

# Evidence supporting the use of *Combretum nigricans* as an antimalarial agent in ethnomedicine

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**Background:** *Combretum nigricans* (Combretaceae) is a small, smooth bark tree used in traditional medicine for the treatment of prurigo, dysentery, fever and other symptoms of acute malaria in North-Central Nigeria. **Materials and Methods:** The antimalarial activity of *C. nigricans* hydro-methanol crude leaf extract was evaluated against *Plasmodium berghei* in mice. The Peters' 4-day suppressive test against early malaria infection and Rane's curative test against established malaria were employed in assessing the antimalarial activity of the crude extract. In each test model, five groups containing five mice each were used. Group 1 mice were administered with 10 ml distilled water/kg b.w. p.o., groups 2 - 4 were administered with 200, 400, and 800 mg extract/kg b.w. p.o. respectively, while group 5 mice were administered with artesunate 10 mg/kg b.w. p.o. **Result:** All doses of *C. nigricans* extract employed for the study (200 - 800 mg/kg b.w. p.o.) gave significant ( $P < 0.05$ ) chemosuppressive effect against *P. berghei*, this effect was observed to be dose-related; while the 400 mg/kg extract dose gave the highest curative effect. Compared to control, the extract also prolonged the mean survival time at all doses, and as well prevented the characteristic decrease in body temperature elicited by *P. berghei* in mice. The LD<sub>50</sub> of the extract was >5000 mg/kg b.w. p.o. in mice. **Conclusion:** The result from the study indicates that *C. nigricans* crude leaf extract is safe orally and possesses significant antimalarial activity.

**Keywords:** *Combretum nigricans*, Antimalarial, In vivo, *Plasmodium berghei*

## INTRODUCTION

Malaria remains a major public health problem (especially in most developing countries), with about 250 million new cases and over 600,000 mortalities annually.<sup>[1 - 4]</sup> About 40% of the world's population is currently at risk of being infected with malaria.<sup>[5]</sup> Africa however account for most of the global malaria cases (about 88%), and mortality (about 90%). In the sub-Saharan African region, most of the recorded severe cases and mortalities occur in pregnant women and children less than 5 years of age.<sup>[6]</sup> Though several agents are currently available for treatment and prophylaxis against malaria; it is still one of the greatest global health threats, maintaining its ranking among the top five causes of mortality among children less than 5 years of age. This can be largely attributed to the increasing malaria parasite resistance to currently available antimalarial agents, including quinine, mefloquine, chloroquine, atovaquone and sulfadoxine/pyrimethamine.<sup>[7 - 10]</sup> This challenge have therefore necessitated the need for the development of new antimalarial agents.

Medical chronicles have shown that plants are a vital source for the development of new drugs, including antimalarials.<sup>[11, 12]</sup> As a matter of fact, some of the currently used antimalarial agents were derived from plant sources; these includes the premiere antimalarial agent, quinine (derived from the stem-bark of cinchona plant), and the current standard antimalarial drug, artemisinin (derived from *Artemisia annua*).<sup>[13]</sup> These success, coupled with the fact that several other plants are currently being used traditionally in various parts of the world for the management of malaria, has actuated more research into medicinal plants for the purpose of developing new antimalarial agents. About 80% of the African population still depends on herbs for the management of several ailments including malaria.<sup>[14, 15]</sup> *Combretum nigricans* is one of such plants, it is a small tree with smooth bark found in North-Central Nigeria, and is traditionally used for the treatment of malaria fever. The seed have been reported to possess hypotensive and central nervous system depressant activities,<sup>[16]</sup> while the anti-leishmaniasis activity has also been reported.<sup>[17]</sup> The plant has also been reported to possess antifungal activity,<sup>[16]</sup> as well as cytotoxic effect.<sup>[18]</sup> In this light, the aim of this study was to evaluate the antimalarial activity of *C. nigricans* crude extract in mice, and hence seek if there is a scientific justification for its traditional application for the management of malaria.

## **MATERIALS AND METHODS**

### **Plant materials**

Fresh leaves of *Combretum nigricans* were collected from Jos-North Local Government Area, Plateau state, Nigeria. It was identified and authenticated by Mr. Jeffrey Azila, a taxonomist at the Federal College of Forestry, Jos, Plateau state, Nigeria. A voucher specimen (FHJ223) was prepared and deposited at the Federal College of Forestry herbarium for future reference.

