MICROBIOLOGY
INDONESIA
ISSN 1978-3477, eISSN 2087-8575
Vol.9, No.2, June 2015, p 82-88

Available online at
http://jurnal.permi.or.id/index.php/mionline
DOI: 10.5454/mi.9.2.5

Chemical Constituens of an Endophytic Fungus *Aspergillus* Sp (Sbd5) Isolated from Sambiloto (*Andrographis paniculata* Nees)

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Medicinal plants and their endophytes are important resources for discovery of natural products. Endophytic fungi isolated from medicinal plants more likely exibit pharmaceutical potentials. In the present study, an endophytic fungus *Aspergillus* sp (SbD5) was isolated from leaves of sambiloto (*Andrographis paniculata*). The fungus isolate was cultivated in Potato Dextrose Broth (PDB) medium for 7 weeks in static, then extracted with ethyl acetate followed by Thin Layer Chromatography (TLC) test. The results displayed three major spots. Ethyl acetate extract was further separated by column chromatography and recrystallization to obtain three pure compounds. Their structures were determined on the basic of spectroscopic analysis. The compounds is one new benzochromen derivative, 1-(3,8-dihydroxy-4,6,6-trimethyl-6H- benzochromen-2-yloxy)propan-2-one (**1**), together with two known compounds 5-hydroxy-4-(hydroxymethyl)-2H-pyran-2-one (**2**) and (5-hydroxy-2-oxo-2H-pyran-4-yl)methyl acetate (**3**). The antibacterial activities of the compounds were tested using the disc diffusion method against *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Shigella dysenteriae* and *Salmonella typhi.* Compound **1** has the highest antibacterial activity followed by compound **2** and **3**.

Key words: *Andrographis paniculata,* antibacterial activity, chemical constituens, endophytic fungus

Tumbuhan obat dan jamur endofitiknya merupakan sumber penting untuk mendapatkan senyawa organik dari bahan alam. Jamur endofitik yang diisolasi dari tumbuhan obat lebih berpotensi untuk dikembangkan dalam bidang farmasi. Dalam penelitian ini, sebuah jamur endofitik *Aspergillus* sp (SbD5) telah diisolasi dari daun tumbuhan sambiloto (*Andrographis paniculata*). Isolat jamur tersebut telah dikultivasi dalam medium Potato Dextrose Broth (PDB) selama 7 minggu dalam keadaan statis, kemudian diekstraksi dengan etil asetat yang dilanjutkan dengan uji pada kromatografi lapis tipis (TLC). Hasilnya menunjukkan bahawa terdapat tiga noda mayor pada plat TLC. Ekstrak etil asetat selanjutnya dipisahkan dengan kromatografi kolom dan rekristalisasi hingga diperoleh tiga senyawa murni. Struktur molekul senyawa-senyawa tersebut ditentukan berdasarkan analisis spektroskopi. Diperoleh satu senyawa baru turunan benzokromen yaitu 1-(3,8-dihidroksi-4,6,6-trimetil-6H-benzokromen-2-iloksi)propan-2-on (**1**), dan dua senyawa yang telah dikenal yaitu 5-hidroksi-4-(hidroksimetil)-2H-piran-2-on (**2**) dan (5-hidroksi-2-okso-2H-piran-4-il)metil asetat (**3**). Aktivitas antibakteri senyawa-senyawa tersebut diuji dengan metode difusi cakram terhadap bakteri uji *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Shigella dysenteriae,* dan *Salmonella typhi.* Senyawa **1** memiliki aktivitas antibakteri paling tinggi yang diikuti oleh senyawa **2** dan **3**.

Kata kunci: aktivitas antibakteri, *Andrographis paniculata*, jamur endofitik, kandungan kimia

Endophytic microorganisms exist within the living tissues of most plant species. Endophytic fungi, which colonize plants internally without apparent adverse effects, occur ubiquitously in plants and do not have pathogenic effects on its hosts. They are important sources of bioactive natural products with enormous potential for the discovery of new molecules for drug discovery, industrial use and agricultural applications (Strobel 2006; Qadri *et al*, 2013; Ramesha and Srinivas 2014).

Strobel *et al.* (2005) mentions several criteria must be considered in plant selection strategy. One of the strategy: plant that have an ethnobotanical history (use by indigenous peoples) that are related to the specific uses or applications of interest are selected for study. One of the interesting facts about the endophytic fungus is its ability to produce the bioactive compounds, same with the host or not (Elfita *et al*. 2011a; Elfita *et al*. 2012a).

