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Antibacterial activity of *Cordyline fruticosa* leaf extracts and its endophytic fungi extracts

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Abstract. *Elfita, Mardiyanto, Fitrya, Larasati JE, Julinar, Widjajanti H, Muharni. 2019. Antibacterial activity of* Cordyline fruticosa *leaf extracts and its endophytic fungi extracts. Biodiversitas 20: 3804-3812.* Endophytic fungi live by forming colonies in plant tissues without harming the host plant. *Cordyline fruticose* has been used as traditional medicine for the treatment of pathogenic bacterial infections; therefore, its endophytic fungi are expected to have similar activity. In this study, *C. fruticosa* leaves were extracted with gradient solvents and evaluated for their antibacterial activity using the Kirby–Bauer method against Gram-negative (*Salmonella typhi, Escherichia coli*) and Gram-positive (*Staphylococcus aureus, Bacillus subtilis*) bacteria. The extracts of endophytic fungi from *C. fruticosa* leaves were evaluated for their antibacterial activity, as well. The endophytic fungus with good antibacterial activity was identified by molecular and phylogenetic methods. Chemical compounds from endophytic fungus DB1 were isolated by chromatography and subsequently determined by spectroscopy. The methanol extract of *C. fruticosa* leaves showed strong antibacterial activity equivalent to the endophytic fungus (DB1). Molecular identification and analysis through the phylogenetic tree showed that the DB1 fungus has a high level of similarity to *Neurospora tetrasperma* strain APBSDSF108. The antibacterial compounds (compound 1 and 2) isolated from the endophytic fungus DB1 were identified as 4-hydroxy-5-phenylpenta-1,3-dien-1-yl acetate and ergosterol, respectively.

Keywords: Antibacterial activity, *Cordyline fruticosa*, endophytic fungi, *Neurospora tetrasperma*

INTRODUCTION

The World Health Organization (WHO) has reported that 90% of bacteria strains are resistant to antibiotics of first choice (Nwakanma et al. 2016). The emergence of pathogenic bacterial resistance to commercial antibiotics has become a serious and worrying health problem throughout the world as new antibiotic-resistant organisms emerge and spread globally every day. This situation encourages researchers to find new and efficient antibacterial metabolites. Therefore, there is a growing interest in screening the antibacterial activity of medicinal plants and their endophytic fungi.

Endophytic fungi are an important reservoir of therapeutically active compounds (Liang et al. 2012; Deshmukh et al. 2015; Pinheiro et al. 2017). Some endophytic fungal species live in specific uninfected plant tissues, such as in the leaves, stems, roots, fruits, flowers, seeds, or rhizomes, and they do not cause damage to the host plant but have mutualistic symbiotic interactions. Endophytic fungi obtain nutrients, protection, and growing sites from host plants. Several endophytic fungal species have been reported to produce antibacterial, antifungal, antiviral, anti-inflammatory, and antitumor bioactive compounds that can protect host plants (Hussain et al. 2014; Bungtongdee et al. 2018; Praptiwi et al. 2018; Ding et al. 2019).

One plant-based traditional medicine for the treatment of infectious diseases is a decoction of *Cordyline fruticosa* leaves. *Cordyline* genus consists of more than 480 species spread throughout tropical and subtropical regions around the world. Based on ethnobotanical information, *C. fruticosa* is used as a hemostatic and also used to treat diarrhea, sore throat, neck pain, toothache, laryngitis, and mammary gland infections (Fouedjou et al. 2014; Fouedjou et al. 2016). Plants used in traditional medicine around the world are studied for their ability as host for endophytic fungi that have antimicrobial potential, and recent developments reported that endophytic fungi are a potential reservoir of bioactive compounds useful for medicinal exploitation (Alshaibani et al. 2017; Liang et al. 2012; Chi et al. 2019).

Endophytic fungi have a tremendous ability to produce chemical compounds as their host plant, and the biological activity of compounds from endophytic fungi are often found to correspond to the biological activity of its host's compounds (Budiono et al. 2019; Fadhilla et al. 2019). In the previous research, two compounds from endophytic fungi associated with sambiloto (*Andrographis paniculata* Nees), i.e., 7-hydroxypiranopyridine-4-one and 1- (3,8-dihydroxy-4,6,6-trimethyl-6H-benzochromene-2-yloxy) propane-2-one, have stronger antimalarial and antibacterial activity than the sambiloto extracts (Elfita et al. 2011; Elfita et al. 2015). Continuing our important work on

screening for antibacterial activity in medicinal plant extracts and their endophytic fungi, we found that ethyl acetate (EtOAc) extract from endophytic culture of *Neurospora tetrasperma* isolated from medicinal plant *C. fruticosa* showed antibacterial activity equivalent to methanol extract of the host plant. Details of the bioassay, isolation of pure compounds, and identification of their molecular structures are described in this paper.

MATERIALS AND METHODS

Material

Fresh leaves of *Cordyline fruticosa* were collected in November 2018 from Inderalaya, Ogan Ilir, South Sumatra, Indonesia, and identified at the Botany Laboratory, University of Sriwijaya, Indonesia (specimen number VIC 2685).

Extraction of *Cordyline fruticosa* leaves

The dried powder of *C. fruticosa* leaves (50 g) was extracted by maceration in a gradient solvent system, namely n-hexane, ethyl acetate (EtOAc), and methanol, for 24 hours each. Maceration was carried out three times, and each extract was evaporated with a rotary evaporator to obtain concentrated extracts.