### **Extraction**

The leaf samples were rinsed, air-dried (for two weeks) and pulverized into fine powder using a plant milling machine. Using standard procedure as illustrated by Handa *et al.*,<sup>[19]</sup> the pulverized leaves (1.6 kg) were macerated in methanol-water (1:1) v/v for 48 h with intermittent vigorous shaking. After 48 h, the mixture was filtered using muslin cloth followed by Whatman filter paper (No.1). The obtained crude extract was concentrated and stored at 4°C in a refrigerator till use.

### **P hytochemical analysis**

The crude extract was subjected to phytochemical analysis for constituent identification using standard procedures illustrated by Harborne<sup>[20]</sup> and Evans,<sup>[21]</sup> to test for the presence of alkaloids, saponin, glycosides, carbohydrates, flavonoids, tannins, terpenoids, resins, proteins, reducing sugar, quinine and steroids.

### **Animals**

Swiss albino mice of both sexes (weighing 22±1.08 g) housed at the animal facility of the Department of Pharmacology and Toxicology, University of Nigeria, Nsukka, were used. The animals were housed in standard cages under standard laboratory conditions at room temperature and humidity with a 12-h light/dark cycle; and ad libitum access to mice feed and water. All animal experiments were conducted in accordance with the NIH guidelines for laboratory animal care and use (Revised 1985)<sup>[22]</sup> and the University of Nigeria regulations for laboratory animal use.

### **Acute toxicity test (LD 50 )**

The oral acute toxicity of the *C. nigricans* crude extract was carried-out on Swiss albino mice using Lorke's method.<sup>[23]</sup> A total of 12 mice of either sex were fasted overnight prior to the study. The study involved two phases. In the first phase, nine mice were randomly divided into three groups of

three mice each, and were administered the extract doses of 10, 100 and 1000 mg/kg body weight orally respectively. They were observed critically for the first 4 h after dosing and subsequently for 24 h for signs of toxicity and mortality. In the second phase, three fresh mice were divided into three groups, one per group, and were administered extract doses of 1600, 2900 and 5000 mg/kg body weight respectively. The animals were observed for signs of toxicity and mortality for the first 4 h and subsequently for 24 h. they were further observed for 2 weeks for delayed toxicity. The LD<sub>50</sub> (acute toxicity dose) was calculated using the following formula:

$$LD_{50} = \sqrt{(\text{highest non-lethal dose} \times \text{least lethal dose})}$$

### **Rodent parasite [ *Plasmodium berghei* (NK- 65) ]**

The rodent parasite was sourced from the Faculty of Veterinary Medicine, University of Nigeria, Nsukka and maintained alive in mice by serial intra-peritoneal passage of blood from donor mouse to another mouse. The re-infected mice were kept at the University of Nigeria, Department of Pharmacology and Toxicology animal house unit, where the study was conducted.

## **In vivo anti malarial studies**

### **Parasite inoculation**

Standard inoculum consisting of about  $1 \times 10^7$  of *Plasmodium berghei* (NK-65) parasitized erythrocytes per ml from donor mouse in 0.2 ml normal saline was made. Each experimental mouse was inoculated intraperitoneally with 0.2 ml of infected blood suspension containing *Plasmodium berghei* NK-65 parasitized erythrocytes.

### **Activity on early malaria infection (suppressive test)**

The Peters'4-day suppressive test against *Plasmodium berghei* (NK-65) infection in mice was employed to test for the suppressive activity of the crude extract.<sup>[24]</sup> On the first day (D<sub>0</sub>), twenty-five Swiss albino mice of both sex were inoculated intraperitoneally (I.P) with 0.2 ml of standard inoculum of parasitized erythrocyte as described above. The mice were divided into 5 groups of 5 mice each (i.e. 5 mice per group) and treated for 4 consecutive days (D<sub>0</sub> - D<sub>3</sub>). Group I mice (control) were administered 10 ml distilled water/kg bodyweight daily. Groups 2, 3 and 4 were administered with 200, 400 and 800 mg extract/kg body daily respectively. Administration was done orally using intra-gastric cannula. Group 5 mice were treated with artesunate 10 mg/kg body weight daily. On the 5th day of study (D<sub>4</sub>), blood from the tail of each mouse was smeared on microscope slide to make a thin film. The films were stained with Giemsa stain and examined under a microscope as illustrated by Huang *et al*,<sup>[25]</sup> to determine parasitaemia and percentage suppression.

