# In vitro antimalarial activity of *Calophyllum bicolor* and hemozoin crystals observed by Transmission Electron Microscope (TEM)

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**Abstract.** *Objective* : In continuation of our antimalarial candidate drug discovery program on Indonesia medicinal plants especially from stem bark of *Calophyllum bicolor. Metode* : We extracted of bioactive crude extract with hexane, acetone and methanol from stem bark of *Calophyllum bicolor* and evaluated their antimalarial activity by using parasite *Plasmodium falciparum in vitro. Results:* Methanol fraction showed most active and potent antimalarial activity dose dependent in *in vitro* experiments with $IC_{50} = 0.52$ ppb, and hexane fraction showed moderate antimalarial activity, but dose not dependent, while acetone fraction have not antimalarial activity. TEM (Trasmission Electron Microscopy) analysis confirmed a remarkable reduction of hemozoin in the presence of the bioactive fraction. *Conclusion*: The results suggest that the antimalarial activity of *Calophyllum bicolor* is due, methanol fraction showed the best *in vi*tro antimalarial activity then another fraction.

**Keywords:** *Calophyllum bicolor, Plasmodium falciparum*, *in-vitro*, TEM (Transmition Electron Mecroscope)

## 1 Introduction

Malaria is a serious parasitic disease transmitted by the bites of the female *Anopheles* mosquites , there are 4 species of plasmodium, e.i. *Plasmodium falciparum, P vivax, P malaria,* and *P ovale,* which *P falciparum* the most lethal [1, 2, 3]. Parasites belonging to *Phyllum* protozoa and genus *Plasmodium* are the most important causal pathogens and cause several human infection with globally massive impact[4]. Malaria remain one of the most significant world-wide public health problem, especially in tropical and subtropical regions [5]. Malaria is endemic to 90 counties world health and effects nearly 40% of the the world's population. Malaria is responsible for the death of 1-2 million each year with more than 90% of cases found in sub Saharan Africa and $\geq 1$ case per 1000 population, and 80% of such cases are concentrated in 13 cauntires, over half in Negeria, Congo, Ethiopia, Tanzania and Kenya. [6, 7, 1]. Nigeria accounts for a quarter of all malaria cases in Africa. In the southern part of the country, transmission occurs all year round while in the north it is more seasonal [1].

One major virulence factor of this parasite is the highly variant *P. falciparum* erythrocyte membrane protein-1 (PfEMP) family. The malaria parasite in its blood stage is a voracious consumer of the globin component of hemoglobin, which causes the release of copious amounts (> 4000 mM) of toxic heme. Proteolysis of hemoglobin yields amino acid for protein synthesis as well as toxic heme. However, the parasite withstands heme toxicity by employing efficeinets mechanisms of heme detoxification. The conversion of heme to the relative benign heme polymer, hemozoin, is achieved by a process akin to template-mediated biomineralization. Templates for heme polymerization include performance hemozoin, histidin rich protein, and lipid or parasite cannot enzymatically cleave the porphyrin ring, heme is'detoxified' by conversion to an insolubly polymer, hemozoin [8 ,9].

The emergence and rapid spread of multidrug-resistant strains of *Plasmodium*, particularly *Plasmodium falciparum* has led to serious problem for prophylaxis and treatment malaria, which becomes more difficult and limits the ability to control this disease and also limits the choice of drugs used. This has been identified as the current primary cause of control failure, this highlights the need to develop quickly more effective and less toxic new antimalarial drugs with different mechanism of action [9. 10]. It has been suggested that hemozoin formation is inhibited by the extract *Calophyllum*.
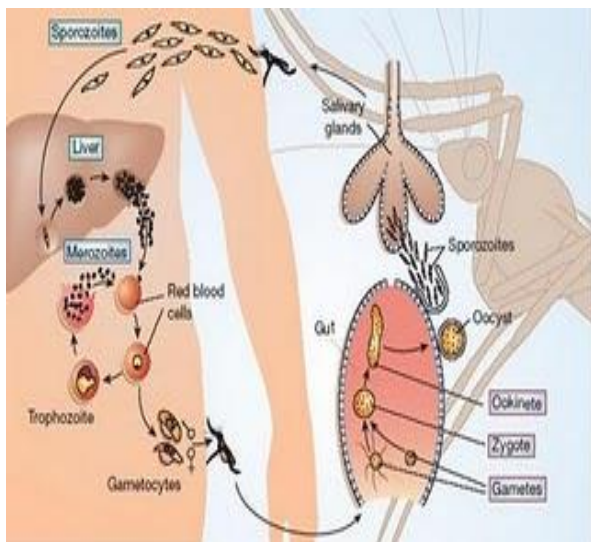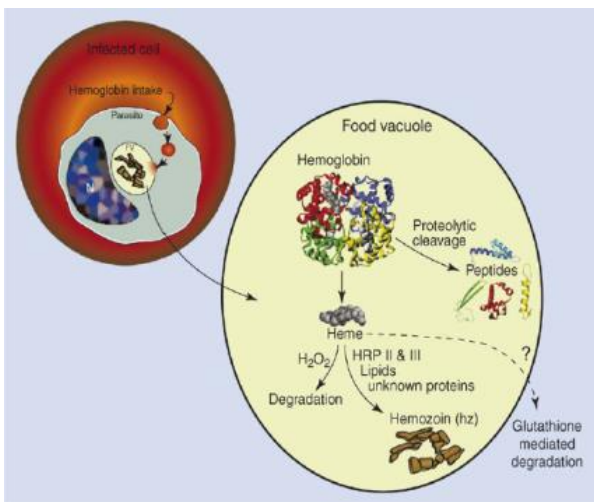
Fig 1. Live cycleof *P.falciparum* parasite



