The antimalarial activity of the extract of the neem leaves (*Azadirachta indica*, A. Juss) on *Plasmodium falciparum* in vitro

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Abstract. Malaria is a life-threatening disease due to the development of resistance by the most lethal causative parasitic species mainly *Plasmodium falciparum*. One of a great challenges in malaria controlling program is *P.falciparum* that has been resistant to the most commonly available antimalarials. New drugs with specific structures and mode of actions are urgently required to treat sensitive and drug-resistant strains of malaria parasites. This study was designed to know the antimalarial activity of the extract of the neem leaves (*Azadirachta indica* A.Juss) on the growth stages of *P. falciparum* FCR-3. The experimental laboratoric study used "post-test only with control design". RPMI 1640 used as culture medium for cultivation of *P. falciparum*. Treated drug was the extract of neem leaves dissolved in dimethylsulfooxide and prepared into 7 levels concentration (3,125; 6,25; 12,5; 25; 50; 100 and 200 ug/mL). Negative control was culture medium with the malarial parasites. After cultured, synchronized, micromalarial culture were divided into control and treated groups then incubated in CO_2 Candle Jar at 37^0 C for 72 hour. Each 8 hour the percentage of parasitemia were measured for observing the activity of the extract on the growth stages of *P. falciparum*. After incubation, supernatant fluid was removed without disturbing the erythrocyte layer. Parasitemia was calculated by made the thin blood smear from the erythrocyte layer and stained with 10% Giemsa for 30 minutes. The antimalarial activity of the extract was calculated by counted the fifty percent of growth inhibition 50 (IC_{50}) using probit analysis. The result showed that the neem leaves extract can inhibit the growth of *P. falciparum FCR-3* on mature schizont stage and the fifty percent inhibitory concentration (IC_{50}) of the extract was 3,86 µg/ml after 32 hour incubating. The result indicated that the extract has an antimalarial activity on *P. falciparum* FCR-3 in vitro.

Key words: *Azadirachta indica A.Juss,* antimalarial*, Plasmodium falciparum*

Introduction. The spread of malaria as the major global health problem, the progressive drug resistance and limited number of effective drug available have underlined the importance of new antimalarial drugs discovery. According to the World Health Organization (WHO), in 2008 there were approximately 247 million cases of malaria, causing nearly one million deaths, mostly among young children in Africa. Although malaria is preventable and curable, it is estimated that in Africa, a child dies every 45 seconds caused by the disease. Malaria represents the most serious infection that cause the increasing morbidity and mortality in the disease therefore the solution have to be serious to manage it.

Indonesia health departement (2010) reported in 2008 there were 544.470 cases of clinical malaria in Indonesia, 1.100.000 cases in 2009 and in 2010 increased to be 1.800.000 cases. In any area of Indonesia, the prevalence of malaria in pregnant women is 18%, so the prediction of babies were born with low body weight risk is two fold greater than pregnant women without malaria.The disease affects humans health especially in the pregnant women, geriatric with complicated diseases, breast feeding mothers, babies and children. In social economic perspective malarial affects the productivity, income of individual and public expenditure.

Malaria infection is transmitted via the bite of an infected female mosquitoes of genus *Anopheles*. Mosquitoes have been infected through a previous blood meal taken on infected human. Infection mosquito caused by the protozoan parasites of the genus *Plasmodium* and the species of them can produce malaria in human. Four species of malarial parasites are *P. falciparum, P. vivax, P. ovale and P. malariae.* Among them *P. falciparum* is the most widespread and the dangerous parasite, it can causes severe anemia, kidney failure, hypoglycaemia and brain damage especially in among children and babies (Sutanto, *et al*., 2008). The great problems in malaria control programmes are parasite resistance to the most commercially available antimalarial, and vector mosquitoes resistance to insectisides.

The increasing global spread of malarial parasites that resistant to the most available and affordable antimalarial drugs is a great challenge which requires innovative strategies to combat. This situation has stimulated research for new drugs active against multidrug-resistant *P. falciparum* parasites. Intensive efforts have been underway for decades to produce vaccines which can enhance levels of protective individual immunity. However chemoprophylaxis and chemotherapy still play the central role in combating malaria infections. Discovery a new antimalarial drugs, preferably with radically different structures and mode of action are urgently required to treat sensitive and resistant strains of malaria to decrease the morbidity and mortality rate.

