

# Antimalarial Activity of Ethanol Extract of Kelakai Leaves (*Stenochlaena palustris*) to Parasitemia and Splenomegaly in BALB/c Mice Infected with *Plasmodium berghei* ANKA

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## ABSTRACT

**Introduction:** Malaria is one of global health problems. Splenomegaly is one of malaria symptoms. Antimalarial drug resistance had been reported. Alternative treatment is by using traditional medicinal plants such as kelakai (*Stenochlaena palustris*). Kelakai contains alkaloid and flavonoid which had been reported to have antimalarial activity. The aim of this study was to discover antimalarial activity of ethanol extract of kelakai leaves to parasitemia and splenomegaly of *Plasmodium berghei* ANKA in infected BALB/c mice.

**Methods:** This study was based on a modified Peter test using BALB/c mice infected with *P. berghei* ANKA treated with ethanol extract of kelakai leaves, with chloroquine diphosphate as a positive control. The negative control was *P. berghei* ANKA infected mice without any additional treatment. Administration of ethanol extract of kelakai leaves was performed for 4 days with a serial doses of 100, 10, and 1 mg/kg body weight. The positive control was given chloroquine diphosphate 20 mg/kg body weight. Parasitemia was observed daily prior to the calculation of the percentage of parasite growth and parasite growth inhibition. At the end of the test, the mice were sacrificed and spleens were isolated to measure their sizes. Probit analysis was performed to obtain ED<sub>50</sub> to find the effect of extract in parasite killing by 50%. Spearman test was performed to analyze the correlation of doses of extract and splenomegaly.

**Results:** Parasitemia growth inhibition was directly proportional to the dose. Higher parasitemia inhibition was obtained at higher doses and vice versa. Result of probit analysis showed an ED<sub>50</sub> was 77.05 mg/kg body weight. Statistical analysis resulted in insignificant correlation between doses and splenomegaly  $p = 1.0$  (significance  $< 0.05$ ).

**Conclusion:** Ethanol extract of kelakai leaves possessed good antimalarial activity and there was no correlation between extract doses and splenomegaly in *Plasmodium berghei* ANKA-infected mice.

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## Introduction

Malaria is one of global health problems which occurs in tropical and subtropical countries. *Anopheles* mosquito as a malaria vector can grow and multiply.<sup>1</sup> Splenomegaly is caused by phagocytosis of infected erythrocytes and hemozoin by splenocytes.<sup>2</sup> Splenomegaly is usually found in human chronic malaria infection.<sup>3</sup>

In 2017, 219 million malaria cases were found and 435,000 people died. Malaria prevalence in Africa was 92%, in Southeast Asia was 5%, and in Eastern Mediterania area was 2%.<sup>4</sup> The incidence of malaria in Indonesia, according to Baseline Health Research (Riskesdas) 2013, was 0.35% or 3.5 of 1,000 population. It was found that 3 provinces with the highest incidence were Papua (6.1%), West Papua (4.5%), and East Nusa Tenggara (2.6%).<sup>5</sup>

In Indonesia, parasite resistance against antimalarial drug such as Chloroquine and Sulfadoksin-Pirimethamin was reported. The first resistance to Chloroquine occurred in 1973, which was found in *Plasmodium falciparum* parasites. In 1990, there was resistance to *P. falciparum* parasites against Chloroquine from all provinces in Indonesia. In addition, resistance was also found in *Plasmodium* against Sulfadoksin-Pirimethamin (SP) in several regions in Indonesia.<sup>6</sup> Alternative of malaria treatment is needed, one of them is using medicinal plants. One of them is Kelakai (*Stenochlaena palustris*). Kelakai is a plant that grows wildly in South Kalimantan. This plant is easy to be obtained because it can be found in traditional markets in South Kalimantan. Dayak Kenyah tribe use it to treat anemia, relieve fever, and skin aches. Kelakai leaves extract contain flavonoids, alkaloids and steroids.<sup>7</sup> Alkaloids and flavonoids have antimalarial effects. The effect of alkaloid is inhibit heme polymerase and causes heme deposit which is toxic to *Plasmodium*.<sup>8</sup> Flavonoid is able to inhibit the biosynthesis of fatty acids (FAS II) in parasites, inhibit the entry of L-glutamine and myoinositol into erythrocytes.<sup>9</sup>

