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Original Article

METHYL 10-*EPI*-PHEOPHORBIDE A FROM MCF-7 CELLS ACTIVE LAYER OF THE INDONESIAN *FICUS DELTOIDEA* JACK LEAVES

ANGGIA MURNI[1], NOVRIYANDI HANIF[2*], MASAKI KITA[3], LATIFAH K. DARUSMAN[1,2]

[1]Tropical Biopharmaca Research Center, Bogor Agricultural University, Jalan Taman Kencana No. 3, Bogor 16128, Indonesia, [2]Department of Chemistry, Faculty of Mathematics and Natural Sciences, Bogor Agricultural University, Gedung Kimia Wing 1 Lantai 3, Jalan Tanjung Kampus IPB Darmaga, Bogor 16680, Indonesia, [3]Department of Applied Molecular Biosciences, Graduate School of Bioagricultural Sciences, Nagoya University, Furo-cho, Chikusa, Nagoya 464-8610, Japan
Email: nhanif@apps.ipb.ac.id

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ABSTRACT

Objective: To isolate and elucidate a cytotoxic principle against breast tumor MCF-7 cells of the Indonesian terrestrial plant *Ficus deltoidea* Jack leaves.

Methods: *F. deltoidea* leaves collected at National Park of mount Gede-Pangrango, Indonesia have been subjected to chemical and biological work. *F. deltoidea* leaves were extracted with 96% aqueous ethanol (EtOH) and was then partitioned into three layers *n*-hexane, dichloromethane (CH_2Cl_2), and *n*-butanol (*n*-BuOH). All layers were checked for their activity against breast tumor MCF-7 cells using MTT assay method. A portion of the most active layer was purified using open column chromatography to give fraction that has toxicity against zebra fish embryos. Based on the assay-guided isolation, compound 1 was isolated. The chemical structure of **1** was elucidated using nuclear magnetic resonance (NMR) and high-resolution mass spectrometry (HRMS) data as well as comparing data with literature.

Results: The CH_2Cl_2 layer of *F. deltoidea* leaves was found to inhibit breast tumor MCF-7 cells with IC_{50} 10 µg/ml which was the most toxic among the layers. A portion of the most active layer was purified using open column chromatography to give 7 fractions. The fraction 5 showed toxicity against zebrafish embryos (LC_{50} 35 µg/ml, 48 hpf). This fraction was purified using high performance liquid chromatography (HPLC) octadecylsilyl (ODS) column with gradient elution 70% aqueous acetonitrile (MeCN) to 100% MeCN (linear gradient) for 40 min with UV detection at 254 nm (t_R = 30.99 min) to give compound 1. The chemical structure of **1** was revealed as a chlorin-type compound named methyl 10-*epi*-pheophorbide A.

Conclusion: Methyl 10-*epi*-pheophorbide A was isolated for the first time from the active fraction of the Indonesian *F. deltoidea* leaves or tabat barito. The chemical structure including absolute stereochemistry was elucidated using NMR and HRMS data as well as by comparison with the literature values. The ^{13}C NMR data has been added to complete the previous report.

Keywords: *Ficus deltoidea*, NMR, HRMS, Cytotoxic, Zebrafish embryos, Alkaloid, Chlorin, Conformational analysis

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INTRODUCTION

Breast cancer is notorious. Among the cancer diseases, breast cancer is the second most common cancer worldwide and the fifth most common cause of cancer death as well as the leading cause of cancer death in women [1]. Terrestrial natural products have proven their activity for curing childhood leukemia, testicular teratoma and Hodgkin's disease by using vinblastine and vincristine, the alkaloids isolated from terrestrial plant *Catharanthus roseus*. Taxol, the most significant drug, isolated from the Pacific yew Taxus brevifolia has efficacy against various solid tumours (lung, colon, skin, kidney, ovary, brain, breast and prostate). The library of significant natural products against breast cancer is still a few, therefore search for anticancer drugs is required combining with elucidating biologically system of cancer cells [2]. The use of herbal remedies on the basis of ethnopharmacology study for treatment cancer disease is required because the substances can be hoped to overcome the current side effect such as hair loss, bone marrow suppression, vomiting, rash, and inflammation of the mouth [3, 4].

