Research Article

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Antiplasmodial Activity of Stigmastane Steroids from *Dryobalanops oblongifolia* Stem Bark

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Abstract: Three stigmastane steroids: 6-hydroxystigmast-4-en-3-one (1), stigmast-4-en-3-one (2), and 3-hydroxystigmast-5-en-7-one (3) were successfully isolated from the acetone extract of *Dryobalanps oblongifolia* stem bark. The structural determination of isolated compounds was carried out on the basis of data analysis of NMR and MS spectra. In order to identify the antiplasmodial activity, the isolated compound was put to test against *Plasmodium falciparum* 3D7. Antiplasmodial activity of the isolated compounds showed that the IC_{50} values of 6-hydroxystigmast-4-en-3-one were 37.29 µg/mL while the IC_{50} values of stigmast-4-en-3-one were 43.54 µg/mL and the IC_{50} values of 3-hydroxystigmast-5-en-7-one were 13.34 µg/mL (chloroquine phosphate was used as a positive control, IC_{50} 0.006 µg/mL). Judging from the results, the isolated compounds were proven to demonstrate mediocre antiplasmodial activity. Compound (**3**) indicated a better antimalarial activity than compound (**1**) and (**2**), even though there was no satisfactory activity that indicated its ability to combat chloroquine. Therefore, it might not be developed as an antimalarial drug.

Keywords: Antiplasmodial; *Dryobalanops oblongifolia*; *Plasmodium falciparum;* Stigmastane steroid.

1 Introduction

Malaria is one of the infectious diseases that has become a major problem of health. It is found in nearly most of all tropics, particularly developing and poor countries. *Plasmodium*, a parasitic protozoa genus, is what causes malaria in humans. The parasite that derived from the genus namely *Plasmodium falciparum* is the lethal part that causes acute infection worldwide with an annual death toll of 1-2 million people [1, 2].

Quinine which isolated from cinchona tree has been widely used to cure malaria, yet it is still powerless to comprehensively break the life cycle of *Plasmodium* parasites [3]. Artemisinin, a sesquiterpene lactone, is reported as a potential antimalarial drug and have the ability to kill all phases of the parasites' life cycle through interaction with heme, yet animal experiment shows neurotoxic and cardiotoxic effect [4]. Development of synthesized drugs, such as chloroquin, pyrimethamine, cycloguanil, and sulfadoxine, have indicated the decline of effectivity caused by the resistance of *Plasmodium* [3,5,6,7]. Therefore, it is crucial to develop alternative medicines from plants by constituent exploration as potential antimalarial drugs.

Dryobalanops oblongifolia belongs to the family of Dipterocarpaceae and is widely found in Indonesia and Malaysia [8].The phytochemical screening of fruit of *D. oblongifolia* revaled the presence of steroids compounds in this species[9]. *Dryobalanops* is known to produce oligostilbene constituents with various interesting activity such as antibacterial, antioxidant, antimalarial and cytotoxic [10, 11, 12, 13, 14]. In continuation for searching bioactive compounds from Indonesia's plants, a study towards *D. oblongifolia* was conducted by isolating the agents and examining the antiplasmodial activity against *Plasmodium falciparum* 3D7. Based on our knowledge this three stigmastane steroids (**1-3**) were first report from family Dipterocarpaceae and these isolated metabolites expressed only moderate antiplasmodial activity.

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2 Experimental section

2.1 General procedures

Firstly, $CDCl_3$ was used to dissolve ^1H and ^{13}C NMR and 2D NMR of stigmastane steroids spectra. While using TMS as the internal standard, JEOL J-500 spectrometer was used to record and it was utilized in $CDCl_3$ at 125 MHz (^{13}C) and 500 MHz (^1H). On a TSQ Quantum Access MAX Triple Quadrupole Mass Spectrometer, mass spectrometry was analyzed. Gravitation column chromatography (GCC) was conducted with Merck Si gel 60 (700-200 mesh). Vacuum liquid chromatography (VLC) and radial chromatography were conducted using Si gel 60 PF_{254} and Si gel 60 GF_{254}. The analysis of Thin Layer Chromatography (TLC) was done on Merck kieselgel 60 $GF_{254,}$ precoated Si gel plates, with a thickness of 0.25 mm. This research used already distilled analytical and technical grade solvents.

