IN VITRO ANTIMALARIAL POTENCY OF ELEUTHERINE BULBOSA AND ITS EFFECT ON THE MORPHOLOGY OF PLASMODIUM FALCIPARUM

LUH SESOTYANING N1, MELVA LOUISA2, HENDRI ASTUTY3*, DIN SYA'RUDIN4, PUJI BUDI SETIA ASIH4

1Department of Biomedical Science, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia. 2Department of Pharmacology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia. 3Department of Parasitology, Faculty of Medicine, Universitas Indonesia, Jakarta, Indonesia. 4Malaria and Vector Resistance Unit, Eijkman Institute for Molecular Biology, Dr. Cipto Mangunkusumo National General Hospital, Jakarta, Indonesia. Email: astutiek@yahoo.com

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ABSTRACT

Objective: This study aimed to determine the potential inhibitory effects of Eleutherine bulbosa on the growth of Plasmodium falciparum and its toxic effects on lymphocyte cells.

Methods: We performed a 50% inhibition concentration (IC_{50}) test of E. bulbosa against P. falciparum, used transmission electron microscopy (TEM) to observe Plasmodium morphology after exposure to E. bulbosa, and determined E. bulbosa’s cytotoxic concentration 50% (CC_{50}) against lymphocytes.

Results: The IC_{50} value of E. bulbosa was 0.62 μg/ml. We observed crystals in the mitochondria of parasites under TEM. CC_{50} values at 24 h and 72 h were 85.14 μg/ml and 707.94 μg/ml, respectively.

Conclusion: The main content of E. bulbosa is naphthoquinones that are suspected of having a mitochondrial target on the malaria parasite. E. bulbosa is not toxic to lymphocytes; therefore, it has a potential as an antimalarial therapeutic.

Keywords: Eleutherine bulbosa, Antimalarial, Toxicity, 50% Inhibition concentration.

INTRODUCTION

Malaria is a life-threatening disease that has killed many children aged <5 years and pregnant women [1]. Globally, the number of malaria cases decreased by 18% from 262 million cases in 2000 to 214 million cases in 2015, whereas the mortality rate decreased by 48% from 839,000 cases in 2000 to 438,000 cases in 2015 [2]. Although malaria prevalence appears to be decreasing, the emergence of parasites that are resistant to antimalarial drugs and vector resistance to insecticides does threaten this trend. Therefore, drug efficacy should be monitored periodically because new therapies should be developed [3].

Currently, the standard method for malaria treatment in Indonesia is artemisinin-based combination therapy [4]. However, the strains of Plasmodium falciparum which are resistant to artemisinin have been detected in five countries in the Melong region, including Cambodia, Laos, Myanmar, Thailand, and Vietnam [2]. To overcome the problem of resistance to antimalarial drugs, looking at the history of the discovery of quinine from the cinchona tree and artemisinin from Artemisia annua L. (Asteraceae) may lead to the discovery of new antimalarial drugs derived from plants [5].

Research to obtain new antimalarial drugs, either synthetic or sourced from natural ingredients, from plants is currently being performed. A study conducted by Widawatyawanty and Zaini reported that dichloromethane extract obtained from Artocarpus champeuden stem bark could inhibit the growth of P. falciparum with an IC_{50} of 0.99 μg/ml. This plant contains compounds such as flavonoids that have antimalarial activity. Flavonoids are suspected to be able to inhibit the growth of P. falciparum through the following two main mechanisms: (1) New permeation pathways, by inhibiting the nutrient transport for parasites, and (2) inhibiting hemoglobin degradation and heme detoxification in parasite food vacuoles [6]. In another study that used methanol extract obtained from Averrhoa bilimbi L. leaves, it was reported to be able to inhibit the growth of P. falciparum with an IC_{50} of 2.805 μg/ml. A. bilimbi L. leaves contain many luteolin compounds, which are flavonoids. Luteolin shows potential in inhibiting the fatty acid biosynthesis I (FabI) Plasmodium enzyme. The FabI enzyme is a part of fatty acid synthase Type II, also known as enoyl-ACP reductase, and is involved in the final stage of the elongation of the Plasmodium fatty acid chain [7].

