South Asian Journal of Research in Microbiology

2(2): 1-10, 2018; Article no.SAJRM.44440

Antiplasmodial Activity of Ethanolic Leaf Extract of *Eucalyptus citriodora* in Swiss Albino Mice Infected with *Plasmodium berghei* NK 65

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Authors' contributions

This work was carried out in collaboration between all authors. Authors DM and EOD designed the study, performed the statistical analysis, wrote protocol and wrote the first draft of the manuscript. Authors DM, MM, EIJ and VIU managed the analyses of the study. Authors DM and EOD managed the literature searches. All authors read and approved the final manuscript.

Article Information

DOI: 10.9734/SAJRM/2018/v2i229251
Editor(s):
(1) Dr. Chamari Hettiarachchi, Senior Lecturer, Department of Chemistry, University of Colombo, Sri Lanka.
(2) Dr. Osunsanmi Foluso Oluwagbemiga, Department of Biochemistry and Microbiology, University of Zululand, South Africa.
Reviewers:
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(3) S. Murugesan, Pachaiyappa's College, University of Madras, India.
Complete Peer review History: http://www.sciencedomain.org/review-history/26973

Original Research Article

Received 07 August 2018
Accepted 22 October 2018
Published 01 November 2018

ABSTRACT

Malaria is a life-threatening disease and emergence of malaria parasite resistance to antimalarial drugs, has necessitated the need for discovery and development of an alternative to malaria medicine. This study assessed the ethanolic leaf extract of *Eucalyptus citriodora* for the presence of bioactive components qualitatively and efficacy of the extract against the malaria parasite. Standard methods were used to determine the bioactive components of the leaf extract. Twenty (20) albino mice of body weight between 18-25 g were randomised into 5 groups of four mice each for acute toxicity test, while twenty-four (24) mice were randomised into six groups of four mice each (group 1,

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2, 3, 4, 5 and 6) for antiplasmodial activity. All the groups were infected with *P. berghei,* except group 3 (normal control). Group 4, 5 and 6 were treated with 0.2 mL of 200, 400 and 800 mg/kg body weight of extract respectively. Group 2 (positive control) were treated with 0.2 mL of 5 mg/kg body weight of chloroquine. Group 1 (negative control) were administered with 0.2 mL of normal saline, while group 3 (normal control) were administered with 0.2 mL of normal saline for four consecutive days. Phytochemical screening revealed the presence of alkaloids, saponins, tannins, anthraquinone, flavonoids and cardiac glycosides and the extract was found safe and nontoxic. The antiplasmodial investigation revealed a decrease in percentage parasitaemia level in mice of group 4, 5 and 6 (extract treated group) compared with mice of group 1 (infected and not treated). The parasitaemia reduction was higher in group 6 (800 mg/kg). This significant decrease ($P<0.05$) in percentage parasitaemia level in the study was dose and time-dependent. The study revealed the potency of *E. citriodora* leaf extract as a future herbal candidate for the treatment of human malaria infection.

Keywords: Eucalyptus citriodora; phytochemical; Plasmodium berghei; antiplamodium; albino mice.

1. INTRODUCTION

Malaria is still top 10 among the causes of mortality despite reduction by 48% of incidence rate and 44% of mortality between 2010 and 2016 in the Southeast Asian region [1]. It is the most dangerous infection in the world and contributed to major socioeconomic problems, which lead to global instability and poverty [2].

Human malaria is caused by *Plasmodium* species (*P. falciparum, P. malariae, P. ovale, P. vivax and P. knowlesi*) and transmitted by the bite of infected females of *Anopheles* mosquitos [3]. Malaria caused by *P. falciparum* and *P. vivax* represents the majority of malaria health burden worldwide, with estimated incidences of 207 million and 8.5 million cases respectively in 2016 [4]. *P. falciparum* is the most dominant and pathogenic species, responsible for almost all mortality caused by malaria in tropical and sub-tropical countries [5]. *P. berghei* is a practical model organism in the laboratory for the study of human malaria aimed at developing a new management measure for the control and prevention of malaria [5]. This parasite (*P. berghei*) is transmitted to rodents, by the bites of an infected mosquito (*Anopheles dureni*),

The current efforts to reduce the global burden of malaria are threatened by the rapid emergence and spread of *P. falciparum* resistance to ACTs including artemisinin derivatives and their partner drugs [6]. The possible source of malaria treatment appears in the uses of traditional herbal medicine. Traditional medicines have been the most available, affordable and cheap sources of malaria treatment for most communities [7].

