IN VITRO AND *IN VIVO* ANTIPLASMODIAL ACTIVITY AND CYTOTOXICITY OF EXTRACTS OF *PHYLLANTHUS NIRURI* L. HERBS TRADITIONALLY USED TO TREAT MALARIA IN INDONESIA

Mustofa[1], Eti Nurwening Sholikhah[1] and Subagus Wahyuono[2]

[1]Department of Pharmacology and Toxicology, Faculty of Medicine, Gadjah Mada University, Yogyakarta; [2]Department of Biology Pharmacy, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia

Abstract. In endemic areas where malaria is prevalent, medicinal plants are often used to treat malaria. This study was conducted to evaluate the *in vitro* and *in vivo* antiplasmodial activity and cytotoxicity of extracts of meniran (*Phyllanthus niruri* L.) herb traditionally used to treat malaria in Indonesia. Three extracts *viz* aqueous, methanolic and chloroformic extracts were obtained by maceration of the herbs. A radioactive method was used to evaluate the *in vitro* antiplasmodial activity of the extracts on chloroquine-resistant (FCR-3) and chloroquine-sensitive (D-10) strains of *Plasmodium falciparum*. *In vitro* antiplasmodial activity was expressed by the concentration inhibiting 50% of parasite growth (IC_{50}). Cytotoxicity was estimated on Hela cells and the Cytotoxicity Index (CI = IC_{50} on HeLa cells/IC_{50} on FCR-3 strain) was calculated to evaluate the safety of tested extracts. A standard 4-day test on *P. berghei* infected mice was used to evaluate the *in vivo* antiplasmodial activity of the extracts showing strong *in vitro* antiplasmodial activity, for both the methanolic and aqueous extracts. The *in vivo* antiplasmodial activity was expressed by the dose inhibiting 50% of parasite growth (ED_{50}). The IC_{50} values obtained for these extracts against *P. falciparum* ranged from 2.3 to 202.4 µg/ml. The methanolic extract was the most active *in vitro* extract with an IC_{50} that ranged from 2.3 to 3.9 µg/ml and a CI that ranged from 41.3 to 57.5. This was also the most *in vivo* active extract with an ED_{50} of 9.1 mg/kg/d. Further study will be conducted to isolate and purify active compounds presented in the methanolic extract.

INTRODUCTION

Malaria is one of the most important health problems in sub-tropical and tropical countries. The World Health Organization estimates that 2,300 million people, or 41% of the total world population, live in areas with malaria risk. More than 300 to 500 million clinical cases are reported annually resulting in at least 1.5 to 2.7 million deaths. Approximately 1 million deaths among children under 5 years old are attributed to malaria alone or in combination with other diseases (WHO, 1997, 1998). In Indonesia alone, 87 million people live in areas at risk for malaria and it is estimated that 6 million cases are reported annually. The National Household Health Survey of 1995 estimated that 32,000 deaths, or 2% of deaths, were caused by malaria annually. A significant increase in malaria occurred during 1997-2001 due to the monetary crisis in Indonesia. During that time, the incidence of malaria increased significantly each year, becoming 0.62 per 1,000 people with an annual

Correspondence: Mustofa, Department of Pharmacology and Toxicology, Faculty of Medicine, Gadjah Mada University, Sekip Utara, Yogyakarta 55281, Indonesia.
Tel: 062-274-511103; Fax : 062-274-511103
E-mail: mustofajogja@yahoo.com

incidence rate in Java-Bali in the region of 0.12-11.73 (Achmadi, 2003).

Choloquine is still recommended as the first line drug for the treatment of chloroquine-sensitive Plasmodium malaria in Indonesia, while sulfadoxin-pyrimethamine is used as a second line drug against chloroquine-resistant Plasmodium malaria. However, the resistance of *Plasmodium* species, especially *P. falciparum,* to chloroquine has been reported in certain provinces of Indonesia since 1970 (Dondero *et al*, 1974; Ebisawa and Fukuyama, 1975; Baird *et al*, 1996; Fryauff *et al*, 1998). The antimalarial resistance of Plasmodium has initiated numerous studies aimed at identifying new antimalarial agents.