Traditional medicines are potential rich source of new drugs against malaria and the investigation of the chemical components of traditional medicinal plants could lead to the development of new efficacious antimalarial drugs. In addition the chemical component could be modificated to get potential and safe antimalarial drugs. Pharmacologycal and chemistry approaches are needed to use these chemical components as templates for designing new derivates with improved properties. Searching for continueous antimalarial drugs from plants source must be done to combat the malarial problems in the future.

In this regard, we show the neem leaves (*Azadirachta indica* A. Juss), in the Meliaceae family, as a unique plant species. It has been used for treatment of fever, malaria, as antiinflammatory, antidiabetic, analgetic, antihypertension and hepatoprotector (Lewis and Lewis, 1977). The other references reported that the neem leaves can be used as antipyretic, antiarthritic, antimalarial, antiseptic, insecticide, antifungal, antioxidant, anthelmentic and anticancer (Perry, 1980; Sofowara, 1982; Ntalli *et al*., 2010; Connoly and Hill, 2003). Many biologically active compounds can be extracted from neem leaves including triterpenoids, phenolic compounds, carotenoids, steroids and ketones. The extract of the neem leaves contains gedunin, nimbin, nimbolide and many more limonoids (Roy and Saraf, 2006).

It has been identified for their in vitro antimalarial activity of gedunin, dihydrogedunin and nimbolide were found to be most effective against *P. falciparum* sensitive strains. The study of antimalarial activity of the oil neem fruits which was given orally on mice showed that the effective dose 50 (ED_{50}) which could inhibited the growth of *P. berghei* was 200 mg/kg of Body Weight (Mulyaningsih and Sudarsono, 2001). Nimbolide is the most active compound which inhibited the *P. falciparum* K1(IC_{50} = 0,95 ng/ml), gedunin (IC_{50} = 720 ng/mL, *P. falciparum* D6) and its dihydro derivates were also found to be active in vitro (IC_{50} = 2630 ng/mL) (Rochanakij et al, 1985, Mackinon et al, 1997). The aqueous extract (IC_{50} = 115 µg/ml) and ethanolic extract (IC50 = 5 µg/ml) (Rochanakij et al., 1985).

Neem is a traditional herb that has an exotic structure, mode of action and efficacious against chloroquine sensitive parasites. Neem leaves extract showing the highest activity against the chloroquine sensitive parasite, but there has not been any reports on its effect in chloroquin resistant parasite. The aim of this study was to find the antimalarial activity of the neem leaves extract on the growth stages of *P. falciparum* chloroquine resistant in vitro.

This study acts to component which work in the blood stages of the malarial infection. In the future the components with acceptable criteria would be continued for in vivo efficacy test and after toxicity studies successful antimalarial candidate could be continued to clinical studies.

Materials and Methods

Materials

Fresh neem leaves (*A.indica* A.Juss) were collected in Darussalam region Banda Aceh and were identified. *P.falciparum* FCR-3 was obtained from Laboratory of Parasitology Medical Faculty Gadjah Mada University. Red blood cells and human serum (O^+), RPMI-1640 (Sigma), giemsa 10% (Merck), sorbitol 5% (Merck), ethanol 96% (Merck), aqua bidestillata steril (KF), absolute methanol (Merck), gentamisin (Merck), dimetilsulfoxide (Merck), $NaHCO_3$ (Merck), NaCl (Merck), immersion oil (Merck).

Instrument

Incubator (37^0C), candle Jar (desiccator with CO_2), culture flask, centrifuge, micropipet, microplate, blue and yellow tip, drying rack for blue and yellow tip, conical tube, eppendorf tube, object glass and microscope.

The preparation of neem leaves extract

The fresh neem leaves were cut into small pieces and extracted three times for 24 hour with ethanol 80% (1 : 5). The solvent is replaced every 24 hour and the ethanolic solutions were evaporated at 40^0C under reduced pressure (Santos *et al*., 1978). The thick extracts subjected to the antimalarial in vitro test.

