IN VITRO ANTIMALARIAL ACTIVITY OF BELIMBING WULUH (Averrhoa Bilimbi) LEAVES EXTRACT ON Plasmodium falciparum

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INTRODUCTION

Malaria is an infectious disease caused by parasites of the genus Plasmodium and is transmitted through the bite of the Anopheles mosquito (World Health Organization, 2011). In humans, Plasmodium parasites multiply in the liver and infect red blood cells. Symptoms caused by this infection may include fever, sweats, headaches, nausea, vomiting, and pain throughout the body. Moreover, in the case of severe infection, P. falciparum-infected human can experience organ failure and blood abnormalities, manifestations may include cerebral malaria, severe anemia due to hemolysis, hemoglobinuria, pulmonary edema, abnormalities in blood coagulation, thrombocytopenia, cardiovascular collapse and shock (CDC 2010). Approximately 3.3 billion people, or about half the world's population living in malaria-endemic areas and are at risk to develop the disease. There are about 250 million new cases of malaria worldwide and nearly 1 million people die from malaria each year. People who live in poor countries have the greatest risk of infection of this disease (World Health Organization 2009). In Africa, malaria has become a very serious problem, where each one of the five children died of this disease. From malaria in Africa, children may develop a fever from 1.6 to 5.4 times annually and every thirty seconds a child dies (World Health Organization 2009). In Indonesia, nearly half the population or about 90 million people living in malaria endemic areas, especially in rural areas and among the poor (R & D Department of Health, 2008, UNICEF 2009, UNDP 2011). The number of clinical cases of malaria were reported in
Indonesia in 2009 were 1,143,024 people and the number of positive cases found by laboratory tests are 199,777 people (Ministry of Health, Republic of Indonesia, 2010). There are an estimated 30 million cases of malaria each year, only about 10 percent of which will be treated in health facilities (UNICEF, 2009, UNDP 2011). The most malarial regions located outside Java, especially the eastern region of Indonesia, of East Nusa Tenggara to Maluku and Papua. In Central Java and West Java, malaria is reappearing (reemerging diseases). According to data from the health facilities in 2001, an estimated prevalence of malaria was 850, 2 per 100,000 people, with rates as high as 20 percent in Gorontalo, 13 percent and 10 percent in the province of Papua. The survey results predict mortality specific from malaria in Indonesia is 11 per 100,000 for men and 8 per 100,000 for women (UNDP 2011).

Treatment with malaria therapy is one right way to reduce the high prevalence of malaria. Prompt and effective treatment are key in controlling malaria (World Health Organization 2011). In the eradication program, the Department of Health has treatment standards in accordance with the area and the sensitivity of P. falciparum to antimalarial drugs. Standardization is useful to prevent the development of resistance to the case of antimalarial drugs other (Tjitra 2004). In the development of the treatment of malaria in Indonesia, it has been reported that drug resistance is recommended the Department of Health. Drug resistance was first reported in 1974 in East Kalimantan on the type of drug chloroquine in P. falciparum infection. The case of chloroquine resistance continues to spread and up to 1996 there have been reports from all provinces in Indonesia (Tjitra et al, 1997, Laihad & Gunawan 2000). Other types of drug-resistant cases have also been found in various provinces in Indonesia, including resistance to sulfadoxine pyrimethamine (Marwoto 1984, Radloff et al 1996), and resistance to mefloquine (Hoffman 1986). To fix this, please note other antimalarial drugs that can be used as alternative medicine (Tjitra 2004).

The combination of the plant Artemisia annua drug from the synthetic drugs are often called ACTs (artemisinin-Based Combination Therapies) is the recommended treatment (Ministry of Health, Republic of Indonesia, 2010). But the recent publication of a failure to report high to ACTs, as well as in vitro drug sensitivity data, which indicate the possible presence of artemisinin resistance along the Thai-Cambodian border area (Vijaykadga et al 2006, Jambou et al, 2005). WHO reported that the parasites against artemisinin resistance in Thai-Cambodian border could derail the success of efforts to control malaria globally (World Health Organization 2009).