**Fig 2**. Heme degradation and hemozoin formatoion

Transmission electron microscope (TEM). TEM is one of type of electron microscope that has three essential systems: (1) an electron gun, generating the electron beam, and the condenser system, which focusing the beam onto the object, (2) the image-producing system, consisting of the objective lens, movable specimen stage, and intermediate and projector lenses, focusing the electrons passing through the specimen to form a real, highly magnified image, and (3) the image-recording system, converting the electron image toward some form perceptible to the human eye. The image-recording system usually covers of a fluorescent screen for showing and focusing the image and a digital camera for permanent records. Bisides,on, a vacuum system, consisting of pumps and their associated gauges and valves, and power supplies are needed.see Figure 4 [11]
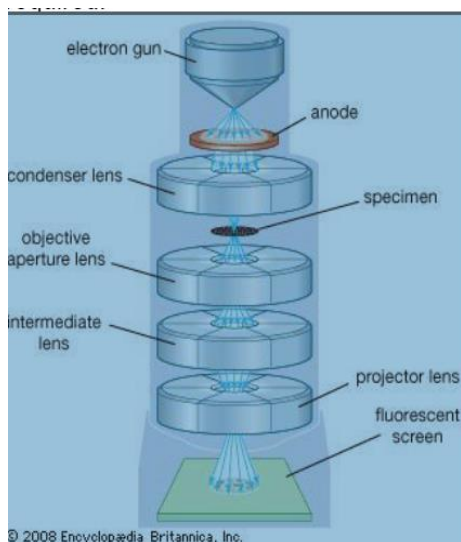


**Fig 3**. Transmission electron microscope (TEM)

## 2 Material and Methods

### 2.1 Material

#### 2.1.1 General experimental procedures

TEM (transmission electrone microscope), maserator, evaporator,UV lamp.

#### 2.1.2 Sample

*C. bicolor* P.F Stevent (2.858 kg) and *C. europhyllum* (1.555 kg) were collected in Bulungan, Kalimantan Island, in Indonesia. The plant were determined by staff from Herbarium Research Centre for Biology - LIPI and voucher specimen was deposited at the Herbarium Research Centre for Biology-LIPI. Indonesia.

#### 2.1.3 Chemical material

RPMI media and glutamin were obtained from Nissui, $NaHCO_3$ was purchased from E. Merck. Silica gel 200-300 mesh, TLC plates were purchased from E. Merck and all organic solvent (methanol, hexane, $CH_2Cl_2$, ethyl acetate) were purchased from local market. RPMI media contain 2,5 μg/mL gentamicin, 50 μg/mL hypoxanthin, 25 mM buffer HEPES (N-2-*hydroxyethylpiperazine-N'-2- ethanne sulfonic acid, (GibcoBRL),* 25 mM natrium bikarbonat, 10% human serum AB+, 5 % hematokrit, human erythrocytes, and chloroquin was used as a positif control. DMSO were used to dilute of the samples

### 2.2 Methods

#### 2.2.1 Extraction

The air-dired *C. bicolor* P.F Stevent (2.858 kg) and *C. europhyllum* Lauter (2,555 kg) were successively extracted with *n*-hexane, acetone and methanol at room

temperature for 4 day  and such was repeated three times, the solvent were evaporated under reduced pressure to give hexane extract.  The residue was extract successively with acetone (10  lt x 3) and  methanol (10  lt x 3), then acetone and methanol were  evaporated under reduced pressure to give a acetone and methanol extracts.

### 2.2.2 Antimalarial test

Erythrocytes infected  with  *Plasmodium falciparum* strain 3D7, from cultures obtained using the method Trager and Janson up to density  of parasites 2%, were suspended in complete culture medium at a hematocrit of 5%. The suspension parasite was distributed in 96-well micro titer plates (200 µl per well). To determine the antimalarial activity against  *plasmodium falciparum*, each extract  (5 mg)  was first dissolved in dimethyl sulfoxide (100 µl DMSO mix with vortek until all samples dilution completely, and then diluted with  completed medium (RPMI) to obtain the desired concentration (5 mg x $10^{-2}$ µl until  x 5 mg x$10^{-9}$ µl), total volume in each well plate are 1 ml) and cloroquin  was used  as a positive control.

Compounds were tested in duplicate   in 2% parasitemia cultures mostly  at ring stage. For each assay,

a parasite culture was incubated  with the compound at 37 ºC for 48 h in 5% $CO_2$ at 95%  relative  humidity and frozen until the  biochemical  assay  could be run. After 48 hours of incubation ach well was harvested to a glass were then fixation by methanol, also added Geimsa reagent.

Concentration of extract required to inhibit 50% parasite growth ($IC_{50}$) was determined by computer. Parasit amount was monitoring by microscope, and computer by linear program  Sigma-plot ($IC_{50}$) was used for calculated of $IC_{50}$. The antimalarial activity was expressed as the $IC_{50}$ value (ppm), which was defined as the  concentration of extract required to inhibit 50% parasite growth after 48-h incubation at 38ºC.

