https://doi.org/10.48048/tis.2024.8498

Steroids Produced by *Cladosporium Anthropophilum*, an Endophytic Fungus, Isolated from *Avicennia Marina* (Forssk.) Vierh and Their Antibacterial Activity

Yeni Mulyani[1,2], Al Arofatus Naini[3], Kindi Farabi[1,4], Fajar Fauzi Abdullah[1], Wahyu Safriansyah[1], Siska Elisahbet Sinaga[4], Azmi Azhari[5], Sari Purbaya[6], Asri Peni Wulandari[7], Sofa Fajriah[3], Yoshihito Shiono[8] and Unang Supratman[1,4,*]

[1]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Jatinangor, West Java 45363, Indonesia
[2]Department of Marine Science, Faculty of Fisheries and Marine Science, Universitas Padjadjaran, Jatinangor, West Java 45363, Indonesia
[3]Research Center for Pharmaceutical Ingredients and Traditional Medicine, National Research and Innovation Agency (BRIN), Complex Cibinong Science Center-BRIN, Cibinong, West Java 16911, Indonesia
[4]Central Laboratory, Universitas Padjadjaran, Jatinangor, West Java 45363, Indonesia
[5]Department of Chemistry Education, Faculty of Tarbiyah and Teacher Training, Institut Agama Islam Negeri Syekh Nurjati, Cirebon 45132, Indonesia
[6]Department of Chemistry, Faculty Science and Informatics, Universitas Jenderal Achmad Yani, Bandung, Jawa Barat 40525, Indonesia
[7]Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Padjadjaran, Jatinangor, West Java 45363, Indonesia
[8]Department of Food, Life, and Environmental Science, Faculty of Agriculture, Yamagata University, Tsuruoka, Yamagata 997-8555, Japan

([*]Corresponding author's e-mail: unang.supratman@unpad.ac.id)

Received: 28 May 2024, Revised: 25 June 2024, Accepted: 2 July 2024, Published: 20 October 2024

Abstract

This work was conducted to isolate the steroid compounds from an endophytic fungus *Cladosporium anthropophilum* and determine their effects against 4 strains of bacteria. The fungi were afforded from a mangrove plant *Avicennia marina* (Forssk.) Vierh of Acanthaceae family, growing in West Java, Indonesia. The fermentation and isolation of *C. anthropophilum* yielded 3 ergostane-type steroids including a highly oxidized one, identified as penicisteroid A (1), ergosterol (2), and ergosterol-5,8-peroxide (3), together with 2 stigmastanes, characterized as stigmasterol (4), and stigmasterol-5,8-peroxide (5). The elucidation structure of all isolated steroids was performed by extensive spectroscopic measurements (MS, IR, 1D, and 2D NMR) and supported by a comparison of previously reported spectral data. Subsequently, the potential of the 5 steroids as well as methanol, *n*-hexane, and ethyl acetate extracts were evaluated against 2 Gram-positive bacteria, *Staphylococcus aureus* ATCC 29213 and *Enterococcus faecalis* ATCC 29212 and 2 Gram-negative bacteria, *Vibrio harveyi* ATCC 5339 and *Escherichia coli* ATCC 25922. The results showed that none of the isolated steroids were active, while the 3 extracts exhibited significant inhibition against

all the tested bacteria's growth and enabled us to propose the synergistic effects between active compounds in these extracts and their antibacterial activity.

Keywords: Steroid, Endophytic fungi, *Cladosporium anthropophilum*, *Avicennia marina*, Antibacterial activity

Introduction

Steroids are a group of lipids that are widely present and produced by almost all eukaryotes including animals, fungi, and plants. They include diverse variations in structure and play a vital importance to life, such as antibiotics, corticoid hormones, sex hormones, cholesterols, and vitamin D. Andostrane, pregnane, and estrane series of steroids are responsible for essential biological functions in cells and exhibit various hormonal effects [1,2]. Nowadays, drugs from steroid-derived are the 2nd largest group in the market and are commonly applied as immune suppressors or anti-inflammatory agents to treat various health concerns associated with the immunological conditions, including cancers, arthritis, asthma, and rheumatoid [3,4]. Another global health concern related with to the emergence of strains of multidrug-resistant bacteria has become a call to discover new antibiotics with high efficacy and good tolerability [5-7]. Meanwhile, the potential of steroid derivatives in antibacterial activity as a family of the sustainable sources from nature remains unexplored [7,8].

Mangroves are ecologically important plants that grow in coastal brackish or saline waters in muddy or rocky soils. Mangroves are called halophytes since they are salt-tolerant and are easily adapted to harsh coastal conditions due to their buttress root system [9]. In recent pharmacological investigations, extracts from mangroves exhibited promising medicinal effects against a wide array of plant, animal, and human diseases [10]. Among 84 mangroves plant species belonging to 16 families and 24 genera, *Avicennia* species was known as an ethnomedicinal plant in the ancient literature to treat a variety of digestive disorders such as rheumatic pain, haemorrhoids, diarrhea, and so on [11,13]. Furthermore, a recent study also showed that genus *Avicennia* had medicinal properties as anticancer, HIV, hepatitis, inflammation, and oxidative stress related disease. Many reports from this genus possessed some unique secondary metabolites of varied classes including alkaloids, terpenoids, steroids, phenolics, and saponins, which may be responsible for their intriguing pharmacological activities [14,15]. Subsequently, mangrove forests are biodiversity hotspot for marine fungi, and fungi derived from mangrove called manglicolous fungi [13,16]. Mangrove-associated fungi have been reported to produce a wide variety of structurally unique and biologically active compounds [17].

Endophytic fungi are endosymbiont microorganisms that have ecological benefits as their hosts. Interactions between plants and endophytes promote better nutrient absorption and result in protection against pathogens as well as increasing the plant's ability to adapt to biotic and abiotic stresses, leading to various secondary production [18-22]. The genus *Cladosporium* is one of the most worthwhile sources of chemodiversity and the largest genus of dematiaceous hyphomycetes belonging to the Cladosporiaceae family [23-25]. Moreover, plants-associated *Clasdosporium* species has attracted attention chemists and pharmacologists owing to their ability to yield a diverse of metabolites with versatile bioactivities such as anticancer, antiviral, insecticidal, antimalarial, and antimicrobial [24,26].

