The Phenolic Compound from *Kalanchoe blossfeldiana* (Crassulaceae) Leaf and Its Antiplasmodial Activity against *Plasmodium falciparum* 3D7

Yenny Febriani Yun[1,2,*], Faizal Hermanto[3], Lilis Siti Aisyah[1,2], Tri Reksa Saputra[1], Arif Rahman Hakim[1], Ade Kania Ningsih[2], Tati Herlina[1], Euis Julaeha[1], Achmad Zainuddin[1], and Unang Supratman[1]

[1]*Department of Chemistry, Padjadjaran University, Jl. Bandung-Sumedang Km 21 Jatinangor, Sumedang 45363, Indonesian*

[2]*Department of Chemistry, Jenderal Achmad Yani University, Jl. Terusan Jendral Sudirman PO BOX 148 Cimahi - West Java, Indonesia*

[3]*Department of Pharmacy, Jenderal Achmad Yani University, Jl. Terusan Jendral Sudirman PO BOX 148 Cimahi - West Java, Indonesia*

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ABSTRACT

Various species of Kalanchoe plants have been widely used as raw materials in traditional medicines. *This study was the continuation of the researches on secondary metabolites from Indonesia Kalanchoe plants, focused on Kalanchoe blossfeldiana*. *Fresh K. blossfeldiana leaf* was *extracted with methanol at room temperature to obtain the concentrated extract*. *The concentrated methanol extract was dissolved in water and then partitioned successively with n*-hexane *and ethyl acetate*. *The methanol*, *n*-hexane, *ethyl acetate* extracts *were tested using antiplasmodial assay against Plasmodium falciparum* 3D7. The *IC_{50} of methanol,* n-*hexane,* and *ethyl acetate extract* were *13.002; 2.807*, *and 11 nM*, respectively. *Ethyl acetate extract* was *separated by the combination of chromatography on silica and ODS. This process produced* the *yellow solid*. *The chemical structure of the compound* was *determined based on UV*, *IR*, *MS*, *^1H*-NMR, and *^{13}C*-NMR analyses *and* the *comparison of data obtained from the literature and identified as phenolic compound*, namely *3,3'*,*4'*,*5,7-pentahydroxyflavone or quercetin (**1**)*, and displayed antiplasmodial activity with *IC_{50}* $3.97x10^{-2}$ nM.

Keywords: *Kalanchoe blossfeldiana; quercetin; antiplasmodial; Plasmodium falciparum 3D7*

ABSTRAK

Kalanchoe blossfeldiana (Crassulaceae) merupakan tanaman sukulen yang termasuk ke dalam genus Kalanchoe. Berbagai spesies tanaman Kalanchoe telah digunakan secara luas untuk bahan baku obat tradisional. Penelitian ini merupakan penelitian lanjutan dari pencarian senyawa metabolit sekunder dari tumbuhan Kalanchoe Indonesia. Daun segar K. blossfeldiana diekstraksi dengan metanol pada temperatur kamar sehingga diperoleh ekstrak pekat. Ekstrak pekat metanol selanjutnya dilarutkan dalam air dan dipartisi berturut-turut dengan n-heksana dan etil asetat. Ekstrak metanol, n-heksana, dan etil asetat dilakukan uji antiplasmodium terhadap Plasmodium falciparum dengan IC_{50} untuk ekstrak metanol, n-heksana, dan etil asetat, masing-masing 13,002, 2,807, dan 11 nM. Ekstrak etil asetat dipisahkan dengan kombinasi kromatografi pada silika dan ODS menghasilkan padatan kuning. Struktur kimia senyawa ditentukan berdasarkan analisis UV, IR, MS, ^1H-NMR, dan ^{13}C-NMR serta perbandingan data yang diperoleh dari literatur dan diidentifikasi sebagai senyawa fenolik yaitu 3,3',4',5,7-pentahidroksiflavon atau kuersetin (1), dan memberikan aktivitas antiplasmodium dengan IC_{50} = $3,97x10^{-2}$ nM.

