come from natural sources or can be chemically synthesized. These substances act as boosters or immune boosters which can be obtained by using medicinal plants which are believed by the public to be able to cure diseases [15].

Disease occurs due to the presence of antigens [foreign substances such as fungi, bacteria, or viruses], freeradicals reaction in the body, and inflammation of tissue [16,17]. Antioxidants and antibacterials are naturally obtained from foods and plants. Many people do not know that these contain antioxidants and antibacterials, so they buy supplements and antibiotics [18–20]. Sungkai (*Peronema canescens*) is a medicinal plant that is believed, traditionally used by the people of South Sumatra, Indonesia, to be a fever medicine that can reduce the risk of severe covid-19 if water boiled with parts of this plant, including the stem bark, is consumed.

Sungkai contains metabolites, such as terpenoids, alkaloids, flavonoids, anthraquinone, quercetin, polyphenols, betulinic acid, and stigmasterol [19]. Studies have revealed that these have bioactivity as antibacterials and antioxidants [20–23]. Due to the potency of its bioactive compounds, parts of this plant such as the stem bark, can be used in traditional medicine to support the immune system. The use of these raw materials is increasing, along with the high demand for traditional medicines that are believed by many to have no side effects. This decreases resource availability [24,25]. Therefore, other alternatives are needed that can preserve nature but do not reduce the role of natural bioactive compounds in supporting public health, specifically the use of endophytic fungi.

Endophytic fungi are capable to produce different or identical compounds from their host plants because they can copy and modify the compounds from their host plants via the coevolution process [26–28]. Extraction and isolation of compounds of fungal endophytes is efficient because of the short cultivation time [29,30]. The results of the researches describe that metabolites of fungal endophytes isolated from medicinal plants have shown that most of their secondary metabolites have unique chemical structures and they can act as antibacterials [32-33], antioxidants [34-36], antifungals [37–39], anticancers [40], antidiabetics, antihyperlipidemics [41], antimalarials [43], antihypertensives [44], and antihypercholesterolemics [45,46]. Studies on endophytic fungi isolated from sungkai plant parts have also been reported, such as the leaves and roots. Compound 3-(2,6-dihydroxyphenyl)-2-hydroxyacrylic acid isolated from the endophytic fungus *P. oxalicum* from *P. canescens* leaves has strong antibacterial activity with MIC values of 31.25 μg/mL and 62.5 μg/mL against *E. coli*, *B. subtilis*, and *S. aureus*, and also a strong antioxidant with IC50 = 31.33 μg/mL [36]. Furthermore, the compound 3-hydroxy-4(hydroxy(4-hydroxyphenyl)methyl)-γ-butyrolactone isolated from the endophytic fungus *Lasiodiplodia theobromae* from *P. canescens* leaves showed strong antibacterial activity with MIC < 64 μg/mL and very strong antioxidant with IC50 = 20.9 μg/mL. From the same endophytic fungus, 3-methyl-3,4-dihydro-1H-isochromene-1,8(7H)-dione was also produced which showed strong antibacterial activity with MIC = 128 μg/mL) and was not active as an antioxidant [42]. Then, the endophytic fungus *Penicillium janczewskii* isolated from the root bark of sungkai (*P. canescens*) showed strong antioxidant activity (IC50 = 21.02 μg/mL) and very strong antibacterial whose chemical compounds have not been reported [31]. Based on this research, endophytic fungi isolated from the bark of Sungkai stems are thought to show antioxidant and antibacterial activity.

2. RESULTS

2.1 Isolation andIIdentification of Endophytic Fungiifrom Stem Bark of Sungkai

The results of endophytic fungi residing in the stem bark of Sungkai revealed 20 isolates (codes RB1 to RB20). They varied in microscopic and macroscopic characteristics (Figure 1 and Figure 2). the colonies colour that appeared were predominantly white, gray, and black, while some were yellow and pink. These characteristics of endophytic fungi can be seen in Table 1 and Table 2.

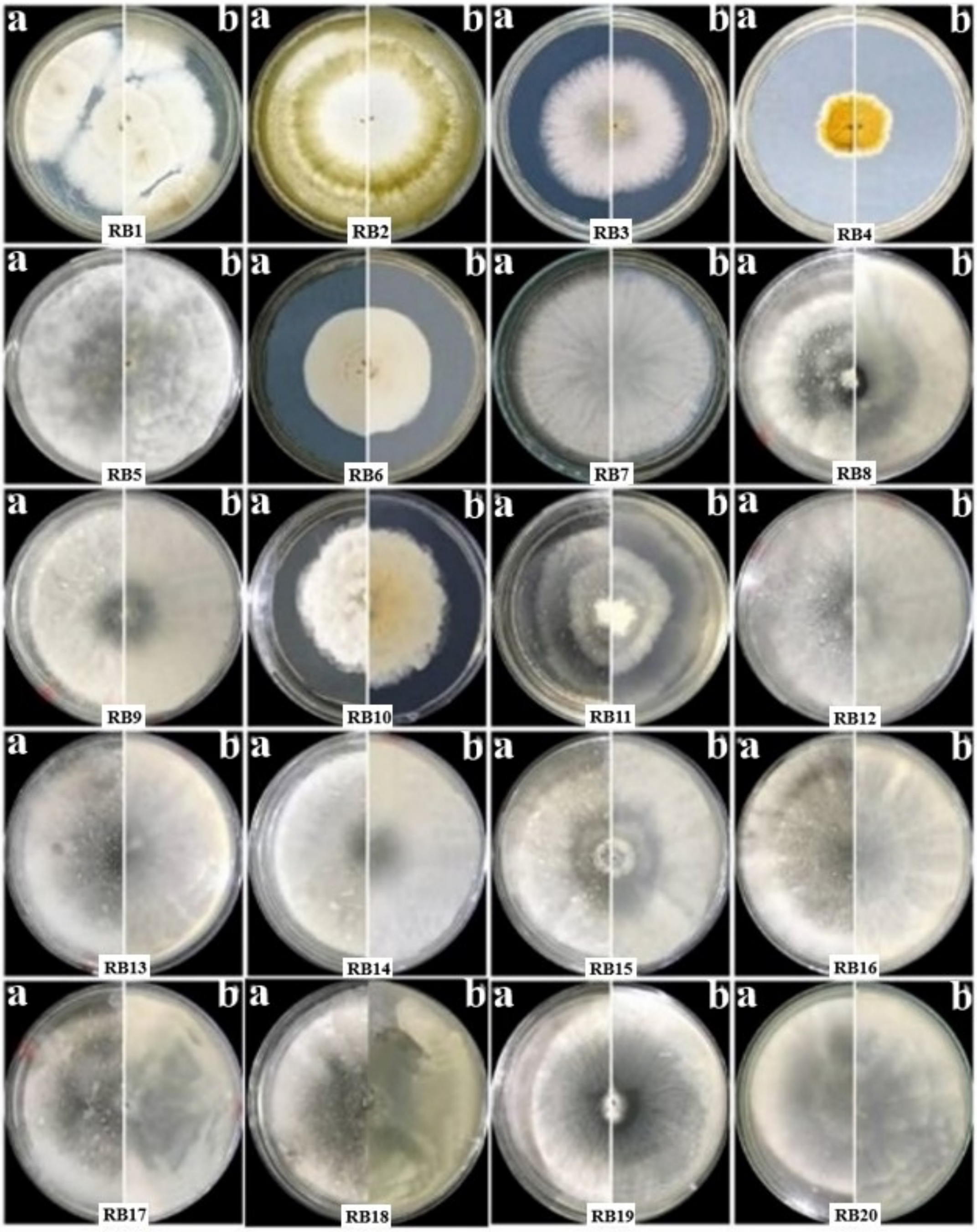

Figure 1. Macroscopic characteristic of fungal endophytes isolated from stem bark of sungkai seven days old in PDA medium (Surface view (a); Reverse view (b))

Table 1 and Table 2 reveal the morphologically of the endophytic fungi residing in the stem bark of sungkai. The genus of endophytic fungal found in the stem bark was Pythium (10 isolates: RB5, RB9, RB10, RB12, RB13, RB14, RB15, RB16, RB17, RB18,), Trichoderma (2 isolates: RB3, RB7), Mortierella (2 isolates: RB19, RB20), and 1 of each isolate for Genus Cladosporium (RB1), Gliocladium (RB2), Colletotrichum (RB6), Curvularia (RB4), Plectospira (RB8), and Alternaria (RB11). Based on these characteristics (macroscopic and microscopic) that showed, 20 isolates from the stem bark of sungkai were identified.

