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In vitro and *in vivo* Antiplasmodial Activities of Stem Bark Extracts of *Garcinia parvifolia* Miq (Guttiferae)

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Abstract: *In vitro* and *in vivo* antiplasmodial activities of stem barks extracts of *Garcinia parvifolia* Miq, medicinal plant traditionally used to treat malaria in Indonesia, have been evaluated. The IC_{50} value of extracts was determined. Extracts of this plant (n-hexane, ethylacetate and n-buthanol extracts) were tested *in vitro* for their antiplasmodial activity on 2 strains of *Plasmodium falciparum*, FCR-3 (chloroquine-resistant strain) and 3D7 (chloroquine-sensitive strain) using a visual method. Cytotoxicity of these extracts were performed on HeLa cells *in vitro* using MTT method. The *in vivo* antiplasmodial testing used a standard 4-days test on *P. berghei* infected mice and acute toxicity testing on mice were also conducted. The IC_{50} values of the plant extracts were in the range of 4,83-40,10 μg mL^{-1}. Significant *in vitro* antiplasmodial activity and higher selective toxicity were observed by n-hexane extract (IC_{50} = 4,11 μg mL^{-1} and C/A = 48,23). The n-hexane extract was the most active *in vivo* against *P. berghei* (ED_{50} = 19,95 mg kg^{-1}BW) and least toxic in mice (LD_{50}= 1060,47 mg kBW^{-1}). These results suggest that n-hexane extract is promising extract for further investigation for new antimalarial agents.

Key words: *Plasmodium falciparum*, *Garcinia parvifolia*, traditional plant, antimalarial drug, *in vitro*, *in vivo*, *Plasmodium berghei*

INTRODUCTION

Malaria placing at risk some 41% of the world's populations has been an important health problem in sub-tropical and tropical countries. More than 250 million clinical cases resulting at least in 1-2 million deaths are reported annually (Winstanley, 2003). In the endemic area where malaria prevails, medicinal plants are often used for antipyretic therapy. However, very little scientific information is available to evaluate the efficacy of these plants. Therefore, it is important to investigate the efficacy of the antimalarial activities of these medicinal plants in order to determine their potential as sources in the development of new antimalarial drugs (Park *et al*., 2003). *Garcinia parvifolia* Miq is one of the medicinal plants traditionally used to treat malaria in Indonesia. The genus *Garcinia*, which belongs to the family *Guttiferae*, is known to be rich in prenylated xanthones (Chamahasathieh *et al*., 2003). Xanthones constituents have been reported to possess several biological activities, such as antibacterial activity (Grasvenol *et al*., 1995), cytotoxicity (Mackeen *et al*., 2000), antioxidant activity (Likhitwatayuid *et al*., 1998) and antiplasmodial activity (Likhitwatayuid *et al*., 1998; Hay *et al*., 2004). The present study is aimed, to evaluate the antiplasmodial activity of stem bark extracts of *G. parvifolia* Miq and also their toxicity.

MATERIALS AND METHODS

Plants extracts: The plants of *G. parvifolia* Miq were collected in Nang Kalis, Borneo, Indonesia, June 2005 and were identified by comparison with authentic specimens. A voucher specimen was deposited at the herbarium of Research Center for Biology, Indonesia Institute of Science. The part of the plant tested (400 g of stem bark) was extracted by maceration with 1000 mL n-hexane for 24 h at room temperature. This 24 h extraction was repeated three times and then the three aliquots were pooled and concentrated by a rotary evaporator to obtained n-hexane extract. The extract was then gradual extraction with EtOAc (3×1000 mL) and with n-BuOH (3×1000 mL) to yield n-hexane (0.88%), EtOAc (2.89%) and n-BuOH (6.89%) fractions.

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***In vtro* antiplasmodial activity assay:** The FCR-3 (chloroquine-resistant with IC_{50}> 200 ng mL^{-1}) and 3D7 (chloroquine-sensitive with IC_{50} <40 ng mL^{-1}) strains of *P. falciparum* were cultured continuosly according to Trager and Jensen (1978). The antiplasmodial activity of plants extracts was evaluated by a visual method, as eser conducted by Van Huyssen and Rieckmann (1993) with modifications. Extract testing was performed three times in triplicate in 96-well culture plates with culture mostly at ring stages at 0, 5-1% parasitemia (hematocrit 1%). One hundred microliter of parasite culture were distributed in well plates and 100 μL of culture medium containing extracts at various concentrations were added. Parasite culture and extract were then incubated for 48 h. The incubation of parasitemia was calculated by visual counting of 2000 erythrocytes on thin Giemsa stained smears. Parasite culture without extract used as control and its parasitemia was referred to as 100%. For this visual method, IC_{50} values were determined graphically (concentration v/s % inhibition) analyzed by a linier regression function (SYSTAT Sigma Plot Software).