In our continuous studies of phytochemical investigations to discover a sustainable compound class from natural products, in particular mangrove-derived fungi, we isolated steroid compounds from *Cladosporium anthropophilum* derived from stem bark of *Avicennia marina* (Forssk.) Vierh, a traditional and folk use medicine for many centuries, distributed in the tropical and subtropical regions of Indo-West-Pacific area. This study aimed to report the structure elucidation of 5 isolated steroids, including 3 ergostanes established as penicisteroid A (1) that shows a poly oxygenated with 4 hydroxyls and one

acetyloxy moeity, ergosterol (2), and ergosterol-5,8-peroxide (3), as well as 2 stigmastanes identified as stigmasterol (4) and stigmasterol-5,8-peroxide (5). Furthermore, the bioactivity of 3 different polarity extracts afforded by fermentation of *C. anthropophilum* and all the 5 steroids against 2 Gram-positive bacteria, *S. aureus* and *E. faecalis* and 2 Gram-negative bacteria, *V. harveyi* and *E. coli* were also described. The extracts of fermented *C. anthropophilum* should be considered as their potent activity for antibacterial.

Materials and methods

General experimental procedures

The IR spectra were recorded on a KBr plate with a Perkin Elmer Spectrum 100 FT-IR spectro (Perkin Elmer, Shelton, USA). The mass spectra were obtained by the high-resolution time-of-flight mass analyzer (HR-TOFMS) on a Water Xevo Q-TOF direct probe/MS system using ESI mode and microchannel plates MCPs detector (Milford, MA, USA) and an ultra-high performance liquid chromatography-tandem mass spectrometry (UPLC-MS/MS) on a Xevo TQ-XS Triple Quadrupole Mass Spectrometer (Waters, Zellik, Belgium) equipped ESI mode. Optical rotations were calculated with an ATAGO AP-300 automatic polarimeter (ATAGO, Saitama, Japan). NMR spectra were recorded on a JEOL JNM-ECX500R/S1 spectrometer (JEOL, Tokyo, Japan) and a Bruker Topspin spectrometer (Karlsruhe, Germany), both using 500 MHz for ^{1}H and 125 MHz for ^{13}C using TMS as an internal standard. Column chromatography (CC) was performed on silica gel 60 (70 - 230 and 230 - 400 mesh, Merck, Darmstadt, Germany). and octadecyl silane (Chromatorex® C_{18} DM1020 M, 200 - 400 mesh, Fuji Sylisia, Tokyo, Japan). Thin-layer chromatography (TLC) plates were precoated with silica gel GF_{254} (0.25 mm, Merck, Darmstadt, Germany), and spot detection was obtained by spraying with 10 % H_2SO_4 in EtOH, followed by heating.

Fungal material

The strain of *Cladosporium anthropophilum* (GenBank Accession number OR892620) was isolated from healthy and fresh stem bark of mangrove plant *Avicennia marina*, which was collected from the Mangrove Reserve at Karawang District, West Java, Indonesia. This endophytic fungus was identified by molecular analysis of the internal transcribed region (ITS) and has been deposited at Laboratory of Biological Activity, Central Laboratory, Universitas Padjadjaran, Indonesia [27].

Fermentation and Isolation

C. anthropophilum was fermented on sterilized unpolished brown rice (total 7.5 kg, 30 g/flask x 250) at room temperature for 42 days. The rice culture was extracted with ethyl acetate (EtOAc) and evaporated in a vacuum to yield an extract of 240 g. Subsequently, the EtOAc extract was dissolved in distilled water (500 mL) and partitioned using separating funnel at room temperature with *n*-hexane and EtOAc, respectively. Each extract was then concentrated under vacuum to afford *n*-hexane extract (100 g) and EtOAc extract (16 g).

The *n*-hexane extract (100 g) was separated on vacuum liquid chromatography (VLC) with a stepwise gradient of *n*-hexane: EtOAc: MeOH (100:0-0:100-100:0) to yield 14 fractions (Fr. A - N). Fr. D (5 g) was chromatographed on silica gel CC using a stepwise gradient of *n*-hexane: EtOAc (100:0-0:100) to yield 8 fractions (Fr. D - D8). Fr. D5 (1.5 g) was subjected to a silica gel column with *n*-hexane: CH_2Cl_2: EtOAc (8:1:1) to afford 3 (5.6 mg) and 4 (5 mg). Fr. D6 (1.3 g) was subjected to a silica gel column with *n*-hexane: acetone (15:1) to afford 5 (6 mg).

Fr. E (3.5 g) was chromatographed on silica gel CC using a stepwise gradient of *n*-hexane: EtOAc (100:0-0:100) to yield 9 fractions (Fr. E1 to E9). Fr. E4 (746 mg) was subjected to a silica gel column with

a stepwise gradient of DCM: EtOAc (100:0-50:50) to yield 4 fractions (Fr. E4.1 - E4.4). Fr. E4.2 (116 mg) was subjected to ODS CC using H_2O-MeOH (70:30) to afford 2 (4.6 mg).

The ethyl acetate extract (16 g) was separated on VLC with a stepwise gradient of *n*-hexane: EtOAc: MeOH (100:0-0:100-100:0) to yield 8 fractions (Fr. 1 - 8). Fr. 5 (256 mg) was subjected to CC silica gel using $CHCl_3$: EtOAc (1:1) to yield 5 fractions (Fr. 5.1 - 5.5). Fr. 5.4 (72 mg) was subjected to CC silica gel with chloroform: acetone (8:2) to yield 1 (4 mg).

Spectroscopic data

Penicisteroid A (1): White powder; m.p. 160 - 162 °C; $[\alpha]_D^{24}$ - 41.1 (*c* 0.1, $CHCl_3$); IR (KBr) ν_{max}: 3384, 2957, 1710, 1268, 1037 cm^{-1}; ^1H and ^{13}C NMR data are shown in **Table 1**; ESI-MS *m/z* 506.3682 $[M]^+$ (calcd for $C_{30}H_{50}O_6$, 506.3607).

Ergosterol (2): White crystalline; m.p. 159 °C; IR (KBr) ν_{max}: 3387, 2952, 2868,1689,1605, 1365, 1025 cm^{-1}; ^1H and ^{13}C NMR data are shown in **Table 1**; HR-ESI-MS *m/z* 397.3500 $[M+H]^+$ (calcd for $C_{28}H_{45}O$, 397.3470).

Ergosterol-5,8-peroxide (**3**): White crystalline; m.p. 181 - 183 °C; IR (KBr) ν_{max}: 3,299, 2,954, 2,920, 2,853, 1,722, 1,458, 1,377, 1,082 cm^{-1}; ^1H and ^{13}C NMR data are shown in **Table 1**; HR-ESI-MS *m/z* 451.3179 $[M+Na]^+$ (calcd for $C_{28}H_{44}O_3Na$, 451.3188).

