ANTIPLASMODIAL EVALUATION OF ONE COMPOUND FROM *Calophyllum flavoranulum*

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ABSTRACT

Calophyllum flavoranulum (Clusiaceae family) is a big tree and medical plants from the tropical rain forest of the Indonesian continent. The organic extracts from stem bark yielded phenyl coumarin. The structure was elucidated with the aid of spectroscopic technique. Antiplasmodial activities of isolated compound was tested in vivo against Plasmodium berghei parasite and showed the best antiplasmodial activity. New flavoranulum coumarin compound showed activity against P. berghei parasite with inhibition growth 0, 31.33, 40, 30.67, 46 and 34% at dosage $1.1x10^{-10}$, $1.1x10^{-9}$, $1.1x10^{-8}$, $1.1x10^{-7}$, $1.1x10^{-6}$, and $1.1x10^{-5}$ mg/mL, respectively. Flavoranulum coumarin very active as antiplasmodial, and at 3 and 4 day incubation all of parasite were died.

Keywords: *Calophyllum flavoranulum; Plasmodium berghei; antiplasmodial activity*

ABSTRAK

Calophyllum flavoranulum (termasuk familia Clusiaceae) merupakan tumbuhan tingkat tinggi dan tumbuhan obat dari hutan hujan tropis kepulauan Indonesia. Ekstrak organik dari kulit batang menghasilkan senyawa fenil koumarin. Elusidasi struktur dilakukan dengan menggunakan alat spektroskopi. Senyawa hasil isolasi diuji aktivitas antiplasmodial secara in vivo menggunakan parasit Plasmodium berghei dan menunjukkan aktivitas antiplasmodial yang sangat bagus. Senyawa flavoranulum koumarin menghambat pertumbuhan parasit P. berghei dengan hambatan 0, 31,33, 40, 30.67, 46 dan 34% pada konsentrasi sampel berturut-turut $1,1x10^{-10}$, $1,1x10^{-9}$, $1,1x\ 10^{-8}$, $1,1x10^{-7}$, $1,1x10^{-6}$, dan $1,1x10^{-5}$ mg/mL. Senyawa flavoranulum kumarin sangat aktif sebagai antiplasmodial karena pada hari ke 3 dan ke 4 inkubasi semua parasit telah mati.

Kata Kunci: *Calophyllum flavoranulum; Plasmodium berghei; aktivitas antiplasmodial*

INTRODUCTION

Malaria is a major health problem with hundreds of millions of people being infected, mostly-but not only in tropical and subtropical countries, with an incidence of 500 million cases per year globally. Despite the importance of this disease, treatment investment and malaria control are modest, especially in Africa where it kills over 2.5 million children per year, according to the WHO data [1], In Brazil, it is estimated that 500.000 cases occur annually, mainly in Amazon Rain Forest. The appearance of drug resistant *Plasmodium falciparum* since 1960 has made the treatment of malaria increasingly problematic and apparently the battle against malaria has not been successful [2]. Drugs which are employed in the treatment of malaria are becoming increasingly ineffective due to the spread of resistant parasite strains threaten to increas the annual death tool [3-4].

As the anopheles vector has developed resistance to insecticides, the situation appears definitely alarming. Reduced sensitivities of *Plasmodium* are also observed toward quinine and mefloquine, inducing recurrences of parasitaemia. Today only artemisinin and its derivatives remain fully effective, except for some recrudescences which may appear when they are used in monotherapy [5].

The development of novel drug for the prophylactic and curative treatment of malaria is highly necessary but has proven to be very difficult. *Calophyllum* is one of the most important medicine plants, because *Calophyllum* as a source of new active compound for treatment of malaria [2-4]. Four species of *Plasmodium: P. falciparum, P. vivax, P. ovale* and *P. Malariae* can produce malaria in human. Among the four species, *P. falciparum* is the most widespread and dangerous [3].

