Artikel Penelitian

Chemical Constituents and Antibacterial Activities of Leaves of Sumatran King Fern (*Angiopteris evecta* G. Forst HOFFM.)

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ABSTRACT: *4,5-dihydro-4-hydroxy-5-methyl-1H-pyran-1-one* ***(2)****, angiopteroside* ***(3)*** *and well known stigmast-5-en-3-β-ol* ***(1)*** *have been isolated from the leaves of Sumatran King fern Angiopteris evecta G. Forst HOFFM. and investigated for their antimicrobial activities towards some human pathogenic bacteria. Angiopteroside* ***(3)*** *showed potential activities to inhibit the growth of Bacillus subtilis.*

Keywords: *Angiopteris evecta, 4,5-dihydro-4-hydroxy-5-methyl-1H-pyran-1-one, angiopteroside, antibacterial*

ABSTRAK: 4,5-dihidro-4-hidroksi-5-metil-1H-piran-1-one **(2)**, angiopteroside **(3)** dan senyawa yang umum stigmast-5-en-3-β-ol **(1)** berhasil diisolasi dari daun tumbuhan paku gajah *Angiopteris evecta* G. Forst HOFFM. dan diuji aktivitas antibakterinya terhadap beberapa bakteri patogen manusia. Angiopteroside **(3)** menunjukan aktivitas daya hambat terhadap pertumbuhan bakteri *Bacillus subtilis*.

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Kata kunci: *Angiopteris evecta*, 4,5-dihidro-4-hidroksi-5-metil-1H-piran-1-one, angiopteroside, antibakteri

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INTRODUCTION

The ferns (Pteridophyta) are spread worldwide, especially in the humid tropics area. On the earth grows about 10.000 species of ferns and from that amount, Malaesian area which mostly consists of Indonesian islands is estimated to have approximately 1.300 species (1,2).

During ethnobotanical survey of Sumatran plants last year, one fern locally called "Paku Gajah" (Elephant fern, *Angiopteris evecta*) was found. Interestingly, traditional use of this fern is different from one location to another. *A. evecta* also known as *Polypodium evectum* G. Forst (3) belongs to the family Angiopteridaceae, a common, large terrestrial herb (Pteridophyte plant) which can be found in a well shaded and humid areas of forests near water sources. *A. evecta* is traditionally used for a folk medicine in many places. In Malaysia, the leaves is used for cough (4) and in India, the roots is used for scabies (5).

There were some studies about its bioactivities such as antibacterial (6), antibacterial for cutis diseases (7), antifungal (8) and antituberculosis (9), but only one reference found in literature reporting isolation of its chemical constituent from its rhizomes which was identified as angiopteroside **(3)** and this compound exhibited significant activity to inhibit the growth of HIV-1 Reverse Transcriptase (IC_{50} 91 μM) (10).

In this study, we tried to isolate the major compounds of the leaves and to investigate their antibacterial activities. Interestingly, in our work we found that angiopteroside (3) gave significant antibacterial activity against *Bacillus subtilis* and so far, this is the first report of antibacterial activities from this compound.

MATERIAL AND METHODS

Materials

Plant material: leaves (2.8 kg) of *A. evecta* were collected in Maek, Payakumbuh, West Sumatera in January 2013. Herbarium specimen (13-002-003-01-07) was identified by Prof. Dr. Syamsuardi from Herbarium Andalas University (ANDA).

Testing microbes : *Bacillus subtilis* ATCC 6633, *Enterococcus faecalis* ATCC 29212, *Eschericia coli* ATCC 25922, *Micrococcus luteus* ATCC 10240, *Pseudomonas aureginosa* ATCC 27853, *Salmonella typhosa* NCTC 786, *Salmonella typhimurium* ATCC 14028, *Staphylococcus aureus* ATCC 25923, *Staphylococcus epidermidis* ATCC 12228, *Streptococcus mutans* ATCC 25175 and *Vibrio cholerae* Inaba, were provided by Indonesian Food and Drug Administration (BPOM) Padang and Pekanbaru offices and Microbiology Laboratory of Medical Faculty of Andalas University and were pre-cultured before being used.

All solvents were distilled before being used, nutrient agar (NA) (Merck), paper disc (Whatman), dimethyl sulfoxide (DMSO) (Merck) and chloramphenicol (Sigma). TLC was carried out using silica gel $60F_{254}$ (Merck) and visualized under UV light (254 nm). Column chromatography was performed on silica gel 60 (0.063-0.200 nm) (Merck).

