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Constituents of *Euphorbia milii*

Hye Sook Yun-Choi*, Jing Ling Jin, Sung Won Hong, Yong Yook Lee, and Jo Hyung Lee**

Natural Products Research Institute, Seoul National University, Seoul 110-460, Korea

Abstract – The methanol extract of *Euphorbia milii* exhibited strong inhibitory effect on platelet aggregation in the course of our search for anti-platelet component from succulent plants. Two components, components **1** and **2** were isolated from this plant. **1** was the mixture of 72% of 1-octacosanol (**1a**) and 28% of 1-triacontanol (**1b**), and **2** was identified as β-sitosterol. **2** (IC_{50}: 195 μM, and 170 μM respectively) was about two fold stronger than ASA (IC_{50}: 420 μM and 340 μM respectively) on both collagen and U46619 induced aggregation, while the effect of **1** to platelets was negligible.
Keywords – *Euphorbia milii*, 1-octacosanol, 1-triacontanol, β-sitosterol, anti-platelet effects

Introduction

Euphorbia milii Ch. des. Moullins (Euphorbiaceae), which is also known as its synonym, *Euphorbia splendens* Bojer, is a succulent shrub originated from Madagascar. This plant is about 1-3 meters high, with 1-2.5 cm long thorns on the stems as its common name, crown of thorns, suggested, and the flowers are red or orange. These days, it is widely cultivated as an ornamental plant in many countries. The stem, root and latex of *E. milii* have long been used as herbal remedies for hepatitis and abdominal edema in China (Dictionary of Chinese Crude Drugs, 1997). The isolation of potent antileukemic macrolide (Lee *et al.*, 1982) and high molluscicidal milliamine (Nascimento *et al.*, 1999) from this plant were reported. In the course of our searching for anti-platelet components from succulent plants, the MeOH extract of *E. milii* was observed to have potent anti-platelet effect. And two components, components **1** and **2** were isolated from this plant. **1** was the mixture of 72% of 1-octacosanol (**1a**) and 28% of 1-triacontanol (**1b**), and **2** was identified as β-sitosterol. The inhibitory effects of **1** and **2** were evaluated on rat platelet aggregation induced by various stimulators.

Experimental

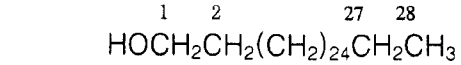
Plant materials – *E. milii* was cultivated at Koyang Cactus Experiment Station (KCES), Koyang, Korea, and identified by Chang-Hui Choi, the Research Scientist of the KCES. The lyophilized whole plants were kindly supplied from KCES in August 2001. The voucher specimens (LYY-030629) were deposited at the Herbarium of Natural Products Research Institute, Seoul National University.

General experimental procedures – Melting point was determined on a Mitamura-Riken melting point apparatus and uncorrected. ^{1}H- and ^{13}C- NMR spectra were taken at 300 MHz and 75.5 MHz respectively with a Varian Gemini-2000 spectrometer. GC/MS spectra were taken with a Hewlett Packard model 5989 B GC/MS system coupled with a 5890 Series II gas chromatograph. Platelet count was determined on an Excell 18 Hematology Analyzer (MWI, Inc., Dallas, Texas, USA). Platelet aggregation was measured on a platelet aggregometer (500VS, Chrono-Log Corp., U.S.A.).

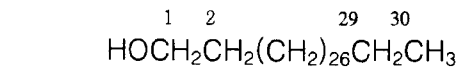
Reagents and animals – Collagen and ADP (adenosine 5'-diphosphate dicyclohexylammonium salts) were purchased from Chrono-Log Corp. (U.S.A.). Epinephrine, sodium arachidonate (AA) and U46619 (9,11-dideoxy-11,9-epoxy-methanoprostaglandin $F_{2\alpha}$) were obtained from Sigma Chem. Co. (U.S.A.). The rats (Sprague-Dawley; 250±20 g) were fed with animal chow and tap water and were housed at 20±2°C and 55±5% humidity in a 12 hours light-dark cycle in accordance with the Guide for the Care and Use of Laboratory Animals by Seoul National University.

Extraction and isolation – The lyophilized and powered *E. milii* (2 kg) were extracted three times with methanol for 3 hours. The MeOH extract was partitioned between $CHCl_3$ and H_2O, and the $CHCl_3$ layer after concentration was again partitioned between hexane and 90% MeOH to obtain hexane fraction and MeOH fraction.

