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Heme Polymerization Inhibitory Activities of Xanthone from *G. parvifolia* (Miq) Miq Stem Bark as an Antimalarial Agent

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A study had been conducted to isolate, to identify and to test heme polymerization inhibitory activities of *G. parvifolia* (Miq)Miq stem bark active compounds with antimalarial properties. UV, IR, RMI 1D (^{1}H NMR and ^{13}C NMR) and RMI 2D (COSY, HMQC and HMBC) and mass spectra were used for identification. Heme polymerization inhibitory activity assay was conducted *in vitro* by measuring β-hematin formation using *ELISA reader*. The study revealed that the active isolates derived from xanthone compounds, 1,3,6-trihydroxy-2-(3-methylbut-2-enyl)-7-methoxy-8-(3-methylbut-2- enyl)xanthen-9-on. HPIA assay showed that the compounds acted by inhibiting weak heme polymerization activities.

Key Words: *G. parvifolia* (Miq)Miq bark, Xanthone, Antimalaria, Heme polymerization.

INTRODUCTION

Malaria is one of the leading public health problems in both developing world and developed world. According to World Health Organization[1], around 41 % of the world's population lived in endemic areas with high risk of malaria infection. It was estimated that 300-500 millions of world's population were infected with malaria. Around 1.5-2.7 million patients, particularly children under the age of five years and pregnant women, died each year. During the period of 1998-2001, extraordinary incidents of malaria in 11 provinces of Indonesia, which included 13 districts or 93 villages, were 20.000 cases with 74 deaths[2]. *Annual parasite incidence* or malaria in Java and Bali islands decreased from 0.19 per mile/year to 0.06 per mile in 1995[3]. Outside Java and Bali islands, the situation was more worrying. *Annual malaria incidence* (AMI) decreased from 20.3 per mile in 1993 to 19.13 per mile in 1995.

The efforts of identifying new antimalarial agent were aimed particularly at finding new compounds with different activities[4]. *In vitro* and *in vivo* antiplasmodial assays for **A-4** isolates indicated moderate antiplasmodial activities. The study was conducted to determine the mechanism in which the compounds served as an antiplasmodial agent by observing their effects on heme polymerization inhibitory activities (HPIA) and by identifying their molecular structures.

EXPERIMENTAL

Isolation and identification: *N*-hexane extracts were obtained by partitioning using methanol as eluent. The resulted extracts were isolated to obtain the portion dissolved in methanol (phase A) and the proportioned un-dissolved in methanol (phase B). The potential phase (phase A) was firstly weighed, grinded with celite and then aired. Silica gel was put into column chromatography, followed by phase A-eluate with solvent system of *n*-hexane-acetone slope to obtain some fractions. The solvent first used as eluent was *n*-hexane to prevent layers from descending. Then, 5 % acetone was added into *n*-hexane to enhance polarity. With acetone added, polarity of *n*-hexane increased to 10, 15, 20 and 30 %. All fractions resulted from fractionation using column chromatography were put into tubes; the solvents were steamed to obtain appropriate viscosity by using an evaporator. The resulted fractions were assayed with TLC to find out the profile. Fractions that showed similar patterns were put into one.

Fractions that has resulted 1 spot were purified with 2-dimension TLC by using the first eluate solution (*n*-hexane-acetone = 7:3) and the second eluate solution (chloroform-

TABLE-1
MEAN HEMATIN LEVEL. PERCENTS OF HEME POLYMERIZATION INHIBITION OF A-4 ISOLATES ISOLATED FROM *G. parvifolia* (Miq)Miq STEM BARK

Test compound	Concentration (µg/mL)	Mean hematin level (mM)	Mean inhibition (%)	IC_{50} value (µg/mL)
Isolate A-4	200	5.83 ± 0.23	53.5 ± 12.31	185.98
	100	8.23 ± 0.42	27.8 ± 16.32	
	50	8.98 ± 0.21	19.5 ± 11.21	
	25	9.23 ± 0.32	9.8 ± 4.21	
	12.5	10.11 ± 0.41	2.4 ± 1.21	
	6.25	10.26 ± 0.51	1.34 ± 0.98	
Chloroquine	9.664	0.98 ± 0.23	98.21 ± 12.32	0.19
	4.832	1.22 ± 0.78	87.21 ± 11.21	
	2.416	2.41 ± 0.31	78.87 ± 9.67	
	1.208	3.83 ± 1.23	73.42 ± 8.32	
	0.302	4.21 ± 1.21	62.98 ± 2.34	
	0.1511	5.21 ± 1.24	49.23 ± 12.11	

