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Research Article

ANTIMALARIAL ACTIVITY OF HEXANE EXTRACT OF NEEM LEAVES (*AZADIRACHTA INDICA* A.JUSS) ON MICE (*MUS MUSCULUS*)

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ABSTRACT
This investigation was focussed on in vivo antimalarial activity and the compounds of hexane extract of neem leaves (*Azadirachta indica*, A Juss). This investigation should be given for strong science based in using neem leaves as antimalarial drugs. The extract was prepared by maceration in hexane at room temperature (25^0C) over the period of 72 hours. By using completely randomized design, 55 mice have been divided in 5 groups (n = 11 each group) for treated and control group. The mice were infected by *P. berghei* on day 0 and on day 1 until day 4 they gave treated drug with doses 0; 100; 200; 400; 800 mg/kg BW/day orally. On day 5 were made thin blood smear by fixation with methanol and stained with giemsa 10% in 30 minutes. The inhibitory of *P. berghei* growth by the extract were counted and the ED_{50} obtained by using regression probit analysis. The result shown the inhibitory of *P. berghei* growth by the hexane extract of neem leaves 78, 35% at level dose 800 mg/kg BW/day and the ED_{50} was 355,45 mg/kg BW/day. The compunds in hexane extract of neem leaves are triterpenoid, steroid and fenolic compounds.
KEY WORDS: Antimalarial activity, *Azadirachta indica* A.Juss, ED_{50}

INTRODUCTION

Malaria is the most life-threatening disease and easyly widespread, causing 350−500 million cases and approximately two million deaths annually[1,2]. The disease is caused by four Plasmodium species (i.e., *P. falciparum, P.vivax, P. ovale, and P. malariae)* that are transmitted to humans during the bite of the female anopheles mosquito[3]. The development of drug resistance to affordable antimalarial drugs has contributed to global increase in number of deaths caused of malaria. Therefore discovery a new antimalarial

drugs, with unique chemical structures and mechanism of action are urgently required to treat sensitive and resistant strains of malarial *parasites*. The important consideration in discovery and development new antimalarial drugs are low-cost, highly effective, safe, well-tolerated, effective used in short time and have to delay the spread of resistant malarial parasites.

In this regard, we show neem known as *Azadirachta indica* A.Juss (Meliaceae family) has been used orally for treatment of malarial infection. This medicinal plant is widely used for the treatment of antifeedant, antibacterial, antihypertension, antiinflammatory, analgetic, hepatoprotector and antidiabetic[4]. The other references reported that the neem leaves can be used as antipyretic, antiarthritic, antimalarial, antiseptic, insecticide, antifungus, antioxidant, anthelmentic and anticancer[5,6,7,8].

Many biologically active compounds can be extracted from neem leaves, they are triterpenoids, phenolic compounds, carotenoids, steroids, flavonoids, alkaloids and ketones. The extract of the neem leaves contains gedunin, nimbin, nimbolide and many more limonoids[9,10]. Nimbolide possess anti-malarial activity by inhibiting the growth *of P. falciparum* and also shows antibacterial activity. Gedunin has been reported to possess both antifungus and antimalarial activities. Azadirachtin have strong antifeedant activity and antimalarial activity [11,12].

The aquaeous leaf extract of neem some times called infusum or decoctum prepared by boiling fresh leaves in water for 15-30 minutes and have been taken orally in single and multiple doses for treatment of malaria[6]. A few studies have been reported that the antimalarial activity of crude extract caused of nimbolide, nimbin, gedunin, dihydrogedunin and azadirachtin[13]. Nimbolide is a mayor compound that responsible for antimalarial activity with moderate potency IC_{50} 0,95 µg/ml and another compound nimbidine has IC_{50} approximately 0,5 – 3,0 µg/ml against *P. falciparum*[13,14].

In this study we used hexane extract of neem leaves for evaluated their in vivo antimalarial activity on *P. berghei* in *Mus musculus,* identified and characterized the active compound of this extract. In the future, may be the active compound is needed to use these agents as templates for designing new derivatives with improved properties.

MATERIALS AND METHODS

Simplicia collecting

Neem's leaves (*Azadirachta indica* A.Juss) were collected on Januari 2010 in Darussalam region, Banda Aceh in Indonesia and identified by Departemen of

Biology Pharmacy, Faculty of Pharmacy University of Gadjah Mada. The fresh leaves were picked from plant with more than 1 year in age and more than 2 meters in high. Leaves taken 1.5 meters height from land without differentiate old or young leaves.