According to Rakholiya and Chanda [8] *Eucalyptus citriodora* Hook (Family: Myrtaceae) is a tall, evergreen and graceful tree which is cultivated for essential oil, fuel, timbers and medicinal purposes. Husain and Ali [9], and Kharwar et al. [10], reported that the leaves of *E. citriodora* produce fragrant volatile oil with known antibacterial, anti-inflammatory, antiseptic, analgesic, deodorant, diuretic, expectorant activities. The present study is aimed at determining the antiplasmodial effect of ethanolic leaf extracts of *E. citriodora* in Swiss albino mice infected with *Plasmodium berghei.*

2. MATERIALS AND METHODS

2.1 Plant Leaf Collection

E. citriodora leaves were collected in November 2017 from the Kogi State University, Anyigba, Nigeria. It was identified and authenticated by an expert, and the voucher specimen number of the plant Bio/ FUTA/ 70 was left in the herbarium of the Federal University of Technology, Akure, Ondo State, Nigeria.

2.2 Extraction and Phytochemical Screening of the Leaves

The leaves were washed, air dried at room temperature for three weeks and pulverised using mortar and pestle. Five hundred grams (500 g) of the pulverised leaf powder was dissolved in 4500 ml of 75% ethanol for 72 hours and then filtered using Millipore (pore size 0.7 µm) filter paper. The filtrate was concentrated to recover the crude extract using rotary evaporator at a reduced temperature of 40°C [11].

2.3 Determination of Phytochemicals

Phytochemical analysis of the ethanolic leaf extract of *E. citriodora* was carried out using standard procedures adopted by Dickson et al. [12] and Dada and Oloruntola [11]. Frothing test was used to check for the presence of saponins in the extract. Borntrager's test was used for the detection of anthraquinones. A blue-black precipitate after the addition of ferric chloride reagent to extract solution was checked for the presence of tannins. A brown ring at the interface which showed the presence of cardenolides was checked for the presence cardiac glycosides after the addition of 0.5 g of dried extract into 2.0 ml of glacial acetic acid with a drop of ferric chloride solution and underlaid with 1.0 ml of concentrated H_2SO_4. A reddish-brown precipitate which confirmed the presence of alkaloids was checked after the extract was warmed in 2% sulphuric acid with two drops of Wagner's reagents. A yellow colouration at the layer of ammonia which indicated the presence of flavonoids was checked after 0.5 g of the extract was heated in 10 ml of ethyl acetate on boiling water, filtered and shaken with 1 ml of ammonia solution.

2.4 Preparation of Leaf Extracts Dosage

The dosages of the extract administered to the mice were prepared by dissolving 0.4 g ,0.8 g and 1.6 g of the extract in 20 ml of distilled water each in the sterile universal bottle based on the body weight and a total number of mice per group to obtain 200,400 and 800 mg/kg body weight respectively [13].

2.5 Assemblage of Experimental Mice

Forty-four (44) Swiss albino mice of body weight between 18-25 g were obtained from the Animal House, Institute for Advance Medical Research and Training (IMRAT), University College Hospital, University of Ibadan, Nigeria. The animals were housed in cages with sawdust bedding at room temperature and were fed with standard diet (Grand cereal) and water ad libitum acclimatised for 7 days before the study. *P. berghei* NK 65 in a donor mouse was obtained from IMRAT.

2.6 Grouping of Animals

The method described by Berhan et al. [14] was used to group the experimental mice.