Mice which are often use as a model for immunology is BALB/c mice.<sup>10</sup> In malaria studies, mice are used for infection with *P. berghei* infection which is able to reach high parasitemia.<sup>11</sup> *Plasmodium berghei* ANKA is one of the parasites that causes malaria in rodents, including mice.<sup>12</sup> *P. berghei* ANKA can be used as a model of human malaria study in mice due to its similar characteristics to *P. falciparum*. Virulence of *P. berghei* ANKA is similar to *P. falciparum* which infects all ages of erythrocytes and causes pathological abnormalities in organs.<sup>13</sup>

Ethanol is a polar non-toxic solvent and has a relatively low price in the market. These are the reasons for using ethanol as a solvent to prepare the extract of kelakai leaves.<sup>14</sup> In addition, compounds in plants when extracted using ethanol solvents will be more stable because ethanol solvents have antimicrobial effect. Ethanol solvents can dissolve polar and non-polar compounds in almost all organic compounds from plants and can open the cell wall

of the plant and draw almost all the compounds from the extracted plant out of the cell.<sup>15,16</sup>

Based on the relation between parasitemia and splenomegaly, the effect of extract doses on parasitemia could also be studied in relation to splenomegaly. The aim of this study was to discover antimalarial activity of ethanol extract of kelakai leaves against parasitemia and splenomegaly of *Plasmodium berghei* ANKA in infected BALB/c mice.

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## Methods

### Research Design

This was a laboratory experimental study. The independent variables in this study were the dosage of ethanol extract of the kelakai leaves 100, 10, and 1 mg / kg body weight. The dependent variable in this study were the percentage of parasitemia and the mean of splenomegaly of the mice which were given ethanol extract of kelakai leaves. This study was performed from March to June 2018.

### Research Material

Ethanol extract of kelakai leaves were obtained from Laboratory of Parasitology, Faculty of Medicine, Universitas Airlangga, Surabaya. The ethanol extract of Kelakai leaves was made with 96% ethanol as solvent.

### Parasites and Animal

*Plasmodium berghei* in this study was ANKA strain obtained from the Laboratory of Parasitology, Faculty of Medicine, Universitas Airlangga, Surabaya. The mice used in this study were 8 weeks BALB / c male mice with 20-25 grams body weight obtained from Experimental Animal Unit of Department of Biochemistry, Faculty of Medicine, Universitas Airlangga, Surabaya. 25 mice were divided into 5 groups. They were treated with the doses of 1; 10; 100 mg / kg body weight, positive controls were given 20 mg / kg body weight chloroquine diphosphate, and negative controls were only infected with *Plasmodium berghei* ANKA without any additional treatment. Every mice was injected with *P. berghei* ANKA  $1 \times 10^6$  of *P. berghei* ANKA infected erythrocytes or 200  $\mu$ l infected blood / mice intraperitoneally.

### Antimalarial Activity Test

The test was based on a modification of Peter test.<sup>17</sup> Administration of ethanol extract of kelakai leaves was performed for 4 consecutive days. Parasitemia was observed daily of blood smears stained with 10% Giemsa. It was calculated per 1000 erythrocytes. Based on parasitemia, the percentage of parasite growth and the percentage of parasite growth inhibition were calculated. The percentage of parasite growth and the percentage of parasite growth inhibition were calculated using the following formula:<sup>18</sup>

$$\% \text{ growth} = \frac{\sum[D_x - D_{x-1}]}{n-1}$$

Where (D<sub>x</sub>-D<sub>x-1</sub>): % parasitemia on x-day minus % parasitemia on day before n: number of observation day

$$\% \text{ inhibition} = 100\% - \left( \left| \frac{X_e}{X_k} \right| \times 100\% \right)$$

Where X<sub>e</sub>: percentage of the average parasite growth in the group given the test solution, X<sub>k</sub>: percentage of the average parasite growth in the negative control.