Eleutherine bulbosa (Miller) Urban is a plant belonging to the Iridaceae family that originates from South America; this plant is widely grown in Asia and Africa [8]. In Indonesia, this plant is known as dayak onion and is planted in Kalimantan, where the local population uses it as a traditional medicine for the treatment of breast cancer, heart disease, tumors, inflammation, and bleeding and as an immunostimulant [9]. E. bulbosa tubers are known to contain many naphthoquinones and its derivatives such as eucalenine, eukellerin, eleutherine, and eleutherone. In addition, E. bulbosa tubers contain alkaloids, steroids, glycosides, flavonoids, phenolics, saponins, triterpenoids, tannins, and quinones [10]. Allegedly, the mechanism of the inhibition of P. falciparum growth is similar to that of atovaquone, an antimalarial drug containing hydroxynaphthoquinones, because the main content of E. bulbosa comprises compounds belonging to the naphthoquinone group. Hydroxynaphthoquinones act as competitive inhibitors of ubiquinol, especially by inhibiting the electron transport of Plasmodium mitochondria in the bc_{1} complex [11]. Studies on E. bulbosa as an antibiotic and antinecancer drug have been widely conducted, but those on E. bulbosa as an antimalarial agent are rare. Therefore, we are interested in examining E. bulbosa as an antimalarial agent by testing its antimalarial activity with a 50% inhibition concentration test (IC_{50}) analyzing the inhibition target of E. bulbosa through the observation of Plasmodium morphology with transmission electron microscopy (TEM), and testing its toxic effects against lymphocyte cells with a 50% cytotoxic concentration (CC_{50}) test.
METHODS

Preparation of sample
The sample used was E. bulbosa which was obtained from the Department of Pharmacology, Faculty of Medicine, University of Indonesia. Dimethyl sulfoxide was added to the sample, which was then vortexed until completely dissolved.

Preparation of P. falciparum parasite culture
The P. falciparum parasite used was from the 3D7 strain, originated from Tokyo University, Japan (routinely cultured at the Eijkman Molecular Biology Institute). The parasite was cultured in Roswell Park Memorial Institute 1640 medium containing 10% AB serum until the percentage of parasitemia reached 2% with a hematocrit level of 3%. The culture was performed using the Trager and Jensen procedure.

IC₅₀ test
The test was performed using a 96-microwell plate; 180 µl of parasite culture was added to each well. The first well containing the control sample was not given any treatment. In the last well, 20 µl of the sample was added and gradual dilution performed up to second well. The microwell plate was inserted into a jar and kept in an incubator at 37°C for 48 h. After incubation, the plate was taken out of the jar, the medium was removed, and a thin blood smear was made from the sediment, followed by staining with 10% Giemsa. The percentage of parasitemia was calculated using a light microscope with 1000× magnification. To determine the IC₅₀, a graph was created using Microsoft Excel.

Preparation of TEM sample
TEM samples used parasite cultures with 5% parasitemia and 3% hematocrit level. Overall, 900 ml of the parasite culture was added into each well of the 24-microwell plate. The first well containing the control sample was not treated; to the second well, 20 µl of the E. bulbosa sample with IC₅₀ concentration was added. Then, it was incubated in a culture jar at 37°C for 48 h. After the incubation period, the solution was transferred into a 1.5 ml tube and centrifuged to remove the supernatant. The sediment was washed by adding 500 µl of cacodylate buffer and homogenized. It was then left to stand for 15 min at 4°C and shaken using the shaker, followed by centrifugation. The washing process was repeated 3 times, followed by incubation of the homogenate for 48 h at 4°C with shaking.

Preparation of TEM block sample
At this stage, the sediment was added with 500 µl of glutaraldehyde (2.5%) and then homogenized and stored for 48 h at 4°C with shaking. It was then centrifuged at 3000 rpm for 5 min, the supernatant was removed, and the sediment was washed with 500 µl of buffer cacodylate 0.1 M + 3% sucrose, shaken for 15 min at 4°C centrifuged at 3000 rpm for 5 min, and then washed 3 times. Then, the sediment was supplemented with 500 µl 2% osmium tetroxide and 2.5% K₂Fe(CN)₆, incubated for 2 h at 4°C with shaking, and then washed with cacodylate buffer 0.1 M and 3% sucrose 3 times. Dehydration was performed using gradual concentration ethanol. After the dehydration stage, the sediment was infiltrated by adding 500 µl of pure propylene oxide and incubated for 1 h at room temperature. Furthermore, the processes of embedding, cutting, and coloring were performed to prepare the sample for observation by TEM.