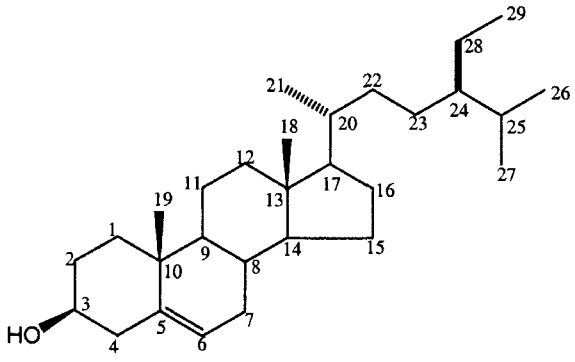
*Author for correspondence
Fax: +82-2-766-7818, E-mail: hsyun@snu.ac.kr
**On leave from Institute of Bioimmunology, Yanbian University College of Medicine, Yanji 133000, China



1-Octacosanol (1a)



1-Triacontanol (1b)



β-sitosterol (2)

The MeOH fraction (15 g) of *E. milii* was chromatographed on a silica gel column (68×6 cm) packed with 780 g of silica gel, eluted with hexane-EtOAc (10:0→4:6) yielding fr. II-1~fr. II-10. Component **1** (24 mg) and component **2** (53 mg) were obtained from fr. II-4 and fr. II-6 respectively upon recrystallization from the mixture of hexane and EtOAc.

Component 1 [mixture of 72% of 1-octacosanol (1a) and 28% of 1-triacontanol (1b)] – milky crystals, m.p: 83~84°C. EIMS: *m/z* 420 [M (**1b**)-H_2O]$^+$, 392 [M (**1a**)-H_2O]$^+$. ^{1}H-NMR ($CDCl_3$): δ 0.88 [3H, *t*, *J* = 6.9 Hz, H-28 (**1a**), H-30 (**1b**)], 1.1~1.5 (*m*), 1.55 (2H, *qt*, *J* = 6.9 Hz, H-2), 3.64 (2H, *t*, *J* = 6.6 Hz, H-1). ^{13}C-NMR ($CDCl_3$): δ 14.1 [C-28 (**1a**) and C-30 (**1b**)], 22.7 [C-27 (**1a**) and C-29 (**1b**)], 25.7 (C-3), 29.4 [C-25 (**1a**) and C-27 (**1b**)], 29.7 [C-4~24 (**1a**) and C-4~26 (**1b**)], 31.9 [C-26 (**1a**) and C-28 (**1b**)], 32.8 (C-2), 63.1 (C-1).

GC-MS analysis of component 1 – A capillary column HP-5 (30 m×0.25 mm×0.25 μm film thickness) was employed for GC. Helium was used as the carrier gas and the oven temperature was kept initially at 50°C for 3 min, programmed to increase up to 250°C at the rate of 10°C/min and then was held at 250°C for 60 more min. The peak of component **1a** appeared at 27.5 min, the peak of component **1b** was observed at 42 min.

Component 2 (β-sitosterol) – colorless needles, m.p: 130~135°C. EIMS: *m/z* 414 [M]$^+$, ^{1}H-NMR ($CDCl_3$): δ 5.35 (1H, *br d*, *J* = 5.4 Hz, H-6), 3.52 (1H, *m*, H-3), 1.01 (3H, *s*, H-19), 0.92 (3H, *d*, *J* = 6.6 Hz, H-21), 0.87 (3H, *t*, *J* = 7.6 Hz, H-29), 0.83 (3H, *d*, *J* = 7.3 Hz, H-26), 0.81 (3H, *d*, *J* = 6.9 Hz, H-27), 0.68 (3H, *s*, H-18). ^{13}C-NMR ($CDCl_3$): δ 140.8 (C-5), 121.7 (C-6), 71.8 (C-3), 56.7 (C-14), 56.0 (C-17), 50.1 (C-9), 45.8 (C-24), 42.3 (C-13), 39.8 (C-12), 39.5 (C-4), 37.2 (C-1), 36.5 (C-10), 36.1 (C-20), 33.9 (C-22), 31.9 (C-7), 31.7 (C-8), 29.6 (C-2), 29.1 (C-25), 28.2 (C-16), 26.0 (C-23), 24.3 (C-15), 23.0 (C-28), 21.1 (C-11), 19.8 (C-26), 19.4 (C-27), 19.0 (C-19), 18.8 (C-21), 12.0 (C-29), 11.9 (C-18)

