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***In vivo* Antiplasmodial Activity and Acute Toxicity of the Fraction of the *Garcinia parvifolia* Miq. Stem Bark**

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Abstract: The study of *in vivo* antiplasmodial activity and acute toxicity of the active fraction of *Garcinia parvifolia* Miq. has been conducted. The fraction was obtained by maceration of n-hexane extract with methanol. A standard 4-day test on *P. berghei* infected Swiss mice was used to evaluate the *in vivo* antiplasmodial activity after an oral administration of the fraction in series dose of 25 to 200 mg kg⁻¹ b.wt. once daily for 4 consecutive days. The *in vivo* antiplasmodial activity was expressed by the dose inhibiting 50% of parasite growth (ED₅₀). Acute toxicity was evaluated in Swiss mice after oral administration of the active fraction in series dose of 2000 to 8000 mg kg⁻¹ b.wt. The acute toxicity was expressed by the dose leading 50% deaths (LD₅₀). The results showed that the active fraction of *G. parvifolia* Miq. was active against *P. berghei* in mice with an ED₅₀ of 74.45 mg kg⁻¹ b.wt. day⁻¹. In addition, the active fraction was also relatively safe as expressed by the LD₅₀ of 8000 mg kg⁻¹ b.wt.

Key words: Antiplasmodial, acute toxicity, active fraction, *Garcinia parvifolia* Miq.

INTRODUCTION

Malaria is one of the most important parasitic diseases of subtropical and tropical countries. Malaria is responsible globally for 300-500 million cases of clinical diseases and it presents a public health problem for 2.4 billion people, representing over 40% of the world's population in over 90 countries. Estimates of malaria mortality vary from 1.5-2.7 million deaths worldwide per year with fatality rate being extremely high among young children below 5 years old and pregnant women (Hoffman *et al.*, 2002; Phillips, 2001). In addition, since resistance of *Plasmodium falciparum* to currently used antimalarial such as chloroquine is spreading rapidly, the need to develop new and effective antimalarial agents is urgent. Plant-derived natural products are expected to play a seminal role in this regard, especially in view of the success with two important antimalarial agent quinine and artemisinin, both of which are derived from plants and are used in clinic.

In the endemic area in Indonesia where malaria prevails, medicinal plants are often used for malaria therapy such as *Brucea javanica* (L.) Merr., *Carica papaya* Linn., *Phyllanthus niruri* L., *Eurycoma longifolia* Jack., *Swietenia mahagoni* Jacq., *Azadirachta indica* Juss., *Alstoea scholaris* (L.) R. Br. and

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Garcinia sp. (Mustofa *et al.*, 2007; Murningsih *et al.*, 2005; Leaman *et al.*, 1995). Although some of *Garcinia* sp. viz. *Garcinia cowa*, *G. dulcis* and *G. viellardi* have been reported to possess antiplasmodial activity (Likhitwitayawuid *et al.*, 1998a, b; Hay *et al.*, 2004), none of these studies involved *G. parvifolia* Miq.

In our continuing search for natural compounds with antimalarial activities that could provide alternative to choloquine, we have evaluated the *G. parvifolia*, Miq. (Clusiaceae) for its *in vitro* antiplasmodial activity. In the preliminary study, the activity of three extracts of *G. parvifolia*, Miq. stem bark viz. n-hexane, ethylacetate and n-butanol extracts have been evaluated. Among these three tested extracts, the n-hexane extract exhibited the highest antiplasmodial activity against both *P. falciparum* chloroquine-resistant (FCR-3) and sensitive (D10) strains with the IC₅₀ ranging from 4.83 to 6.41 $\mu\text{g mL}^{-1}$ (Syamsudin *et al.*, 2007). In this study, we reported *in vivo* antiplasmodial activity and acute toxicity of an active fraction isolated from n-hexane extract of the *G. parvifolia* Miq. stem bark.

MATERIALS AND METHODS

Plant Material

The stem barks of *G. parvifolia* Miq. were collected in Nang Kalis village, Borneo, Indonesia, June 2005 and were identified by comparison with authentic specimens at the Herbarium Bogoriense, Research Center for Biology, Indonesian Institute of Science. A voucher specimen is kept in Department of Pharmacology, Faculty of Pharmacy, Pancasila University, Jakarta, Indonesia.