methanol = 6:4). The resulted crystals were brownish red solvable in ethyl acetate and acetone. Repeated washing of *n*-hexane with 2 drops of acetone produced yellow powder. The resulted powder was subject to HPLC to determine the purity. Pure isolates were identified using spectrometry of UV, IR, RMI 1D (^{1}H NMR and ^{13}C NMR) and RMI 2D (COSY, HMQC and HMBC) and mass spectra.

Heme polymerization inhibitory activities assay: Heme polymerization inhibiting activities (HPIA) assay was conducted using a method as follows. 100 mL of 4 mM hematin in 0.1 M NaOH was added into 96-well micro-culture and added with 50 mL assay solution with various concentrations into each well. A portion of solution with test solution was used as a control. 50 mL of glacial acetate acid (pH 2.6) was added into the microculture to initiate heme polymerization reaction. The microculture was then incubated under 37 ºC for 24 h to obtain perfect polymerization. After the period of incubation, the microculture was centrifuged and the resulted deposits were washed three tines using 200 mL of dimethyl sulfoxide (DMSO). The deposits resulted in each well of microculture was subsequently added with 200 mL of 0.1 M NaOH; OD values were read using *ELISA reader* with a wavelength of 405 nm. The value of heme polymerization inhibitory activities was expressed[5] in IC_{50}.

RESULTS AND DISCUSSION

Results of heme polymerization inhibitory activities assay: Hemoglobin is composed on heme and globin. Heme is a complex compound *i.e.*, a porphyrin with an iron atom in the middle; while globin is a protein. During hemoglobin degradation, free heme is released and oxidized from stable Fe^{2+} to hematin Fe^{3+}. For forms of heme and hematin are toxic compounds for parasites. Both forms are subjected to polymerization to become an inert crystal substance called hemozoin. Hemozoin is a malaria pigment, which in microscopic view, could indicate or predict malaria.

Heme polymerization inhibitory activities assay was conducted *in vitro* by measuring β-hematin formation using *ELISA reader*. β-Hematin was dissolved in 0.1 NaOH. N would be subject to the process of splitting iron-carboxylate bond into hematin. Addition of glacial acetate acid might initiate polymerization reaction. With high pH (> 6), hematin became more dominant in stimulating polymerization reaction. Centrifugation produced free-form aggregate hematin and hematin produced by β-hemozoin polymerization. Washing with DMSO could dissolve aggregate hematin but could not dissolve β-hematin. β-Hematin was dissolved using 0.1 N NaOH. β-Hematin is the polymer of interrelated hematin units. β-Hematin was identical to hemozoin in *P. falciparum* infected erythrocyte, while β-hematin was identical with hem. Polymerization of hematin to be β-hematin or hem to be hemozoin constituted a physical-chemical reaction.

Heme polymerization inhibitory activities of the compounds were tested with a modified method suggested by Bassilico *et al.* Heme polymerization inhibitory activities were determined by comparing hematin absorbance with test compound and hematin level in a standard curve[5]. Comparison of the increase in test compound concentration and increase in heme polymerization inhibition is presented in Table-1.

Table-1 shows that isolate **A-4** has a IC_{50} value of (185.98 µg/mL) weaker than chloroquine (0.19 µg/mL). Heme polymerization inhibitory activities assay showed that isolate **A-4** acted by inhibiting the weak heme polymerization.

Identification of xanthone: Pure compounds resulted from isolation were then identified by using spectrometer of UV, IR, RMI 1D (^{1}H NMR and ^{13}C NMR) and RMI 2D (COSY, HMQC and HMBC) mass spectra. Analysis of UV spectra for isolate **A-4** provided maximum wavelength of λ 315; 243; and 208 nm. Specific chromosphore group for the wavelength above was a conjugated benzene ring. IR spectrum that provided absorption on a wavelength of 2966 cm^{-1} was an stretching vibration of methyl group (-CH_3); 2858 cm^{-1} was a bending vibration of methyl group (-CH_3); 1284 cm^{-1} m was a bending vibration of hydroxyl group (-OH); 1090 cm^{-1} was a bending vibration of methoxyl group (-OCH_3) and benzene ring at 1719, 1460 and 1603 cm^{-1}. Mass spectra for isolate **A-4** provided molecular ion peak with *m/z* 410 $[M]^+$ as molecular weight. Molecular ion $[M]^+$ was subject to fragmentation, resulting in ion fragments at *m/z* 395 (*m/z* 410-150) with *m/z* 367 as basic peak, *m/z* 339 (*m/z* 410-71), *m/z* 331, *m/z* 207; *m/z* 69 and *m/z* 41.

