Isolation and Identification of Antiplasmodial Compound from Methanol Extract of *Calophyllum bicolor* P. F. Steven

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Abstract

Calophyllum bicolor (Clusiacea) is a big tree from Indonesian rain forest in Palangkaraya, Central Kalimantan. *Calophyllum* or bintagor is one of many sources of natural bioactive compounds that can be used in the fields of health and pharmaceuticals. The aim of this research was to explore the antiplasmodial activity of methanol extract of *Calophyllum bicolor* P.F. Steven against *Plasmodium falciparum*. The methanol extract was purified by colomn chromatography system, hexane – ethyl acetate was used as solvent with increasing polarity. One pure compound was obtained and was elucidated based on the 1H-&13C-NMR and 2D-NMR, [COSY, HMBC and HMQC] data and the isolated compound was identified as xanthone. Methanol extract showed antiplasmodial activity growth inhibition against *P. falciparum* with IC_{50} 5.2 ppm and the new 5-methoxy trapezifolixanthone compound have maximum inhibition at concentration 0.11 nMol.

Keywords: *Calophyllum bicolor* P. F. Steven, *Plasmodium falciparum, antiplasmodial activity, coumarin*

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Introduction

Malaria is a disease caused by infection of red blood cells with protozoan parasites that lives part of its life in humans and part in mosquitoes. Malaria remains an important cause of illness and death in children and adults in countries in which it is endemic. Malaria threatening the lives of more than one third of the world's population. It thrives in the tropical areas of Africa, Asia, and Central and South America. WHO estimate that 3.3 billion people were at risk of malaria in 2011, with populations living in sub-Saharan Africa having the highest risk of acquiring malaria, approximately 80 % of cases and 90 % of deaths are estimated occur in the WHO African Region (WHO, 2012).

The Center for Disease Control and Prevention (CDC) estimates 1,200 cases of malaria are diagnosed each year in the United States. Malaria is caused by a single-celled parasite from the genus *Plasmodium*. More than 100 different species of *Plasmodium* exist. They produce malaria in many types of animals and birds, as well as in humans. Four species of *Plasmodium* commonly infect humans. Each one has a distinctive appearance under the microscope, and each one produces a somewhat different pattern of symptoms. Two or more species can live in the same area and infect a single person at the same time. *Plasmodium falciparum* is responsible for most malaria deaths, especially in Africa (NIAID, 2007).

Nowadays, *P. falciparum* and *Plasmodium vivax* species have different drug resistance patterns in different geographic regions {(CDC (Centers for Disease Control), at 2013)}. Resistance to antimalarial drugs is a major threat to the control and elimination of malaria. The greatest problem with antimalarial drug resistance is with *P. falciparum.* All geographical areas are affected, and the worst affected is mainland South-east Asia. Resistance to chloroquine in *P. falciparum* has spread across most of the world and caused millions of deaths. Resistance to antifolate drugs and atovaquone arises frequently (*eg*. antifolate resistance rose

to high levels within 2 years of the initial deployment of proguanil in peninsular Malaya in 1947), and it can be induced readily in both *P. falciparum* and *P. vivax.* Mefloquine resistance arose over 6 year period on the north-west border of Thailand Antermisinin derivatives resistance *P. falciparum* has emerged recently in South-West Asia. Piperaquine resistance has begun to emerge in Cambodia and high level resistance to chloroquine is prevalent in Indonesia and Papua New Guinea (WHO, 2015).

In the absence of resistence to the drug, several new antimalarial drugs or new combinations have been important recently. In general, when there are no satisfactory alternaties, newly registered drugs may be recommended (WHO, 2015). There are many research that found the plant extract having antimalarial activity, such as ethanolic extract of *Tetrapleura tetraptera* fruit (Okokon *et al*., 2007). Medical plants as Monimiaceae, *Siparuna aspera, Renealmia thyrsoidea, Renealmia alpinia, Piper aduncum L, Piper sp,* and the leaves *of Jacaranda copaia* (Celine *et al*., 2009) from Yanesha (Peru) have selected as antimalaria. *Calophyllum* (Clusiaceae) is one of the genera that get growing interest by the scientific community, because of its promising chemical contents that potential for drug development (Cechinel, *et al*., 2009).

