

ORIGINAL ARTICLE

Antimalarial Activity of Ethanol Extract of Noni Leaves (*Morinda citrifolia*) towards Parasitemia, Splenomegaly, and Hepatomegaly in *Plasmodium berghei* ANKA Infected Mice

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ABSTRACT

Introduction: Malaria is one of the infectious diseases found in tropical countries and sub-tropical countries. In 2016 there were an estimated 445,000 people died to malaria. Alternative medicine is needed, such as natural based ingredient. *Morinda citrifolia* or noni plant is a medicinal plant found in all parts of Indonesia which has many benefits, such as antibacterial, analgesic, anticancer, antioxidant, and anti-inflammatory. The aims of this study were to determine the antimalarial activity of ethanol extract of noni leaves and its effect on splenomegaly and hepatomegaly.

Methods: Extract of noni leaves was prepared by maceration using ethanol solvent. In vivo experiments were conducted using *Plasmodium berghei* infected BALB/c mice treated with the doses of 100, 10, 1 mg/kg body weight(BW) orally of ethanolic extract of noni leaves. Then, the percentage of parasitemia was calculated from day 1 to day 4 after treatment and at the end of the test, mice were sacrificed then spleen and liver were collected.

Results: The highest parasite growth was found in the group treated with noni leaves ethanol extract at a dose of 1 mg/kg WB and vice versa. Probit analysis resulted in ED50 was 0.882 mg/kg WB. Spearman test showed there was no correlation between doses and the size of splenomegaly with $p=0,2$ and between doses and the size of hepatomegaly with $p=0,6$.

Conclusion: Ethanol extract of noni leaves possessed antimalaria activity and there was no correlation between doses of extract and the splenomegaly and hepatomegaly.

Introduction

Malaria is an infectious disease which has spread to almost all over the world with tropical and sub-tropical climate.¹ This disease is caused by parasites from the genus *Plasmodium* and transmitted by the bite of female *Anopheles mosquitoes*. Physical examination results found in patients are generally fever, pale on the conjunctiva or palms, spleen enlargement (splenomegaly), and liver enlargement (hepatomegaly).² Malaria is an endemic disease in various poor countries and tropical developing countries such as Africa, Asia, and Latin America. The World Malaria Report even states that malaria is in 106 countries in the world. In 2015, in the world there were 429,000 deaths from malaria. The following year in 2016 there were 216 million cases of malaria and 445,000 people died. However, the heaviest cases occur in Africa because 90% of deaths in the region are caused by malaria, and more than two-thirds of deaths occur in children under 5 years

of age.³ In Indonesia, the regions located in the eastern part have high Annual Parasite Incidence (API) compared to other regions, API is the number of malaria positive cases per 1000 inhabitants in one year.⁴

Parasite resistance to antimalaria drugs such as quinine, chloroquin, and pyrimethamine-sulfadoksin has occurred in 3 of the 5 types of malaria that attack humans, namely *Plasmodium falciparum*, *Plasmodium vivax*, and *Plasmodium malariae*. This can be caused partly by irrational use of antimalarial drugs and patient non-compliance with the drug until it runs out. The latest malaria treatment uses a combination drug derived from Artemisinin known as Artemisinin based Combination Therapy (ACT). This combination is given to increase effectiveness and prevent resistance.

To overcome this resistance problem, one of which is the need for other alternative medicines that can play a role in curing malaria. The use of natural ingredients as anti-malarial



drugs is one of the right choices, considering that there are many herbal plants that have medicinal properties in Indonesia. Medicinal plant one of them is noni (*Morinda citrifolia*) or pace in Indonesian language is a plant that is often found in all regions of Indonesia and now extends to other tropical countries. Noni plants have many benefits for human health, such as antibacterial, analgesic, anticancer, antioxidant, anti-inflammatory, and can enhance immunity.⁵ Noni leaves can act as antibacterial because they contain chemical compounds such as anthraquinone, alkaloids, saponins, flavonoids, and terpenoid.⁶ Ethanol extract of noni leaves was stated to have antimalarial activity against *P. falciparum* 3D7 strain with an IC value of 22.68 µg/mL.⁷