Table 1. Colonies characteristic of fungal endophytes isolated from stem bark of sungkai seven days old in PDA medium

Isolates	Surface of colony	Reverse of colony	Structure	Elevation	Pattern	Exudate Drops	Radial line	Concentric circle
RB 1	White	White	Cottony	Rugose	flowery	-	-	-
RB 2	white yellow	white yellow	Cottony	Rugose	Radiate	-	√	-
RB 3	white with yellowish green	white with yellowish green	Cottony	Rugose	Radiate	-	√	-
RB4	Yellow	Yellow	Yellow	Umbonate	Radiate	-	√	-
RB5	white yellow	white yellow	Cottony	Rugose	Radiate	-	√	-
RB6	white pinkish	white pinkish	white pinkish	Umbonate	Radiate	-	√	-
RB 7	white green	white green	Cottony	Rugose	Radiate	-	√	-
RB8	White	White	Cottony	Umbonate	Radiate	-	√	√
RB9	white	white	Cottony	Rugose	Radiate	-	√	-
RB10	Milky white	white yellow	velvety	Umbonate	flowery	-	-	√
RB11	White	White	Cottony	Umbonate	Zonate	-	-	√
RB12	White	White	Cottony	Umbonate	Radiate	-	√	-
RB13	White	White	Cottony	Umbonate	Radiate	-	√	-
RB14	White	White	Cottony	Umbonate	Radiate	-	√	-
RB15	White	White	Cottony	Rugose	Radiate	-	√	-
RB16	White	White	Cottony	Rugose	Radiate	-	√	-
RB17	White	White	Cottony	Rugose	Radiate	-	√	-
RB18	White	White	Cottony	Rugose	Radiate	-	√	-
RB19	gray	gray	Cottony	Rugose	Radiate	-	√	-
RB20	gray	gray	Cottony	Rugose	Radiate	-	√	-

2.2 Antibacterial and antioxidant activity of endophytic fungi extract

The extracts of fungal endophytes isolated from stem bark of sungkai using ethyl acetate showed potential as antibacterials and antioxidants (Table 3). Four extracts of endophytic fungi showed potential contrary to *E. coli*, *S. thypi*, *S. aureus*, and *B. subtilis*. Endophytic fungi extracts also revealed very strong (IC_{50} < 20 µg/mL), strong (IC_{50} < 100 µg/mL), and moderate (IC_{50} < 100-500 µg/mL) antioxidant activity.

Table 3 shows the fungal endophyte extracts isolated from the stem bark of sungkai, which constrain the growth of the bacterial test indicated by the inhibition zone. Methanol extract of the stem bark of sungkai showed strong activity against the four tested bacteria, equal to the extracts of isolates RB1, RB3, RB4, and RB6. There were some extracts of endophytic fungi isolates with moderate or even weak antibacterial activity. Moreover, most of the endophytic fungi extracts also exhibited strong antioxidant activity, relate to the host plant. The IC_{50} of the endophytic fungal extract was still lower than the IC_{50} value of ascorbic acid. However, the IC_{50} values of isolates RB4 and RB6 were close to the IC_{50} of antioxidant standard, 11.426 µg/mL and 15.338 µg/mL.

Table 2. Endophytic fungi isolated from stem bark of sungkai Microscopically

Isolates	Spore	Shape of spore	Hyphae	Characteristics	Result of identification
RB1	sporangia	cylindrical	Septate	Conidiophores pale brown, conidia blastosporrous, hyaline pale brown, cylindrical.	*Cladosporium cladosporiodes*
RB2	sporangia	Globose	Coenocytic	Conidiophores hyaline, especially in metula, conidia pale green, subglobose, chlamydospores globose.	*Gliocladium virens*
RB3	sporangia	Subglobose	Septate	Conidiophores hyaline, branched, pale green, subglobose	*Trichoderma pseudokoningi*

Isolates	Spore	Shape of spore	Hyphae	Characteristics	Result of identification
RB4	Conidia	Subglobose	Septate	Conidiophores pale brown, simple, bearing conidia apically and laterally.	*Curvularia intermedia*
RB5	Conidia	Globose	Septate	Oogonia terminal, borne on thick oogoniumphores, Sporangia hypa-like, globose	*Pythium* sp.
RB6	sporangia	Globose	Septate	Conidiophores hyaline, simple, inflated globosely, pale brown to yellowish brown,	*Colletotrichum cliviicola*
RB7	sporangia	Subglobose	Coenocytic	Hyalin conidiophore, phialides verticillate, clamidospores light brown, subglobose.	*Trichoderma hamatum*
RB8	sporangia	Globose	Septate	Oogonia, Sporangia simple, branched	*Plectospira myriandra*
RB9	Conidia	Globose	Coenocytic	Oogonia terminal, borne on thick oogoniumphores, Sporangia hypa-like, globose	*Pythium* Sp.
RB10	Conidia	Globose	Septate	Oogonia terminal, borne on thick oogoniumphores, Sporangia hypa-like, globose,	*Pythium* Sp.
RB11	Conidia	Globose	Septate	Conidia catenulate, conidiophores pale brown, branched, cylindrical or spindle-shaped	*Alternaria alternata*
RB12	Conidia	Globose	Septate	Oogonia terminal, borne on thick oogoniumphores, Sporangia hypa-like, globose,	*Pythium* sp.
RB13	sporangia	Globose	Septate	Oogonia terminal, borne on thick oogoniumphores, Sporangia hypa-like, globose,	*Pythium* sp.
RB14	Conidia	Globose	Septate	Oogonia terminal, borne on thick oogoniumphores, Sporangia hypa-like, globose,	*Pythium* sp.
RB15	Conidia	Globose	Septate	Oogonia terminal, borne on thick oogoniumphores, Sporangia hypa-like, globose,	*Pythium* sp.
RB16	Conidia	Globose	Septate	Oogonia terminal, borne on thick oogoniumphores, Sporangia hypa-like, globose	*Pythium* sp.
RB17	Conidia	Globose	Septate	Sporangia hypa-like, globose, oogonia terminal	*Pythium* sp.
RB18	Conidia	Globose	Septate	Oogonia terminal, borne on thick oogoniumphores, Sporangia hypa-like, globose	*Pythium* sp.
RB19	sporangia	Globose	Septate	Oogonia terminal, borne on thick oogoniumphores, Sporangia hypa-like, globose	*Mortierella* sp.
RB20	sporangia	Globose	Septate	Sporangia terminally, Sporangia many-spored,hyaline and globose	*Mortierella* sp.