Instrumentation

Melting points were measured on a Sybron Thermolyne Melting Point Apparatus MP-12615 and are uncorrected. FT-IR spectra on Thermo Scientific Nicolet iS10 Smart OMNI-Transmission, UV spectra recorded on Shimadzu Spectrophotometer UV-Vis Pharmaspec 1700, ^{1}H and ^{13}C NMR spectra were recorded with an Agilent DD2 system (Agilent Technologies, Santa Clara, CA, USA) operating at 500 (^{1}H) and 125 (^{13}C) MHz using residual and deuterated solvents as reference standard. TLC was carried out using silica gel $60F_{254}$ (Merck) and visualized under UV light (254 nm). Column chromatography was performed on silica gel 60 (0,063 – 0,200 nm) (Merck).

Methods

Extraction and Isolation

Fresh leaves (2.8 kg) of *A. evecta* were chopped and macerated with methanol (MeOH) (10 L) for 7 days. After separation of solvents, the process

was repeated twice more. The combined MeOH extracts were evaporated *in vacuo* and succesively partitioned into hexane, ethyl acetate (EtOAc), and butanol (BuOH), then evaporated to give hexane (8.23 g), EtOAc (6 g), and BuOH (9 g) fractions.

The hexane fraction were preadsorbed on SiO_2 then column chromatographed on the same adsorbent and eluted with step gradient polarity solvents started from hexane, hexane-EtOAc. Fractions having similar TLC pattern were combined and recrystallized from hexane-EtOAc to give colorless needles (21.9 mg). Based on its spectroscopic data this compound was identified as stigmast-5-en-3-β-ol **(1)**, MP: 130-132^{0}C, Undepressed the mixed melting point with the authentic sample. The ^{13}C NMR data can be seen on Table 1.

The EtOAc fraction was preadsorbed on SiO_2 then column chromatographed on the same adsorbent and eluted with step gradient polarity solvents started from hexane, hexane-EtOAc, EtOAc, EtOAc-MeOH to give 7 subfractions (AELE 1-7). Subfractions (AELE 5) which have the same behavior on TLC were combined and rechromatographed as above. Fractions having similar

Table 1. ^{13}C NMR data of compound **(1)** (125 MHz/ $CDCl_3$) and Stigmast-5-en-3-β-ol (12)

No.	Function Group	δC (ppm)	
		Compound 1	Stigmast-5-en-3-β-ol
1	CH_2	37.3	37.28
2	CH_2	29.2	31.69
3	CH	71.8	71.82
4	CH_2	42.3	42.33
5	C	140.8	140.74
6	CH	121.7	121.72
7	CH_2	31.7	31.69
8	CH	31.9	31.93
9	CH	50.1	50.17
10	C	36.5	36.52
11	CH_2	21.1	21.1
12	CH_2	39.8	39.8
13	C	42.3	42.31
14	CH	56.8	56.79
15	CH_2	23.1	24.37
16	CH_2	26.1	28.25
17	CH	56.1	56.09
18	CH_3	11.9	11.86
19	CH_3	19.4	19.4
20	CH	36.2	36.52
21	CH_3	18.8	18.79
22	CH_2	34	33.98
23	CH_3	26.1	26.14
24	CH	45.8	45.88
25	CH	28.3	28.91
26	CH_3	19.8	19.8
27	CH_3	19	18.79
28	CH_2	21.1	23.1
29	CH_3	12.0	11.99

Table 2. NMR data of Compound **(2)** and 4,5-dihydro-4-hydroxy-5-methyl-1H-pyran-1-one (13)

No.	Compound 2 ($CDCl_3$)		4,5-dihydro-4-hydroxy-5-methyl-1H-pyran-one (CD_3OD)	
	C (125 MHz)	H (500 MHz)	C (125 MHz)	H (500 MHz)
1	164	-	167.1	-
2	122.7	6.11(1H, d, J=9.5 Hz)	123.2	6.1 (1H, dd, J=9.7, 2 Hz)
3	144.6	7.02 (1H, dd, J=9.5, 5.5 Hz)	147.9	7.12 (1H, dd, J=9.7, 2.3 Hz)
4	63	4.03 (1H, s, br)	63.7	4.1 (1H,m)
5	77.2	4.55 (1H, dq, J= 6.5, 4 Hz)	79.3	4.62 (1H,m)
6	15.8	1.56 (3H, d, J=6.5)	16.8	1.45 (3H,d, J=6.7 Hz)