Fraction Preparation

Five hundred gram of the stem bark was air dried for 10 days at 33°C and powdered. The extract was then prepared by maceration with 1500 mL n-hexane for 24 h at room temperature. This maceration was repeated three times and then the three aliquots were pooled and concentrated by a rotary evaporator to obtain 8 g of n-hexane dry extract. The n-hexane extract was then fractionated to obtain 7.2 g of methanol soluble fraction. This active fraction was then refreshed at 4°C until it was analyzed. At the moment of use, the fraction was weighed and reconstituted in gum acacia solution (2%) as vehicle at the final concentration shown in the results.

In vivo Antiplasmodial Activity Tests

The *in vivo* antiplasmodial activity of the fraction was determined by the classic 4-day suppressive test, as described by Peters *et al.* (1975) against *P. berghei*, NK 65 strain infections in mice. The ANKA 65 strain of *P. berghei* was obtained from Research and Development Center for Pharmacy and Biomedicine in Jakarta. Six groups of 5 Swiss female mice with 25±2 g body weight (b.wt.) were used in this study. Each mouse was inoculated with 1×10^6 *P. berghei* infected red blood cells intraperitoneally on day 0. At 2 h after inoculation, the mice on group 1 to 4 were administered orally with 0.2 mL of fraction tested in water solution at different concentration (25 to 200 mg kg⁻¹ of b.wt. per day). The mice on group 5 as positive control and on group 6 as negative control were administered orally with 0.2 mL of chloroquine (5 mg kg⁻¹ of b.wt. per day) and vehicle, respectively. The administration of the fraction and control solutions was repeated for 3 consecutive days from day 0 of parasite inoculation. At the 1st to 5th day after parasite inoculation, blood films were taken from the tail blood and the level of parasitemia was determined on Giemsa stained smears by counting 2000 erythrocytes. Parasite growth inhibition was compared to the level of parasitemia of control group. For the 4th day suppressive method, the ED₅₀, the dose leading to 50% parasite growth inhibition compared to the negative control, was determined from % of growth inhibition versus the log dose.

Acute Toxicity Tests

The acute toxicity of the fraction was evaluated according to General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine (WHO, 2000) after single intraperitoneal injection of the fraction. Fifty Swiss mice of both sexes, weighing 20 to 25 g, were divided randomly into five groups of 10 animals each (five males and five females). The tested fraction was prepared for suitable dose levels in gum acacia solution (2%). The test groups received a single dose of fraction in various doses (25 to 200 mg kg⁻¹ of b.wt. per day). The control group was administered orally with gum acacia solution (2%). The animals were observed continuously for 1 h intermittently for 6 and 12 h and then after 1 day, 2 days, 3 days, 7 days and 14 days. The dying animal during the period of observation and those that survived up to the end of the period (14 days) were autopsied and subjected to histopathological analysis. Histopathological studies were made on four organ viz. intestines, heart, lungs and kidneys. The number of mice that died within the period of observation was noted for each group. Subsequently, the LD₅₀, which corresponded to the dose leading to 50% deaths, was calculated by probit analysis.

RESULTS

The mean percent parasitemia of the negative control mice increased drastically within 4 days after treatment, from 27.7% on the day 1 to 47.9% on the day 4 after treatment. A single oral administration of test fraction (50 to 200 mg kg⁻¹ of b.wt. per day) reduced the parasitemia in mice by more than 59% compared to that in control mice on day 4 after treatment. However, such suppression of the fraction is still inferior to that of chloroquine as positive control producing more than 97% inhibition parasitemia on day 4 after treatment. Table 1 shows the percent parasitemia in *P. berghei* infected mice after 4 days of treatment with fraction isolated from n-hexane extract of *G. parvifolia* Miq.

The fraction produced a dose dependent chemosuppressive effect although the chemosuppression was never complete. The average percentages of suppression of parasitaemia were 43.27, 49.10, 55.83 and 59.75% at the doses of 25, 50, 100 and 200 mg kg⁻¹ of b.wt. per day, respectively. Chloroquine at 5 mg kg⁻¹ of b.wt. per day produced 97.10% chemosuppression. The ED₅₀ value obtained of the fraction was 74.45 mg kg⁻¹ of b.wt. per day. Table 2 shows the parasite growth inhibition after 4 days of treatment with fraction isolated from n-hexane extract of *G. parvifolia* Miq. on *P. berghei* infected mice.