Compounds content of extracts that can be extracted by solvents during the extraction process, play a role in various pharmacological activities. Therefore, the selection of solvents is important in the extraction process. In this study, the solvent used was ethanol. Ethanol is a non-toxic solvent that can dissolve the chemical components found in plants, both polar and non-polar.⁸

This current study will use ethanol extract of noni leaves against parasitemia, splenomegaly, and hepatomegaly in BALB/c mice infected with *Plasmodium berghei* ANKA. The BALB/c mice are used as an experimental animal because the similarities in the reaction towards the disease, treatment, and physiological condition with human.⁹ The *P. berghei* ANKA parasites have been widely used for research on the development of anti-malarial drugs with various plant extracts.^{10,11}

This research was conducted considering the number of cases of resistance of plasmodium parasites to anti-malarial drugs such as chloroquine pyrimethamine-sulfadoxine and quinine.¹¹

Methods

Study design

This research was laboratory experimental which aims to determine the effect of ethanol extract of noni leaves on parasitemia, splenomegaly, and hepatomegaly. The study was conducted from February to June, 2018.

Ekstrak

The ethanol extract of noni (*Morinda citrifolia*) leaves used in this study was obtained from Batu who was identified at the Center for Materia Medica, East Java. The ethanol extract of noni leaves is made by soaking 1 kg of dry leaf simplicia with 96% ethanol solvent at least twice the volume of powder, shaken, stored for 24 hours then filtered. Repeat the process 2 times. Then the extraction is evaporated in a water bath at 40°C until the stability of the extract is achieved.

Experimental method

The parasite used in this study were the *Plasmodium berghei* ANKA strains obtained from the Department of Parasitology, Universitas Airlangga, Surabaya. The parasite was present in the frozen blood of mice. The frozen blood infected with *P. berghei* ANKA was thawed at 37°C and then inoculated in 5 donor mice as much as 200 µL intraperitoneally. After parasitemia reaches 15-20% in donor mice, the mice are operated on and blood is drawn from the heart using a syringe. Blood is put into a test tube containing EDTA anticoagulant and mixed for parasite homogenization. Blood was then inoculated 0.2 ml each of the test mice intraperitoneally.

The experimental animals used in this study were 8 weeks old male BALB/c strain mice weighting 20-25 grams obtained from the experimental Animal Unit of the Department of Biochemistry, Faculty of Medicine, Universitas Airlangga. Mice were acclimatized for 7 days, given special pellet feed for mice and given mineral water every day at 1 pm.

The 25 of mice were injected with 0.2 ml each of the test mice intraperitoneally of *P. berghei* ANKA infected erythrocyte and divided into 5 groups with each group consisted of 5 mice. Based on drug and extract treatment, Group 1, was a positive control group which treated with 20 mg/kg BW orally chloroquin diphosphate; Group 2, was a negative control group, without any additional treatment; Group 3,4,5, test groups which were given the ethanol extract of noni leaves at a doses of 100, 10, and 1 mg/kg BW orally. The test was carried out by giving ethanol extract of noni leaves for 4 consecutive days starting from the 5th day after infection of the test mice. This antimalarial activity test was carried out in vivo using a modified Peter method, where the treatments were done for 4 consecutive days (D1-D4).¹² Blood of mice were taken everyday from the tip of the tail to prepare blood smear, then stained with 10% Giemsa prior to parasitemia observation. The parasitemia was calculated by counting the number of infected erythrocytes in 1000 erythrocytes. Based on the percentages of parasitemia, then the percentages of parasitemia growth and percent inhibition were calculated using the following formula:¹³

$$\% \text{ growth} = \frac{\sum (Dx - Dx - 1)}{n-1}$$

Where: (Dx-Dx-1) = % parasitemia on x-day minus % parasitemia on day before, n = number of observation day.

$$\% \text{ inhibition} = \left(\left| \frac{Xe}{Xk} \right| \times 100\% \right)$$

Where: Xe = percent growth of the average parasite in the group given the test solution, Xk = percent of average parasite growth in negative control.

Probit analysis using the SPSS 20 program was used to determine ED50 or Effective Dose 50 which was able to inhibit 50% of *P. berghei* ANKA growth in mice. Spearman test to analyze the relationship between the ethanol extract dose of Noni leaves with splenomegaly and hepatomegaly. The data were correlated significantly if p < 0.05.