One of the strategies in the search for new antimalarial compounds is a study of active constituents of medicinal plants. In malaria endemic areas of Indonesia, medicinal plants, such as makasar fruit (*Brucea javanica* (L.) Merr.), papaya leaves (*Carica papaya* Linn.), pasak bumi roots (*Eurycoma longifolia* Jack.), mahoni leaves (*Swietenia mahagoni* Jacq.), mimba leaves (*Azadirachta indica* Juss.), pule seeds (*Alstonea scolaris*) and meniran herb (*Phyllanthus niruri* L.) are often used to treat malaria (Anonymous, 1990; Sudarsono *et al*, 1996). However, scientific information about the antimalarial activity of these plants is very limited. It is important, therefore, to investigate the antimalarial activities of these medicinal plants in order to determine their potential as sources of new antimalarial agents.

Phyllanthus niruri, locally named meniran, is one of the medicinal plants traditionally used to treat malaria in Indonesia. *Phyllanthus niruri* has been used traditionally to treat various illnesses, including renal stones, gastrointestinal disturbances, cough, hepatitis, gonorrhea, fever and malaria. This plant was reported to possess hypoglycemic activity (Hukuri *et al*, 1988), angiotensin-converting enzyme inhibition (Ueno *et al*, 1988), lipid lowering activity (Khanna *et al,* 2002), anticancer activity

(Giridharan *et al*, 2002) and anti-HIV activity (Qian-Cutrone *et al*, 1996). However, very little scientific information is available about its activity against *P. falciparum* although this plant is extensively used to treat malaria.

In our attempt to find new natural compounds with antimalarial activity that may provide an alternative to chloroquine, we report here on the *in vitro* antiplasmodial activity and cytotoxicity of extracts of *P. niruri* herb. In this preliminary study, chloroformic, methanolic and aqueous extracts of *P. niruri* were evaluated for its antiplasmodial activity against *P. falciaprum* and cytotoxicity of these extracts was investigated on the HeLa cell line. For the most active extracts, *in vivo* antiplasmodial activity in *P. berghei* infected mice was also investigated.

MATERIALS AND METHODS

Plant extracts

The plant *P. niruri* was collected in its natural habitat in Sleman, Yogyakarta and identified by comparison with reference specimens in the Laboratory of Pharmacognocy, Faculty of Pharmacy, Gadjah Mada University (GMU), Yogyakarta. The herb was air-dried and ground to provide a fine powder. Extracts were then prepared by maceration of the powder with chloroform, methanol and distilled water, sequentially. Two hundred grams of the powder was macerated with 1,000 ml of chloroform for 24 hours. After stirring for 3 hours, the chloroform was separated by filtration and then maceration was repeated three times on the residue. The three macerates were pooled and concentrated by a rotary evaporator to obtaine a chloroformic extract. The residue left was remacerated with methanol then distilled water in the same manner as maceration was performed with chloroform. Upon evaporation under reduced pressure, chloroformic, methanolic and aqueous extracts were obtained.

Parasite strains and *in vitro* culture

The FCR-3 strain of *P. falciparum*, chloroquine-resistant with an $IC_{50} > 150$ ng/ml and D-10 chloroquine-sensitive strain with an $IC_{50} < 50$ ng/ml were cultured continuously according to Trager and Jensen (1978) with modifications described by Van Huyssen and Rieckmann (1993). The parasites were maintained *in vitro* in human erythrocytes ($O^{\pm}$), diluted to 1% hematocrit in RPMI 1640 (Sigma) supplemented with 25 mM HEPES and 30 mM $NaHCO_3$ and complemented with 5% human $O^{\pm}$ serum. Parasites cultures were incubated at 37ºC in candle jars with a daily change of medium. Parasite cultures were synchronized with 5% D-sorbitol given every 48 hours as reported by Lambros and Vanderberg (1979).

Parasite and mice strains for *in vivo* antiplasmodial assay

The Swiss mice were bred at the Department of Pharmacology and Toxicolology, Faculty of Medicine GMU, Yogyakarta. The ANKA strain of *P. berghei* was obtained from the Department of Parasitology, Faculty of Medicine GMU, Yogyakarta, Indonesia.