Table 1 ^1H (500 MHz) and ^{13}C NMR (125 MHz) Data of 1-3 in $CDCl_3$ (δ in ppm).

No of C	Compound 1		Compound 2		Compound 3	
	δc (mult.)	**δ_H (∑H, m, *J*(Hz))**	**δc (mult.)**	**δ_H (∑H, m, *J*(Hz))**	**δc (mult.)**	**δ_H (∑H, m, *J*(Hz))**
1	38.6 (t)	1.03 (1H, m) 1.80 (1H, m)	38.4 (t)	1.29 (2H, m)	34.7 (t)	1.92 (1H, m) 1.68 (1H, m)
2	31.1 (t)	1.48 (1H, m) 1.88 (1H, m)	32.0 (t)	1.86 (2H, m)	30.1 (t)	1.82 (1H, m) 1.52 (1H, m)
3	71.4 (d)	3.63 (1H, m)	70.5 (d)	3.62 (1H, m)	66.5 (d)	3.95 (1H, tt, 10.5, 5.0)
4	34.7 (t)	1.62 (1H, m) 2.02 (1H, m)	40.8 (t)	2.26 (1H, m) 2.45 (1H, m)	36.9 (t)	2.10 (1H, m) 1.89 (1H, m)
5	46.5 (d)	1.17 (1H, m)	139.8 (s)	-	82.2 (s)	
6	77.4 (d)	3.68 (1H, dd, 3.8, 2.4)	119.6 (d)	5.55 (1H, dd, 5.5, 3.0)	135.5 (d)	6.22 (1H, d, 8.5)
7	77,0 (d)	3.26 (1H, dd, 9.5, 3.8)	116.3 (d)	5.37 (1H, dd, 5.4, 2.5)	130.8 (d)	6.48 (1H, d, 8.5)
8	34.9 (d)	2.01 (1H, m)	141.4 (s)	-	79.4 (s)	-
9	56.4 (d)	0.76 (1H, dd, 11.3, 3.5)	46.3 (d)	1.96 (1H, m)	51.1 (d)	1.47 (1H, m)
10	35.1 (s)	-	37.1 (s)	-	37.0 (s)	-
11	68.3 (d)	4.29 (1H, brs)	21.1 (t)	1.67 (2H, m)	23.4 (t)	1.49 (1H, m) 1.20 (1H, m)
12	48.7 (t)	1.39 (1H, dd, 13.7, 2.6) 2.20 (1H, dd, 13.7, 3.2)	39.1 (t)	1.46 (2H, m)	39.4 (t)	1.94 (1H, m) 1.22 (1H, m)
13	42.8 (s)	-	42.8 (s)	-	44.6 (s)	-
14	55.3 (d)	1.08 (1H, dt, 7.9, 4.6)	54.6 (d)	1.88 (1H, m)	51.7 (d)	1.55 (1H, m)
15	37.6 (t)	1.50 (1H, m)	23.0 (t)	1.70 (2H, m)	20.7 (t)	1.58 (1H, m)

No of C	Compound 1		Compound 2		Compound 3	
	δc (mult.)	**δ_H (∑H, m, *J*(Hz))**	**δc (mult.)**	**δ_H (∑H, m, *J*(Hz))**	**δc (mult.)**	**δ_H (∑H, m, *J*(Hz))**
		2.66 (1H, m)				1.38 (1H, m)
16	75.3 (d)	5.05 (1H, m)	28.3 (t)	1.28 (2H, m)	28.7 (t)	1.75 (1H, m) 1.34 (1H, m)
17	59.9 (d)	1.15 (1H, m)	55.7 (d)	1.25 (1H, m)	56.2 (d)	1.18 (1H, m)
18	15.6 (q)	1.14 (3H, s)	12.1 (q)	0.95 (3H, s)	12.9 (q)	0.79 (3H, s)
19	19.2 (q)	1.24 (3H, s)	16.3 (q)	0.63 (3H, s)	18.2 (q)	0.86 (3H, s)
20	34.4 (d)	2.52 (1H, m)	40.5 (d)	2.04 (1H, m)	39.7 (d)	2.00 (1H, m)
21	21.2 (q)	1.07 (3H, d, 6,9)	21.1 (q)	1.02 (3H, d, 7.0)	20.9 (q)	0.97 (3H, d, 6.5)
22	135.2 (d)	5.16 (1H, t, 4,6)	135.6 (d)	5.20 (1H, m)	135.2 (d)	5.11 (1H, dd, 15.2, 8.2)
23	133.0 (d)	5.14 (1H, t, 4,6)	132.0 (d)	5.16 (1H, m)	132.3 (d)	5.20 (1H, dd, 15.2, 8.2)
24	43.3 (d)	1.78 (1H, m)	42.8 (d)	1.84 (1H, m)	42.8 (d)	1.80 (1H, m)
25	33.1 (d)	1,30 (1H, m)	33.1 (d)	1.58 (1H, m)	33.1 (d)	1.46 (1H, m)
26	20.2 (q)	0,79 (3H, d, 7)	19.7 (q)	0.83 (3H, d, 7.5)	19.7 (q)	0.79 (3H, d, 6.7)
27	19.7 (q)	0.79 (3H, d, 7)	20.0 (q)	0.81 (3H, d, 7.5)	20.0 (q)	0.81 (3H, d, 6.8)
28	18.2 (q)	0.85 (3H, d, 7)	17.6 (q)	0.91 (3H, d, 7.0)	17.6 (q)	0.89 (3H, d, 6.8)
1’	170.4 (s)	-				
2’	21.6 (q)	1.91 (3H, s)				

Stigmasterol (4): White solid; m.p. 161-163 °C; IR (KBr) ν_{max}: 3401, 2937 1457 1052 cm^{-1}; ^1H and ^{13}C NMR data are shown in **Table 2**; ESI-MS *m/z* 451.3148 $[M]^+$ (calcd for $C_{29}H_{48}O$, 451.31704).

Stigmasterol-5,8-peroxide (5): White solid; IR (KBr) ν_{max}: 3471, 2955, 2869, 1458, 967 cm^{-1}; ^1H and ^{13}C NMR data are shown in **Table 2**; HR-ESI-MS *m/z* 465.3288 $(M+Na)^+$ (calcd for $C_{29}H_{46}O_3Na$, 465.3279).

Table 2 ^1H (500 MHz) and ^{13}C NMR (125 MHz) Data of 4 and 5 in $CDCl_3$ (δ in ppm).

