

# Antibacterial Activity of Traditional Medicine *Scurrula atropurpurea* (BL) DANS and their Endophytic Fungi

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## Antibacterial Activity of Traditional Medicine *Scurrula atropurpurea* (BL) DANS and their Endophytic Fungi

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**Abstract.** *Scurrula atropurpurea* known as benalu is a medicinal plant that has been used for the treatment of various diseases such as antibacterial. Plants with ethnomedicine history use to cure pathogenic bacterial infections and their endophytic fungi is a promising source of antibacterial compounds. This study aimed to compare the antibacterial activity of *S. atropurpurea* leaves and their endophytic fungi. The secondary metabolites were isolated from the leaves of *S. atropurpurea* and their endophytic fungi by the chromatography method. The antibacterial activity test was carried out by Kirby Bauer method against *Salmonella typhi* (IPCCB.11.669) and *Escherichia coli* (ATCC 25922) as Gram (-) and *Bacillus subtilis* (ATCC 6633) and *Staphylococcus aureus* (ATCC 25923) as Gram (+). The antibacterial compound from *S. atropurpurea* was determined by spectroscopy analysis as Quercetin-3-O- $\alpha$ -L-rhamnopyranoside, while the antibacterial compound from endophytic fungi (strain BB1) as a lactone. Phylogenetic tree of strain BB1 has the highest homology with *Neopestalotiopsis surinamensis* strain CBS 450.74.

### Introduction

Antibiotics are medications used to prevent and treat bacterial infections. Antibiotic resistance occurs when bacteria change in response to the use of these medications. The resistance causes increased mortality to very dangerous levels in the whole of the world. The list of infections is increasing like pneumonia, tuberculosis, blood poisoning, and gonorrhea. This infection is difficult to treat because antibiotics are less effective [1].

Antibiotic resistance is a global health problem [2, 3]. Research has shown that microbes have developed resistance to antibiotics through various molecular mechanisms (through various molecular mechanisms). This global problem causes an increase in research-oriented to the discovery of new antibiotic compounds. These compounds can be sourced from medicinal plants [4-6] or endophytic fungi that live symbiosis in the tissue of the medicinal plants. Endophytic fungi, especially those isolated from medicinal plants that have traditionally been used to cure infectious diseases, has potential as a source of new antibiotics [7].

Endophytic fungi are microorganisms that live in all healthy plant tissues (roots, fruits, stems, seeds, and leaves) without causing disease or morphological changes in the plant life cycle. These microbes live symbiosis mutually beneficial with their host plants and can jointly produce specific secondary metabolites [8,9]. The latest investigation has reported the potential of endophytic fungi in producing bioactive compounds such as taxol (anticancer), podophyllotoxin (antitumor), diorthothrome A and diorthothrome B (antimalarials), and Cytochalasin H (antibacterial). Endophytic fungi are a good source of producing new antibiotics [10-13]. In this paper reported the antibacterial activity and chemical content of *S. atropurpurea* and its endophytic fungi.

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### Experimental Section

**Sampling and identification.** *Scurrula atropurpurea* leaves fresh are taken from around the Sriwijaya University campus, Indralaya, Ogan Ilir district, South Sumatra. Plant identification is carried out at the Botany Laboratory, FMIPA, Sriwijaya University.

**Extraction of *S. atropurpurea*.** *Scurrula atropurpurea* leaves fresh (650 g) were cleaned, cut into small pieces, then dried at room temperature until the weight is constant, then milled so that it is obtained dry powder weighed 312 g. The dry powder (300 g) was macerated using solvents with polarity increases, namely hexane, ethyl acetate, and methanol.

**Kirby-Bauer method.** Antibacterial activity test using the Kirby Bauer method with four tested bacteria. Determination of the antibacterial activity of the compounds tested was carried out by comparing the inhibitory zones in each concentration with inhibitory zones in three standard antibiotics. Antibacterial activity was expressed as inhibition percentage of a sample of antibiotics that are classified as strong (inhibition of  $\geq 70\%$ ), moderate (inhibition 50-70%), and weak (inhibition  $< 50\%$ ) [14].