Sambiloto (*Andrographis paniculata*) is a plant that has been widely used for traditional medicine in Indonesia. Rao *et al*. (2004) and Jada *et al*. (2007) write in their publication that *Andrographis* is a genus of the Acanthaceae family comprising of about 40 species several members of which enjoy a reputation in

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traditional medicine. *Andrographis paniculata* Nees is a herb commonly used in India, China, and Southeast Asia for the treatment of a large variety of illnesses, which include meningitis, acute hepatitis, acute inflammatory, influenza, dysentry, malaria, antipyretic, detoxicant, and analgesic agent for the treatment of acute infections of the gastrointestinal tract, respiratory organ and urinary system.

As part of a phytochemical study of our research, many bioactive and/or new compounds were isolated. Elfita *et al*. (2011b) was reported one alkaloid 7-hidroksipiranopiridin-4-on which is active as antimalarial from an endophytic fungus which was isolated from the stem of sambiloto. The paper was reported the isolation of nine endophytic fungi from the stems and leaves of sambiloto, but the *Aspergillus* sp (SbD5) was not found in the study. The purpose of the present study was to isolate and elucidate three compounds produced from *Aspergillus* sp (SbD5) an endophyitic fungus from leaves of sambiloto and how antibacterial activity of this compounds.

MATERIALS AND METHODS

Material and Chemicals. The leaves of sambiloto were collected on January 2014 from the Indralaya, Ogan Ilir, South Sumatra. Material for isolation and cultivation of endophytic fungi using Oxoid: potato dextrose agar (PDA), chloramphenicol, and potato dextrose broth (PDB), for sterilization: ethanol 70% and NaOCl. Material for separattion: analytical thin layer chromatography (TLC) using Merck (Art.5554) silica gel 60 F_{254}, and column chromatography using Si gel 60 (70-230 mesh). Organic solvents (hexane, ethyl acetate, methanol) were technical grade and distilled before use and ampicillin (Sigma Chemical).

Instrumentations. The apparatus in the research were colony counter, autoclave, incubator, water bath, microscope, magnetic hotplate, UV lamp, column chromatography and general apparatus in organic and microbiology laboratory, melting point, UV-Shimadzu, FTIR-Perkin Elmer-Spectrum One, and NMR –Agilent DD2 500 MHz (^{1}H) and 125 MHZ (^{13}C), UV light at 254 nm and 365 nm.

Isolation and Identification of Endophytic Fungi. The fungi was isolated from the leaves of *sambiloto* collected from the Indralaya, Ogan Ilir, South Sumatra using the reported methods of Barik *et al*. (2010) and Elfita *et al*. (2013). The endophytic fungal strain was identified based upon colony morphology and microscopic observation of mycelia and sexual spores according to the method described in the literature (Liu *et al*. 2010).

Cultivation of Pure Fungal Strain. Thirty bottles (1 L) containing 300 mL PDB medium per bottle were autoclaved for 45 min at 121 °C. The purified fungi (a small park) were transferred under sterile conditions to the bottles under sterile condition. The cultures were then incubated at room temperature (no shaking) for 7 weeks (Elfita *et al.* 2011c; Elfita *et al*. 2012b).

Extraction, Isolation, and Structure Elucidation of Secondary Metabolites. Mycelium biomass was separated and culture broth repeatedly extracted with EtOAc (1:1). The extract was filtered and concentrated using rotary evaporator at a temperature ± 45 °C. The EtOAc extract (2 g) was applied to column chromatography on silica gel (40 x 1 cm) and eluted with n-hexane- EtOAc-methanol gradient and collected in 86 vials each containing 10 ml. The TLC analysis showed the presence of seven column fractions (F1-F7). Fraction F2, F3, and F6 showed a major compound and further separation by column chromatography. Fraction F3 (0.24 g) was rechromatographed over silica gel (30 × 0,7 cm) and eluted with n-hexane-EtOAc (5:5) to yield compound **1** (26 mg). Fraction F6 (0.58 g) was rechromatographed over silica gel (30 × 0,7 cm) and eluted with n-hexane-EtOAc (4:6) to yield compound **2** (120 mg). Fraction F2 (0.35 g) was rechromatographed over silica gel (30 × 0,7 cm) and eluted with n-hexane-EtOAc (7:3) to yield compound **3** (33 mg). The isolation of the compounds from ethyl acetate extract of *Aspergillus* sp (SbD5) from the leaves of sambiloto is described in Figure 1. Structures of the afforded compounds were confirmed on the bases of different spectroscopic means (UV, IR, ^{1}H-NMR, ^{13}C-NMR, HMQC, HMBC, and COSY) and comparison (Elfita *et al*. 2014).