No of C	Compound 4		Compound 5	
	δc (mult.)	**δ_H (∑H, m, *J*(Hz))**	**δc (mult.)**	**δ_H (∑H, m, *J*(Hz))**
1	37.3 (t)	1.08 (1H, m) 1.84 (1H, m)	34.7 (t)	1.92 (1H, m) 1.67 (1H, m)
2	31.9 (t)	1.49 (1H, m) 1.81 (1H, m)	30.1 (t)	1.81 (1H, m) 1.51 (1H, m)
3	71.8 (d)	3.51 (1H, m)	66.5(d)	3.94 (1H, tt, 11.2, 5.0)
4	42.3 (t)	2.28 (1H, dd, 2.0;5.2) 2.30 (1H, dd, 2.0;5.2)	36.9 (t)	2.08 (2H, m) 1.88 (1H, m)
5	140.8 (s)	-	82.2 (s)	
6	121.8 (d)	5.34 (1H, dd, 1.7, 3.5)	135.2 (d)	6.23 (1H, d, 8.5)
7	33.9 (t)	1.54 (1H, m)	130.8 (d)	6.49 (1H, d, 8.5)

No of C	Compound 4		Compound 5	
	δc (mult.)	**δ_H ($\sum$H, m, *J*(Hz))**	**δc (mult.)**	**δ_H ($\sum$H, m, *J*(Hz))**
		1.96 (1H, m)		
8	31.9 (d)	1.46 (1H, m)	79.5 (s)	
9	50.1 (d)	0.94 (1H, m)	51.1 (d)	1.47 (1H, m)
10	36.2 (s)	-	36.9 (s)	
11	21.1 (t)	1.46 (1H, m) 1.49 (1H, m)	23.4 (t)	1.48 (1H, m) 1.20 (1H, m)
12	39.8 (t)	1.15 (1H, m) 1.95 (1H, m)	39.4 (t)	1.92 (1H, m) 1.20 (1H, m)
13	42.3 (s)	-	44.6 (s)	-
14	56.8 (d)	1.03 (1H, m)	51.7 (d)	1.52 (1H, m)
15	24.3 (t)	1.07 (1H, m)	20.7 (t)	1.59 (1H, m)
16	28.3 (t)	1.56 (1H, m) 1.26 (1H, m) 1.67 (1H, m)	29.1 (t)	1.39 (1H, m) 1.75 (1H, m) 1.34 (1H, m)
17	56.1 (d)	1.13 (1H, m)	56.2 (d)	1.20 (1H, m)
18	12.0 (q)	0.68 (3H, s)	12.9 (q)	0.79 (3H, s)
19	19.4 (q)	1.01 (3H, s)	18.2 (q)	0.86 (3H, s)
20	39.8 (d)	2.02 (1H, m)	39.8 (d)	2.01 (1H, m)
21	19.1 (q)	0.97 (3H, d, 7)	21.1 (q)	0.98 (3H, d, 6.6)
22	138.4 (d)	5.12 (1H, dd, 8.0, 15.5)	137.6 (d)	5.12 (1H, dd, 8.5)
23	129.3 (d)	5.22 (1H, dd, 5.0, 15.5)	130.2 (d)	5.02 (1H, dd, 15.1, 8.6)
24	50.1 (d)	1.53 (1H, m)	51.2 (d)	1.49 (1H, m)
25	31.7 (d)	1.45 (1H, m)	32.0 (d)	1.51 (1H, m)
26	21.1 (q)	0.79 (3H, d, 6.5)	21.1 (q)	0.82 (3H, d, 6.3)
27	19.9 (q)	0.80 (3H, d, 6.5)	19.3 (q)	0.76 (3H, d, 6.7)
28	25.5 (t)	1.15 (1H, t, 3.2)	24.9 (t)	1.38 (1H, m) 1.14 (1H, m)
29	12.0 (q)	0.89 (3H, d, 7)	12.8 (q)	0.77 (3H, t, 6.9)

Antibacterial activity assay (MIC)

The assay was conducted using a suitable medium Mueller Hinton Broth. Two Gram-positive bacteria, *S. aureus* and *E. faecalis* and 2 Gram-negative bacteria, *V. harveyi* and *E. coli* were cultured in accordance with manufacturing instructions. The positive control used in this experiment was ampicillin, while 2 % DMSO was used as a negative control. Three extracts, including methanol, *n*-hexane, and ethyl acetate, as well as 5 steroid compounds, were dissolved in 2 % DMSO at various concentrations of 1,000, 500, 250, 125, 62.5, 31.3, 15.6, 7.8, 3.9, 1.9 and 0.9 µg/mL, were incubated on a 96-well microplate at 37 °C up to 24 h. The absorbance was observed for up to 0.125 at 550 nm with dilution process using sterile saline. A further 10-fold dilution of the cell suspension was performed to produce a concentration of

approximately 10^7 cfu/mL (cfu: Colony forming unit). Subsequently, 10 µL of the organism suspension was inoculated into each well, resulting in a final cell density of 10^4 cfu/mL. The absorbance at 550 nm was subsequently measured using a spectrophotometer to determine the effects of the concentration variants on bacterial growth [28].