Kata Kunci: *Kalanchoe blossfeldiana; kuersetin; antiplasmodium; Plasmodium falciparum 3D7*

INTRODUCTION

Indonesia has high plant biodiversity and ecosystems [1]. Some of the plants, such as those from genus *Kalanchoe*, are used as herbal medicines by the society. They are usually used, with the characteristics of thick and watery leaves. These plants are very popular because of easy cultivation, low water needed, and the flower colors [2]. From the point of ethnopharmacology, *Kalanchoe* plants are used as traditional medicines to cure headache, cough, chest pain, ulcer and skin diseases. They cure fever, fix the irregular menstruation, heal wound, hypertension and are utilized, not only in Indonesia but also almost sure

* Corresponding author. Tel/Fax : +62-22-6631556
Email address : yennyfy@gmail.com

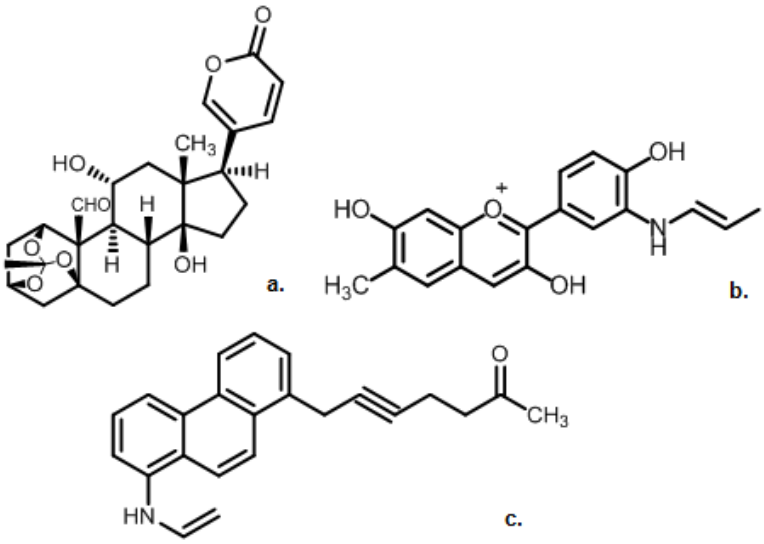

Fig 1. (a) bufadienolide, **(b)** phenolic, and **(c)** alkaloid compounds from *Kalanchoe* plants [6-16]

everywhere in the world [3-4]. Traditional medicines are potential sources for new drugs. Some researches reported that *Kalanchoe* plants contain bufadienolide [5-6], triterpenoid [7], and flavonoid [8-9], and display biological activities such as antileismanial, antiinflamatory, cytotoxic, and inhibitor tumor cell growth [10]. Biological activity is related to the presence of active groups of *Kalanchoe* plants [11] (Fig. 1), like *ortho* acetate and α-phyrone in bufadienolide [6-12], hydroxyl in phenolic compounds [13-15] and amine in alkaloid compounds [16] . The relevance of a functional group of secondary metabolites and their activity are very interesting to study, especially phenolic compounds which are the largest secondary metabolite in *Kalanchoe* [5].

In particular, the variety of plant species is enormous; therefore Indonesian plants are a very important source of new biologically active compounds. However, thus so far there is no phytochemistry and antiplasmodial compounds reported from *K. blosfeldiana*. During the course of our continuing search for antiplasmodial compounds from Indonesian *Kalanchoe* plants, the methanolic extract of *K. blossfeldiana* leaf was found to show antiplasmodial activity against *Plasmodium falciparum* 3D7.

Malaria is a major global public health problem and responsible for mortality of over 1 million people annually, where 1-10 malaria cases of 1000 population were found in Indonesia [17-18]. The development of chloroquine as an antimalarial drug and the subsequent evolution of drug-resistant *Plasmodium* strains had major impacts on global public health [19]. *Plasmodium falciparum* chloroquine resistance is a major cause of worldwide increases in malaria mortality and morbidity [20]. In endemic countries, traditional medicinal plants are frequently used to treat malaria. Therefore, screening of medicinal plants for antiplasmodial activity seems to be promising, and recently, considerable antiparastic activities have been identified in plants from various malaria-endemic regions [21]. As a part of our studies on antiplasmodial candidate compounds from Indonesia *Kalanchoe*, we isolated one phenolic compound from *K. blossfeldiana* and identified its structure as well as its antiplasmodial activity.

EXPERIMENTAL SECTION

Materials

The leaf of *K. blossfeldiana* were collected from Lembang, West Bandung area, West Java, Indonesia, and were identified at Herbarium Bogoriense, Biology Research Center of *Lembaga Ilmu Pengetahuan Indonesia* or LIPI, The Indonesian Institute of Sciences, Cibinong, Bogor, West Java, Indonesia.