2.7 Acute Toxicity Test

A total of 20 healthy mice were randomised into 5 groups of 4 mice per group. Each mouse in groups 1 2, 3 and 4 were treated with 500 mg/kg, 1000 mg/kg, 1500 mg/kg and 2000 mg/kg body weight of the extract, respectively. Group 5 mice, the control group received normal saline. They were observed for signs of toxicity and general behaviour such as reduced activity, licking a paw, body weakness, convulsion, sleeping, salivation and mortality for seven days (7 days).

2.8 Preparation of Inoculums

The donor mouse of 20% parasitemia was anaesthetized with chloroform, blood containing *P. berghei* infected erythrocytes were withdrawn through the cardiac puncture with a syringe, transferred into a screw cap sterile plastic tube containing normal saline to obtain 1_X10^7 *P. berghei* infected erythrocytes.

2.9 Administration of Extract and Drugs

The method of Ogundolie et al. [5] was used to administer the extract and drug.

2.10 *In vivo* Antiplasmodial Activity of the Extract

Twenty-four (24) mice were randomised into six groups of four mice each. They were infected intravenously with 0.2 ml of 1_X10^7 standard inoculum of chloroquine-sensitive *P. berghei*. Three (3) hours after infections, 0.2 ml of 200, 400 and 800 mg/kg body weight of leaf extract were administered orally to group 4, 5 and 6 respectively as treatment dose once daily for four consecutive days. Group 2 (positive control) were treated with 0.2 ml of 5 mg/kg body weight of chloroquine, group 1 (negative control) were given 0.2 ml of normal saline, and group 3 (normal control) received 0.2 ml of normal saline but were not infected with *P. berghei* [13].

2.11 Determination of Packed Cell Volume

The packed cell volume (PCV) of each mouse was measured before and after infection. Blood was collected from the tail of each mouse in heparinised capillary tubes, up to ¾ of the entire length. The tubes were sealed using crystal sealant and placed in a microhematocrit centrifuge with the sealed end outwards. The

blood sample was centrifuged at 12,000 rpm for 5 minutes. The result was read using the microhaematocrit reader. The volume of the total blood and the volume of erythrocytes were measured, and PCV was calculated as;

PCV = Volume of erythrocytes in a given volume of blood X 100 Total blood volume.

2.12 Determination of Parasitemia

On day five, the parasitaemia level of the mice was determined by collecting a drop of blood on a microscope slide from each mouse by venesection of the tail. Thin and thick blood smear was made and allowed to air dry at room temperature. It was fixed with methanol for two minutes before staining with 10% Giemsa for 15 minutes. The slides were allowed to air-dried, examined and counted under a light microscope at X 100 magnification using oil-immersion. The parasitaemia was determined by counting a minimum of three fields per slide with 100 RBC per field [15].

Parasitemia = (Number of parasitised RBC x100 / Total Number of RBC examined.

2.13 Statistical Analysis

All data were expressed as Mean ± SEM. One-way analysis of variance was used to analyse data. P< 0.05 was considered a significant difference between means (Duncan's multiple range test).

3. RESULTS

3.1 Percentage Yield of the Ethanolic Leaf Extract of *Eucalyptus citriodora*

Percentage yield (Table 1) of the ethanolic leaf extract of *Eucalyptus citriodora* was 9.37% (46.83/500 g)

3.2 Phytochemicals Screening of Ethanolic Leaf Extract of *E. citriodora*

Phytochemical Screening of ethanolic leaf extract of *E. citriodora* revealed the presence of alkaloids, saponins, tannins, anthraquinone, flavonoid and cardiac glycosides (Table 2).

3.3 Acute Toxicity of *E. citriodora* Leaf

The result of the toxicological test of *E. citriodora* leaf extract in mice showed no noticeable sign of toxicity such as salivation, reduced activity, licking paw, body weakness, convulsion, jumping, hyperactivity in the mice and no death or mortality recorded for all the doses tested. It indicated that the LD_{50} is greater than 2000 mg/kg body weight.

3.4 Body Weight of Mice before and after Infection and Treatment

The mice of group 1 and 4 (infected untreated and infected treated with 200 mg/kg) showed decrease of body weight after 4 days of treatment (Fig. 1). However, the mice infected and treated with 400 mg/kg and 800 mg/kg (group 5 and 6) as well as those treated with 5 mg/kg chloroquine (group 2) also showed decreased body weight after 4 days of treatment, but not as that of infected untreated mice and infected treated with 200 mg/kg. Mice in group 3 (normal saline) experienced increased in body weight.

