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IN VIVO ANTIMALARIAL ACTIVITIES OF *CROTON ZAMBESICUS* LEAF FRACTIONS AGAINST *PLASMODIUM BERGHEI* INFECTION IN MICE

Martin O. Anagboso*[1], Jude E. Okokon[2] and Emmanuel I. Etim*[3]

[1]Department of Microbiology, Madonna University Nigeria, Elele campus, Rivers State, Nigeria.
[2]Department of Pharmacology and Toxicology, Faculty of Pharmacy, University of Uyo, Uyo, Nigeria.
[3]Department of Pharmaceutical and Medicinal Chemistry, Faculty of Pharmacy University of Uyo, Uyo, Nigeria.

***Corresponding Author: Martin O. Anagboso**
Department of Microbiology, Madonna University Nigeria, Elele campus, Rivers State, Nigeria.

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ABSTRACT
Croton zambesicus Muell Arg. (Euphorbiaceace) a medicinal plant used traditionally in the treatment of malaria was screened for antiplasmodial activity. The leaf fractions (hexane, dichloromethane, ethyl acetate and methanol; 150 mg/kg) were investigated for suppressive, prophylactic, and curative antiplasmodial activities against chloroquine-sensitive *Plasmodium berghei* infections in Swiss albino mice. Chloroquine (5 mg/kg) and pyrimethamine (1.2 mg/kg) were used as positive controls. Thin films made from tail blood of each mouse were used to assess the level of parasitaemia of the mice. The leaf fractions progressively reduced parasitaemia induced by chloroquine-sensitive *P. berghei* infection in suppressive (58.16-74.82%), prophylactic (32.75 – 46.79%) and curative (65.87–76.33%) models in mice with ethyl acetate fraction exhibiting the highest suppressive and curative activities, while DCM fraction followed by methanol fraction exerted the highest prophylactic effect. These reductions were statistically significant (p<0.01–0.001).They also improved significantly (p<0.01–0.001) the mean survival time (MST) from 10.03 to 26.06 d in suppressive, 10.06 to 20.01 in prophylactic and 10.12 to 25.33 d in curative models relative to respective controls. The activities of the leaf fractions were not comparable to that of the standard drugs used (chloroquine and pyrimethamine) in all the models. The leaf of *C. zambesicus* may possess antiplasmodial effect which may in part be mediated through the chemical constituents of the plant.

KEYWORDS: Croton zambesicus; Plasmodium berghei, medicinal plant.

INTRODUCTION
Malaria is one of the devastating infectious disease in the world. Despite enormous effort gearing towards the eradication of the disease, World Malaria report of 2021 estimated that malaria caused 241 million clinical sequences and 627,000 deaths. In 2020, malaria caused an estimated 95% deaths in the World Health Organisation report for African region.[1] It is recorded that the most vulnerable are persons with none or little immunity against the disease. Nigeria alone carries 26.6% and 31.3% of all cases of malaria and death globally.[1] In 2021, 76% of malaria deaths were recorded in children under five years, particularly African children with limited access to health care. Pregnant women are at high risk because in 2020, an estimated forty million pregnancies across 38 malaria-endemic African countries of which 13.3 million (32%) were exposed to malaria.[2] Being the leading cause of death and disease in many developing countries, malaria is one of the most severe public health problems worldwide. It imposes substantial cost to both individual and government. Despite significant improvement in its management, it is still threatening particularly in Nigeria. Plants have been proven to provide an important source of anti-malarial drugs. Therefore, a continuous search for a cheaper, readily available and a more active and effective anti-malarial drug is urgently needed.

Croton zambesicus Muell Arg. (Euphorbiaceace) (syn *C. amabilis* Muell. Arg. *C. gratissimus* Burch) is an ornamental tree grown in villages and towns in Nigeria. It is a Guineo–Congolese species widely spread in tropical Africa. Traditionally, the leaf decoction is used as anti-hypertensive, anti-microbial (urinary infections)[3], antimalarial[4], and antidiabetic.[5] The roots are also used as anti-malarial, febrifuge and antidiabetic by the Ibibios of Niger Delta region of Nigeria.[6] The ethanol leaf extract has been reported to possess antiplasmodial[4], anti-inflammatory, analgesic and antipyretic activities[7] and *in vivo* alpha amylase and alpha glucosidase inhibitory activities[8] Boyom *et al.*, reported that the essential oils from the leaves are rich in monoterpenes.[9] Aderogba *et al.*, further isolated quercetin-3-O-*p*-600 (p-coumaroyl) glucopy-ranoside-30-methyl ether, helichrysoside-30-methyl ether, along with kaempferol-3-O-*p*-600 (p-coumaroyl)

glucopyranoside, tiliroside and apigenin-6-C-glucoside, isovitexin as the antioxidant constituents from the leaf of the plant.[10] We report in this study the effect of leaf gradient fractions of the plant on *Plasmodium berghei*-infection in mice.