The chemicals used in the isolation process were technical solvents (redistilled), *of n*-hexane, methanol, ethyl acetate, acetone, and pro-analysis grades, solvents of dichloromethane and chloroform. Chromatographic separations were carried out on silica gel 60 (70-230 mesh and 230-400 mesh). The TLC plates were precoated with silica gel GF254 (Merck, 0.25 mm) and detection was observed by spraying with 10% of $AlCl_3$ in ethanol, followed by heating.

Some chemicals were used for antiplasmodial activity test, i.e: K3EDTA anticoagulant (Vacculab), Roswell Park Memorial Institute (RPMI) 1640 (Sigma Aldrich), dimethyl-sulfoxide (DMSO, Merck), HEPES buffer (Sigma Aldrich), $NaHCO_3$ (Merck), gentamicin sulfate 50 mg/mL, (Sigma Aldrich); AB serum; cells of

human blood group O; aquabidest sterile (Otsuka), Giemsa stain (Merck), immersion oil (Merck), alcohol 70% (Brataco), and methanol (Brataco).

Instrumentation

The instruments used were laboratory glasswares, macerator, rotary evaporator (Buchi, R-200), UV lamp (Vilbert Luomart, λ 254 and 365 nm), UV-Vis spectrophotometer (Shimadzu, Hp 8452 A), IR spectrophotometer (Perkin-Elmer, 1760X FT-IR in KBr), HR-ESI TOFMS Spectrometer (JEOL JMS-700), Nuclear Magnetic Resonance (NMR) Spectrometer (JEOL JNM A-500) with TMS as standard.

Procedure

Extraction and isolation

The fresh *K. blossfeldiana* leaf (18 Kg) was grinded, extracted with methanol, and then concentrated. The obtained methanol extract (385.83 g) was dissolved in water and respectively partitioned respectively using *n*-hexane and ethyl acetate, yielding *n*-hexane extract (32 g) and ethyl acetate extract (25 g). The ethyl acetate extract was fractioned using vacuum liquid chromatography (VLC) column packed with silica gel 60 by gradient elution, resulted in 7 fractions. The fractions were analysis with thin layer chromatography under the UV lamp 254 nm and with stain-displaying reagent 10% of $AlCl_3$ in ethanol to detect the presence of flavonoid compound. Out of the 7 fractions, the fraction 5 was further fractioned. Fraction 5 was purified using column chromatography using silica G60, with the eluent of *n*-hexane:ethyl acetate (3:7), isocratically, which resulted in yellow solid of 95.5 mg. This solid was further fractioned using column chromatography using the octadecilsylane (ODS) silica, with the eluent of methanol:water:acetone (1:2:1), isocratically, which resulted compound (**1**) as yellow powder isolate (7.1 mg).The isolate was analyzed using UV, IR, NMR, and MS spectrometers.

The compound (**1**): yellow powder, m.p. 315-317 °C; UV (MeOH) λ_{maks} nm: 255, and 371; UV (MeOH+$AlCl_3$) λ_{maks} nm: 268, and 340; IR (KBr) ν_{maks} cm^{-1}: 3413; 1608; 1492; 1081; and 813; ^{1}H-NMR (acetone-d_6, 500 MHz) δH (ppm): see Table 1; ^{13}C-NMR (acetone-d_6, 125 MHz) δC ppm: see Table 1**;** HR-ESI TOFMS (negative ion mode) *m/z* $[M-H]^-$ 301,0355, (calcd. for $C_{15}H_{10}O_7$ *m/z* 302,0355).

Antiplasmodial assay continuous culture towards P. falciparum 3D7

P. falciparum 3D7 (IC_{50} artemisinin 3.97 nM ± 0.08) was cultured in well with the Medium RPMI 1640 containing the human red blood cell with 5% of haematocrit, 25 mM of HEPES buffer, 10% of AB serum and 30 mM of $NaHCO_3$ was subsequently maintained at 37 ^oC in a candle jar for 24 h, according to Trager and Jensen technique [22-23]. [3H] hypoxanthine
(0.5 µCi) was subsequently added to each well and parasites were maintained for further 24 h.

Extracts and isolate were dissolved in DMSO (20 mg/mL) and diluted in medium to obtain final concentrations between 100 and 1.56 µg/mL, to be continued on the antiplasmodial assay.

Parasite synchronization

The culture containing the *P. falciparum* 3D7 was transferred into Falcon tube 15 mL, centrifuged at the speed of 1.500 rpm for 5 min. The supernatant was discarded. The solution of 5% of sorbitol (5 mL) was added into the yielded packed cell. The Falcon tube was tenderly agitated few times then put in the incubator for 5 min. The culture was centrifuged at 1.500 rpm for 5 min, then the supernatant was discarded and the packed cell was washed using 5 mL of basic medium (RPMI). The packed cell was centrifuged at 1,500 rpm for 5 min, and then the supernatant was discarded before the culture medium (RPHS) was added. The parasite was ready to be recultured and had been in the ring stadium.