2.2 Plant Material

Mount Mali was the place where *D. oblongifolia* Dyer stem bark was originally collected. The mount is located in Tempunak, Sintang, West Kalimantan of Indonesia. The researchers then proceeded to the identification step by sending the plant specimen to the Biological Research Center of LIPI in Bogor, Indonesia. A voucher specimen was put in safekeeping at the herbarium.

2.3 Extraction and Isolation

At room temperature, as much as 5 kg of *D. oblongifolia* stem bark powder was pulped twice in acetone. It is meant to afford the extraction of brownish gummy after the process of vacuum evaporation. The extract was subsequently separated into 2 fractions: 1 fraction is able to be dissolved in acetone – diethyl ether while the other fraction is insoluble. As much as 48 g of the soluble fraction was divided into fractions using vacuum liquid chromatography (VLC) (*n*-hexane – ethyl acetate, enhancing polarity) to give four main fractions which are fraction A-D. By using radial chromatography techniques and Gravitation Colum Chromatography (GCC), as much as 1.7 g of Fraction B was separated and purified. The process led to the production of compound **1** with a total of 6.5 mg and compound **2** with a total of 3 mg. In order to isolate both compounds and enhance the polarity, *n*-hexane and ethyl acetate mixtures were used. As much as 1.6 g of Fraction C was separated and refined by using the same chromatography techniques and solvent mixtures, resulting in the production of compound **3** with a total of 3.4 mg.

2.3.1 *In Vitro* Antiplasmodial Assays

The antiplasmodial activity of compound 1-3 was determined in the Tropical Disease Institute of Universitas Airlangga, which is located in Surabaya, Indonesia. In this part, the method used was equivalent with the former method described by Widyawaruyanti et al. [15]. The dissolution of these samples was conducted in DMSO and they were stored at –20°C until use. A culture plate with 24 wells was used to cultivate the *P. falciparum* clone. The concentration range of each compound was 100, 10, 1, 0.1, and 0.01 µg/mL. As a positive control, a drug with antimalarial characteristics namely Chloroquine phosphate was used. The antiplasmodial activity measurement of compound **1**-**3** and chloroquine phosphate was calculated in replica. The monitoring process of parasitaemie was conducted when 48 hours had passed by firstly making a blood test fixed with MeOH and spattered with Geimsa (Merck). With the aim to determine the parasitaemia average ratio and average inhibition, the researchers calculated the total number of infected erythrocytes from originally 1000 healthy erythrocytes. The researchers used IC_{50} value to state the antiplasmodial activity of compound **1-3**. IC_{50} value is the concentration of compounds that causes 50% inhibition of the parasite growth. The IC_{50} value was obtained by using probit analysis processed by the SPPS program.

Ethical approval: The conducted research is not related to either human or animal use.

3 Result and Discussion

The acetone extracted from *D. oblongifolia* stem bark was fractionated and purified using radial chromatography, gravitation column chromatography, and vacuum liquid chromatography. It was intended to produce three stigmastane steroid compounds, specifically 6-hydroxystigmast-4-en-3-one (**1**), stigmast-4-en-3-one (**2**), and 3-hydroxystigmast-5-en-7-one (**3**). The isolated compound structures were identified on the basis of ^1H- and ^{13}C-NMR spectral data, and 2D NMR experimentations and contrast with the reported data and MS spectral data.

Table 1: Avarage Inhibition of isolated compounds (1-3) and Chloroquine phosphate against *P. falcifarum* 3D7.