The genus *Calophyllum* of the Guttiferae/Clusiaceae family is a large group of tropical trees consisting of approximately 180-200 different species. The genus *Calophyllum* is primarily found in the Indo-Pacific region, particularly Malaysia [6]. In

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Indonesia, this plant is commonly known as nyamplung [7]. *Calophyllum* is one of the most important traditional medicine plants .The balsams from the bark of *C. inophyllum* Alexandrian Laurel used as a cicatrisan, infusion or decoction of the leaves has been traditionally used as an eye remedy in Asian medicine [8]. Bioactivity of xanthon from Calophyllum as antihypoglycemic, antiplatelet, antimicrobial [9], antiinflammatory, antifungal, inhibition of lipid peroxidase [8-10], prenylcoumarin have activity as antitumor [11], class of coumarin (such as costatolide, soulatrolide, calanolide A) that the presence of a benzylic alcohol at C10 was important for anti-HIV activity [12]. Polyisoprenylated ketone, enervosanone from the stem bark of *C. enervosum* have activity as antimicrobial [13], and other compouns have activity as antibacterial.

Calophyllum species also content cromene acid, most of these acids possess a phloroglucinol ring system, such as the isocordato-oblogic acid from the *n*-hexane extract of the stem bark of *C. cordato-oblongum* [14]. These spesies are also particularly rich in coumarin derivates, calanolide F, soulattrolide and costatolide and other coumarin were isolated from the leaf, bark, latex and twig of *Calophyllum* [15].

According to the reference [8], the large tree of *C. flavoranulum* M.R Henderson up to 45 m tall, with bole up to 160 cm in diameter. It grows up to 800 m altitude. The outer layer of the fruit is edible but fibrous. Distribution in Southern Thailand, Peninsular Malaysia, Singapore, Sumatra and Kalimantan Islands. *C. flavoranulum* M.R Henderson & Wyatt-Smith grows in mixed dipterocarp forest up to 450 altitude and fairly large tree up 38 m tall with bole up to 90 cm in diameter. Distribution in Peninsular Malaysia and Riau Archipelago, perhaps also in Sumatra [4].

C. flavoranulum synonyms is *C. parvifolium* Vesque. Distribution in Peninsular Malaysia and Northern Kalimantan. The outer layer having large air space under the skin outer bark is yellowish to greywish but the innerbark is pink with a lot of yellow latex. *C. flavoranulum* is small tree and have 12 m tall hight and with bole up to 15 cm diameter and grown in sandy soils up to 1900 m altitude, *C. flavoranulum* is one of the most important traditional medicine plants. Antioxidant activity of six *Calophyllum* medicinal plants from Indonesian tropical forest using DPPH (1,1-diprenyl-2-picrylhydrazyl) was reported by Abbas, J et al. [17]. Recently, calocoumarin compound was isolated isolate from *C. incrasaptum* which has antiplasmodial activity and cytotoxic activity [18].

Xanthones as antimalarial agenst have been studied by Marina, V. [19]. Phenyl coumarin from *C.teysmannii var. inophylloide* was isolated by Cao Shu-Geng [15-16], that phenyl coumarin compound was used as reference compound to phenyl coumarin from *C. flavoranulum*. The IR spectrum of reference compound showed at 3420 cm^{-1} for OH,1740 and 1609 cm^{-1} for C=O group. According to HR-MS data of reference compound established the molecular formula $C_{28}H_{24}O_7$ with molecular weight 470. The IR and UV spectra of reference compound was very similar to isolated compound from *C. flavoranulum,* thus suggesting the structure of an oxygenated coumarin. Also the ^{1}H and ^{13}C–NMR spectra of reference compound (Table 1) was similar with flavoranulum coumarin from *C. flavoranulum*. Phenyl coumarin from *C. teysmannii var. inophylloide* have cytotoxit activity, in this study phenyl coumarin compound from *C. flavoranulum* will be test as antiplasmodial activity.

EXPERIMENTAL SECTION

Materials

The stem bark of *C. flavoranulum* was collected from the Palangkaraya forest Kalimantan Island in Indonesia and samples was identified by Mr. Ismail Rahman is a botanis from Herbarium Research Centre – LIPI (Indonesian Institute of Sciences). A voucher specimens were deposited at the Herbarium Research Centre – LIPI Bogor, Indonesian. Ethanol (75%), ethyl acetate, butanol chloroform, silica gel for column chromatography (silica gel 60, 200 mesh) and for preparative plates were purchased from Merck (Darmstadt, Germany). Solvents were distilled before used.

