

In Vitro and In Vivo Antiplasmodial Activities of *Garcinia Parvifolia* (MIQ) MIQ Leaves From Indonesia

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Abstract: The objective of this research is to determine in vitro and in vivo antiplasmodial activities of *G. parvifolia*(Miq) Miqleaves. Methods: in vitro antiplasmodial test by microscopic and Malaria SYBR Green I-based Fluorescence (MSF) assay is indicated by the value of IC₅₀; meanwhile, in vivo antiplasmodial assay used suppression method for 4 hours in *P.berghei*-infected mice. Leaves of *G. parvifolia*(Miq)Miq were extracted by means of chloroform and methanol solvent, and then the anti-plasmodium activity is tested in vitro by means of microscopic and Malaria SYBR Green I-based Fluorescence (MSF) methods. Results: In microscopic method, the value of IC₅₀ was obtained from chloroform extract i.e. 33.17 µg/mL in D6 strain and 0.55 µg/mL in W2 strain. In Malaria SYBR Green I-based Fluorescence (MSF) method, the IC₅₀ value was obtained from methanol extract i.e. 48.00µg/mL in D6 strain and 56.37µg/mL in W2 strain. In vivo assay found that chloroform extract had an ED₅₀ value of 156.22mg/kgBW, stronger than that of methanol extract with an ED₅₀ value of 412.92mg/kgBW. Conclusions: The study concludes that chloroform and methanol extracts of *G. parvifolia*(Miq) Miq leaves have anti-plasmodium activity against *P. falciparum* culture of D6 strain, W2 strain on microscopic and Malaria SYBR Green I-based Fluorescence (MSF) methods just like in vivo assay in *P. berghei*-infected mice does. The assay found that chloroform extract has stronger anti-plasmodial activity than methanol extract.

Keywords: *G. parvifolia* (Miq)Miq leaves, in vivo antiplasmodial activity, in vitro antiplasmodial activity

I. Introduction

Malaria is still one of the major health problems in the developing countries as well as in developed countries. The 2010 World Malaria Report summarizes information received from 106 malaria-endemic countries/areas and other partners and it updates the analyses presented in the 2009 Report [1]. The World Health Organization estimated 2.5 million clinical attacks in Indonesia for that year. According to Malaria Atlas Project (MAP), the annual number of clinical attacks of *P. falciparum* alone was over 12 million, and the number of Indonesians at risk of *P. falciparum* in 2007 was 151 million [2]. In 2006, Indonesia reported 347,597 confirmed and suspected malaria cases to the World Health Organization (WHO), and most of which had a laboratory-confirmed diagnosis [3]. One of the main problems in malaria eradication is the existence of malaria vector, *Anopheles* mosquitoes, which are resistant to insecticides and malaria parasites. However, they are not affected by the existing antimalarial drug. The resistance of *P.falciparum* has become a serious and disturbing problem nowadays since it causes failures in treating malaria and eventually results to deaths. This encourages researchers to find new antimalarial drugs to replace the ineffective ones. One of the efforts to discover new antimalarial drugs is to explore active compounds from medicinal plants traditionally used by natives to cure malaria in various endemic areas in Indonesia.

Natural products are the direct or indirect sources of most of the drugs introduced in the past 30 years. Natural products from plants are a rich source of lead compounds for the development of new drugs against protozoan parasitic diseases such as malaria [4]. Nitidine was first isolated in 1959 from *Zanthoxylum nitidum* (Rutaceae) and it can be considered a potential anti-malarial lead compound. Its ability to complete haem and inhibit b-haematin formation suggests a mechanism of action similar to that of chloroquine [5]. Some plants from Clusiaceae family have been known to have relatively good antimalarial activity. Nine phenolic compounds isolated from *Cratoxylum maingayi* and *C. cochinchinense* (Clusiaceae) were evaluated for anti-malarial activity against *Plasmodium falciparum* while formoxanthone C was found to be the most active against *Plasmodium falciparum* and vismione B had the highest activity against *Plasmodium falciparum* [6]. Plants of Clusiaceae family contains much xanthone compound with relatively good antimalarial activity. *Garcinia*, a genus in the Clusiaceae, is a source of antiparasitic and antimalarial phenolic secondary metabolites, including benzophenones and xanthenes [7]. The antimalarial potency of the xanthenes correlated well with their ability to inhibit in vitro heme polymerization, suggesting that these compounds exert their antimalarial