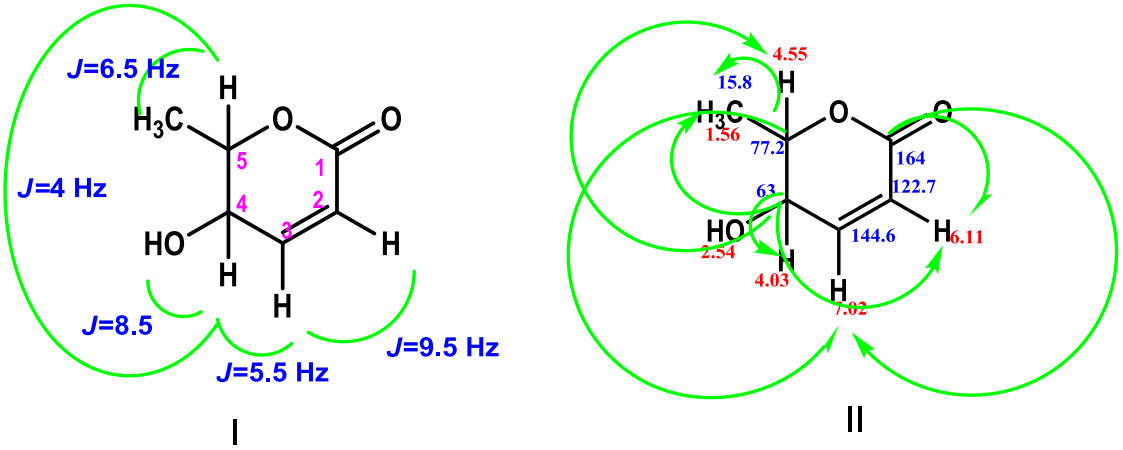

Figure 1. COSY (I) and HMBC (II) of Compound **(2)**

pattern were combined, evaporated, then passed through sephadex LH20 column and finally purified by using preparative HPLC to yield colorless gum **(2)** (16 mg). IR (KBr) spectrum showed absorptions at 3412 (O-H), 2989 (C-H), 1709 (C=O), 1386 (C-H), 1266 (C-O-C), 1060 (C-O) (cm^{-1}). The UV spectrum showed absorbance at λ_{max} (nm, log ε): 376 (4.85), 371 (4.37), 354 (4.68), 322 (5.00), 306 (2.11), 276 (2.77), 207 (4.62). Based on its spectroscopic data this compound was identified as 4,5-dihydro-4-hydroxy-5-methyl-1H-pyran-1-one. The ^{1}H dan ^{13}C NMR data can be seen on Table 2.

In order to confirm its structure this compound **(2)** (10 mg) was acetylated with acetic anhydride (1 ml) in pyridine (1 ml) by stirring the mixture overnight using magnetic stirrer. Ice (about 2 g) was added and let it stand until the ice melt then the mixture was evaporated *in vacuo* to dryness. The acetylated product compound **(2a)** showed one spot on TLC and obtained as colorless gum (12 mg). IR (KBr) spectrum showed absorbtions at 3463 (O-H), 2991 (C-H), 1736 (C=O), 1228 (-OCH_3), 1061 (-C-O-), 825 (C=C-H). The UV spectrum showed absorbance at λ_{max} (nm, log ε): 342 (1.81), 306 (2.01), 276 (2.68), 207 (4.53). Based on its spectroscopic data this compound was identified as 4,5-dihydro-4-acetyl-5-methyl-1H-pyran-1-one. The chemical shifts as well as correlation of its proton and carbon atoms can be seen on Figure 2.

BuOH fraction was preadsorbed on SiO_2 then column chromatograped on the same adsorbent and eluted with step gradient polarity solvents from 5% acetic acid in EtOAc, then with 5% acetic acid MeOH to give 9 subfractions. Subfractions 4