Antibacterial Activity Test. Each of the three compounds dissolved in ethanol (concentration: 500 g mL^{-1}). The MHA (15 mL) was poured into petri dishes and inoculated with 100 μL of the suspension containing 1 × 10^{5} CFU mL^{-1} of bacteria. Ampicillin was used as the positive control and discs treated with ethanol were used as the negative control. Sterile paper discs (6 mm) loaded with 20 μL of the samples were placed onto the surface of the agar. The plates were then placed in an incubator at 37 °C for 24 h, after which the diameter of the zone of inhibition around each of the discs was measured and recorded. Each experiment was performed in triplicate (Choi *et al*. 2011).

RESULTS

The fungal strain was identified as *Aspergillus* sp (SbD5) by the Sekolah Ilmu dan Teknologi Hayati, Institut Teknologi Bandung, Indonesia. The fungus *Aspergillus* sp (SbD5) was cultivated on 9 L of PDB medium for 7 weeks at room temperature, the culture broth extracted by solvent partition with EtOAc (1:1), followed by evaporation. From the results obtained by evaporation of 10.8 g of EtOAc extract. The extract showed three major spot on TLC. The isolation of the compounds from ethyl acetate extract of *Aspergillus* sp (SbD5) from the leaves of sambiloto is described in Fig 1.

Compound **1** (1-(3,8-dihydroxy-4,6,6-trimethyl-6H-benzochromen-2-yloxy)propan-2-one) was obtained as a white crystal, mp. 150-152 °C. UV (MeOH) λ_{max} (log ε) nm: 220 (4.31), 273 (4.35). IR (KBr) ν_{max} cm^{-1}: 3446.8; 3429.4 (OH), 3016.7 (CH-aromatic), 2927.9 (CH-aliphatic), 1735.9; 1701.2 (C=O), 1633.7 (C=C aromatic), 1045.4 (C-O alcohol): ^{1}H NMR ($CDCl_3$, 500 MHz) δ_H ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ_H ppm (Table 1).

Compound **2** (5-hydroxy-4-hydroxymethyl-2H-pyran-2-one) was obtained as a white crystal, mp. 155-156 °C; IR (KBr) ν_{max} cm^{-1}: 3267.4 (OH), 3097.7 and 3070.7 (CH-aromatic), 2924.1 and 2854.7 (CH-aliphatic), 1658.8 (C=O esther), 1626.0-1579.7 (C=C aromatic), 1224.8 (C=O esther), 1072.4 (C-O alcohol): ^{1}H NMR (CD_3OD, 500 MHz) δ_H ppm; ^{13}C NMR (CD_3OD, 125 MHz) δ_C ppm (Table 2).

Compound **3** (5-hydroxy-2-oxo-2H pyran-4-yl)methyl acetate) was obtained as a white crystal, mp. 88-89 °C; IR (KBr) ν_{max} cm^{-1}: 3348.4 and 3263.6 (OH), 3109.2 (CH-aromatic), 2960.0 (CH-aliphatic), 1730.2, 1662.6 (C=O esther), 1629.8 (C=C aromatic), 1255.7 and 1236.4 (C=O esther), 1037.7 (C-O alcohol); ^{1}H NMR ($CDCl_3$, 500 MHz) δ_H ppm; ^{13}C NMR ($CDCl_3$, 125 MHz) δ_C ppm (Table 3).

The antibacterial activity of three compounds was produced from *Aspergillus* sp (SbD5) an endophyitic fungus from leaves of sambiloto against *S. aureus, E. coli, S. dysenteriae,* and *S. typhi* was evaluated by the disc diffusion method via determination of the diameter zones of inhibition. The antibacterial activity of the compounds was determined by the disc diffusion method (Table 4).