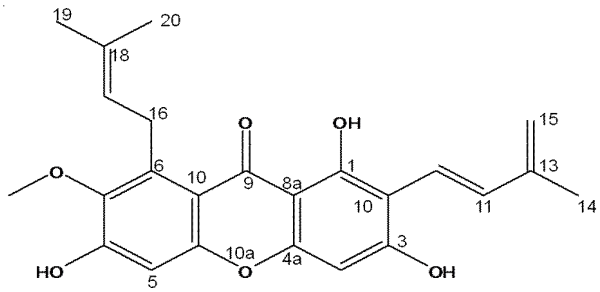
Investigation of proton RMI on high-field areas for isolate **A-4** revealed 4 (four) methyl groups at δ_H 1.69 (s); 1.77 (s); 1.83 (s) and 1.84 ppm (s). Methoxyl group was observed during the chemical shift of δ_H 3.83 (s) and methylene group (-CH_2-) on δ_H 3.45 and 4.08 ppm. Investigation of low-field

areas revealed that chemical shift on δ_H 5.29, 5.26, 6.29 and 6.82 ppm implied the existence of olefinic group and benzen ring substituted by oxygen atom. The chemical shift (δ) was a product of electron in a molecule that provided shielding effect on core spin since it has a conflicting magnetic field. An atom with a d value on low field (near to TMS) was called high shielded field. On the other hand, if δ value is farther to TMS, it would be called low shielded field.

Measurement of spectra ^{13}C NMR and DEPT could produce primary, secondary and thirstier and even quaternary carbons (CH_3, CH_2, CH, C, O-C, C=O, H-C=O, -CONH, -COOH and -COOR) depending on the degree of chemical shift. Investigation on carbon's chemical shift for Isolate **A-4** produced 24 signal carbons. DEPT analysis on the 24 carbons revealed that they consisted on 5 carbons for methyl group (4 carbons for CH_3 and 1 carbon for OCH_3); 2 methylene carbons (-CH_2-); 4 methine carbons (=CH-) and 13 quaternary carbons (12 for =C= and 1 for =C=O).

Analysis of 2D HMQC RMI spectra for isolate **A-4** compounds revealed that there was a correlation between carbon and its geminal proton. Proton of methyl group δ_H 1.77 was found to be correlated with δ_C 26.04; 1.84 with 18.11; 1.83 with 18.41; 1.69 with 26.02 and 3.83 with 62.23 ppm for methoxyl group. Likewise, a correlation of methylene proton δ_H 3.45 with 21.63 and 4.08 and 26.72 was also found. Methine proton δ_H 5.24 was found to be correlated with 21.66 as was 5.26 with 123.33; 6.82 with 101.75 and 6.29 with 93.46 ppm. In view of UV spectra data showing a conjugated benzene ring; IR spectra data showing carbonyl OH group; RMI proton, carbon, DEPT and HMQC showing that 24 carbon atoms had an empirical formula of $C_{24}H_{26}O_6$ and mass spectra data showing a molecular weight of 410, isolate **A-4** was determined as a compound derived from xanthone. Therefore, isolate **A-4** was rationally 1,3,6-trihydroxy-2-(3-methylbut-2-enyl)-7-methoxy-8-(3- methylbut-2-enyl)xanthen-9-on.

Elucidation of chemical structure of isolate **A-4** based on analysis of UV-VIS spectra, FT-IR, NMR and KG-MS as compared with literature on the compounds derived from *G. parvifolia* (Miq)Miq stem bark as found by Xu *et al.*[5] concluded that isolate **A-4** was of xanthone group, namely 1,3,6-trihydroxy-2-(3-methylbut-2-enyl)-7-methoxy-8-(3-methylbut-2-enyl)xanthen-9-on.