Isolation of endophytic fungi

Isolation of endophytic fungi follows the protocols described by Liang et al. (2012) and Kalyanasundaram et al. (2015). Fresh leaves of *C. fruticosa* were rinsed with tap water, and the surface was sterilized by immersion in 70% ethyl alcohol (EtOH) for 2 minutes, followed by immersion in 1% sodium hypochlorite for 1 minute and then rinsed three times with sterile water. The fractions of leaves (0.5 × 0.5 cm) were placed on Petri plates containing potato dextrose agar (PDA) medium supplemented with 150 mg/L of chloramphenicol to inhibit bacterial growth. The plates were incubated at 27 ± 2 °C in the dark for 1–2 weeks. The hyphal tips were transferred to new PDA plates and then subcultured on a fresh PDA plate to obtain pure morphotype colonies.

Cultivation of endophytic fungi strains

All strains of the endophytic fungi were cultivated in potato dextrose broth (PDB). Six pieces of mycelia agar plugs (5 x 5 mm^2) were inoculated with ose needles into 200 mL potato dextrose broth (PDB) medium, and then incubated for four weeks at room temperature under static conditions. After the incubation period, mycelia were separated from the broth culture using filter paper. Then, 200 mL of EtOAc was added into the culture medium, and the broth was partitioned; this was repeated three times. Finally, the EtOAc extract was evaporated under reduced pressure using a rotary evaporator to obtain concentrated extracts (Marcellano et al. 2017).

Antibacterial activity of *Cordyline fruticosa* leaf extracts and its endophytic fungi extracts

The antibacterial activity of the extracts was evaluated using the agar disc diffusion method against four bacteria, which were *Salmonella typhi* (IPCCCB.11.669), *Escherichia coli* (ATCC 25922), *Staphylococcus aureus* (ATCC 25923), and *Bacillus subtilis* (ATCC 6633). All of the bacterial suspensions were equivalent to 1.5 x 10^8 CFU/mL, based on the McFarland 0.5 standard. One hundred µl of the bacteria suspensions at a density of 5 x 10^5 CFU/mL were inoculated on Petri plates containing 20 ml Mueller–Hinton agar (MHA). Paper discs (6.0 mm) were impregnated with concentrated extracts at 400 µg/disk in EtOH were placed on inoculated agar plates. Tetracycline 30 µg/disk was used as a positive control. Petri plates were incubated at 37 °C for 24 h. After the incubation period, clear zone inhibition was measured and clear zone inhibition of the extracts compared to standard antibiotics as the activity percentage. Antibacterial activity was classified as strong (inhibition zone ≥ 70%), moderate (inhibition zone 50-70%), or weak (inhibition zone < 50%). The minimum inhibitory concentration (MIC) values of the pure compounds were evaluated by dilution in EtOH to obtain series concentrations of 256, 128, 64, 32, 16, 8, and 4 µg/mL. The inhibition zone > 9 mm indicated that the compound had antibacterial activity at the corresponding concentration (Ding et al. 2019).

Molecular identification of endophytic fungi strain

Endophytic fungi that showed antibacterial activity were identified based on molecular analysis at the Research Center for Biology, Indonesian Institute of Sciences, Cibinong, Indonesia. Total DNA was extracted from the endophytic fungi strain following the protocol described by Hermansyah et al. (2017). The internal transcribed spacer (ITS) region of rDNA was amplified with PCR amplification using primer ITS-4 (5' TCC TCC GCT TAT TGA TAT GC 3') and primer ITS-5 (5' GGA AGT AAA AGT CGT AAC AAG G 3'). The PCR products were purified and sequenced using an automated DNA sequencer (ABI Prism 3130 Genetic Analyzer; Applied Biosystems, Foster City, CA, USA). The identified sequences were compared with the Basic Local Alignment Search Tool (BLAST) database (http://www.ncbi.nlm.nih.gov/BLAST/) to determine taxon/species. The phylogenetic trees were constructed using the neighbor-joining method with PHYLIP version 3.695, and phylogenetic trees were visualized by using the TreeView program with 1,000 bootstrap replicates (Budiono et al. 2019).

Cultivation and isolation of pure compounds from selected endophytic fungi

The endophytic fungi showed the highest antibacterial activity was re-cultivated to obtain more extract. The fungal strain was cultured in 5 x 200 mL of PDB medium, and after the cultivation period, the mycelium was separated from the liquid media. The liquid media was partitioned with EtOAc three times and evaporated to obtain concentrated EtOAc extract (5.7 g). The mycelium was dried in an oven at 60 °C to constant weight, and the mycelium dry powder was extracted by soxhlet with methanol. The methanol extract was evaporated to obtain concentrated methanol extract (4.9 g)

The concentrated EtOAc extract (5 g) of the broth culture was chromatographed on a silica gel column (70-230 mesh, 50 g) and eluted with a gradient solvent system, n-Hexane-EtOAc (10:0→0:10) and ethyl acetate methanol (EtOAc-MeOH) (10:0→0:10), The concentrated MeOH extract (4 g) of the dried biomass was chromatographed on a silica gel column (70-230 mesh, 60 g) and eluted with a gradient solvent system of n-Hexane - EtOAc (10:0→0:10) and EtOAc - MeOH (10:0→0:10), The chemical structures of the compounds were determined by ultraviolet (UV) spectra (Beck DU-7500 UV-visible scanning spectrophotometer; Beckman Coulter, Brea, CA, USA); infrared (IR) spectra (PerkinElmer Fourier transform IR [FT-IR] spectrometer; PerkinElmer, Waltham, MA, USA); nuclear magnetic resonance (NMR) spectra, including ^{1}H-NMR, ^{13}C-NMR, HMQC, and HMBC (Agilent DD2 ^{1}H-500 MHz and ^{13}C-125 MHz; Agilent Technologies, Santa Clara, CA, USA); and mass spectroscopy (MS) (LC-MS ESI positive ion; PerkinElmer, Waltham, MA, USA).