Assay for *in vitro* antiplasmodial activity

The antiplasmodial activity of plant extracts on the two strains of *P. falciparum* was evaluated according to the semiautomatic microdilution technique of Desjardins *et al* (1979). Each extract was tested in triplicate in three independent experiments. Testing was performed in 96-well culture plates with culture mostly at the ring stage at 0.5-1% parasitemia (hematocrit, 1%). One hundred µl of parasite culture was distributed into each well plate and 100 µl of culture medium containing extracts at various concentrations was added. The parasite cultures and extracts were then incubated over two time intervals, 24 and 72 hours, before adding [³H]-hypoxanthine (0.25 µCi per well). Following incubation, the parasites were harvested onto fiber glass filters using an automatic cell har-

vester. Incorporation of [³H]-hypoxanthine was determined by a liquid scintillation counter. Parasite growth was estimated by [³H]-hypoxanthine incorporation and compared to controls. The control parasite culture freed from extract was referred to as 100% growth. The IC_{50} values, concentration required to inhibit parasite growth by 50%, were determined by linear interpolation from the growth inhibition curves generated for each extract-parasite combination.

Assay for cytotoxicity

Cytotoxic properties of the extracts was estimated on Hela cells. Cells were cultured under the same conditions as for *P. falciparum*, except the 5% human serum was replaced by 5% fetal bovine serum. To determine the toxicity of the *in vitro* extracts, cells were distributed into 96-well plates at 2×10^4 cells per well in 100 µl, then 100 µl of culture medium containing extract at various concentrations was added. The cell culture and extract were then incubated for 24 and 72 hours the same as the *P. falciparum* contact period. Cell growth was estimated by [³H]-hypoxanthine incorporation and compared to control cultures without extract. The IC_{50} values were determined by linear interpolation from the growth inhibition curves.

Assay for *in vivo* antiplasmodial activity

The *in vivo* antiplasmodial activity was evaluated by the classical 4-day suppressive test (Peters *et al*, 1975). Male Swiss mice (20-25 g) were inoculated intraperitoneally with 10^7 *P. berghei*-infected erythrocytes, resuspended in RPMI 1640 medium on day 0 to a volume of 0.2 ml. Fifty-four mice were divided into 9 groups. The first 4 groups received 25, 50, 100, and 200 mg/kg BW/d of aqueous extract, respectively. The second 4 groups received 12.5, 25, 50, and 100 mg/kg BW/d of methanolic extract, respectively. The other group without compound was the control. Each dose of extract was administered once

daily for 4 consecutive days, beginning on the day of infection, starting two hours after inoculation until day 3. The level of parasitemia was determined the day following the last treatment. The ED_{50}, which is the dose leading to 50% parasite growth inhibition compared to growth in the control, was evaluated from a plot of activity (expressed as a percentage of the activity in the control) versus the log dose. These experiments were conducted in accordance with the Experimental Animal Guidelines of Laboratory Method on Toxicology GMU (Ngatidjan, 1991).

Statistical analysis

Comparison of antiplasmodial activity (IC_{50}s) among three extracts tested after 24 hours and 72 hours of incubation on the two *P. falciparum* strains was performed using the Kruskal Wallis test, followed by the Mann-Whitney *U* test.

RESULTS

The IC_{50} values obtained with extracts of various plants against FCR-3 and D-10 strains using the radioactive method are summarized in Table 1. Data are expressed as the median and range. The IC_{50} ranged from 2.3 to 200.4

μg/ml. Among the three extracts tested in the present study, the methanolic extract showed highest antiplasmodial activity in comparison with other extracts tested with IC_{50} values ranging from 2.3 to 3.9 μg/ml. The IC_{50} values were significantly different ($p < 0.05$) compared to the chloroformic extract (IC_{50} ranged from 132.6 to 200.4 μg/ml). However, no significant differences were observed ($p > 0.05$) when they were compared with aqueous extract (IC_{50} ranged from 2.9 to 4.1 μg/ml).