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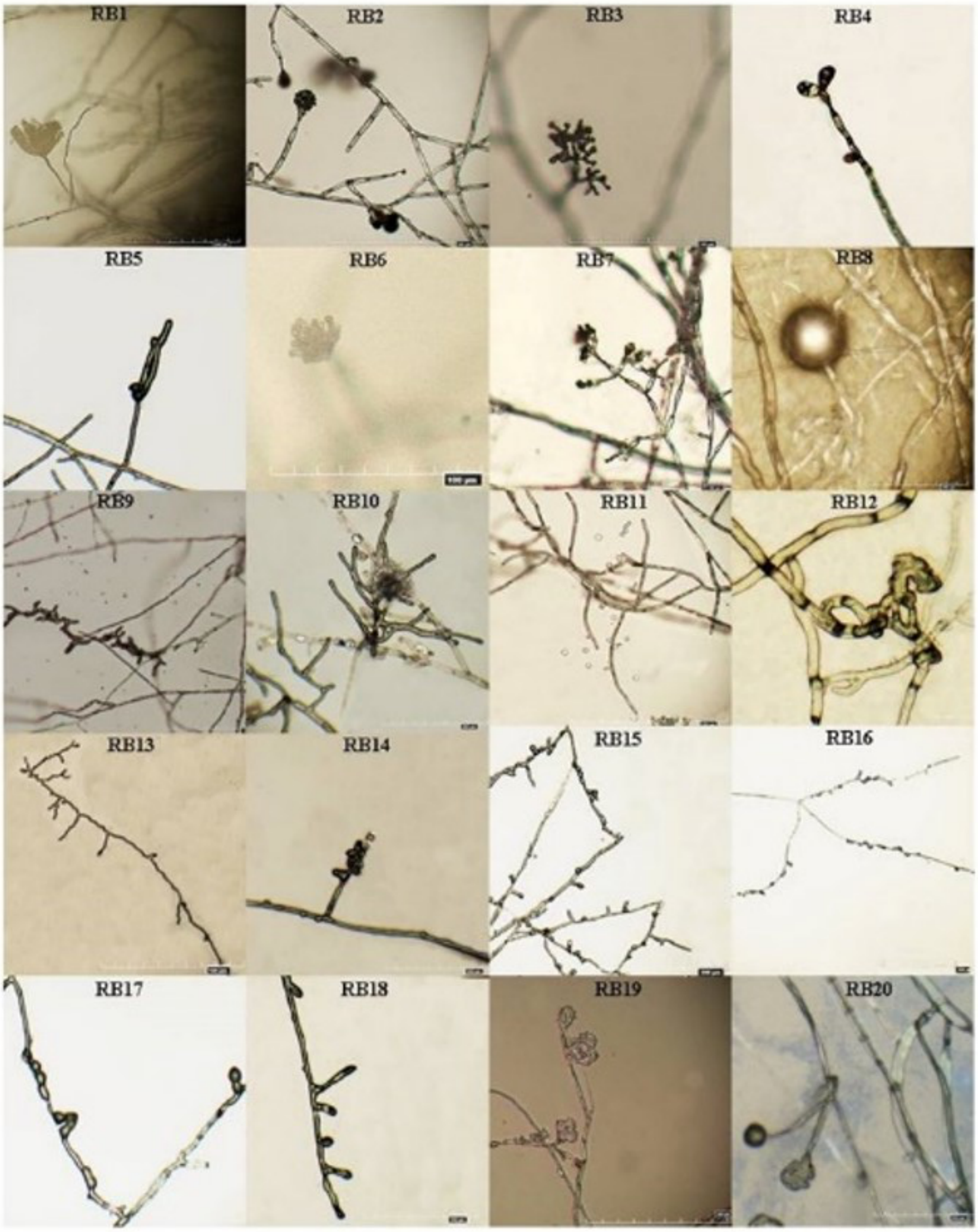

Figure 2. Microscopic appearance of endophytic fungi isolated from stem bark of sungkai (1000X magnification)

Table 3. Antibacterial activity percentage and IC_{50} endophytic fungi extract isolated from stem bark of sungkai with ascorbic acid and tetracycline as a standard

Sample	Extract	% Antibacterial Activity ± SD				Antioxidant Activity IC_{50} (μg/ml)
		E. coli	*S. aureus*	*S. thypi*	*B. Subtilis*	
Host Plant	Methanol of Sungkai Stem Bark	71,4 ± 0,51 ***	73,3 ± 0,42 ***	71,6 ± 0,54 ***	74,2 ± 0,37 ***	13,940 ****
Endophytic Fungi	RB1 [*Cladosporium cladosporiodes*]	71,8 ± 0,48 ***	78,7 ± 0,56 ***	78,4 ± 0,17 ***	98,1 ± 0,89 ***	26,548 ***
	RB2 [*Gliocladium virens*]	63,0 ± 0,16 **	76,8 ± 0,31 ***	78,1 ± 0,65 ***	62,1 ± 0,48 **	24,831 ***
	RB3	70,3 ± 0,44 ***	74,5 ± 0,31 ***	89,2 ± 0,60 ***	72,7 ± 0,39 ***	66,268 ***

[*Trichoderma pseudokoningi*]					
RB4 [*Curvularia intermedia*]	74,8 ± 0,18 ***	83,4 ± 0,50 ***	87,8 ± 0,40 ***	88,6 ± 1,68 ***	11,426 ****
RB5 [*Pythium* sp.]	62,3 ± 0,89 **	67,9 ± 0,35 **	74,9 ± 0,34 ***	78,9 ± 0,68 ***	64,877 ***
RB6 [*Colletotrichum cliviicola*]	71,3 ± 1,10 ***	76,4 ± 0,51 ***	81,1 ± 0,38 ***	72,1 ± 0,74 ***	15,338 ****
RB7 [*Trichoderma hamatum*]	62,9 ± 0,90 **	63,7 ± 0,36 **	73,8 ± 0,69 ***	80,1 ± 0,64 ***	29,506 ***
RB8 [*Plectospira Myriandra*]	61,4 ± 0,78 **	66,1 ± 1,18 **	73,7 ± 0,50 ***	69,8 ± 0,73 **	136,440 **
RB9 [*Pythium* sp.]	63,4 ± 1,18 **	71,5 ± 0,31 ***	78,6 ± 0,25 ***	81,1 ± 0,66 ***	85,815 ***
RB10 [*Pythium* sp.]	64,0 ± 0,27 **	66,5 ± 0,28 **	69,8 ± 0,15 **	71,1 ± 1,05 ***	119,462 **
RB11 [*Alternaria Alternata*]	73,3 ± 0,88 ***	65,3 ± 0,39 **	85,3 ± 0,91 ***	84,2 ± 0,27 ***	84,938 ***
RB12 [*Pythium* sp.]	59,6 ± 0,43 **	68,3 ± 0,63 **	72,2 ± 1,12 ***	70,1 ± 0,44 ***	120,605 **
RB13 [*Pythium* sp.]	57,1 ± 0,43 **	67,7 ± 0,47 **	73,6 ± 1,23 ***	66,1 ± 1,27 **	143,832 ***
RB14 [*Pythium* sp.]	52,0 ± 1,91 **	52,3 ± 0,29 **	70,6 ± 1,32 ***	64,7 ± 1,74 **	30,978 ***
RB15 [*Pythium* sp.]	44,3 ± 0,69 *	52,2 ± 1,44 **	61,9 ± 0,35 **	69,4 ± 0,46 **	66,268 ***
RB16 [*Pythium* sp.]	32,2 ± 0,25 *	39,8 ± 0,40 *	53,1 ± 0,88 **	43,2 ± 0,67 *	136,440 **
RB17 [*Pythium* sp.]	38,0 ± 0,84 *	48,7 ± 0,75 *	56,2 ± 1,30 **	50,3 ± 0,91 **	64,877 ***
RB18 [*Pythium* sp.]	47,4 ± 0,66 *	65,8 ± 0,58 **	57,2 ± 1,05 **	72,7 ± 0,72 ***	37,957 ***
RB19 [*Mortierella* sp.]	53,7 ± 0,44 **	60,1 ± 0,80 **	65,9 ± 0,62 **	67,2 ± 1,40 **	29,506 ***
RB20 [*Mortierella* sp.]	62,3 ± 0,73 **	36,2 ± 0,13 *	45,4 ± 0,63 *	37,7 ± 1,28 *	28,375 ***
Positive Control	Tetracyclin 100 ***	Tetracyclin 100 ***	Tetracyclin 100 ***	Tetracyclin 100 ***	Ascorbic Acid 10,083 ****

Note: Antibacterial activity percentage: *** strong (≥ 70%), **moderate (50-70%), and *weak (< 50%)
antioxidant activity IC50 (µg/mL): ****very strong < 20 µg/mL ***strong < 100 µg/mL; **moderate 100-500 µg/mL; * weak > 500 µg/mL

2.3 Molecular Identification of Endophytic Fungi

The isolates of RB4 and RB6 were tested for molecular identification. Both endophytic fungi isolates showed strong antioxidant and antibacterial activity compared to other extracts. The result of Molecular identification can be seen in Figure 3. The sequence of ITS rDNA isolates of RB4 was as follows:
ATATGCTTTAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCAACCTTGAGAAAAGTTCAGAAGGTT
CGTCCGGCGGGCGACGCCAACCGCTCCAAAGCGAGGTGTATTCTACTACGCTTGAGGGCTGAACAG
CCACCGCCGAGGTCTTTGAGGCGCGTCCGCAGTGAGGACGGTGCCCAATTCCAAGCAGAGCTTGAG
GGTTGTAATGACGCTCGAACAGGCATGCCCCCCGGAATACCAAGGGGCGCAATGTGCGTTCAAAGA
TTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGATGCCA
GAACCAAGAGATCCGTTGTTGAAAGTTTTAGTTTATTAACTTGTTTATCAGACGTCTGCGTTTACTGA
CTGGAGTTTGAAGGTCCTTTGGCGGCCGGAGCCGCCAAAGCAACAGAGGTACGTTCACAAAGGGTG
GGAGAGTCGAGCCGGAGCTCGAAAACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGAACCCTT
GTT