Instrumentation

Liquid chromatography : silica gel 60 (particle size 70-230 mesh or 0.063-0.200 mm) and silica H. TLC silica gel precoated aluminium plates. (Merck, silica gel 60 F_{254}). Spot were visualized by 10% H_2SO_4, and by UV lamp at (λ_{254max} and $\lambda_{365\ nm}$). Spectra UV-VIS were recorded on a Hitachi spectrophotometer (λ in nm). IR spectra were measured on a Shimadzu Prestige 21 FTIR Spectrometer in KBr pellet; (υ in cm^{-1}). NMR spectra JEOL–JNM ECA 500 (500 ^{1}H and 125 ^{13}C) instruments using $CDCl_3$ or (D_6) acetone as solvent, otherwise state chemical shift (δ) in ppm, coupling constant (*J*) in Hz. LC-MS Mariner Bio Spectrometry was used for determinate of molecular weight. Melting points for isolated crystals were measured on a Fisher Scientific serial 903N0056 apparatus.

Procedure

Extraction plant materials

Stem bark of *C. flavoranulum* was dried at room temperature then at oven 50 °C and powdered. The

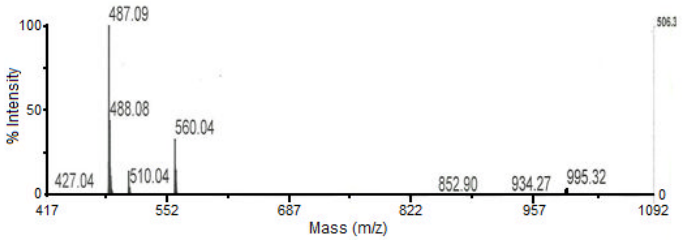

Fig 1. LC-MS spectra of new compound B (flavoranulum coumarin)

dried powdered 3.2 kg *of C. flavoranulum* was successively extracted with ethanol 70%. Evaporation of solvent gave ethanol extract, then suspended in water, then partitioned by using hexane, EtOAC and butanol to give -hexane, EtOAC and butanol crude extract.

Isolation of EtOAC crude extract

EtOAC crude extract was separated by vacuum column chromatography (silica gel Merck 800 g), eluting with *n*-hexane and a gradient of EtOAC was added up to 100%, followed by $CHCl_3$-MeOH in order of increasing polarity up to (1:1). Total chloroform extract (22 g) was fractionated by column chromatography using silica 200 mesh as solid phases (95 g, column 60 length x 5 cm id and eluent EtOAC–MeOH, in order of increasing polarity to give 35 fraction. From fraction 16 were then crystallized by CH_2CL_2–MeOH to yield a new flavoranulum coumarin compound.

Determination of the antimalarial activity

Antimalarial activity of the isolated compounds was determinated *in-vivo*. In brief each flavoranulum compound 1.1 mg (BW 486.09) was dissolved in 100 ul DMSO to obtained 10^{-2} as stock solution and kept at -20 °C until used. Antimalarial activity was done *in-vivo* against *P. berghei* using mice strain Swiss, inoculation as intraperitoneal (ip) in mice, were parasite up to 20%, then inject coumarin to the mice were the take the blood from the vena of the mice's tail after zero day (Do), 1 day (D1), 2 days (D2), 3 days (D3) and 4 days (D4) and calculate % parasite by making a blood smear fixed with methanol and stained with Giemsa stain and number of the infected red blood cells were counted under the microscope. Calculation of infected red cell by parasite was done at Eijkman Institute for Molecular Biology. Chloroquine was used as a positive control. The concentration response parasite growth data were analyzed by a linear regression function using the Sigma-plot 2000 computer program to determine the 50% inhibitory concentration (IC_{50}). The IC_{50} value is defined as the concentration of compound producing 50% parasite growth inhibition relative to untreated control.

Total parasitaemia was calculated as the number of parasites observed (number of the infected erythrocyte by parasite), was divide by the total erythrocyte multiplied by 100%

$$\% \text{ parasetemia} = \frac{\text{Number of the infected erythrocyte by parasite}}{\text{Total erythrocyte}} \times 100\%$$

$$\% \text{ Inhibition growth} = \frac{\text{Total parasite in control - total of parasite in sample}}{\text{Total of parasite in control}} \times 100\%$$

Culture of *P. berghei*

The parasites were maintained in continuous in vitro culture as described by Trager and Jensen (1976) [18]. The chloroquine-susceptible D6 clone of *P. berghei* has been previously described. The parasite were cultured in Group A^- human erythrocytes, suspended at a 2% hematocrit in RPMI-1640 (pH 7.4 by addition natrium carbonate solution) which containing 3 g/L glucose, 2,5 µg/mL getamicin (Sigma), 50 µg/mL hypoxanthin (Sigma), getamicin (Sigma), 25 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethannesulfonic acid, GibcoBRL) 10% human serum and maintained at 37 °C in a gas mixture of 5% O_2, 5% CO_2 and 90% N_2 [14].

