

THE EFFECT OF MALACCA LEAVES (*Phyllanthus emblica*) ETHANOLIC EXTRACT ON *Plasmodium falciparum* GROWTH IN VITRO

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ABSTRACT

The aim of this research was to find out in vitro antiplasmodium activity of malacca leaves (*Phyllanthus emblica*) ethanolic extract against *Plasmodium falciparum* growth. In this study, Plasmodium culture contained 5% parasitemia in ring stage was cultured using candle jar method and antiplasmodial activity test was carried out using microculture. The treatments were divided into 7 groups with four repetitions. K₁ as negative control group was given Roswell Park Memorial Institute (RPMI), while K₂ as positive control group was given artesdiaquine. Groups K₃, K₄, K₅, K₆, and K₇ were added with 100 µg/mL, 75 µg/mL, 50 µg/mL, 25 µg/mL, and 5 µg/mL of malacca leaves ethanolic extract, respectively. Antiplasmodial activity was determined by inhibition concentration of 50% parasite growth (IC₅₀). The data were analyzed using ANOVA and followed by Duncan test. The average of parasitemia level in group K₁, K₂, K₃, K₄, K₅, K₆, and K₇ were 55.25±15.62, 8.50±2.52, 8.50±3.00, 9.25±0.95, 9.00±2.70, 9.79±2.06, and 10.75±2.22, respectively. The average of inhibition percentage in group K₁, K₂, K₃, K₄, K₅, K₆, and K₇ were 0.00±0.00%, 84.62±4.55%, 84.62±5.43%, 83.26±1.73%, 83.71±4.90%, 82.35±3.73%, and 80.54±6.83%, respectively (P<0.01). The results showed that the administration of malacca leaves ethanolic extract significantly affect (P<0.01) the inhibition of Plasmodium growth as compared to group K₁ (negative control). Probit analysis reveals the IC₅₀ value was 3.889 µg/mL. In conclusion, all doses of malacca leaves ethanolic extract used in this study was able to inhibit *Plasmodium falciparum* growth with IC₅₀ value was 3.889 µg/mL.

Key words: antiplasmodial, malaria, *Phyllanthus emblica*, *Plasmodium falciparum*

ABSTRAK

Penelitian ini bertujuan mengetahui pengaruh ekstrak etanol daun malaka (*Phyllanthus emblica*) terhadap pertumbuhan *Plasmodium falciparum* secara in vitro. Penelitian ini menggunakan kultur *Plasmodium stadium cincin* dengan konsentrasi parasitemia 5%. Kultur *Plasmodium* menggunakan metode candle jar dan uji aktivitas *Plasmodium* dilakukan dengan metode mikrokultur. Kelompok perlakuan dibagi menjadi 7 kelompok dengan 4 kali pengulangan. Kelompok K₁ sebagai kontrol negatif diberikan Roswell Park Memorial Institute (RPMI), sedangkan K₂ sebagai kontrol positif diberikan artesdiakuin. Kelompok K₃, K₄, K₅, K₆, dan K₇ masing-masing diberikan ekstrak etanol daun malaka dengan dosis 100, 75, 50, 25, dan 5 µg/ml. Aktivitas antiplasmodium dinyatakan dengan nilai inhibition concentration (IC₅₀) yaitu kemampuan menghambat 50% pertumbuhan *Plasmodium*. Data yang diperoleh dianalisis dengan analisis varians (ANOVA) dan dilanjutkan dengan uji Duncan. Rerata parasitemia pada K₁; K₂; K₃; K₄; K₅; K₆; dan K₇ masing-masing adalah 55,25±2,75; 8,50±2,52; 8,50±3,00; 9,25±0,96; 9,00±2,71; 9,75±2,06; dan 10,75±2,22. Rerata persentase hambatan pada K₁; K₂; K₃; K₄; K₅; K₆; dan K₇ masing-masing adalah 0,00±0,00%; 84,62% ±4,55; 84,62%±5,43; 83,26%±1,73; 83,71%±4,90; 82,35%±3,73; dan 80,54%±6,83 (P<0,01). Uji lanjut menunjukkan bahwa perlakuan K₂, K₃, K₄, K₅, K₆, dan K₇ berpengaruh sangat nyata (P<0,01) terhadap penghambatan pertumbuhan plasmodium dibandingkan dengan kelompok K₁. Hasil analisis probit menunjukkan bahwa nilai IC₅₀ sebesar 3,889 µg/ml. Disimpulkan bahwa semua dosis ekstrak etanol daun malaka dapat menghambat pertumbuhan *Plasmodium falciparum* dengan nilai IC₅₀ value adalah 3.889 µg/ml.