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The sequence of ITS rDNA isolates of RB6 was as follows:
TTAAGTTCAGCGGGTATCCCTACCTGATCCGAGGTCAACCTGGTTAAGATTGATGGTGTTCGCCGGC
GGGCGCCGGCCGGGCCTACAGAGCGGGTGACGAAGCCCCATACGCTCGAGGACCGGACGCGGTGC
CGCCGCTGCCTTTCGGGCCCGCCCCCCGGAAGCGGGGGGCGAGAGCCCAACACACAAGCCGTGCTT
GAGGGCAGCAATGACGCTCGGACAGGCATGCCCCCCGGAATACCAGGGGGCGCAATGTGCGTTCA
AAGACTCGATGATTCACTGAATTCTGCAATTCACATTACTTATCGCATTTCGCTGCGTTCTTCATCGA
TGCCGGAACCAAGAGATCCGTTGTTGAAAGTTTTAACTGATTTAGTCAAGTACTCAGACTGCAATCT
TCAGACAAGAGTTCGTTTGTGTGTCTTCGGCGGGCGCGGGCCCGGGGGCGGATGCCCCCCGGCGGC
CGTGAGGCGGGCCCGCCGAAGCAACAAGGTACGATAAACACGGGTGGGAGGTTGGACCCAGAGGG
CCCTCACTCGGTAATGATCCTTCCGCAGGTTCACCTACGGAAACCTT.
Based on the test, RB4 and RB6 revealed one clade with *Curvularia intermedia* and *Colletotrichum cliviicola.*

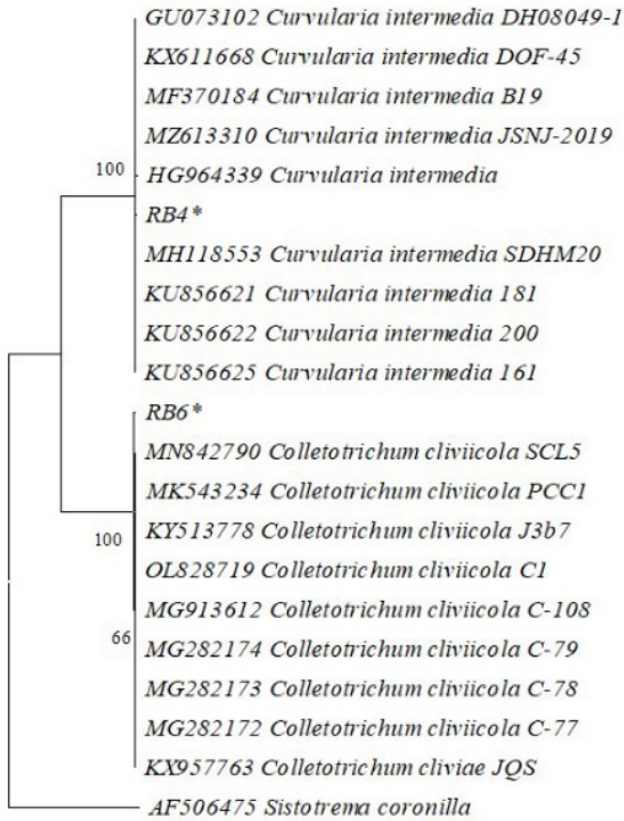

Figure 3. Phylogenetic tree from sequences data of RB4 and RB6 (signed *) constructed by using Neighbor-Joining method (bootstrap value = 1000).

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2.4 Isolation and Identification of Compound 1 and Compound 2

2.4.1 Compound 1

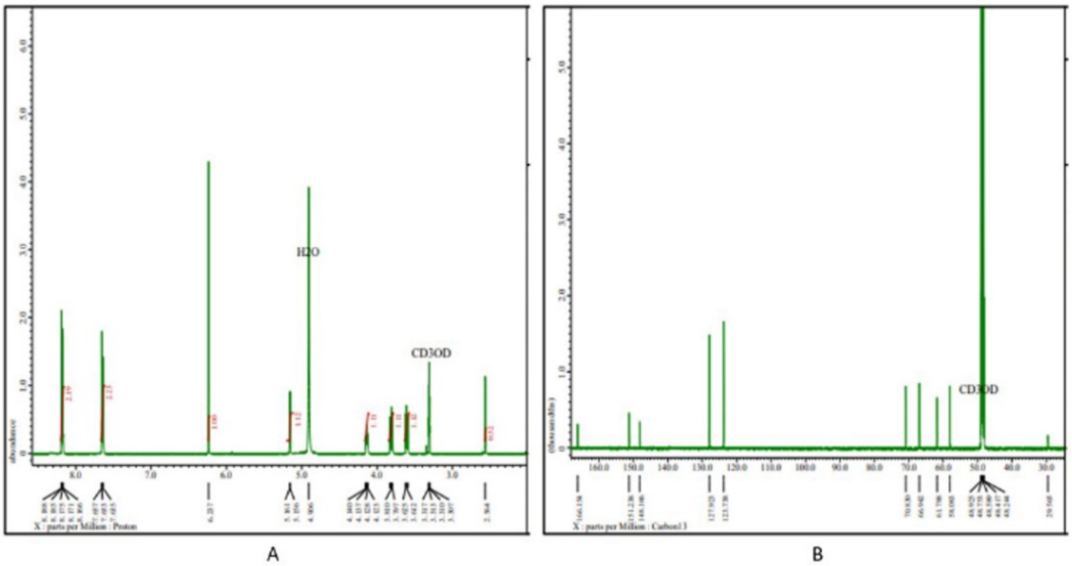

Figure 4. The ^{1}H-NMR (A) and ^{13}C-NMR (B) spectral of compound 1 (^{1}H-500 MHz; ^{13}C-125 MHz in CD_3OD)

Ethyl acetate extract of RB4 (2 g) which had been preabsorbed with silica gel in a ratio of 1:1 and column chromatography using silica gel as stationary phase with eluent graded *n*-hexane-ethyl acetate (10:0→0:10) to ethyl acetate-methanol (10:0→0:10) obtained six subfractions (F1-F6). Based on the pattern on TLC, the F4 subfraction was column chromatographed again with *n*-hexane-ethyl acetate eluent (5:5 → 0:10) and gave three subfractions (F4.1-F4.3). The subfraction F4.2 was rinsed *n*-hexane-ethyl acetate (3:7) to obtain compound 1 (48.5 mg).

The NMR spectra of compound 1 (Figure 4A) showed the appearence of seven proton signal consisting of two aromatic signals (δ_H 7.00-8.50 ppm) each had two proton integration and doublet fission (capling constant J = 9.0 Hz). Next there was a proton signal methine sp^3, and four proton signals bound to the oxygenated carbon atom (at H 3.50-6.30 ppm). The ^{13}C-NMR spectral of compound 1 (Figure 4B) exhibited the existence of nine carbon signals. Five signals of carbon appear at δ_C > 100 ppm as sp^2 carbon and four carbon signals at δ_C 55-75 ppm as sp^3 carbon. In the sp^2 carbon region there are two signals that are in the low field, namely at δ_C 166.2 as carbonyl ester carbon and at δ_C 151.2 ppm as oxyaryl carbon. Analysis of the ^{1}H-NMR and ^{13}C-NMR spectral in Figure 4 demonstrates that compound 1 is a aromatic compound with para-substituted by a hydroxyl group and a group having a cyclic ester substituted group of hydroxyl.