MATERIALS AND METHODS

Plants collection

The plant material *Croton zambesicus* (leaves) were collected in compounds in Uruan area, Akwa Ibom State, Nigeria in June 2021. The plant was identified and authenticated by a taxonomist in the Department of Botany and Ecological Studies, University of Uyo, Uyo, Nigeria.

Extraction

The leaves were washed and shade-dried for two weeks. The dried plants' materials were further chopped into small pieces and reduced to powder using electric grinder. The powdered leaves material (1.5 kg) was successively and gradiently macerated for 72 h in each of these solvents (2 x 5L), n-hexane, dichloromethane, ethyl-acetate and methanol to give corresponding fractions of these solvents. These were thereafter filtered and the liquid filtrates were concentrated and evaporated to dryness *in vacuo* at 40°C using a rotary evaporator (BuchiLab, Switzerland). The fractions were stored in a refrigerator at -4°C, until used for the proposed experiments.

Microorganism (parasite)

The parasite strain used in this study was chloroquine-sensitive strain of *Plasmodium berghei* which was obtained from the National Institute of Medical Research (NIMER), Yaba Lagos, Nigeria and maintained by subpassage in mice.

Parasite inoculation

Each mouse used in the experiment was inoculated intraperitoneally with 0.2 mL of infected blood containing about 1.0 x 10^7 *P. berghei* parasitized erythrocytes collected from an infected mice with 20-30% parasitaemia. The inoculums consisted of 5 x 10^7 *P. berghei* infected erythrocytes per milliliter prepared by determining both the percentage parasitemia and the erythrocytes count of the donor mouse and diluting the blood with isotonic saline in proportions indicated by both determinations.[11.12] Parasitemia was monitored by standard methods; thin blood smears were made on glass slides, fixed using methanol, and stained using Giemsa stain, and parasitemia was counted using a microscope and was calculated as a percentage of infected red blood cells (RBCs) relative to the total number of cells in a microscopic field at ×100 magnification according to the formula of Peters and Robinson[13] as given below:

$$\text{Parasitemia (\%)} = \frac{\text{Total number of parasitised RBCs}}{\text{Total number of RBCs}} \times 100.$$

Experimental animals

Swiss albino mice (19-25 g), male and female, used in the study were obtained from the University of Uyo's animal house. They were kept in standard plastic cages in a well ventilated room and left to acclimatized for a period of 10 days before the experiments. The mice were fed on standard pellete diet and water *ad libitum*. The care and use of animals were conducted in accordance with the National Institute of Health Guide for the Care and Use of laboratory Animals.[14] Approval for the study was obtained from the University of Uyo's Animal Ethics Committee.

Drug administration

The extract, fractions, chloroquine and pyrimethamine that were used in the study were administered orally with the aid of a stainless metallic feeding cannula.

Evaluation of the *in vivo* antimalarial activities of leaf fractions of *Croton zambesicus*

Evaluation of suppressive activities of leaf fractions of *Croton zambesicus* (4-day test)

The suppressive activities of the leaf gradient fractions and chloroquine against early *P. berghei berghei* infection in mice was carried out using the method described by Okokon *et al.*, 2019.[15] The study involved thirty-six (36) mice which were infected with the parasite on the first day (D0) and randomly divided into six groups of six mice each. The mice in groups 1-4 were given 150 mg/kg of n-hexane, dichloromethane (DCM), ethyl acetate and methanol fractions respectively, while groups 5 was given 5 mg/kg of chloroquine (positive control) and group 6 given 10 mL/kg of distilled water (negative control) for four consecutive days (D_0-D_3) between 8am to 9am. Thin films were made from the tail blood of each mouse on the fifth day($_4$). The films were stained with Giemsa stain to reveal parasitized erythrocytes out of 500 in a random field of the microscope. The average suppression of parasitemia was calculated as follows: (average % parasitemia positive control – average % parasitemia negative control) / (average % parasitemia negative control). The mean survival time of the mice in each treatment group was determined over a period of 29 days (D_0-D_{28}), as follows:

(No. of days survived) / (total No. of days (29) x 100

Evaluation of prophylactic or repository activities of leaf fractions of *Croton zambesicus*