3.5 Pack Cell Volume (PCV) before and after Infected and Treatment

The PCV of group 1 and 4 (infected untreated and infected treated with 200 mg/kg mice) decrease significantly ($p<0.05$) after 4 days of treatment (Fig. 2). However, group 5 and 6 (mice infected treated with 400 mg/kg and 800 mg/kg) as well as those of group 2 (treated with 5 mg/kg chloroquine) also showed a decrease in PCV values, but not like those of groups 1 and 4. Mice in group 3 (normal control) recorded an increase in value of PCV.

3.6 Percentage Parasitaemia Count

Figs. 3 to 7 expressed the results of the percentage parasitaemia counts in mice of all groups. Mice in group 6 (800 mg/kg), group 2 (chloroquine treated) and group 3 (normal control) had zero parasitaemia counts for day 1, 2 and 3. However, for day1, groups 4 and 5 (200 and 400 mg/kg of the extract) recorded low percentage parasitaemia counts compared with group 1 (infected not treated). Also, from day 2, the percentage parasitaemia counts of mice in group 4 and 5 were significantly lower compared with mice of group 1, however, mice of group 5 (400 mg/kg) showed lower counts compared with mice of group 4 (200 mg/kg). Comparative observations of group 4 and 5 mice for day 1 and day 2 revealed a significant decrease ($P<0.05$) in the percentage parasitaemia counts from

Table 1. Percentage yield of ethanolic leaf extract of *Eucalyptus citriodora*

Plant species	Plant part	Weight of powder (g)	Volume of solvent (ml)	Yield (g)	% Yield
E. citriodora	Leaf	500	4500	46.83	9.37

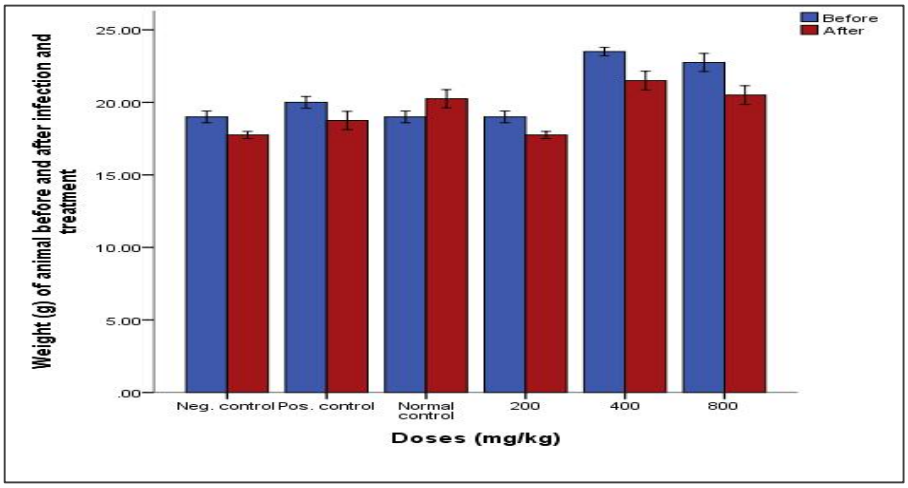

Fig. 1. Body weight of mice before and after infection and treatment

day1 to day2. Day 3 observations revealed a significant increase (P<0.05) in percentage parasitaemia in group 1, 4 and 5 compared with day1 and 2. This might be due to the parasitic nature of the extract. Observation from day 4 revealed a significant increase (P<0.05) in percentage parasitaemia for group 2, 4, 5 and 6 compared with day 1, 2 and 3. This also might be due to the suppressive nature of the extract. After 5 days of treatment, the percentage parasitaemia counts were significantly low (P<0.05) in mice treated with 800 mg/kg body weight of the extract compared with other extract treated groups, thus indicating a dose-dependent relationship. However, percentage parasitaemia was significantly lower (P<0.05) in mice treated with chloroquine compared with mice treated with the highest dose of the extract (800 mg/kg).

