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Effect of Ethyl Acetate Extract of Malacca Leaf (*Phyllanthus emblica*) on the Growth of Plasmodium falciparum in Vitro

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ABSTRACT

This study aimed to determine the antiplasmodium activity of ethyl acetate extract of malacca leaf (Phyllanthus emblica L.) on Plasmodium falciparum in vitro. In this study, five treatment groups with extract dosage of 100, 75, 50, 25, and 5 µg / ml were used. For positive control, antimalarial drug artesdiaquine was given. Plasmodium culture used the candle-jar method and the antiplasmodium activity test was carried out by microculture method in vitro. This research used a complete randomized design (RAL), data was analyzed using analysis of variance (ANOVA) and continued with Duncan's tests. The inhibition concentration (IC50) of malacca leaf extract was analyzed using PROBIT SPSS. Antiplasmodium activity was expressed by IC50 value which is the ability to inhibit 50% of Plasmodium growth. The results showed that the IC50 value of the extract was 17,849 µg/ml. It can be concluded that the ethyl acetate extract of malacca leaf had good activity as antiplasmodium and the best concentration was 25 µg / ml.

Keywords: *Phyllanthus emblica L., Plasmodium falciparum, antiplasmodium, malaria*

INTRODUCTION

Malaria is a disease caused by intracellular obligate protozoa of the genus Plasmodium, naturally transmitted by a female Anopheles mosquito (Arsin, 2012). Malaria is still a health problem with a high morbidity and mortality. Malaria can be found almost all over the world, especially tropical and subtropical countries (Novita, 2009; Ramdja, 1997). Indonesia is one of the countries located in the tropics and is endemic to malaria, and has a quite high risk (Ishak, 2005). According to WHO data from 2001-2012, Indonesia's health profile was ranked 16th as a country with a mortality rate above 80% due to malaria. The percentage of deaths in toddlers reached 90% and is in 9th place (WHO 2013).

There are several types of Plasmodium that cause malaria in Indonesia, namely Plasmodium falciparum, P. vivax, P. malariae, and P. ovale (Minister of Health RI, 2011). P. falciparum is the most dangerous type compared to other types of Plasmodium that infect humans (Panggabean, 2010), with a percentage of 86.4% in 2008 (Minister of Health RI, 2011). Resistance of P. falciparum to antimalarial drugs is a problem in endemic areas. The increase in parasite resistance to existing drugs is one of the causes of high rates of morbidity and mortality due to malaria (Olliara and Bloland, 2001). Resistance of parasites to antimalarial drugs such as chloroquine (CQ) and sulfadoxin-primetamine (SP) almost occurs in all provinces in Indonesia (Tarigan, 2007).

The use of natural remedies, such as herbal remedies to treat diseases is very common in Asian regions and developing countries including Indonesia, this is due to the fact that they are cheaper and more efficient than synthetic drugs (Juliana et al., 2010). Research on the plant has made very beneficial progress for anticancer and antimalarial therapy. Malacca or Phyllanthus emblica L. or Emblica officinalis Gaertn. known as Indian gooseberry or Amla, is one of the most important medicinal plants in the

system of traditional Indian medicinal plants. Malacca is also the most studied plant and is reported to contain secondary metabolite compounds such as tannins, alkaloids, and phenolics (Kamdod, 2012). According to Saxena et al. (2003), other secondary metabolites present in malacca are benzenoids, furanolaktans, diterpenoids, triterpenoids, flavonoids, sterols, and carbohydrates. Various flavonoid and terpenoid compounds have been reported to be active as antimalarials (Saxena et al., 2003).

Pinmai et al. (2010) reported that the infusion of malacca leaves showed antiplasmodium activity (IC50 14.37 g/ml) while Ravikumar et al. (2012) stated that the ethanol extract of malacca leaves showed activity as the most effective antiplasmodium (IC50 35.09 μg/ml).

The understanding of the biological phenomenon of P. falciparum increased with the discovery of the culture method in vitro. The calculation of the percentage of parasitemia in red blood cells is one way to find out the response of P. falciparum to antimalarial drugs and drug resistance. The calculation of parasitemia is carried out by manually calculating the number of red blood cells infected with parasites and the total number of red blood cells, using a light microscope. The process of calculating the percentage of parasitemia manually is a process that takes a long time and requires expertise to determine parasitemia in red blood cells (Sio et al., 2007; Ramadhani and Nurhayati, 2012).