Cultivation of P. falciparum and in vitro antimalarial activity test

The steps of in vitro antimalarial activity covered: sterilization of all instruments and substances, preparation of normal erythrocite and human serum, medium culture for cultivation, thawing of *P. falciparum,* culture synchronized with sorbitol 5% and microculture for antimalarial activity test.

Plasmodium falciparum FCR-3 (IC_{50} for chloroquin = 0,050 mM) were maintained in continuous culture in human red blood cells ($O^{+)}$ added 1% hematocrit in RPMI 1640. Medium supplemented with 25 nM HEPES, 30nM NaHCO3 and 10% Human O^+ serum (Trager & Jensen, 1976). The ethanolic extracts were dissolved in dimethylsulfooxide (DMSO) to make a stock solution (1mg/ml) and diluted in medium (RPMI 1640) to final concentration. Before testing the extract in medium, sterilized by filtration (pore size = 0,22um). Antimalarial activity were performed in 96 well microculture plates. Briefly, 100 µL treated drugs were added to each well in microculture plate and 100 µL of paratized culture (haemotocrit 1%, 0,5% parasitaemia) were added to each well in microculture plate and incubated in CO_2 candle jar for 72 hour at 37^0C (Trager & Jensen, 1976). Every 8 hour the percentage of parasitemia were measured for observing the activity of the extract on the growth stages of *P. falciparum* FCR-3. After incubation contents of the wells were harvested and stained. The growth stages of parasites was monitor by making a thin blood smear, fixed with methanol and stained with Giemsa 10% for 30 minutes. All test were performed in triplicate.

The parasitemia was examined under microscope with magnification 1000 by adding immersion oil. Parasitemia was calculated by formula:

$$\% \text{ parasitemia} = \frac{\text{Number of infected red blood cells}}{\text{Total red blood cells}} \times 100$$

The percentage of the growth inhibition of the parasites was calculated by formula:

$$\% \text{ inhibition} = \frac{\text{Parasitemia in control} - \text{parasitemia in treated group}}{\text{Parasitemia in control}} \times 100\%$$

The percentage of the growth stages inhibition of the parasites at each concentration was determined by the mean of at least three IC_{50} parasites viability. The fifty percent of growth stages inhibition was calculated using probit analysis.

Results and Discussion

The result of in vitro antimalarial activity of the extract of the neem leaves (*A.indica* A.Juss) on *P.falciparum* FCR-3 were obtained. The percentages of parasitemia and the percentages of growth stages inhibitory of *P.falciaparum calculated* in every 8 hour interval to 72 hour shown in the Tabel 1, 2 and Figure 1, 2.

Tabel 1. The percentage of parasitemia at different concentrations of neem leaves extract after cultivation in 8 hour interval to 72 hour

Concentration (ug/mL)	% Parasitemia								
	Interval of cultivation (hour)								
	8	16	24	32	40	48	56	64	72
0,000	8,166	11,04	12,67	16,22	15,87	16,36	15,660	15,920	16,38
3,125	6,128	8,011	8,040	9,510	11,53	11,20	14,790	13,61	11,21
6,250	5,215	7,329	6,670	6,260	8,960	11,03	13,690	12,390	10.42
12,500	5,039	7,021	5,590	5,770	7,120	10,71	13,020	12,170	9,090
25,000	3,541	5,626	5,070	5,150	6,210	9,570	12,210	11,580	7,870
50,000	2,566	5,300	4,670	4,670	5,080	6,770	10,500	10,220	6,040
100,000	2,229	4,248	4,270	3,400	4,040	5,520	9,150	8,160	5,570
200,000	1,701	3,027	3,690	2,610	3,350	3,790	4,090	5,010	3,920