**Exploration and structure elucidation of antibacterial compound from *S. atropurpurea* leaves.** Isolation of pure compound from the antibacterial active fraction, carried out by column chromatography, using silica gel G60 (70-230 mesh) as stationary phase, column length 45 cm, and diameter 2 cm. The extracts (5 g) were preabsorbed with silica gel (1:1), then eluted using gradient elution ( $\pm 30$  drops/minute). Eluates were collected in vials (10 mL) and used to monitor the eluates by TLC. Fractions that provide the same stain pattern were combined into one column fraction. Fractions that have potential stains are then purified. A pure compound from isolation results tested for antibacterial activity at a concentration of 400  $\mu\text{g}/\text{disk}$ . Furthermore, the molecular structure was identified by spectroscopic methods, including  $^1\text{H-NMR}$ ,  $^{13}\text{C-NMR}$ , HMQC, and HMBC.

**Isolation of endophytic fungi from *S. atropurpurea* leaves.** Isolation of endophytic fungal strains follows the method described in with slight modifications [15].

**Cultivation and antibacterial activity test of endophytic fungi extracts.** The cultivation of endophytic fungal strains was carried out by the method described in Elfita et al. [16]. Each fungal strain was cultured in 5 x 300 mL of PDB liquid medium. The extracts were tested the antibacterial activity with the Kirby Bauer method. Endophytic fungi extract with the highest antibacterial activity was continued to the separation of pure compound.

**Identification of endophytic fungi.** Endophytic fungi that provide the highest antibacterial activity was identified molecularly and analyzed by the construction of phylogenetic trees [14].

**Exploration of antibacterial compound from endophytic fungi and structure elucidation.** The active extract of the endophytic fungi was separated using column chromatography with silica gel as a stationary phase (ratio 1:10). Samples that have been prepared by preabsorption was injected into the column and was eluted using eluent with polarity increases. Eluates were stored in bottles every 10 ml, and analyzed by thin layer chromatography for grouped into the column fractions. Each column fraction was evaporated with a rotary evaporator and further separated and purified by re-chromatography and recrystallization techniques to obtain pure compound. The molecular structure of the compound was determined by spectroscopic analysis includes UV, IR,  $^1\text{H-NMR}$ ,  $^{13}\text{C-NMR}$ , HMQC, and HMBC.

### Results and Discussion

**Exploration of antibacterial compounds from *S. atropurpurea*.** Extraction of dried powder of *S. atropurpurea* leaves (300 g) in a gradient using n-hexane, ethyl acetate, and methanol produced concentrated extracts of 21.13, 29.87, and 30.24 g, respectively. Each fraction was tested for antibacterial activity against four bacteria, namely *S. typhi*, *B. subtilis*, *E. coli*, and *S. aureus*. Antibacterial activity of *S. atropurpurea* extracts compare to standard antibiotics showed to Table 1. These antibiotics can be used as a standard if they provide inhibitory zones with susceptible until intermediate categories when tested on an inoculum equivalent to McFarlan 0.5. If the antibiotic inhibition zone is categorized as resistant, it cannot be used as a standard [17].

**Table 1.** Antibacterial activity of fraction from *S. atropurpurea* leaves.

Sample	Fraction (400 µg /disk)	Test bacteria	Inhibition zone (mm)	Antibacterial activity percentage of ethyl acetate extracts compare to standard antibiotics (%)		
				Inhibition zone of Tetracycline (mm)	Inhibition zone of Streptomycin (mm)	Inhibition zone of Penicillin (mm)
<i>S. atropurpurea</i> leaves	n-hexane	<i>S. typhi</i>	0 ± 0	0	0	0
		<i>B. subtilis</i>	0 ± 0	0	0	0
		<i>E. coli</i>	0 ± 0	0	0	0
	Ethyl acetate	<i>S. aureus</i>	6.3 ± 0.61	30.4*	33.3*	37.1*
		<i>S. typhi</i>	0 ± 0	0	0	0
		<i>B. subtilis</i>	0 ± 0	0	0	0
		<i>E. coli</i>	10.1 ± 2.11	52.6**	54.3**	56.4**
		<i>S. aureus</i>	11.6 ± 1.64	56.0**	61.4**	68.2**
		<i>S. typhi</i>	7.2 ± 0.98	36.4*	36.7*	40.7*
		Methanol	<i>B. subtilis</i>	11.3 ± 2.30	57.9**	61.4**
	<i>E. coli</i>		13.6 ± 1.86	70.8***	73.1***	76.0***
	<i>S. aureus</i>		14.9 ± 2.11	72.0***	78.8***	87.6***

Note: \*\*\* strong: inhibition of ≥ 70% \*\* moderate: inhibition 50-70% \* weak: inhibition < 50%

The methanol extract showed the highest antibacterial activity against *E. coli* and *S. aureus*. Lim et al. (2016) reported that several *Scurrula* plant members, such as *S. atropurpurea* had good antibacterial activities against bacteria of Gram-positive and negative [18].