$$\text{Percentage suppression} = (A - B / A) \times 100$$

Where;

**A**=mean parasitaemia in the negative control group

**B**=mean parasitaemia in the test/standard group.

### **Activity on established malaria infection (Rane test)**

The curative activity of *C. nigricans* crude extract against established malaria infection was evaluated following the procedure outlined by Ryley and Peters.<sup>[26]</sup> Twenty five Swiss albino mice of both sex were weighed and grouped into five groups of five mice each. The animals were inoculated intraperitoneally (I.P) on the first day (D<sub>0</sub>) with 0.2 ml of standard inoculum of parasitized

erythrocyte and left untreated until the fourth day ( $D_3$ ) of study. On the fourth day ( $D_3$ ), blood from the tail of each mouse was smeared on microscope slide to make a thin film. The blood films were stained with Giemsa stain and examined under a light microscope as to determine pre-treatment parasitemia. The animals were treated for four days ( $D_3 - D_6$ ). Group I mice (control) were administered with 10 ml distilled water/kg bodyweight per oral. Groups 2 - 4 mice were administered with 200, 400 and 800 mg extract/kg body weight orally. While Group 5 mice were administered with artesunate 10 mg/kg body weight orally. Blood was collected from each mouse by tail bleeding on  $D_7$ , and thin blood film was made on microscope slide. The films were stained and examined under a microscope as described previously, to determine post-treatment parasitemia level. The percentage parasite clearance was calculated using the following formula:

$$\text{Parasite clearance (\%)} = (X - Y / X) \times 100$$

Where;

**X** = pre-treatment ( $D_3$ ) mean parasitaemia

**Y** = post-treatment ( $D_7$ ) mean parasitaemia

### Mean survival time

The animals were monitored to ascertain the mean survival time (days) across each group. The mice in each group were monitored daily for mortality from the first day of infection ( $D_0$ ), this was continued after the treatment period till mortality of all the animals. The mean survival time was recorded for each group as described by Saidu *et al.*<sup>[25]</sup>

### Temperature determination

The rectal temperature of each mouse in all groups was taken before infection ( $D_0$ ), during treatment ( $D_3 - D_6$ ) and after treatment ( $D_7$ ). The temperature was taken using a digital thermometer. The change in rectal temperature of treated groups was compared with the control group.

### Statistical analysis

The data obtained was expressed as mean standard error of mean (Mean  $\pm$  SEM). One way analysis of variance (ANOVA) followed by Dunnett's post hoc test was used to test for significance.  $P < 0.05$  was considered significant. GraphPad Prism for windows (version 7.0), San Diego California USA was used for the analysis.

## RESULTS

### Phytochemical screening

The results obtained from the phytochemical screening of *C. nigricans* crude leaf extract revealed the presence of alkaloids, cardiac glycosides, terpenoids, saponins, flavonoids, quinines, steroids, carbohydrate, tannins, proteins and reducing sugar; while resins were absent.

### Acute toxicity (LD 50 )

The mice appeared normal and no mortality was recorded at all the doses used for the study after 24 h, and throughout the two weeks observation period for delayed toxicity [Table 1]. The oral median lethal dose ( $LD_{50}$ ) of *C. nigricans* in mice was estimated to be greater than 5000 mg/kg body weight.