No	Compound	% Avarage Inhibition (µg/mL)					IC_{50} µg/mL	IC_{50} µM
		100	10	1	0,1	0,01		
1	6-hydroxystigmast-4-en-3-one	60.78	35.14	18.40	10.26	1.36	37.29	86.91
2	Stigmast-4-en-3-one	58.06	34.76	20.28	11.17	1.51	43.54	105.36
3	3-hydroxystigmast-5-en-7-one	72.40	42.08	26.09	12.07	1.51	13.34	31.08
4	Chloroquine phosphate	99.13	89.38	82.01	65.73	58.55	0.006	0.01

***** The IC_{50} value was obtained by using probit analysis of SPPS program.

Compound 1 was isolated as an achromatic formless powder. $C_{29}H_{48}O_2$ was established as the molecular formula by means of ESI-MS ($[M+H]^+$ ion at *m/z* 429.076). There were a total of 6 methyl groups that were found to be present according to the ^{1}H-NMR spectrum. They were 2 groups of tertiary methyl [δ_H 0.74 (H-18) and 1.38 (H-19)], 3 groups of secondary methyl [δ_H 0.92 (H-21), 0.81 (H-26), and 0.84 (H-27)], and 1 group of primary methyl δ_H 0.85 (H-29). Furthermore, 1 oxygenated methine proton was seen at δ_H 4.35 (H-6), while 1 sp^2 methine was seen at δ_H 5.81(H-4). Meanwhile, it appeared that there was an overlap in the proton peaks of methylene and methine groups. There were 29 carbon signals revealed by the ^{13}C-NMR spectrum that contained an oxygenated secondary carbon at δ_C 73.3 (C-6), a carbonyl carbon at δ_C 200.4 (C-3), and 2 olefinic carbons [δ_C 126.3 (C-4) and 168.5 (C-5)]. There was an indication that the carbon signals that were present at δ_C 126.3, 168.5, and 200.4 signified an α,β-unsaturated carbonyl system that was present in compound **1**. It indicated a suggestion that **compound 1** was stigmastane steroid, especially when looking at the data of ^{1}H-NMR and ^{13}C-NMR. There were many correlations that have been pointed out by the spectra of compound 1 Heteronuclear Multiple Bond Correlation (HMBC) particularly between H-21/C-17, H-21/C-20, and H-21/C-22; H-22/C-23; H-25/C-23, H-25/C-24,H-25/C-27, and H-25/C-28; H-26/C-24, H-26/C-25, and H-26/C27; H-27/C-25 and H-27/C-26; H-29/C-28. The structure of side chain was established by these correlations. The presence of pentenoperhydrophenanthrene nucleus was indicated by the correlations between H-18/C-12, H-18/C-13, and H-18/C-14; H-19/C-1, H-19/C-5, H-19/C-9, and H-19/C-10, they signified the tetracyclic. In addition, there were indications showed by the HMBC links between H-4 / C-2, H-4 / C-6, H-4 / C-10, and H-6 / C-4, H- 6 / C-8, H-6 / C-10 that there are 2 different locations for the α, β-unsaturated carbonyl system and the hydroxyl group. The location of the former is in ring A, while the latter in ring B (Table 1). Aside from the analysis of the HMBC spectrum, the determination of hydroxyl group location can also be achieved by doing a TOCSY test. Compound **1** structure was indicated as 6-hydroxystigmast-4-en-3-one (Figure 1) [16].

Compound 2 was isolated as an achromatic formless powder with a $[M+H]^+$ ion at *m/z* 413.244. It was isolated during ESI-MS analysis and it corresponded to $C_{29}H_{48}O$ molecular formula. Compound **2** was discovered by the NMR data as a steroid with stigmastane skeleton. Although compound **2** has a high resemblance with compound **1** in terms of ^{1}H-NMR and ^{13}C-NMR spectrum chemical shifts, compound **2** does not possess any hydroxyl group (Table 1). Compound **2** structure was indicated as stigmast-4-en-3-one (Figure 1) [17, 18].

Compound 3 was isolated too as an achromatic formless powder that has $C_{29}H_{48}O_2$ molecular formula (ESI-MS, $[M+H]^+$ ion at *m/z* : 429.227). Compound **3** has high resemblance with compound **1** and **2** in terms of NMR spectrum chemical shifts, exposing that compound **3** was a stigmastane steroid. There were 29 carbon signals displayed by ^{13}C-NMR and DEPT spectra. These carbon signals consisted of 6 methyl carbons, 10 methylene carbons, 9 methine carbons, and 3 quaternary carbons, and carbonyl ketone. The HMBC spectra proved that 1 hydroxyl group and the α, β-unsaturated carbonyl system were present with links between H-4/C-2, H-4/C-3, H-4/C-5, H-4/C-6, H-4/C-10 ; H-6/C-4, H-6/C-8, and H-6/C10 ; H-8/ C-7, H-8/C-9. There are indications initiated by the HMBC analysis that the hydroxyl group and the α, β-unsaturated carbonyl system was located on different positions. The location of the former was at the position of C-3 in ring A, while the location of the latter was in ring B (Table 1). Compound **3** structure was identified as 3-hydroxystigmast-5-en-7-one. The confirmation can be seen through a contrast with stigmast 3-hydroxy-5-en-7-one chemical shifts, which is similar to the previously published research (Figure 1) [19, 20].

