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ANTI-PLASMODIUM FALCIPARUM IN VITRO ACTIVITY OF CALOPHYLLUM BICOLOR EXTRACT: Morphology and Ultra Structure

[1]Martha Marie Kaseke, [2]Veny Hadju, [2]Syafruddin Karim, [2]Armyn Nurdin

[1]Faculty of Medicine, Sam Ratulangi University, Manado-Indonesia
[2]Faculty of Medicine, Hasanuddin University, Makassar-Indonesia

Background: Anti-malarial resistance of Plasmodium, such as chloroquine becoming a health problem worldwide. This research aims to evaluate the in vitro anti-plasmodium activity of hexane fraction extract of Calophyllum bicolor (C. bicolor) against culture of Plasmodium falciparum (P. falciparum) 3D7 and determine its inhibitor concentration of 50% (IC_{50}); to observe the microscopic changes of P. falciparum after exposed to the hexane fraction of C. bicolor extract and to observe the ultra structure changes of P. falciparum 3D7 after being exposed to hexane fraction of C. bicolor extract. **Method:** This research applied quasi experimental with post-test only control group design. Anti malarial activity test of hexane fraction of C. bicolor extract towards Plasmodium falciparum 3D7 2% was undertaken over 48 hours with in vitro incubation technique. The level of parasitemia was observed using binocular optical microscope with 1000x magnification by counting the infected erythrocytes with Giemsa color technique. Then it was analyzed to determine the level of inhibitor concentration of 50% (IC_{50}) of the C. bicolor extract. The next step is to observe the changes of parasite's morphologic and ultra structure after the 24 and 48 hours incubation of the parasite P. falciparum 3D7 with hexane fraction of C. bicolor, IC_{50}. Then, the morphologic change of P. falciparum 3D7 was observed with optical binocular microscopy and the ultra structure changes with Transmission Electron Microscopy (TEM). The changes of morphologic and ultra structure were analyzed qualitatively. **Results:** The research revealed that hexane fraction of the C. bicolor extract inhibited the growth of the parasite P. falciparum 3D7 with the value of -49.00±2.54, 12.53±3.13, 23.01±1.10, 27.68±4.23, 48.65±18.71, 70.82±4.67, 80.52±6.17% with the hexane extract concentration of 0, 0.001, 0.01, 0.1, 1, 10, 100, and 1000 µg/mL. This research also found that the inhibitor concentration of 50% (IC_{50}) of hexane fraction of C. bicolor extract was 3.94 µg/mL in 10^{-7} which could change the morphologic and ultra structure of P. falciparum 3D7. There were several changes in morphology of the parasite over 24 hour incubation compared with the control group, i.e. the nucleus of young tropozoid became thicker, darker, and smaller; the skizon were condensate, thicker, darker, karyorrhexis and demolition of membrane. Hexane fraction of C. bicolor extract also changes the ultra structure over 24 hour incubation, i.e. vacuole membrane boundary and hemozoin were unclear. Over 48 hours of incubation the nucleus and cytoplasm bigger than those in the control group. **Conclusion:** Hexane fraction of the C. bicolor extract showed a strong anti plasmodium activity towards the in vitro culture of P. falciparum 3D7 at the level of (IC_{50}) 3.94 µg/mL. The Hexane fraction of C. bicolor extract could change the morphology and ultra structure of P. falciparum 3D7 in vitro.

Keywords:Calophyllum bicolor; Plasmodium falciparum 3D7, Activity; anti plasmodium

INTRODUCTION

Malaria is an infectious disease cause by Plasmodium and become one of the most frequent health burden in subtropical and tropical countries, including Indonesia. Around 3.2 billion people in the world in 107 tropic countries have exposed to the risk of Malaria (The World Malaria Report 2010).[1] Based on data from WHO, the prevalence of Malaria were 350-500 million per annual with a number of death of 2.5 million people.[2,3] Indonesia is one of malaria endemic area. Malaria causes several million infections and about 10,000 deaths each year in Indonesia.[4]

Correspondence:
Martha Marie Kaseke,
Department of Anatomy and Histology, Faculty of Medicine, Sam Ratulangi University
Manado,Indonesia.
Telp:HP:089698129912; Home:0431-811817
E-mail: kaseke_marie@yahoo.com