Phase	Extract dose (mg/kg)	No. of mortality after 24 hours	No. of mortality after 14 days
Phase 1	10	0/3	0/3
		100	0/3
		1000	0/3
	Phase 2	1600	0/1
			2900
			5000

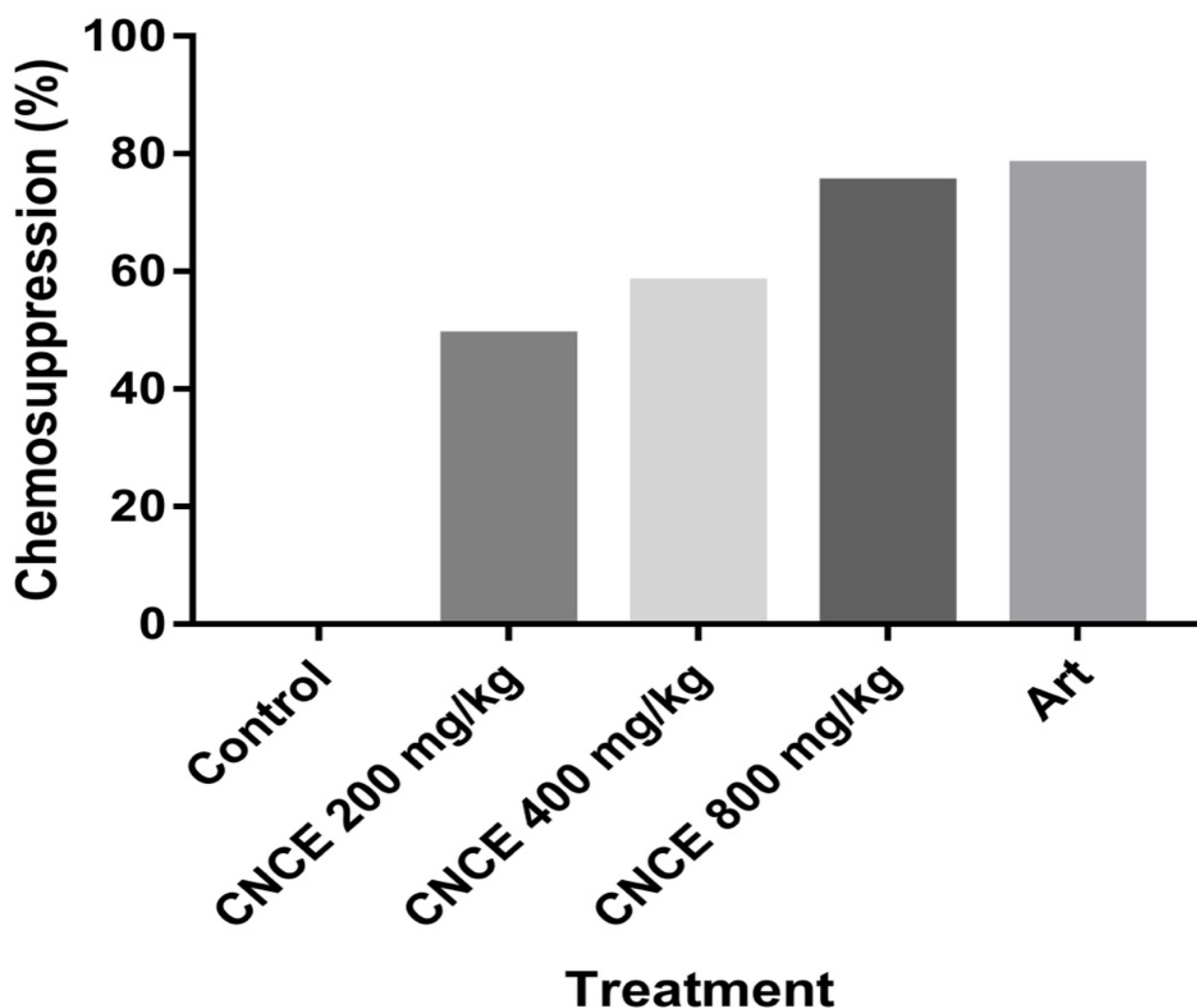
**Table 1.** Oral acute toxicity of *Combretum nigricans* crude leaf extract

### Activity on early malaria infection (suppressive test)

All doses of *C. nigricans* crude leaf extract gave significant ( $P < 0.05$ ) chemosuppressive effect against *P. berghei*, this effect was observed to be dose-related [Table 2]. The percent chemosuppression were 49.1, 57.5 and 75.5% for 200, 400 and 800 mg/kg doses respectively, while artesunate gave 78.3% [Figure 1].