action by preventing hemozoin formation [8]. As part of our continuing efforts to identify antimalarial agents from medicinal plants, particularly those from Clusiaceae family, an in vivo and in vitro antimalarial assay was conducted on the extracts of *Garcinia parvifolia* (Miq)Miq leaves.

II. Materials And Methods

Sample collection: Samples of leaves of *G. parvifolia* (Miq)Miq were collected from Sei Kepayang, Asahan District of North Sumatera, Indonesia in June 2013.

Preparation of Crude Extracts

G. parvifolia (Miq)Miq leaf was collected in and around Nang Kalis, West Kalimantan, Indonesia. *G. parvifolia* (Miq)Miq leaf was washed with tap water and shade dried at room temperature. An electrical blender powdered the dried plant materials (leaves). 1 kg of the powder of the leaf was extracted with 4 L of organic solvents of methanol for 8 h using a Soxhlet apparatus. The extracts were filtered through a Buchner funnel with Whatman number 1 filter paper. The crude plant extracts were evaporated to dryness in rotary vacuum evaporator to obtain the methanolic extract (ME). Another portion (1.4 kg) was extracted with chloroform using Soxhlet extractor to obtain crude chloroform extract (CE). The crude chloroform extract was concentrated with rotary evaporator.

Culture of *P. falciparum*

Two strains of *P. falciparum*, one sensitive to chloroquine (D6) and the other resistant to chloroquine (W2), were obtained from the Laboratory of Parasitology, Naval Medical Research (NAMRU), Jakarta. The cultures was maintained in our laboratory at 5% hematocrit (human type O-positive red blood cells) in complete RPMI 1640 medium (RPMI 1640 medium supplemented with 25 mM HEPES, 370 μ M hypoxanthine, 40 μ g/ml gentamycin, 0.25 μ g ml⁻¹ Fungizone and 0.5% [wt/vol] AlbuMax II) in 60 mm petri dish by modified candle jar method [9].

In vitro Antiplasmodial Assay

Standard drug (chloroquine) and extracts (at different concentrations of 4, 20, 100, 200, and 400 μ g/ml) were prepared in distilled water (chloroquine; Sigma) and DMSO (test extracts); and then, it was diluted to achieve the required concentrations. The synchronized culture with parasitaemia of 1.5% and 3% haematocrit were incubated in 96-well microtitre plate predisposed with multiple concentrations of compounds/extracts for 48 hrs at 37°C in candle jar. The study used a 96-well micro-culture. The first column was filled with 12.5 μ L of RPMI 1640; the second to the sixth columns were filled with 12.5 μ L of chloroform extracts; and the seventh to the eleventh columns were filled with 12.5 μ L of methanol extracts. Each well was filled with 100 μ L of culture. The microculture was then placed into a candle jar. To get optimal gas concentration, the candle jar was tightly capped for certain period until the candle inside the jar turned off. For microscopic method, the upper part of the suspension was removed. The thick suspension was used for thick blood smear preparation by piping the thick suspension, and then dropping it into a glass object. However, the blood smear was desiccated for 5-10 minutes. Then Giemsa was subsequently washed using tap water and desiccated. The smear that had been formed could be viewed using a microscope (100 x magnification) by adding immersion oil. The result was presented as the percentage (%) of parasitemia. In Malaria SYBR Green I-based Fluorescence (MSF) method, 100 μ L of lysis buffer that included SYBR Green I was fed into each well. The micro-well plate was incubated in a dark room for 1 hour and read using a fluorescence plate reader. The data was presented in terms of a table of percentage (%) growth inhibition. The Inhibition concentration of 50% (IC₅₀) was determined using probit analysis based on the correlation of log concentration and probit value. In Malaria SYBR Green I-based Fluorescence (MSF) assay, the data was presented in a log concentration and fluorescent linearity. Inhibition concentration of 50% (IC₅₀) was obtained by putting data between the log concentration and fluorescent linearity into Microsoft Excel program. Then, the data was analyzed using non-linearity regression supplied by Graph Prism software (San Diego, CA) [10].