Preparation of neem leaves extract

Neem fresh leaves 1000 g were macerated three times with n-hexane (1: 2,5) at room temperature (25^0C) over the period of 72 hours. Every 24 hour the solvent was changed and filtered through whatman paper. After collecting all the solvent was vacum evaporated at 40^0C. Final extract was a dark green semisolid in percentage weight of 15%. This hexane extract was kept in deep freezer at 20 C° until use.

Identification of the compounds in hexane extract of neem leaves

The extract was dissolved in acetone and impregnated into silica G_{60} (1 : 30). The impregnated sample was filled in vacum liquid column (VLC) and eluted by hexane – ethyl acetate in graded polarity. The fraction were received in 50 ml and after concentrating was splattered on Aluminium Silica F_{254} plate for thin layer chromatography and eluted by hexane-ethyl acetate in graded polarity. Analyzing the compound by using UV light at 254 and 366 nm. The spots that have same retention factor value (Rf) were collected and analyzed by using visualizing reagent that habitually using in analysis of triterpenoid, flavonoid, steroid, phenolic compound and alkaloid.

Animals

Fifty five male Swiss mice age around 2.5 – 3,0 months, 20 – 30 g of body weight were used in study of antimalarial activity test. Fifty male and female mice of the same strain used for studying the acute toxicity test. The animals were acquired from the animal centre of Medical Faculty University of Gadjah Mada. All animals were kept in a room maintained under environmentally controlled conditions of 23 ±1C° and 12 h light12 h dark cycle. All animals had free an access to water and standard food ad libitum. They were acclimatized at least one week before the experiments were started. The animals submitted to oral administration of the extracts that were fasted for 18 h before the experiment done (water was available).

Acute toxicity test

Four groups of mice of both sexes (n=10, 5 male and 5 females), received oral single doses of the extract (250; 500; 1000; 2000 mg/kg BW). The one control group received the mixture of distilled water and dimethyl sulfooxide (DMSO).

After a single dose administration, mice were placed in group clear plastic cages and all animals were observed for possible mortality cases (24 hour), behavioural changes and followed by periodic body weight monitoring for 14 days. The number of death mice in treated groups were used for measuring the lethal dose $_{50}$. The lethal dose 50 (LD_{50}) values were determined as described by Weil C.S (1952) method[15].

Inoculation of *Plasmodium berghei* in mice

Swiss mice were maintained and bred at the animal facility of Medical Faculty University of Syiah Kuala. At first, blood of the infected mice was taken from its tail and counted the percentages of parasitemia**.** All the mice were infected intravenously with 1×10^7 parasitized erythrocytes from a homologous donor mouse, which had been infected with frozen of *P. berghei* strain ANKA. Then the blood for infecting all mice taken from the infected mice source through heart using spuit injection 3 ml with anticoagulant and then dilute with RPMI 1640 until concentration of *P. berghei* was $1x10^7$. On day 0 (D_0), 0.2 mL blood of mice which contained *P.berghei* $1x10^7$ are injected by intraperitoneal route to all mice and kept together for 24 hour after inoculation. On day 1 (D_1) infected mice are randomly distributed in groups of eleven mice per cage (control and treated groups).

In vivo antimalarial activity test

On day 1 until day 4, mice in group were given 0.2 ml of 0 mg/kg BW/day (aqua destillata + DMSO, group I); treated drug 100 mg/kg BW/day (group II); 200 mg/kg BW/day (group III); 400 mg/kg BB/day (group IV) and 800 mg/kg BW/day (group V). Everyday the blood were taken for each mice by cutting the tail to determined parasitemia until day 4 (D_4).

Preparation of thin blood smear

One drop of blood taken from the tip of the tail of the mice, then placed on the right edge of the glass object and immediately the other end of the glass objects were placed with a slope 45^0. Position of glass object was tilted slightly shifted to the right until it touches the blood drop, and drops of blood will fill the second meeting of a glass object. The glass object is shifted to the left in order to obtain a thin and flat stock. Blood were dried, fixed in absolute methanol for about 5 minutes and washed with running water. After drying thin blood smear were stained with giemsa 10% solution for 30 minutes, washed with running water and dried again at room temperature.

Observation

The percentage of parasitemia of control and treated groups were examined by counting infected red blood cells in 1000 red blood cells under a light microscope with strong magnification (ocular and objectif lens 10 x 100 times). The efficacy of the treated drug was evaluated by determining the drug doses that reduced parasite growth by 50% (ED_{50}). The ED_{50} value come from the percentage of parasitemia and inhibition of parasites growth data by using regression probit analysis[16].