The plant of *F. deltoidea* Jack known as tabat barito (Indonesia), mas cotek (Malaysia) or kangkalibang (Africa) is a medicinal plant widely distributed in Southeast Asia especially in Indonesia and Malaysia. Botanically, *Ficus* genus belongs to the family of Moraceae and is one of the biggest genera of Angiospermae [5]. The plant has been reported as traditional medicine to prevent and cure diseases such as diarrhea, sores lung watery, diabetes, high blood pressure, weak heart, cardiovascular and cure skin diseases [6]. Research showed that the extracts of *F. deltoidea* have effects as antioxidant [7, 8], antidiabetic [7, 9], anti-inflammatory [10, 11], antinociceptive [12], antibacterial [13-15], anti melanogenic [16], antiherpes simplex virus type 1 (HSV-1), antiproliferation in 3T3-L1 adipocytes [17, 18] and antiangiogenic effect [19] to name a few. In our previous research, the result revealed that extracts of *F. deltoidea* leaves inhibited the growth of breast tumor in mice and inhibit enzyme tyrosine kinase activity [20].

A few chemical metabolites have been isolated and elucidated from *F. deltoidea* leaves. Moretenol and *epi*-lupeol [21], terpenoid-type compounds, were isolated from Malaysian *F. deltoidea* leaves. The latter was also found in West Sumatera [15]. In addition, vitexin and isovitexin, flavonoid-type compounds were obtained from *F. deltoidea* leaves collected at Tapah, Perak, Malaysia [22]. The molecules showed *in vivo* α-glucosidase inhibition. Moreover, the *Ficus* genus contained alkaloid such as chlorin as ficusmicrochlorin A-C [23] and ficuschlorin A-D [24] have been discovered from Taiwanese *Ficus microcarpa*. All the compounds were elucidated using detailed analysis of 1D and 2D NMR spectroscopy as well as MS data. Chlorins are known as natural pigments and have been used a traditional medicine and therapeutic purpose for a long time [25]. Their special structure, which are composed of cyclic tetrapyrroles, exhibited as photosensitizers for use in photodynamic therapy (PDT), a physical treatment for cancer with a combination of photosensitizers and light [26]. Mechanistic studies showed that pheophorbide A, a chlorin from *Scutellaria barbata* induced apoptosis in Hep3B cells, a viral-induced hepatoma cell line. However, it was found to be non-toxic in normal human liver cells WRL-68. In Chinese clinics, *S. barbata* has been used in the treatment of liver cancer, digestive system cancers, lung cancer and breast cancer [27].

Pharmaceutical and phytochemical studies have verified the traditional uses of *F. deltoidea*, nevertheless, there is a necessity to investigate the chemical or bioactive compounds that responsible

for certain properties. Currently, there is a great interest in the phytochemical content and potential beneficial effect of Indonesian medicinal plants including *F. deltoidea*. In our continuing interest on bioactive molecules from terrestrial plant [28], the Indonesian plants *F. deltoidea* or tabat barito leaves were examined.

MATERIALS AND METHODS

Materials

The leaves of sample collected at National Park of mount Gede Pangrango, Cibodas Indonesia in June 2013 were identified as *Ficus deltoidea* Jack by Herbarium Bogoriense, Research Center for Biology, Indonesian Institute of Science (LIPI). A voucher of the specimen (NYH-01-A) was deposited in Department of Chemistry, Bogor Agricultural University.