The methanol extract (5 g) was carried out to column chromatography over silica gel 60 (70-230 mesh). A total of 81 eluates were collected. The same fraction was combined to obtain six fractions (F1-F6). The antibacterial activity of column fractions shows that F-2 was more active compared to other column fractions. The F2 fraction showed a major compound under a UV lamp at λ 254 nm, and it was further to the purification to yield compound **1** as a yellow crystal (96 mg). The results of the antibacterial activity test on 4 test bacteria showed that compound **1** (at a concentration 400 µg/disk) had a moderate category against *E. coli*.

Phytochemical tests showed the compound **1** as a flavonoid group. The <sup>1</sup>H-NMR spectrum revealed 11 signals with 13 numbers of protons. The presence of five aromatic protons attached to rings A and B of flavonoids. The appearance of five proton signals in a chemical shift around 3-5 ppm indicated that compound **1** has a glucoside group. These signals in the area often appear overlapping. The characteristics of the glucoside group are the presence of anomeric protons (H1) with multiplicity of doublet in δ<sub>H</sub> 6-4 ppm. The small coupling value of H1 and H2 (1.3 Hz) indicated the glucoside is an anomeric α. The type of rhamnosyl group attached at the compound is the appearance of a methyl (3H, d) signal at δ<sub>H</sub> around 1 ppm (J = ±7 Hz).

The <sup>13</sup>C-NMR spectrum showed the presence of 21 carbon signals, 15 carbon for the flavonol skeleton, and six for the rhamnosyl group. This compound **1** as flavonol rhamnoside indicated the presence of carbonyl carbon signals at δ<sub>C</sub> 179.7 ppm. The spectrum of compound **1** revealed seven

oxyaryl (= C-O) carbon atoms at  $\delta_c$  136-166 ppm. The signals indicated the presence of four hydroxyl groups attached to flavonoids, two hydroxyl groups in the A and C rings, respectively. The hydroxyl group in the C ring is substituted with the rhamnosyl group.

Based on spectrum analysis and after compared with literature [19] (Table 2), it was concluded that compound **1** as quercetin-3-O- $\alpha$ -L-rhamnopyranoside. The molecular formula  $C_{21}H_{20}O_{11}$ , with DBE = 12. The HMBC correlation and  $\delta$ -assignment of compound **1** showed in Fig. 1.

**Table 2.**  $^1H$  and  $^{13}C$  NMR data of compound **1** recorded at  $^1H$ -500 MHz,  $^{13}C$ -125 Mhz, in  $CD_3OD$  dan  $1''$  recorded at  $^1H$ -400 MHz,  $^{13}C$ -100 Mhz, in DMSO, ppm [19]

No. C	$\delta_c$ (ppm) 1	DEPT 1	$\delta_H$ (ppm), $\Sigma H$ , mult, J (Hz) 1	HMBC 1	COSY 1	$\delta_c$ (ppm) 1'	$\delta_H$ (ppm) 1'
2	159.4	C				157.9	
3	136.3	C				134.9	
4	179.7	C				178.3	
5	163.3	C				161.8	
6	99.9	CH	6.20 (d, 2.0)	163.3; 166.0; 94.8; 106.0	6.37	98.4	6.21 br,s
7	166.0	C				164.5	
8	94.8	CH	6.37 (d, J=2.0)	158.6; 166.0; 99.9; 106.0	6.20	93.4	6.33 br,s
9	158.6	C				157.1	
10	106.0	C				104.6	
1'	122.9	C				121.6	
2''	117.0	CH	7.33 (d, J=2.6)	123.0; 149.9; 159.4; 146.5		115.6	7.32 m
3''	146.5	C				145.0	
4''	149.9	C				148.4	
5''	116.4	CH	6.91 (d, J=8.4)	146.5; 149.9; 123.0	7.30	115.0	6.91 (d, J=8.4)
6''	123.0	CH	7.30 (dd, J=8.4; 2.6)	117.0; 149.9; 159.4	6.91	121.5	7.32 m
1''	103.6	CH	5.35 (d, J=1.3)	136.3; 72.1	4.22	102.2	5.30 br, s
2''	71.9	CH	4.22 (m)	73.3;	5.35; 3.75	70.8	4.22 m
3''	73.3	CH	3.34 (m)	72.2		70.7	3.20-3.76 m
4''	72.2	CH	3.42 (m)	73.3	0.94	70.7	3.20-3.76 m
5''	72.1	CH	3.75 (dd, J=3.25; 6.50)	73.3	4.22; 3.44	70.6	3.20-3.76 m
6''	17.7	CH <sub>3</sub>	0.94 (d, J=6.50)	72.2	3.42	16.4	0.93 (d, J=5.4)