Data Analysis

The percentage of parasitemia was calculated based on the formula below:

$$\text{Percentage of parasitemia} = \frac{\text{Number of infected parasites}}{\text{1000 red blood cells}} \times 100\%$$

The percentage inhibition of parasites growth was calculated using formula below:

$$\text{Percentage inhibition of parasites growth} = \frac{\%\text{Parasitemia of control group} - \%\text{ Parasitemia of treated group}}{\%\text{ Parasitemia of control group}} \times 100\%$$

The lethal dose 50 (LD_{50}) was counted by using Weil C.S (1952) method on the formula: $\mathbf{Log\ LD_{50} = LogDa + d\ (f + 1)}$; Da is low dose , d is logarithm of multiple dose and f is the value in Weil CS (1952) table. The differences of body weight between treated groups were analyze by analysis of variance (ANOVA).

RESULTS AND DISCUSSION

The results showed the inhibitory of parasites growth was 78, 35% at level dose 800 mg/kg BW/day and the effective $dose_{50}$ (ED_{50}) was 355,45 mg/kg BW/day. This investigation shown the hexane extract of neem leaves has a moderate antimalarial activity[17,18]. When it is compared to the previous investigation by using neem oil seed obtained the ED_{50} value 973,93 mg/Kg BB/day in mice[19], so the hexane extract of neem leaves is better than neem oil seed. The next challenge is isolation of promise compounds in hexane extract that show the best potency for any properties. The percentage of parasitemia and inhibition of parasites growth of *P. berghei* after treating with hexane extract of neem leaves (*Azadirachta indica* A. Juss) on Day 4 were shown on Table 1 and Figure 1.

Table 1. The Percentage of Parasitemia and Inhibition of Parasites Growth of *Plasmodium berghei* After Treating with Hexane Extract of Neem Leaves (*Azadirachta indica* A. Juss) on Day 4.

Doses of Hexane Extract of Neem Leaves (mg/kg BW/day)	Percentage of Parasitemia	Percentage of Inhibition of Parasites Growth
0	13,86 ± 1,62	-
800	3,00 ± 1,15	78,35
400	6,34 ± 1,91	54,26
200	10,04 ± 1,68	27,56
100	12,07 ± 8,08	12,91

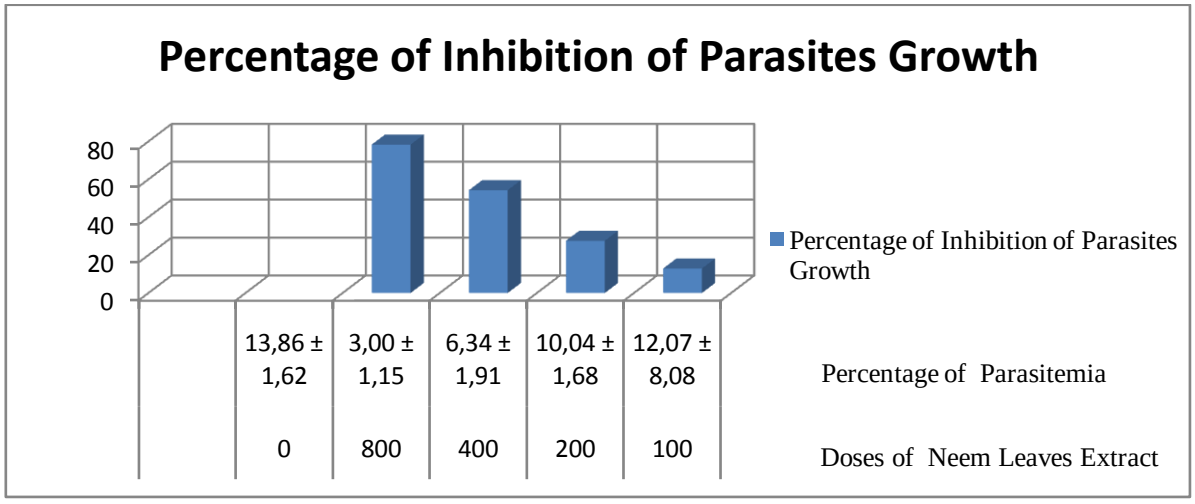

Figure 1. The Percentage of Inhibition of Parasites Growth, Parasitemia and The Doses of Hexane Extract of Neem Leaves

The onset of acute toxicity symptoms have been seen 30 minutes after oral administration at dose 1000 mg/kg BW like respiratory disturbance, reduced of locomotoric activity and convulsion. The mortality was happened 24 hour after administration at the doses 1000 - 2000 mg/ kg/day. The severity of these effects were increased within 2 hour and mortality was 100% at dose 2000 mg/kg BW/day. According to Weil CS (1952) we found the lethal dose $_{50}$ (LD_{50}) = 1000 mg/kg or

approximately between 732,82 – 1364,58 mg/kg BW with confidence limits of 95%. Table 2 shown the number of death mice in 24 hour after administration the hexane exract of neem leaves.