1,3,6-trihydroxy-2-(3-methylbut-dienyl)-7-methoxy-8-(3-methylbut-2-enyl)xanthen-9-on or isolate **A-4**

Fig. 1. Chemical structure of xanthone from *G. parvifolia* (Miq)Miq

The chemical structure Fig. 1 was confirmed by an interpretation on the data of 2-dimensional RMI spectra (COSY and HMBC) as presented by Fig. 2.

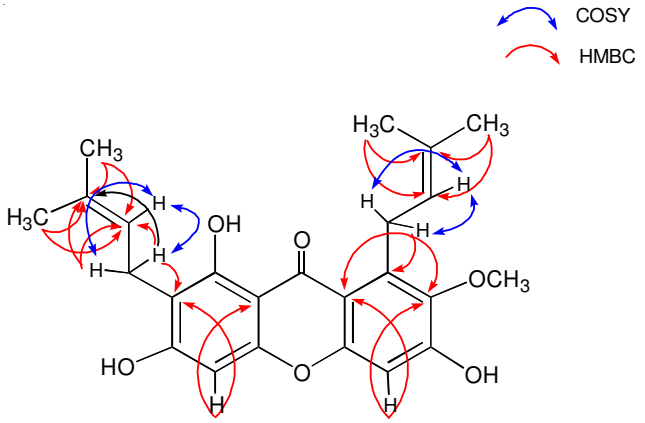


Fig. 2. Estimated correlation between proton and carbon atom of 1,3,6-trihydroxy-2-(3-methylbut-dienyl)-7-methoxy-8-(3-methylbut-2-enyl)xanthen-9-on

Heme polymerization inhibitory activities assay revealed that isolate **A-4** acted by inhibiting polymerization of hemes, like chloroquine. The results of the recent study seemed consisted with those of a study conducted by Ignatuschenko *et al.*[6] that antiplasmodial activities of xanthone compounds were probably the result of hydroxyl cluster on position 4 and 5[7]. The xanthone compounds probably acted by interacting with heme monomer: (1) interaction between Fe^{3+}-heme and carbonyl oxygen, (2) interaction between both aromatic systems; and (3) interaction between side-chain carboxyl group of heme and xanthone on position 4 and 5[7].

Heme detoxification may occur through the polymerization of heme to hemozoin or malarial pigments. One of the malaria's targets is preventing the formation of hemozoin pigments[8]. Hemoglobin consisted more than 90 % of total protein in the erythrocyte. In the case of malaria infection with 20 % parasitemia in trophozoite stadium, more than 75 % could be digested within 48 h.

The essential source of amino acid, plasmodium, is derived from uptake of free amino acid. Most synthesis of amino acid by plasmodium originated from hemoglobin digestion obtained from endocytosis contained in dietary vacuole[9,10]. Dietary vacuole of plasmodium was acidic organelle with pH of 5-5.4. Hemoglobin degradation was preceded by enzyme plasmepsin I that hydrolyzed 33 phenylalanine-34 leucine bonds on the parts playing role in the integrity of hemoglobin tetramer. Plasmepsin and falcipain activities produced peptide that subsequently formed amino acid[10]. Heme or ferriprotophorephyrin IX (FP IX) itself was made of hemoglobin degradation that was toxic to parasites. Toxic effects of FP IX might be in the form of protease inhibition, which further inhibited the formation of amino acid needed by parasites and damaged cell membrane of parasites. In the body of parasites, a mechanism of heme detoxification took place after converting heme into non-toxic metabolites called hemozoin or malaria pigment.

The process of free heme detoxification by plasmodium involved incorporation of free heme to form a complex called hemozoin. Identification of the pathway was a target of some antimalarials. Steele *et al.* conducted a research on 42 chemical compounds isolated from 9 plants used as antimalarials in North America by using heme polymerization inhibitory

activities assay. Heme polymerization inhibitory activities method was determined based on b hematin formation and constituted an appropriate method to determine antimalarial action of the test compounds[10].

Conclusion

The xanthone found in *G. parvifolia* (Miq) Miq stem bark, which was heme polymerization inhibitory activity is 1,3,6-trihydroxy-2-(3-methylbut-dienyl)-7-methoxy-8-(3- methylbut-2-enyl)xanthen-9-on.

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