DISCUSSION

1-(3,8-dihydroxy-4,6,6-trimethyl-6H-benzochromen-2-yloxy)propan-2-one (1). Compound **1** was isolated from the EtOAc extract of liquid cultures of *Aspergillus* sp (SbD5) as brown crystal (26 mg). The structures was determined on the basic of spectroscopic analysis including UV, IR, ^{1}H-NMR, ^{13}C-NMR, HMQC, and HMBC. It displayed UV absorbances at λ_{max} (MeOH) 273 nm (log ε = 4.35) indicating the presence of aromatic chromophore. The IR spectrum of 1 exhibited absorptions for hydroxyl (3446.8; 3429.4 cm^{-1}), C-H aliphatic (2927.9 cm^{-1}), C-H aromatic (3016.7 cm^{-1}), carbonyl (1735.9; 1701.2

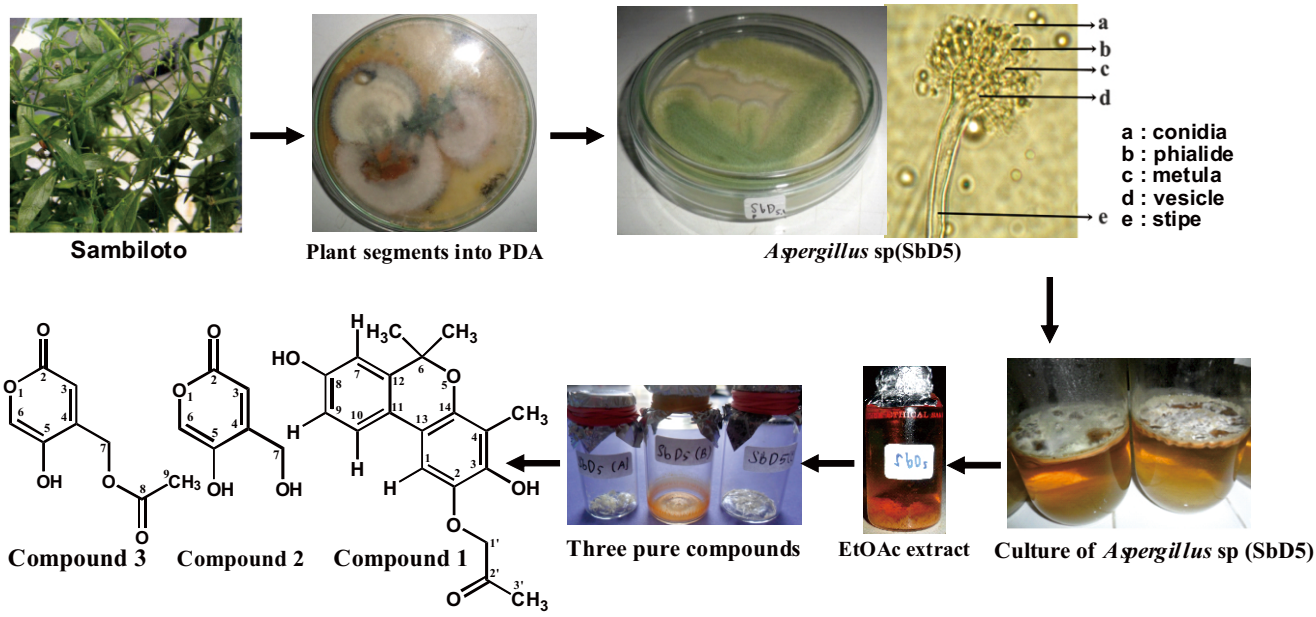

Fig 1 Isolation of the compounds from ethyl acetate extract of *Aspergillus* sp (SbD5) from the leaves of sambiloto.

Table 1 The NMR data of compound **1**, recorded at ^{1}H-500MHz; ^{13}C-125 MHz in $CDCl_3$

No. C	δC (ppm)	δH (ppm), ΣH, multiplicity, *J* (Hz)	HMBC
1	131.5	7.57 (1H; s)	141.6
2	167.8		
3	165.0		
4	132.3		
6	66.1		
7	123.2	8.03 (1H; s)	66.1 ; 120.3 ; 125.6 ; 126.4
8	164.8		
9	120.3	7.60 (1H; d; 6 Hz)	105.0
10	105.0	6.90 (H; d; 6 Hz)	120.3
11	126.4		
12	152.7		
13	125.6		
14	141.6		
1’	54.1	4.25 (2H; s)	167.8 ; 201.7
2’	201.7		
3’	30.4	2.38 (3H; s)	201.7 ; 54.1
4-CH3	26.5	3.46 (3H; s)	141.6 ; 165.0
6-CH3 (A)	26.9	1.92 (3H; s)	152.7 ; 26.9 ; 66.1
6-CH3 (B)	26.9	1.92 (3H; s)	152.7 ; 26.9 ; 66.1

Table 2 The NMR data of compound **2** recorded at ^{1}H-500MHz; ^{13}C-125 MHz in CD_3OD