RESULTS AND DISCUSSION

Antibacterial activity

Extraction of the dried powder (50 g) of *C. fruticosa* leaves produced concentrated extracts of n-Hexane (4.2 g), EtOAc (2.8), and EtOH (4.6 g). The antibacterial activity of the EtOH extract (Table 1) shows that it had strong antibacterial activity against three of the tested bacteria (*S. typhi*, *E. coli*, and *S. aureus*).

Six endophytic fungi strains were isolated from *C. fruticosa* leaves, but one isolate did not grow well in PDB medium. Five isolates were labeled as DB1–DB5. Each fungus isolate was cultivated in PDB medium for four weeks, and broth cultures were partitioned using EtOAc to obtain secondary metabolites. Screening for antibacterial activity of these extracts was tested against *S. typhi, E. coli, S. aureus,* and *B. subtilis* (Table 1).

Phylogenetic analysis

Homology sequences of nucleotides were compared with BLAST (http://www.ncbi.nlm.nih.gov). The ClustalW program was used to align nucleotide sequences. Visualization and editing of sequences were carried out with the GeneDoc program. The phylogenetic trees (Figure 1) were constructed using the PHYLIP program and visualized by the TreeView program (Yohandini et al. 2014). The DB1 strain had the highest homology with *Neurospora tetrasperma* strain APBSDSF108. Sequence data of strain DB1 was submitted to GenBank and deposited with the accession number MN341013.

The results of molecular identification in the form of DNA sequencing of Strain DB1 (Contig-DB1) is as follows:

ACAAGGTCTCCGTTGGTGAACCAGCGGGAGGGATCAT
TACAGAGTTGCAAAACTTCCCCACAAACCATCGCGAATC
TTACCCGTACGGTTGCCTCGGCGCTGGCGGTCCGGAAAG
GCCCTCGGGCCCTCCCGGATCCTCGGGTCTCCCGCTCGC
GGGAGGCTGCCCGCCGGAGTGCCGAAACTAAACTCTTGA
TATTTTATGTCTCTCTGAGTAAACTTTTAAATAAGTCAA
AACTTTCAACAACGGATCTCTTGGTTCTGGCATCGATGA
AGAACGCAGCGAAATGCGATAAGTAATGTGAATTGCAGA
ATTCAGTGAATCATCGAATCTTTGAACGCACATTGCGCT
CGCCAGTATTCTGGCGAGCATGCCTGTTCGAGCGTCATT
TCAACCATCAAGCTCTGCTTGCGTTGGGGATCCGCGGCT
GTCCGCGGTCCCTCAAAATCAGTGGCGGGCTCGCTAGTC
ACACCGAGCGTAGTAACTCTACATCGCTATGGTCGTGCG
GCGGGTTCTTGCCGTAAAACCCCCCATTTCTAAGGTTGA
CCTCGGATCAAGTAGGAATACCCGCTGAACTTAAGCAT.

Table 1. Antibacterial activity percentage of *C. fruticosa* leaf extracts and its endophytic fungi extracts (at a concentration of 400 µg/disk) compared to a standard antibiotic (tetracycline)

Sample	Extracts	Antibacterial activity [a]			
		S. typhi	***B. subtilis***	***E. coli***	***S. aureus***
C. fruticosa leaves	n-hexane	7.1 ± 0.8 (36.6*)	6.3 ± 0.2 (30.6*)	9.1 ± 1.4 (46.7*)	11.2 ± 1.7 (53.1*)
	Ethyl acetate	9.6 ± 1.1 (49.5*)	11.3 ± 2.3 (54.8**)	12.5 ± 1.9 (64.1**)	13.8 ± 2.2 (65.4**)
	Methanol	15.1 ± 1.4 (77.8***)	12.6 ± 0.9 (61.2**)	16.7 ± 3.2 (85.6***)	17.0 ± 2.6 (80.6***)
Endophytic fungi	DB1	14.2 ± 1.1 (73.2***)	14.7 ± 2.9 (71.4***)	16.0 ± 2.5 (82.0***)	14.3 ± 0.7 (67.8**)
	DB2	11.3 ± 2.3 (58.2**)	11.8 ± 1.2 (57.3**)	10.1 ± 0.7 (51.8**)	9.8 ± 0.8 (46.4*)
	DB3	-	-	7.2 ± 1.1 (36.9*)	-
	DB4	7.7 ± 1.3 (39.7*)	-	8.1 ± 1.3 (41.5*)	-
	DB5	-	7.3 ± 2.4 (35.4*)	-	-
Antibiotic	Tetracycline[b]	19.4 ± 3.2	20.6 ± 2.4	19.5 ± 1.9	21.1 ± 3.3

Note: [a]Antibacterial activity percentage of extracts compared to tetracycline (%): inhibition zone (mm) of extract/ inhibition zone (mm) of tetracycline: *** strong (≥ 70%), **moderate (50-70%), and *weak (< 50%) against *S. typhi, B. subtilis, E. coli,* and *S. aureus.* [b]positive controls (30 µg/disk). (-) no inhibition zone