In this study, it was revealed that the antibacterial activity decreased from the methanol fraction to pure compound. This might be due to the synergistic effect in the methanol fraction, which strengthens its antibacterial activity. Amin et al. (2015) have reported the antibacterial activity of flavonoid compounds, namely quercetin, morine, and rutin. The quercetin, morine, and rutin (at 500  $\mu g$  / disk) against *S. aureus* (ATCC 43300) showed the inhibition zones  $13.5 \pm 0.21$ ; 0.0; and 0.0 mm, respectively [20]. When the three compounds are combined, namely quercetin + morin + rutin, the inhibition zone increases to  $16.5 \pm 0.21$  mm. Inhibition zones also increase when flavonoids are combined with resistant antibiotics. Flavonoids, alone or in combination, also damage bacterial cell membranes [20].

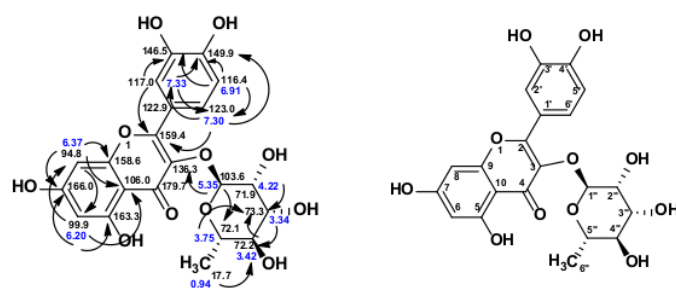


Figure 1. The HMBC correlation and  $\delta$ -assignment of compound 1

Antibacterial activity test of endophytic fungi extracts from *S. atropurpurea* leaves. Four strains of endophytic fungi were isolated from *S. atropurpurea* leaves, namely BB1-BB4. Each isolate has been cultivated in 5x300 mL of PDB medium for 6 weeks. The medium has been partitioned with ethyl acetate and evaporated to remove the solvent. The crude extract of each endophytic fungus is BB1 = 2.1 g; BB2 = 2.4 g; BB3 = 1.6 g; and BB4 = 1.9 g. The inhibition zone of all ethyl acetate extracts from endophytic fungi against four test bacteria compared to standard antibiotics is shown in Table 3.

Antibacterial activity of endophytic fungi extracts (Table 3) showed that BB1 isolates had strong activity against four bacteria (*S. typhi*, *B. subtilis*, *E. coli* and *S. aureus*), but has moderate activity against *S. aureus* by comparing tetracycline. Isolate BB1 generally has broad-spectrum. Table 3 reveals that BB4 provides moderate activity against four test bacteria. Two other fungal isolates (BB2 and BB3) have weak to inactive activity.

Table 3. Antibacterial activity of endophytic fungi extracts from *S. atropurpurea* leaves