### 2.2.3 TEM (Transmission electron microscope)

Brief, small amount of extract were placed on the grid (formvar carbon coated Cu messed 300), at the acceleration voltage of 80 kV, using Zeiss. Model EM 10c (Jeol Japan). In addition, the apparatus was used for imaging at two different scales 24 and 30 nm.  i*n-situ* and after isolation was used for detect hemozoin
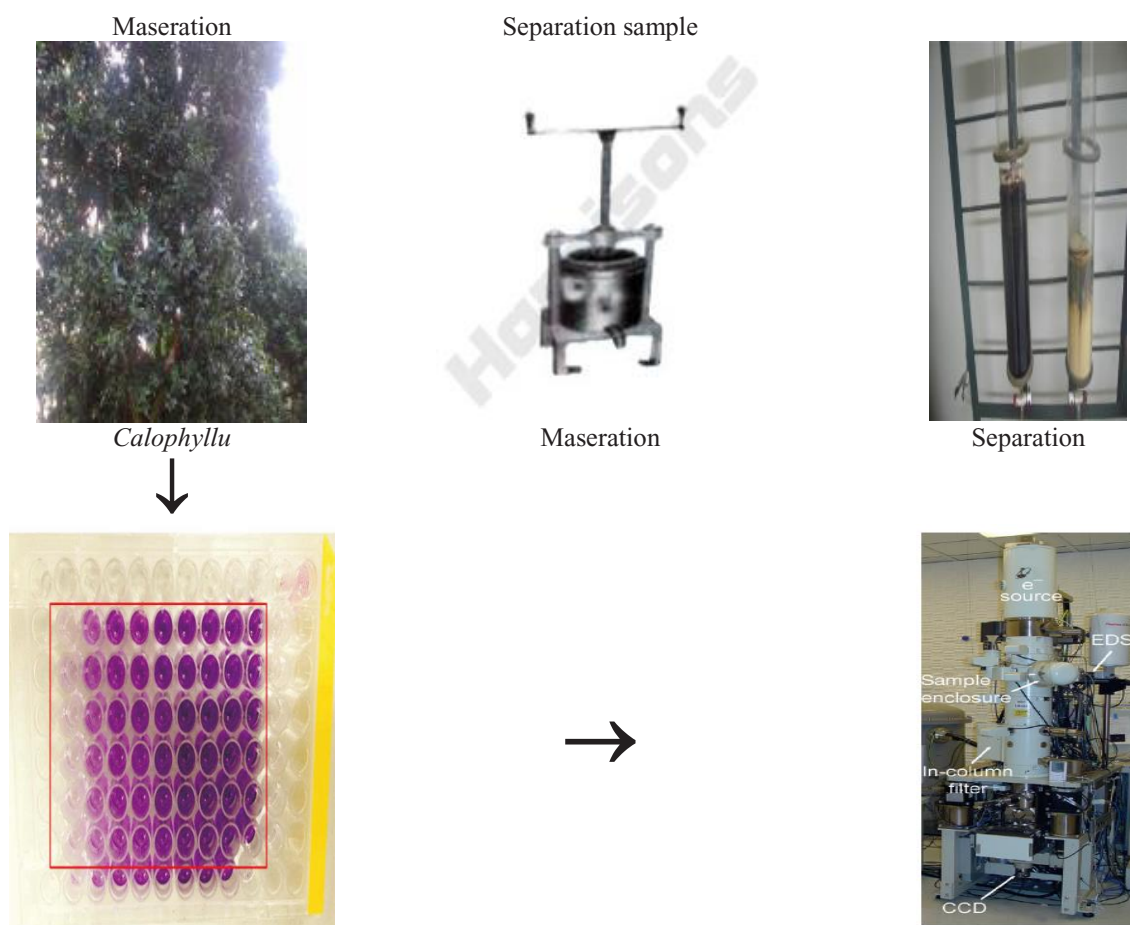
### 2.2.4 Flow chart of methode



**Fig. 4** . Flow chart of  isolation and TEM analysis

# 3 Results and Discussion

## 3.1 TEM result

### 3.1.1 TEM analysis of the fraction containing bioactive material showed significan hemozoin reduction.

In TEM, fixed-illumination mode, individual spectra are obtained from whichever area of the sample is illuminated. To control the sampling volume, the microscopist converges the beam to illuminate only the region of interest uses postspecimen apertures to select the divergence angle of electrons reaching the spectrometer. Because the sample and beam are fixed relative to each other, spectra, EDS and/or EELS, can be obtained in TEM mode

### 3.1.2 TEM imaging- Produccing system.

The specimen grid is conducted in a small holder in amoved specimen stage,. The objective lens is usually of short focal length 1-5 mm (0.04-0.2 inch) and results a real intermediate image that is furher magnified by the projector lenses. A single projector lens will provide a range of magnification of 5:1. For practical reasons of image stability and brightness, the microscope is operated to give a final magnification of 1,000-250,000 x on the sreen to show the hemozoin crystal.

Reduction of hemozoin crystal formation in the present of bioactive fraction had investigated by using TEM (Transmition Electrone Microscope) technich *in-situ* and after isolation. The identification of spesific area of hemozoin an image, or pixels with specified characteristic,

To furher characterize the antimalarial activity of *Calophyllum*, methanol extract (from the late sate of hemozoin formation) in the present sample after 1x24 hr incubation and in present sample after after 2x24 hr incubation were examined by TEM. There existed numerous hemozoin with diameter 1µm after 1x24 incubated, but there are not hemozoin after 2x24 hr incubation).