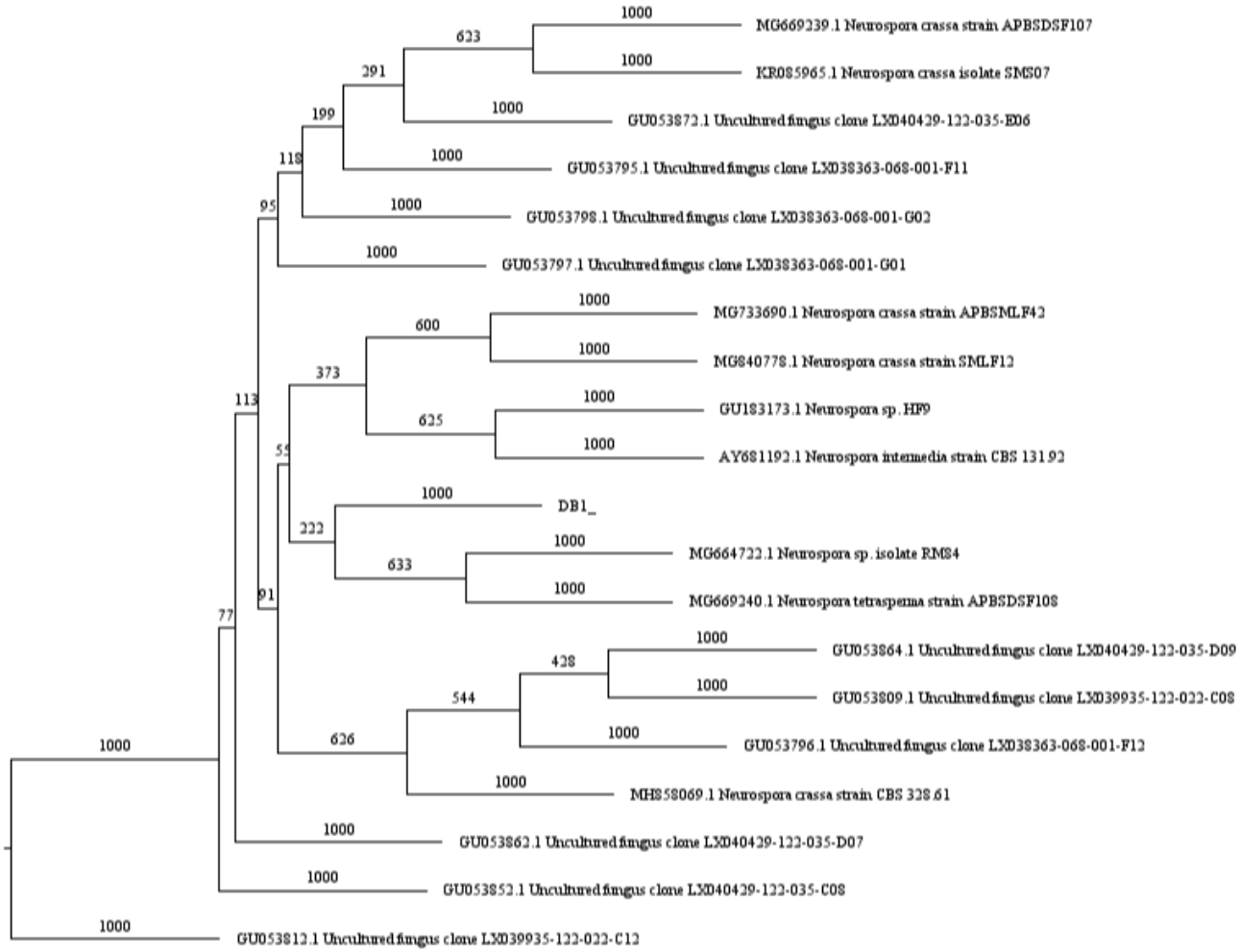

Figure 1. Phylogenetic tree of the DB1strain

Chemical compounds from endophytic fungi

Neurospora tetrasperma

Separation of concentrated EtOAc extract (5 g) by column chromatography, yielded five subfractions (A–E). Fraction C (1.1 g) was subjected to a silica gel column (70-230 mesh, 20 g) with a solvent system n-Hexane - EtOAc (5:5→0:10) as the eluent and yielded four fractions (C1–C4). Fraction C3 (120 mg) was subjected to a silica gel column (70-230 mesh, 10 g) and eluted with n-Hexane - EtOAc (3:7) to yield compound **1** (32 mg).

Separation of concentrated MeOH extract (4 g), yielded four subfractions (A–D). Subfraction B (0.83 g) was separated over a silica gel (230-400 mesh, 20 g) and eluted with a solvent system of n-Hexane - EtOAc (8:2→3:7) to give four fractions (B1–B4). Subfraction B3 (126 mg) was subjected to a silica gel column (70-230 mesh, 10 g) to give three fractions (B3.1–B3.3). Subfraction B3.2 (58 mg) was purified with re-crystallization to yield compound **2** (white crystals, 41 mg).

The result of column chromatography of the EtOAc extract from the endophytic fungi broth culture was successfully isolated compound **1** that revealed as an aromatic ester. Column chromatography of the MeOH extract from the biomass successfully isolated compound **2** and based on the spectroscopic data, compound **2** was identified as steroid ergosterol. The MIC values of the pure compounds were shown in Table 2.