Table 2. Phytochemical screening of ethanolic leaf extract of *E. citriodora*

Phytochemicals	Results
Alkaloids	+
Saponins	+
Tannins	+
Anthraquinone	+
Flavonoids	+
Cardiac Glycoside	+

Present = + and absent = -

4. DISCUSSION

The percentage yield of the ethanolic leaf extract is low (9.32%) compare to the one obtained by Yaya et al. [16], with a high yield of the ethanolic and hydroethanolic leaves extracts of *E. citriodora* respectively 20.8 and 23.4%. The observed differences in the yield might either be due to the age of the plant or period of the harvest of the leaf and to the nature of the soil.

Phytochemicals screening of the ethanolic leaf extract of *E. citriodora* which revealed the presence of alkaloids, saponins, cardiac glycosides, tannins, flavonoids and anthraquinone, agrees with Yaya et al. [16]. The presence of alkaloids in this extract could be responsible for the reduced parasitaemia level observed in this study, this agreed with David et al. [17], that the presence of alkaloids blocks protein-synthesis of *Plasmodium* species.

The acute toxicity test of the extract in mice, which revealed no death and a general sign of toxicity is expected. This is in line with the findings of Bello et al. [18], who indicated that herbal extracts with LD_{50} above 3000 mg/kg/oral may be considered safe and nontoxic.