Table 2. The Number of Death Mice on Acute Toxicity Test of Hexane Extract of Neem Leaves ((*Azadirachta indica* A. Juss)

Single Doses of Hexane Extract of Neem Leaves (mg/kg BW)	Number of Death Mice
0	0
250	0
500	0
1000	3
2000	6

Observation on mice body weight were done on day 0 (before infected), day 5; 10 and day 14 after giving single dose of treated drugs orally. The change of body weight was presented in Table 3 and Figure 2. Lowering of body weight is an indicator of toxicity effect of the extract, but in treated mice groups could be prevented the weight loss[20].

Table 3. The Change of Mice Body Weight on Acute Toxicity Test of Hexane Extract of Neem Leaves

Single Doses of Hexane Extract of Neem Leaves (mg/kg BW)	Body Weight of Mice			
	Day 0	Day 5	Day 10	Day 14
0	23 ± 0,35	25 ± 0,30	27 ± 0,55	27± 0,85
250	25 ± 0,20	25 ± 0,45	25 ± 0,55	26 ± 0,45
500	27 ± 0,40	27 ± 0,55	28 ± 0,35	28 ± 0,65
1000	24 ± 0,60	25 ± 0,30	25 ± 0,45	27 ± 0,25
2000	27 ± 0,50	27 ± 0,65	28 ± 0,15	28 ± 0,25

The amount of hexane extract of neem leaves was obtained 15% after 72 hour of maceration and isolation of antimalarial compounds in hexane extract of neem leaves shown several compounds that detected under UV light 254 nm and 365 nm (after reacted with specific visualizing reagents). The result shown in Table 4.

Table 4. Thin Layer Chromatography of Hexane Fraction Neem Leaves (HFNL) and Phytochemistry Analysis

HFNL	Rf value	Visualizing reagents	Colours	Standard
Flavonoid	-	Ammonia vapour	-	Rutin
Steroid	0.84	Lieberman-Burchard	Violet	Beta-sitosterol
Terpenoid	0.21; 0.24; 0.33; 0.81 and 0.96	Vanilin-sulphuric acid	Red brown - violet	Carvone
Alkaloid	-	Dragendorf	Orange-brown	Quinin
Fenol	0.75	Ferri chloride	Dark green	Fenol

Based on thin layer chromatography and phytochemistry analysis indicated that hexane fraction of neem leaves contained terpenoids, steroid and fenolic compounds. Observation to terpenoids under UV light 365 nm showed 5 terpenoid compounds which could be seen as red brown-violet spots after reacted with vanilin-sulphuric acid by using carvone as standard of terpeneoid. Beside that we found one spot was same to beta-sitosterol (the standard of steroid), but we did not know the kind of steroid presented in the fraction.

Detection of alkaloid under UV light 254 nm was not found any compounds classified to alkaloid, while the result after reacting with Dragendorf reagent showed one spot with different compared to quinin as the standard. One more substance, shown as a dark green spot, was detected as fenolic compound after been sprayed with ferri chloride (Stahl,1985; Harbone,1987). Observation of flavonoid under UV light 254 nm showed no compound that have same to Rutin (the standard of flavonoid). The same result also was shown after the plate was sprayed with ammonia vapour and then observed under UV light 365 nm. This indicated that

there was no flavonoid extracted in hexane (Harbone & Christian, 2000).

CONCLUSION

The hexane extract of neem leaves has inhibitory of parasites growth 78, 35% at level dose 800 mg/kg BW/day and effective dose$_{50}$ (ED_{50}) 355,45 mg/kg BW/day. The onset of acute toxicity symptoms have been seen after oral administration at dose 1000 mg/kg BW like respiratory disturbance, reduced of locomotoric activity and convulsion were observed by 30 minutes after giving orally. The lethal dose $_{50}$ (LD_{50}) = 1000 mg/kg or between 732,82 – 1364,58 mg/kg BW. The hexane fraction of neem leaves contained terpenoids, steroid and fenolic compounds.

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