Chemicals and equipment

Nuclear magnetic resonance (NMR) spectra were recorded on a Bruker Biospin AVANCE 600 spectrometer (600 MHz for ^{1}H and 150 MHz for ^{13}C) and on JEOL JNM-ECA500 (500 MHz for ^{1}H NMR) spectrometer. Chemical shifts (δ) were referenced to TMS or $CDCl_3$ signals (^{1}H δ 7.26, ^{13}C δ 77.16). All chemical shifts (δ) are given in ppm and coupling constants (*J*) are in Hz. The ^{13}C NMR data were determined by HSQC and HMBC experiments. HRESIMS mass spectra were measured on AccuTOF CS spectrometer (JEOL) with reserpine as internal standard (*m/z* 609.28121 for [M+H]). High-performance liquid chromatography separations were carried out on a Shimadzu UFLC-20 AD intelligent pump equipped with an SPD-M20A diode array detector. A column used for HPLC were Shim-pack VP-ODS (4.6 x 250 mm). Analytical thin layer chromatography (TLC) was performed on Merck silica gel 60 F254 plates and monitored with anisaldehyde and a UV lamp at 254 nm. All solvents used were of analytical grade.

Extraction and isolation

The *F. deltoidea* fresh leaves (1 kg) were macerated in 96% aqueous EtOH for 24 h and repeated for three times. The EtOH extract (16g) was then partitioned between ethyl acetate (EtOAc)-water (H_2O) (2:1) then EtOAc layer partition between hexane and 90% methanol (MeOH)-H_2O. The aqueous MeOH was partitioned between CH_2Cl_2 and 50% MeOH-H_2O. The H_2O layer from partition EtOH extract was then extracted with *n*-BuOH. The three extracts (hexane, CH_2Cl_2, and *n*-BuOH) were checked their activity against MCF-7 tumor cells.

The CH_2Cl_2 layer (0.48 g) was purified using open column chromatography with silica gel as the stationary phase and eluted with increasing gradient polarity, hexane-CH_2Cl_2-MeOH to give 7 fractions. Major fraction was tested toxicity assay against zebra fish embryos. In addition, a portion of the fifth fraction was checked its ^{1}H NMR and then separated by HPLC with a C18 column (4.6 x 250 mm) using gradient elution 80% aqueous MeOH to 100% MeOH for 45 min to give 5 subfractions. The fourth subfraction (5.2 mg) was purified using a C18 column (4.6 x 250 mm) with gradient elution with 70% aqueous MeCN to 100% MeCN for 40 min at flow rate 1 ml/min UV detection 254 nm to give 0.8 mg of pure methyl 10-*epi* pheophorbide A (1) (t_R = 30.99 min).

Cytotoxicity assay against breast tumor cells MCF7 cells

Growing cells of MCF 7 (ATCC® HTB22) were suspended in DMEM containing fetal bovine serum and penicillin-streptomycin. Samples dissolved in acetone were added into the 96 wells tissue culture plate with 5000 cells/well. The cells were incubated at 37 °C for 48 h in a CO_2 incubator with a humidified atmosphere containing 5% CO_2. The cell numbers were counted by the MTT method. Optical density at 565 nm was measured with a microplate reader.

Table 1: NMR data of 1[a]

No.	δC, multiplicity	δH, multiplicity, (J in Hz)
1	141.8, C	
2	131.9, C	
2^1	12.1, CH_3	3.40, s
3	136.4, C	
3^1	129.0, CH	8.00, dd (17.8, 11.5)
3^2	122.6, CH_2	6.30, dd (17.8, 1.2)
		6.19, dd (11.5, 1.2)
4	134.5, C	
5	97.3, CH	9.40, s
6	155.8, C	
7	136.1, C	
7^1	11.1, CH_3	3.25, s
8	145.4, C	
8^1	19.7, CH_2	3.70, q (7.8)
8^2	17.8, CH_3	1.70, t (7.8)
9	150.2, C	
10	104.2, CH	9.54, s
11	137.5, C	
12	132.0, C	
12^1	12.1, CH_3	3.69, s
13	129.4, C	
13^1	189.5, C	
13^2	61.0, CH	6.25, s
13^3	170.0, C	
13^4	52.6, CH_3	3.87, s
14	149.7, C	
15	105.0, C	
16	161.5, C	
17	50.5, CH	4.20, ddd (9.1, 3.2, 2.0)
17^1	31.2, CH_2	2.62, m
		2.50, m
17^2	30.2, CH_2	2.22, m
17^3	17.3, C	
17^4	52.0, CH_3	3.56, s
18	50.1, CH	4.46, dq (2.0, 7.3)
18^1	23.1, CH_3	1.80, d (7.3)
19	172.2, C	
20	93.2, CH	8.56, s

[a]600 MHz ^{1}H NMR and 150 MHz ^{13}C NMR in $CDCl_3$.