The antiplasmodial assay of the methanol, n-hexane, ethyl acetate extracts, and isolate of K. blossfeldiana Leaf

The antiplasmodial assay was performed by laboring the parasitemia. This test was performed using the culture of synchronized *P. falciparum* 3D7. The culture of *P. falciparum* 3D7 was placed into 24 well plates, each filled with 1 mL of ±1% parasitemia culture in the medium of RPHS. The medium of RPHS was replaced by medium of RPHS, containing the methanol, *n*-hexane, ethyl acetate extracts, and isolate of *K. blossfeldiana* leaf with various concentrations ($1x10^{-1}$ to $1x10^{-7}$ mg/mL). The cultures were incubated at 37 °C, under 5% of CO_2 atmosphere for 48 h before cultivation [23]. Then the blood smears were prepared, followed with Giemsa staining. Then the percentages of parasitemia *P. falciparum* 3D7 were calculated by calculating the number of infected erythrocytes from 1000 erythrocytes. The data were analyzed statistically using of probit analysis to calculate the 50% parasite inhibition (IC_{50}).

RESULT AND DISCUSSION

The leaf of *K. blossfeldiana* were grounded and successively extracted with *n*-hexane, ethyl acetate, and methanol. All of the extracts were evaluated their

Table 1. ^{1}H-NMR (acetone-D6, 500 MHz) and ^{13}C-NMR (acetone-d_6, 125 MHz) Compound **1** and Quercetin [25]

No.	^{1}H-NMR δ(ppm) (I^1; m^2; J^3=Hz) Compound 1	^{13}C-NMR δ (ppm)	^{1}H-NMR δ (ppm) (I^1; m^2; J^3=Hz) Quercetin	^{13}C-NMR δ (ppm)
1	-	-	-	-
2	-	157.8	-	156.6
3	-	136.7	-	136.2
4	-	176.5	-	176.3
5	-	162.1	-	161.2
6	6.23 (1H, d, 1.9)	99.1	6.37 (1H, d, 2.5)	98.8
7		164.9	-	164.5
8	6.49 (1H, d, 1.9)	94.3	6.14 (1H, d, 2.5)	93.7
9	-	148.8	-	148.2
10	-	104.1	-	103.4
1’	-	121.4	-	120.4
2’	7.80 (1H, d, 1.9)	115.7	7.64 (1H, d, 2.5)	116.1
3’	-	145.8	-	145.5
4’	-	146.9	-	147.2
5’	6.97 (1H, d, 8.4)	116.2	6.85 (1H, d, 8.5)	115.5
6’	7.79 (1H, dd, 1.9; 8.4)	123.7	7.49 (1H, dd, 2.5; 8.5)	122.4

I^1 = integration, m^2 = multiplicity, J^3 = coupling constant (Hz)

Table 2. Antiplasmodial activity of extracts and compound **1** against *P. Falciparum* 3D7

Extracts/Compound	IC_{50} (µg/mL)	IC_{50} (nM)
Methanol extract	13.02	13,002
Hexane extract	2.8	2,807
Ethyl acetat extract	0.01	11
Compound **1**	$3.97x10^{-5}$	$3.97x10^{-2}$

artemisinin (IC_{50} 3.97 nM)

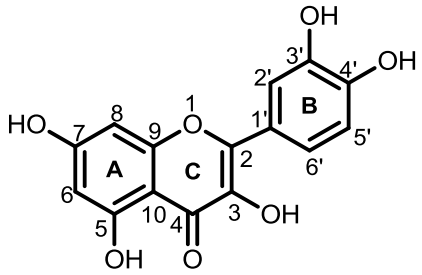

Fig 2. The chemical structure of quercetin **(1)**

antiplasmodial activity against *P. falciparum* 3D7 and the ethyl acetate showed strongest antiplasmodial activity (Table 2). Subsequent phytochemical analysis was therefore focused on the ethyl acetate extract. The ethyl acetate extract was fractioned using various chromatography methods to afford compound **1** (Fig. 2).