Results and discussion

Compound 1 was obtained as a white amorphous powder. Its molecular formula was established as $C_{30}H_{50}O_6$ on the basis of mass spectrum analysis. The ESIMS displayed a *quasi*-molecular ion peak at *m/z* 506.3682 $[M]^+$, manifesting 6 degrees of unsaturation. The IR spectrum of 1 showed absorption bands for hydroxyl (3,384 cm^{-1}) and carboxyl (1,725 cm^{-1}) functionalities. The ^1H NMR (**Table 1**) exhibited 3 tertiary methyls including one acetyl proton at δ_H 1.15 (s, CH_3-18), 1.24 (s, CH_3-19), and 1.91 (s, CH_3-2'), 4 secondary methyls at δ_H 1.07 (d, J = 7 Hz, H-21), 0.79 (d, J = 7 Hz, H-26), 0.79 (d, J = 7, H-27), and 0.85 (d, J = 7 Hz, H-28), 5 oxygenated methines including one proton substituted carboxyl group at δ_H 3.63 (m, H-3), 3.68 (dd, J = 3.8, 2.4, H-6), 3.26 (dd, J = 9.5, 3.8, H-7), 4.29 (brs, H-11), and 5.05 (dt, J = 7.8, 4.6, H-16), and 2 olefinic protons at δ_H 5.16 (m, H-22) and 5.14 (m, H-23). The ^{13}C NMR and DEPT data (**Table 1**) of 1, along with its HMQC spectrum, suggested a total of 30 carbons that include 3 quaternary carbons involving one carbonyl ester (δ_C 37.1, 42.8, 170.4), 15 methines including 5 oxygenated and 2 olefinic (δ_C 33.1, 34.4, 34.9, 43.3, 46.5, 55.3, 56.4, 59.9, 68.3, 71.4, 75.3, 77.0, 77.4, 133.0, 135.2), 5 methylenes (δ_C 31.1, 34.4, 37.6, 38.6, 48.7), and 7 methyls (δ_C 15.6, 18.2, 19.2, 19.7, 20.2, 21.2, 21.6). The primary data exhibited the presence of 2 unsaturated degrees, indicating 4 additional ring systems. The existence of 2 tertiary methyls and 2 non-oxygenated quaternary carbons, as well as 4 secondary methyls positioned in the side chain suggested that compound 1 was ergostane-type steroid. According to the 2D NMR spectrum, a paired olefinic carbon of -CH=CH- was located at C_{22}-C_{23}, supported by HMBC correlations observed from H-20/H-24 to C-22, C-23, and cross-peak from 2 vicinal protons H-22/H-23 of ^1H-^1H COSY data. HMBC correlations from H-5 to C-3, C-6, C-7 and from H-8/H-9 to C-11, along with the spin systems from ^1H-^1H COSY correlations of H-2/H-3/H-4/H-5/H-6/H-7 and H-9/H-11/H-12 confirmed the attachment of 4 hydroxyls at C-3, C-6, C-7, and C-9. In addition, the presence of acetyloxy substituted at C-17 was deduced by strong HMBC correlations from H-17 and CH_3-2' to an ester carbon of C-1'. Therefore, the planar structure of 1 was elucidated as shown in **Figure 1**.

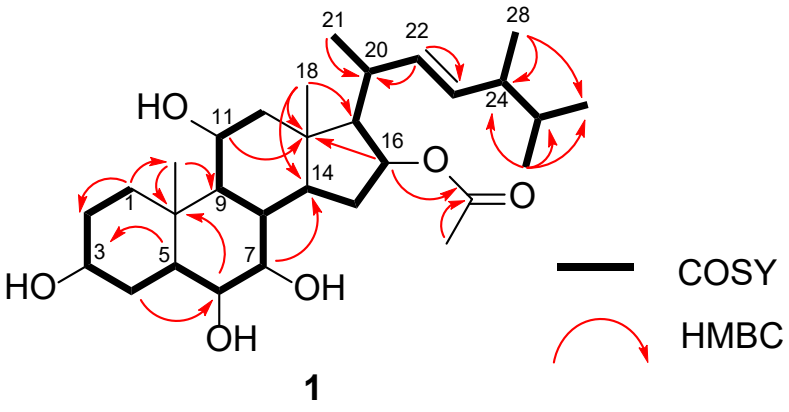

Figure 1 Key ^1H-^1H COSY (black bold bonds) and HMBC (red arrows) correlations of Compound 1.

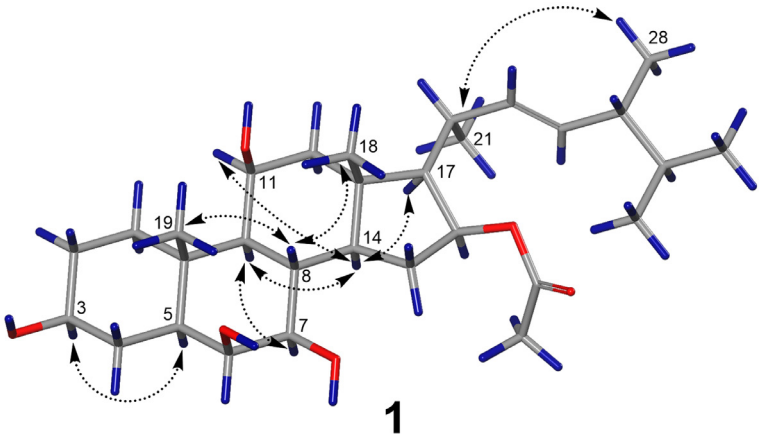

Figure 2 Key NOESY (black dashed arrows) correlations of compound 1.

The relative configuration of 1 was determined by analyses of coupling constants, ^{1}H-^{1}H NOESY correlations, and by comparison with related literature data. The strong NOE correlations (**Figure S(8)**) of H-5/*α* to H-3, H-9, H-9/*α* to H-7, H-14, and H-14/*α* to H-11, H-17 clearly suggested the co-facial orientations of these protons with *α*-orientation (**Figure 2**). Conversely, the cross-peaks of H-8/*β* to CH_3-18 and CH_3-19 confirmed the orientations of these protons on the opposite face. These assignments were then consistent with the large coupling constants ($J_{7,8}$ = 9.5 Hz) denoted the 1,2-diaxial relationship between H-7 and H-8, while the small coupling constant ($J_{9,11}$ = 3.5 Hz) represented the *cis* arrangement between H-9 and H-11. Therefore, the 3 hydroxyls at C-3, C-7, and C-11, and acetyloxy at C-17 were *β*-oriented. The remaining one hydroxyl at C-6 was also determined to be *β* orientation due to the small coupling constants observed in H-5/H-6 ($J_{5,6}$ = 2.4 Hz) and H-6/H-7 ($J_{6,7}$ = 3.8 Hz), allowing the *cis*-relationship between hydroxyls at C-6 and C-7. In addition, the observed NOE correlation between CH_3-21 (δC 21.1) and CH_3-28 (δC 18.2) was not enough to confirm the orientation of these 2 protons owing to the nature free rotation of the single bond in the side chain. The *α* orientations of CH_3-21 (δ_C 21.1) and CH_3-28 (δ_C 18.2) were then elucidated as the same as its analogs of those reported ergostanes [CH_3-21*α* (δ_C 21.0-21.6) and CH_3-28*α* (δ_C 17.8 - 18.5)] by an unambiguous ORTEP representation of X-ray experiment [29,30]. Subsequently, further analysis and literature investigation showed that the 1D-NMR data of 1 (**Table 1**) had high similarity to penicisteroid A from the culture extract of *Penicillium chrysogenum* QEN-24S, resulting in complete structure elucidation of 1 as 16*β*-acetoxy-3*β*,6*β*,7*β*,11*β*-tetrahydroxyergost-22*E*-ene [31]. To the best of our knowledge, penicisteroid A (1), as a highly oxidized ergostane-type steroid was first ever reported from *Cladosporium anthropophilum*, an endophytic fungus isolated from mangrove plant *Avicennia marina* (Forssk.) Vierh.