When the two incubation times are compared, two kinds of results are observed. For the aqueous and methanolic extracts, no cumulative effect was observed. The IC_{50} values of these two extracts 24 hours after contact between the parasites and extracts were not significantly different ($p > 0.05$) compared to 72 hours after contact. In contrast, on the tested chloroformic extract, the IC_{50} values 72 hours after contact revealed a cumulative effect expressed by the lower 72-hour IC_{50} values than the 24-hour values ($p < 0.05$).

In regard to chloroquine sensitivity of the *P. falciparum* strain with the aqueous and IC_{50} for the chloroquine-resistant (FCR-3 strain) and -sensitive (D-10) strains were similar ($p > 0.05$). Thus it appears the potential antima-

Table 1

In vitro antiplasmodial activity (IC_{50} in μg/ml)[a] against *P. falciparum* culture (FCR-3 and D-10 strains) and cytotoxicity (IC_{50} μg/ml) against Hela cell line for extracts of meniran (*P. niruri*) herb at 24-hour and 72-hour incubation.

| | Strain/cell | | | | | | | |
| | FCR-3 strain | | D-10 strain | | HeLa cell | | CI[b] | |
Extract	24-hour	72-hour	24-hour	72-hour	24-hour	72-hour	24-hour	72-hour
Aqueous	4.1	3.3	3.4	2.9	435.2	99.2	106.8	31
	(3.6-4.5)	(2.7-3.9)	(2.8-3.9)	(2.5-3.3)	(392.8-477.6)	(90.5-107.9)	(95.8-116.5)	(28.3-33.7)
Methanolic	4	2.3	3.2	2.5	167.7	134.1	41.3	58.3
	(2.7-5.2)	(1.8-2.7)	(2.2-3.4)	(1.9-3.1)	(148.0-174.2)	(122.2-146.0)	(37.9-44.7)	(53.1-63.5)
Chloroformic	202.3	166.5	161.2	132.5	111	262.9	0.5	1.6
	(192.1-212.5)	(155.8-177.1)	(147.6-174.8)	(120.0-145.0)	(98.8-123.2)	(243.5-282.3)	(0.5-0.6)	(1.5-1.7)

[a]median (range); [b]CI, Cytotoxicity Index: IC_{50} against Hela cells/IC_{50} against FCR-3 strain.

Table 2

In vivo antiplasmodial activity (ED_{50} in mg/kg/d) of extracts for meniran (*P. niruri*) herb against *P. berghei* infected mice.

Extract	Dose tested (mg/kg/d)	Parasitemia (%)	Growth inhibition (%)	Mortality (n/N)[b]
Aqueous	50	42 ± 7	77.2 ± 11.7	0/6
Estimated ED_{50} : 20.0 mg/kg BW/d	100	42 ± 6	78.2 ± 11.5	0/6
	200	43 ± 5	79.0 ± 12.6	0/6
	400	51 ± 3	95.1 ± 6.0	2/6
Methanolic	12.5	41 ± 6	75.2 ± 13.8	0/6
Estimated ED_{50} : 9.1 mg/kg BW/d	25	34 ± 11	62.9 ± 19.6	0/6
	50	52 ± 8	95.8 ± 14.6	1/6
	100	51 ± 4	93.9 ± 7.6	2/6
Control[a]	-	54 ± 15	100	0/6

[a]RPMI; [b]Mortality is defined as n/N, where n is the number of dead mice and N is the number of mice in each group

larial compounds contained in these extracts may have interfered with *P. falciparum* growth by a different mechanism than that of chloroquine. However, for the chloroformic extract, the IC_{50} was higher for the chloroquine-resistant strain than for chloroquine-sensitive strain. The extracts of *P. niruri* also showed different levels of cytotoxicity (Table 1). The IC_{50} values of the various extracts of this plant on HeLa cells ranged from 111.0 to > 200 µg/ml depending on the kind of extract and incubation time. The methanolic extract exhibiting the highest antiplasmodial activity was also more toxic (median IC_{50}, 167.7 µg/ml after 24 hours and 134.1 µg/ml after 72 hours) than the aqueous extract after 24-hour incubation (IC_{50}, 435.7 µg/ml) but it was less toxic after 72-hour incubation (IC_{50}, 99.2 µg/ml) (p < 0.05). The mean Cytotoxicity Index (CI = IC_{50} against HeLa/IC_{50} against the FCR-3 strain) of methanolic extract after 24-hour incubation (median CI, 41.3) was lower than that of the aqueous extract after 24-hour incubation (CI, 106.8) but was higher after 72-hour incubation (CI, 31.0). Inversely, in comparison with chloroformic extract (IC_{50}, 111.0 µg/ml after 24 hours and 262.9 µg/ml after 72 hours), the