In order to evaluate the possible toxicity of the fraction of the *G. parvifolia* Miq., acute toxicity test has been conducted after oral administration of Swiss mice. The results indicated that male mice were better able to tolerate the lethal of the fraction than the male ones since there was no mortality observed in male mice. In contrast, the mortality was observed in female mice after a single dose ingestion of the fraction at 4000 and 8000 mg kg⁻¹ of b.wt. The oral administration of moderate doses of the fraction (4000 mg kg⁻¹ of b.wt.) indicated only mild Central Nervous System (CNS) stimulation.

Table 1: Percent parasitemia in *P. berghei* infected mice after 4 days of treatment with fraction isolated from n-hexane extract of *G. parvifolia* Miq.

Treatments	Dose (mg kg ⁻¹ of b.wt. per day)	No. of mice tested	% Parasitemia (mean±SD) on the following day after treatment			
			D1	D2	D3	D4
Fraction	25	5	7.74±1.02	16.22±1.29	26.93±1.40	26.84±0.93
	50	5	5.69±0.65	14.75±0.72	23.83±0.93	24.41±1.05
	100	5	4.04±1.15	13.19±2.12	23.01±1.70	21.72±1.78
	200	5	1.80±0.67	18.64±0.49	18.90±1.21	19.92±0.58
Chloroquine	5	5	0.18±0.04	1.25±0.07	2.29±0.15	1.40±0.25
Control	-	5	7.95±0.68	11.79±1.04	40.71±1.05	47.84±1.36

Table 2: *In vivo* antiparasmodial activity (ED₅₀ in mg kg⁻¹ of b.wt. per day) of fraction of *G. parvifolia* Miq. on *P. berghei* infected mice

Treatments	Dose tested (mg kg ⁻¹ of b.wt. per day)	Parasitemia (%±SEM)	Suppression (%±SEM)	Mortality (n/N) ^b
Fraction	25	26.84±0.93	43.27±1.17	0/6
Estimated ED ₅₀ : 74.45 mg kg ⁻¹ of b.wt. per day	50	24.41±1.05	49.10±1.15	0/6
	100	21.72±1.78	55.83±1.26	0/6
	200	19.92±0.58	59.75±2.10	0/6
Chloroquine	5	1.40±0.25	90.70±1.08	0/6
Control ^a	-	47.84±1.36	0.00	0/6

^a: Vehicle, ^b: Mortality is defined as n/N, where n is the number of dead mice and N is the number of living mice in each group

Table 3: The mortality rate of male and female mice after oral administration of *G. parvifolia* Miq. in various doses

Dose of fraction (mg kg ⁻¹ of b.wt.)	Male mice			Female mice		
	Dead	Living	Mortality rate	Dead	Living	Mortality rate
2000	0	5	0/5	0	5	0/5
4000	0	5	0/5	2	3	2/5
8000	0	5	0/5	2	3	2/5
Control	0	5	0/5	0	5	0/5

Table 4: Histopatological description of mice organs after oral administration of *G. parvifolia* Miq.

Organ	Doses of fractions (mg kg ⁻¹ of b.wt.)			
	Control	2000	4000	8000
Intestines	No change (normal)	No change (normal)	Flaking of villi epithel and debris of the lumen	Flaking of vili epithel and debris of the lumen
Liver	No change (normal)	No change (normal)	Bleeding of the liver cells	Bleeding of the liver cells
Kidneys	No change (normal)	No change (normal)	Bleeding of the tubulus and mild leucocytes infiltration	Bleeding of the tubulus and mild leucocytes infiltration
Heart	No change (normal)	No change (normal)	No change (normal)	No change (normal)
Lungs	No change (normal)	A mild thickening of alveolus	Thickening of alveolus	Thickening of alveolus

However, at the highest doses (8000 mg kg⁻¹ of b.wt.), the intraperitoneal oral administration caused rapid respiration, twitchy, writhing, tremor and generalized convulsion. In addition, at these highest doses the mortality rate was less than 50% or 2 of 10 male and tested female mice (Table 3) indicated that the LD₅₀ of the fraction was more than 8000 mg kg⁻¹ of b.wt.