Calophyllum genus is composed of the *ca.* 180 – 200 species (Crane *et al*., 2005). Review on *Calophyllum* species by Su *et al*. (2008) show that from this genera 243 compounds have been isolated and classified in 4 major groups of compounds which were coumarins (84 compounds), xanthones (82 compounds), chromanones (45 compounds), steroids and triterpenoids (27 compounds), besides some other compounds (5 compounds) (Su *et al*., 2008).

Calophyllum bicolor P. F. Steven is one of *Calophyllum* species that belongs to Indonesian biodiversity. In our on going study there are five species of *Calophyllums* evaluated for their antimalaria activity, wich were *C. lowii. C. teysmannii, C. bicolor, C. europhyllum* and *C. canon* (Abbas J., *et al* 2010; 2011; 2013 and 2014). These species that are used to cure symptoms that can be posibbly related to malaria crisis such as sever headache, chills, vomits, fever that associated or not with diarrhoea and liver pains.

Although there are many publications from other *Calophyllum* species, however *C. bicolor* has not been much studied so far, and we aware there is no study on bioactivities were reported from this plant. Therefore the aim of this project were to study the antiplasmodial activity from *C. bicolor* P. F. Steven and to isolate and identification isolated compound from the active extract.

Materials and Methods

Materials. Chemicals were purchased from Sigma-Aldrich (USA) such as ethyl acetate, n-buthanol, chloroform, silica gel for column chromatography (silica gel 60,200 mesh) and Preparative plates were purchased from Merck (Darmstadt, Germany). Technical grade solvents were distilled before used.

Plant Materials. The stem barks of *C bicolor* used in this study were collected forest area of Palangkaraya, Central Kalimantan Island Indonesia, in January 2013. Samples were identified by Mr. Ismail Rahman, Herbarium, Research Centre for Biology, Indonesian Institute of Sciences (LIPI). The voucher specimens were deposited at the Herbarium Bogoriense.

Instruments. UV spectra were recorded on a agilent Technologies, Cary 60 UV-Vis spectrophotometer. ^{1}H-&^{13}C-NMR, DEPT, and 2D NMR experiments (COSY, HMBC, HMQC) spectra were recorded in $CDCl_3$ solution on a JEOL JNM 500 MHz instrument, using TMS as the internal standard, otherwise state chemical shift (δ) in ppm and coupling constand (*J*) in Hz. Melting points were determinated on an electrothermal Fisher melting point (scientific serial 903N0056 apparatus). IR spectra were recorded on a FTIR Prestige 21 Shimadzu spectrophotometer KBr pellet; υ in cm-^{1}). Mass spectrometry analysis were performed on a Mariner Bio spectrometry to determine molecular weight. Silica Kieselgel 60 (230-400 mesh 0.04-0.063 mm) was used for column chromatography and precoated Si gel plates (Merck, SIL G 25, UV 254, 0.25 mm) were used for preparative TLC and analytical TLC. Spot were visualized by 10 % H_2SO_4, and after 24 h period sprayed by vanillin/H_2SO_4 reagent. Fine chemicals A. sephadex LH-20 also used to purifiy the

isolated compound.

Preparation of Plant Extract. The stem barks of *C. bicolor* were cut into small pieces, dried at room temperature, then dried in oven at 50 ^{o}C and grinded. The dried powder of *C. bicolor* (5 kg) was maserated at room temperature with *n*-hexane to get dry extract 40 gr, Me_2CO 231.6 gr and methanol 95.7 gr successively. Antiplasmodial activity of each extract were evaluated by using *P. falciparum,* bioassay *in vitro*. The active extract (methanol extract = 40 gr) was subjected to silica gel column chromatography (C_6H_{14}-Me_2CO system) to give 11 fractions, followed by EtOAc-MeOH (1:1) to obtain 11 fractions. Compound 1 (xanthone) were obtained by recrystallization using dichloromethane – methanol (8:2) from fraction 3 (EtOAc-MeOH =8:2)