Ethical Clearance

This study was approved by Health Research Ethics Committee, Faculty of Medicine, Universitas Airlangga, Surabaya with ethical number 341/EC/KEPK/FKUA/2017.

Results

Percentages of Parasitemia

Percentage of parasitemia in BALB/c mice infected with *P. berghei* ANKA given a 4-day test solution are listed in Table 1.

Percentages of Growth and Inhibition of Parasitemia Growth

Percentages of growth and inhibition of parasitemia in group treated with ethanol extract of noni leaves, positive control and negative control are shown in Table 2.

The highest growth was found in the group treated with ethanol extract of noni leaves at 1 mg/kg BW, whereas, the lowest growth was in the group treated with dose of 100 mg/kg BW. The higher inhibition was found in the group treated with ethanol extract of noni leaves with dose of 100 mg/kg BW, whereas, the lowest inhibition was found in the group treated with dose 1 mg/kg BW. The effect of ethanol extract of noni leaves showed that the higher doses of extract gave the lower percentages of parasitemia growth, inversely gave the higher percentages of inhibition of parasitemia growth.

Comparison of percentages of parasitemia growth in mice treated with ethanol extract of noni leaves and negative control showed that, the percentages of parasitemia growth in extract-treated mice were lower than that in negative control indicated that ethanol extract of noni leaves influenced the parasitemia growth during the 4 day course of treatment. Percentages of inhibition of parasitemia growth were lower than positive control, indicated that the extract was able to inhibit parasit growth, although less potent than Chloroquin,

since the *P. berghei* ANKA is chloroquin sensitive parasites.

Effective Dose (ED50)

Probit analysis resulted in ED50 of 0.882 mg/kg BW. The ethanol extract of noni leaves was able to kill 50% infected erythrocyte at the dose of 0.882 mg/kg BW. The antiplasmodium value in vivo has very good activity if ED50 <10 mg/kg; good if ED50 10-100 mg/kg; moderate if ED50 is 100-1000 mg/kg; and the antimalarial activity is not defined if ED50 >1000 mg/kg.¹⁷

Analysis of Splenomegaly and Hepatomegaly

Enlargement size was compared with the size of those organs in normal size where the size of spleen was 0.72 cm² - 1.25 cm² and liver was 4.4 cm² - 5.06 cm². The averages size of spleen and liver after treated with ethanol extract of noni leaves compared with positive control were summarised in Table 3.

The normality test of the data using Shapiro-Wilk test showed that the data was abnormally distributed. The analysis data was followed by Spearman correlation test. Spearman test resulted from correlation between spleen and doses were p= 0.2 indicated there was no significant correlation, as well as the correlation between liver and doses were p=0.6 indicated there was no significant correlation too.

Table 1. Average of parasitemia (%) in BALB/c mice infected with *P. berghei* ANKA with treatment

Treatment	Doses(mg/kgBW)	Day 0(%)	Day 1(%)	Day 2(%)	Day 3(%)	Day 4(%)
Group 1	1	13.8	19.29	22.75	29.59	22.04
Group 2	10	14.44	18.8	21.98	26.19	20.22
Group 3	100	15.1	17.95	18.94	19.83	16.22
PC	20	14.09	5.57	3.16	3.65	0.78
NC	-	11.34	15.91	20.43	26.61	29.69

Table 2. Averages percent of growth and percent inhibition of *P. berghei* ANKA after administration ethanol extract of noni's leaves

Treatment	Doses(mg/kgBW)	% growth	% inhibit
Group 1	1	2.06	55.08
Group 2	10	1.44	68.50
Group 3	100	0.28	93.92
PC	20	-	100
NC	-	4.59	-

Table 3. Average of liver and spleen size of treated mice

Treatment	Doses (mg/kg BW)	Size (cm ²)	
		Liver	Spleen
Group 1	1	6.90	1.94
Group 2	10	5.99	2.24
Group 3	100	4.90	1.95
PC	20	4.94	1.77
NC	-	5.75	1.73

PC : Positive control

NC : Negative control

Discussion

The results of this study showed an ED50 of ethanol extract on *P. berghei* ANKA was 0.882 mg/kg BW. Thus, it can be said that the smaller the value of ED50 a dose of the test material, the higher the antimalarial activity of the test material. The relatively small ED50 value indicated that ethanol extract of noni leaves is potential to be developed as an antimalaria drug in the future.¹⁵