Treatment	Dose (mg/kg)	Mean parasite count
Control	10 ml/kg	$21.2 \pm 0.80$
<i>C. nigricans</i>	200	$10.8 \pm 1.02^*$
	400	
	800	
Artesunate	10	$4.6 \pm 0.24^*$

**Table 2.** Chemosuppressive activity of *C. nigricans* crude leaf extract in *P. berghei* infected mice (Values expressed as Mean  $\pm$  SEM, where n=5, \*significant at  $P < 0.05$ )



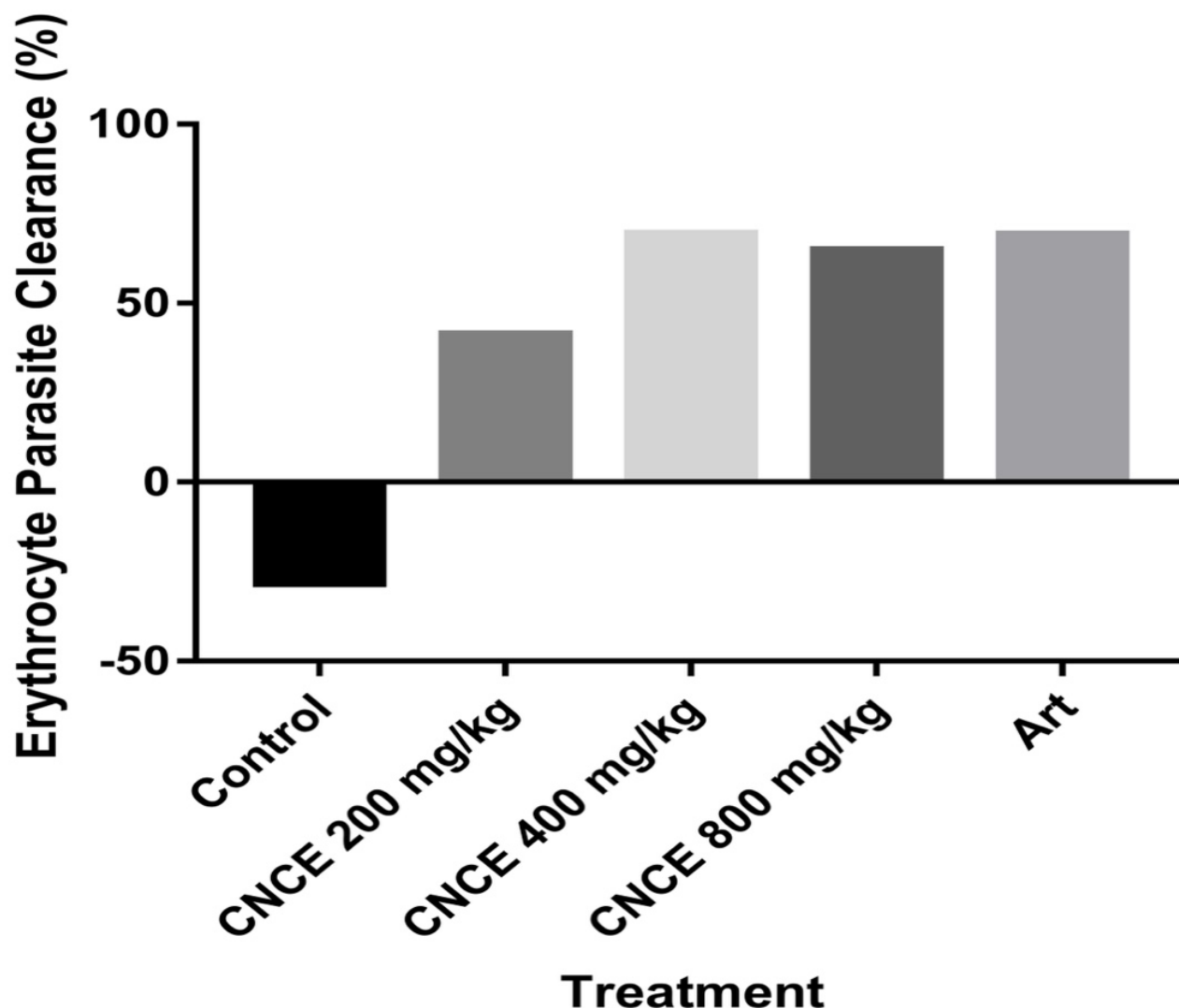
**Figure 1.** Percent chemosuppressive activity of *C. nigricans* crude extract against *P. berghei* in mice. Art = artesunate, CNCE= *Combretum nigricans* crude leaf extract.