Compound **1** was obtained as a yellow powder. The HR-ESI TOFMS spectrum showed m/z $[M-H]^-$ m/z 301.0355, (calcd. m/z 302.0355) which corresponded the molecular formula of $C_{15}H_{10}O_7$. Thus requiring eleven degrees of unsaturation, originating from seven C double bond, one C=O and the remaining of tricyclic flavonoids. Thin layer chromatogram of compound **1** showed the yellow spot using aluminium chloride and fluorescenced under UV light at 254 and 365 nm, indicating the presence of conjugated double bonds, and flavonoid compounds. The UV spectra showed that the compound (**1**) has the absorbance at λ_{maks} 0f 370.8 nm (band 1) and 255.4 nm (band 2) which are the characteristics of flavonol compound. Band 1 indicated the absorbance related to the resonance of cynamoil group that involved ring B. Band 2 indicated the absorbance related to the resonance of benzoyl group that involved ring A of flavonoid. UV spectra using $AlCl_3$ as shift reagent, showed the presences of 3-OH, 5-OH, 7-OH, 3'-OH, and 4'-OH [24]. The IR spectrum showed absorption peaks at 3413, 1608, 1492, 1081, and 813 cm^{-1} suggesting the presences of hydroxyl, carbonyl group, C=C olefin ring, symmetric and asymmetric C-O-C as well as substituted benzene, respectively. The ^{1}H-NMR (acetone-d_6, 500 MHz) spectrum showed the presences of five proton signals as methine groups, resonating at δ_H (ppm) 6.23 (1H, d, *J* = 1.9 Hz, H-6), 6.49 (1H, d, *J* = 1.9 Hz, H-8), 6.97 (1H, d, *J* = 8.4 Hz, H-5'), 7.79 (1H, dd, *J* = 1.9;8.4 Hz, H-6'), and 7.80 (1H, d, *J* = 1.9 Hz, H-2'). Three proton signals resonating at δ_H (ppm) 6.97 (1H, d, *J* = 8.4 Hz, H-5'), 7.79 (1H, dd, *J* = 1.9;8.4 Hz, H-6'), and 7.80 (1H, d, *J* = 1.9 Hz, H-2') indicated the presence of an ABX coupling system. This showed that 2', 5', and 6' were trisubtituted on the B-ring. Two *meta*-protons of A-ring resonated at δ_H (ppm) 6.23 (1H, d, *J* = 1.9 Hz, H-6), and 6.49 (1H, d, *J* = 1.9 Hz, H-8). The ^{13}C-NMR (acetone-d_6, 125 MHz) spectrums showed the presence of 15 carbon signals, which was assigned by their chemical shift and Distortionless Enhancement by Polarization Transfer (DEPT) spectra as 5 sp^2 methine, 9 quaternary sp^2 and one carbonyl. These functionalities were accounted for eight of total eleven degrees of unsaturation. The

remaining three degrees of unsaturation was consistent of tricyclic flavonoid structure [25].

From the data of UV, IR, NMR and MS, it could be predicted that the compound **1** had the molecular formula $C_{15}H_{10}O_7$. These functionalities accounted eight of the total eleven degrees of unsaturation from C double bond, and the remaining three degree of unsaturation consistent to three rings of flavonoid. A comparison of the NMR data of **1** with these of quercetin (Table 1) [25], revealed that the chemical shifts of the two compounds were very similar, compound **1** was identified as 3,3',4',5,7-pentahydroxyflavone or quercetin (Fig. 2).

The antiplasmodial assay of the methanol, *n*-hexane, ethyl acetate extracts, and compound **1** againts *P. Falciparum* 3D7 were conducted according to the method described in previous paper [20-21] and were used artemisinin (IC_{50} 3.97 nM ± 0.08) as a positive control [21]. The antiplasmodial activities of the methanol, *n*-hexane, ethyl acetate extracts, and compound **1** were shown in Table 2. Activity of quercetin (**1**) was influenced by hydroxyl group in ring A, B, and C that have role as hydrogen bond donors that can be improve the reactivity of **1**, towards hydrogen atoms attached to a relatively electronegative atom gain a positive partial charge which makes them very reactive. The more hydroxyl group can be found in aromatic ring, the more hydrogen bond donors are available and the higher is the reactivity [26].

CONCLUSION

Flavonoid of 3,3',4',5,7-pentahydroxyflavone or quercetin was isolated from ethyl acetate extract of *K. blossfeldiana* and displayed antiplasmodial activity with IC_{50} $3.97x10^{-2}$ nM. The antiplasmodial assay against *P. falciparum* 3D7 showed that the IC_{50} values for methanol, *n*-hexane, and ethyl acetate extracts were 13.002; 2.807, and 11 nM, respectively.

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