Starfruit (Averrhoa bilimbi L) as a tropical plant that grows in Indonesia is still not widely used. The use traditionally to treat hypertension and diabetes mellitus has been scientifically proven. The content of starfruit leaves composed of several flavonoids that possess a wide variety of roles in biological life. One flavonoid found in many starfruit leaves are Luteolin (Mian & Mohamed 2001). Luteolin is a flavonoid member that has been identified as an antimalarial agent. Luteolin showed potential in inhibiting Fab I enzyme Plasmodium. Fab I enzyme is part of the Type II Fatty Acid Synthase (FAS II) which is also known as enoyl-ACP reductase, involved in the final reduction step of the cycle extension fat plasmodium acid chain (Tasdemir 2006). In a study conducted by Lehane and Saliba (2008) mentions that Luteolin as the most active compound in inhibiting the growth of Plasmodium strains 3D7 and 7G8 compared to other types studied flavonoids (kaempferol, myricetin, quercetin, acacetin, apigenin, baikalein, chrysins, naringenin, genistein) with inhibitory concentration IC50 (inhibitory Concentration 50) the minimum is 11 ± 1 for strain 3D7 and 12 ± 1 for 7G8. Character of starfruit leave extract potential is expected to be used as a growth inhibition of P. falciparum in vitro. The purpose of this study was to analyze the effect of the leaf extract of starfruit (Averrhoa bilimbi L) on the inhibition of P. falciparum growth in vitro.

MATERIALS AND METHODS

This research is a kind of experimental laboratory research. The study population was a blood smear on 0 and 48 hours after incubation with various concentrations of methanol extract of leaves of starfruit (Averrhoa bilimbi L). The sample was a thin blood smears were prepared from each well in the microplate were tested with the methanol extract of leaves of starfruit (Averrhoa bilimbi L). From each well made one blood smear done while each concentration of two replications. The concentration used for the test is 100; 10; 1; 0.1 and 0.01 ug/ml. A positive control was 2 so well with the negative control 2 well, and the blood smear for hours 0, so that the total number of blood smear sample is 15. Samples were taken at the end of the test in hours to 48 by way of as much as 2 mL of blood is taken and made smear a blood thinner and then painted with 5% Giemsa. The process of implementation of starfruit leaf extract extraction performed at the Laboratory of Phytochemistry-section of Nature Materials Sciences Faculty of Pharmacy, Airlangga University, Surabaya. Breeding, testing of P. falciparum parasitaemia malaria parasite strains 3D7 in vitro carried out in the Department of Parasitology, Faculty of Medicine Airlangga University, Surabaya.
The study was conducted from September 2011 to December, 2011.

Fresh leaves from Surabaya cleaned, dried at room temperature, and not exposed to direct sunlight for 2 weeks. The dried leaves are ground into a coarse powder. The leaf powder was suspended in a solution of 90% methanol until all the leaves are submerged with a volume of about 4 liters of methanol for 4 days. After that, the suspension is filtered so that the results obtained clear filtrate, then evaporated at rotavapor at 60°C until all the alcohol and starfruit leaf extracts obtained was separated. Extracts were stored at 5 °C prior to use for testing extracts. Testing setup procedures refer to the workings of Trager and Jensen (1976) referred to by Pharm and Afshar (1982), and Azizah et al (2010).

For a test antimalarial activity, necessary parasites in a state that is synchronous ring stage. Synchronization is done by using 5% sorbitol (Lambros and Vanderberg 1979). Parasite suspension consists of complete medium, IRBC, RBC comprising 10% complete medium, 1% IRBC, and 5 HCT. As the test material is starfruit leaf methanol extract at a concentration of 100; 10; 1; 0.1; 0.01 µg/ml. Way of providing this solution prepared in aseptic conditions.

To test antimalarial activity performed in sterile micro well plates, these plates consist of 96 wells consisting of 8 lines (AH) dan12 column. For the preparation of positive control, pipette solution with a micropipette from Chloroquin diphosphate 20,000 µg/ml, 10 mL and diluted with 990 µL complete medium (2000 µG/ml) was then pipetted to 20 mL of Chloroquin diphosphate in 2000 µg/ml and dissolved in 180 (complete medium, IRBC, RBC) and then inserted into the test wells F1. This procedure is performed replication and put in test wells F2. Negative controls for the preparation of the micropipette pipette solution complete medium as much as 20 mL then added with 180 mL (complete medium, IRBC, RBC) and then inserted into the test wells G1. This procedure is performed replication and put in test wells G2.