The transmission electron microscope (TEM) image in Figure 5 showed hemozoin crystal extracted from the sample previously used for the MO measurement. The typical length of the extracted hemozoin crystal range 500 nm. For comparison., a TEM image of synthetic hemozoin crystal in our MO study, is showen in panel A and B. The synthetic crystal have more elongated shape in typical length 500 nm. Panel A and B display TEM images of intact infected erythrocytes in the schizont stage. Distinct component of the parasite and erythrocyte can be observed including the erhythrocyte membrane (EM), parasite membrane (PM). Food vacuole membrane (FVM), merozoite (M), hemoglobin transport vesicles (HbTV) knons (9K) and hemozoin (Hz).Figure 5 and Figure 6.
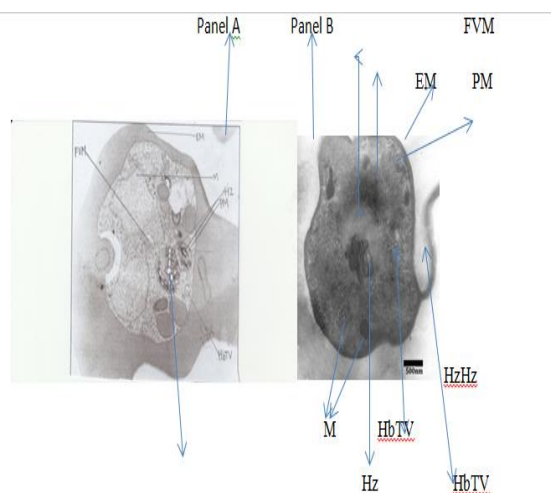
### 3.1.3 Figure from TEM results



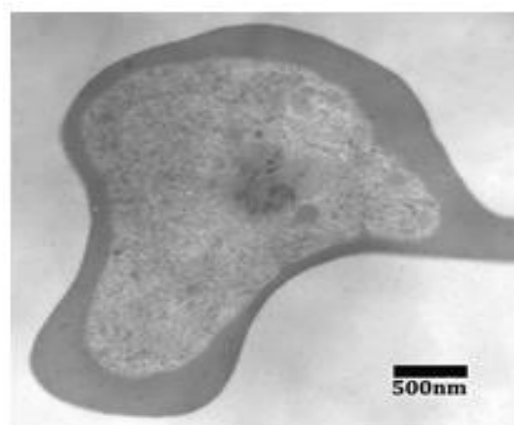**Fig 5**. Hemozoin crystal showed in figure as white crystal after 1x24 incubattion



**Fig 6**. Hemozoin crystal have not showed in figure after 2x24 incubattion

## 3.2 Sepration results

The present investigation for antimalarial principles from *C. bicolor* has led to the finding of three fraction, e.i, hexane (208.29 g), acetone (413.62 g) and methanol 149.8 g.

## 3.3 Antimalarial activity results

According to the previous paper [12] which it's explained that antimalarial activity of methanol fraction of *C. bicolor* have better than hexane fraction, and hexane fraction have better than acetone fraction while the acetone fraction have not antimalarial activity, these data showed in Figure 7.
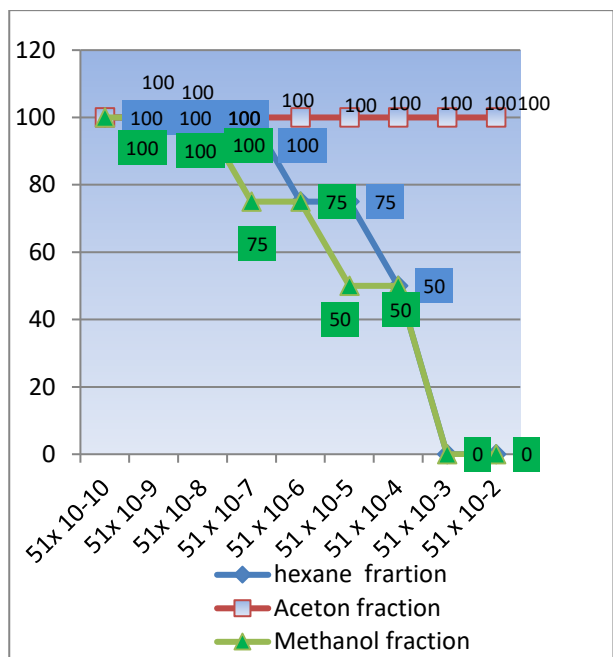
**Fig 7**. Grafik antimalarial activity of hexane,acetone and methanol fraction of C bicolor.