Cytotoxicity assay: Cytotoxicity of the extracts was estimated on HeLa cells using the MTT assay reported by Mosmann (1983). Cells were cultured in the same conditions as for *P. falciparum*, except for the 5% human serum, which was replaced by 5% fetal bovine serum. For the determination of extracts *in vitro* toxicity, cells were distributed in 96-wells plates at 2×10^4 cells per well in 100 μL, then 100 μL of culture medium containing extracts at various concentrations were added. Cells culture and extract were then incubated for 48 h exactly as for *P. falciparum* contact period. In the following incubation the medium was removed and the cells were resuspended in RPMI 1640 medium, 10 μL of 5 mg mL^{-1} MTT, (3-9,4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) and then further incubated for 4 h. The reaction was stopped by adding 100 μL 0,04 HCl-isopropanolol and the OD was measured in an ELISA plate reader at λ_{max} 550 nm. OD values were directly proportional to the number of 5 cells. The OD values in the presence of extract were compared with that of control cultures without extract. For this MTT method, IC_{50} values were determined graphically (concentration v/s % of inhibition curves) analyzed by a linier regression function (SYSTAT Sigma Plot Software).

***In vivo* antimalarial activity assay:** The *in vivo* antimalarial activity of the extracts was determined by the classic 4-days suppressive test against *P. berghei*, NK 65 strain, in mice (Peters, 1980). Thirty Swiss male mice (20±2 g) were inoculated with 1×10^6 infected red blood cells intraperitoneally on day 0. At 2 h after inoculation, eight mice were injected intraperitoneally with 0,1 mL of extracts tested in water solution at different concentration (50- 400 mg kg^{-1} of body weight day^{-1}). The injection of the extracts was repeated for 3 consecutive days from day 0 of parasite injection. The control group treated with distilled water were used in all extracts testing. At the 5th day after parasite inoculum, 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 ED_{50} values were determined graphically (dose v/s % of growth inhibition) curves.

Acute toxicity assay: The acute toxicity of the extracts was determined according to General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine (2000). Fifty Swiss mice of both sexes weighing 20-25 g were divided randomly into 5 groups of 10 animal each (5 males and 5 females). Extracts tested were prepared to suitable dose levels in water solution. The test groups received a single dose intraperitoneally of the extracts at different concentration. The control group treated with distilled water used in all extracts testing. The LD_{50} was expressed as the 50% lethal dose, which corresponds to the dose leading 50% deaths, 14 days after extract administration.

RESULTS AND DISCUSSION

The *Garcinia* genus, particulary *G. dulcis* and *G. cowa* has been reported to exhibit antiplasmodial activity (Likhitwatayuid *et al*., 1998a, b) but none of the studies involved *G. parvifolia*. In order to explore the possibility of other *Garcinia* species having antiplasmodial activity, extract of *G. parvifolia* was evaluated on *P. falciparum* culture. The IC_{50} values obtained of three extracts of *G. parvifolia* stem barks on FCR-3 and 3D7 strains of *P. falciparum* with visual method are summarized in Table 1. The data are expressed as the mean±Standard Deviation (S.D) of the IC_{50} of at least three independent experiments

Table 1: *In vitro* antiplasmodial and cytotoxic activity of crude extracts from stem barks of *G. parvifolia* Miq

	Extracts					
	N-hexane		Ethlacetate		N-buthanol	
	FCR-3	3D7	FCR-3	3D7	FCR-3	3D7
IC_{50} in *Plasmodium*	6.41	4.83	40.1	50.2	18.69	15.18
IC_{50} in Hela cells	198.24		89.41		173.46	
Selectivity Index (SI)	48.23		2.22		16.74	

Concentration is in μg mL^{-1}

Table 2: *In vivo* antiplasmodial and acute toxixicy of crude extracts from stem bark of *G. parvifolia*

	Extracts		
	N-hexane	Ethlacetate	N-buthanol
ED_{50} (mg kg^{-1})	19.95	51.06	87.69
LD_{50} (mg kg^{-1})	1060.47	399.97	749.89
Therapeutic Index (TI)	53.17	7.83	8.55

on different days for each strain. The IC_{50} ranged from 4.83-40.1 μg mL^{-1} depending on kind of extract and strain tested.