Recently, there is a problem of anti malarial resistance of Plasmodium such as cloroquine. The anti malarial resistance of Plasmodium has been reported in the majority of endemic areas in the world including Indonesia. Therefore this problem has initiated researchers to identify the new anti malarial drugs to substitute the resistant drug. WHO also encourage researcher to find the effective anti malarial drugs with the new mechanism in order to prevent cross resistance. The chemical compound of the natural or medicinal plants has been studies to identify their active compounds that may become anti malarial agents. There are some examples of anti malarial that come from the extract of medicinal plant such as quinine are extracted from cinchona sp. and artemisinine are from Artemisia Annua. These two drugs are recently successfully used for severe malaria.[5] The other medicinal plants that have been used as antimalarial agents in Indonesia are Carica papaya,[6] Phyllantus niruri,[7] and Alstonea scolaris.[8] C. bicolor PF Stevens or Bintagor is one of the species of genus Calophyllum (Clusiaceae family) that traditionally used as a treatment of various diseases.[9] Calophyllum has 180-200 species that grow in the coastal areas in Kalimantan and Papua. Various biological activities from Calophyllum extract was reported as anti-HIV, anti-cancer, insecticide agent, antibacterial, and antimalaria.[10-14] Calophyllum consists of active compound such as ksanton, kumarin, benzodipirin, kromanon, bioflavonoid, trirerpenoid and steroid.[15]

Therefore, it is worth to study the hexane fraction of C. bicolor to examine the anti plasmodium activity.

METHODS

This research applied quasi experimental with post-test only control design and 48 hours in vitro incubation technique for anti malarial activity test. The sample comprises of the extract of hexane fraction C. bicolor (yellow liquid) and P. falciparum 3D7 were obtained from the Malaria Laboratory, Biology Molecular Eijkman in Jakarta. The anti plasmodium activities test has 3 steps namely thawing P. falciparum 3D7, maintain culture in vitro of P. falciparum 3D7 and anti plasmodium activities test of hexane fraction extract C. Bicolor. The IC_{50} value was determined by linear interpolation from the growth inhibition curves.

Thawing Plasmodium falciparum 3D7

P. falciparum 3D7 that was stored in liquid nitrogen at -196^{0}C was placed at room temperature for 1-2 minutes, then placed in a 50 mL tube and gently add 5 mL NaCl 3.5% between 5-10 minutes and shaking the tube slowly to homogenize the culture. After that it was centrifuged at 1500 rpm within 5 minutes at temperature of 20^{0}C. The sediment was washed with RPMI with the volume ratio of 1:9, centrifuged within 5 minutes at temperature of 20^{0}C. This step was repeated 2 times. Complete medium 10 mL was added to the remained sediment in the tube and homogenized them with the pipe, then placed it in the flash with atocrit. The flash was placed in the incubator and changed the complete medium 10% everyday.

The parasite culture was maintained by change 10% complete medium every day. The level of paracitemia was monitored every day.

Antiplasmodium activity test of hexane fraction extract C. bicolor

The anti plasmodium activity test was complete after obtained the level of parasitemia of 2%. The testing was performed in the 8 well (duplication test). A number of 180 µL of 2% parasites and 20 µL hexane fraction extract C. bicolor were distributed to each well. Every well contained different concentration of the C. bicolor extract. The control group did not receive the C. bicolor extract.

The parasite and the extract were incubated within 48 hours and harvested. The level of parasitemia was examined by counting the infected erythrocytes with Giemsa coloring technique, and used binocular optical microscope with 1000x magnification. After determine the level of parasitemia, the level of inhibitor concentration of 50% (IC_{50}) of the C. bicolor extract was determined by the logarithmic curve.

Microscopic changes of P. falciparum after exposed to the hexane fraction of C. bicolor extract

The next step is to observe the changes of parasite morphologic after the 24 hours incubation of the parasite P. falciparum 3D7 with hexane fraction of C. bicolor, IC_{50} of 3.94 µg/mL, 10^{-7}. Morphology change of P. falciparum 3D7 was observed with the optical binocular microscopy and the level of parasitemia.

Ultra structure changes of P. falciparum after exposed to the hexane fraction of C. bicolor extract

The changes of parasite ultra structure was observed over the interval times of 24 and 48 hours incubation of the parasite P.falciparum 3D7 with hexane fraction of C. bicolor, IC_{50} of 3.94 µg/mL in $10^{-7.}$

The TEM sample preparation was prepared by several steps namely fixation, dehydration, infiltration and embedding. The fixation process comprise of 3 different steps. Firstly immerse the sample into 2.5% Glutaraldehyde within 24 hour at the temperature of 4^{0}C while shacking and it was centrifuged with the speed of 3000 rpm. The next step was to rinse the sample with 0.1 coca-dilate

and 3% of sucrose then it was centrifuged (repeated 3 times). The third step was fixing the pellet in 2% osmium tetraoxide and 2.5% $K_3Fe(CN)_6$ in buffer within 2 hours, while keep shaking it in the temperature of 4^0C. After that the second step process using 0.1 coca-dilate and 3% of sucrose was repeated 3 times.