Compound 2 was isolated as a white crystal and its molecular formula was established as $C_{28}H_{44}O_3$ by ^{13}C NMR data and a protonated *quasi*-molecular ion $[M + H]^+$ at *m/z* 397.3500 (calcd. for $C_{28}H_{45}O$, 397.3470) in the HRESIMS spectrum, referencing 7 degrees unsaturation. Its NMR spectroscopic data also showed an ergostane-type steroid, similar to those of 1, except the additional of one degree unsaturation by the presence of 2 paired olefinic carbons at δ_C 139.8/C-5, 119.6/C-6 (δ_H 5.51, dd, J = 5.5, 3.0 Hz, H-6), δ_C 116.3/C-7 (δ_H 5.37, dd, J = 5.4, 2.5 Hz, H-7), 141.4/C-8, as well as the absence of acetyloxy at C-16. Since the 2 hydroxyls at C-6 and C-7 in 1 were absent in 2, together with the coupling constants of H-6 (J = 5.5, 3.0 Hz) and H-7 (J = 5.5, 3.0 Hz), enabled the assignment of a conjugation system by 2 paired olefinic carbons at C_5 = C_6-C_7 = C_8. Furthermore, the detachment of another hydroxyl at C-11 and acetyloxy at C-16 in compound 2 was confirmed by combining the HR-ESIMS data and the presence of 2 new methylenes

at δ_C 21.1/C-11 (δ_H 1.67, m, H-11) and 28.3/C-16 (δ_H1.28, m, H-16). The chemical shift values of 2 from 1D-NMR data (**Table 1**) were identical to the published data [32]. The configuration of 3-OH was deduced as *β*-oriented and other stereocenter carbons were assigned to be the same as ergostane-type. Thus, compound 2 was identified as an ergostane-type steroid known as ergosterol.

Compound 3 was purified as a white crystal. A sodium adduct *quasi*-molecular ion $[M+Na]^+$ at *m/z* 451.3179 (calcd. for $C_{28}H_{44}O_3Na$, 451.3188) suggested that 3 possessed 7 degrees unsaturation. According to the ^1H and ^{13}C-NMR data (**Table 1**), compound 3 had similarity to the ergosterol (2). However, compound **3** had only one additional olefinic pair at δ_C 135.5/C-6 (δ_H 6.22, d, J = 8.5 Hz, H-6) and 130.8/C-7 (δ_H 6.48, d, J = 8.5 Hz, H-7) and 2 quaternary oxygenated carbon's presence at δ_C 82.2/C-5 and 79.4/C-8. The peroxide bridge between C-5 and C-8 was established by 2 remaining oxygen atoms and 1 additional ring to satisfy the unsaturation demand, while the 2 olefinic methines (-$\underline{CH_6}$=$\underline{CH_7}$-) was proved by large coupling constant of *cis*-relationship of H-6/H-7 ($J_{6,7}$ = 8.5 Hz). The NMR data (**Table 1**) of 3 were in accordance with the published data [33]. Consequently, the 3-OH was *β*-oriented, peroxide bridged at C-5 and C-9 was *α*-oriented, and other stereocenter carbons were assigned to be the same as ergostane-type. Thus, compound 3 was elucidated as a 5*α*,8*α*-epidioxy-22*E*-ergosta-6,22-dien-3*β*-ol known as ergosterol-5,8-peroxide.

Compound 4 was obtained as a white solid, which showed the molecular formula of $C_{29}H_{48}O$ as determined by its ion molecular $[M]^+$ at *m/z* 412.3748 (calcd. for $C_{29}H_{48}O$, 412.3704). Detailed analysis of its 1D-NMR data (**Table 2**) implied that compound 4 was a stigmastane-type steroid. The presence of one primary methyl at δ_C 12.0/C-29 (δ_H 0.89, t, J = 7 Hz, H-29) and additional one methylene δ_C 25.5/C-28 (δ_H 1.15, t, J = 3.1 Hz, H-28), as well as the olefinic bonds at C-22 (138.4)/C-23 (129.3) confirmed the side chain moiety of stigmastane skeleton. Moreover, the attachment of hydroxyl at C-3 (δ_C 71.8) and another double bond pair at C-5 (δ_C 140.8)/C-6 (δ_C 121.8) permitted the whole structure of 4. A comparison of the NMR of compound 4 with the previous literature showed nearly identical which enabled us to identify as (22*E*,24*S*)-24-ethyl-cholesta-5,22-dien-3*β*-ol known as *β*-stigmasterol [34].

Compound 5, as a white powder, gave a molecular formula of $C_{29}H_{46}O_3$ established by HR-ESITOFMS (*m/z* 465.3279 $[M+Na]^+$, calcd. for $C_{29}H_{46}O_3Na$, 465.3288). Its NMR data (**Table 2**) was similar to those of 3 with the obvious differences of the substituent at C-24. Secondary methyl (C-28) in the compound 3 was replaced by an ethyl group at C-24 in the compound 5. This motif was confirmed by the observed signals for one primary methyl at δ_C 12.8/C-29 (δ_H 0.77, t, J = 6.9 Hz, CH_3-29) and one methylene δ_C 24.9/C-28 (δ_H 1.38, m; 1.14, m, H-28). Furthermore, the peroxide bridge at C-5/C-9, olefinic bonds at C-6/C-7, and oxymethine by hydroxyl at C-3 were also present in 5, generating the stigmasterol-type with peroxide moiety. The structure of 5 was then identified as (22*E*,24*S*)-5*α*,8*α*-epidioxy-24-ethyl-cholesta-6,22-dien-3*β*-ol known as stigmasterol-5,8-peroxide [35]. Since their 1D-NMR data were akin including the coupling constant values (**Table 2**), the configuration of each carbon stereocenter was also established the same as the comparison compound.

Table 3 Antibacterial activity (MIC, μM).