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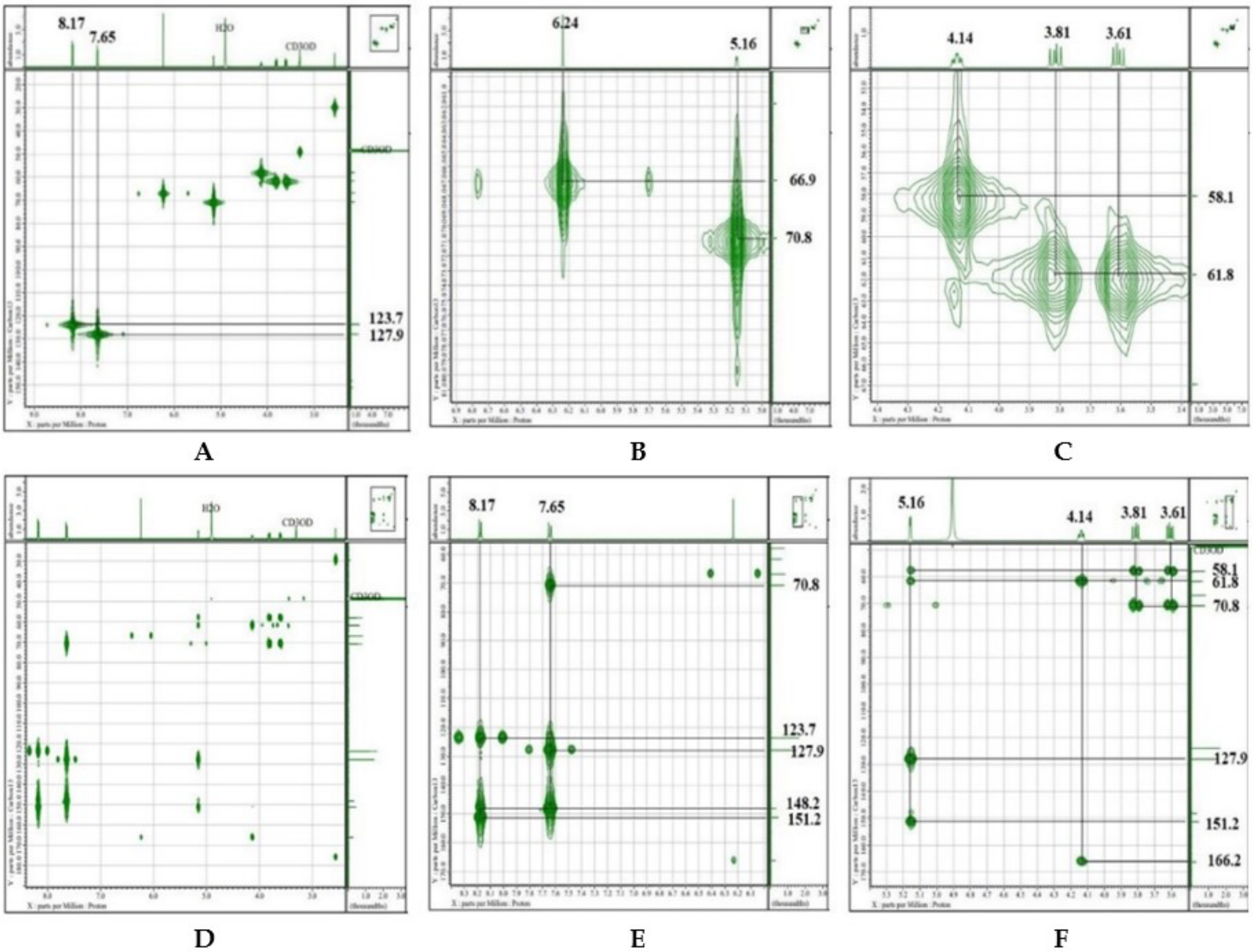


Figure 5. The HMQC spectrum of compound 1 (A, B, C); The HMBC spectra of compound 1 (D, E, F)

The HMQC spectral [Figure 5A, 5B, 5C] revealed a ^{1}H-^{13}C correlation through one bond. There are seven correlations consisting of two aromatic ^{1}H-^{13}C signal correlations, four proton signal correlations on oxygenated carbon consisting of two methine ^{1}H-^{13}C signal correlations and two methylene ^{1}H-^{13}C correlations with different chemical shifts to the same carbon atom. In addition, there is a correlation of a ^{1}H-^{13}C methine sp^3 proton to a tertiary carbon atom. This correlation indicates that the aromatic ring substituent of compound 1 consists of a hydroxyl group and a cyclic ester group that binds a hydroxyl group.

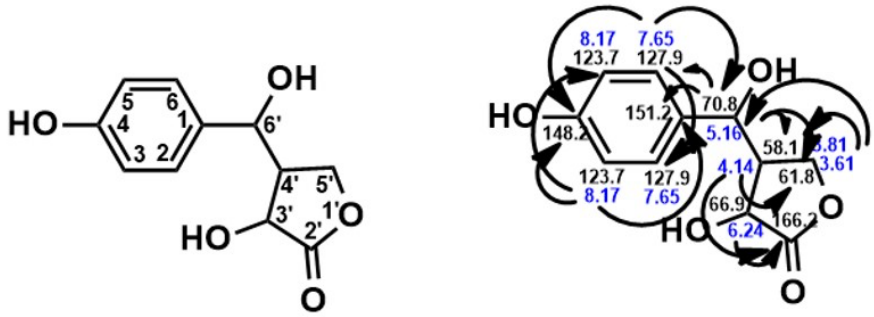
The HMBC spectral [Figure 5D, 5E, 5F] demonstrated the correlation of protons to carbon atoms through two or three bonds. There are seven correlations, one of which is the correlation of two pairs of protons with aromatic equivalent to their equivalent carbon atoms. In addition, a pair of aromatic protons equivalent to δ_H 7.65 ppm was seen to be correlated with an oxyaryl carbon atom at δ_C 148.2 ppm and an oxygenated carbon atom bonded to a para hydroxyl substituent at δ_C 70.8 ppm. This reveals that the methine oxygenated carbon atom is precisely bonded to the benzene ring which is strengthened by the proton correlation δ_H 5.16 ppm to aromatic methine carbon at δ_C 127.9 ppm and aromatic quaternary carbon atom at δ_C 151.2 ppm. Similarly, for a pair of aromatic protons at δ_H 8.17, two more correlations are seen, namely to the oxyaryl carbon atom δ_C 148.2 ppm and the quarternary aromatic carbon atom δ_C 151.2 ppm. Furthermore, proton-carbon correlation which is part of the para hydroxyl substituent is seen, namely the correlation of methine proton at δ_H 5.16 ppm with methine carbon at C 58.1 ppm and an oxygenated methylene carbon at δ_C 61.8 ppm. The presence of the substituent in the form of ring 5 cyclic ester is supported by the correlation of methin proton at δ_H 4.14 ppm with oxygenated methylene carbon at δ_H 61.8 ppm and carbonyl ester carbon at δ_H 166.2 ppm. It was further strengthened by the correlation of an oxygenated methine proton with a carbonyl ester carbon at a δ_H of 166.2 ppm. The 1D and 2D NMR spectrum data of compound 1 are exhibited in Table 4. The HMBC correlation of compound 1 was shown in Figure 7.

Table 4. The NMR data of compound 1 (^{1}H -500 MHz, ^{13}C -125 MHz in CD3OD) and 1* (^{1}H -500 MHz, ^{13}C -125 MHz in $CDCl_3$)

No. C	δ_C ppm 1	Type of C 1	δ_H ppm (ΣH. Multiplicity, Hz) 1	HMBC 1	δ_C ppm 1*	δ_H ppm (ΣH. Multiplicity, Hz) 1*
1	151.2	C			150.3	
2	127.9	CH	7.65 (^{1}H, d, J= 9.0 Hz)	70.8; 127.9; 148.2	127.0	7.65 (^{1}H, d, J= 9)
3	123.7	CH	8.17 (^{1}H, d, J= 9.0 Hz)	123.7; 148.2; 151.2	122.8	8.18 (^{1}H, d, J= 9)
4	148.2	C			147.2	
5	123.7	CH	8.17 (^{1}H, d, J= 9.0 Hz)	123.7; 148.2; 151.2	122.8	8.18 (^{1}H, d, J= 9)
6	127.9	CH	7.65 (^{1}H, d, J= 9.0 Hz)	70.8; 127.9; 148.2	127.0	7.65 (^{1}H, d, J= 9)
2′	166.2	C			165.5	
3′	66.9	CH	6.24 (^{1}H, s)	166.2	66.0	6.24 (^{1}H, s)
4′	58.1	CH	4.14 (^{1}H, m)	61.8; 166.2	57.2	4.15 (^{1}H, m)
5′	61.8	CH2	A. 3.81 (^{1}H, m)	58.1; 70.8	60.9	3.82 (^{1}H, m)
			B. 3.61 (^{1}H, m)	58.1; 70.8		3.62 (^{1}H, m)
6′	70.8	CH	5.16 (^{1}H, d, J= 2.5 Hz)	58.1; 61.8; 127.9; 151.2	70.0	5.16 (^{1}H, d, J= 2.5)

* ref. [70]

According to 1D and 2D NMR spectrum analysis, compound 1 was an aromatic compound which had a benzene ring substituted for a para hydroxyl with a 3-hydroxy-4-(hydroxymethyl)-ɣ-butyrolactone substituent. Thus, the proposed compound 1 structure was 3-hydroxy-4(hydroxy(4-hydroxyphenyl)methyl)-ɣ-butyrolactone as revealed in Figure 6.