Parasite Cultivation and Crude Preparation. *P. falciparum* (strain 3D7) were cultivated *in vitro* essentially according to the standard procedure published previously by Trager & Jensen (1976). The cultivation used RPMI-1640 (GibcoBRL) media supplemented with 2.5 µg/mL gentamicin, 50 µg/mL hypoxanthin, 25 mM N-2-hydroxethylpiperazine-N-2-ethane sulfonic acid (HEPES), buffer, 25 mM natrium bicarbonat and 10 % human serum AB^+ and maintained at 5 % hematocrit pH 7.4 and incubated at candle jar incubator at 37 °C. The samples were solubilized.

Antiplasmodial activity. Antiplasmodial activity of extract was determined against the chloroquine-resitant 3D7 strain of *P. falciparum* (IC_{50} chloroquine = $3x10^{-9}$ M). *P. falciparum* was maintained continuously in culture on human erythrocytes as described by Trager & Jensen (1976). Stock solutions of chloroquine and all extracts (hexane, acetone, and methanol extract) and isolated compound were prepared in sterile, distilled water and DMSO (5.5 mg/100 µL in DMSO as stock solution), then serially diluted by culture medium up to 10^{-10} to the expected concentrations.

Parasite cultures were added (2 % parasetemia and 0.5 % final hematocrit) and put in 96-well microplates and plates were maintained for 48 h at 37 ^{o}C in incubator. Chloroquine was used as positive control. Giemsa-stained thick blood film were prepared for each well and percentage of inhibition of parasite growth was determined under microscope by comparison of the number of ring, trophozoities and schizonts with total of 100 parasites with that of control well containing no extract. The growth inhibition for each well was determined by regression linear program Sigma – plot (IC_{50}) and compared to chloroquine standard. Percent growth inhibition of the parasite was calculated by the following formula:

$$\% \ of \ inhibition = \left\{ \frac{Parasetimia \ control - parasetimia \ in \ extract}{Parasetimia \ control} \right\} x100$$

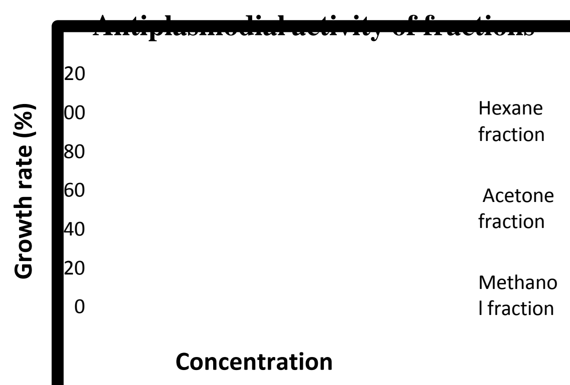
The concentration that inhibit 50 % of the parasite growth (IC_{50}) was calculated after evaluating percent growth inhibition at different concentrations. Reference compound was chloroquine. The DMSO concentration never exceeded 0.1 % and did not inhibit the parasite growth (Hay, 2004)

Results and Discussion

Antiplasmodial Acitivity

Antiplasmodial activity of all extracts is given in Figure 1 and Table 1. Figure 1 showed that hexane extract and methanol extracts exhibited antiplasmodial activity, but acetone extract has no antiplasmodial activity.

One xanthone isolated had been tested on a chloroquino-resistent strain of *P. falciparum*. The result of these test are presented in Table 2 and Figure 2).



Dosage: 5.1 mg/100 µL dilute 10^{-2} until 5.1 mg/100µL x 10^{-10}).