Kata kunci: antiplasmodium, malaria, *Phyllanthus emblica*, *Plasmodium falciparum*

INTRODUCTION

Malaria is an infectious disease caused by intracellular protozoa named Plasmodium, which is transmitted through the bite of a female Anopheles mosquito (Arsin, 2012). Malaria can cause death, especially in high-risk groups, i.e.: infants, toddlers, and pregnant women, it also directly cause anemia and reduce work productivity. In 2013, the national malaria prevalence rate was 6.0%, with 15 provinces having a prevalence above the national average including Aceh and the highest prevalence was found in Eastern Indonesia, i.e.: Papua (28.6%), East Nusa Tenggara (23.3%), West Papua (19.4%), Central Sulawesi (12.5%), and Maluku (10.7%) (Departemen Kesehatan RI, 2013).

The resistance of malaria treatment to conventional drugs such as chloroquine and sulfadoxine-pyrimethamine leads to further complicates efforts to eradicate malaria. The report on malaria treatment resistance to previous drugs (chloroquine, sulfadoxin-pyrimethamine, and quinine) occur in more than 25% of provinces in Indonesia in the last 10 years, therefore new steps are needed in the treatment of malaria (Harijanto, 2011). One of the solutions that should be taken is to conduct research to find a new drug substance in plants which inhibit or kill malaria-causing parasites. The fruit, leaves, stems or plant roots has been known to contain secondary metabolites which can be used as antimalaria.

Malacca (*Phyllanthus emblica*) is a plant that can cure various diseases (Khan, 2009; Kaur *et al.*, 2013).

Previous studies mentioned that malacca can be used as an anti-oxidant (Charoenteeraboon *et al.*, 2010), hepatoprotector (Malar and Mary, 2009), analgesic and antipyretic (James *et al.*, 2004), anti-cancer (Singh *et al.*, 2015), anti-inflammatory (Nicolis *et al.*, 2008; Dang *et al.*, 2011), and also can reduce blood glucose levels (Qureshi *et al.*, 2009). Pinmai *et al.* (2010) reported that malacca fruit extract has anti-Plasmodium activity.

The phytochemical content of malacca leaves ethanolic extract includes alkaloids, flavonoids, tannins, saponins, phenols, and glycosides (Ravikumar *et al.*, 2012). The extract of malacca leaf is also effective as chemo-protector (Singh *et al.*, 2011), anti-tumor (Ngamkitidechakul *et al.*, 2010; Sumalatha, 2013), antimicrobial, antibacterial, antifungal (Malliga *et al.*, 2015; Singh *et al.*, 2015), antiviral, antimutagenic, anti-allergic (Khopde *et al.*, 2001), and antimalaria (Ravikumar *et al.*, 2012). This study was designed to find out in vitro antiplasmodium activity of malacca leaves (*Phyllanthus emblica*) ethanolic extract against *Plasmodium falciparum* growth.

MATERIALS DAN METHODS

This study used a complete randomized design (CRD) with 7 treatment groups and 4 repetitions. The K1 group as a negative control was administered with Roswell Park Memorial Institute (RPMI), while group K2 as a positive control was administered with artesiasquine. Groups K3, K4, K5, K6, and K7 were administered with ethanolic extract of malacca leaves at doses of 100, 75, 50, 25, and 5 µg/mL, respectively.

Malacca Leaf Extraction

Malacca leaves used in this study were obtained from Aceh Besar area, which has been identified by Biological Herbarium of Mathematics and Natural Science Faculty, Universitas Syiah Kuala. Leaves that grow from the base to the tip of the branch were collected and air dried. The leaves were then cleaned from twigs and foreign objects, grinded and sieved with a 40 mesh sieve. As much as 1.5 kg malacca leaf powder was macerated using ethanol solvent for 24 hours until a clear solution was obtained. The macerated solution was filtered using filter paper, then the filtrate was collected and evaporated using a rotary evaporator until all the solvents evaporated and paste form extract was obtained. 10 mg ethanolic extract of malacca leaves was dissolved in 5 mL sterile double distilled water, homogenized and stored as a stock solution. The solution was then diluted into various doses of test compounds with the concentrations of 100, 75, 50, 25, and 5 µg/mL.

Medium Preparation

Roswell Park Memorial Institute media was made by mixing 10.4 g RPMI (Gibco1640, powder) and 5.94 g hydroxyethyl piperazineethanesulfonic acid (HEPES), then 1000 mL distilled water was added, stirred with the stirrer, and added 50 mg/L gentamycin.

The solution was then sterilized with a 0.22 µm filter, divided into 4 bottles and stored in a refrigerator. The transport medium was made by mixing 100 mL of RPMI media with 5% NaHCO₃, then homogenized and sterilized using a 0.22 µm filter. Roswell Park Human Serum (RPHS) culture medium was prepared from 100 mL RPMI medium, which was added with 4.2 mL of NaHCO₃ and 11.5 mL of AB blood type serum, then sterilized with a 0.45 µm filter and stored in a refrigerator.