Sample	MIC			
	Gram positive bacteria		Gram negative bacteria	
	S. aureus	***E. feacalis***	***V. harveyi***	***E. coli***
Methanol Extract (μg/mL)	31.25	62.5	31.25	31.25
n-hexane Extract (μg/mL)	31.25	31.25	62.5	31.25
Ethyl acetate Extract (μg/mL)	15.625	15.625	31.25	15.625
Compound 1 (μg/mL)	> 500	> 500	> 500	> 500
Compound 2 (μg/mL)	> 500	> 500	> 500	> 500
Compound 3 (μg/mL)	> 500	> 500	> 500	> 500
Compound 4 (μg/mL)	> 500	> 500	> 500	> 500
Compound 5 (μg/mL)	> 500	> 500	> 500	> 500
Ampicillin (μg/mL)	15.6	15.6	31.25	31.25

The results of the antibacterial activity assay of the 3 extracts, 5 isolated steroids (1-5), and ampicillin as the positive control are shown in **Table 3**. The antibacterial activity was defined as a minimum inhibitory concentration (MIC). The MIC results showed that the steroid compounds (1-5) showed no inhibitory effects against all the tested bacteria. These results are agreed by other previous researchers, which reported that ergosterol (2), ergosterol peroxide (3), and stigmasterol (4) were inactive against Gram-positive *S. aureus* and Gram-negative *E. coli* [35]. Meanwhile, the highly oxidized steroids, identified as penicisteroid A (1) and stigmasterol peroxide (5) have not been reported their antibacterial effects, although compound 1 showed potent activity against pathogenic fungus *Aspergillus niger*, and *Alternaria brassicae* in previous report [36]. Beyond that, the antibacterial assay in this study revealed the synergistic effects produced by cumulative ingredients with similar or related outcomes, since all the 3 tested extracts had greater inhibitory effects than the single compound. The ethyl acetate crude extract exhibited the most potent with MIC value at 15.6 μg/mL against all the tested bacteria, except for *V. harveyi* bacteria was 2-fold greater at 31.3 μg/mL, while methanol and *n*-hexane extracts showed MIC values between 31.3 and 62.5 μg/mL to suppress the growth of 2 Gram-positives (*S. aureus* and *E. faecalis*) and 2 Gram-negatives (*V. harveyi* and *E. coli*).

Conclusions

The endophytic fungus *Cladosporium anthropophilum* from a mangrove plant *Avicennia marina* (Forssk.) Vierh of Acanthaceae family resulted 5 steroids. The 5 isolated steroids were elucidated as penicisteroid A (1), a highly oxygenated ergostane-type, together with ergosterol (2), ergosterol-5,8-peroxide (3), stigmasterol (4), and stigmasterol-5,8-peroxide (5) by extensive spectroscopic methods (MS, IR, 1D and 2D NMR) and supplemented by comparison of previously reported spectral data. The antibacterial activity showed that methanol, *n*-hexane and ethyl acetate extracts from *C. anthropophilum* fermentation had promising inhibition, while all the isolated steroids were inactive against 2 Gram-positive bacteria, *S. aureus* ATCC 29213 and *E. faecalis* ATCC 29212 and 2 Gram-negative bacteria, *V. harveyi* ATCC 5339 and *E. coli* ATCC 25922. Hence, the synergistic effects of active compounds in these extracts might affect to their antibacterial activity. However, further work has to be done to explore the potential of steroids as an active ingredient, as well as to discover antibacterial drugs from steroid-based compounds.

Acknowledgements

This research was financially supported by National Postgraduate Education Scholarship (BPPDN) from the Ministry of Education, Culture, Research, and Technology of the Republic of Indonesia and Universitas Padjadjaran, Indonesia through Academic Leadership Grant (No: 1549/UN6.3.1/PT.00/2023 by Unang Supratman.

References

[1] HN Bhatti and RA Khera. Biological transformations of steroidal compounds: A review. *Steroids* 2012; **77**, 1267-90.

[2] AG Mensah-Nyagan, L Meyer, V Schaeffer, C Kibaly and C Patte-Mensah. Evidence for a key role of steroids in the modulation of pain. *Psychoneuroendocrinology* 2009; **34**, S169-S177.

[3] Ericson-Neilsen, William and AD Kaye. Steroids: Pharmacology, complications, and practice delivery issues. *Ochsner J.* 2014; **14**, 203-7.

[4] J Xu, Winkler and H Derendorf. A pharmacokinetic/pharmacodynamic approach to predict total prednisolone concentrations in human plasma. *J. Pharmacokinet. Pharmacodyn*. 2007; **34**, 355-72.

[5] ARM Coates and Y Hu. Novel approaches to developing new antibiotics for bacterial infections. *Br. J. Pharmacol.* 2007; **152**, 1147-54.

[6] B Spellberg, J Bartlett, R Wunderink and DN Gilbert. Novel approaches are needed to develop tomorrow's antibacterial therapies. *Am. J. Respir. Crit. Care Med.* 2015; **191**, 135-40.

[7] MA Cataldo, G Granata and N Petrosillo. *Clostridium difficile* infection: New approaches to prevention, non-antimicrobial treatment, and stewardship. *Expert Rev. Anti. Infect. Ther.* 2017; **15**, 1027-40.

[8] NS Nadaraia, LS Amiranashvili, M Merlani, ML Kakhabrishvili, NN Barbakadze, A Geronikaki, A Petrou, V Poroikov, A Ciric, J Glamoclija and M Sokovic. Novel antimicrobial agents' discovery among the steroid derivatives. *Steroids* 2019; **144**, 52-65.

[9] SN Bibi, Z Gokhan, J Rajesh and MF Mahomoodally. Fungal endophytes associated with mangroves - Chemistry and biopharmaceutical potential. *S Afr. J. Bot.* 2020; **134**, 187-212.

[10] J Wu, Q Xiao, J Xu, M Li, JY Pan and MHYang. Natural products from true mangrove flora: Source, chemistry and bioactivities. *Nat. Prod. Rep*. 2008; **25**, 955-81.

[11] A Rege, RY Ambaye and R Deshmukh. *In-vitro* testing of anti-HIV activity of some medicinal plants. *Indian J. Nat. Prod. Resour.* 2010; **1**, 193-9.

[12] M Shafie, A Forghani and J Moshtaghiyan. Anti-inflammatory effects of hydro-alcoholic of mangrove (*Avicennia marina*) and vitamin C on arthritic rats. *Bull Environ. Pharmacol. Life Sci.* 2013; **2**, 32-7.

[13] H Thatoi, D Samantaray and SK Das. The genus *Avicennia*, a pioneer group of dominant mangrove plant species with potential medicinal values: A review. *Front Life Sci.* 2016; **9**, 267-91.

[14] MN Sharief, A Srinivasulu, B Chittibabu and UM Rao. Identification of bioactive principles of *Avicennia officinalis* fruit extract in methanol and screening for antibacterial activity. *Int. J. Pharm. Sci. Drug Res*. 2015; **7**, 73-7.