### Activity on established infection ( Rane test )

The crude extract exhibited significant ( $P<0.05$ ) curative effect in established infection [Table 3]. The erythrocyte parasite clearance was 41.3%, 69.4%, and 64.8% for 200, 400 and 800 mg/kg respectively, while artesunate gave 69.2%. No parasite clearance was observed in the control group, but rather there was an increase by 28.2% [Figure 2].

Treatment	Dose (mg/kg)	Mean p parasite count	
		Day 3	Day 7
Control	10 ml/kg	28.0 $\pm$ 1.30	35.9 $\pm$ 1.78
<i>C. nigricans</i>	200	25.2 $\pm$ 0.58	14.8 $\pm$ 1.93*
	400	26.8 $\pm$ 1.66	8.2 $\pm$ 1.07*
	800	26.4 $\pm$ 1.21	9.3 $\pm$ 0.25*
Artesunate	10	26.0 $\pm$ 3.54	8.0 $\pm$ 0.58*

**Table 3.** Curative activity of *C. nigricans* crude leaf extract in *P. berghei* infected mice Values expressed as Mean  $\pm$  SEM, where n=5, \*significant at  $P<0.05$

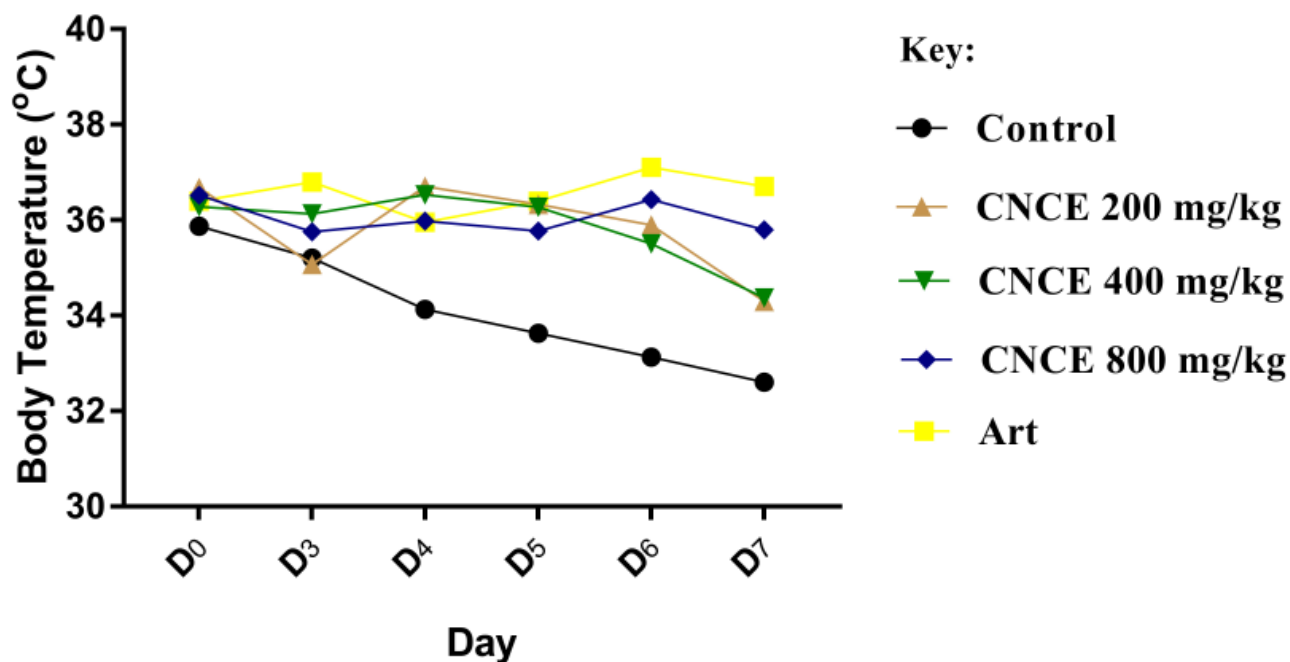


**Figure 2.** Erythrocyte clearance of *P. berghei* by *C. nigricans* crude leaf extract in mice. Art = artesunate, CNCE= *Combretum nigricans* crude leaf extract.