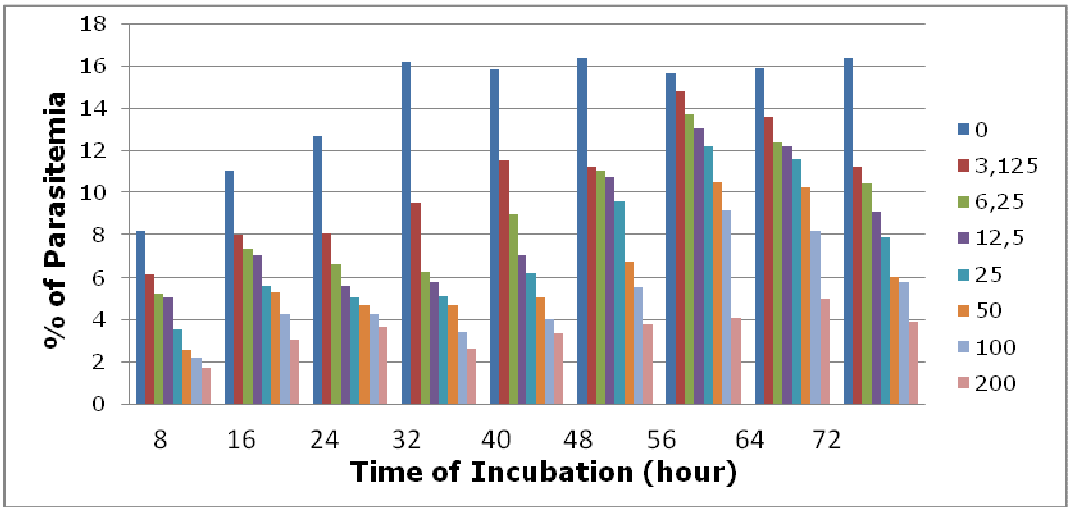

Figure 1. The percentage of parasitemia at different concentrations of neem leaves extract after cultivation in 8 hour interval to 72 hour

Tabel 2. The percentage of growth inhibition of *P. falciparum* at different concentrations of neem leaves extract after cultivation in 8 hour inteval to 72 hour.

Concentration (ug/mL)	% Growth Inhibition of P.falciparum Interval of cultivation (hour)								
	8	16	24	32	40	48	56	64	72
0,000	-	-	-	-	-	-	-	-	-
3,125	24,96	27,44	36,54	41,38	27,35	31,54	5,56	14,51	31,56
6,250	36,14	33,61	47,37	61,42	43,57	32,58	12,58	22,17	36,39
12,500	38,29	36,40	55,88	64,40	55,77	34,53	16,86	23,56	44,50
25,000	56,64	49,04	59,94	68,23	60,86	41,48	22,03	27,26	51,97
50,000	68,58	51,99	63,14	71,19	68,02	58,61	32,95	35,80	63,16
100,000	72,70	61,52	66,28	79,02	74,56	66,28	41,55	48,72	64,78
200,000	79,17	72,58	70,86	89,83	78,88	76,81	73,84	68,56	76,06

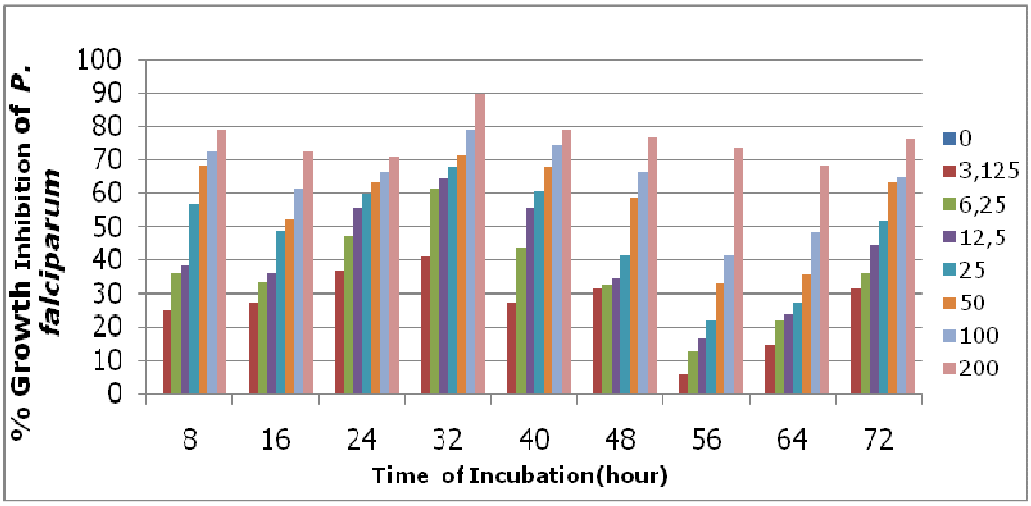

Figure 2. The percentage of growth inhibition of *P. falciparum* at different concentrationsof neem leaves extract after cultivation in 8 hour inteval to 72 hour.