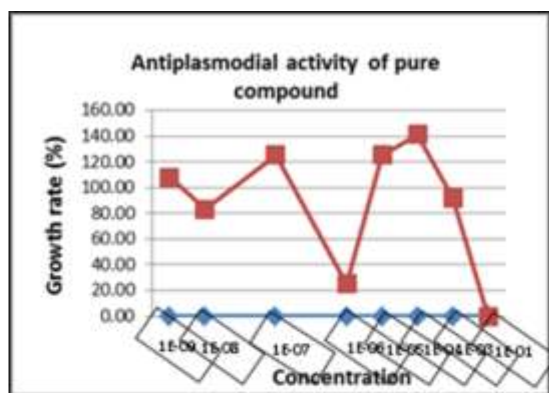
Figure 1. Antiplasmodial activity of hexane, acetone and methanol fraction of *C. bicolor*

Table 1. Antiplasmodial activity of hexane, acetone and methanol extracts of *C.bicolor*

No	Concentration	Stage			Total	Growth rate (%)	Activity
		Ring	Trop	Schizont			
		5.1 mg+100 µl DMSO (stock hexane's fraction solution)					
1	$1x10^{-10}$	0	4	1	5	100	Active but Dose not dependent
2	$1x10^{-9}$	1	1	1	3	60	
3	$1x10^{-8}$	1	1	1	3	60	
4	$1x10^{-7}$	1	2	0	3	60	
5	$1x10^{-6}$	1	1	0	2	40	
6	$1x10^{-5}$	1	1	1	3	60	
7	$1x10^{-4}$	0	3	0	3	60	
8	$1x10^{-3}$	0	0	0	0	0	
9	$1x10^{-2}$	0	0	0	0	0	
		5.1 mg+100 µl DMSO (stock acetone's fraction solution)					
1	$1x10^{-10}$	0	1	1	2	100	Have not active
2	$1x10^{-9}$	1	0	1	2	100	
3	$1x10^{-8}$	1	0	1	2	100	
4	$1x10^{-7}$	1	0	1	2	100	
5	$1x10^{-6}$	1	0	1	2	100	
6	$1x10^{-5}$	0	1	1	2	100	
7	$1x10^{-4}$	1	1	0	2	100	
8	$1x10^{-3}$	1	1	0	2	100	
9	$1x10^{-2}$	1	1	0	2	100	
		5.2 mg+100 µl DMSO (stock solution)					
1	$1x10^{-10}$	0	2	2	4	100	Active but Dose not dependent
2	$1x10^{-9}$	0	2	2	4	100	
3	$1x10^{-8}$	0	2	2	4	100	
4	$1x10^{-7}$	1	0	2	3	75	
5	$1x10^{-6}$	0	2	1	3	75	
6	$1x10^{-5}$	1	1	0	2	50	
7	$1x10^{-4}$	1	1	0	2	50	
8	$1x10^{-3}$	0	0	0	0	0	
9	$1x10^{-2}$	0	0	0	0	0	

Table 2. Antiplasmodial activity of isolated compound of *C. bicolor*

No	Concentration 5 mg+100 µl DMSO (isolate compound)	Stage				Total	Means	Growth rate (%)	IC50
		Ring	Trop	Schizont	Gametosit				
1	$1x10^{-9}$	4	2	1	0	7	6.5	108	Active but Dose not dependent
		4	1	1	0	6			
2	$1x10^{-8}$	4	1	1	0	6	5	83	
		2	1	1	0	4			
3	$1x10^{-7}$	3	2	1	0	6	7.5	125	
		4	4	1	0	9			
4	$1x10^{-6}$	1	0	0	0	1	1.5	25	
		2	0	0	0	2			
5	$1x10^{-5}$	3	3	1	0	7	7.5	125	
		4	2	2	0	8			
6	$1x10^{-4}$	4	2	1	0	7	8.5	141	
		6	3	1	0	10			
7	$1x10^{-3}$	5	0	0	0	5	5.5	92	
		6	0	0	0	6			
8	$1x10^{-1}$	-				0	0	0	
		-				0			
9	Control	2	2	1	0	5	6	100	
		2	3	2	0	7			



Dosage: 5 mg/100 µL dilute 10^{-2} until 5 mg/100µLx10^{-9}.
Maximum inhibition at concentration 0.11 nMol.
Figure 2. Antiplasmodial activity of isolated compound of *C. bicolor*

