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ANTI-MALARIAL COMPOUND FROM THE STEM BARK OF *Erythrina variegata*

Tati Herlina[1*], Unang Supratman[1], Ukun M.S. Soedjanaatmadja[1], Anas Subarnas[2], Supriyatna Sutardjo[2], Noor Rain Abdullah[3], and Hideo Hayashi[4]

[1]*Department of Chemistry, Faculty of Mathematics and Natural Sciences, Padjadjaran University, Jatinangor 45363, Sumedang, Indonesia*

[2]*Faculty of Pharmacy, Padjadjaran University, Jatinangor 45363, Sumedang, Indonesia*

[3]*Herbal Medicine Research Center, Institute for Medical Research, Jalan Pahang 50588,Kuala Lumpur, Malaysia*

[4]*Laboratory of Natural Products Chemistry, Division of Applied Biological Chemistry, Graduated School of Agriculture and Life Sciences,Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka 599-8531, Japan*

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ABSTRACT

During the course of our continuing search for novel anti-malarial compounds from Indonesian plants, the methanol extract of the bark of E. variegata showed significant anti-malarial activity toward Plasmodium falciparum in vitro using the lactate dehydrogenase (LDH) assay. The methanol extract of the bark of E. variegata was separated by using bioassay-guide fractionation. The ethyl acetate fraction showed the most activity, exhibiting equipotency against both strains of parasite with IC_{50} of 23.8 µg/mL against 3D7 and 9.3 µg/mL against K1. Furthermore, by using the anti-malarial activity to follow separation, the ethyl acetate fraction was separated by combination of column chromatography to yield an active compound. The chemical structure of active compound was determined on the basis of spectroscopic evidences and comparison with those previously reported and identified as an isoflavonoid, warangalone. The warangalone showed anti-malarial activity against both strains of parasite used with IC_{50} of 4.8 µg/mL against 3D7 and 3.7 µg/mL against K1.

Keywords: *Antimalarial, Erythrina variegata, warangalone*

INTRODUCTION

Malaria is an endemic disease in many parts of Asia, Africa, Central and South America. World Health Organization (WHO) estimates that each year 200-300 million people suffer from the case of malaria, resulting in up to 2.7 million deaths. The appearance of drug-resistance *Plasmodium falciparum* since 1960 has made the treatment of malaria increasingly problematic, and apparently the battle has not been successful [1]. This could be attributed in part to the widespread problem of parasite drug resistance [2]. Recently, attention was focused on medicinal plants to provide new and novel anti-malarial agents.

Erythrina variegata (Leguminosae) is a famous medicinal plant widely distributed in tropical and subtropical region of the world. This plant is locally known as “dadap ayam” in Indonesia and the leaves of *E. variegata* are used as an antimalarial agent [3-5]. Previous studies of the leaves of *E. variegata* have shown significant antimalarial against on *P. bhergei in vivo* and *P. falciparum in vitro* [6]. The activity of flavonoid and triterpenoid from the ethyl acetate fraction of the leaves of *E. variegata* showed an antimalarial against on *P. falciparum in vitro* [7,8]. As part of our continuing search for novel antimalarial compound from Indonesian *Erythrina* plants, we report isolation, structure elucidation and its antimalarial activity from the stem bark of *E. variegata*.

EXPERIMENTAL SECTION

General Experimental Procedure

Melting points (mp) were uncorrected. The IR spectra were recorded with a Perkin-Elmer 1760 X FT-IR spectrophotometer, and the UV spectra were recorded with a Hitachi model U-3210. Mass spectra were recorded with JEOL JMS-DX300 instrument. The ^{1}H- and ^{13}C-NMR spectra were obtained with JEOL JNM GX 270 and JNM A-500 spectrometer. Chemical shifts are given on a δ (ppm) scale with TMS as an internal standard. Column chromatography was carried out using Merck Kieselgel 60 (70-200 mesh), and thin layer chromatography (TLC) analysis was performed on precoated Si Gel plates (Merck Kieselgel GF_{254}, 0.25 mm 20 x 20 cm).

Plant material

Samples of the stem bark of *E. variegata* was collected on June 2004, in Bandung District, West Java, Indonesia. The plant was identified by a staff at

* Corresponding author. Tel/Fax : +62-227794391
Email address : tatat_04her@yahoo.com

the Laboratory of Plant Taxonomy, Department of Biology, Bandung Insitute of Technology, Bandung, Indonesia, and a voucher specimen has been deposited at the hebarium.

Parasite strain

In this study two strains of *P. falciparum* were used, culture of 3D7 (chloroquine sensitive) and K1 (chloroquine resistant).

Drug dilution

The lactate dehydrogenase (LDH) method was performed using 96-well microtitreplates (flat bottom). The initial concentration of the extract, chloroquine (Sigma Chemical, USA) and artemisinin (Sigma Chemicals, USA) were 1000 µg/mL and 1 µg/mL, respectively. The samples were then serially diluted in culture medium supplemented with 10% human serum 19 times.