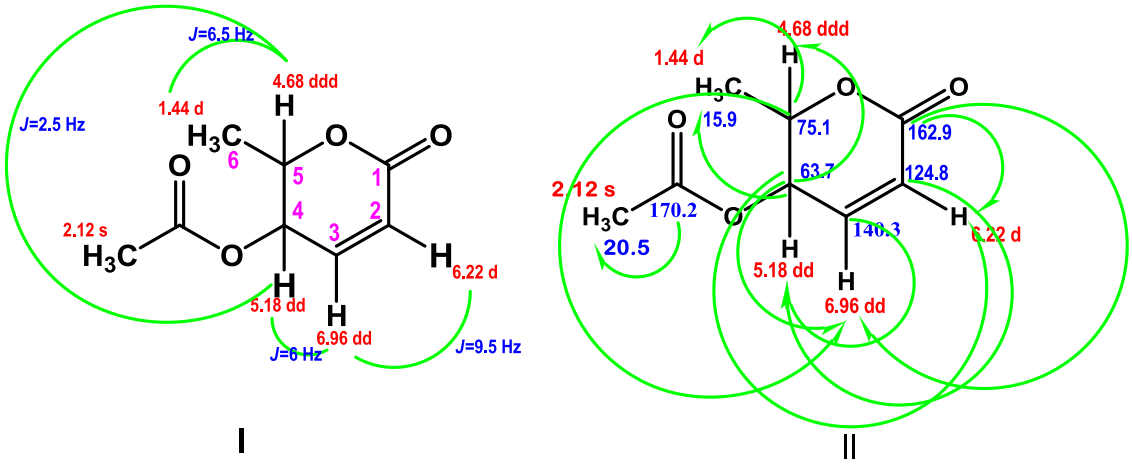

Figure 2. COSY (I) and HMBC (II) NMR data of Compound **(2a)**

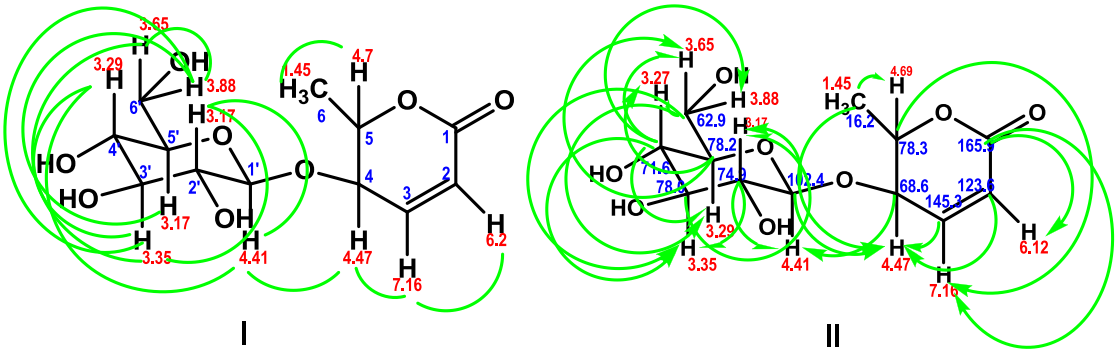

Figure 3. COSY (I) and HMBC (II) of Compound **(3)**

Table 3. ^{1}H and ^{13}C NMR data of compound **(3)** and angiopteroside (6)

No.	Compound 3 (CD_3OD)		Angiopteroside (DMSO-*d*6)	
	C (125 MHz)	H (500 MHz)	C (125 MHz)	H (500 MHz)
1	165.9	-	165.9	-
2	123.6	6.2 (d, *J*=10 Hz)	123.6	6.12 (d, *J*=9.9 Hz)
3	145.3	7.16 (dd, *J*=5 and 10 Hz)	145.3	7.16 (dd, J=4.9 and 9.8 Hz)
4	68.6	4.47(dd, *J*=3.5 and 6.5 Hz)	68.7	4.47 (dd, *J*=3.7 and 4.9 Hz)
5	78.3	4.7 (ddd, *J*=3.5 and 6.5 Hz)	78.3	4.7 (ddd, *J*=3.7 and 6.4 Hz)
6	16.2	1.45 (d, *J*=6.5 Hz)	16.2	1.45 (d, *J*=6.4 Hz)
1’	102.4	4.41 (d, *J*=8 Hz)	102.5	4.41 (d, *J*=7.6 Hz)
2’	74.9	3.17 (dd, *J*=8 and 9 Hz)	74.9	3.17 (dd, *J*=7.6 and 8.5 Hz)
3’	78.0	3.35 (t, *J*=9 Hz)	78.0	3.33 (t, *J*=8.5 Hz)
4’	71.6	3.27 (t, *J*=9 Hz)	71.7	3.25 (t, *J*=8.5 Hz)
5’	78.2	3.29	78.2	3.29 (obsecured by DMSO-*d6*)
6’	62.9	3.65 (dd, *J*=5.5 and 11.5 Hz) 3.88 (dd, *J*=2 and 11.5 Hz)	62.9	3.65 (dd, *J*=5.8 and 11.6 Hz) 3.88 (d, *J*=11.6 Hz)