Isolation and preparation of lymphocyte cells
Blood was added into a tube containing ethylenediaminetetraacetic acid; the lymphocyte cells were isolated using Ficoll gradient reagent. The lymphocyte cell sediment was supplemented with 1 ml of complete medium (Dulbecco’s Modified Eagle Medium, 10% fetal bovine serum, and 1% Penicillin-Streptomycin) and stained with trypan blue; the lymphocyte cell number was calculated with a hemocytometer. The lymphocyte cell solution was prepared with a density of 100,000 cells/200 µl.

IC₅₀ test
Five wells (96-microwell plates) were inoculated with 180 µl of lymphocyte cell stock solution. The well 1 (control) was left untreated. To wells 2, 3, 4, and 5, 20 µl of E. bulbosa with concentrations of 10⁻², 10⁻³, 10⁻⁴, and 10⁻⁵ was added, respectively. Homogenization was then carried out. Two plates were made along with a duplicate for each plate. Plate 1 was incubated in an incubator at 37°C, 5% for 24 h, whereas plate 2 was incubated for 72 h. After the incubation period, the solution from each well was transferred into 1.5 ml tubes and centrifuged at 12000 rpm for 10 min; the supernatant was removed, 200 µl PBS was added, and the sample was homogenized. Next, 10 µl of solution was taken and stained with trypan blue; the living and dead cells were enumerated using hemocytometer. The percentage cell viability was calculated using the following formula:

Number of living cells/total number of cells × 100%.

RESULTS

Analysis of the potential of E. bulbosa in the inhibition of P. falciparum growth
The result of the IC₅₀ test of E. bulbosa on the growth of P. falciparum is presented in Table 1 and Fig. 1.

| Concentration (µg/ml) | % Parasitemia | % Growth | % Inhibition |
|----------------------|---------------|----------|-------------|
| 0                    | 3.8           | 100.0    | 0.0         |
| 3.367 × 10⁻²          | 2.8           | 74.3     | 25.7        |
| 3.000367              | 2.5           | 65.9     | 34.1        |
| 0.003367              | 2.3           | 61.1     | 38.9        |
| 0.03367               | 2.1           | 54.9     | 45.1        |
| 0.3367                | 2.0           | 52.2     | 47.8        |
| 3.367                 | 1.7           | 46.0     | 54.0        |
| 33.67                 | 1.3           | 35.0     | 65.0        |
| 336.7                 | 0.7           | 18.6     | 81.4        |
| 3367                  | 0.0           | 0.0      | 100.0       |

Table 1: IC₅₀ of E. bulbosa

IC₅₀: 50% inhibition concentration, E. bulbosa: Eleutherine bulbosa
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Fig. 1: A 50% inhibition concentration graph of the effect of *Eleutherine bulbosa* on *Plasmodium falciparum*

Fig. 2: The morphology of *Plasmodium falciparum* (control group). The mitochondria of the parasite can be seen with crystals in it (blue arrow)

Fig. 3: The morphology of *Plasmodium falciparum* after exposure to *Eleutherine bulbosa* at a concentration of 0.62 µg/ml for 48 h. The mitochondria are seen without crystals (m)

### Table 2: CC$_{50}$ of *E. bulbosa* to lymphocyte cells after 24 h

| Concentration (µg/ml) | Cell viability (%) |
|-----------------------|--------------------|
| 0                     | 100                |
| 0.003367              | 100                |
| 0.03367               | 99.165             |
| 3.367                 | 97.48              |
| 33.67                 | 89.78              |

CC$_{50}$: 50% cytotoxic concentration, *E. bulbosa*: *Eleutherine bulbosa*

### Table 3: CC$_{50}$ of *E. bulbosa* at higher concentrations to lymphocyte cells after 24 h

| Concentration (µg/ml) | Cell viability (%) |
|-----------------------|--------------------|
| 1347                  | 37.5               |
| 2020                  | 30.56              |
| 2694                  | 23.08              |
| 3367                  | 21.62              |