MATERIALS AND METHODS

Malacca leaves were collected in Aceh Besar area, plant confirmation was carried out at the Herbarium of Biology, Faculty of Mathemathics and Natural Sciences, Universitas Syiah Kuala. Extraction was carried out at Pharmacology Laboratory of Faculty of Veterinary Medicine, Universitas Syiah Kuala. Plasmodium culture was carried out at the Health Research and Development Agency (Balitbangkes) Jakarta, and examination was carried out at the Clinical Laboratory of Faculty of Veterinary Medicine, Universitas Syiah Kuala.

The method used in this study was a complete randomized design with seven treatment groups, consisted of positive control (artesdiaquine), negative control (RPMI 1640 media), and malacca leaf ethyl acetate extract with doses of 100 μg / ml, 75 μg / ml, 50 μg / ml, 25 μg / ml and 5 μg / ml, respectively. Each treatment was conducted in quadruple.

Extraction of Malacca Leaf

The stems of malacca were cut, dried, then the leaves were collected, blended into powder, and sifted to a size of 40 mesh. Malacca leaf powder was weighed and macerated using ethyl acetate solution for 24 hours. The macerated solution was then filtered using filter paper, and the filtrate was evaporated using a vacuum rotary evaporator at 40 ºC. The extract obtained was then prepared for further analysis.

Preparation of Culture Media

RPMI media was prapared by mixing RPMI (Gibco 1640, powder) 10.4 g, HEPES 5.94 g, aquadest 1000 ml and stirred with a stirrer. Then gentamicin 50 mg / l was added and sterilized with 0.22 μm filter. Transport media was prepared by mixing 100 ml of RPMI media with 4.2 ml of NaHCO3 5%, then homogenized and sterilized using a 0.22 μm filter. Culture media (RPHS) was prepared by mixing RPMI media 100 ml, NaHCO3 4.2 ml and

11.5 ml human blood serum 10%, then sterilized with 0.45 μm filter.

Erythrocyte preparations

Blood type O was prepared in a tube containing cpd anticoagulants, and homogenized slowly. The blood was put in a 15 ml centrifuge tube and centrifuged at 1500 rpm for 15 minutes. The supernatant was discarded and the pellets were washed two times by centrifugation at 1500 rpm for 15 minutes. Then the supernatant was discarded and the pellets were mixed with RPHS (1:1), and stored in the refrigerator.

Culture preparation

The stock of P. falciparum was thawed using a water-bath, and then transferred into a 15 ml centrifuge tube, and washed with a 3.5% NaCl solution with double volume of the stock. Then it was centrifuged for 10 minutes at 1500 rpm, the supernatant was discarded, and the pellets were washed using sterile AB blood serum of the same volume. The pellets were washed again with transport media 3 times, and then the hematocrit value was calculated.

Antiplasmodial Test of Malacca Leaf Extract

In this study, the life cycle of P. falciparum used was the ring stage. To obtain a ring stage, Plasmodium was synchronized using sorbitol solution. Antiplasmodial test was conducted using a well plate which was prepared with 10 μl of extract and 190 μl of culture medium which contained parasites with 5% parasitemia and 5% hematocrit. Artesdiaquin was used as positive control, and culture medium as negative control. The dosages of the extract were 100, 75, 50, 25, 5 μg/ml. The plate then covered and stirred gently to mix properly. The plate was put in a candle-jar (Trager and Jensen, 1976) and incubated at 37 ºC for 48 hours.

Giemsa Staining

A thin blood preparation was carried out on object glass using samples in each well plate. The blood was smeared on glass object, dried, washed with PBS, and fixated with glutaraldehyde, and dred on an incubator. Giemsa 5% was dripped and let sit for 30 minutes, then washed off with aqueous and dried.

Parasitemia counting

The number of parasitemia was calculated in 1000 erythrocytes using a light microscope OLYMPUS CX21FS1 with 1000x magnification. Parasitemia is calculated at trophozoite stage (ring stage and advanced stage) and schizont. The percentage of parasitemia and resistance were calculated using the following formula:

$$\text{Percentage of Parasitemia} = \frac{\text{The number of parasitemia in 1000 erythrocyte}}{1000} \times 100\%$$

$$\text{Inhibition Percetage} = 100\% - \frac{(Xe - 100\%)}{Xk}$$

Xe= percent of the average growth of parasites given a specific dose of test material
Xk= percent of the average growth of parasites on negative control.