RESULT AND DISCUSSION

Antioxidant Activity

From 3.2 kg of stem bark of *C. flavoranulum* was given 104.9 g, 92.9 g and 152.9 g hexane, ethyl acetate and *n*-butanol extract respectively, According to the result the antioxidant activity, that ethyl acetate fraction have good antioxidant activity by IC_{50} = 214.03 ppm [16], Antioxidant activity are more 500 ppm for hexane extract and 4.70 ppm for butanol extract and in this study we hope ethyl acetate extract also have good antiplasmodial activity.

Elucidation Structure by Nucleur Magnetic Resonance (H and C-NMR) Spectroscopy

One new compound B (*flavoranulum coumarin*) from sub fraction ethyl acetate of *C. flavoranulum* was isolated as yellow crystal. This structure was established by spectroscopic means. From LC-MS spectra showed the molecular ion $(M+1)^+$ at m/z = 487.09 and molecular ion (2M + Na) at m/z = 995.18, compound B have molecule weight 486.09 with formula is $C_{29}H_{26}O_7$.

In the IR spectrum, a absorption bands attributable for OH (3300.20 cm^{-1} broad) and C=O groups from coumarin moiety (1703 and 1610 cm^{-1} for C-O), aromatic ring (C=C) at 1442 and 1492 cm^{-1} and C-H stretching methyl group at group at 2922cm^{-1} and also C-H bending methyl at 1192 cm^{-1}. Showed IR

Table 1. ^{1}H-NMR and ^{13}C-NMR data new compound B (*flavoranulum coumarin*)

C-Number	^{13}C-NMR Reff	^{13}C-NMR C. *depressiner vosum*	^{1}H-NMR Ref.*	^{1}H-NMR C. *depressiner vosum*
C(2)	159.1 (s)	158.1 (s)		
C(3)	113.5 (*d*)	110.5 (*d*)		
C(4)	155.2 (*s*)	154.2(*s*)	6.0 (*s*) C-H	6.4(*s*) C-H
C(4a)	160.0 (s)	161.0 (s)		
C(5)	156.6 (s)	156.9 (s)		
C(5a)	115.9(s)	117.0,(s)		
C(6)	71.2 (d)	70.,4 (d)	5.55 (*d, J* = 6.1)	5.50 (*d. J* = 5,5)
C(7)	90.3 (d)	90.3 (d)	4.28 (*d. J* = 7.8)_	4.28 (*d. J* = 7.50)
C(8)	-	-		
C(8a)	16.6 (s)	1610 (s)		
C(9)	106.2 (s)	102.2 (s)		
C(9a)	155.6 (s)	154.6 (s)		
C(10)	190.0 (s)	192.0 (s)		
C(11)	72.8 (s)	71.85 (s)	7.30-7.88	7.30-7.85
C(12)	24.7 (q)	24.2 (q)	1.14 (CH_3)	1.00(CH_3)
C(13)	28.3 (q)	27.0 (q)	1.27 (CH_3)	1.58 (CH_3)
C(14)	-	**22.6 (q)**	1.27	**3.40 (OCH_3)**
C(1')	139.8 (*s*)	139.38 (*s*)		3.80 (s)
C(2',6')	126.9 (d)	127.9 (d)	7.30 (*m*) (C-H)	7.30-7.88 (C-H)
C(3',5')	128.0(d)	128.5(d)	7.41	7.30-7.88 (C-H)
C(4')	127.6(d)	126.6(d)	7.41 (*m*)(C-H)	7.14 (*m*) C-H)
C(1')	137.6 (s)	138.4(s)	7.89 (*dd, J* =7.9 & 1.2	7.25 (*dd.J* =7.9 & 1.2)
C(2',6')	129.7 (*d*)	129.3 (*d*)	7.47 (*t. J* = 8)	7.15 (*t J* = 8)
C(3',5')	128.6 (*d*)	126.6 (*d*)	7.59 (*t. J* = 7,9 &1.2)	7.55 (*t. J* = 7 &1.5)
C(4')	133.8 (*d*)	131.8 (*d*)	7.60 (*m. J* = 8 &1.2)	7.60 (*m., J* =7.5 &1.2)
MeO (C-15)	59.4	58.0	3.60 (s)	**3.75 (s) (OCH_3)**