The use of ethanol extract of noni leaves in this study was based on active compounds those are anthraquinone, alkaloids, saponins, flavanoids, and terpenoids.⁶ Noni (*M. citrifolia*) is a member of Rubiaceae family with 9 other sub-families namely Rubia, Cinchona, Coffea, Ixora, Mussaenda, Gardenia, Uncaria, Uragoga, and Pausingstalia.¹⁴ Another famous Rubiaceae member is used as an anti-malarial drug, Cinchona. Antimalarial activity originates from alkaloid compounds especially kinin and kinidin isolated from the bark of the Cinchona or the quinine tree.¹⁵ Based on the chemotaxonomic approach, namely an approach that states the similarity of anatomical, histological, morphological, and contained substances, plants originating from the same taxon has a strong kinship in the content of compounds, especially at the level of family, genus, and species. Based on the same approach, several plant species from different families including Meliaceae, Piperaceae, Acanthaceae, Myrtaceae, Myrsinaceae, Solanaceae and Fabaceae were also stated to have antimalarial activity.¹⁶

Research on noni leaves was previously carried out in vitro at the Laboratory of Tropical Diseases, Airlangga University. Among them stated that the ethanol extract of noni leaves had anti-malaria activity against *P. falciparum* 3D7 strain with an IC value of 22.68 µg/mL.⁷ In this case, the main components that have an antimalarial effect on noni leaves are flavonoids and alkaloids. Another study using noni leaves juice proved to inhibit the growth of *E. coli* bacteria because it also contains active flavonoids, alkanoids and atrakuinon.⁶

The ethanol extract of noni leaves in this study was given for 4 days. Based on the percentages growth and inhibition after being given ethanol extract of noni leaves, it was found that the extract dose tested was directly proportional to the percent inhibition and inversely proportional to the percentage of parasite growth. Mice that given higher doses of noni leaves extract showed higher percent inhibition and lower percent growth, and vice versa.

Splenomegaly and hepatomegaly as the clinical signs of malaria infection that caused by numbers of infected erythrocytes phagocytosed by macrophages in the spleen and Kupffer cells in the liver.^{17,18} Malaria infection involve a humoral and cellular immune response in the host to eliminate parasites. Spleen plays a role in cellular immunity to filter infected erythrocytes. The increased number of infected erythrocytes activates immune cells, such as macrophages for phagocytosis. Macrophage cells in the spleen also phagocytose hemozoin (malaria pigment). Spleen and liver in malaria infection are not only enlarged but also become dark brown due to ingestion of hemozoin and hemosiderin. The hemozoin pigment originate from changes in hemoglobin from the

ingestion of erythrocytes by parasites, while hemosiderin, the brown substance of ferritin oxide that remains from the breakdown of hemoglobin.¹⁹

In this study, the spleen and liver from mice were measured on the last day of observation of parasitemia, which was the fourth day. The result of spleen and liver measurements had no correlation with the dose of the extract either splenomegaly or hepatomegaly. This means that splenomegaly and hepatomegaly that occur are not affected by the dose of extract. It was proved that in the mice with the largest test dose treatment that was 100 mg/kg BW also experienced splenomegaly and hepatomegaly with an organ size that was almost the same as the smaller test dose treatment.

In this study, chloroquine-treated mice in positive control also showed splenomegaly and hepatomegaly, despite parasitemia was decreased since administration of chloroquine. Chloroquine is classified as a blood schizonticidal drug, therefore, its effect does not reach the parasites in the tissues. Splenomegaly and hepatomegaly in all treated mice, thus the ethanol extract of noni leaves has a similar mechanism of action with chloroquine which affects parasites that are circulating in the blood but not in tissues.²⁰

Conclusion

Ethanol extract of noni leaves has very good antimalarial activity in inhibiting the growth of *P. berghei* ANKA with similiar mechanism to chloroquin with ED50 values of 0.882 mg/kg BW. Therefore, ethanol extract of noni leaves has the potential to be further developed as an antimalarial.

Conflict of Interest

The author stated there is no conflict of interest

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