Antimalarial activity of the metanol fraction best compare hexane and acetone fraction, these methanol fraction are devided further into five sub fraction (e.i sub fraction 9 :1 ; 8 :2 ; 7 :3 ; 6 :4 ; 5 : 5. The data antimalrial activity explaned in Table 1

**Table 1.** Antimalarial activity of sub fraction methanol

| DOSAGE | C.Bi MeOH 9:1 | C.Bi MeOH 8:2 | C.Bi MeOH 7:3 | C.Bi MeOH 6:4 | C.Bi MeOH 5:5 |
|---|---|---|---|---|---|
| 1.00E-09 | 83 | 145 | 65.2 | 111 | 100 |
| 1.00E-08 | 92 | 118 | 43.75 | 100 | 100 |
| 1.00E-07 | 92 | 136 | 43.75 | 133 | 127 |
| 1.00E-06 | 117 | 127 | 37.5 | 122 | 127 |
| 1.00E-05 | 141 | 136 | 31.25 | 100 | 127 |
| 1.00E-04 | **75** | 136 | 75 | 88 | 118 |
| 1.00E-03 | 83 | 127 | 62.5 | 122 | 81 |
| 1.00E-02 | 83 | 118 | 37.5 | 55 | 9 |
| 1.00E-01 | 16 | 18 | 31.25 | 0 | 0 |

## 4 Discussion

The present investigation for antimalarial principal from methanol fraction of *C. bicolor* has led to the finding of five sub fraction e.i sub fration MeOH 9 :1 ; MeOH 8 :2 ; MeOH 7 :3 ; MeOH 6 :4 and MeOH 5 :5. Among the sub fraction which had weak-to-moderate antimalarial activities on *P. falcioparum* parasite. Antimalarial activities of sub fraction methanol e.i sub fraction MeOH 9 :1 ; MeOH 8 :2 ; MeOH 7 :3 ; MeOH 6 :4 and MeOH 5 :5 were elustrated in Figure 8.9
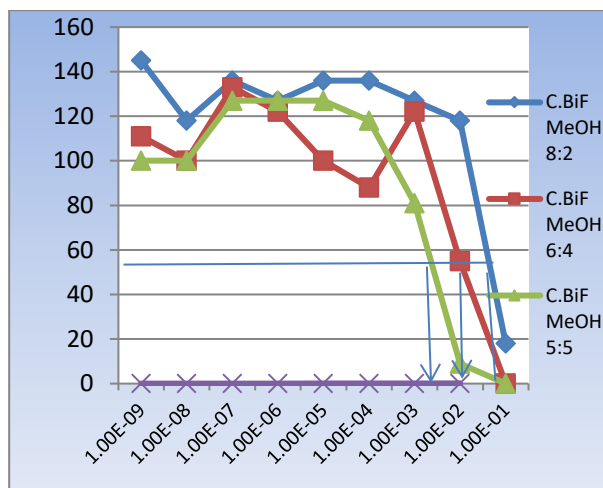


**Fig 8**. Antimalarial activity of sub fraction MeOH 8 :2 ; 6 :4 and 5 :5

Percentage of parasite *P. falciparum* growth versus sample concentration ($10^{-9}$ to $10^{-1}$ mg/mL) was observed. The increasing in sample concentration should decrease the parasite growth percentage. Increasing sample concentration from $10^{-9}$ to $10^{-8}$ mg/mL leads to a decrease the percentage of parasite growth from 145 to 111 : 65.2 to 43.75 and 111 to 100% for sub fraction 8 :2 ; 6 :4 and 5 :5 respectively.

Unfortunately,In the fact state that the increasing sample concentration from $10^{-8}$ to $10^{-7}$ mg /mL did not cause a decreasing percentage of parasite growth, this illustrates that the percentage decreasing parasite growth does not depend on the sample concentration (see Figure 8).

According to the Figure 8, we can calculated the $IC_{50}$ of sub faction MeOH 8 :2 ; 6 :4 and 5 :5 samples are 50 $x10^{-1}$ mg/mL, 50 $x10^{-2}$ mg/mL and 50x $10^{-3}$ mg/mL respectively (equivalent 5000 ppm, 500 ppm and 50 ppm respectively).
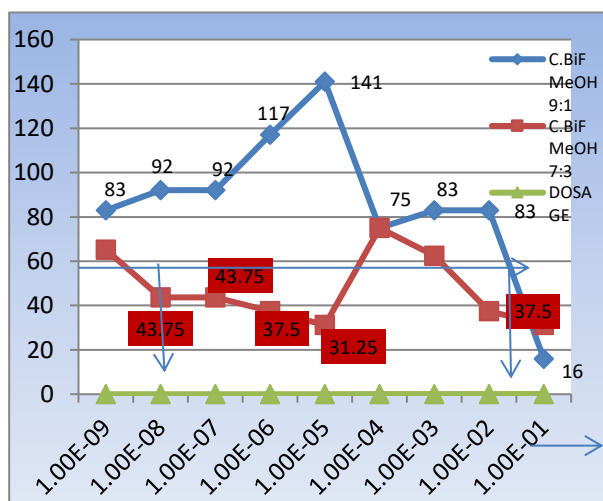


**Fig 9**. Antimalarial activity of sub fraction MeOH 9 :1 ; and 7 :3

The antimalarial activity of sub fraction 9: 1 and 7: 3 are described in Figure 9. In case of sub fraction 7 :3 sample, increasing the sample concentration from $10^{-9}$ to $10^{-8}$ ; $10^{-7}$ to $10^6$ ; $10^{-6}$ to $10^{-5}$ mg/mL leads to a decrease

the percentage of parasite growth from 65.2 to 43.75 %: 43.75 to 37.5 and 37.5 to 31.24 % respectively. Unfortunately increasing sample concentration from $10^{-5}$ to $10^{-4}$ mg /mL did not cause a decreasing percentage of parasite growth.