Antimalarial activity was determined by fifty percent of growth stages inhibitory of *P. falciparum* (IC_{50}). In this investigation we found each 8 hour interval to 72 hour are presented in Table 3 and Figure 3 which was calculated using probit analysis.

Tabel 3. The IC_{50} value of neem leaves extract on the growth of *P. falciparum* after cultivation in 8 hour inteval to 72 hour.

Observation (hour)	**IC_{50} (µg/mL)**
8	18,73
16	31, 72
24	9,77
32	3,86
40	12,08
48	27,53
56	99,30
64	92,93
72	19,42

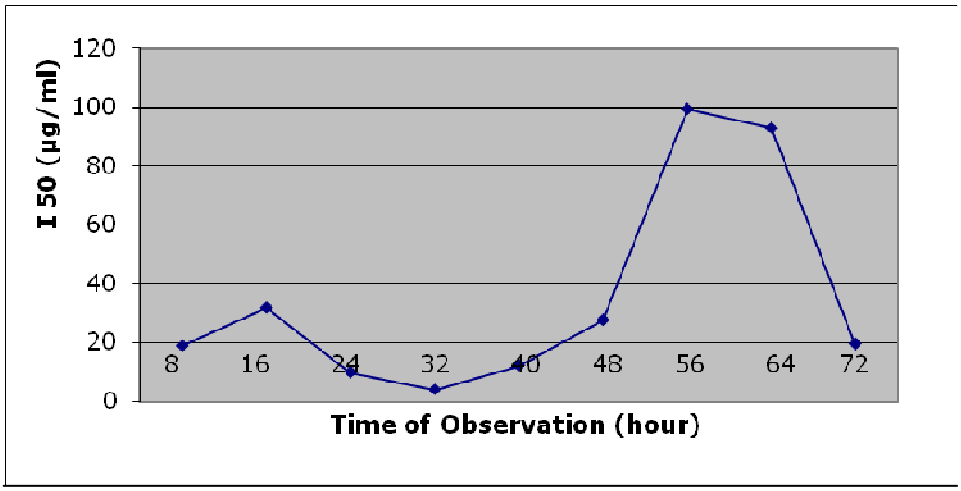

Figure 3. The IC_{50} value of neem leaves extract on the growth of *P. falciparum* after cultivation in 8 hour inteval to 72 hour.

Discussion

Determining of antimalarial activity based on the fifty percent of growth inhibitory (IC_{50}) of treated extract. The IC_{50} is the concentration of crude treated drug which inhibite the growth of *Plasmodium* until fifty percent. In vitro antimalarial activity could be categorized as follow as the value of $IC_{50.}$ In this investigation the most interesting in vitro antimalarial activity was found in the concentration inhibition 50 (IC_{50} = 3,86 µg/mL) of the ethanolic extract of neem leaves on mature schizont stage, but Alshawsh *et al*, (2007) was found the concentration inhibition 50 (IC_{50} = 2, µg/mL) of the aqueous of the neem leaves extract.

In this investigation we found the most potent in vitro antimalarial activity IC_{50} = 3,86 µg/ml $\leq$ 10 µg/ml at 32 hour. Miscrospically, the fifty percent of growth *P. falciparum* inhibition on mature schizont in value 81,66% and on young tropozoit 18,34% after 32 hour cultivation.

The IC_{50} which found in these investigation were different to the previous reseacher (IC_{50} of ethanolic extract of neem leaves = 5 µg/ml). The condition may be caused by the coumpound in the leaves (Munoz *et al*., 2000). As we known the in vitro antimalarial activity was not described as the in vivo antimalarial activity, such a thing resulted in the differences of its metabolic agent.

Conclusions

The result showed that neem leaves extract has in vitro antimalarial activity on *P. falciparum* FCR-3 with IC_{50} 3, 86 µg/ml. The IC_{50} categorized as the most potential antimalarial activity after 32 hour cultivation. The percentage of growth inhibitory is 81,66% on mature schizont and on young tropozoit 18,34% after 32 hour cultivation.

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