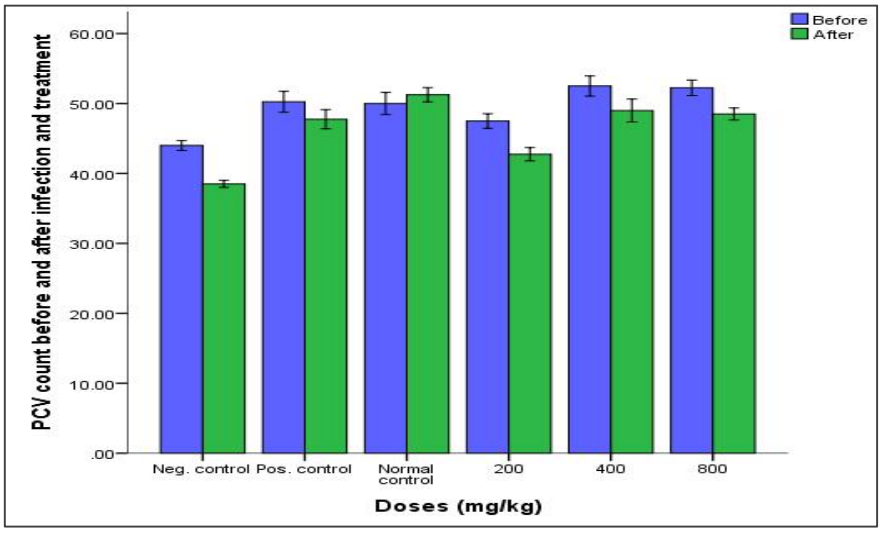

Fig. 2. PCV of Mice before and after infection and treatment

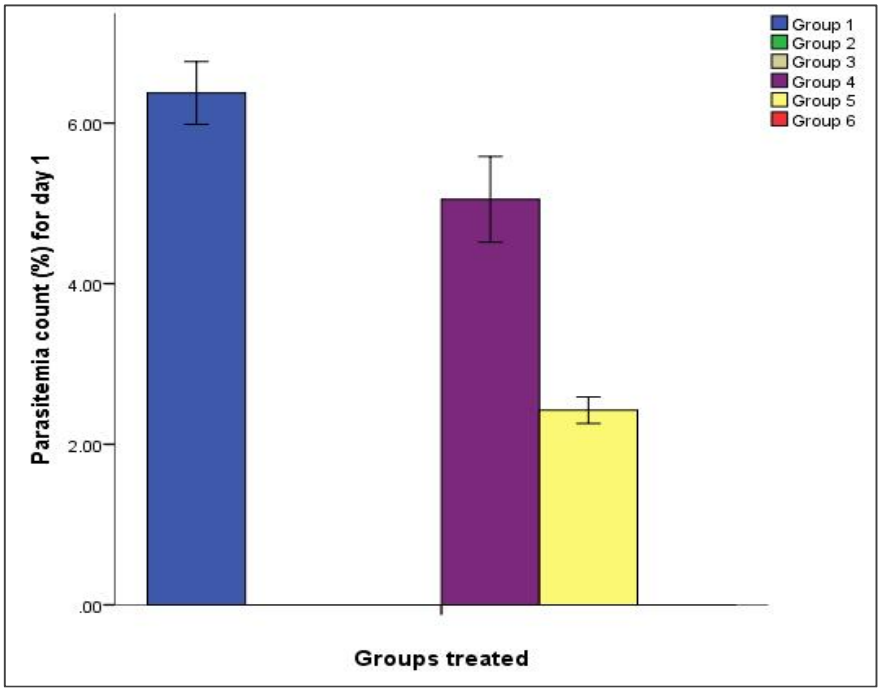

Fig. 3. Parasitemia count (%) for day 1

Group 1: P. berghei + 0.2 ml normal saline, group 2: P. berghei + 5 mg/kg body weight Chloroquine, group 3: 0. 2 ml normal saline, group 4:P. berghei + 200 mg/kg body weight leaf extract, group 5: P. berghei + 400 mg/kg body weight leaf extract and group 6: P. berghei + 800 mg/kg body weight leaf extract.

Decreased body weight observed in infected treated and infected not treated mice compared to the normal control group, could be due to loss of appetite and increased in the metabolic rate. Also decreased body weight of infected and treated mice could be caused by reduced feed conversion efficiency. This corroborates with the report of Aglal et al. [19]. Also, body weight loss observed in this study after infection and treatment could be reason advanced by Langhorne et al. [20], that body weight loss is a sign of malaria-infected mice.

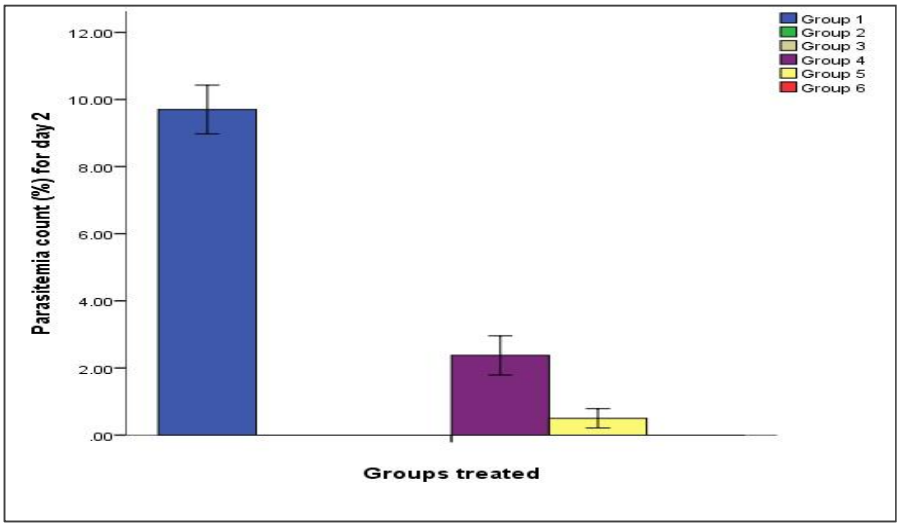

Fig. 4. Parasitemia count (%) for day 2

Group 1: P. berghei + 0.2 ml normal saline, group 2: P. berghei + 5 mg/kg body weight Chloroquine, group 3: 0. 2 ml normal saline, group 4:P. berghei + 200 mg/kg body weight leaf extract, group 5: P. berghei + 400 mg/kg body weight leaf extract and group 6: P. berghei + 800 mg/kg body weight leaf extract.

