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Research Article

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

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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 851.14 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.

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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 Mekong 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 Widyawaruyanti and Zaini reported that dichloromethane extract obtained from *Artocarpus champeden* 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 elecanacine, eleutherol, eleutherine, and eleutherinone. 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 anticancer 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.

IC_{50} 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 candle and kept in an incubator at 37°C for 48 h. After incubation, the plate was taken out of the candle 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 IC_{50}, a graph was created using Microsoft Excel.

Preparation of TEM sample

TEM samples used parasite cultures with 5% parasite 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 IC_{50} concentration was added. Then, it was incubated in a candle 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% $K_3Fe(CN)_6$, 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.

CC_{50} 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^{-4}, 10^{-6}, and 10^{-8} 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, CO_2 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 1200 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 IC_{50} test of *E. bulbosa* on the growth of *P. falciparum* is presented in Table 1 and Fig. 1.

The IC_{50} concentration of *E. bulbosa* was determined to be 0.62 µg/ml.

Observation of *P. falciparum* morphology under TEM

The morphological differences of untreated and treated *P. falciparum* parasites are observed in Figs. 2 and 3.

Analysis of the toxicity effect of *E. bulbosa* on lymphocyte cells

In the toxicity test of *E. bulbosa*, lymphocyte cells were used because blood is exposed to chemical compounds/drugs consumed. Therefore, lymphocyte cells are often used by researchers to determine the toxic effects of a new chemical and drug compound [12].

In this test, lymphocyte cells with a density of 100,000 cells per well were incubated with *E. bulbosa* for 24 and 72 h. These two different incubation conditions were used to determine whether the toxic effects were affected only by the magnitude of the compound concentration or also affected by the duration of exposure to the compound. The concentrations of *E. bulbosa* used were 336.7 µg/ml, 3.367 µg/ml, 3367×10^{-5} µg/ml, and 3367×10^{-7} µg/ml. For these concentrations, the cell viability percentages obtained after incubation for 24 h were 89.78%, 97.48%, 99.165%, and 100%, respectively. From the results, the CC_{50} value was not obtained because the highest cell viability percentage obtained was 89.78%. Incubation for 72 h with the same concentrations of *E. bulbosa* resulted in the following cell viability percentages of 84.865%, 90.64%, 92.23%, and 93.75%, respectively. At the highest concentration of 336.7 µg/ml, the percentage of cell viability obtained was 84.865. By comparing the means of the 24-h and 72-h incubation groups by *t*-test using Microsoft Excel, P value obtained was 0.02 ($p<0.05$); this denoted that there was a significant difference between the two groups. The test was repeated with higher concentrations of 3367 µg/ml, 2694 µg/ml, 2020 µg/ml, and 1347 µg/ml because CC_{50} had not been determined. The cell viability percentages after incubation for 24 h were 21.62%, 23.08%, 30.56%, and 37.5% for concentrations 336.7 µg/ml, 3.367 µg/ml, 3367×10^{-5} µg/ml, and

Table 1: IC_{50} of *E. bulbosa*

Concentration (µg/ml)	% Parasitemia	% Growth	% Inhibition
0	3.8	100.0	0.0
3.367×1^{-5}	2.8	74.3	25.7
0.0003367	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