Blood Serum Preparation

A total of 50 mL vein blood was collected and put into a sterile tube, then stored in an incubator at 37° C in an inclined state (forming a 15° angle) for 1-2 hours. Then, the blood was put into the refrigerator for 12 hours. The blood was centrifuged at a speed of 1500 rpm for 10 minutes. The serum taken and inserted in a test tube, then inactivated in a water bath at a temperature of 56° C for 1 hour. The serum was stored at -20° C until it was ready to use.

Preparation of Fresh Erythrocyte

A total of 10 mL of fresh blood was collected into a tube that had been given citrate phosphate dextrose (CPD) anticoagulant, then homogenized slowly and centrifuged at 1500 rpm for 15 minutes. The supernatant was discarded, and the sediment (blood objects) was washed with transport media 2 times, then shaken slowly and centrifuged for 15 minutes at a speed of 1500 rpm. After that, the supernatant was removed and the precipitate was mixed with RPHS (1:1) and stored in a refrigerator.

Culture Preparation

Plasmodium falciparum stock was thawed by placing in waterbath or rubbed by hand. Then it was put into a 15 mL centrifuge tube and washed with 3.5% NaCl, centrifuged for 10 minutes with a speed of 1500 rpm. The supernatant was removed and the pellet obtained was washed with AB blood serum. Subsequently, the pellet was washed again with transport media 3 times. Finally, the package cell volume (PCV) was calculated.

Antiplasmodium Potential Test for Malacca Leaves Extract

Plasmodium falciparum culture used was at ring stage with 5% parasitemia concentration and 5% hematocrit. The ring stage was obtained by synchronizing using a 5% sorbitol solution. Each well plate was filled with 10 µL of test extract and 190 µL of culture medium containing Plasmodium at ring stage. Negative control was given RPMI, positive control was given artesiasquine, and the other five groups given ethanolic extract of malacca leaves with the dose of 100, 75, 50, 25, and 5 µg/mL. The plate was subsequently closed and stirred slowly to mix perfectly. Finally, the plate was put in a candle jar and incubated at 37° C for 48 hours.

Giemsa Staining

Thin blood smear was carried out on the object glass for each plate. Then dried, washed with PBS, and fixed with glutaraldehyde, and dried in an incubator. Then, 5% Giemsa was added and let dry for 30 minutes, washed with distilled water and dried.

Parasitemia Count

The amount of parasitemia was observed and counted under a microscope with 1000x magnification (Sardjono and Fitri, 2007). The percentage of parasitemia and the percentage of the inhibition were obtained using the following formula:

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

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

X_e = Percentage of the average growth of parasites in each treatment

X_k = Percentage of the average growth of parasites in negative control.

Data Analysis

The data obtained were analyzed using one way analysis of variance (ANOVA) and continued with Duncan multiple range tests. Determination of IC50 values was performed by probit analysis.

RESULTS AND DISCUSSION

The number of parasitemia in group K1, K2, K3, K4, K5, K6, and K7 are shown in Table 1. Based on Table 1, the mean of parasitemia numerically increased as the dose of malacca leaves ethanolic extract decreased. The highest mean of parasitemia was observed in K7 which given the lowest concentration of malacca leaf extract, while the lowest mean of parasitemia was observed in K3 which given the highest dose of the extract. The results showed that all the treatments were significantly different ($P < 0.01$)

Table 1. Mean of parasitemia in 1000 erythrocytes

Groups	Mean (\pm SD)
K1 (Negative control)	55.25 \pm 2.75 ^a
K2 (Positive control)	8.50 \pm 2.52 ^b
K3 (Ethanolic extract of malacca leaves dose 100 μ g/mL)	8.50 \pm 3.00 ^b
K4 (Ethanolic extract of malacca leaves dose 75 μ g/mL)	9.25 \pm 0.96 ^b
K5 (Ethanolic extract of malacca leaves dose 50 μ g/mL)	9.00 \pm 2.71 ^b
K6 (Ethanolic extract of malacca leaves dose 25 μ g/mL)	9.75 \pm 2.06 ^b
K7 (Ethanolic extract of malacca leaves dose 5 μ g/mL)	10.75 \pm 2.22 ^b

^{a,b}Different superscripts within the same column indicate significant differences ($P < 0.01$)

Table 2. Percentage of parasitemia and inhibition of *Plasmodium falciparum* after given ethanolic extract of malacca leaves in vitro