The prophylactic activities of the leaf fractions were assessed using the method earlier described by Peters, 1965.[16] and modified by Williams *et al.* 2022.[17] The mice were divided into six groups of six mice each and treated as follows; groups 1-4 were respectively administered with 150 mg/kg each of n-hexane, dichloromethane, ethyl acetate and methanol fractions orally. Group 5 was given 1.2 mg/kg of pyrimethamine (positive control) and group 6 given 10 mL/kg of distilled water (negative control). Administration of the fractions and pyrimethamine continued for three consecutive days (D_0-D_2). On the fourth day (D_3), the

mice were inoculated with *P. berghei berghei*. The parasitemia level was assessed by blood smears 72 hours later. The mean survival time of the animals were calculated over a period of 29 days.

Evaluation of the curative activities of the leaf fractions of *Croton zambesicus*

Curative activities of the fractions and chloroquine in established plasmodial infection were investigated using the modified method of Ryley and Peters 1970,[18] as reported by Williams *et al.*, 2022.[17] The 36 mice used in this study were infected intraperitoneally with *P. berghei berghei* on the first day *(D_0)*. Seventy two hours post-infection (D_3), the mice were divided into six groups of six mice each. The leaf fractions (n-hexane, ethyl acetate, dichloromethane, and methanol) 150 mg/kg each were respectively administered orally to groups 1- 4. Group 5 was given 5 mg/kg chloroquine (positive control) and group 6 was given 10 mL/kg distilled water (negative control). The leaf fractions and chloroquine were administered once daily for 5 days. Giemsa stained thin smears were prepared from tail blood samples collected on each day of treatment to monitor the parasitemia level. Rectal temperature of each mouse were taken on the first day (Day 0), and on days 3 and 7 of the study. The mean survival time (MST) of the mice in each group was determined over a period of 29 days (D_0-D_{28}).

Statistical analysis

Data collected were analyzed using one way analysis of variance (ANOVA) followed by Tukey's multiple comparison post-test (Graph pad prism software Inc. La Jolla, CA, USA). Values were expressed as mean ± SEM and significance relative to control were considered.

RESULTS

Suppressive test

The results of suppressive activities of the leaf fractions are shown in Table 1. The leaf fractions (n-hexane, DCM, ethyl acetate and methanol; 150 mg/kg), exerted significant ($p<0.001$) reduction in the percentage parasitaemia of the treated mice when compared to the control group in the study. There was a significant ($p<0.05$-0.001) decrease in parasitaemia level of groups treated with DCM, ethyl acetate and methanol fractions when compared to the control with the ethyl acetate fraction showing the highest activity. The chemosuppression were 66.74, 69.42, 74.82 and 58.16% for n-hexane, dichloromethane, ethyl acetate and methanol respectively. This was however lower than that of chloroquine the standard drug as shown in table 1.

The standard drug, chloroquine showed relatively higher chemosuppression (92.12%). The extract and fractions exerted considerable protection to the animals as shown in the significantly ($p<0.05$-0.001) prolonged MST of the treated mice with the ethyl acetate fraction having higher MST values of 26.06±1.54 d (Table 1).

Prophylactic Test

The prophylactic activities of leaf fractions are shown in Table 2. The leaf fractions (150 mg/kg) showed significant ($p<0.001$) reductions in parasitaemia levels with 12.19±1.21, 10.12±1.10, 12.79±0.56, and 11.54±0.45% for n-hexane, dichloromethane, ethyl acetate and methanol respectively, and 2.28±1.6% for pyrimethamine. There were significant ($p<0.001$) decreases in the parasitaemia level of all the fractions-treated groups compared to the control with the dichloromethane fraction showing the highest activity followed by methanol fraction though less than that of the standard drug, pyrimethamine, as shown in Table 2. The extract and fractions further demonstrated prominent protection on the treated infected mice with dichloromethane and methanol fractions treated group having elongated MST of 20.01±0.96 and 19.11±1.5 respectively (Table 2).

Curative Test Result (Rane's Test)

Daily reductions in parasitaemia levels of the lea fractions-treated groups of infected mice were observed in the study from day 0 to day 7 (Figure 1). The fractions exhibited statistically significant ($p<0.001$) chemotherapeutic effects when compared to control on day 7 following treatment with the fractions (150 mg/kg) with ethyl acetate fraction showing the highest activity, compared to the control, though lower than that of the standard drug (chloroquine), as shown in figure 1.