According to the Figure 9, we can calculated the $IC_{50}$ of sub faction MeOH 7 :3 is $50 \times 10^{-8}$ mg/mL (equivalent 0.005 ppm), and $IC_{50}$ of sub fraction 9 :1 sample is $50 \times 10^{-2}$ mg/mL (equivalent 0.5 ppm).According to the Figure 8 and 9, conclution taht the sub fraction MeOH 7 :3 is better as antimalarial compare to any another sub fraction.

One pure compound have isolated from the sub fraction of *C. bicolor* and have antimalarial activity with $IC_{50}$ is $50 \times 10^{-2}$ mg/mL (equivalent is 500 ppm) (see Table 2 and Figure 10

**Table 2.** Antimalarial activity of isolate compound

| No | Conc mg/mL | Stage | | | T | x̄ | Growth rate (%) |
|---|---|---|---|---|---|---|---|
| | Conc | R | Tr | Sc | | | |
| 1 | $50 \times 10^{-9}$ | 2 | 2 | 2 | 6 | | 116 |
| | | 3 | 3 | 2 | 8 | 7 | |
| 2 | $50 \times 10^{-8}$ | 2 | 1 | 1 | 4 | | 67 |
| | | 2 | 1 | 1 | 4 | 4 | |
| 3 | $50 \times 10^{-7}$ | 3 | 1 | 1 | 5 | | 92 |
| | | 2 | 3 | 1 | 6 | 5.5 | |
| 4 | $50 \times 10^{-6}$ | 4 | 4 | 1 | 9 | | 133 |
| | | 3 | 2 | 2 | 7 | 8 | |
| 5 | $50 \times 10^{-5}$ | 3 | 2 | 1 | 6 | 7 | 117 |
| | | 5 | 2 | 1 | 8 | | |
| 6 | $50 \times 10^{-4}$ | 4 | 1 | 1 | 6 | 5.5 | 92 |
| | | 3 | 1 | 1 | | | |
| 7 | $50 \times 10^{-3}$ | 1 | 2 | 1 | 4 | 4 | 67 |
| | | 2 | 1 | 1 | 4 | | |
| 8 | $50 \times 10^{-2}$ | 2 | 5 | 1 | 8 | 7 | 116 |
| | | 1 | 4 | 1 | 6 | | |
| 9 | $50 \times 10^{-1}$ | 1 | 0 | 0 | 1 | 1 | 16 |
| | | 1 | 0 | 2 | 1 | | |
| | Conc = Concentration, R = ring, Tr = Tropozoid, S= Schizont, T=Total, C= Conclusion. NA = Not active | | | | | | |

One pure compound have isolated as trepenoid from stem bark of *C. bicolor*. Triterpen as white powder. LC-MS (Figure 7) displayed a positive molecular ion paek at m/z 387,86 [M+H]$^+$ indicating a molecular formula $C_{27}H_{46}O$.
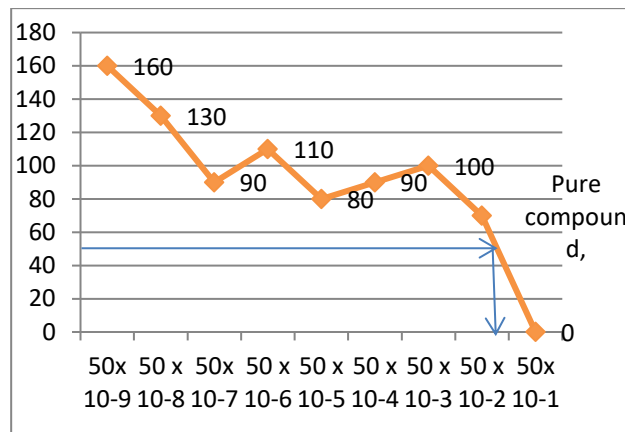

**Fig 10.** Antimalarial activity of pure isolate compound

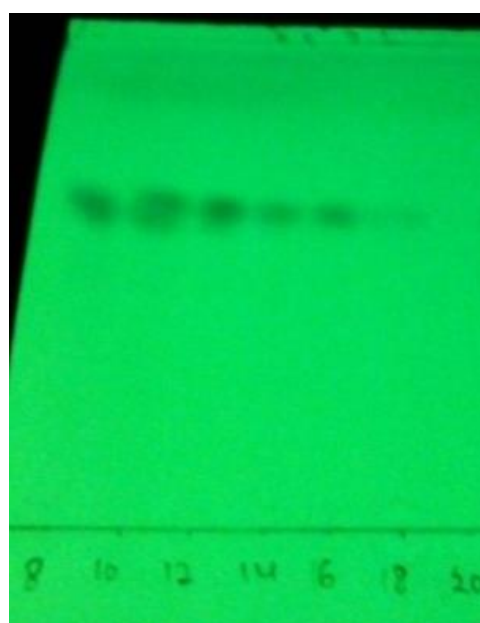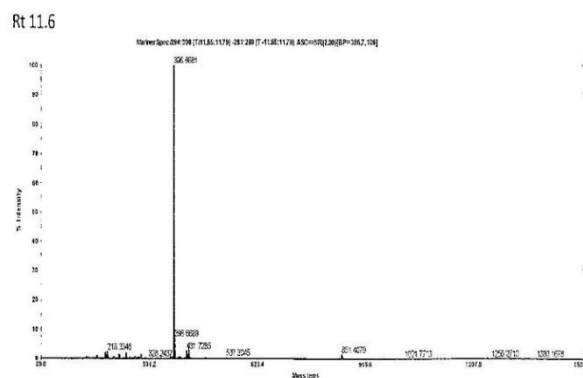Pure isolate compound have tested by using TLC. The TLC result saw single spot ( Figure 11).