Toxicity assay using embryos zebrafish

Wild-type zebrafish *D. rerio* was supplied by the fish local market. The mature zebrafish aged between 5 and 24 mo was used for egg production. The spawning and fertilization took place in the morning within 1 h. The eggs were kept in the water which was used for spawning with a bunch of *Terminalia catappa* as an antibacterial agent. The fertilized and divided normally eggs were selected. Eggs with obvious anomalies or damaged membranes were discarded. Sample stock solution was made in 500 µg/ml and diluted with the appropriate solvent to give a series of concentration 12.5, 25, 50, and 100 µg/ml. DMSO was added when the extract was difficult to dissolve. Ten embryos with 48 h post fertilization (hpf) were added to each well which was previously added of fresh water and sample. The experiment was performed in three times. The multiwell was incubated for 24 h. The dead embryo was counted and its LC_{50} was calculated by using statistical software SPSS 23.0.

Conformational analysis

Conformational searches were performed with Spartan '14 (Wavefunction Inc.) using a commercially available PC. The model was constructed on a graphical user interface considering an equilibrium geometry at ground state using MMFF.

RESULTS

A sample of *F. deltoidea* leaves was thoroughly extracted with EtOH. After concentration, the residue was partitioned with *n*-hexane, CH_2Cl_2, and *n*-BuOH, respectively. The CH_2Cl_2 layer was the most toxic against tumor cells MCF-7 with IC_{50} 10 µg/ml, while the *n*-hexane and *n*-BuOH layer showed activity against the cells with IC_{50}>100 and>1000 µg/ml, respectively. The purification of the CH_2Cl_2 layer was performed using open column chromatography followed by HPLC ODS with gradient elution 70% aqueous MeCN to 100% MeCN (linear gradient) for 40 min with UV detection at 254 nm to give compound **1** as methyl 10-*epi*-pheophorbide A (t_R = 30.99 min) with the following properties: dark green solid, ^{1}H NMR ($CDCl_3$, 600 MHz) and HRESIMS $[M+Na]^+$629.2739 (calcd for $C_{36}H_{38}N_4O_5Na$ 629.2740).

DISCUSSION

The CH_2Cl_2 layer of *F. deltoidea* leaves inhibited breast tumor MCF-7 cells using MTT assay method. The half maximum inhibitory concentration (IC_{50}) was 10 µg/ml which was the most toxic among the layers. The active layer was purified using open column chromatography to give fraction 5 that has toxicity activity against zebra fish embryo. The half maximum lethal concentration (LC_{50}) was 35 µg/ml. Based on assay guided isolation, we found compound 1 after purification using HPLC.

Compound 1 was obtained as a dark green solid. The high-resolution mass spectrometry using electrospray ionization (HR-ESIMS) showed $[M+Na]^+$629.2739 (Δ+0.1 mmu), which was consistent with the molecular formula $C_{36}H_{38}N_4O_5$ indicating 19 degrees of unsaturation number. The ^{1}H NMR of compound 1 showed key signals for three aromatic methine groups [δ_H 9.40 (H-5), 9.54 (H-10), 8.56 (H-20)], one conjugated vinyl group [δ_H 8.00 (H-3^1), 6.30 (H-3^2) and 6.19 (H-3^2)], three aromatic methyl groups [δ_H 3.69 (H-12^1), 3.40 (H-2^1) and 3.25 (H-7^1)], two methyl ester groups [δ_H 3.87 (MeO-13^4), 3.56 (MeO-17^4)], and one ethyl group [δ_H 3.69 (H-8^1) and 1.70 (H-8^2)]. The ^{1}H NMR data of 1 showed close similarity with that of phaeophytin A. Compound 1 possessed additional ^{1}H NMR signal of δ_H 3.56 assigned as methyl ester (H-17^4) replacing the phytyl branch in phaeophytin A. The ^{13}C NMR was determined by HMQC and HMBC spectra due to the small amount of material available. Observed ^{13}C NMR data for 1 was close resemblance with phaeohytin A [29] except for the presence of methoxy group at C-17^4 as in 1.