A B

Figure 6. Chemical structure of compound 1 with numbering of carbon atom (A) and HMBC correlation (B).

2.4.2 *Compound 2*

Ethyl acetate extract of RB6 (2.1 g) was preabsorbed with silica gel (1:1) and detached by column chromatography using silica gel stationary phase and eluted gradient with *n*-hexane-ethyl acetate (10:0→0:10) eluent and continued with ethyl acetate-methanol (10:0→0:10). Based on the stain pattern on TLC, five subfractions were obtained (F1-F5). The F3 subfraction was then separated by column rechromatography with *n*-hexane-ethyl acetate (7:3 → 0:10) eluent until four subfractions were obtained (F3.1-F3.4). The subfraction F3.3 was rinsed with *n*-hexane-ethyl acetate (5:5) to obtain compound 2 (42.3 mg).

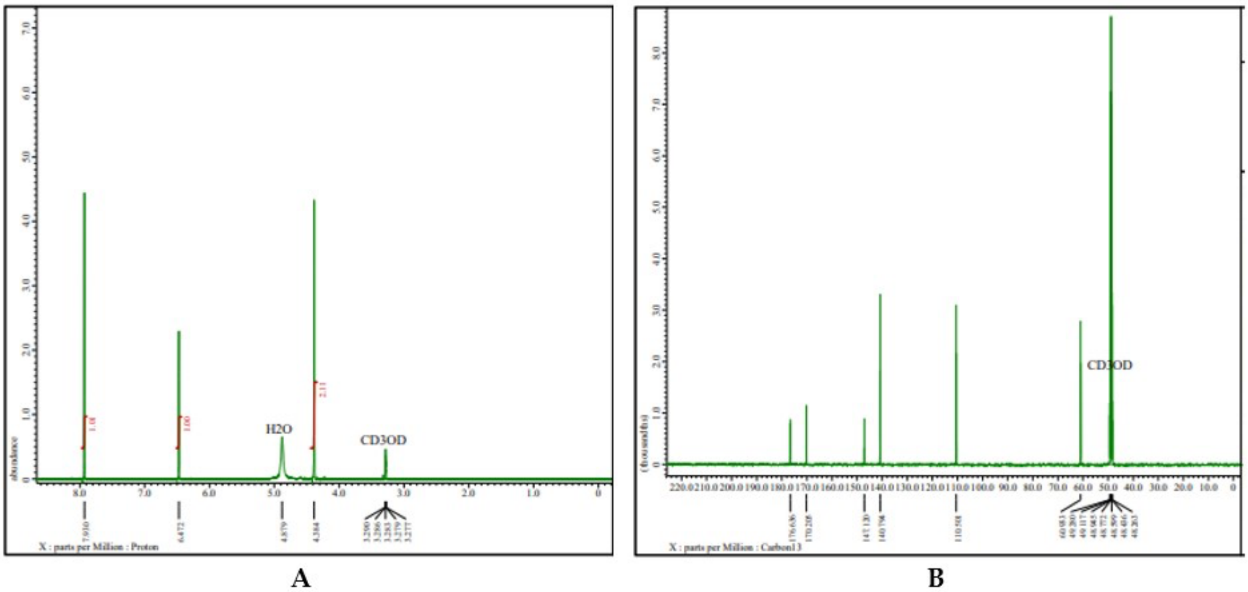


Figure 7. The ^{1}H-NMR (A) and ^{13}C-NMR (B) spectra of compound 2 (^{1}H-500 MHz; ^{13}C-125 MHz in CD_3OD)

The ^{1}H-NMR spectrum (Figure 7A) shows the presence of four protons at three chemical shifts, namely at δ_H 7.93 (^{1}H;s); 6.47 (^{1}H;s); and 4.38 ppm (2H;s). These signals indicate that there are two sp^2 protons bonded to the two carbons and two sp^3 protons bonded to the oxygenated carbon. All three proton signals have a singlet multiplicity indicated that the proton has no neighboring protons. The ^{13}C-NMR spectrum (Fig. 7B) shows that the isolated compound has six carbon atoms at δ_C 176.6; 170.2; 147.1; 140.8; 110.5; and 60.9 ppm. The carbon atom consists of five sp^2 carbons identified as low-field carbonyl ester carbons and oxyvinyl carbons, and one oxygenated sp^3 carbon.

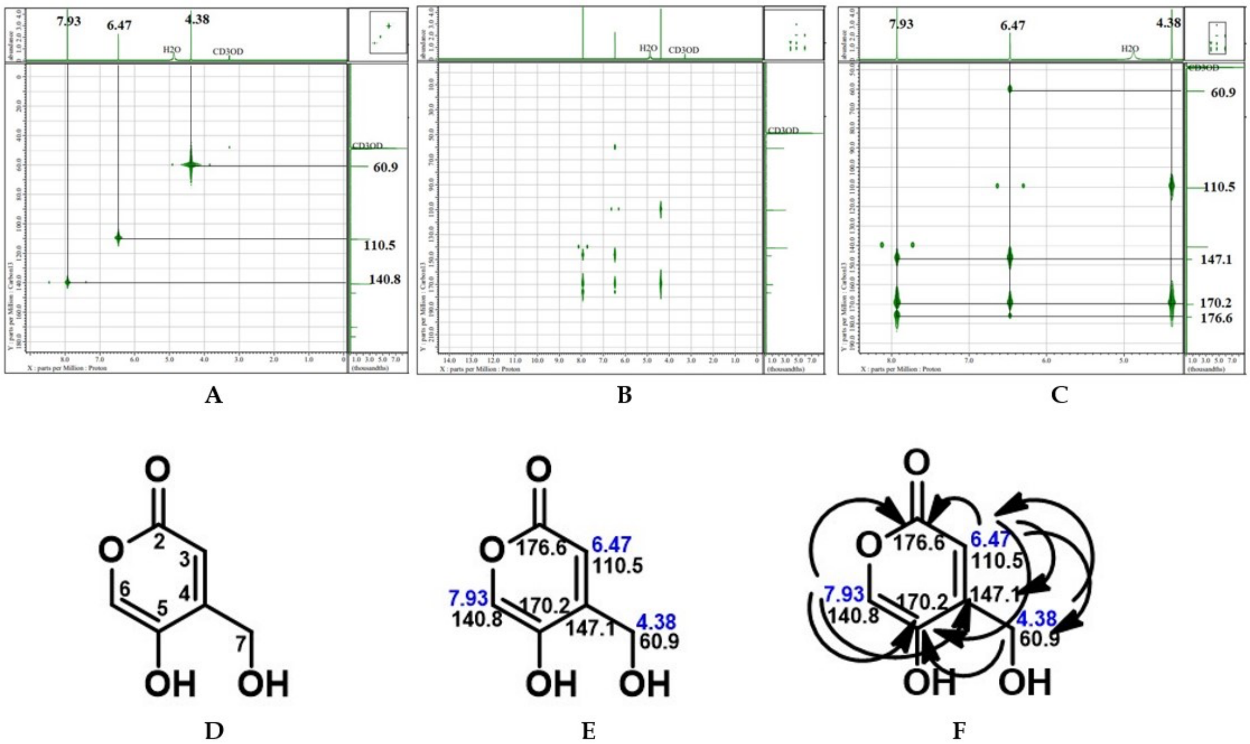


Figure 8. The HMQC spectrum of compound 2 (A); The HMBC spectra of compound 2 (B, C); Structure of compound 2: 5-hydroxy-4-(hydroxymethyl)-2H-pyran-2-on with numbering of carbon atom (D), proton and carbon chemical shift placement (E), and HMBC correlation (F).