Groups	Parasitemia (%)	Inhibition (%)
K1 (Negative control)	5.25 \pm 0.28	0.00 \pm 0.00
K2 (Positive control)	0.85 \pm 0.25	84.62 \pm 4.55
K3 (Ethanolic extract of malacca leaves dose 100 μ g/mL)	0.85 \pm 0.30	84.63 \pm 5.43
K4 (Ethanolic extract of malacca leaves dose 75 μ g/mL)	0.93 \pm 0.10	83.26 \pm 1.73
K5 (Ethanolic extract of malacca leaves dose 50 μ g/mL)	0.90 \pm 0.27	83.71 \pm 4.90
K6 (Ethanolic extract of malacca leaves dose 25 μ g/mL)	0.98 \pm 0.21	82.35 \pm 3.73
K7 (Ethanolic extract of malacca leaves dose 5 μ g/mL)	1.08 \pm 0.22	80.54 \pm 6.83

from negative control. The results of Duncan test showed that K1 (negative control) was significantly different ($P < 0.01$) from K2, K3, K4, K5, K6, and K7, while K3 was not significantly different ($P > 0.05$) from K2, K4, K5, K6, and K7. These results indicate that there are no significant differences among treatment groups administered with ethanolic extract of malacca leaves.

The percentage of parasitemia dan inhibition rate of Plasmodium are presented in Table 2. The inhibition rate of Plasmodium by ethanolic extract of malacca leaves increased with the increasing dose given, except K4 which decreased 0.5% from K5. The highest inhibition rate was 84.62 \pm 5.43% found in K3 (100 μ g/mL), while the lowest inhibition was 80.54 \pm 6.83% found in K7 (5 μ g/mL). The group which was given 100 μ g/mL ethanolic extract (84.63 \pm 5.43%) had similar ability compared to group given artesdiaquine (84.62 \pm 4.55%) in inhibiting Plasmodium growth.

The results showed that the highest the dose of extract that have been used, the highest the inhibition of Plasmodium growth. All doses of malacca leaves ethanolic extract used in this study showed the inhibition rate more than 80% towards Plasmodium growth. Pouplin *et al.* (2007) stated that an extract is considered to have an antiplasmodium properties if it can inhibit more than 30% parasite, indicated that all doses of malacca leaves ethanolic extract used in study have antiplasmodium properties.

To assess the relationship between the increased of concentration and the inhibitory activity on the growth of *Plasmodium falciparum*, the percentage of inhibition data was analyzed using probit analysis to obtain the value of inhibition concentration (IC50). The results of IC50 analysis showed that the ability of ethanolic extract of malacca leaves concentration in inhibiting 50% *Plasmodium falciparum* growth in erythrocytes in vitro was found at a dose of 3.889 μ g/mL.

Ravikumar *et al.* (2012), reported that the antiplasmodium activity of the test substance in vitro was divided into 4, i.e: test substances with very active activity if IC50 < 5 μ g/mL, active activity when IC50

values were between 5-50 µg/mL, very low activity if IC₅₀ values are 50-100 µg/mL, and not active if IC₅₀>100 µg/mL. Based on these findings, it can be concluded that the administration of ethanolic extract of malacca leaves has a very active activity in inhibiting the growth of *Plasmodium falciparum* with IC₅₀ value was 3.889 µg/mL. The IC₅₀ value obtained in this study was different from which was reported by Ravikumar et al. (2012), which found the IC₅₀ was 35.09 µg/mL. This difference is likely to occur due to differences in the area where malacca grows and the method of extraction used.

Malacca leaves contains secondary metabolites, such as alkaloids, flavonoids, tannins, saponins, phenols, glycosides, or carbohydrates which have ability to inhibit the growth of Plasmodium (Dhale and Mogle, 2011; Ravikumar et al., 2012). According to Tasdemir et al. (2007), plant secondary metabolites, such as alkaloids, can inhibit biosynthesis of fatty acid and inhibit the biocrystallization of hemozoin in *Plasmodium falciparum* (Dubar et al., 2010). Carbohydrates also can inhibit the invasion of Plasmodium merozoites into erythrocytes (Adams et al., 2005), and interfere with the formation of the *Plasmodium falciparum* rosette (Rowe et al., 1994).

Plant secondary metabolite compound also have antiplasmodium activity which increase the oxidation of red blood cells or inhibit protein synthesis (Phillipson and Wright, 1991). According to Rinidar et al. (2013), the in vitro antiplasmodium activity occur probably caused by the presence of terpenoids in plants which are thought to prevent the division of the trophozoite nucleus into schizont in Plasmodium after incubated for 24-36 hours. However, the most active phytochemical compounds in malacca leaves that act as antiplasmodium and its mechanism have not been evaluated in this study. Therefore, further study is needed to find out the mechanism of malacca metabolites in vivo.

CONCLUSIONS

All doses of malacca leaves ethanolic extract used in this study were able to inhibit *Plasmodium falciparum* growth with IC₅₀ value was 3.889 µg/mL.

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