Effect of leaf extract/fractions on mean survival time of infected mice

There were prominent improvement in the mean survival times of the leaf fractions-treated groups. The fractions (150 mg/kg) increased the mean survival time from 10.12 to 25.33 days when compared to the control with the ethyl acetate fraction-treated group demonstrating the longest mean survival time. However, it was shorter when compared to that of the standard drug, chloroquine (29.44 days) as shown in Table 3.

Effect of leaf extract/fractions on rectal temperatures of infected mice

Administration of the leaf fractions of *Croton zambesicus* and chloroquine to *P. berghei*-infected mice did not cause any significant difference ($p>0.05$) in the rectal temperatures of the treated mice when compared with that of control on days 3 and 7 (Table 4).

Table 1: Suppressive activities of leaf gradient fractions of *Croton zambesicus* during early *Plasmodium berghei* infection in mice.

Treatment	Dose (mg/kg)	Parasitaemia	Chemosuppression (%)	MST
Control	-	36.14 ±1.54	-	10.03±0.46
n-hexane	150	12.02 ±1.58^{c}	66.74	21.40±1.12^{b}
Dichloromethane	150	11.05± 1.53^{c}	69.42	22.33±1.43^{b}
Ethyl acetate	150	9.10± 1.22^{c}	74.82	26.06±1.54^{c}
Methanol	150	15.12± 1.36^{c}	58.16	20.30±1.04^{b}
Chloroquine	5	1.04 ± 0.33^{c}	97.12	29.50±0.12^{c}

Values are expressed as mean ± SEM. Significant relative to control. ^{a}p<0.05; bp<0.01; cp<0.001. n = 6

Table 2: Prophylactic activities of of leaf fractions of *Croton zambesicus.*

Treatment	Dose (mg/kg)	Parasitaemia	Chemo-suppression (%)	MST
Control	-	19.02 ±1.91	-	10.06 ±0.28
n-hexane	150	12.19±1.21^{c}	35.90	18.24 ±1.04^{a}
Dichloromethane	150	10.12± 1.10^{c}	46.79	20.01 ±0.96^{b}
Ethyl acetate	150	12.79± 0.56^{c}	32.75	16.64 ±1.45
Methanol	150	11.54± 0.45^{c}	39.32	19.11±1.05^{a}
Pyrimethamine	1.2	2.28 ± 1.06^{c}	88.01	26.62 ±1.02^{c}

Values are expressed as mean ± SEM. Significant relative to control. ^{a}p<0.05; bp<0.01; cp<0.001. n = 6

Table 3: Mean survival time of mice treated with leaf fractions of *Croton zambesicus* during established *Plasmodium berghei* infection in mice.

Treatment/ Fractions	Dose (mg/kg)	Mean Survival Time (Days)
Control	-	10.12 ±0.16
n-hexane	150	23.01±0.81^{c}
Dichloromethane	150	21.04±0.46^{c}
Ethyl acetate	150	25.33± 0.68^{c}
Methanol	150	20.66 ±1.57^{c}
Chloroquine	5	29.44± 1.64^{c}

Values are expressed as mean ± SEM. Significant relative to control. ^{a}p<0.05; bp<0.01; cp<0.001. n = 6.

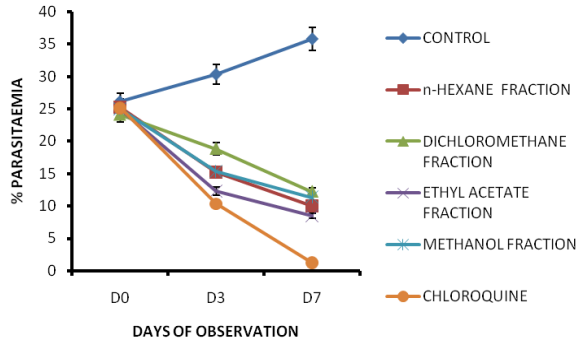

Figure 1: Effect of leaf fractions of *Croton zambesicus* on parasitaemia during established *Plasmodium berghei* infection in mice.

Table 4: Effect of leaf fractions of *C. zambesicus* on rectal temperatures of mice infected with *Plasmodium berghei* during established infection.

Treatment	Dose (mg/kg)	Rectal Temperature (˚C)		
		D0	D3	D7
Control	-	35.01±0.25	35.42 ±0.08	35.67±0.05
n-hexane	150	34.88±0.29	35.23 ±0.11	35.71 ±0.16
Dichloromethane	150	34.31±0.45	35.32± 0.05	35.68 ±0.04
Ethyl acetate	150	34.92±0.84	35.33± 0.04	35.71 ±0.10
n-butanol	150	35.02±0.71	35.25± 0.06	35.70 ±0.11
Chloroquine	5	35.05 ±0.19	35.31 ± 0.08	35.312±0.13

Values are expressed as mean ± SEM.