No. C	δC (ppm) 2*	δC (ppm) 2	δH (ppm), ΣH, multiplicity, *J* (Hz) 2	HMBC 2
2	176.8	176.8		
3	110.7	110.7	6.50 (1H; s)	147.3; 170.4
4	147.3	147.3		
5	170.4	170.4		
6	141.0	141.0	7.95 (1H; s)	147.3; 170.4; 176.8
7	61.2	61.2	4.41 (2H; s)	110.7; 170.4

* ref. Elfita *et al.,* (2014)

Table 3 The NMR data of compound **3** recorded at ^{1}H-500MHz; ^{13}C-125 MHz in $CDCl_3$

No. C	δC (ppm) 3*	δC (ppm) 3	δH (ppm), ΣH, multiplicity, *J* (Hz) 3	HMBC 3
2	173.9	173.9		
3	111.2	111.2	6.50 (1H; s)	61.4; 145.9; 162.8
4	145.8	145.9		
5	162.9	162.8		
6	137.9	138.0	7.85 (1H; s)	145.9; 162.8; 173.9
7	61.4	61.4	4.93 (2H; s)	111.2; 162.8; 169.8
8	169.8	169.8		
9	20.6	20.6	2.16 (3H; s)	169.8

* ref. Elfita *et al.* (2014)

cm^{-1}), C=C aromatic (1633.7 cm^{-1}), and C-O alcohol (1045.4 cm^{-1}). The ^{13}C NMR spectrum, and was supported by HMQC spectrum showed 19 signals consisting af four methyls, one methylene, four methines, and ten quarternary carbons. The HMQC spectrum supplied complete assignment of all protonated carbons. The fourth methyl group include the presence of gem dimethyl groups that supported the existence of a signal at δ_H 1.92 ppm (6H, s) attached to δ_C 26.9 ppm. Two of these signal were assigned to

Table 4 Antibacterial activity (as inhibition zone diameters) of three compounds was produced from *Aspergillus* sp (SbD5) an endophyitic fungus from leaves of sambiloto and ampicillin against *S. aureus, E. coli, S. dysenteriae,* and *S. typhi*

Samples (500 µg mL^{-1})	Diameter of clear zone (mm)			
	S. aureus	*E. coli*	*S. dysenteriae*	*S. typhi*
Compound 1	11.6 ± 0.3	12.3 ± 0.4	11.8 ± 0.4	12.1 ± 0.3
Compound 2	11.2 ± 0.1	10.1 ± 0.3	9.3 ± 0.3	9.7 ± 0.2
Compound 3	9.3 ± 0.2	8.9 ± 0.2	8.1 ± 0.3	8.2 ± 0.3
Ampicillin (positive control)	16.8 ± 0.3	18.5 ± 0.4	17.9 ± 0.3	18.1 ± 0.3
Ethanol (negative control)	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

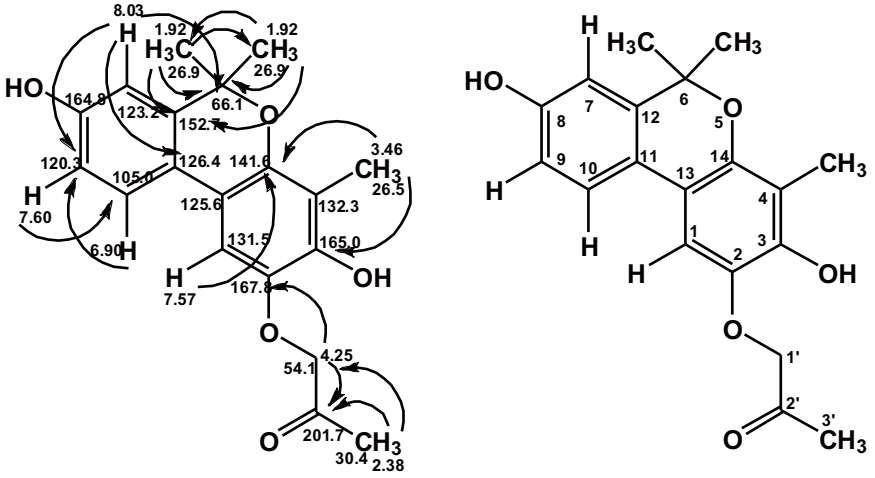

Fig 2 The HMBC correlation and δ-assignment of compound **1**.

carbon atoms bearing hydroxyl aromatic groups (δ_C 164.8 and 165.0 ppm) and one quarternary carbon binding cyclic ether group at δ_C 66.1 ppm. One of the signal is the aromatik carbon bearing O-ether group as side chains (δ_C 167.8 ppm) and one signal is the carbonyl carbon at the side chains (δ_C 201.7 ppm). Thirteen are in the chemical shifts above 100 ppm indicate the presence of seven double bonds, one of the double bond carbonyl carbon and six double bond of two aromatic rings. These data indicate that compound **1** has a structure of two aromatic rings and a cyclic ether in which the side chain attached to the aromatic ring which has ether groups and carbonyl groups. Two hydroxyl and one methyl groups is also bonded to the aromatic ring. While the other two methyl groups attached to the quaternary carbon in the cyclic ether ring.