methanolic extract was less toxic by 24-hour incubation but more toxic by 72-hour incubation (p < 0.05). However, the mean CI of the methanolic extract was higher than the chloroformic extract, they are measured both at 24-hour (CI, 0.5) and 72-hour incubation (CI, 1.6) (p < 0.05).

Of the three extracts tested in the *in vitro* study, two extracts (methanolic and aqueous) displayed strong antiplasmodial activity. These two extracts were then evaluated for their *in vivo* antiplasmodial activity on *P. berghei* infected mice. The results of this study show the methanolic extract was more active *in vivo* (ED_{50} = 9.1 mg/kg BW/d) than the aqueous extract (ED_{50} = 20.0 mg/kg BW/d) as shown in Table 2. However, this methanolic extract was not well tolerated in mice compared with the aqueous extract, since loss was observed by 4 days of treatment at 50 mg/kg BW/d.

DISCUSSION

The IC_{50} values obtained for the aqueous and methanolic extracts of this plant are interesting when they were compared with the results from other plant extracts reported in

the literature. *Artemisia annua* (the source of artemisinin) and *Azadirachta indica* (Neem) have an IC_{50} of 3.9 μg/ml and 2.3 to 12.5 μg/ml, respectively (O'Neill *et al*, 1985; Benoit-Vical *et al*, 1996). Both plants are considered as reference medicinal plants by numerous authors due to their wide use in the treatment of malaria (Benoit-Vical *et al*, 1996).

Gessler *et al* (1994) recommended if the extract displayed an IC_{50} less than 10 μg/ml, antiplasmodial acitivity was very good, from 10 to 50 μg/ml, the antiplasmodial acitivity was moderate and over 50 μg/ml the extract was considered to have low activity. Based on this recommendation, the aqueous and methanolic extracts of *P. niruri* with IC_{50} values from 2.9 to 4.1 μg/ml and from 2.3 to 4.0 μg/ml, respectively, can be concluded as having very good antiplasmodial activity. These results suggest more active compounds may be extracted with a polar solvent and should be evaluated for *in vivo* antiplasmodial activity. For this purpose, a 4-day suppressive test was performed on male Swiss mice using *P. berghei*.

The results summarized in Table 2 show the methanolic and aqueous extracts were active *in vivo* with ED_{50} of 9.1 and 20.0 mg/kg BW/d, respectively. The *in vivo* antiplasmodial activity can be classified as moderate, good and very good activity if the extract displays a percent growth inhibition equal to or greater than 50% at a dose of 500, 250 and 100 mg/kg BW/d, respectively (Munoz *et al,* 2000). Base on this classification, the two tested extracts exhibited very good antiplasmodial activity. These results are consistent with the values obtained from the *in vitro* antiplasmodial study.

ACKNOWLEDGEMENTS

REFERENCES

Achmadi UF. Malaria and poverty in Indonesia : 1997-2001 situational review of malaria and poverty in Indonesia. Jakarta: Center for Data and Information, Ministry of Health, Republic of Indonesia, 2003.

Anonymous. Traditional medicine of Kalimantan people. Jakarta: Department of Education and Culture, Republic of Indonesia, 1990.

Baird JK, Sustriayu Nalim MF, Basri H, *et al*. Survey of resistance to chloroquine by *Plasmodium vivax* in Indonesia. *Trans R Soc Trop Med Hyg* 1996; 90: 409-11.

Benoit-Vical F, Valentin A, Pélissier Y, *et al*. *In vitro* antimalarial activity of vegetal extracts used in West African traditional medicine. *Am J Trop Med Hyg* 1996; 54: 67-71.