Compound 1. White crystals; mp. 111-113ºC; UV (MeOH) λ_{max} 216, 248 nm; UV (MeOH + NaOH) λ_{max} 216, 248 nm; IR (KBr) ν_{max} cm^{-1}: 3371.6 (OH), 3064.9 (CH-aromatic), 2922.2, 2852.7 (CH-aliphatic), 1664.9 (C=O esther), 1604.8 (C = C aromatic), 1259.5 (C = O esther); ^{1}H, ^{13}C NMR : see Table 3.; LCMS –ESI m/z= 219.13 $[M + H]^{+}$.

Table 2. MIC values of tetracycline, compound **1** and **2** against four tested bacteria

Compound	MIC values (µg/mL)			
	S. typhi	***B. subtilis***	***E. coli***	***S. aureus***
1	128	128	64	> 256
2	> 256	> 256	> 256	> 256
Tetracycline	4	4	4	4

The inhibition zone > 9 mm indicated that the compound has antibacterial activity at the corresponding concentration

Compound 2. White crystal; mp. 126-128°C ; UV (MeOH) λ_{max}: no absorption; IR (KBr) ν_{max} cm^{-1}: 3429.4 (OH), 29586.9 and 2870.1 (CH-aliphatic), 1627.9 (C=C), 1460.1 (bending CH_2 cyclic) and 1373.3 for $–CH_2$ (CH_3)2γ), 1033.85 (cycloalkane); NMR (^{1}H-500 MHz; ^{13}C-125 MHz; in $CDCl_3$) see Table 4.

The ^{1}H-NMR data (Table 3) showed the presence of three signals in the aromatic region at δ_H 7.2–7.7 ppm for five protons. The signals indicated the presence of an aromatic group with mono substitution. Other signals appeared at δ_H 7.67 (1H; d; J = 5.85 Hz); 6.27 (1H; dd; J = 2.45; 5.85 Hz); and 6.14 ppm (1H; d; J = 2.45 Hz), which indicate a side chain that has three vinyl protons located at C = C conjugated. The appearance of a signal at δ_H 3.81 ppm (2H; s) suggested that the side chain was connected through a methylene group attached directly to the aromatic ring. The signal at δ_H 1.25 ppm (3H; s) was a methyl ester group in the side chain.

The C-NMR and HMQC spectra (Table 3 and Figure 2) showed the presence of 11 signals for 13 carbons, two of which were C sp^3. The signal of two carbons at δ_C 129.1 and 129.2 ppm is carbon equivalent to four carbon methines. The presence of one other methine carbon signal at δ_C 127.7 ppm indicated that compound **1** was in the aromatic group with mono substitution. The spectrum also revealed the presence of a carbon carbonyl ester at δ_C 179.3 ppm and two oxyarylcarbons at 155.3 and 168.2 ppm. The two oxyaryl carbons consisted of oxyaryl to ester and oxyaryl to hydroxyl groups. These signals indicated the presence of ester side chain conjugates with two double bonds (C = C).

The HMBC spectrum (Table 2 and Figure 2) showed the presence of a ^{1}H-^{13}C correlation between the proton signal at δ_H 3.81 ppm (methylene signal) and the aromatic carbon δ_C 129.2; 134.6; 115.7 ppm. It indicates that the methylene group in the side chain was attached directly to the aromatic ring. Two bonds also separated the methylene proton with oxyaryl carbon (δ_C 168.2) and the three bonds with carbon methine (δ_C 115.7 ppm). The oxyarylmethine proton (δ_H 7.67 ppm) showed a long-distance correlation with oxyaryl carbon δ_C 168.2 ppm. Based on the spectroscopic analysis, compound **1** was identified as 4-hydroxy-5-phenylpenta-1,3-dien-1-yl acetate. The correlation between the structures of compound **1** and HMBC is shown in Figure 3.

The steroid characteristics of compound **2** (Figure 4 and Table 4) can be identified in the H-NMR spectrum by the presence of six methyl signals at δ_H 0.5-1.0 ppm. These signals include two methyl singlets found in the steroids skeleton and four methyl doublets found in the side chains. The two vinyl protons at δ_H 5.38 (1H, d, J = 8.4 Hz) and 5.57 ppm (1H, d, J = 8.4 Hz) were mutually coupled in the steroids skeleton. Furthermore, two vinyl protons appear at δ_H 5.19 (1H, m) and 5.22 (1H, m) attached to the side chain. The typical proton signal for steroids is shown by the presence of an oxygenated methine at δ_H 3.63 ppm (1 H, m) at C3.

Table 3. ^{1}H and ^{13}C NMR data of compound **1** (**(**^{1}H-500 MHz, ^{13}C-125 Mhz, in $CDCl_3$), **δ** in ppm, J in Hz

No. C	δ_C 1	δ_H (ΣH, multiplicity, *J*) 1	HMBC
1	155.3	7.67 (1H; d; J=5.85 Hz)	116.8; 168.2; 179.3
2	116.8	6.27 (1H; dd; J=2.45; 5.85 Hz)	155.3
3	115.7	6.14 (1H; d; J=2.45 Hz)	168.2
4	168.2		
5	40.2	3.81 (2H; s)	129.2; 134.6; 115.7; 168.2
1’	134.6		
2’	129.2	7.23 (1H; m)	127.7; 129.1; 40.2
3’	129.1	7.35 (1H; m)	129.2; 134.6
4’	127.7	7.30 (1H; m)	129.2
5’	129.1	7.35 (1H; m)	129.2; 134.6
6’	129.2	7.23 (1H; m)	127.7; 129.1; 40.2
1”	179.3		
2”	29.8	1.25 (3H; s)	