Identification and Structure Elucidation of Purified Compound

Barks of *C. bicolor* collected from Palangkaraya, Indonesia were divided into bark and wood. Barks were air-dried, grinded and extracted successively with hexane, acetone, and methanol. All extracts were analized by *P. falciparum* assay to know which fraction is more active as antiplasmodial. Methanol extract showed the antiplasmodial activity compared to acetone extract but does not dependent. Methanol extract of the bark was chromatographed on silica gel and Sephadex LH-20 to give compound 1 and the known xanthones. The chromatographic separation of the methanol extract from *C. bicolor* (in order of increasing polarity on silica gel) was obtained as yellow amourpous powder with melting point 115-116 °C.

Derivate xanthone as the new 5-methoxy trapezifolixanhone was isolated an yellow powder from the methanol extract of the stem bark of C. bicolor. LC-MS displayed a positive molecular ion peak at m/z 393.3 [M+H]+ spectrum correspondent to C24H24O5, with MW=392.3The UV spectra were suggestive of a derivate xanthone as the new 5-methoxy trapezifolixanthone Its UV spectrum with λ_{max} 274, 249 and 215 nm. Compound 1 gave typical xanthone. IR spectrum showed bands ascribed to hydroxyl group (V_{max} 3508.52 cm-1), 2954-2870 cm^{-1} (sp2 and sp3 CH), an a conjugated carbonyl group (C=O, V_{max} 1724 cm^{-}1) and an unsaturated lactone (C-O, V_{max} 1621-1598 cm^{-1}). The ^{1}H-NMR spectrum revealed the presence of chelated hydroxyl proton signal at ∂ 13.08 (*s*, 1H). five vinylic proton aromatic signal at ∂ 7.76 (1H, *dd*, *J* = 1.3 and 7.8 Hz); 7.24 (1H, *t*, *J* = 7.8 Hz); 7.3 (1H, *dd, J* = 1.3 and 7.8 Hz); 5.62 (1H, *d*, *J* = 9.8 Hz) and 6.75 (1H, *d, J*= 9.8) for H-8, H-7, H-6, H-11, and H-10 respectively.

One methylene signals at ∂ 3.42 (2H, *d* H-1'). One methine signals at ∂ 5.25 (*1H, t H-2).* Four methyl signals at ∂ 1.72 (*s*,1 Me), 1.87 (*s*,1 Me) and 1.48 (*s,* 2×Me) were also observed

The ^{13}CNMR spectrum and DEPT experiment (Figure 4) showed the presence of twelve quaternary carbons at ∂ 158.5 (C-1), 103.5 (C-2), 156.2 (C-3), 108.0 (C-4), 159.4 (C-4a), 163.8 (C-5), 121 (C-8a), 181.1 (C-9), 104.9 (C-9a), 144.6 (C-10a), 78.5 (C-12), and 131.9 (C-3'). Six methyne signals at ∂ 116.9 for (C-8), 124.2 (C-7), 119.9 (C-6), 115.9 (C-10), 127.7 (C-11) and 129.9 ppm (C-2') respectively. One methylene signals at ∂ 22.7 (C-1') and also have four methyl signals at ∂ 25.8 (C-4'), 18.14 (C-5') and ∂ 25.8 x 2 for (C-13, C-14) respectively. 13 CNMR spectrum also indicated a carbonyl at ∂ 181.1 ppm.

Structure as also elucidated by HMBC spectral analysis after the assignment of the protons to their direct bonding carbons by the HMQC spectrum The. chelated protons at ∂ 13.08 due to hydrogen-bonding with the carbonyl group (∂ 181,1 C=O) which resulted in the deshielding effect. The 3 *J* and 2 *J* connectivity of the chelated hydroxyl proton at ∂ 13.08 with ∂ 158.5 (C-1), 103.5 (C-2) and 104.9 (C-9a) confirmed OH (Hydroxyl group) locations at C-1 (Figure 4). HMBC correlations depicted in Figure 3 confirmed the assignment as 1-hydroxyl xanthone with subtitle at position C-2, C3 and C-4