Desjardins RE, Canfield CJ, Haynes JD, Chulay JD. Quantitative assessment of antimalarial activity *in vitro* by a semiautomated microdilution technique. *Antimicrob Agents Chemother* 1979; 16: 710-8.

Dondero TJ Jr, Kosin E, Parsons RE, Tann G, Lumanuaw FH. Preliminary survey for chloroquine resistant malaria parts of Nort Sumatra, Indonesia. *Southeast Asian J Trop Med Public Health* 1974; 5: 574-8.

Ebisawa I, Fukuyama T. Chloroquine resistance of *Plasmodium falciparum* in West Irian and East Kalimantan. *Ann Trop Med Parasitol* 1975; 69: 275-82.

Fryauff DJ, Soekartono, Tuti S, *et al*. Survey resistant in vivo to chloroquine of *Plasmodium falciparum* and *P. vivax* in North Sulawesi, Indonesia. *Trans R Soc Trop Med Hyg* 1998; 92: 82-3.

Gessler MC, Nkunya MHN, Mwasumbi LB, Heinrich M, Tonner M. Screening Tanzanian medical plants for antimalarial activity. *Acta Trop* 1994; 55: 65-7.

Giridharan P, Sumasundaram ST, Perumal K, *et al.* Novel substituted methylenedioxy lignan suppresses proliferation of cancer cells by inhibiting and activation of c-myc and caspases leading to apoptosis. *Br J Cancer* 2002; 87:

98-105.

Hukeri VI, Kalyani GA, Kakrani HK. Hypoglycemic activity of flavonoids of *Phyllanthus frametus* in rats. *Fitoterapia* 1988; 59: 68-70.

Khanna A, Rizvi F, Chander R. Lipid lowering activity of *Phyllanthus niruri* in hyperlipemic rats. *J Ethnopharmacol* 2002; 82: 19.

Lambros C, Vanderberg JP. Synchronisation of *Plasmodium falciparum* erythrocytic stages in culture. *J Parasitol* 1979; 64: 418-20.

Munoz V, Souvain M, Bourdy G, *et al.* The search for natural bioactive compounds through a multidisciplinary approach in Bolivia: Part II. Antimalarial activity of some plants used by Mosetena Indians. *J Ethnopharmacol* 2000; 139-55.

Ngatidjan. Laboratory method for toxicology. Yogyakarta: Gadjah Mada University, 1991.

O'Neill MJ, Bray DH, Boardman P, Phillipson JD, Warshut DC. Plant as sources of antimalarial drugs part. 1. *In vitro* test method for the evaluation of crude extracts from plants. *Planta Med* 1985; 52: 394-8.

Peters WJ, Portus JH. Robinson BL. The chemotherapy of rodent malaria XXII. The value of drug-resistant strains of *P. berghei* in screening for blood schizontisidal activity. *Ann Trop Med Parasitol* 1975; 69: 155-71.

Qian-Cutrone J, Huang S, Trimble J, *et al.* Niruriside, a new HIV RE/RRE binding inhibitors from *Phyllanthus niruri. J Nat Prod* 1996; 59: 196-9.

Sudarsono, Agus P, Didik G, Subagus W, Imono AD, Ngatidjan. Medicinal plant. Yogyakarta: Research Center for Traditional Medicine, Gadjah Mada University, 1996.

Trager W, Jensen JB. Cultivation of malarial parasites. *Nature* 1978; 273: 621-2.

Ueno H, Horie S, Nishi Y, *et al.* Chemical and pharmaceutical studies on medical plants in Paraguay. Geraniin, an angiotensin-converting enzyme inhibitor from Paraparai Mi *Phyllanthus niruri*. *J Nat Prod* 1988; 51: 357-9.

Van Huysenn W, Rieckmann KH. Disposable environment chamber for assessing the drug susceptibility of malaria parasites. *Trop Med Parasitol* 1993: 44: 329-30.

WHO. The situation of malaria in the world in 1994. *Wkly Epidemiol Rec* 1997; 72: 269-92.

WHO. Roll Back Malaria. A global partnership. Geneva: World Health Organization, 1998.