[15] M Sumithra, J Anbu, S Nithya and R Velayutham. Anticancer activity of methanolic leaves extract of *Avicennia officinalis* on Ehrlich ascitis carcinoma cell lines in rodents. *Int. J. Pharmtech. Res.* 2011; **3**, 1290-2.

[16] CA Shearer, E Descals, B Kohlmeyer, J Kohlmeyer, L Marvanová, D Padgett, D Porter, HA Raja, JP Schmit, HA Thorton and H Voglymayr. Fungal biodiversity in aquatic habitats. *Biodivers. Conserv.* 2007; **16**, 49-67.

[17] ZF Zhou, XH Yang, HL Liu, YC Gu, BP Ye and YW Guo. A new cyclic peptide and a new steroid from the fungus *Penicillium* sp. GD6 isolated from the mangrove *Bruguiera gymnorrhiza*. *Helv. Chim. Acta* 2014; **97**, 1564-70.

[18] T Suzuki, NR Ariefta, T Koseki, H Furuno, E Kwon, H Momma, D Harneti, R Maharani, U Supratman, K Kimura and Y Shio. New polyketides, paralactonic acids A-E produced by *Paraconiothyrium* sp. SW-B-1, an endophytic fungus associated with a seaweed, *Chondrus ocellatus* Holmes. *Fitoterapia* 2019; **132**, 75-81.

[19] S Das, D Kumar, D Samantaray, JK Patra, L Samanta and H Thatoi. Antidiabetic potential of mangrove plants: A review. *Front. Life Sci.* 2016; **9**, 75-88.

[20] Azhari A and Supratman U. The chemistry and pharmacology of fungal genus *Periconia*: a review. *Sci. Pharm*. 2021; **89**, 34.

[21] GL Maria, KR Sridhar and RN Seetharam. Antimicrobial and enzyme activity of mangrove endophytic fungi of southwest coast of India. *J. Agr. Tech.* 2005; **1**, 67-80.

[22] S Purbaya, D Harneti, W Safriansyah, Rahmawati, AP Wulandari, Y Mulyani and U Supratman. Secondary metabolites of biscogniauxia: Distribution, chemical diversity, bioactivity, and implications of the occurrence. *Toxins* 2023; **15**, 686.

[23] L Wang, X Han, G Zhu, Y Wang, A Chairoungdua, P Piyachaturawat and W Zhu. Polyketides from the endophytic fungus *Cladosporium* sp. isolated from the mangrove plant *Excoecaria agallocha*. *Front. Chem.* 2018; **6**, 344.

[24] HM Manjunath and CG Joshi. Characterization, antioxidant and antimicrobial activity of silver nanoparticles synthesized using marine endophytic fungus-*Cladosporium cladosporioides*. *Process Biochem.* 2019; **82**, 199-204.

[25] MM Salvatore, A Andolfi and R Nicoletti. The genus *Cladosporium*: A rich source of diverse and bioactive natural compounds. *Molecules* 2021; **26**, 3959.

[26] X Han, XF Bao, CX Wang, J Xie, XJ Song, P Dai, GD Chen, D Hu, XS Yao and H Gao. Cladosporine A, a new indole diterpenoid alkaloid with antimicrobial activities from *Cladosporium* sp. *Nat. Prod. Res.* 2021; **35**, 1115-21.

[27] Y Mulyani, AP Wulandari, SE Sinaga, W Safriansyah, A Azhari, S Purbaya, FF Abdullah, K Farabi, Y Shiono and U Supratman. Antibacterial activities and molecular identification of endophytic fungi isolated from mangrove *Avicennia marina*. *Biodivers. J.* 2023; **24**, 6923-33.

[28] SE Sinaga, M Fajar, T Mayanti and U Supratman. Bioactivities screening and elucidation of terpenoid from the stembark extracts of *Lansium domesticum* Corr. cv. Kokosan (Meliaceae). *Sustainability* 2023; **15**, 2140.

[29] Y Yaoita, K Amemiya, H Ohnuma, K Furumura, A Masaki, T Matsuki and M Kikuchi. Sterol constituents from five edible mushrooms. *Chem. Pharm. Bull.* 1998; **46**, 944-50.

[30] RQ Mei, FJ Zuo, XY Duan, YN Wang, JR Li, CZ Qian and JP Xiao. Ergosterols from Ganoderma sinense and their anti-inflammatory activities by inhibiting NO production. *Phytochem. Lett.* 2019; **32**, 177-80.

[31] S Gao, X Li, C Li, P Proksch and B Wang. Penicisteroids A and B, antifungal and cytotoxic polyoxygenated steroids from the marine alga-derived endophytic fungus *Penicillium chrysogenum* QEN-24S. *Bioorg. Med. Chem. Lett.* 2011; **21**, 1, 2894-7.

[32] M Martinez, ST Alvarez, MG Campi, JA Bravo and JL Vila. Ergosterol from the mushroom *Laetiporus* sp.: Isolation and structural characterization. *Rev. Boliv. Quim.* 2015; **32**, 90-4.

[33] M Kobori, M Yoshida, M Ohnishi-Kameyama, T Takei and H Shinmoto. 5α, 8α-Epidioxy-22E-ergosta-6, 9 (11), 22-trien-3β-ol from an edible mushroom suppresses growth of HL60 leukemia and HT29 colon adenocarcinoma cells. *Biol. Pharm. Bull.* 2006; **29**, 4, 755-9.

[34] JM Carvalho, LKO da Paixão, MF Dolabela, PSB Marinho and AMR Marinho. Phytosterols isolated from endophytic fungus *Colletotrichum gloeosporioides* (Melanconiaceae). *Acta Amazonica* 2016; **46**, 69-72.

[35] E Ioannou, AF Abdel-Razik, M Zervou, D Christofidis, X Alexi, C Vagias, MN Alexis and V Roussis. 5α,8α-Epidioxysterols from the gorgonian *Eunicella cavolini* and the ascidian *Tridemnum inarmatum*: Isolation and evaluation of their antiproliferative activity. *Steroids* 2009; **74**, 73-80.

[36] U Supratman, AP Sari, N Nurlelasari, R Maharani, A Azhari, D Harneti, R Maharani, T Mayanti, K Farabi, D Darwati, S Fajriah, MN Azmi and Y Shiono. New Ergostane-type sterol produced by an endophytic fungus *Fusarium phaseoli* isolated from *Chisocheton macrophyllus* (Meliaceae). *Rec. Nat. Prod*. 2022; **16**, 1-8.