Reference [(Coa S.G, et al., 1998)]

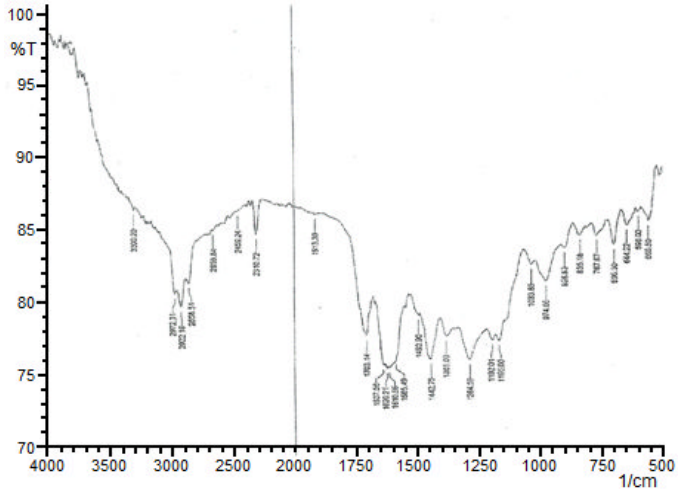

Fig 2. IR spectra of compound B (flavoranulum coumarin)

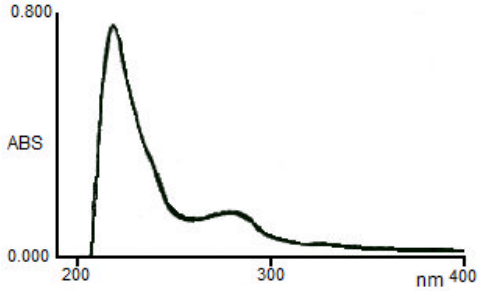

Fig 3. UV spectra of compound B (flavoranulum coumarin)

spectrum of reference compound at 3420 cm^{-1} for OH, 1740 and 1609 cm^{-1} for C=O group, similar phenyl coumarin from *C. flavoranulum*.

UV absorption maximum at λ 215.5 nm with absorbance 0.315 typical of an oxygenated coumarin and λ 274.5 nm with absorbance 0.15 and λ 381.5 nm absorbance is 0.025, (C 10 ppm in methanol). These UV spectrum showed the typical absorptions of a phenylcoumarin nucleus at λ_{max} 215.5 & 274.5 and 381.5 nm (Reff at λ_{max} 228 & 286 and 324 nm).

^{1}H-NMR spectrum revealed signal assign able to a unsubstituted phenyl group, with ten aromatic proton (δ 7.30-7.88) and additional one olefinic proton (δ 6.0) specific at position C-3.Two MeO groups (δ 3.75 and 3.80), two Me groups (δ 1.58 and 1.40) and two CH protons (δ 5.56 and 4.30) belong to isopropyl group were present at Table 1. The only difference in the ^{13}C-NMR spectra of compound B (*Flavoranulum coumarin)* from stem bark of *C. flavoranulum* with reference was the peak at *∂* 22,6 ppm (C-14).

The ^{1}H and ^{13}C-NMR spectra of compound B (*Flavoranulum coumarin)* was similar to ^{13}C-NMR spectra of compound in reference (phenyl coumarin, see Table 1 and Fig. 4a and 4b), except for the ^{13}C-NMR signal of the compound B at δ 22.6 (C-14) arising from the MeO group. The HMBC spectrum of compound B (*Flavoranulum coumarin)* confirmed the position of the phenyl group at C(4).