The HMQC spectrum of compound 2 (Figure 8A) shows that the three proton signals bonded to three carbon atoms are two proton signals bonded to two sp^2 carbons and a proton signal bonded to an sp^3 carbon.

This indicated that compound 2 was a cyclic ester compound with a hydroxyl group and a hydroxymethyl group as substituents.

The HMBC spectrum of compound 2 (Figure 8B, 8C) describes that vinylic protons at δ_H 6.47 ppm correlate with two and three bonds with carbon at δ_C 60.9; 147.1; 170.2; 176.6 ppm indicating that the proton is a neighbor of the carbonyl ester carbon, the quaternary sp^2 carbon, the sp^2 oxyvinyl carbon, and the oxygenated methylene carbon. Protons at δ_H 7.93 ppm correlated with two and three bonds with carbon at δ_C 147.1; 170.2; 176.6 ppm which indicates that the proton is a neighbor of the quaternary sp^2 carbon, oxyvinyl sp^2 carbon and ester carbonyl carbon. Furthermore, the methylene proton signal at δ_H 4.38 ppm correlates with three bonds with carbon at δ_C 110.5 and 170.2 ppm, which are vinylic carbon and oxyvinyl carbon, respectively. The 1D and 2D NMR spectrum data for compound 2 were shown in Table 5.

Table 5. The NMR data of compound 2 (^{1}H -500 MHz, 13C-125 MHz in CD3OD) and 2* (^{1}H -500 MHz, 13C-125 MHz in DMSO)

No. C	δ_C ppm 2	Type of C 2	δ_H ppm (ΣH. Multiplicity, Hz) 2	HMBC 2	δ_C ppm 2*	δ_H ppm (ΣH. Multiplicity, Hz) 2*
2	176.6	C			176.8	
3	110.5	CH	6.47 (^{1}H, s)	60.9; 147.1; 170.2; 176.6	110.7	6.49 (^{1}H, s)
4	147.1	C			147.3	
5	170.2	C			170.4	
6	140.6	CH	7.93 (^{1}H, s)	147.1; 170.2; 176.6	141.0	7.94 (^{1}H, s)
7	60.9	CH2	4.38 (2H, s)	110.5; 170.2	61.2	4.40 (2H, s)

* ref. [71]

Based on the 1D and 2D NMR spectroscopy data of compound 2, it was known that compound 2 was a six-ring cyclic ester compound that bind two substituents, namely the hydroxylmethyl group on C-4 and the hydroxyl group on C-5 with the molecular formula $C_6H_6O_4$, Double Bond Equivalent (DBE) were 4, namely one each for the cyclic, one for the carbonyl group, and two for the carbon-carbon double bond. Thus, the molecular structure was proposed, specifically 5-hydroxy-4-(hydroxymethyl)-2H-pyran-2-on. This compound have ever been found from *Trichoderma* sp isolated from root of brotowali (*Tinaspora crispa*). The proposed structure of compound 2 is compared with the same compound from the literature, namely compound 2* [70] shown in Table 5. The molecular structure of compound 2 which was equipped with the numbering of carbon atoms, the placement of the chemical shifts of protons and carbons, and the HMBC correlation is shown in Figure 8D, 8E, and 8F.

2.4.3 Bioactivity of Secondary Metabolites from Endophytic Fungi

Antibacterial and antioxidant activity of EtOAc extract and pure compound from endophytic fungi *Curvularia intermedia* and *Colletotrichum cliviicola* compared to tetracycline and the ascorbic acid as standard can be seen in Table 6.

Table 6. MIC and IC_{50} values of EtOAc extract and secondary metabolites from endophytic fungi compared with tetracycline and ascorbic acid as standard

Sample	MIC Values (µg/mL)				IC_{50} (µg/mL)
	E. coli	*S. aureus*	*S. thypi*	*B. subtilis*	
EtOAc extract RB4	64	32	32	64	11.426
EtOAc extract RB6	32	64	64	32	15.338
Compound 1	32	32	64	64	20.87
Compound 2	64	64	128	128	78,43
Tetracycline[a]	4	4	4	4	
Ascorbic Acid[b]					10,083

[a]Antibacterial positive control; [b] Antioxidan positive control

Table 6 reveals the antibacterial and antioxidant activities of the ethyl acetate extract of the endophytic fungus *Curvularia intermedia* and *Colletotrichum cliviicola* as well as the two compounds isolated from their ethyl acetate extract. The antibacterial test revealed that compound 1 had very strong activity for all test bacteria (MIC ≤ 64 µg/mL) and the antioxidant was in the strong category (IC_{50} = 20 - 100 µg/mL). Likewise with compound 2, it exhibited strong antibacterial activity (MIC = 64 - 128 µg/mL) and strong antioxidant (IC_{50} = 20 - 100 µg/mL). Antioxidants were classified as very strong if IC_{50} < 20 µg/mL [77-79].

3. DISCUSSION

This study found a total 20 isolates of endophytic fungi belonging to nine different genera isolated from the bark of sungkai, namely Cladosporium, Gliocladium, Curvularia, Trichoderma, Colletotrichum, Pythium, Plectospira, Alternaria, and Mortierella. The genus of Pythium was the genus most commonly found on the stem bark of sungkai. Pythium can be found in various ecological conditions, such as soil, grassland, forest, swamp, and water. Generally, temperature can affect spore development (zoospore release). Low temperatures can increase humidity, causing the stem bark of sungkai to become more humid [47–50]. Stem bark that contains water can be a suitable habitat for spores (zoospores), which is characteristic of Pythium, to breed. Zoospores that can move in this condition cause many endophytic fungi, like Pythium, to be found on the stem bark of sungkai. This finding indicates that there are endophytic fungi that can reproduce well in specific tissues and environmental conditions.

The methanol extract of sungkai's stem bark showed strong antioxidant and antibacterial activity. The strong bioactivity of this extract was nearly related to its chemical constituent. Research revealed that the stem bark of sungkai contains phenolic compounds, tannins, alkaloids, steroids, and sapoins. Those secondary metabolites have antioxidant and antibacterial activity [57].

Antioxidant and antimicrobes agents of fungal endophyte extracts isolated from the stem bark of sungkai evidently have strong activity equivalent to the host, even some extracts whose IC_{50} value and inhibition zone were better than the host. RB4 and RB6 showed strong antioxidant and antibacterial activity against the four tested bacteria. Table 3 shows that some endophytic fungi extracts have IC_{50} and inhibition zones smaller than the methanol extracts of their hosts. This is presumably because the chemical components contained in the endophytic fungi extract are less numerous than in the host, causing the bioactive compounds to not synergize properly. This is unlike the methanol extract of the sungkai bark, which contains many chemical contents so that the biological activity of compounds are less concentrated and synergized.

Based on molecular identification, RB4 and RB6 were *Curvularia intermedia* and *Colletotrichum cliviicola*. The extracts produced by them had strong antioxidant and antibacterial activity because they inhibited the four test bacteria. After the isolation of the compounds, they produced each compound, namely 3-hydroxy-4(hydroxy(4-hydroxyphenyl)methyl)-ɣ-butyrolactone (1) and 5-hydroxy-4-(hydroxymethyl)-2H-pyran-2-on (2), which have been found in previous studies [70, 71]. These compounds belong to the phenolic and pyran group. Different compounds were found in these two different endophytic fungi caused by the environmental conditions and stress on the host plant which activates the genes in endophytic fungi to produce certain types of compounds. Research has been found which explains that endophytic fungi can produce the same compound even though the species are different. Unlike the case in this study, it is natural that the compounds found are different in different species of endophytic fungi [28]. However, this can be explained scientifically based on the mechanism of activation of silent genes due to environmental conditions. Silent genes have different activation tolerances in each endophytic fungal species. It could be, in the two species found that the activated silent gene is different so that the mechanism for the production of secondary metabolites is also different. Studies reported that due to the long endophytes coevolution and their host plants, endophytes have adapted to their particular microenvironments with genetic variations, along with the uptake of some DNA into their own genomes. This could lead to the capability of certain endophytes to biosynthesize several secondary metabolites that were originally associated with the host plant. There are "silent gene" clusters or biosynthetic gene clusters that can be activated by certain environmental conditions or stresses so that endophytic fungal can produce different compounds from the same host [58,59].