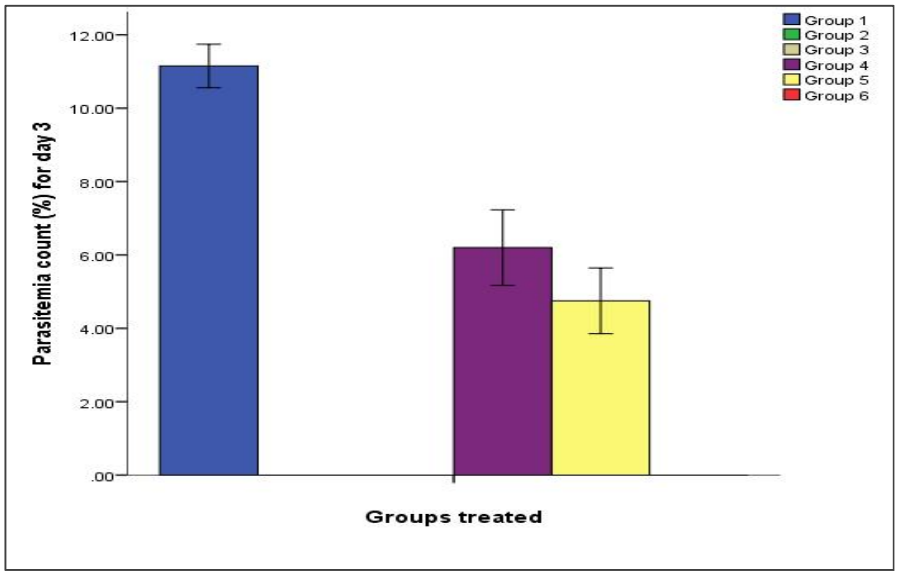

Fig. 5. Parasitemia count (%) for day 3

Group 1: P. berghei + 0.2 ml normal saline, group 2: P. berghei + 5 mg/kg body weight Chloroquine, group 3: 0. 2 ml normal saline, group 4:P. berghei + 200 mg/kg body weight leaf extract, group 5: P. berghei + 400 mg/kg body weight leaf extract and group 6: P. berghei + 800 mg/kg body weight leaf extract.

The decreased PCV values in mice of group 4, 5 and 6 (extract treated groups) compared with normal control (group 3). Mice of group 1 (negative control) showed higher decreased of PCV values compared with mice of group 4, 5 and 6. This decreased PCV values are expected and could be due to anaemia as a result of the destruction of RBC by *Plasmodium*. This is in line with the findings of Kabiru et al. [21], who reported that the presence of *Plasmodium* parasites in the bloodstream results in anaemia due to active lysing of RBC. Similarly, the observed decrease in PCV could also be due to reason advanced by Langhorne et al. [20], that reduction in PCV is a cardinal sign of malaria-infected mice.

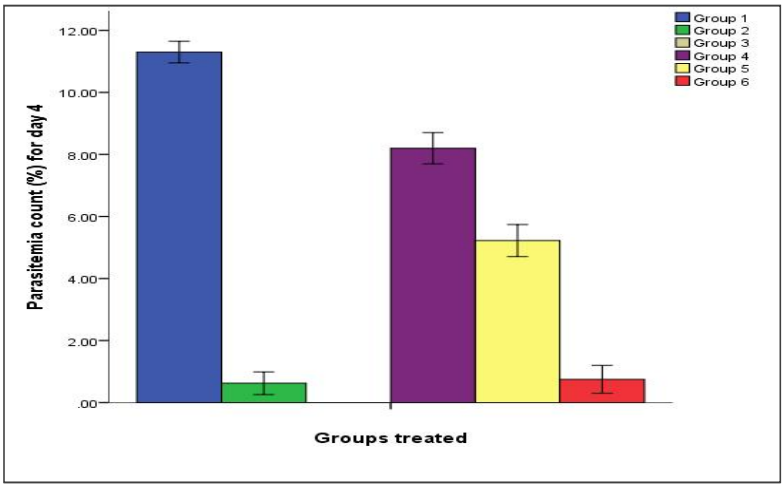

Fig. 6. Parasitemia count (%) for day 4

Group 1: P. berghei + 0.2 ml normal saline, group 2: P. berghei + 5 mg/kg body weight Chloroquine, group 3: 0. 2 ml normal saline, group 4:P. berghei + 200 mg/kg body weight leaf extract, group 5: P. berghei + 400 mg/kg body weight leaf extract and group 6: P. berghei + 800 mg/kg body weight leaf extract.