Anti-platelet aggregating activity – Rat blood was drawn from heart after surgery using syringes containing 0.1 part of 2.2% sodium citrate. Platelet rich plasma (PRP) was prepared by centrifugation of citrated blood at 200×g for 10 min. PRP was diluted with saline to adjust the final platelet number to 400~450×10^6/ml. The degree of aggregation was measured with platelet Aggregometer. After 3 min preincubation of the adjusted PRP, sample (dissolved in DMSO) or vehicle was added and an aggregation inducing agent ADP (2~5 μM), collagen (2~5 μg/ml) or epinephrine (1~4 μM) was added at 30 sec after the sample addition. Epinephrine induced aggregations were measured by the method previously prescribed (Yun-Choi *et al.*, 2000). Briefly, sample was added 30 sec before the addition of the threshold concentration of collagen (0.8~1.0 μg/ml) and epinephrine was added 30 sec after the addition of collagen. The reduction in turbidity of PRP was observed as the aggregation processed. AA (10~40 μM) or U46619 (1~5 μM) induced aggregations were also measured in the presence of the threshold concentration of collagen. The minimum inducer concentration that elicited maximal aggregation was employed as the control for each PRP. The concentration at which each compound causing 50% inhibition (IC_{50}) was determined from the Regression Wizard from the SigmaPlot equation library.

Results and Discussion

The fractionation with solvents and the column chromatographic resolution of the MeOH extract of *E. milii* led to the isolation of components **1** and **2.**

Component **1** gave milky crystals. The ^{1}H-NMR spectrum showed the triplet signals at δ 0.9 (*J*=6.9 Hz) and a large multiplet signals at δ 1.3, which are suggestive of the aliphatic long chain with one terminal methyl group. A triplet at δ 3.6 (*J*=6.6 Hz) suggested the presence of an oxygenated CH_2 group attached to CH_2 group. One terminal methyl carbon signal at δ 14.1, one carbon signal at δ 63.1 and one big carbon signal at δ 29.7 in ^{13}C-NMR spectra data together

Table 1. Platelet anti-aggregating activities of components isolated from *E. milii*

Components	IC_{50} (μM)			
	Collagen[a]	Epinephrine[b,e]	AA[c,e]	U46619[d,e]
ASA[f]	420	53.0	66.0	340
1	>500	>500	>500	>500
2	195	174	145	170

[a]Collagen 2-5 μg/ml, [b]Epinephrine 1-4 μM, [c]Sodium arachidonate 10-40 μM, [d]U46619 1-5 μM, [e]With the threshold concentration of collagen (0.8-1.0 μg/ml), [f]ASA; acetylsalicylic acid.

with ^{1}H-NMR data conformed the structure of a long chain aliphatic alcohol. In the EIMS spectrum, $[M-H_2O]^+$ peak were shown at both *m/z* 420 and 392. Two peaks, with retension times of 27.5 min (72%) and 42 min (28%), appeared in GC spectrum and the first peak gave *m/z* 392 [M (**1a**)-H_2O]$^+$ and the second peak showed *m/z* 420 [M (**1b**)-H_2O]$^+$. The spectral data were compared with the literature values (Gonzalez-Bravo *et al.,* 1996; Ramanarayan *et al.,* 2000) and component **1** was identified as a mixture of 72% of 1-octacosanol (**1a**) and 28% of 1-triacontanol (**1b**) by the GC-MS analysis.

Component **2** was white needle-shape crystal. In the EI-MS spectrum, the molecular ion peak was appeared at *m/z* 414. In the ^{1}H-NMR spectrum, two angular methyl singlets at δ 0.68 and δ 1.01, and three doublets at δ 0.92, δ 0.83 and δ 0.81 were observed. One broad singlet appeared at δ 5.35, while no aromatic signals were observed. The signals at δ 140.8 and 121.7 in the ^{13}C-NMR spectrum suggested the presence of one unsaturated double bond in the structure. The spectral data were compared with the literature values (Rubinstein *et al.,* 1976) and **2** was identified as β-sitosterol.

The inhibitory effects of **1** and **2** on rat platelet aggregation were examined and compared with the effects of acetylsalicylic acid (ASA). Since rat platelets were observed not to aggregate in response to epinephrine, AA, or U46619 in the concentration dependent manner, the aggregations were observed in the presence of threshold concentration of collagen (Yun-Choi *et al.,* 2000). **1** showed only negligible effects to all the stimulators tested. **2** (IC_{50}: 195 μM, and 170 μM respectively) was about two fold stronger than ASA (IC_{50}: 420 μM and 340 μM respectively) on both collagen and U46619 induced aggregation, but less inhibitory than ASA to epinephrine and AA induced aggregation as described previously (Yun-Choi *et al.,* 2003).

Acknowledgements

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