which gave similar TLC pattern were combined and recrystallized from MeOH-EtOAc to yield colorless plates (6.8 mg) **(3)**; MP: 92-95°C. IR (KBr) spectrum showed absorptions at 3447 (O-H), 2360 (C-H), 1728 (C=O), 1389 (C-H), 1264. (C-O-C), 1085. (C-O) (cm^{-1}). The UV spectrum showed absorbance at λ_{max} (nm, log ε): 324 (3.09), 210 (4.92). ^{1}H and ^{13}C data can be seen on Table 4 and correlation of proton and carbon atoms can be seen on Figure 3.

Based on its spectroscopic data this compound was identified as angiopteroside **(3)**.

Antimicrobial properties

Before being used each bacterium was grown separately in nutrient agar (NA) (Merck) and incubated at 37°C for 24 h. These cultures were used for antimicrobial assay by modified agar disc diffusion method of Kirby and Bauer (11). Single colony of the respective testing bacterium was transferred into NA medium and incubated for 24 h. Culture suspension in sterile NaCl 0.9% with 25% transmittance were respectively swabbed onto agar medium. Each compound **(2)** and **(3)** was prepared to the concentration of 2, 1, 0.5, 0.25, 0.125, 0.0625 mg/ml in DMSO. Each 10 µl of the above solution was dropped onto the paper disc (Whatman, 5 mm diameter) and carefully put onto culture media and the test was done in triplicate. Control disc contained chloramphenicol 30 µg/paper disc was similarly prepared in DMSO. Each plate was incubated at 37°C for 24 h. Inhibition zones (including the diameter of disc) were measured and recorded and the inhibition results were reported as means of the triplo data.

Table 4. Antibacterial activities of extract and fractions of leaves of *A. evecta*

Sample	Zone Inhibition (mm) (µg ekstrak/disc)					
	EC	**EF**	**PA**	**SA**	**STi**	**ST**
Methanolic extr.	6(10)	6(20)	6(5)	6(20)	6(5)	6(5)
Hexane fr.	-	6(0.63)	-	8(0.63)	-	-
EtoAc fr.	6 (2.5)	9(0.63)	6(0.63)	8(0.63)	7(2.5)	6(2.5)
Buthanol fr.	6(10)	6(0.63)	6(1.25)	6(0.63)	6(2.5)	6(5)
Chloramphenicol	29 (30)	30(30)	30(30)	28(30)	29(30)	29(30)

EC : *E. coli;* EF : *E. faecalis,;* PA : *P. aureginosa;* SA : *S. aureus;* STi: *S. typhimurium;* ST: *S. typhosa*

Table 5. Antibacterial activities of compound **(2)** and **(3)**

Sample	Concentration (µg ekstrak/disc)						
	20	**10**	**5**	**2.5**	**1.25**	**0.63**	**(+)30**
	Inhibition zone (mm)						
Compund 2							
- *E. faecalis,*	6.5	6	-	-	-	-	30
- *S. aureus*	6	6	-	-	-	-	29
- *S.typhimurium*,	6,5	6	-	-	-	-	29
Compound 3							
- *B. subtilis*	11	9	-	-	-	-	28

+ = positif control (Chloramphenicol)

RESULT AND DISCUSSION

Isolated compounds

Compound **(1)** was obtained as colorless crystals and based on its melting point which was not depressed when mixed with authentic sample as well its ^{13}C NMR data. this compound was identified as stigmast-5-en-3-β-ol **(1)**.

Compound **(2)** was obtained as colorless gum. From COSY NMR data there were clean correlations between protons 3 and proton methyl, proton 4 to OH and 3 , H3 to H2 and H4 and H2 to H3 (Figure 1-I). HMBC data showed correlation between C1 (164 ppm) to H3 (7.02) and H2 (6.11). C3 (144.6) to H4, C4 (63.7) to H2, H3, and H6 (1.56) were also observed. Long range coupling correlation between C5 (77.2) to H2 and H6 was also observed. The HMBC correlations are illustrated on Figure 1-II. Therefore compound **(2)** was elucidated to be 4,5-dihydro-4-hydroxy-5-methyl-1H-pyran-1-one. These data are in line with the properties of compound isolated before from EtoAc fraction of aerial parts of *Angiopteris esculenta* (9).