The secondary metabolites had strong antibacterial agents contrary to all test bacteria. Compound 1 showed MIC value of ≤ 64 µg/ml, and antioxidant agents had IC_{50} of 20.87 µg/ml as well as compound 2 also showed similar results with MIC = 64 - 128 µg/ml and IC_{50} of 78.43 µg/ml including the active category. The lower antioxidant agents of the chemical constituent compared to the methanol extract [based on the IC_{50} value) due to considerable factors, including the existence of another antioxidant compounds which has not been isolated from fungal endophyte extracts. Further research is needed to isolate these compounds. Another factor likely due to the synergistic effect of some compounds contained in the extract providing high antioxidant activity. Thus, to develop it into a source of medicinal source materials, known chemical contents of extracts can be used.

Several studies reveal that *Curvularia* sp. contains 2'-deoxyribolactone, hexylitaconic acid, and ergosterol, while *Colletotrichum* sp. contains 3-methyl 1-butanol [isopentyl alcohol), 4-amino-1-pentanol, p-Hydroxyphenylacetic acid, Pterin-6-carboxylic acid, and d-alaninol [52–56]. The metabolites constituent produced by the two fungal endophytes belong to the phenolic, pyran, flavonoid, sesquiterpene, and naphthalene groups, compounds of which have antioxidant and antibacterial activities [57–60]. References describe that chemical compounds produced by endophytic fungi can be similar or contrasting from their hosts. This is disclose to the role of endophytic fungi in mutualistic associations with their host plants. Endophytic fungal have a function in enhancing host fitness and assist in adaptation to surrounding and biological stresses [61]. In this role, endophytic fungal produce chemical constituent that are distinct from their hosts but can have strong bioactivity. Endophytic fungi have a great convenience to become a source of new bioactive compounds to overcome antibiotic resistance. In addition, novel compounds produced by fungal endophyte can add active ingredients to new drugs to treat diseases.

4. CONCLUSION

Endophytic fungi extract isolated from stem bark of *P. canescens* were obtained the different compound from 2 species of endophytic fungi, specifically 3-hydroxy-4(hydroxy(4-hydroxyphenyl)methyl)-ɣ-butyrolactone and 5-hydroxy-4-(hydroxymethyl)-2H-pyran-2-on. These compounds active as antioxidant and antibacterial. Furthermore, it can be developed for medicinal materials in the future.

5. MATERIALS AND METHODS

5.1 Sample Sterilization and Endophytic Fungi Isolation

The stem bark of the *P. canescens* used was fresh and healthy from Palembang, South Sumatra. Plants were identified in the Biosystematic Laboratory, Universitas Sriwijaya (301/UN9.1.7/4/EP/2021). Stem bark was washed with running water thoroughly ± 5 minutes. Then, surface sterilization was done by soaking for ± 1 minute in 70 % alcohol and rinsing with hygienic distilled water ± 1 minute. The sample was then soaked with sodium hypochloride ± 30 seconds, rinsed again with 70 % alcohol in ± 30 seconds, and rinsed with water destilation for ± 1 minute. The sterilized sample was ± 3 x 0.5 cm aseptically. The sample was inoculated in PDA and incubated at room condition for 3-7 days. It was monitored continuously till the appearance of endophytic fungi. Colonies grew in distinct morphological dimensions and were then purified. The purification was done by moving the colony to new medium of PDA and incubating at room condition for 48 hours. Purified colony was transferred to culture media in room temperature for further observation of macroscopic and microscopic characteristics [36].

5.2 Cultivation and Extraction of Endophytic Fungi

Endophytic fungal isolated was cultivated by placing five blocks (diameter of 5 mm) of purified-culture agar into 300 ml potato dextrose broth, as many as 15 bottles (glass material with 1 L in volume). The culture was incubated for 30 days in static conditions at room temperatures. Separation of mycelia and media were done after incubation using filter paper. Furthermore, ethyl acetate was added to the media (1:1) and extracted. Evaporation of ethyl acetate extract used a rotary evaporator [32,36].

5.3 Characterization and Identification of Endophytic Fungi

Macroscopic characteristic was observed at 3-7 days, which included color of the front and reverse colonies, colony texture (cotton, granules, flour, slimy), the presence of concentric circles, exudate droplets, radial lines, and. The microscopic characteristics were determined by using the Henrici's slide culture method. Observation of microscopic charcteristics included the shape of the spores and the appearence or absence of septa on the hyphae [63]. Identification based on appearing microscopic and macroscopic characteristics compared to literature [63,64].

5.4 Antioxidant Activity Test

The Antioxidant was tested by using DPPH with different concentrations [50]. The absorbances were measured by using spectrophotometer UVVis at 517 nm, and ascorbic acid as positive control. Antioxidant activity was determined via IC_{50} value.

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

A_k = Control absorbance
A_s = Samples absorbance

5.5 Antibacterial Activity Test

Analysis of antibacterial was made using the Kirby-Bauer method in MHA media. The bacteria test used *Bacillus subtilis, Escherichia coli*, *Salmonella typhi,* and *Staphylococcus aureus*. The disc paper was dropped with extract of fungal endophytes at 400 μg/disc. The positive control used tetracycline at 30 μg/disc. The inhibition zone was observed and measured after being incubated for 1x24 hours at 37°C based on the following formula [65]:

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

A: Sample
B: Positive control

5.6 Isolation and Identification of Compounds

The ethyl acetate extracts of RB4 (2.0 g) and RB6 (2.1 g) were analyzed by Thin Layer Chromatography (TLC) using various eluent systems to see the content of secondary metabolites and determine the right eluent for initial separation. Separation using gravity column chromatography (CCG) method. The ethyl acetate extract was dissolved in a suitable solvent and impregnated using silica gel 60 (70-230 mesh) with a ratio of 1:1 between the weight of the sample and the impregnated silica. The mixture was stirred and evaporated to dryness at room temperature. The column for CCG separation was prepared by condensing a quantity of silica gel G 60 (70-230 mesh) in a column containing n-hexane solvent by continuously flowing the solvent. The ratio of the sample weight and the weight of the silica gel used was 1:15. Impregnated RB4 ethyl acetate extract (2.0 g) was put into the CCG column and eluted using n-hexane-ethyl acetate (10:0→0:10) to ethyl acetate-methanol (10:0→0:10) as eluent. The results of the separation with CCG were collected into vials every 10 mL of eluate and analyzed by TLC. Eluates with the same spot pattern were then combined into one fraction and six subfractions were obtained, namely (F1-F6). Subfraction F4 was purified by gravity column recchromatography to obtain compound 1. Impregnated RB6 ethyl acetate extract (2.1 g) was put into the CCG column and eluted using n-hexane-ethyl acetate (10:0→0:10) to ethyl acetate as eluent -methanol (10:0→0:10). The results of the separation with CCG were collected into vials every 10 mL of eluate and analyzed by TLC. Eluate with the same spot pattern was then combined into one fraction and five subfractions were obtained, namely (F1-F5). Subfraction F3 was purified by gravity column rechromatography to obtain compound 2. The chemical structure of compounds 1 and 2 was determined by spectroscopy methods, which included ^{1}H-NMR, ^{13}C-NMR, HMQC, and HMBC.

5.7 Identification of Endophytic Fungi Molecularly

Identification of the most potential bioactivity of endophytic fungal was followed by molecular identification based on area of DNA (rDNA) internal transcribed spacer (ITS). ITS1 and ITS4 primers were used for the amplification process. Forward and reverse primer DNA sequence assemblage was composed using the Bioedit program. The sequences were then entered to the Basic Local Alignment Search Tool at http://blast.ncbi.nlm.nih.gov/. Moreover, the sample and databases sequences were aligned by using the CLUSTAL W method in MEGA11, and the phylogenetic tree was created using the neighbour-joinning tree method with a bootstrapvalue of 1,000 [66].

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Conflict of interest statement: The authors declared no conflict of interest.

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