Table 4. ^{1}H and ^{13}C NMR data of compound **2** (^{1}H-500 MHz, ^{13}C-125 Mhz, in $CDCl_3$) and ergosterol[b] (^{1}H-400 MHz, ^{13}C-100 Mhz, in $CDCl_3$ + CD_3OD, ppm) (Martinez et al. 2015)

No. C	^{13}C-NMR 2	^{1}H-NMR 2	DEPT 2	^{13}C-NMR[b]
1	38.5		CH_2	38.4
2	32.1		CH_2	32.0
3	70.6	3.63 (1H, m)	CH	70.4
4	40.9		CH_2	40.8
5	139.9		C	139.8
6	119.7	5.38 (1H, d, J=8.4 Hz)	CH	119.6
7	116.4	5.57 (1H, d, J=8.4 Hz)	CH	116.3
8	141.5		C	141.3
9	46.4		CH	46.2
10	37.2		C	37.0
11	21.2		CH_2	21.1
12	39.2		CH_2	39.1
13	42.9		C	42.8
14	54.7		CH	54.6
15	23.2		CH_2	23.0
16	28.4		CH_2	28.3
17	55.8		CH	55.7
18	12.2	0.63 (3H, s)	CH_3	12.0
19	17.8	0.94 (3H, s)	CH_3	17.6
20	40.6		CH	40.4
21	21.2	1.04 (3H, d, J=6.6 Hz)	CH_3	21.1
22	135.7	5.22 (1H, m)	CH	135.6
23	132.1	5.19 (1H, m)	CH	132.0
24	43.0		CH	42.8
25	33.2		CH	33.1
26	19.8	0.83 (3H, d, J=7.15 Hz)	CH_3	19.6
27	20.1	0.83 (3H, d, J=7.15 Hz)	CH_3	19.9
28	16.4	0.92 (3H, d, J=6.8 Hz)	CH_3	16.2