CC$_{50}$: 50% cytotoxic concentration, *E. bulbosa*: *Eleutherine bulbosa*

### Table 4: CC$_{50}$ of *E. bulbosa* to lymphocyte cells after 72 h

| Concentration (µg/ml) | Cell viability (%) |
|-----------------------|--------------------|
| 0                     | 95.09              |
| 0.0003367             | 93.75              |
| 0.03367               | 92.23              |
| 3.367                 | 90.64              |
| 33.67                 | 84.865             |

CC$_{50}$: 50% cytotoxic concentration, *E. bulbosa*: *Eleutherine bulbosa*

3.367 × 10$^{-4}$ µg/ml, respectively. After incubation for 72 h, the percentages of cell viability were 4.62%, 17.02%, 21.74%, and 23.91% for the abovementioned concentrations, respectively. The CC$_{50}$ value after 24 h was 851.14 µg/ml and after 72 h was 707.94 µg/ml (Tables 2-5 and Figs. 4 and 5).

**DISCUSSION**

Many studies have used *E. bulbosa* as an antibiotic and anticancer agent, but research on *E. bulbosa* as an antimalarial is still very rare. Therefore, this study was conducted to determine the antimalarial potential.
work mechanism, and toxicity effect of *E. bulbosa*. To determine the antimalarial potential of this plant, a IC50 test was conducted. In other studies, experiments have been conducted with methanol extract of srikaya leaves (*Annona squamosa*) as antimalarial agents, and the IC50 value was found to be 2 μg/ml; an IC50 value of 4.9 μg/ml was obtained with the Zanthoxylum zanthoxyloides extract [13] and 22 μg/ml with the root chloroform extract was obtained from *Andrographis paniculata*. According to Basco et al. and Dolabela et al., criteria of in vitro antimalarial activity can be divided as follows: IC50 < 10 μg/ml which denotes good activity, IC50 10–50 μg/ml which denotes medium activity, IC50 50–100 μg/ml which denotes low activity, and IC50 > 100 μg/ml which denotes no antimalarial activity [7]. According to these criteria, *E. bulbosa* confers good antimalarial activity. Compared with the IC50 value of the dichloromethane extract obtained from *A. champeden* stem bark (IC50 = 0.99 μg/ml), it is evident that antimalarial activities of *E. bulbosa* are higher (IC50 = 0.62 μg/ml).

One way to understand the mechanism of a drug or a compound is to observe the cell morphology of cells affected by the substance using a transmission electron microscope (TEM). TEM has been shown to be useful in the analysis of all cellular components, including the cytoskeleton, membrane system, organelles, cilia, and the special structures involved in cellular development, such as microvilli and synaptonemal complexes [14]. In this study, the morphology of malarial parasites was observed using culture samples of *P. falciparum* that had been exposed to *E. bulbosa* at a concentration of IC50 that was equal to 0.62 μg/ml and compared with cells that had been exposed...

![Fig. 4](image1.png)

**Fig. 4:** A 50% cytotoxic concentration graph of *Eleutherine bulbosa* to lymphocyte cells after 24 h

![Fig. 5](image2.png)

**Fig. 5:** A 50% cytotoxic concentration graph of *Eleutherine bulbosa* to lymphocyte cells after 72 h
to a negative control sample. In the control samples, a mitochondrial form of *Plasmodium* with crystals was observed, whereas, in the treated samples, *Plasmodium* mitochondria without crystals were observed. This occurred possibly as a result of the inhibition of electron transport to the parasite mitochondria. *E. bulbosa* contains many naphthoquinone compounds, and these compounds have been shown to have antimalarial activity. A synthetic compound of the naphthoquinone group, hydroxynaphthoquinone, has been shown to have an antimalarial activity with a mechanism of inhibiting the electron transport of *Plasmodium* mitochondria in complex bc₁ [11]. From the parasite mitochondrial observations, it was seen that the target of *E. bulbosa* was on the parasite mitochondria.

**CONCLUSION**

*E. bulbosa* has potential as an antimalarial agent with an IC₅₀ value of 0.62 μg/ml with an inhibition target in the parasite mitochondria. The concentration of *E. bulbosa* cytotoxic to lymphocyte cells at 24 h incubation was found to be 851.14 μg/ml and at 72 h incubation was 707.94 μg/ml.

**CONFLICTS OF INTEREST**

All authors have none to declare.

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