Sample	Extracts (400 $\mu$ g/disk)	Test bacteria	Inhibition zone (mm)	Antibacterial activity percentage of ethyl acetate extracts compare to standard antibiotics (%)		
				Inhibition zone of Tetracycline (mm)	Inhibition zone of Streptomycin (mm)	Inhibition zone of Penicillin (mm)
				<i>S. typhi</i> (19.8)	<i>S. typhi</i> (19.6)	<i>S. typhi</i> (17.7)
				<i>B. subtilis</i> (19.5)	<i>B. subtilis</i> (18.4)	<i>B. subtilis</i> (18.8)
				<i>E. coli</i> (19.2)	<i>E. coli</i> (18.6)	<i>E. coli</i> (17.9)
				<i>S. aureus</i> (20.7)	<i>S. aureus</i> (18.9)	<i>S. aureus</i> (17.0)
	BB 1	<i>S. typhi</i>	14.7 $\pm$ 1.80	74.2***	75.0***	83.1***
		<i>B. subtilis</i>	14.0 $\pm$ 2.25	71.8***	76.1***	74.5***
		<i>E. coli</i>	16.3 $\pm$ 1.69	84.9***	87.6***	91.1***
		<i>S. aureus</i>	13.8 $\pm$ 1.08	66.7**	73.0***	81.2***
	BB2	<i>S. typhi</i>	0 $\pm$ 0	0	0	0
		<i>B. subtilis</i>	0 $\pm$ 0	0	0	0
		<i>E. coli</i>	8.3 $\pm$ 0.65	43.2*	44.6*	46.4*
		<i>S. aureus</i>	7.2 $\pm$ 1.30	34.8*	38.1*	42.4*
Endophytic fungi	BB3	<i>S. typhi</i>	0 $\pm$ 0	0	0	0
		<i>B. subtilis</i>	8.1 $\pm$ 1.05	41.5*	44.0*	43.1*
		<i>E. coli</i>	0 $\pm$ 0	0	0	0
		<i>S. aureus</i>	10.7 $\pm$ 2.02	51.7**	56.6**	62.9**
	BB4	<i>S. typhi</i>	11.1 $\pm$ 1.24	56.1**	56.6**	62.7**
		<i>B. subtilis</i>	13.0 $\pm$ 1.08	66.7**	70.6***	69.1**
		<i>E. coli</i>	10.7 $\pm$ 1.27	55.7**	57.5**	59.8**
		<i>S. aureus</i>	9.8 $\pm$ 1.41	47.3*	51.8**	57.6**

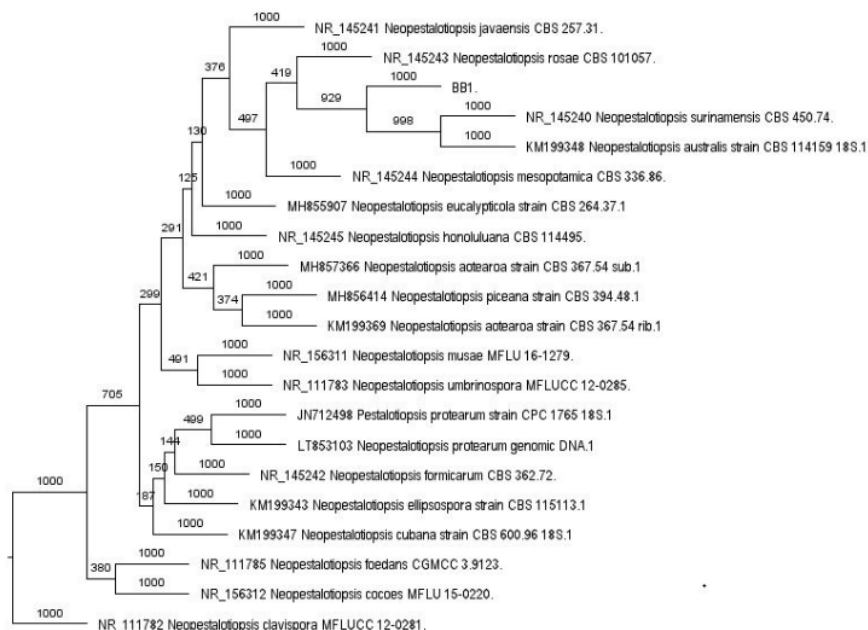
Note: \*\*\* strong: inhibition of  $\geq$  70% \*\* moderate: inhibition 50-70% \* weak: inhibition < 50%

**Identification of endophytic fungal isolates.** Identification of endophytic fungal isolate (BB1) was carried out molecularly based on partial genetic analysis at locus *Internal Transcribed Spacer (ITS) ribosomal DNA* fungi [14].

The sequencing data are then trimming and assembling using the SeqTrace program. The sequence data (contig) that has been assembled [19] then carried out by BLAST with a database that has been registered with the NCBI/National Center for [32] Biotechnology Information (<http://ncbi.nlm.nih.gov/BLAST/>). This aims to determine taxa or species that have the greatest homology/similarity molecularly. The visualization uses the Treeview v1.4.3 program to design the phylogeny tree as shown in Fig 2. Based on the phylogeny tree, the endophytic fungus BB1 has highest homology with *Neopestalotiopsis surinamensis* strain CBS 450.74 [22]

**Isolation of secondary metabolite from BB1.** Ethyl acetate extract of BB1 (2 g) was separated by column chromatography using silica gel 60 (70-230 mesh). A total of 56 eluates were collected consisting of five fractions (F1-F5). Fraction F4 was purified by re-chromatography to obtain compound **2** (32 mg). Antibacterial activity test of compound **2** (at 400 µg/disk) against four bacteria showed that this compound had a moderate category of *E. coli* and *S. aureus*.