Based on the data of the percentage of growth inhibition, the probit analysis was performed to obtain ED<sub>50</sub> which indicated concentration of the extract that killed parasite by 50%.

Observation of splenomegaly was performed at the end of the test. The mice were sacrificed and spleens were removed to measure the length and width of the spleen and then calculated the wide of spleen. Correlation of concentration of the extract and size of spleen was analysed by using Spearman test.

**Ethical Clearance**

This study was approved by Health Research Ethics Committee, Faculty of Medicine, Universitas Airlangga, Surabaya.

**Results**

**Parasitemia**

Observations of blood smear from BALB / c mice infected with *P. berghei* ANKA with a light microscope at 1000 times magnification resulted in percentage of parasitemia as shown in Table 1.

Table 1. The average daily percentages of parasitemia in *P. berghei* ANKA infected BALB/c mice

Treatment	Doses (mg/kg body weight)	Observation Day				
		D0	D1	D2	D3	D4
EE of KL*	100	17.35	20.93	26.79	31.18	26.40
EE of KL*	10	7.42	9.87	13.23	16.09	17.88
EE of KL*	1	8.5	10.86	13.63	16.44	20.63
Chloroquine diphosphate	20	14.09	5.57	3.16	3.65	0.78
None	-	11.34	15.91	20.43	26.61	29.69

\* EE of KL: Ethanol Extract of Kelakai Leaves

Based on the percentage of parasitemia, the percentage of parasite growth and parasite growth inhibition were presented in Table 2.

Table 2. Percentage of growth and growth inhibition of *P. berghei* ANKA after treatments

Treatment	Doses (mg/kg body weight)	Growth (%)	Inhibition (%)
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EE of KL*	100	2.26	50.63
EE of KL*	10	2.61	42.99
EE of KL*	1	3.03	33.90
Chloroquine diphosphate	20	-	100
None	-	4.59	-

\* EE of KL: Ethanol Extract of Kelakai Leaves

The results showed the percentage of parasite growth was inversely proportional to the percentage of parasite growth inhibition. The higher percentage of parasite growth, the lower percentage of parasite growth inhibition. The percentage of parasite growth inhibition was directly proportional to the doses. The percentages of parasite growth in treated mice compared with negative control was lower, indicating that the extract affected parasite growth, while the percentage of parasite growth inhibition in treated mice compared with positive control was lower because Chloroquine is a potent antimalarial and *P. berghei* ANKA is Chloroquine sensitive.

**Effective Dose 50 (ED<sub>50</sub>) of Ethanol Extract of Kelakai Leaves**

Probit analysis of data on *P. berghei* ANKA growth inhibition and doses of ethanol extract of kelakai leaves obtained ED<sub>50</sub> as much as 77.05 mg / kg body weight.

**Correlation of Splenomegaly and Doses of Ethanol Extract of Kelakai Leaves**

Splenomegaly was observed in the mice treated with ethanol extract of kelakai leaves, Chloroquine, and negative control, and then compared to normal mice. The spleens of treated mice were more blackish and larger in size, whereas in normal mice the spleen was fresh red and smaller with the spleen sizes of 0.72 - 1.25 cm<sup>2</sup>.