Data Analysis

The data were analyzed using Analysis of Variance (ANOVA) and continued with Duncan test (Gasperzs, 1997). Determination of IC50 value was carried out using SPSS.

RESULTS AND DISCUSSION

Observation of a thin blood smear under a microscope showed the difference in normal and infected erythrocytes. Plasmodium infected erythrocytes in stage of trophozoites (ring stage and advanced stage) and schizont.

The percentage of parasitemia and the average inhibition of plasmodium growth after treated with malacca leaf extracts can be seen in Table 1. Malacca leaf extract at 25 μg/ml had the lowest percentage of parasitemia and had the highest inhibition percentage. The results of ANOVA test revealed that each treatment was significantly different ($p<0.05$). Duncan test results showed that positive control was not significantly different from malacca leaf extract at 100 μg/ml, 75 μg/ml, 50 μg/ml, and 25 μg/ml, but significantly different from malacca leaf extract at 5 μg/ml and negative control.

Table 1. Percentage of parasitemia and average inhibition of Plasmodium falciparum after treatment with ethyl acetate extract of malacca leaves (±SD)

Malacca leaf extract	Percentage of Parasitemia (%)	Inhibition percentage (%)
Positive control	0.85±0.24^{a}	83.00±4.75^{a}
100 μg/ml	1.3±0.002^{b}	73. 75±3.97^{b}
75 μg/ml	1.1±0.002^{ab}	76.75±3.92^{ab}
50 μg/ml	1.2±0.002^{ab}	78.75±3.41^{ab}
25 μg/ml	1.0±0.002^{ab}	79.75±4.20^{ab}
5 μg/ml	4.2±0,009^{c}	15.75±4.69^{c}

a,b,cDifferent superscripts on the same column showed significant differences ($P<0.05$)

The lowest percentage of parasitemia was observed at 25 μg/ml and the highest was observed at 5 μg/ml. Higher doses of malacca leaf extract did not show better effect in inhibition activity. This was probably due to erythrocyte lysis caused by high saponin in the extract. Lysis of erythrocyte could affect the normal erythrocyte calculated which led to decreasing percentage of parasitemia. (Widodo and Rahayu, 2010).

Pouplin et al., (2007) stated that an extract had antiplasmodium property if the inhibition activity was more than 30%. To determine the relationship between concentration and the amount of inhibitory activity to the growth of P. falciparum, it was analyzed using probit analysis using SPSS to obtain IC50 value. The lower dosage of IC50, the more effective of inhibition activity of the extract.

Probit analysis showed that IC50 value of malacca leaf extract against P. falciparum was 17,849 μg/ml. The antiplasmodium activity is considered the best if IC50 is ≤ 10 μg / ml, good activity when the IC50 value is between 10-50 μg / ml, and considered not good if the IC50 ≥ 50 μg / ml (Gessler,

1994). Based on the statement, the ethyl acetate extract of malacca leaf was in good category where 17,849 µg / ml extract could inhibit 50% of the growth of P. falciparum.

Various flavonoid and terpenoid compounds have been reported to be active as antiplasmodium (Saxena et al., 2003). Flavonoid compounds had potent antiplasmodium activity, through inhibition of hemoglobin degradation and heme detoxification as well as other mechanisms that are not yet known (Nindatu, 2008). Meanwhile, terpenoid group compounds have been known to inhibit the growth of Plasmodium berghei by inhibiting protein synthesis in mammalian cells and also malaria parasites (Pouplin et al., 2007). Alkaloid group compounds have been known to inhibit the growth of parasites by blocking the growth of parasites through choline intracellular transport (Hilou et al., 2006).

The anti-plasmodium activity of malacca leaf extract is likely due to its bioactive compounds such as flavonoids, terpenoids, alkaloids and steroids. Nevertheless, more studies are required to find out the duration of the extract to work properly, and to screen bioactive compounds which play roles as anti-plasmodium.

CONCLUSION

Ethyl acetate extract of malacca leaf (Phyllanthus emblica) had the ability to inhibit the growth of Plasmodium falciparum. The anti-plasmodium activity was categorized as good with an IC50 value was 17,849 µg / ml.

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