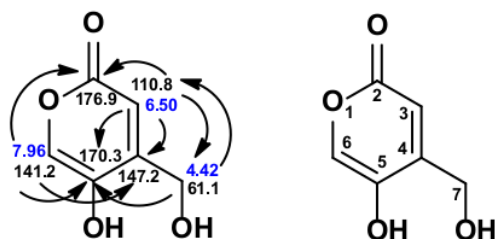
Compound **2** as white crystals. UV (MeOH)  $\lambda_{\max}$  270 nm; U [10] (MeOH + NaOH)  $\lambda_{\max}$  313 nm (bathochromic shift); IR (KBr)  $\nu_{\max}$   $\text{cm}^{-1}$ : 3265 (O-H), 3175 (C-H aromatic), 2922; 2851 (C-H aliphatic), 1661 (C=O), 1470 (C=C aromatic), and 4327 (C-O ester). The  $^1\text{H-NMR}$  spectrum showed the presence of three signals at  $\delta_{\text{H}}$  4.42 (2H, s), 6.50 (1H, s), and 7.96 ppm (1H, s). This signal indicates that compound **2** has one methylene  $\text{sp}^3$  group and two methine  $\text{sp}^2$  groups. The  $^{13}\text{C-NMR}$  spectrum exhibited the presence of six carbons namely one carbon  $\text{sp}^3$  and five carbon  $\text{sp}^2$ . The presence of two oxyaryl (= C-O) carbon atoms exhibited at  $\delta_{\text{C}}$  141.2 and 170.3 ppm. Three signals of carbonyl cyclic-ester at  $\delta_{\text{C}}$  176.9 ppm were assigned to lactone, which was indicated by the presence of oxyaryl carbon at  $\delta_{\text{C}}$  141.2 ppm. Further identification of carbon and protons signals was determined based on 2D NMR spectrum (Table 4). Based on [25] the spectroscopic analysis and compared with literature [21], it was concluded that compound **2** as **5-hydroxy-4-hydroxymethyl-2H-pyran-2-one**. Compound **2** has the molecular formula  $\text{C}_6\text{H}_6\text{O}_4$ , with DBE = 4. Fig. 3 showed the HMBC correlation and  $\delta$ -assignment of compound **2**.



**Figure 2.** The phylogenetic tree of BB1 with 1000 bootstrap replicates

**Table 4.** <sup>1</sup>H and <sup>13</sup>C NMR data of compound **2** recorded at <sup>1</sup>H-500 MHz, <sup>13</sup>C-125 Mhz, in CD<sub>3</sub>OD and 2\* recorded at <sup>1</sup>H-500 MHz, <sup>13</sup>C-125 Mhz, in CD<sub>3</sub>OD [21]

No. C	$\delta_c$ (ppm) 2	$\delta_H$ (ppm), $\Sigma H$ , mult, $J$ (Hz) 2	HMBC 2	$\delta_c$ (ppm) 2*	$\delta_H$ (ppm) 2*
2	176.9			176.8	4.8
3	110.8	6.50 (1H; s)	61.1; 147.2; 170.3; 176.9	110.7	6.49 (1H; s)
4	147.2			147.3	
5	170.3			170.4	
6	141.2	7.96 (1H; s)	147.2; 170.3; 176.9	141.0	7.94 (1H; s)
7	61.1	4.42 (2H; s)	110.8; 170.3	61.2	4.40 (2H; s)



**Figure 3.** The HMBC correlation and  $\delta$ -assignment of compound **2**

Previous studies have found an esterification derivative of compound **2** from *Andrographis paniculata* leaves, namely **3** (5-hydroxy-2-oxo-2H pyran-4-yl) methyl acetate). The inhibition zone in the antibacterial activity test of the compound is lower than that of compound **2**. The esterification of free hydroxyl groups might cause a decrease in antibacterial activity [22]. Thus it is known that free hydroxyl groups affect the antibacterial activity of a compound.

### Summary

Endophytic fungi extract of *Neopestalotiopsis surinamensis* has a higher antibacterial activity than its host. Compound **1** had a moderate category against *E. coli*, and compound **2** had moderate activity against *E. coli* and *S. aureus*. The results of this study recommend that further research should be needed to combine compound **1** and **2** as additives to resistant antibiotics to determine their increased antibacterial activity.

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