The extract displayed IC_{50} values equal or less than 10 μg mL^{-1}, the antiplasmodial activity was categorized as very good, from 10-50 μg mL^{-1} the antiplasmodial activity was moderate and over 50 μg mL^{-1} the extract was considered low activity (Gessler *et al*., 2004). Among three extracts tested, n-hexane extract showed the best among the three tested extracts antiplasmodial activity against both strains with an IC_{50} values ranging from 4.83-6.41 μg mL^{-1}. On the other hand, n-buthanol extract (IC_{50} 30.2-40.1 μg mL^{-1}) and ethylacetate (IC_{50} 15.2-18.7 μg mL^{-1}) showed moderate antiplasmodial activity. The IC_{50} value of n-hexane extract was comparable to that of other species. The antiplasmodial activities of these extract were superior to those reported for various experimental antiplasmodial plants that had IC_{50} higher than 5-10 μg mL^{-1} (Likhitwatayuid *et al*., 1998a, b; Benoit *et al*., 1996). Further study including the purification of the active compounds and search for possible their mechanism of action will be focused on n-hexane extract.

The cytotoxicity study showed that the n-hexane extract also exhibited lower cytotoxicity on HeLa cells having IC_{50} at 198.24 μg mL^{-1}. The mean ratio cytotoxicity to antiplasmodial activity (C/A = IC_{50} on HeLa/IC_{50} on Plasmodium) of n-hexane extract (C/A = 48.23) was higher than that of n-buthanol extract (C/A = 16.74) and ethylacetate extract (C/A = 2.22). The previous study showed that the C/A of n-hexane was lower than the ratio obtained with ethanol extracts of *Terminalia glaucescens* (C/A = 21), *Alchornea cordifolia* (C/A = 19) (Mustofa *et al*., 2000). It indicates that n-hexane extract is more selective on *P. falciparum* than other extracts tested.

The ED_{50} values obtained of the three extracts *G. parvifolia* stem barks on *P. berghei* infected mice are summarized on Table 2. The ED_{50} ranged from 19.95 to 87.69 mg kg^{-1} day^{-1}. The *in vivo* antiplasmodial activity can be classified as moderate, good and very good activity if the extract displayed the percentage of growth inhibition equal or greater than 50% at the dose 500, 250 and 100 mg kg^{-1} day^{-1}, respectively (Munoz *et al*., 2000). Based on the classification, all three extracts tested exhibited very good antiplasmodial activities. The *in vivo* antiplasmodial activity obtained for n-hexane extract was consistent with values taken from *in vitro* antiplasmodial study. The n-hexane extract was also the most active against *P. berghei* infected mice with an ED_{50} of 19.95 mg kg^{-1} day^{-1}.

All extracts were also tested for their acute toxicities after intraperitoneal injection of Swiss mice. The LD_{50} values obtained with three extracts ranged from 399.97 to 1060.47 mg kg^{-1}. Therapeutic indices (Tis) were calculated on the basis of acute LD_{50}/ED_{50} ratio. For the n-buthanol and ethylacetate extracts, the TI achieved 8.55 and 7.83, respectively while the n-hexane achieved 53.17, this indicate that the n-hexane extract is/or was less toxic than other extract tested.

Although a number of xanthones from genus *Garcinia* have been isolated and tested for their antiplasmodial activity, none of these studies involved *G. parvifolia*. Likhitwatayuid *et al*. (1998a) isolated five xanthones from the bark of *G. cowa* and evaluated for their antiplasmodial activity. The results showed that cowaxanthone was the most active against *P. falciparum* with an IC_{50} of 1.5 μg mL^{-1}. The others xanthones from *G. dulcis*, were also evaluated for their antiplasmodial activity. Among five xanthones tested, garcinia xanthone exhibited the most active with an IC_{50} of 0. 96 μg mL^{-1} (Likhit Watayuid, 1998b). It was suggested that antiplasmodial activity of stem bark extracts of *G. parvifolia* was due to its xanthones constituents.

CONCLUSION

The present study provides evidence that n-hexane extract is active both *in vitro* against *P. falciparum* and *in vivo* against *P. berghei* infected mice. The n-hexane extract would be promising extract for further study to discover new antimalarial agents.

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