Signal of compound typical for an oxygenated C atom at δ 70.4 (C6). The HMBC spectrum show the

Table 2. Growth rate inhibition of Plasmodium parasite by six dosage of the flavoranulum coumarin

Dosage (mg/mL)	Parasitaemia (%)			Parasitaemia (%)			Parasitaemia (%)			Growth rate (%)	Inhibition growth (%)
	A Do	B Do	Average (%)	A D1	B D1	Average (%)	A D2	B D2	Average (%)		
$1.1x10^{10}$	3.05	3.0	3.025	3.50	3.40	3.450	4.00	3.50	3.750	100	0
$1.1x10^{-9}$			3.025	1.85	68.67	1.825	2.65	2.50	2.575	68.67	31.33
$1.1x10^{-8}$			3.025	1.50	2.0	1.750	2.20	2.30	2.250	60	40
$1.1x10^{-7}$			3.025	1.55	3.4	2.475	2.40	2.80	2.600	69.33	30.67
$1.1x10^{-6}$			3.025	1.15	1.9	1.525	1.95	2.10	2.025	50	50
$1.1x10^{-5}$			3.025	1.30	1.6	1.450	1.85	3.10	2.475	66	34
					IC_{50} =						$1.1x10^{-6}$ mg/mL

Sample 1.1 mg dissolved in 100 µL DMSO to get a solution in mg/mL as a stock solution

- The highest concentration is $1.1x10^{-2}$ mg/ml and then diluted up to $1.1x\ 10^{-9}$ mg/mL
- IC_{50} of flavoranulum coumarin ($1.1x10^{-6}$ mg/mL) = $2.26x10^{-3}$ mM (BM 486.09)

Table 3. Growth rate inhibition of Plasmodium parasite by six dosage choroquine

Dosage Mol	Parasitaemia (%)			Parasitaemia (%)			Growth rate (%)	IC_{50} (Mol)
	A Do	B Do	Average (%)	A D1	B D1	Average (%)		
$3x10^{-10}$	0.45	0.5	0.475	3.7	4	3.85	100	$3x10^{-9}$ M
$3x10^{-9}$	0.45	0.5	0.475	2.3	2.8	2.55	66.23	
$3x10^{-8}$	0.45	0.5	0.475	0.3	0.8	0.55	14.29	
$3x10^{-7}$	0.45	0.5	0.475	0.08	0.09	0.085	2.21	
$3x10^{-6}$	0.45	0.5	0.475	0.03	0.04	0.035	0.91	
$3x10^{-5}$	0.45	0.5	0.475	0.01	0.0	0.005	0.13	
				$IC_{50} = 3x10^{-9}$ M = $3x10^{-12}$ mM				

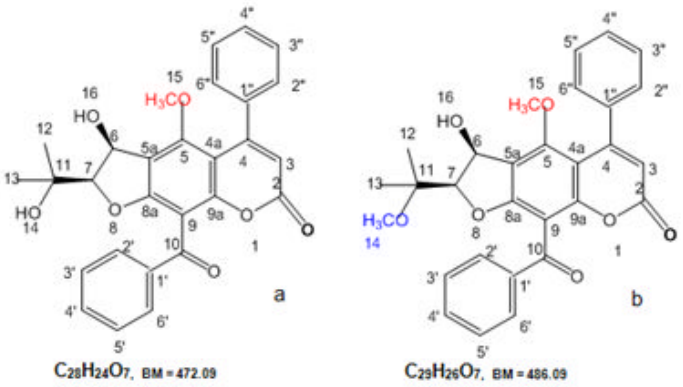

Fig 4. Structure of phenyl coumarin from reference [14] (a), structure of new compound B (*Flavoranulum coumarin*) from *C. flavoranulum* (b)

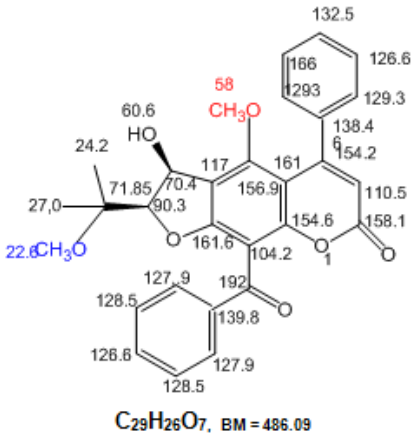

Fig 5. Chemical shifts for the new compound B (*Flavoranulum coumarin*) in ^{13}C-NMR