Extraction and isolation

The dried stem bark (2.2 kg) of *E. variegata* was extracted by maceration technique three times with methanol. The crude methanolic extract was then assayed for anti-malarial activity via the LDH method. Evaporation of the methanol extract gave concentrated aqueous extract, which was extracted with dichloromethane. The resulting dichloromethane extract was partitioned between *n*-hexane and methanol containing 10% water, and then lower layer was concentrated and extracted with ethyl acetate. The ethyl acetate layer was subsequently dried over anhydrous sodium sulfate, filtred, evaporated to dryness, and assayed for anti-malarial activity. The ethyl acetate fraction (15.8 g) was chromatographed on Kieselgel 60 (70-230 mesh) by eluting with *n*-hexane and an increasing ratio of ethyl acetate, and by ethyl acetate and an increasing ratio of methanol to afford the 20% methanol eluate (1.2 g). The fraction (683 mg) eluted with 10% and 20% methanol were further flash-chromatographed on Kieselgel 60 with 5% methanol in chloroform to yield a crude active compound (42.3 mg), which was crystallized from methanol to yield an active compound (25.5 mg).

Screening for anti-malarial activity

In vitro testing of the anti-malarial activity was carried out by measuring the LDH activity of the parasite [9,10]. Briefly, continuous culture of the 3D7 sensitive chloroquine and K1 resistant chloroquine, were maintained in a suspension consisting of RPMI 1640 culture medium supplemented with HEPES, *N*-2-hydroxyethylpiperazine-*N*'-2-ethane-sulfonic acid (25 mM), sodium bicarbonate (0.2%) and gentamycin (40 µg/mL) at pH 7.4, and O type red blood cell [11,12]. For each LDH test, a blood suspension of 1% parasitemia and 2% haematocrit were prepared. Control reading of parasitised red blood cells devoid of plant extracts or drugs and non-parasitised red blood cells were done simultaneously. After the plate has been prepared, it was placed in a candle jar and incubated for 48 h at 37 °C. After 48 h, 100 µl of Malstat (Flow Inc., Portland, OR), was dispensed into a new microtitreplate. To it was added 25 µl of NBT-PES (Sigma Chemicals, USA) mixture. Twenty microliters of blood suspension was transferred into the plate containing the Malstat and NBT-PES. Any air bubbles were eliminated as it could interfere with the absorbance reading. Absorbance was read at 630 nm using an ELISA plate reader (MRX Microplate Reader, Dynex Technologies, USA). Chloroquine and artemisinine functioned well as positive controls.

Analysis of results

Percentage inhibition of parasite viability was determined and the mean of least IC_{50} values was calculated using the curve fitting analysis in Grafit (Grafit v.4.09, Erithacus Sofware Limited).

RESULT AND DISCUSSION

The methanolic extract of dried stem bark of *E. variegata* exhibited an anti-malarial activity against *Plasmodium falciparum* in vitro using the LDH assay. The active methanol extract was partitioned between *n*-hexane and ethyl acetate to afford an active ethyl acetate fraction. By using the LDH assay to follow the separations, the ethyl acetate fraction was separated by combination of column chromatography on Kieselgel 60 to afford an isolate active **1**. The isolate **1** was obtained as pale yellowish needles crystal (*n*-hexane-acetone); mp 163-164 °C; FAB-MS *m/z* (rel. int) 405 $[M+H]^+$, 389 (40), 349 (50), 295 (13), 185 (16), and 93 (27); UV λ_{max} (MeOH) nm (ε) 325 (16,000), 240 (80); IR V_{max} (KBr) 3453, 3077, 1710, 1513, and 782 cm^{-1}; ^{1}H-NMR ($CDCl_3$) 400 MHz and ^{13}C-NMR (100 MHz) are listed in Table 1.

The isolate **1** was shown to have the molecular formula of $C_{25}H_{24}O_5$ by FAB-MS data together with ^{1}H- and ^{13}C-NMR spectral data, indicating that **1** has twelve bond equivalents. The UV absorption maxima of **1** in MeOH were absorved at 325 nm (ε 16,000) and 240 nm (ε 80), suggested the presence of an isoflavonoid skeleton. IR absorption bands due to a hydroxyl, an aromatic ring, and carbonyl groups were observed at 3453, 3077, 1710, 1513, and 782 cm^{-1}, respectively.