**Fig 11**. TLC spot of pure isolate compound

One compound have isolated as trepenoid from stem bark of *C. bicolor*. Triterpen as white powder. LC-MS (Figure 12) displayed a positive molecular ion paek at m/z 387,86 [M+H]$^+$ indicating a molecular formula $C_{27}H_{46}O$.


**Fig 12**. LC-MS spectrum of pure isolate compound

Isolated compound give typical triterpen IR absorption at 3210-3550 cm$^{-1}$ (OH) , 2930 (sp3/CH3

aliphatic and 2864 cm$^{-1}$ sp2/CH$_2$ aliphatic), 1681 (C=C) cm-1 and 1456 cm$^{-1}$ as CH bending (Figure 13)
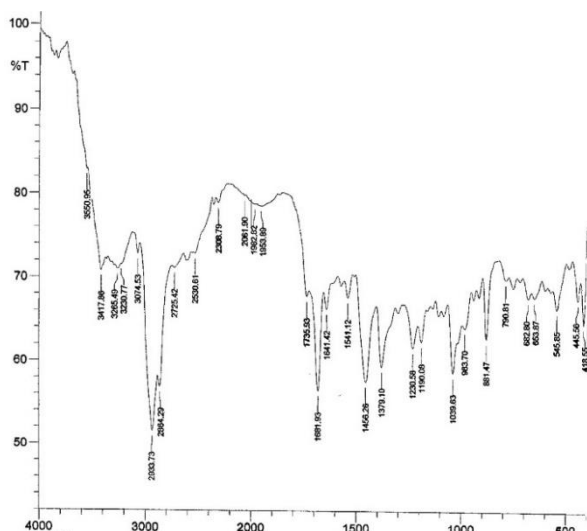


**Fig 13**. Infra Red spectrum of pure isolate compound

UV spectrum of pure isolate compoune was showed in figure 14. Maximum absorption were observed at wave length 233,5 nm as electron transition of π to π* from C=C group and wave length 295 nm as electron transition of n- π* from OH group.
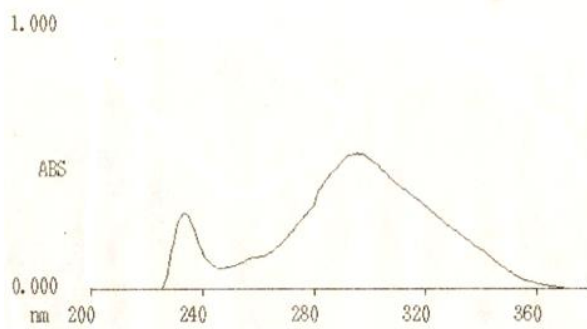


**Fig 14.** UV spectrum of pure isolate compound

The $^{1}$H-NMR spectrum revealed the presence of one hydroxyl proton doublet at $\delta_H$ 12 ppm (1s, OH), one alyllic proton signal at $\delta_H$ 4.72 (dd, 2H, J = 2.6 and 4.56 Hz) for H-21 recpectively. There are 6 six methyls singlet signal at $\delta_H$ 0,75 ppm; 0,85 ppm; $\delta_H$ 0,95 ppm; $\delta_H$ 0,96 ppm; $\delta_H$ 1,01 ppm and $\delta_H$ 1,7 ppm (sp$_3$ CH) (Figure 15)
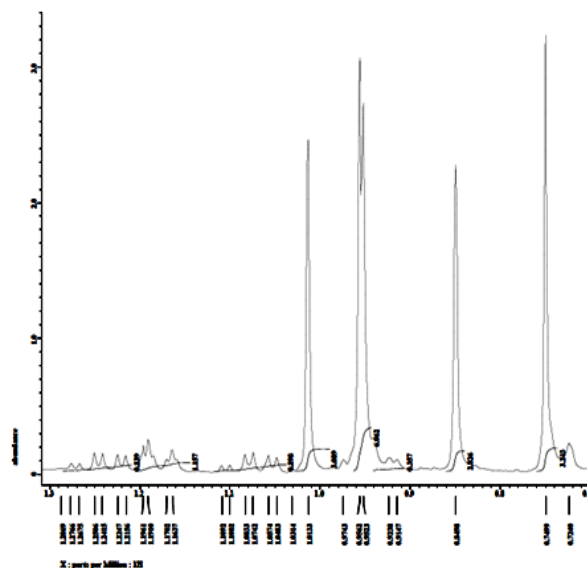


**Fig 15.** $^{1}$H-NMRspectrum of pure isolate compound

There are ten metilen (CH$_2$) signal at $\delta_H$ 1,36 ppm; $\delta_H$ 1,56 ppm; $\delta_H$ 1,91 ppm; $\delta_H$ 1,30 ppm; $\delta_H$ 1,46 ppm; $\delta_H$ 1,43 ppm; $\delta_H$ 1,63 ppm; $\delta_H$ 1,51 ppm; $\delta_H$ 1,48 ppm; $\delta_H$ 3,05 ppm and $\delta_H$ 4,72 ppm (2H, *dd* J 2,6 & 4,92).