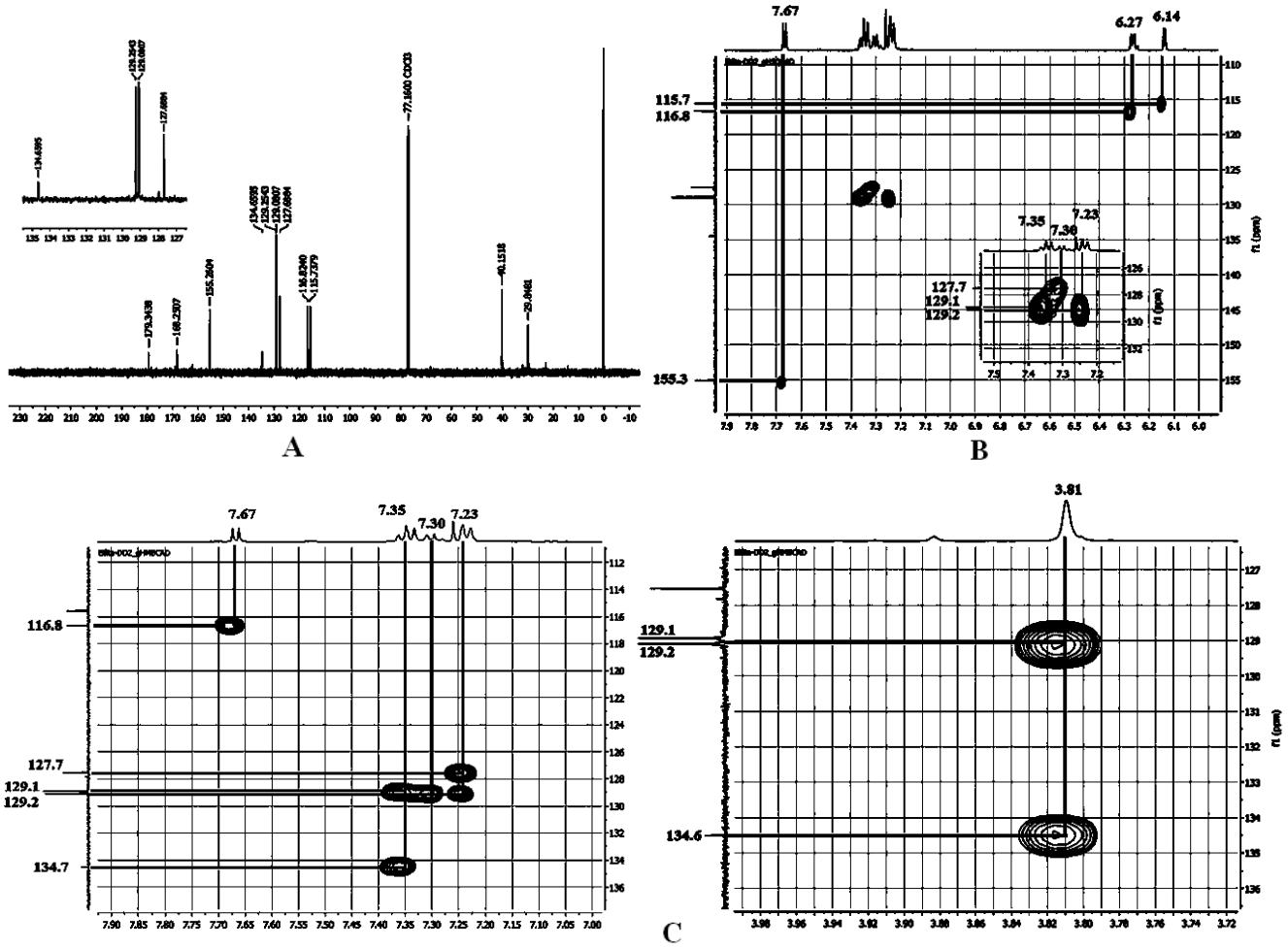

Figure 2. The ^{13}C-NMR (A), HSQC (B), and HMBC (C) spectra of compound **1** (^{1}H-500 MHz, ^{13}C-125 MHz, in $CDCl_3$)

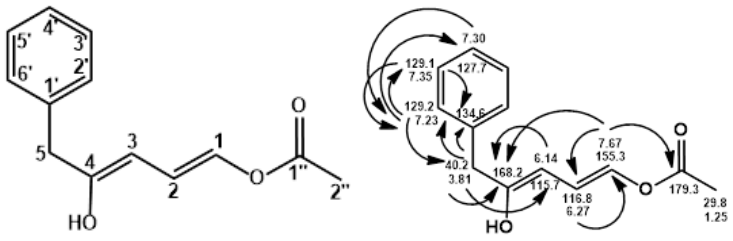

Figure 3. Structure of 4-hydroxy-5-phenylpenta-1,3-dien-1-yl acetate and HMBC correlation

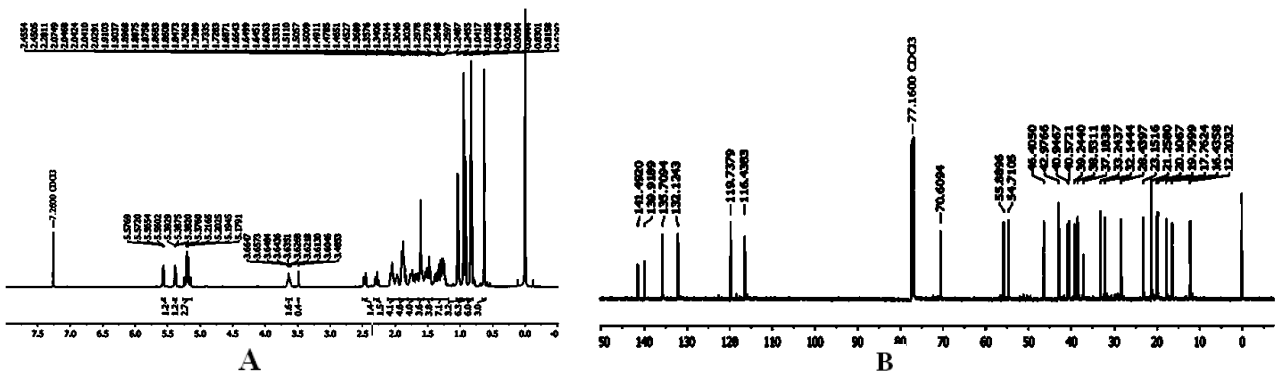

Figure 4. The ^{1}H-NMR (A) and ^{13}C-NMR (B) spectra of compound **2** (^{1}H-500 MHz, ^{13}C-125 MHz, in $CDCl_3$)

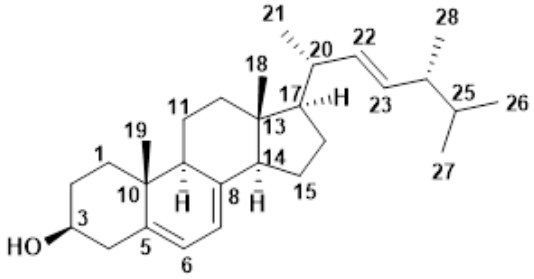

Figure 5. Structure of ergosta-5,7,22-trien-3β-ol (ergosterol)

The C-NMR spectrum showed the presence of 28 carbon signals consisting of six C sp^2 at δ_C 116.4, 119.7, 132.1, 135.7, 139.9, and 141.5 ppm. The signals indicate that steroids have two double bonds conjugating in the skeleton and one double bond on the side chain. Four C sp^2 conjugated in the skeleton consisted of two quaternary carbons and two methyl carbons. The spectrum showed the existence of one carbon bound to heteroatoms (OH groups), specifically, at δ_C 70.6 ppm. Based on the spectroscopic analysis and compared with data in the literature, compound **2** was ergosta-5,7,22-trien-3β-ol (ergosterol). The structure of compound **2** was shown in Figure 5.

Discussion

Antibacterial activity of *C. fruticosa* leaf extracts (Table 1) showed that methanol extract had strong antibacterial activity against *S. typhi*, *E. coli*, and *S. aureus*. The antibacterial activity of methanol extract is related to the chemical content of *C. fruticosa* leaves, namely, the flavonoids farrerol, quercetin-3-O-[6"trans-p-Coumaroyl-β-D-glucopyranoside] (helichrysoside), Apigenin-8-C-glucoside, quercetin-3-O-β-D-glucopyranoside, and quercetin-3-O-α-L-rhamnopyranosyl- (1-6)-β-D-glucopyranoside (rutin) (Fouedjou et al. 2016). Flavonoids are compounds that have a variety of biological activities, including antibacterial (Cushnie and Lamb 2005). Farreror has been commonly used in traditional Chinese medicine as an antibechic and antibacterial agent, and farrerol can inhibit the hemolysis of *S. aureus* by reducing the level of α-toxin production with the MIC value ranged from 4 to 16 μg/mL against 35 *S.aureus* strains (Qiu et al. 2011).