The examination of antiplasmodial activity against *P. falciparum* 3D7 was carried out by in vitro to three stigmastane steroid compounds. The test results showed

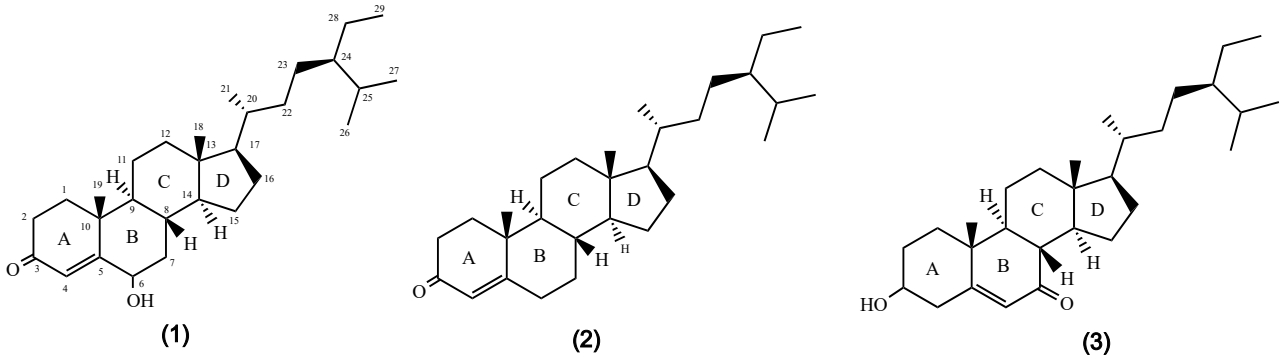

Figure 1: The structure of isolated compounds **1, 2** and **3** from *D. oblongifolia*.

6-hydroxystigmast-4-en-3-on (1)

Tabel 2: Antimalarial activity of 6-hydroxyistigmast-4-en-3-one against *P. falciparum* 3D7.

Dose (µg/ml)	R	% Parasitaemia		% Growth	% Inhibition	% Avarage Inhibition
		0 jam	48 jam			
Kontrol (-)	1	1.17	4.49	3.32	-	-
	2	1.17	4.48	3.31	-	
100	1	1.17	2.45	1.28	61.45	60.78
	2	1.17	2.49	1.32	60.12	
10	1	1.17	3.34	2.17	34.64	35.14
	2	1.17	3.30	2.13	35.65	
1	1	1.17	3.86	2.69	18.98	18.40
	2	1.17	3.89	2.72	17.82	
0.1	1	1.17	4.15	2.98	10.24	10.26
	2	1.17	4.14	2.97	10.27	
0.01	1	1.17	4.45	3.28	1.20	1.36
	2	1.17	4.43	3.26	1.51	
IC_{50}						**37.29 µg/mL**

that the IC_{50} value of 6-hydroxystigmast-4-en-3-one (**1**) was as much as 37.29 µg/mL. Meanwhile, for stigmast-4-en-3-one (**2**), the IC_{50} value was as much as 43.54 µg/mL, whereas the IC_{50} value of 3-hydroxystigmast-5-en-7-one (**3**) was as much as 13.34 µg/mL. Chloroquine phosphate was used a positive control with as much as 0.006 µg/mL IC_{50} (Table 1). Judging from the results, mediocre antiplasmodial activity was found in the three stigmastane steroid compounds [21]. Compound **3** showed better antiplasmodial activity than the others. The structure of stigmastane steroids chemical compound revealed that the presence and position of hydroxyl group can influence their antiplasmodial activity. The location of hydroxyl group in compound **3** is easier to interact with extracellular and intracellular fluids so that it can be easily carried to target molecule [22]. However, compound **3** is considered as lacking the ability to fight against the chloroquine so it may not be promoted as an antimalarial agent.