Table 3. The average spleen size in *P. berghei* ANKA infected BALB/c mice after treatments

Treatment	Doses (mg/ kg body weight)	Spleen sizes (cm <sup>2</sup> )
EE of KL*	100	2.04
EE of KL*	10	1.50
EE of KL*	1	2.11
Chloroquine diphosphate	20	1.77
None	-	1.73

\* EE of KL: Ethanol Extract of Kelakai Leaves

Spearman correlation test showed that there was insignificant correlation between splenomegaly and extract dose with p = 1.0 (significance <0.05).

**Discussion**

The results of this study showed different fluctuations in parasitemia in the treatment group from day 0 to day 4. Based on the results, parasitemia in the test group was



lower than in negative control, indicating that the ethanol extract of kelakai leaves possessed antimalarial activity. Probit analysis showed ED<sub>50</sub> was 77.05 mg / kg body weight and was considered as good antimalarial activity. Herintsoa classification showed that an extract which has antimalarial activity is considered very good if ED<sub>50</sub> <10 mg / kg body weight; considered good if ED<sub>50</sub> 10-100 mg / kg body weight; rated moderate if ED<sub>50</sub> 100-1000 mg / kg body weight; and considered not having antimalarial activity if ED<sub>50</sub> >1000 mg / kg body weight.<sup>19</sup>

In vivo tests of antimalarial activity of ethanol extract of kelakai leaves in BALB / c mice were based on bioactive substances in kelakai leaves which have antimalarial effect. Bioactive substances in kelakai leaves are flavonoids, steroids, and alkaloids. Alkaloids are the most dominant ingredient.<sup>7</sup> Alkaloids have antipyretic, anti-inflammatory, and antimalarial effects.<sup>20,21</sup> Alkaloids inhibit heme polymerase and cause heme deposit in food vacuole which is toxic to *Plasmodium*.<sup>8</sup> The content of flavonoids in kelakai leaves are 14.5 µg/ml.<sup>22</sup> Flavonoids have anti-inflammatory and antimalarial effect. Flavonoids inhibit parasitic fatty acid biosynthesis (FAS II), influx L-glutamine and myoinositol into infected erythrocytes, and disrupt the growth of *Plasmodium*.<sup>9</sup> In addition, kelakai leaves also contain phenolic and anthraquinone.<sup>23</sup>

Malaria infection usually has several symptoms, one of which is splenomegaly as a clinical manifestation of high parasitemia and immune reaction against infection.<sup>13</sup> High parasitemia increases phagocytosis of infected erythrocytes and hemozoin in the red pulp area and hosts immune reaction in white pulp area which causes enlargement of spleen.<sup>2,24</sup> In splenomegaly, spleen also changes color from fresh red to blackish brown. The color change is due to hemozoin and hemosiderin accumulation in the spleen. Hemozoin is a waste product from the digestion of hemoglobin by malaria which has black or brown color. Hemosiderin is the substance produced by the breakdown of brown hemoglobin.<sup>25</sup>

In this study, it was found that the ethanol extract of kelakai leaves had antimalarial activity against parasitemia in the blood, but had no effect on splenomegaly. It can be seen in each group of mice which were given extracts with doses of 1, 10, 100 parasitic growth inhibition and positive control decreasing parasitemia, whereas the size of the spleen was enlarged in groups of mice which given extracts with doses of 1, 10, 100 positive control and negative control. Chloroquine which was given to positive controls was effective in reducing parasitemia, but did not inhibit splenomegaly. It happened because chloroquine only works against parasites in the blood, but it is not effective in tissues.<sup>2</sup> Thus, the mechanism of ethanol extract of kelakai leaves against *P. berghei* ANKA in mice was similar with chloroquine.

## Conclusion

Ethanol extract of kelakai leaves possessed good antimalarial activity with an ED<sub>50</sub> of 77.05 mg / kg body

weight, inhibiting parasitemia growth in a similar mechanism to chloroquine, and there was no correlation between extract doses and splenomegaly in *Plasmodium berghei* ANKA-infected mice.

## CONFLICT OF INTEREST

The author stated there is no conflict of interest in this study.

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