The dehydration process was undertaken several times by rinsed the sample with different concentration of ethanol. Firstly, the ethanol 10% within 5 minutes, while keep shaking at 4^0C and centrifuging, following with same process using 30% ethanol (5 minutes), 50% ethanol (24 hours), 70% ethanol (10 minutes), 90% ethanol (10 minutes) and 2 times with absolute ethanol (20 minutes).

The infiltration process was performed by adding the pure propylene oxide to the sample and kept it 60 minutes at room temperature.

The last step was embedding process, by adding propylene oxide: Spurr's resin (1:1) and homogenised it within 30 minutes at room temperature. The half of mixture was poured out and added equal amount of pure Spurr's resin and homogenised it within 30 minutes at room temperature. After that, all the remained mixture was poured out and replace with pure Spurr's resin, kept it overnight. Then placed the pellet to the block tube or eppendorf and placed them in the vacuum overnight. The sample was immersed in the pure Spurr'd resin mixture and put them into the oven for 24 hours at 70^0C. Then, the sample was cut with microtome, coloured with uranil tripelit, and observed with TEM. The result of TEM was analysed with computer.

RESULTS

The research revealed that hexane fraction of the C. bicolor extract inhibited the growth of the parasite P. falciparum 3D7. The level of inhibitor concentration of 50% (IC_{50}) of the C. bicolor extract was determined by the logarithmic curve. This research also found that the inhibitor concentration of 50% (IC_{50}) of hexane fraction of C. bicolor extract was 3.94 µg/mL in 10^{-7}, which could change the morphologic and ultra structure of P. falciparum 3D7. Table 1 presents the value of parasitemia (%) and the growth inhibitor (%) of P. falciparum after exposed to hexane fraction C. bicolor.

There were several changes in morphology of the parasite over 24 hour incubation compared with the control group, i.e. the nucleus of young tropozoid became thicker, darker, and smaller; the skizon were condensate, thicker, darker, karyorrhexis and demolition of membrane. Figure 1 shows the morphology of P. Falciparum in control and after 24 hour incubation in vitro with Hexane fraction of the C. bicolor extract using the optical microscope.

Tabel 1
Parasitemia (%) and inhibitor growth (%) of P. falciparum after exposed to C. bicolor hexane fraction

Extract Concentration µg/mL	Means Parasitemia levels	Means Growth Inhibition levels
0	4.53±2.70	0
0.001	6.72±3.92	-49.00±2.54
0.01	4.00±2.51	12.53±3.13
0.1	3.50±2.12	23.01±1.10
1	3.22±1.74	27.68±4.23
10	2.25±1.41	48,65±18,71
100	1.25±0.57	70.82±4.67
1000	0.91±0.57	80.52±6.17

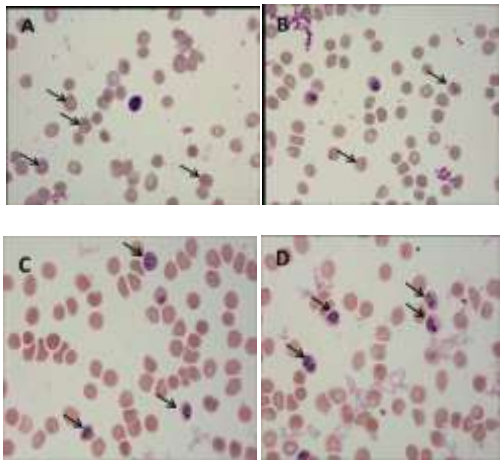

Figure 1
Morphology of P. Falciparum in control and after 24 hour incubation in vitro with Hexane fraction of the C. bicolor extract using the optical microscope. (A)Young tropozoid in control, (B) Young troposoid in treatment, (C) Skizon in control, (D) Skizon in treatment

Hexane fraction of the extract C. bicolor also changes the ultra structure over 24 hour incubation, i.e. vacuole membrane boundary and hemozoin were unclear. Over 48 hours of incubation the nucleus and cytoplasm bigger than those in the control group as can be seen in Figure 2. Figure 2a and c present the ultra structure of old tropozoid after 24 and 48 hours incubation in control using TEM and picture 2b and d shows the ultra structure of old troposoid after 24 and 48 hours incubation in treatment using TEM.