Antimalarial effect in vitro testing procedures are ring-stage parasites with ± 1% parasitaemia were cultured together with test materials and controls using 96-well microplate with each well filled volume of 200 mL. Solution of the test material is inserted into the test well and coupled with complete medium, IRBC, and RBC according to the procedure above. Candles were lit and the desiccator was closed. Having extinguished the candle in a desiccator, a desiccator put incubator and incubated at 37o C for 48 hours.

Observations were made on thin blood smears were painted with 5% Giemsa at hour 0 and hour to 48 to determine the% parasitaemia. Based on the calculated% parasitaemia and then calculated% growth inhibition. IC50 is an effective inhibitory concentration capable of inhibiting 50% growth of P. falciparum in vitro. Based on data% inhibition then performed probit analysis by creating a curve relationship between probit (probability unit) percent inhibition and the logarithm of concentration using a linear regression line equation to determine IC50.

RESULTS

Results of 90% methanol extraction starfruit weighing 2,000 grams from the Institute for Research and Industrial Consultancy Surabaya semi-liquid extracts obtained as much as 20 grams. Painted with a thin blood smear Giemsa dye and observed with a microscope at a magnification of 1000 times (Table 1).

Percentage inhibition data are then used to obtain IC50 values with probit analysis on SPSS 17. From the probit analysis obtained Inhibitory Concentration (IC50) of the methanol extract of the leaves of the pertumbuhanP starfruit. falciparum in 48-hour incubation. The IC50 value indicates the magnitude of the concentration of the extract that can inhibit 50% of parasite growth. The smaller the IC50 value, the greater the effectiveness of the extract on the growth inhibition of the parasite. IC50 of starfruit leaves methanol extract obtained was 2.805 µg/ml.

DISCUSSION

Test antimalarial activity in vitro by using cultured P.falciparum can be used as a preliminary test to evaluate prospective biological materials as an antimalarial. In this study, the methanol extracts of the leaves is used in the testing starfruit antimalarial activity. This is because the active substance contained in the leaves starfruit which has the effect of luteolin on the inhibition of the parasite is much soluble in methanol than other solvents such as water or ethanol (Mean & Mohamed 2001). This study aims to determine the IC50 of the antimalarial activity of methanol extract of leaves of starfruit. IC50 is the concentration of the extract can inhibit the growth of P. falciparum parasti by 50%.
Table 1. Number of *P. falciparum* infected erythrocytes per 1000 erythrocytes in 48-hour incubation

<table>
<thead>
<tr>
<th>Material</th>
<th>Concentration (μg/ml)</th>
<th>Duplicates</th>
<th>Σ Infected Erythrocyte</th>
<th>Σ Total Parasite</th>
<th>% Parasitemia</th>
<th>% Growth</th>
<th>% Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Ring</td>
<td>Trophozoit</td>
<td>Schizont</td>
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<tr>
<td>Negative Control</td>
<td>1</td>
<td>18</td>
<td>19</td>
<td>2</td>
<td>39</td>
<td>4.05</td>
<td>3.45</td>
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<tr>
<td></td>
<td>2</td>
<td>16</td>
<td>22</td>
<td>4</td>
<td>42</td>
<td></td>
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<tr>
<td>Methanol extract of bilimbi leave (μg/ml)</td>
<td>100</td>
<td>1</td>
<td>8</td>
<td>5</td>
<td>1</td>
<td>14</td>
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<td>13</td>
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<tr>
<td></td>
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<td>15</td>
<td>18</td>
<td>2</td>
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<td>18</td>
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<td>Positive Control</td>
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<td>3</td>
<td>0</td>
<td>4</td>
<td>0.45</td>
<td>–</td>
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<tr>
<td></td>
<td>2</td>
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<td>3</td>
<td>0</td>
<td>5</td>
<td></td>
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<tr>
<td>Parasite (D0)</td>
<td>5</td>
<td>1</td>
<td>0</td>
<td>6</td>
<td>6</td>
<td>0.6</td>
<td>–</td>
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</table>

![Probit Transformed Responses](image)

Figure 1. Graph Probit analysis of the inhibition of methanol extract of *belimbing wuluh* leaves of *P. falciparum* at 48 hours incubation
Antimalarial activity assay used in this study were cultured *Plasmodium falciparum* isolates by a modified method of Trager and Jensen (1976). *P. falciparum* isolates were cultured with synthetic medium RPMI 1640, HEPES buffer, sodium bicarbonate solution and then incubated in human serum desiccator containing wax (candle jar) or incubator at 37°C for 48 hours. After incubation for 48 h, thin blood smears were made and stained by Giemsa. The smear was observed with a microscope at a magnification of 1000 times. Then count the number of erythrocytes infected with malaria parasites per 1000 erythrocytes.