HMBC spectrum also exhibited long-range C-H correlations between methylene protons at ∂ 3.42 corelated to carbon at ∂ 108, 122.9 and 131.9 ppm, also methyne protons at ∂.5.25 correlated to carbon at ∂ 22.7 and 131.9 ppm. Aromatic proton at ∂ 7.76 cause cross-peaks with carbons at ∂ 119.9; 144.6 and 181.1 and proton at ∂ 7.24 correlated to carbon at ∂ 119.9; 144.6; 121 and proton t ∂ 7.3 correlated to carbons at ∂ 144.6 and 116.9. The methyl proton (1.48) was further correlated with the carbons at ∂ 127.7 and 78.5. Another proton correlation to carbon was showed in Figure 3.

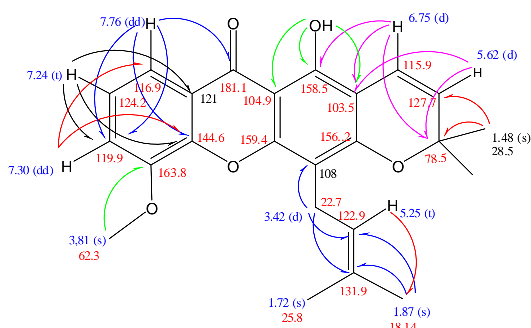


Figure 3. HMBC, HMQC and COSY of new derivate 5-methoxy trapezifolixanthone from *C. bicolor*

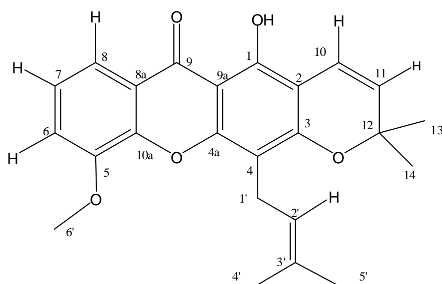


Figure 4. Structure of of new derivate 5-methoxy trapezifolixanthone from *C. bicolor*

From the ^{1}H NMR spectrum showed that isolated compound consist of four methyls singlet at (δ_H 1.48 -2×Me), at (δ_H 1.87 -1 Me) and at (δ_H 1.72 -1 Me) and also one methoxy at (δ_H 3.81 ppm).

COSY correlation between H-10/H-11 and H-11/H-10 and H-6/H-7 and H-7/H-8. Correlation proton to carbon (HMBC) cross peaks from H-11 (5.62 ppm) to C-2 (103.5), correlation proton H-10 (6.75 ppm) to C-1 (158.5), C-3 (156.2) and to C-12 (∂ 78.5).

The HMBC results also demonstrated long-range ^{2}J and ^{3}J correlation between two doublet protons at 5.62 (H-11, J = 9.75 Hz) and 6.75 (H-10, J =9.75 Hz) with the carbon signal at ∂ 78.5(C-12) respectively. The linkages the two aliphatic methyl singlet at ∂ 1.48 (H13 & H-14) to the carbon signals at ∂ 78.5 and 127.7 (C-11) were also seen. These data spectrum analysis together with the COSY suggested the existence of a pyran ring. The pyran ring was proven onto the non-oxygenated carbon C-2 and an oxygenated carbon C-3, as conformed by the long range (^{3}J) correlation of H-10 (∂ 6.75) to C-3 (∂ 156.2) and H-11 (∂ 5.62) correlated to C-2 (∂ 103.5) these proven that pyran ring was attached to C-2 and C-3

The ^{1}H-NMR spectrum of isolated compound also showed two methyls singlet at (∂ 1.72,3H and ∂ 1.87,3H) correlated to C-2' (∂ 122.9), C-3'(∂ 131.9) proven that two methyl protons were located at position C-3', and also proton ∂ 3.42 (H-1') correlated to C-4 (108), C-2' (122.0) and C-3' (131.9) proven that methelene proton were located at position C-1'. The coupling of H-1' and H-2' in the COSY spectrum showed connectivity between ∂ 22.7 (C-1') and ∂ 122.9 (C-2'). The linkage between ∂ 3.42 (H-1') with 108 (C-4) , 122.9 (C-2') and 131.9 (C-3') proven that prenyl group is attached to C-4 (Figure 5 &7, Table 3). The methoxyl group was deducated to be located at C-5 position by HMBC experiment, the cross-peak from methoxyl ∂ 3.81 to C-5 (∂ 163.8).