The methanol extract has higher antibacterial activity than the n-Hexane and EtOAc extract that may be due to the secondary metabolites in methanol extract that are more varied than other extracts. This is because the gradient fractionation causes non-polar and semi-polar compounds to be extracted into the n-hexane and ethyl acetate fractions. In contrast, the polar compounds with more variations are extracted into the methanol fraction. The synergistic effect of several compounds within extract often occurs in the methanol fraction, because the methanol fraction is more varied with secondary metabolite compounds than the n-hexane and ethyl acetate fractions. The more variations of secondary metabolite compounds will be able to increase the potential for synergy. Research conducted by Amin et al. (2015) that combined antibacterial compound quercetin with morin and routine that is not active against *S. aureus* (ATCC 43300) showed that the combination of all three flavonoids could improve antibacterial activity. The structure of the compound influences the synergistic effect, so the researchers combined resistant antibiotics with companion compounds to increase the working power of these antibiotics (Amin et al. 2015).

The antibacterial activity of endophytic fungi extracts from *C. fruticosa* leaves showed that the DB1 extract (at a concentration of 400 μg/disc) exhibited the highest level of growth inhibition (strong: ***) against *S. tiphi*, *B. subtilis*, and *E. coli*. The extract of DB2 had moderate (**) inhibitory effects on the growth of *S. tiphi*, *E. coli*, and *B. subtilis* and was weak (*) against *S. aureus*, while the other three endophytic extracts (DB3-DB5) showed weak antibacterial activity or no growth inhibition against the tested bacteria.

The antibacterial activity of the methanol extract of *C. fruticosa* leaves was comparable to the antibacterial activity of the endophytic fungal extract DB1 which has strong antibacterial against 3 tested bacteria and moderate antibacterial against one tested bacteria. Plants that have strong antibacterial activity may also be a potential source of antibacterial compounds from their endophytic fungus (Elfita et al., data not shown).

Results of the antibacterial assay showed that compound **1** has good antibacterial activity against *E. coli* (MIC 64 μg/ml) and moderate activity against *S. typhi* (MIC 128 μg/ml) and *B. subtilis* (MIC 128 μg/ml), while DB2 showed no activity against the four tested bacteria. Pure compound with MIC value <100 μg/mL categorized as good antibacterial (Pinheiro et al. (2017).

The antibacterial activity of compound **2,** when compared to the antibacterial activity of several other compounds from the ergostanone group produced by *Colletotrichum* isolated from *Ilex canariensis*, showed the differences in antibacterial activity. The six ergostanone compounds showed good antibacterial activity against *E. coli* and *Bacillus megaterium* at 0.05 μg/disc (5 μg/ml) (Deshmukh et al. 2015). Compound **2** and the six ergostanone compounds have the same skeletal structure, but different in the positions of the carbonyl, hydroxyl, and double bond groups, where the double bond and OH groups can affect antibacterial activity (Fouedjou et al. 2014). Compound **1** that isolated from the broth culture, is a new compound. Eight compounds have been reported from *C. fruticosa* leaves such as three steroidal saponins, isoquercitrin, helichrysoside, quercetin 3-rutinoside, apigenin 8- C-b-D-glucopyranoside, and farrerol (Fouedjou et al. 2014). However, the good antibacterial activity of compound **1** correlates with the good activity of the host. The results of this study indicate that endophytic fungi isolated from *C. fruticosa* leaves produce different bioactive compounds than those originating from the host plant, they have similar activities. It is known that some endophytic fungi have the ability to produce bioactive compounds that are the same or similar to compounds

produced by their host plants. For instance, taxol is a well-known and widely used tetracyclic diterpenoid compound as an anticancer agent produced from types of yew trees (*Taxus* spp.). Similarly, podophyllotoxin compound is an aryl tetralin lignan compound with anticancer, antiviral, antioxidant, antibacterial, antirheumatic, and immunostimulating properties produced by *Sinopodophyllum* plant (Zhao et al. 2010; Garyali et al. 2013).

However, there are many compounds from endophytic fungi that are different from the compounds produced by the host plant but they have strong biological activity. The endophytic fungus Alternaria sp. from the medicinal plant *Morinda officinalis* produces 13 compounds that don't have a chemotaxonomic relationship with its host plant. However, these compounds have inhibitory activities against four tumor cell lines and α-glucosidase (Wang et al. 2017; Lee et al. 2017). Calcul et al. (2013) reported the antimalarial compounds produced by endophytic fungi from mangroves. Previously, Ravikumar et al. (2011) also reported the antimalarial activity and the chemical content of mangrove plants is alkaloids, triterpenes, flavonoids, tannins, catechins, anthraquinones, phenols, sugars, and proteins. All of these classes of compounds do not have a chemotaxonomic relationship to the compounds produced by their endophytic fungi.

Results in this study showed that methanol extract of *C. fruticosa* leaves possess good antibacterial activity, and the endophytic fungus DB1 that identified as *N. tetrasperma* also has the potential as an antibacterial source. Therefore, *C. fruticosa* and the endophytic *N. tetrasperma* have the prospect of being developed as an antibacterial agent.

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