DISCUSSION

This research found that IC_{50} value hexane fraction extract C. bicolor was 3.94 µg/mL, which is categorized as a strong anti plasmodial. Classification of the fraction activity or plant extract was classified into 4 groups according their IC_{50} for antiplasmodial activity test.[16-18] Very active

anti plasmodial has IC_{50} less than 5 µg/mL; active anti plasmodial has IC_{50} less 5-50 µg/mL; less active anti plasmodial has IC_{50} less than 51-100 µg/mL ; inactive antiplasmodial has IC_{50} more than 100 µg/mL.

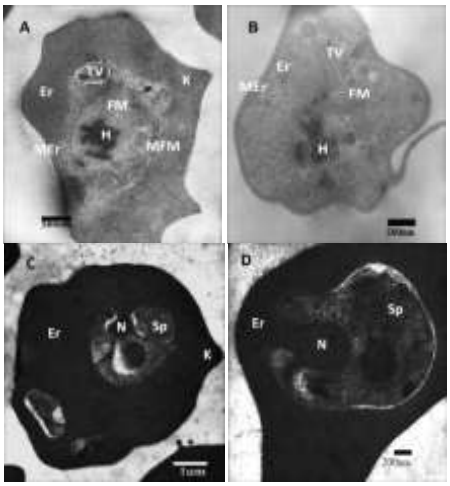

Figure 2
Vacuole Membrane Boundary and Hemozoin Changes

(2a, c) present the ultra structure of old tropozoid after 24 and 48 hours incubation in control using TEM and (2b, d) shows the ultra structure of old troposoid after 24 and 48 hours incubation in treatment using TEM.

There were several publications which study anti plasmodial activity of Calophyllum genus. Hay *et al* (2004) reported the potencial anti plasmodium of Calophyllum caledonicum.[14] Based on their study, there were several xanthon compounds from Calophyllum caledonicum that has strong anti plasmodial activity such as dimetilkalabaksanton and 6-deoksi-y-mangostin with IC50 < 1 µg/mL. Abbas and Syafruddin (2010) successfully isolated the compound of xanthon that is fenil kaumarin from Calophyllum flavoranulum from Kalimantan and reported its antiplasmodial activity invivo in mencit that was infected by Plasmodium Berghei.[19] Rissyelly (2010) also reported antiplasmodial activity of ksanton compound namely [2-(3',3'-dimetilalil)-1,3,7-trihidroksiksanton] from Calophyllum canum.[20]

The changes in morphology and ultra structure of P. falciparum 3D7 after being exposed to hexane fraction extract C. bicolor might be related to the mechanism and target of given anti plasmodial.[21] The morphology changes shows that hexane fraction extract C. bicolor were possibly work by inhibit tropozoid grow to be skizon. When the tropozoid turn to be skizon, the Plasmodium will metabolise haemoglobin to become hem and haemoglobin fragment. Hem is a toxic agent for the plasmodium that will be polymerised by Plasmodium as a mechanism of detoxification, and turn the hem to be hemozoin or malaria pigment. Several antiplasmodial work by inhibit the polymerisation of hem to become hemozoin, such as Cloroquine, artemisin, meflokuin and halofantrin. This mechanism in turn will increase hem as a toxic agent for plasmodium.

It was assumed that C. bicolor consist of Ksanton compound that may inhibit hem polymerisation and work specifically at tropozoid stadium.[22,23]

CONCLUSION

It could be concluded that Hexane fraction of the C. bicolor extract showed a strong antiplasmodial activity towards the in vitro culture of PF 3D7 at the level of (IC50) 3.94 µg/mL. The Hexane fraction of C. bicolor extract could change the morphology and ultrastructure of P. falciparum 3D7 in vitro.

It is recommended for further research to identify the chemical structure or compound and study the antiplasmodial activity of hexane fraction C. bicolor in vitro and in vivo. The mechanism and target actions of anti plasmodial