4 Conclusion

There were three stigmastane steroids that were successfully isolated from the acetone extract derived from the stem bark of *Dryobalanops oblongifolia*. The evaluation on antiplasmodial activity was performed to all of the isolated compounds, specifically 6-hydroxystigmast-4-en-3-one (1), stigmast-4-en-3-one (2), and 3-hydroxystigmast-

Stigmast-4-en-3-one (2)

Table 3: Antimalarial activity of sistigmast-4-en-3-one against *P. falciparum* 3D7.

Dose (µg/ml)	R	% Parasitaemia		% Growth	% Inhibition	% Avarage Inhibition
		0 jam	48 jam			
Kontrol (-)	1	1.00	4.65	3.65	-	-
	2	1.00	4.60	3.60	-	
100	1	1.00	2.53	1.53	57.50	58.06
	2	1.00	2.49	1.49	58.61	
10	1	1.00	3.35	2.35	35.62	34.76
	2	1.00	3.38	2.38	33.89	
1	1	1.00	3.91	2.91	20.27	20,28
	2	1.00	3.87	2.87	20.28	
0.1	1	1.00	4.21	3.21	12.06	11.17
	2	1.00	4.23	3.23	10.28	
0.01	1	1.00	4.58	3.58	1.91	1.51
	2	1.00	4.56	3.56	1.11	
IC_{50}						**43.54 µg/mL**

3-hydroxysistigmast-5-en-7-one (3)

Tabel 4: Antimalarial activity of 3-hydroxystigmast-5-en-7-one against *P. falciparum* 3D7.

Dose (µg/ml)	R	% Parasitaemia		% Growth	% Inhibition	% Avarage Inhibition
		0 jam	48 jam			
Kontrol (-)	1	1.17	4.49	3.32	-	-
	2	1.17	4.48	3.31	-	
100	1	1.17	2.09	0.92	72.29	72.40
	2	1.17	2.08	0.91	72.51	
10	1	1.17	3.08	1.91	42.47	42.08
	2	1.17	3.10	1.93	41.69	
1	1	1.17	3.64	2.47	25.60	26.09
	2	1.17	3.60	2.43	26.59	
0.1	1	1.17	4.10	2.93	11.75	12.07
	2	1.17	4.07	2.90	12.39	
0.01	1	1.17	4.44	3.27	1.51	151
	2	1.17	4.43	3.26	1.51	
IC_{50}						**13.34 µg/mL**

5-en-7-one (3). The antiplasmodial activity indicated that there was a total of 37.29 µg/mL of IC_{50} value in 6-hydroxystigmast-4-en-3-one. Meanwhile, the IC_{50} value of stigmast-4-en-3-one was as much as 43.54 µg/mL while as much as 13.34 µg/mL of IC_{50} value was found in 3-hydroxystigmast-5-en-7-one (chloroquine phosphate was used as a positive control, IC_{50} 0.006 µg/mL). Compound **3** was the most active isolated compounds although there was not enough activity to fight against chloroquine. As the consequence, compound 3 did not meet the standard of an antimalarial drug and may not be developed as a proper medication of the disease.

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Conflict of interest: Authors declare no conflict of interest.