The ^{1}H-NMR and ^{13}C-NMR in combination with DEPT spectra of **1** showed signals assignable to two methyl groups [δ_H 1.68 (3H, s), 1.81 (3H, s); δc 18, 26], and one gem-dimethyl at [δ_H 1.47 (6H, s)]; δc 28], an olefinic proton at [7.89 (1H, s); δc 152.7], a chelated hydroxyl [δ_H 13.1 (1H, s); δc 181.4], one non-chelated hydroxyl [δ_H 9.88, (1H, s); δc 156.9], and two set of ortho couple doublets (J = 8.5 Hz) resonating at δ_H 6.86

Table 1. The NMR data of isolate **1**

Position	$\delta_{C(ppm)}$	$\delta_{H(ppm)}$ [integral, mult., and $J_{(Hz)}$]
2	152.7	7.89 (1H, s)
3	121.0	-
4	181.4	-
5	154.0	-
6	106.0	-
7	156.0	-
8	107.0	-
9	154.0	-
10	105.0	-
1’	123.0	-
2’	130.0	7.37 (1H, d, 8.5)
3’	115.0	6.86 (1H, d, 8.5)
4’	157.0	-
5’	115.6	6.86 (1H, d, 8.5)
6’	130.2	7.37 (1H, d, 8.5)
2”	78.0	-
3”	128.0	5.63 (1H, d, 10.0)
4”	116.0	6.74 (1H, d,10.0)
5”	28.0	1.47 (3H, s)
6”	28.0	1.47 (3H, s)
1”’	21.0	3.40 (2H, d, 7.6)
2”’	120.0	5.17 (1H, dd, 7 and 8)
3”’	131.0	-
4”’	26.0	1.68 (3H, s)
5”’	18.0	1.81 (3H, s)

and 7.37, one carbonyl at δc 171.4, indicating **1** to be tetracyclic structure. The presence of AB type aromatic proton signals were observed in the ^{1}H- and ^{13}C-NMR spectra [δ_H 5.63 (1H, d, *J* = 10 Hz); δc 128 and 6.74 (1H, d, *J* = 10 Hz); δc 116] were typical of 4” and 5”, respectively, indicated the presence of a pyran ring. In the extended spectrum of ^{1}H-NMR indicated that the other prenyl group was observed from the presence of A_2X at 3.40 (2H, d, *J* = 7 Hz) and 5.17 (1H, dd, *J* = 7 and 8 Hz), suggesting that the prenyl group was located at C-8.

Besides these, the signals arising from a methine carbon [δc 152.7 (C-2), 130.2 (C-2’), 115.0 (C-3’), 115.6 (C-5’), 130.2 (C-6’), 116 (C-4”), 128 (C-3”), 122 (C-2”’), and 152 (C-2), one oxygenated methane [δc 78 (d, C-2”) were confirmed by the ^{13}C-NMR and DEPT spectra. These observations together with a detailed comparison of spectral data with those previously reported [13] led us to identify **1** as warangalone, 8(3,3-dimethyl-allyl)-4’-hydroxy-2”’,2”’-dimethylpyran[6,7,b]isoflavon (Fig. 1).

The potency of methanol extract, ethyl acetate fraction, and warangalone against both the chloroquine sensitive and chloroquine resistant strain can be described to be in the following order; warangalone > ethyl acetate fraction > methanol extract. Warangalone was most potent towards the target strains compared to methanol extract and ethyl acetate fraction (Table 2). Its inhibition of the resistant strain (K1) was also much

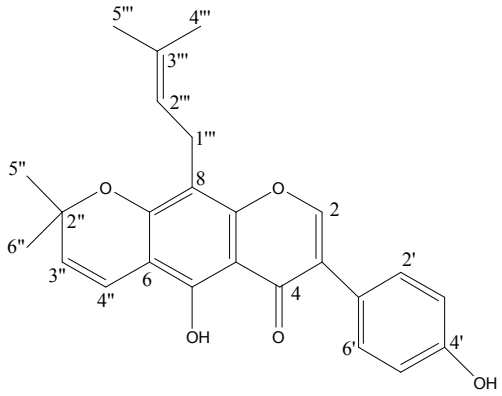

Figure 1. Chemical structure of isolate **1**

Table 2. IC_{50} values of methanol extract, ethyl acetate fraction and warangalone obtained against both the sensitive and resistant strain of *P. falciparum*

Sample	IC_{50} (µg/mL)	IC_{50} (µg/mL)
	3D7	K1
Methanol extract	38.7	20.5
Ethyl acetate fraction	23.8	9.3
Warangalone	4.8	3.7
Chloroquine	0.04	0.04
Artemisinine	0.01	0.01

better compared to its inhibition of the sensitive strain (3D7).

Anti-malarial activity of warangalone against both strains of parasite used with IC_{50} of 4.8 µg/ml against 3D7 and 3.7 µg/mL against K1 were level activity anti-malarial good to moderate (1-10 µg/mL) based on Tresholds for in vitro antiplasmodial activity of antimalarial agents [14]. The activity indicated that warangalone to be potential as anti-malarial agents.

CONCLUSION

Warangalone, 8(3,3-dimethyl-allyl)-4’-hydroxy-2”’,2”’-dimethylpyran[6,7,b]isoflavon had been isolated from ethyl acetate fraction of the stem bark of *E. variegeta*. This results strongly suggested that the warangalone is promising sources for antimalarial agents.

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