In vivo Antiplasmodial Assay

In vivo antiplasmodial assay commonly used is a method that utilizes the model rodents, i.e. mice and rats. For antimalarial filtering, the most commonly used animal models were *P. berghei* Strain ANKA infected mice. The method of in vivo antiplasmodial assay was a modified method developed by Akanbi (2013). The mice were infected by injecting 10⁷ of infected erythrocyte into the peritoneal cavity of the mice. Male mice with body weights ranging from 20-30 g were prepared in a fixation tool; the animals were slowly injected in an Intraperitoneal (IP) way. In vivo assay was conducted on Swiss strain mice infected with *P.berghei* [11]. The blood of donor mice with parasitemia of 30-40% was taken out from the heart. Then, the blood was diluted in such a way that yielded *P.berghei* inoculums of 1 x 10⁷ for every mouse. Inoculation was conducted using an

Intraperitoneal (IP) method by injecting 0,2 mL of inoculums. The mice were assigned into treatment group based on the dosage and positive control group given with chloroquine and negative control. The level of test dosage for chloroform extracts and methanol extracts of *G. parvifolia* (Miq)Miq leaves was 50, 100, 300, and 600mg/kgBW/day, respectively (Table 3). Every group of animal was given oral test preparation and chloroquine based on the dose that had been determined for 4 days after the inoculation of *P. berghei*. Every day, the blood of each mouse was taken for parasitemic assay, up to the fourth day (since D+1 to D+4). Thin blood preparation was prepared for parasitemic assay. Negative doses were used to determine the influence of test preparation on the *P. berghei*-infected mice. Effective Dosage (ED₅₀) was determined and log-probit graphs were plotted [12].

III. Results

In microscopic method, the value of IC₅₀ was obtained from chloroform extract 33.17 µg/mL in D6 strain and 0.55 µg/mL in W2 strain. In Malaria SYBR Green I-based Fluorescence (MSF) method, the IC₅₀ value was obtained from methanol extract 48.00 µg/mL in D6 strain and 56.37 in W2 strain. In vivo assay found that chloroform extract had an ED₅₀ value of 156.22mg/kgBW, stronger than that of methanol extract with an ED₅₀ value of 412.92mg/kgBW. Table 1 shows the results of the growth inhibitory activity of D6 Strain and W2 Strain of *P. falciparum* from methanol extract and chloroform extract of *G. parvifolia* (Miq)Miq leaves using microscopic method. The chloroform extract on D6 Strain indicated a strong antiplasmodial activity and W2 strain indicated a very strong antiplasmodial activity. Methanol extract of D6 strain and W2 strain showed a strong antiplasmodial activity. This implies that chloroform extract had a distinctive mechanism that made it more potent than W2 strain. However, compared to positive control group (chloroquine), chloroform extract and methanol extract had weaker antiplasmodial activities.

There is an increasing trend in the concentration of chloroform extracts and methanol extracts as evidenced by the increased percentage (%) growth inhibition of *P. falciparum*. To get the IC₅₀ value, a curve of linear regression, which is shown in Figure 1, was prepared in advance. In vitro antiplasmodial activity assay used Malaria SYBR Green I-based Fluorescence (MSF) method. Data on the IC₅₀ value are presented in MS Excel sheets. After analysis with Graph Prism (San Diego, Ca) Software, the data were presented in Table 3.