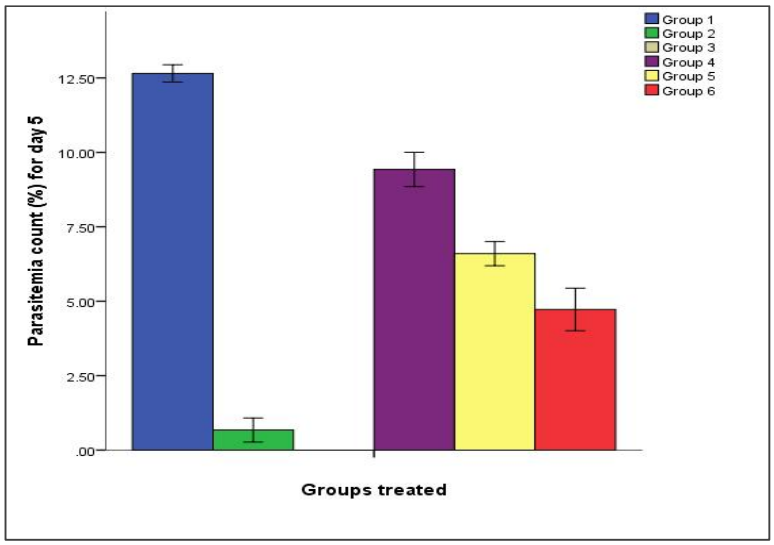

Fig. 7. Parasitaemia count (%) for day 5

Group 1: P. berghei + 0.2 ml normal saline, group 2: P. berghei + 5 mg/kg body weight Chloroquine, group 3: 0. 2 ml normal saline, group 4: P. berghei + 200 mg/kg body weight leaf extract, group 5: P. berghei + 400 mg/kg body weight leaf extract and group 6: P. berghei + 800 mg/kg body weight leaf extract.

The antiplasmodial investigation of the extract that revealed a decrease in percentage parasitaemia of groups 4, 5 and 6 (extract treated group) compared with group 1 (infected and not treated) is expected. The degree of parasite reduction was higher in group 6 (800 mg/kg). This significant decrease ($P<0.05$) in percentage parasitaemia was dose and time-dependent. This agrees with Kabiru et al. [21], who in antiplasmodial activities of the aqueous and methanol extracts of *Eucalyptus* observed a reduction in percentage parasitaemia after 5 days of treatment. Also, the zero parasitaemia counts observed, respectively, for the highest dose (800 mg/kg) of the extract for day1, 2 and 3 treatment could be favourably compared with the chloroquine (group 2). This is in line with similar findings by Akanbi [22], who observed that the parasite growth inhibition in the positive control (chloroquine treated group) was almost similar to

the group treated with the highest dose of 200 mg/kg body weight of *A. leiocarpus*. This finding suggests that *E. citriodora* leaf extract could be used in the treatment of malaria if purified. Also, the reduced percentage parasitaemia level could be attached to the report of Taramelli et al. [23], that saponins, phenols and tannins can inhibit haem polymerisation and the unpolymerised haem is very toxic for intraerythrocytic *Plasmodia.*

5. CONCLUSION

The results of this study have revealed that the ethanolic leaf extract of *E. citriodora* possesses phytochemicals and antiplasmodial potency suitable for treatment of human malaria. Further investigation to determine the pure, active components of the leaf extracts of the *E. citriodora* responsible for these activities and the effect on long-term administration is recommended for further studies.

ETHICAL APPROVAL

The whole experimental management, handling and care were approved by the Research and Ethics Committee of the Department of Microbiology, School of Sciences, The Federal University of Technology, Akure, Nigeria.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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Peer-review history:
The peer review history for this paper can be accessed here:
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