Signal at $\delta_H$ 3,12 ppm (1H, *dd* J 5,28 & 11,65) is signal of mehine (CH) at position C-3 of triterpenoid compound, Isolate compound also have one hydroxil group at C-3. Unfortunately isolate compound have not signal at 6-8 ppm, these indicated that isolate compound have not aromatic proton.

There are six methine (CH) signal at $\delta_H$, 3.12 ppm, 2,35 ppm (1H, *ddd* J 3,25; 2,95, $\delta_H$ 2,24 ppm, $\delta_H$ 1,18 ppm and $\delta_H$ 1,08 ppm, CH signal will be explained in figure 16



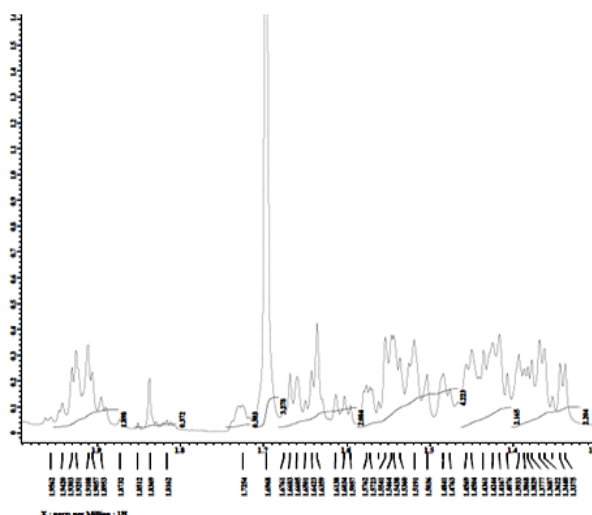**Fig 16.** $^{1}$H-NMR spectrum of pure isolate compound

The $^{13}$C-NMR spectrum and DEPT experiment showed the presence 6 six methyl, ten methylene, six methine five quarterner carbons at ∂ 39.6 (C-4), 56.4 (C-5), 39.1 (C-8), 39.7 (C-10) and 151.7 (C-21) respectively see Figure 17
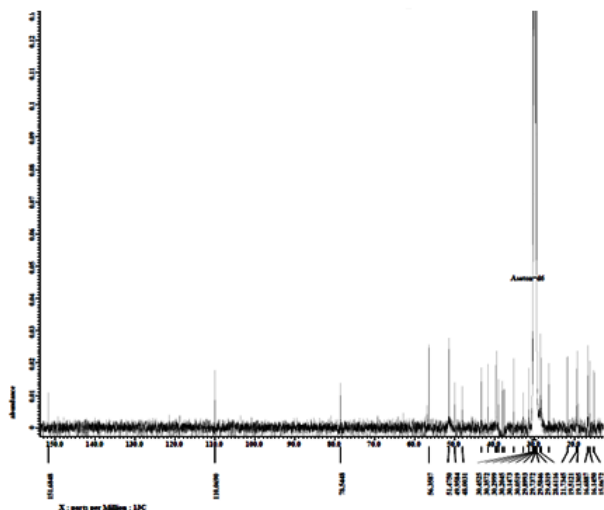
**Fig 17.** ^{13}C-NMRspectrum of pure isolate compound

^{1}H-NMR and ^{13}C-NMR data showed that the isolate compound have 27 carbon atom, fourty six hydrogen and one oxygen, with structure shown in figure 18
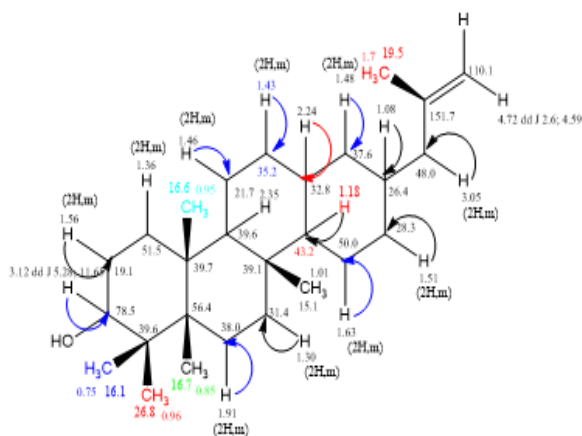


**Fig 18.** Structure terpenoid from C *bicolor* P.F Steven (CDCl$_3$) have antimalarial activity

## 5 Conclusions

1. These data showed the biological activity potency of Indonesian Clusiaceae plant (*C. bicolor*) as a source of antimalarial drugs and also showed that hexane and methanol fraction have antimalarial activity, but acetone fraction have not antimalarial activity, methanol fraction more active then hexane fraction,
2. According to the Figure 8 and 9, conclution that the sub fraction MeOH 7 :3 is better as antimalarial compare to any another sub fraction.
3. Hemozoin crystal were detected by TEM after 24 hours incubation of parasite in extract sample.
4. One triterpen compound have isolated and have antimalarial activity with IC$_{50}$ is 500 ppm

## 6 Equations and mathematics

$$\% \text{ parasetemia} = \frac{\text{Total eritrosit was infected by parasite}}{\text{total eritrosit}} \times 100\% \quad (1)$$

Percentase inhibition was obtained from parasetemia in control positive minus parasetemia in sampel /parasetemia in control x 100% :

$$\text{Inhibisi} = \frac{\text{Parasetemia ( in control - sample )}}{\text{Parasetemia in control positive}} \times 100 \quad (2)$$

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