IV. Discussion

In this present study, IC₅₀ values of chloroform extract and methanol extract of *G. parvifolia*(Miq)Miq leaves using microscopic and Malaria SYBR Green I-based Fluorescence (MSF) methods are presented in Table 2. In vitro antiplasmodial property assay using *P. falciparum* subculture can be used as a preliminary test for biological, natural agents that are prospective as antiplasmodial agents. The study was conducted on the chloroform extract and methanol extract of *G. parvifolia* (Miq)Miq leaves. Chloroform extract was polar that it could attract polar compounds. The study was conducted on Strain D6 (sensitive chloroquine) and Strain W2 (resistant chloroquine) of *P. falciparum*. Both strains had been determined as the standard strains of *P. falciparum* by WHO. They were used to examine the sensitivity of drug or natural agents for their antimalarial properties. Antiplasmodial activity assay utilized two methods, i.e. microscopic and Malaria SYBR Green I-based Fluorescence (MSF) methods.

In vitro antiplasmodial assay using microscopic method, the result in IC₅₀ values of chloroform extract 33.17µg/mL in strain D6 and 0.55µg/mL in strain W2 of *P. falciparum*. Meanwhile, IC₅₀ values of methanol extract were 32.44µg/mL in strain D6 and 41.23µg/mL in W2 strain of *P. falciparum*. As seen in Table 2, Malaria SYBR Green I-based Fluorescence (MSF) method showed IC₅₀ values of chloroform extract as 14.29µg/mL in D6 strain and 0.21µg/mL in W2 strain of *P. falciparum*, meanwhile, Malaria SYBR Green I-based Fluorescence (MSF) method resulted in an IC₅₀ values of 48.00µg/mL in D6 Strain and 56.37µg/mL in W2 Strain of *P. falciparum*.

Several criteria have been proposed for considering a compound as active. Generally, a compound is considered to be inactive when it shows an IC₅₀ > 200 µM, whereas those with an IC₅₀ of 100-200 µM have low activity; IC₅₀ of 20-100 µM, moderate activity; IC₅₀ of 1-20 µM good activity; and IC₅₀ < 1 µM excellent/potent antiplasmodial activity [13]. Therefore, an IC₅₀ value of chloroform extract from W2 strain showed better antiplasmodial activity than that of D6 strain.

Table 3 shows the rates of parasitemia for every treatment group (chloroform extract and methanol extract with dosages of 600, 300, 100 and 50mg/kgBW) and the negative control group through oral administration. Based on values in Table 3, ED₅₀ value of chloroform extract 156.22mg/kgBW and 412.92mg/kgBW of methanol extract of *G. parvifolia* (Miq)Miqleaves given orally could be calculated and compared in terms of antiplasmodial activity of the chloroquine as a standard antimalarial drug, as presented in Table 3

In vivo antiplasmodial activity assay found that treatment group given chloroform extract was stronger than group given methanol extract of *G. parvifolia*(Miq)Miq (*Clusiaceae*) leaves. This is evident in the ED₅₀

values of 156.22mg/KgBW and 412.92 mg/kgBW. High ED₅₀ value indicated higher effectiveness in inhibiting the growth of parasite of the chloroform extract. Some plants from Clusiaceae family known to have antimalarial activities included the ethyl acetate bark extract of *Rheedia acuminata* led to the isolation of the new compound 1,5,6-trihydroxy-3-methoxy-7-geranyl-xanthone have IC₅₀s against *P. falciparum* exceeding 10 μM [14].

A new compound, garcinia-xanthone, was isolated from the roots of *Garcinia polyantha* Oliv. (Clusiaceae), in addition to three known compounds, smeathxanthone A, smeathxanthone B and chefoxanthone. They exhibited antimalarial activity against NF54 with IC₅₀ values ranging from 2.5 to 4.1 mM [15]. Research conducted by Ignatushchenko et al. (1997) explained that the potent antimalarial xanthenes act in a unique fashion to kill *Plasmodium* parasites through formation of soluble complexes with heme, thereby inhibiting the process of heme polymerization [16]. The results of this study indicated that the extract of the *G. parvifolia* (Miq)Miq., possesses antimalarial activity. Further studies are required to determine if the antimalarial activity of *G. parvifolia* (Miq)Miq is attributable to the xanthenes and benzophenone constituents. Meanwhile, the results partly corroborate claims made in traditional medicine of the antimalarial efficacy of this plant

V. Conclusion

In conclusion, chloroform and methanol extracts of *G. parvifolia*(Miq) Miq leaves have anti-plasmodium activity against *P. falciparum* culture of D6 strain, W2 strain on microscopic, and Malaria SYBR Green I-based Fluorescence (MSF) methods.