IC_{50}: 50% inhibition concentration, *E. bulbosa: Eleutherine bulbosa*

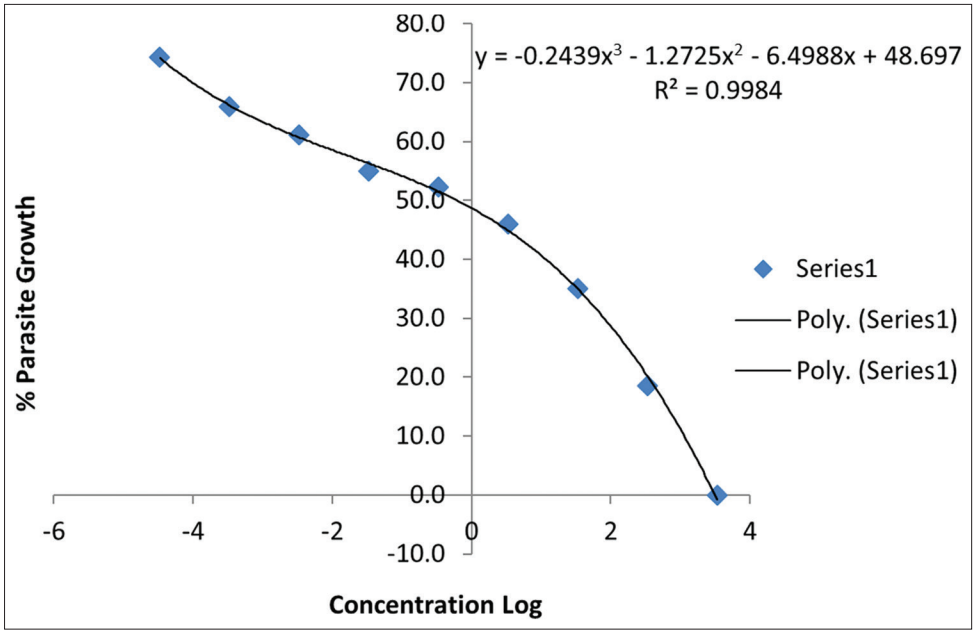

Fig. 1: A 50% inhibition concentration graph of the effect of *Eleutherine bulbosa on Plasmodium falciparum*

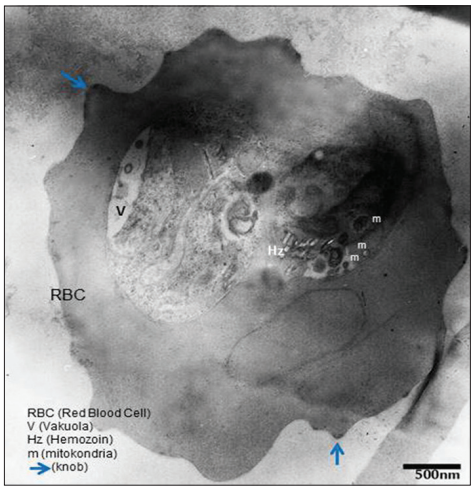

Fig. 2: The morphology of *Plasmodium falciparum* (control group). The mitochondria of the parasite can be seen with crystals in it (blue arrow)

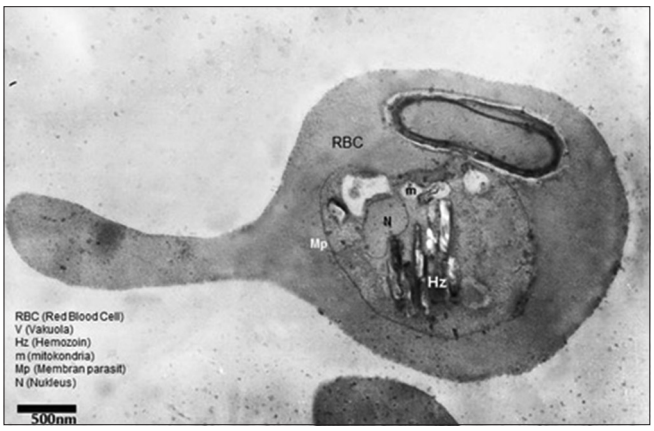

Fig. 3: The morphology of *Plasmodium falciparum* after exposure to *Eleutherine bulbosa* at a concentration of 0.62 µg/ml for 48 h. The mitochondria are seen without crystals (m)

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
336.7	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
336.7	84.865

CC_{50}: 50% Cytotoxic concentration, *E. bulbosa: Eleutherine bulbosa*

3367×10^{-7} 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 IC_{50} test was conducted. In other studies, experiments have been conducted with methanol extract of srikaya leaves (*Annona squamosa*) as antimalarial agents, and the IC_{50} value was found to be 2 µg/ml; an IC_{50} 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: IC_{50} <10 µg/ml which denotes good activity, IC_{50} 10–50 µg/ml which denotes medium activity, IC_{50} 50–100 µg/ml which denotes low activity, and IC_{50} >100 µg/ml which denotes no antimalarial activity [7]. According to these criteria, *E. bulbosa* confers good antimalarial activity. Compared with the IC_{50} value of the dichloromethane extract obtained from *A. champeden* stem bark (IC_{50} = 0.99 µg/ml), it is evident that antimalarial activities of *E. bulbosa* are higher (IC_{50} = 0.62 µg/ml).