REFERENCES

1. WHO. 2010. World Malaria Reports. Global Malaria Programme World Health Organization 20, avenue Appia CH–1211 Geneva 27 http://www.who.int/malaria. e-mail: infogmp@ who.int
2. WHO. 2005a. *World Malaria report* (www.who.malariareport 2005.org).
3. Aty W., Subehan, Kalauni S. K., Awale S., Nindatu M., Zaini N. C., Syafruddin D., Asih P. B. S., Yasuhiro T., Shigetoshi K. 2007. New prenylated flavones from Artocarpus champeden and their antimalarial activity *in vitro*. J Nat Med. 61: 410-413.
4. CDC. 2014. CDC In Indonesia. *CDC-Atlanta*
5. Achan J., Talisuna A. O., Erhart A., Yeka A., Tibenderana J. K., Baliraine F. N. 2011. Quinine, an old anti-malarial drug in a modern world: role in the treatment of malaria. *Malaria J*, 10:144. http://www.malariajournal. com/ content/10/1/144
6. Rehena J. F. 2010. In vitro Activities Test of Papaya Leaf Extract (Carica papaya. LINN) as an Antimalaria Agent. *Jurnal Ilmu Dasar*.11,1, Jan 2010 : 96 –100
7. Mustofa, Sholikah E. N., Wahyuono S. 2007. *In Vitro* and in vivo Anti plasmodial Activity and Cytotoxicity of Extracts of Phyllanthus niruri L. Herbs Traditionally Used to Treat Malaria In Indonesia. *J. Trop Med Public Health*. 38 (4)
8. Keawpradub, N., Kirby, G. C., Steele, J. C., Houghton, P. J. 1999. Antiplasmodial activity of extracts and alkaloids of three Alstonia species from Thailand. 65(8):690:694.

9. Heyne K. 1987. Tumbuhan Berguna Indonesia (Terjemahan), Jilid 3, *Badan Litbang Kehutanan*, Jakarta.
10. Laure F., Raharivelomanana P., Franc J., Butaud O., Bianchini J. P. and Gaydou, E. M. 2008. Screening of Anti-HIV-1 Inophyllums by HPLC–DAD of *Calophyllum inophyllum* Leaf Extracts from French Polynesia Islands. *Analytica Chimica Acta*, 624: 147–153.
11. Mah S. H., Ee G. C. L., Teh S. S., Rahmani M., Lim Y.M., and Go R. 2012. Phylatrin, A New Cytotoxic Xanthone from *Calophyllum soulattri*, *Molecules.*, 17: 8303-8311.
12. Syahputra E., Prijono D., and Dono D., 2007. Sediaan Insektisida *Calophyllum soulattri*; Aktivitas Insektisida dan Residu terhadap Larva Crocidolomia pavonana dan Keamanan pada Tanaman, *J.H.P.T Tropika*,7,1,21-29.
13. Khan M. R., Kihara M. and Omoloso A. D. 2002. Antimicrobial Activity of *Calophyllum soulattri*. *Fitoterapia.*73: 741-743.
14. Hay A. E., Hélesbeux J. J., Duvala O., Red L. M., Grellien P., Richomme P. 2004. Antimalarial xanthones from *Calophyllum caledonicum* and *Garcinia vieillardii. Life Sci*, 75: 3077-3085.
15. Su X. H., Zhang M. L., Li L.G., Huo C. H., Gu Y. C., and Shi Q. W. 2008. Chemical Constituents of the Plants of The Genus *Calophyllum*, *Chemistry and Biodiversity*, 5, 2579-2608.
16. Gessler M, C., Nkunya M. H. H., Mwasumbi L. B., Heinrich M., Tanner M.1994. Screening Tanzanian Medicinal Plants for Antimalarial Activity. *Act Trop* 56:65-77.
17. Karov D., Dicko M. H., Sanon S., Simpore J., Traore A. S. 2003.Antimalarial activity of Sida acuta Burn.f (Malvaceae) and Pterocarpus erinaceus Poir (Fabaceae). J Ethnopharmacol 89:291-294.
18. Bickii J., Tchouya G. R. F., Tchouankeu J. C., Tsamo E. 2007. Antimalarial activity in crude extracts of some Cameroonian medicinal plants. *Afr. J. Trad. CAM*., 4(1):107-111.
19. Abbas J., Syafruddin D. 2014. Antiplasmodial evaluation of one compound from *Calophyllum flavoranulum*. *Indo. J. Chem*., 14 (2): 185-191.
20. Rissyelly, Katrin, Abbas J. 2010. Antimalarial Activity of Xanthone from Stem Bark Callophyllum canum Hook.f). Jurnal Bahan Alam Indonesia ISSN 1412-2855, 7,4: 224-228.
21. Fidock D. A., Rosenthal P. J., Croft S. L. Brun R., Nwaka S. 2004. Antimalarial drug discovery: Efficacy models for compound screening., www.nature.com/reviews/drugdisc.
22. Ignatushchenko M., Winter R., Riscoe. 2000. Xanthones as antimalarial agents. Stage specificity. *Am J Trop Med Hyg* 62 (1): 77-81
23. Ignatushchenko M., Winter R., Baechinger H, P., Hinrich R. J., Riscoe M. K. 1997. Xanthones as antimalarial agents: Studies of a possible mode of action. *FEBS lett* 409:67-73

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