At 48 h incubation, the culture isolates of *P. falciparum* erythrocyte phase will repeat the cycle skizogoni (asexual) so shaped schizont parasite will burst into merozoites. If this meets with erythrocyte merozoites (asexual) so shaped schizont parasite will burst into merozoites. It then will evolve into a new form of cinicin. In Table 5.1 it can be seen that the higher the percentage of inhibition with increasing concentration. It can be seen at a concentration of 0.01 µg/ml, 0.1 µg/ml, 1 µg/ml, 10 µg/ml, and 100 µg/ml. So it looks in the picture graph of percent inhibition of 5.1 will be linear.

In the negative controls were not given the test material indicates the percentage of parasitaemia as much as 4.05%, while the concentration of 0.01 µg/ml of parasitaemia smaller percentage of negative control as many as 3.6%, it indicates that the smallest concentrations are 0.01 µg/ml materials starfruit leaf methanol extract can inhibit the growth of parasites. Greater concentration is 0.1 µg/ml, 1 µg/ml, 10 µg/ml, and 100 µg/ml showed a decrease in the percentage of parasitaemia is directly proportional to the increase in concentration of the extract solution compared to the negative control. This shows the higher concentration of leaf extract starfruit then inhibition against *P. falciparum* is increasing.

Starfruit leaves contained many types of flavonoids luteolin (Miean & Mohamed 2001). Luteolin is an anti-plasmodium because it can inhibit the first enzyme of Plasmodium Fab. Feb I enzyme is part of the Type II Fatty Acid Synthase (FAS II) which is also known as enoyl-ACP reductase, involved in the final reduction step of fatty acid chain extension cycle of plasmodium. This leads to the disruption of energy supplies, nutrition, maturation and membrane biogenesis and parasitophorous vacuole formation required by the parasite so that luteolin dissolved in methanol extracts of leaf starfruit can inhibit the growth of parasites in erythrocytes (Tasdemir 2006).

Observation of malaria parasite-infected erythrocytes per 1000 erythrocytes in the blood smear microscopy at a magnification of 1000 times the obtained results in the form of a percentage parasitaemia. Of the percentage of parasitaemia, will be obtained percentage growth of malaria parasites. Then based on the percentage of growth, the percentage of inhibition can be obtained. Furthermore, the percentage of inhibition will be used in the probit analysis SPSS 17 to obtain IC50 is the concentration of the methanol extracts of the leaves starfruit that have inhibitory effect on *P. falciparum* as much as 50%.

In this study, it is known that inhibition of parasite growth in the methanol extract of leaves starfruit as much as 50% with a 48-hour incubation of 2.805 µg/ml.

A material is said to potent antimalarial if the IC50 of less than 50 µg/ml for extracts whereas the IC50 values for the fraction of less than 25 (Ringwald et al 2000). This indicates that the methanol extract of leaves starfruit show large parasite growth inhibition at concentrations smaller and has strong potential as a new drug for malaria.

Basco et al (1994) and Dolabela et al (2008) divides the criteria in vitro antimalarial activity of extracts into four criteria: IC50 <10 µg/ml for both activities, IC50 10-50 µg/ml for moderate activity, IC50 50-100 µg/ml for the low activity, and IC50 >100 µg/ml inactive. In this study, visible IC50 of starfruit leaf extract on the inhibition of *P. falciparum* is equal to 2.805 µg/ml. It shows starfruit leaf extract was included in the criteria for a good activity because the IC50 <10 µg/ml. Therefore, further research is needed to determine the possible potential produced starfruit leaf extract as antimalarial test them in vivo antimalarial activity. It should also be done fractionation and isolation of luteolin and testing in vitro and in vivo to determine the potential of starfruit leaves as an astringent or new antimalarial herbal medicine.

CONCLUSION

There inhibition on starfruit leaf extract on the growth of *P. falciparum* in vitro. From research antimalarial activity of methanol extract of leaves starfruit against *P. falciparum* in vitro at 48 h of incubation obtained IC50 of 2.805 µg/ml.

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