DISCUSSION

The leaves of *Croton zambesicus* are used in Ibibio traditional medicine as malaria remedy and previous report by Okokon *et al*., 2005a.[4] showed that the leaf extract possesses antimalarial potential against *Plasmodium berghei* infection in mice. This work was therefore designed to identify the fraction(s) with the highest activity where the active compound(s) can be isolated and characterised for further development into a potent antimalaria molecule(s).

The leaf fractions (hexane, dichloromethane, ethyl acetate and methanol) of *Croton zambesicus* were investigated for antimalarial activity against rodent malaria parasite, *P. berghei* infection in mice using standard *in vivo* models. The results revealed that the leaf fractions significantly reduced the parasitaemia in prophylactic, suppressive and curative models with ethyl acetate fraction exhibiting the highest suppressive and curative activities, while DCM fraction followed by methanol fraction exerted the highest prophylactic effect confirming the antimalarial potential of this extract. The leaf fractions also prolonged the MST of the mice suggesting protective potentials to the mice. This activity could have resulted from plasmodicidal or plasmodistatic activity of the fractions. The results of this study corroborate earlier report by Okokon *et al*., 2005a.[4] which significant antimalarial activity was reported on the leaf extract. These results validate the use of the leaf extract decoctions as malaria remedy. The findings of this study corroborate previous report of Okokon *et al*., 2005a.[4]

The leaves of *C. zambesicus* have been reported to contain monoterpenes[9], quercetin-3-O-*p*-600 (p-coumaroyl) glucopy-ranoside- 30-methyl ether, helichrysoside-30-methyl ether, along with kaempferol-3-O-*p*-600 (p-coumaroyl) glucopyranoside, tiliroside and apigenin-6-C-glucoside, isovitexin[10] which are also the antioxidant constituents from the leaf of the plant. Some secondary metabolites of plants such as alkaloids, flavonoids and triterpenoids have been reported previously to have antiplasmodial properties.[19,20,21] Antiplasmodial activities of monoterpenes[22] and flavonoids[22,23,24] have been documented. These compounds mentioned above to be present in the leaves and active fractions maybe responsible for the observed antiplasmodial activities. Antioxidant property of flavonoids such as quercetin has been suggested to be responsible for its antiplasmodial activity[23,24,25] as elevated free radical levels are associated with malaria disease and responsible for the pathogenesis of severe malaria complications. Scavenging of these free radicals could be one of the mechanisms of action of this extract. Other proposed mechanisms of antiplasmodial activity for flavonoids are chelation of nucleic acid base pairing of the parasite[26], modulation of host immunity to tackle disease and inhibition of plasmodial enoyl-ACP reductase (FAB I enzyme) – a key regulator of type II fatty synthases (FAS-II) in *P. falciparum*[27,28] and binding to parasite's serinethreonine kinase with high affinity thereby affecting its development.[29] The leaf fractions may be acting through one of these mechanisms. These compounds present in these leaf fractions may in part have contributed to the plasmodicidal activity of this extract/fraction.

The findings of this study further support the antimalarial activity of the leaves of *Croton zambesicus* which is due to the activities of its phytochemical constituents, thereby authenticating its use as malarial remedy in folkloric medicine.

Although fever is one of the common symptoms of malaria especially in humans, *P. berghei* infection in mice is reported to be associated with hypothermia rather than pyrexia.[30] Results indicated that rectal temperatures of the infected mice in this study (curative test), showed no significant difference between the mean temperature values of both the fraction-treated and untreated infected mice pre- and post treatment, suggesting that the mice were hypothermic. This may have resulted from physiological activities of the malaria parasite in the host, leading to body heat loss and ultimately death of mice.[31] Moreso, malaria parasite affect negatively the metabolism of host's carbohydrate, lipid, and protein.[32] Low body temperatures have been correlated with reduced metabolic rates of *P. berghei*-infected mice.[33] The leaf fractions lacked the potentials to prevent such processes, thus hypothermia.

CONCLUSION

The results of this study show that the leaf fractions of *Croton zambesicus* possess *in vivo* antimalarial potentials which may be attributed to the activities of their phytochemical constituents.

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COMPETING INTERESTS

The authors have not declared any conflict of interests.

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