Conflict of interest statement

We declare that we have no conflict of interest

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Table 1. Growth inhibition of *P. falciparum* (%) of chloroform extracts and methanol extracts of *G. parvifolia* (Miq)Miq leaves in various ranges of concentrations by using microscopic method

Extract Concentration (µg/mL)	% Growth Inhibition			
	P. falciparum strain D6		P. falciparum strain W2	
	Chloroform extract	Methanol extract	Chloroform extract	Methanol extract
4	42.93	14.04	27.98	4.97
20	36.48	10.49	63.32	8.84
100	73.55	85.45	82.87	60.66
200	88.95	93.37	83.50	90.34
400	96.50	97.88	92.66	91.28

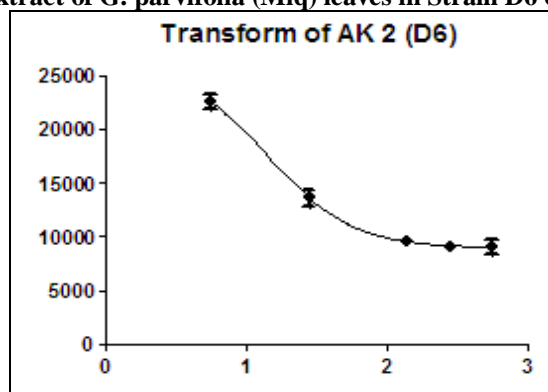
Table 2. IC₅₀ values (µg/mL) of chloroform extract and methanol extract of *G. parvifolia*(Miq)Miq leaves using microscopic and Malaria SYBR Green I-based Fluorescence (MSF) methods

Sample	Microscopic method		MSF method	
	Strain D6	Strain W2	Strain D6	Strain W2
Chloroform extract	33.17	0.55	14.29	0.21
Methanol extract	32.44	41.23	48.00	56.37
Chloroquine	0.012	1.08	0.026	0.23

Table 3. Rate of parasitemia and % growth inhibition of chloroform extract and methanol extract of *G. parvifolia* (Miq)Miq leaves given orally

Dose (mg/kgBW)	Rate of Parasitemia		% inhibition	
	Chloroform extract	Methanol extract	Chloroform extract	Methanol extract
600	4.45	10.37	87.00	71.60
300	11.85	27.06	65.50	26.00
100	24.46	30.57	28.70	16.40
50	25.48	33.02	25.80	9.70
control	34.35	36.57	0	0

Chloroform extract of *G. parvifolia* (Miq) leaves in Strain D6 of *P. falciparum*



Chloroform extract of *G. parvifolia* (Miq) leaves in strain W2 of *P. falciparum*

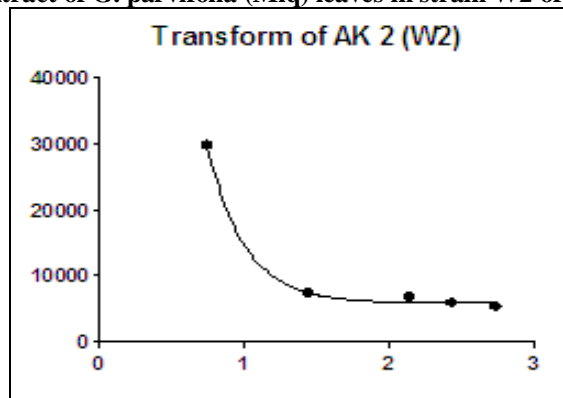


Figure 1. Relationship between chloroform extract and methanol extract of (*G. parvifolia* (Miq)Miq) Leaves with fluorescent linearity in Strain D6 and W2