The ^{13}C, DEPT and HMQC, HMBC and NMR spectra of isolated compound exhibited 24 carbon signals, with revealed to presence four methyls, one methoxyl, one methylene, six methines, twelve quarternary carbons including one carbonyl group and one prenyl group. NMR spectra revealed signal assignable to pyran at C-2 and C-3 and prenyl group at C-4, one hydroxyl (OH) group at C-1 and one methoxyl group at C-5. Based on elucidation of 1D and 2D NMR results, the isolated compound was identified as a xanthone from *C. bicolor.*

The isolation *of* trapezifolixanthone was reported from stem bark of *C. soulattri* by Lien EE (2011) which its content hydroxyl group at position C-5, in these research has isolated new compound because isolated compound content methoxy group at positon C-5 of trapezifolixanthone.

These new 5-methoxy trapezifolixanthone showed in vitro as antiplasmodial activities to *P. falciparum* parasite in our assay with have maximum inhibition at concentration 0.11 nMol.

Since some xanthones were found to be antiplasmodial from *C caledonicum* (Hay 2004), antiplasmodial studies of xanthones isolated from *C. bicolor* P. F. Steven were carried out. Our study clearly revealed that 1-hydroxy -5-methoxy -4-prenylated xanhtone or another nama was new 5 methoxy trapezifolixanthone isolated from steam bar of *C. bicolor* P. F. Steven possessed antiplasmodial activity (the xanthone significant inhibition of growth of *plasmodium falciparum* 3D7 clone *in vitro*..

Conclusion

The hexane and methanol extract of *C. bicolor* showed antiplasmodial activity with IC_{50} value 5.2 ppm and 0. 11 nMol ppm, against *P. falciparum* which suggest that this plant is a potential source for antiplasmodial drug. A pure compound isolated from this plant extract was identified as new 5 methoxy trapezifolixanthone.

Acknowledgments

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Table 3. Data HMBC, COSY ,chemical shift (ppm) and J in Hz of xanthone from *C. bicolor*

No	C	H-NMR (δ_H) ppm	HMBC H–C	COSY
1 C-OH	158.5	13,08	C-1	
2	103.5	-	-	-
3	156.2	-	-	-
4	108	-	-	-
5	163.8	-	-	-
6	119.9	7.3 (1H, *dd, J* = 1.3 & 7.8 Hz)	C-8, C-10a	H6 - H7, H6 - H8
7	124.2	7.24 (1H, *t, J* = 7.8 Hz)	C-6, C-8a,C-10a	H7 - H6, H7 - H8
8	116.9	7.76 (1H, *dd, J*= 1.3 & 7.8 Hz)	C-6, C-9, C-10a	H8 - H7, H-8 –H6
9	181.1	C=O		
4a	159.4	-		
8a	121.0	-		
9a	104.9	-		
10a	144.6	-		
10	115.9	6.75 (1H, d, *J* = 9.75 Hz)	C-1, C-2, C-12	H10 - H11
11	127.7	5.62 (1H, d, *J* = 9.75 Hz)	C-2, C-12	H-11 – H10
12	78.5	-	-	-
13 & 14	25.8	1.48 (6H, *s*)	C-11, C-12	
1’	22.7	3.42 (2H, *d, J* = 1,3 Hz)	C-4, C-2’, C-3’	H-1’-H-2’
2’	122.9	5.52 (1H, *t* =)	C-1’, C-3’	H-2’ – H1’
3’	131.0	-	-	-
4’	25.8	1.72 (3H, *s*)	C-2’, C-3’	
5’	18.14	1.87 (3H, *s*)	C-2’, C-3’	-
6’	62.3	3.81 (3 H, methoxy, s)	C-5	-

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