First, confirmation of the almost planar structure of porphyrin moiety was explained. The NOESY correlation observed between H-18 (δ_H 4.46) and H-17^2 (δ_H 2.50 and 2.22) was inferred that H-18 was the same face as H-17^2. Another key NOESY signal between H-17 (δ_H 4.20) and H-13^2 (δ_H 6.30) was observed, which proved that two methyl esters were the same β-orientation. Moreover, the configuration at C-13^2 was determined by Inhoffen method [30, 31]. The method described the deshielding effect of a functional group side chain at C-13^2 against H-17 leading to the determination of its configuration *R* or *S*. If the 13^2-CO_2Me is located on the same side of the molecular plane as 17-H, it gives the deshilded effect and has the *R* configuration. On the other hand, when the 13^2-CO_2Me has the opposite configuration *S*, the 17-H signal is little affected (Δδ 0.1) by the presence of the functional group.

The ^{1}H NMR signal of H-17 is distinctly little affected by the presence of the 13^2-CO_2Me and shows the chemical shift around δ_H 4.20 ($CDCl_3$). The shift observed for H-17 was very small (Δδ 0.01) compared with the literature [29] and therefore, it was assigned as *S** [30, 31]. The structure of 1 was concluded as in fig. 1.

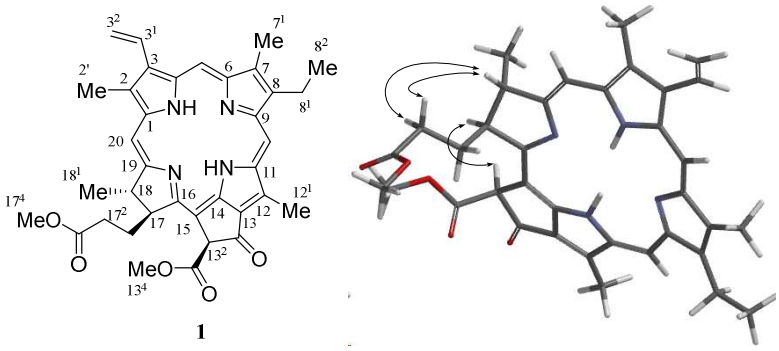

Fig. 1: Structure and key NOESY signals of 1 (3D model) measured in $CDCl_3$

As a result, the structure of compound **1** was identical to methyl 10-*epi*-pheophorbide A that was originally isolated from spinach leaves [32] and later in *Ficus microcarpa* leaves [23, 24]. Meanwhile, this is the first report of methyl 10-*epi*-pheophorbide A from *F. deltoidea*. We also supplied the complete ^{13}C NMR data for 1 to equip the previous report [32]. Due to a minute amount of the compound (0.8 mg), we could not establish the cytotoxicity against MCF-7 tumor cells.

CONCLUSION

Methyl 10-*epi*-pheophorbide A (**1**) was isolated from a MCF-7 cell active layer of the Indonesian *Ficus deltoidea* Jack leaves or tabat barito. The chemical structure including absolute stereochemistry was elucidated using NMR and HRMS data and by comparison with the literature values. This type of compound could be considered as an antitumor agent from *F. deltoidea* based on reported of its activity as antiproliferative on hepatoma Hep3B cells.

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AUTHOR'S CONTRIBUTION

AM performed extraction, purification using open column chromatography and HPLC, MCF-7 cell assay, interpretation of the data, and wrote the paper. NH contributed for sample collection, conceived design experiments, interpretation of the data, supervised the laboratory work, and wrote the paper. MK contributed for HRMS and NMR measurement as well as searching structure databases. LKD initiated for research theme. All the authors have read the final manuscript and approved the submission.

CONFLICT OF INTERESTS

The authors declare no conflict interest

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