Table 5: CC_{50} of *E. bulbosa* at higher concentrations to lymphocyte cells after 72 h

Concentration (µg/ml)	Cell viability (%)
1347	23.91
2020	21.74
2694	17.02
3367	4.62

CC_{50}: 50% cytotoxic concentration, *E. bulbosa: Eleutherine bulbosa*

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 IC_{50} that was equal to 0.62 µg/ml and compared with cells that had been exposed

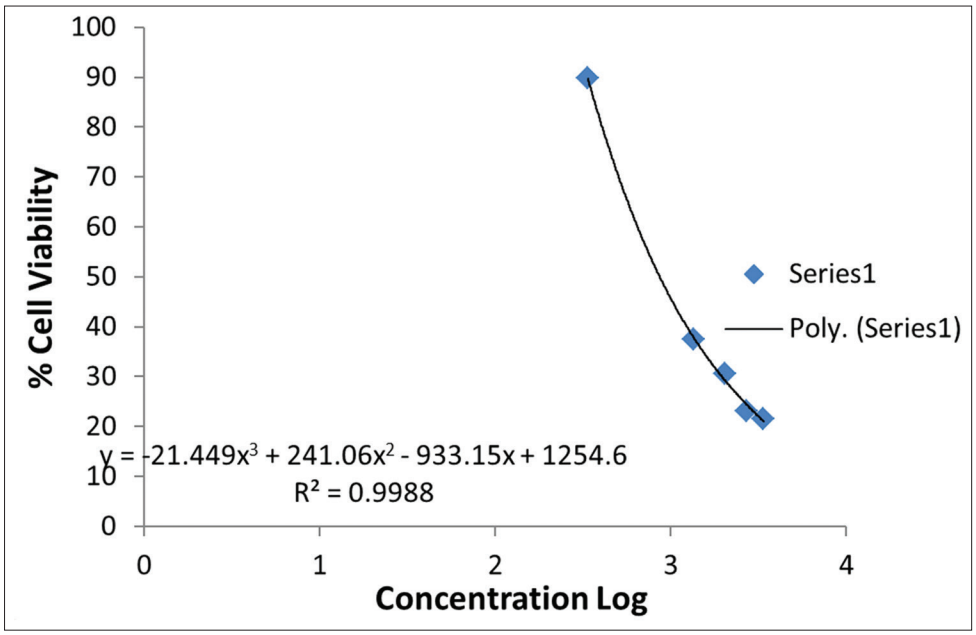

Fig. 4: A 50% cytotoxic concentration graph of *Eleutherine bulbosa* to lymphocyte cells after 24 h

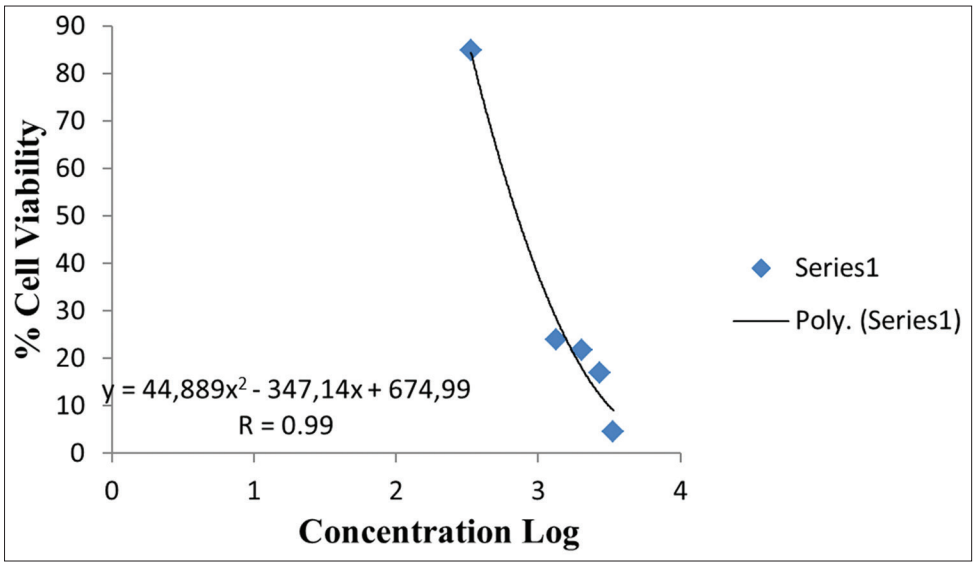

Fig. 5: A 50% cytotoxic concentration graph of *Eleutherine bulbosa* to lymphocyte cells after 72 h

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 bc_1 [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 IC_{50} 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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