Compound **(2a)** was obtained as colorless gum and the ^{1}H NMR spectrum showed the presence of six protons at δ_H 1.44 (3H, d, *J*= 6.5 Hz, H-6), 4.68 (1H, dq, *J*=6.5, 2.5 Hz, H-4), 5,18 (1H, dd, *J*= 6.5, 2.5 Hz, H-5), 6,22 (1H, d, *J*=9.5 Hz, H-3), 6.96 (1H, dd, *J*=6, 9.5 Hz, H-2) and signal at 2.13 (s) indicated the methyl group from acetyl chain. Signal of 5 ^{13}C atom at δc 162.9, 140.3, 124.8, 75.1 and 63.7 ppm indicated the presence of six membered of lactone ring and signal at 15.8 ppm indicated the signal of atom C from methyl group. Signal at 170.2 indicated the carbonyl carbon and 22.3 it indicated the acetyl chain.

From COSY NMR data there were couplings among its protons as shown on Figure 2-I. From HMBC NMR data there were correlations (J_{2CH} couplings) among C and H (ppm): C (carbonyl) (170.2) to CH_3 of acetyl (2.12), C1 (162.9) to H3 (6.96) and H2 (6.22). Other HMBC correlation of C3 (140.3) and C2 (124.8) to H4, C4 (63.7) to H2, H3, H5 and H6, and C5 to H3 (6.96) and H6 (1.44). The HMBC correlation are illustrated on Figure 2-II. Therefore compound **(2)** was elucidated to be 4,5-dihydro-4-acetyl-5-methyl-1H-pyran-1-one.

Compound **(3)** was obtained as colorless needles and identified as angiopteroside which have isolated before from ethyl acetate fraction of rhizome of *Angiopteris evecta* (6).

Assignment of chemical shift and correlations between protons and carbon of this molecule was made by using HSQC and correlation among protons and was based on COSY NMR spectrum.

Following HMBC data there were correlations among C-H (ppm), C (carbonyl) (165.9) to H3 (7.16) and H2 (6.12). Other HMBC correlation of C3 (145.3) and C2 (123.6) to H4 (4.47), C4 (68.6) to H1' (4.41), C5 (78.3) to H2 (6.12), C6 (16.2) to H5(4.69) and H4 (4.47), other correlation in glucose group are C1' (102.4) to H4 (4.47) and H2' (3.17), C2' (74.9) to H1' (4.41) and H3' (3.35), C3' (78.0) to H2' (3.17), C4' (71.6) to H H3' (3.35), H5' (3.29), H6' (3.65 and 3.88), C5' (78.2) to H4' (3.27) and C6' (3.65), and C6' (62.9) to H3' (3.35), H4' (3.27) and H5' (3.29). The HMBC correlation are illustrated on Figure 3-II. Therefore compound **(3)** was elucidated to be angiopteroside.

Attempts to obtain molecular ions of these lactones by using M^{-1} and M^{+1} LCMS methode were unsuccessful, probably due to the instabilty of their molecular ions.

Antimicrobial properties

Antibacterial activity of extracts and fractions of leaves was tested against *B. subtilis, E. faecalis, E. coli, M. luteus, P. aeruginosa, S. typhosa, S. typhimurium, S. aureus, S. epidermidis, S. mutans and V. cholerae.* EtOAc and BuOH fractions showed significant inhibition activities especially toward *E. faecalis*, and *S. aureus*. Interestingly, in our study we found that angiopteroside gave significant activities toward *B. subtilis* and this is the first report of its antibacterial activity but there were no inhibition toward other bacteries. On the other hand, compound **(2)** did not show significant activities towards *E. faecalis*, *S. aureus* and *S. typhimurium*, and there were no inhibition againts other bacteries.

CONCLUSION

The isolated compounds from the leaves of *A. evecta* were identified as stigmast-5-en-3-β-ol **(1)**, 4,5-dihydro-4-hydroxy-5-methyl-1H-pyran-1-one **(2)**, and its derivative 4,5-dihydro-4-acetyl-5-methyl-1H-pyran-1-one **(2a)** and angiopteroside **(3)**.

Angiopteroside gave significant inhibition toward the growth of *B. subtilis*. 4,5-dihydro-4-hydroxy-5-methyl-1H-pyran-1-one did not show significant activites against *E. faecalis, S. aureus and S. typhimurium*.

The EtOAc fraction of the leaves of A. evecta showed significant activities to inhibit the growth of used testing microbes. However, the isolated compounds did not give significant inhibition, this will be works investigated further.

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