The histopathological studies microscopically of intestines, heart, lungs and kidneys (Table 4) revealed that only mild thickening of alveolus was observed in lungs of the mice after they received 2000 mg kg⁻¹ of b.wt. doses of the fraction. However, mice that received 4000 and 8000 mg kg⁻¹ of b.wt. doses showed flaking of vili epithel and debris of lumen in intestines, bleeding of cells liver in livers, bleeding of tubulus and leucocytes infiltration in kidneys and thickening of alveolus in lungs, although there were no abnormalities observed in heart after receiving doses of the fraction.

DISCUSSION

The classic 4-day suppressive test using the *P. berghei* infected mice model has been widely used as a preliminary test for the *in vivo* antiparasmodial activity of potential antimalarial agents, as it provides a preclinical indication of any *in vivo* potential bioactivity as well as possible toxicity of the tested sample. Munoz *et al.* (1999) classified the *in vivo* antiparasmodial activity of an extract or a fraction as moderate, good and very good activity if the extract or the fraction displayed the percentage of suppression equal or greater than 50% at the dose 500, 250 and 100 mg kg⁻¹ of b.wt. per day. The

results from this study strongly indicate that the tested fraction exhibited very good antiplasmodial activity. In addition, this fraction was well tolerated in mice since there is no mortality observed after 4 days of treatment at the maximum dose of ingestion 200 mg kg^{-1} of b.wt. per day. This result supports the traditional use of the *G. parvifolia* Miq. for the treatment of malaria by traditional healers in endemic areas in Indonesia.

The genus *Garcinia*, which belongs to the family Guttiferae, is known to be rich in active constituents of prenylated xanthenes (Linuma *et al.*, 1994; Xu *et al.*, 2001; Chamahasathien *et al.*, 2003). Xanthenes have been reported to possess several biological activities such as antibacterial (Grasvenol *et al.*, 1995; Mackeen *et al.*, 2000), antitumour, antioxidant (Mackeen *et al.*, 2000) and antiplasmodial (Likhitwitayawuid *et al.*, 1998a, b). In their study, concerning antiplasmodial activity of the genus *Garcinia*, Likhitwitayawuid *et al.* (1998a) isolated five xanthenes from the bark of *G. cowa* and evaluated their antiplasmodial activity. The results showed that cowaxanthone was the most active against *P. falciparum* with an IC_{50} of $1.5 \mu\text{g mL}^{-1}$. The other xanthenes from *G. dulcis*, were also evaluated for their antiplasmodial activity. Among the five tested xanthenes, garcinia xanthone exhibited the most active with an IC_{50} of $0.96 \mu\text{g mL}^{-1}$ (Likhitwitayawuid *et al.*, 1998b). It is suggested that antiplasmodial activity of the fraction of *G. parvifolia* Miq. is due to its xanthenes constituents. Purification of the xanthenes from the active fraction and evaluation its antiplasmodial activity will be undertaken.

The LD_{50} values obtained more than 8000 mg kg^{-1} of b.wt. indicated that the fraction of *G. parvifolia* Miq. can be grouped as weak toxic substance (Homburger, 1989). This result is supported by the Therapeutic Index ($\text{TI} = \text{LD}_{50}/\text{ED}_{50}$) value of the fraction showing more than 107.5. Although the fraction did not induce the mortality up to the dose level of 2000 mg kg^{-1} of b.wt., at high doses the fraction appears to exert their toxic effects on the CNS. Increasing doses of the fraction have graded CNS effects such as twitchy, writhing, tremor, convulsion and finally death. In addition, mice that died after intraperitoneal injection of high doses of the fraction showed pathological change in the intestines, livers, kidneys and lungs.

In conclusion, these studies demonstrated the very good *in vivo* antiplasmodial activity with weak toxic effect of fraction isolated from n-hexane extract of the *G. parvifolia* Miq. stem bark. Efforts will be undertaken to continue the bioassay guided fractionation in order to isolate and identify the active compounds, as well as to understand the mechanism of action.

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