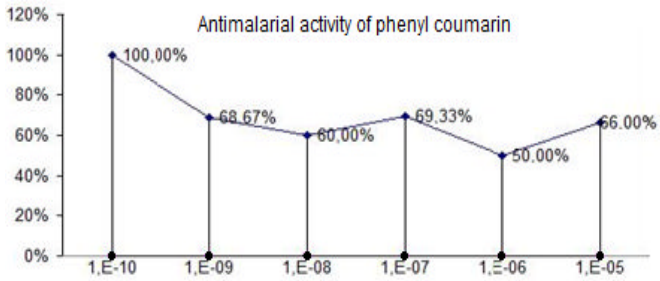

Fig 6. Determination IC_{50} of flavoranulum coumarin

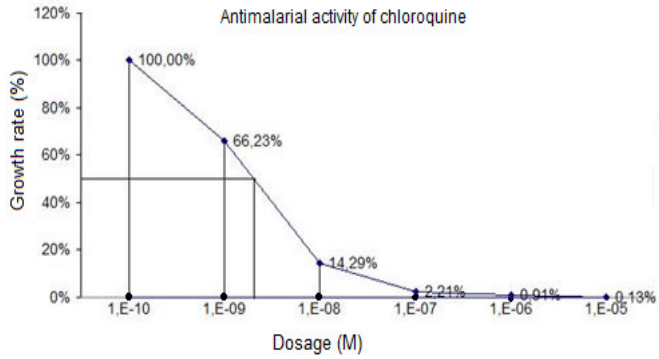

Fig 7. Determination IC_{50} of chloroquine

corelation between C(1') (δ 139.38) and H-C(3) (δ 6.0) and H-C (3',5') (δ 7.41) allowed the position of the phenyl group at C4. According to their coupling constant (*J* = 6.1 Hz), H-C(6) and H-C(7) were cis arranged and benzoil group at (C-9) was confirmation with the NMR data (see Fig. 4a and Fig. 5).

Antimalarial Activity of Phenyl Coumarin from C. flavoranulum

We got new compound B (Flavoranulum coumarin) from *C. flavoranulum*, our new compound content methoxy (OCH_3) at position C-14, phenyl coumarin from *C. inophyllum* have not methoxy (OCH_3) at position C-14. New compound B (Flavoranulum coumarin) have antiplasmodial activity with growth rate 100%, 68.67%, 60.0%, 69.33%, 50% and 66% or inhibition growth 0%, 31.33%, 40%, 69.33%, 50% and 34% with sample concentration $1.1x10^{-10}$, $1.1x10^{-9}$, $1.1x10^{-8}$, $1.1x10^{-7}$, $1.1x10^{-6}$, $1.1x10^{-5}$ mg/mL, respectively (see Table 2 and Fig. 6).

From the Table 2 and Fig. 6 displayed inhibition growth rate of parasites *Plasmodium berghei* were not depend of sample concentration and showed the potent activity (IC_{50} = $2.2x10^{-3}$ mM) which is lower than the well-know antimalarial drug, chloroquine (IC_{50} = $3x10^{-12}$ mM) see Table 3 and Fig. 7 (antimalarial activity of chloroquine as positive control).

CONCLUSION

The chloroquine showed activity against *Plasmodium* with dosage $3x10^{-10}$; $3x10^{-9}$; $3x10^{-8}$; $3x10^{-7}$; $3x10^{-6}$ and $3x10^{-5}$ could inhibit growth rate of parasite 0%; 33.77%; 85.71%; 97.79%; 99.09% and 99.87% respectively. Growth rate inhibition of *Plasmodium* parasite by chloroquine are depend of dosage of chloroquines. The chloroquine with IC_{50} = 3 x 10^{-9} Mol or = 3 x 10^{-6} mM or = 0.03 µM, but growth rate inhibition of *Plasmodium* parasite by *flavanium coumarin* are not depend of dosage. The sample new flavoranulum coumarin showed activity against *P. berghei* with inhibition growth 0%, 31.33%; 40%; 30.67%; 46% and 34% at concentration $1.1x10^{-9}$; $1.1x10^{-8}$; $1.1x10^{-7}$; $1.1x10^{-6}$; and $1.1x10^{-5}$ mg/ml, respectively. Flavoranulum coumarin very active as antiplasmodial, with IC_{50} 1.1 $x10^{-6}$ mg/mL equal with $2.26x10^{-3}$ mM (BM 486.09). From the observed antimalarial activities, we concluded that the present of the phenyl group at C-4 and hydroxyl group at C-6 plays the important role for antimalarial activity. The inhibition activity exhibited by this *flavanium coumarin* could support the traditional use of the bark of *C. flavoranulum* as an antimalarial drug.

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