The HMBC spectrum showed that the gem dimethyl correlated each other and bonded to quaternary carbon at δ_C 66.1 ppm and another one methyl group attached to the aromatic ring were correlated with C-5 and C-14. Correlation from H-9 (7.60 ppm; d; 6 Hz) to C-10 and H-10 (6.90; d; 6 Hz) to C-9 showed that both the aromatic protons in the ortho position. While the other two aromatic protons, namely H-1 (7,57 ppm; s) correlated with C-14 and H-7 (8,03ppm; s) correlated with C-6, C-9, C -11, and C-13. Further HMBC correlation from methylene proton H-1' (4.25 ppm; s) to C-2 and C-2' and methyl proton H-3' (2.38 ppm; s) to C-2' and C-1' indicated that the yloxypropan-2-one group bound to the C-2. The HMBC correlation and δ-assignment of compound **1** showed Figure 2.

The compound **2** (5-hydroxy-4-hydroxymethyl-2H-pyran-2-one) and compound **3** (5-hydroxy-2-oxo-2H pyran-4-yl)methyl acetate) were identified by IR, 1D and 2D NMR and in comparison with the literature data (Elfita *et al*. 2014). The NMR data of compound **2** and **3**, recorded at ^{1}H-500MHz; ^{13}C-125 MHz in DMSO and $CDCl_3$ and the literature 3 data showed (Table 2, 3).

The discovery of three secondary metabolites from an endophytic fungal species of medicinal plants sambiloto suggests that an endophytic fungus has a high ability to synthesize more than one secondary metabolites. Seen that endophytic fungi can modify the structure of the resulting secondary metabolites such as compound **3** is the result of esterification of compound **2** according to the mode of enzymatic esterification.

Compound **2** and **3** also produced from endophytic fungus *Trichoderma* sp of brotowali (*Tinaspora crispa*) (Elfita *et al*. 2014). This fact indicates that the two compounds are not specifically produced by endophytic fungi of sambiloto, whereas compound **1** is a benzochromen derivative that may be produced by a typical from endophytic fungi of sambiloto. Compound **1** was not have the same biogenesis pathway with compounds **2** and **3** so that it is possible to find other secondary metabolites from endophytic fungi of different species of the sambiloto.

Antibacterial activity test of all three compounds showed that they all have antibacterial activity below ampicillin activity as positive control. Harlina *et al*. (2013) reported on the interpretation of antibacterial activity as followed: the diameter of clear zone > 15.0 mm was considered as strong; 10.0 to 14.5 mm as moderate and <10 mm as weak. Among the three compounds, compound **1** has moderate antibacterial activity and the highest of the compounds **2** and **3**. This fact may result from molecular structure of compound **1** which is identified as derivative of phenolic compound. Phenolic derivative is well known for high antibacterial activity. Compounds **2** and **3** have a moderate to weak antibacterial activity. The lowest activity is showed by compound **3** which appears that it is due to compound **3** was derived from compound **2** precursors that undergoes esterification. The test result above indicate that reduce of free OH groups of a compound can decrease its antibacterial activity.

Compared to similar compound mevalolactone (isolated from endophytic fungus *Aspergillus* sp EJC08 of *Bauhinia guianensis)*, that has high antibacterial activity against *Escherichia coli, Pseudomonas aeruginosa, Bacillus subtilis*, and *Staphylococcus aureus* (Pinheiro *et al*. 2013). The compound has a saturated ring are suggested to responsible for increase in antibacterial activity. Other similar compounds, vermopyrone (isolated from unidentified fungus No.2533 of *Avicennia marina* Forssk) is inactive antibacterial (Gunatilaka 2006). This compound has a unsaturated ring and no hydroxyl group.

ACKNOWLEDGMENT

The authors are grateful to the Directorate General of Higher Education for research funding through the Hibah Fundamental Grant in 2014.

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