References

[1] WHO. World Malaria Report (www.who.int/malaria); 2015.
[2] WHO. Global Report on Antimalarial Drug Afficacy[MOU1] and Drug Resistance; 2010.
[3] Marcus B. Malaria. Deadly Diseases and Epidemics; 2009.
[4] Balint GA. Artemisinin and its derivatives: an important new class of antimalarial agents. Pharmacol Ther. 2001 May-Jun;90(2-3):261–5.
[5] Shakya S, Kasturi K, Rao KR. Dihydrofolate reductase: A target for antimalaria drug. Pharmanest. 2010;1(1):6–10.
[6] Bwijo B, Kaneko A, Takechi M, Zungu IL, Moriyama Y, Lum JK, et al. High prevalence of quintuple mutant dhps/dhfr genes in Plasmodium falciparum infections seven years after introduction of sulfadoxine and pyrimethamine as first line treatment in Malawi. Acta Trop. 2003 Mar;85(3):363–73.
[7] Happi CT, Gbotosho GO, Folarin OA, Akinboye DO, Yusuf BO, Ebong OO, et al. Polymorphisms in Plasmodium falciparum dhfr and dhps genes and age related in vivo sulfadoxine-pyrimethamine resistance in malaria-infected patients from Nigeria. Acta Trop. 2005 Sep;95(3):183–93.
[8] Bawa KS. 1998 CIFOR (www.cifor.org)
[9] Liliwirianis N, Musa NL, Zain WZ, Kassim J, Karim SA. Premilinary studies on phytochemical screening of ulam and fruit from malaysia. E-J Chem. 2011;8(s1 S1):S285–8.
[10] Indriani; Yoshiaki T.; Puspaningsih, N.N.T.; Aminah, N.S.; (-)-Ampelopsin F, Dimerstilben compound from Dryobalanops oblongifolia dyer (Dipterocarpaceae) and Antimalarial activity test. Chem Nat Compd. 2017;53:559–61.
[11] Syah YM, Aminah NS, Hakim EH, Aimi N, Kitajima M, Takayama H, et al. Two oligostilbenes, cis- and trans-diptoindonesin B, from Dryobalanops oblongifolia. Phytochemistry. 2003 Aug;63(8):913–7.
[12] Aminah NS, Syah YM, Hakim EH, Aimi N, Achmad SA, Niwa M. A dimer resveratrol from Dryobalanops oblongifoli. Indonesia Journal Chemistry. 2006;6(1):79–81.
[13] Muhtadi H. E.H.; Juliawaty, L.D.; Syah, Y.M.; Achmad, S.A.; Latip, J.; Ghisalberti, E.L. Cytotoxic resveratrol oligomers from the tree bark of Dipterocarpus hasselti. Fitoterapia. 2006;77:550–5.
[14] Wibowo A, Ahmat N, Hamzah AS, Sufian AS, Ismail NH, Ahmad R. R.; Jaafar, F.M.; Takayama, H. Malaysianol A, a new trimer resveratrol from the stem bark of Dryobalanops aromatica. 2011. Fitoterapia. 2011;82:676–81.
[15] Widyawaruyanti A. Subehan, Kalauni,S.K.; Awale,S.; Nindatu, M.; Zaini, N.C.; Syafruddin, D.; Setia Asih, P.B.; Tezuka, Y.; Kadot, S. New prenylated flavones from Artocarpus campeden and their antimalarial activity in vitro. J Nat Med. 2007;61:410–3.
[16] Aguilar-Gonzalez AR, Mena-Rejón GJ, Padilla-Montaño N, Toscano A, Quijano L. Triterpenoids from Hippocratea excelsa. The crystal structure of 29-hydroxytaraxerol. Zeitschrift für Naturforschung. 2005;60(5):577–84.
[17] Fathaiya J, Suhaila M, Nordin L. Hypoglycaemic effect of stigmast-4-en-3-one from Parkia speciosa. Food Chem. 1994;54:9–13.
[18] Barla A, Birman H, Kultur S, Oksuls S. Secondary metabolites from Euphorbia helioscopia and their vasodepressor activity. Turk J Chem. 2006;30(3):325–32.
[19] Kovganko NV, Kashkan ZN. Synthesis of (24R)-3β-Hydroxystigmast-5-en-7-one. Chem Nat Compd. 1999;35(4):433–6.
[20] Siddiqui BS, Ara I, Faiz S. Non-terpenoidal constituents of Azadirachta indica root bark. J Indian Chem Soc. 2013;90:1569–672.
[21] Batista R, Silva AJ Jr, de Oliveira AB. Plant-derived antimalarial agents: new leads and efficient phytomedicines. Part II. Non-alkaloidal natural products. Molecules. 2009 Aug;14(8):3037–72.
[22] Siswandono.; Soekardjo, B. Kimia Medisinal, Jilid I, Edisi II, Surabaya: Airlangga University Press. [MOU1]Efficacy; 2000.