There was progressive decrease in mice body temperature of the control from study D<sub>3</sub> through D<sub>7</sub>. This observed decrease in body temperature was as low as 32.6 °C on D<sub>7</sub>. *C. nigricans* extract substantially prevented the decrease of temperature in a dose-related manner, with *C. nigricans* 800 mg/kg dose giving the highest effect. The standard drug artesunate also had the same effect of preventing body temperature decrease [Figure 3].

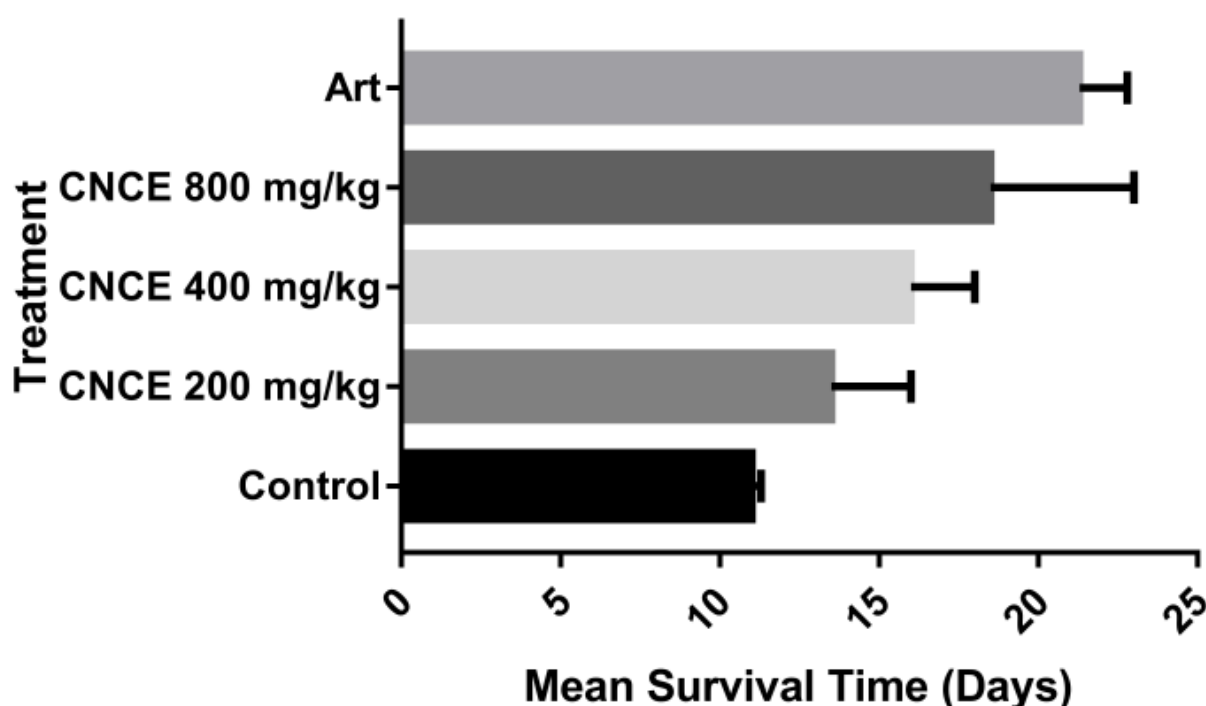
There was a prolongation in the mean survival time of the extract treated groups, as well as the artesunate treated group compared to the control. This effect was also dose-dependent and was significant ( $P<0.05$ ) in all the treated groups except the group treated with 200 mg extract/kg in which the effect was not significant ( $P<0.05$ ). The mean survival time of the control group was 11.0

days, while that of the groups administered crude extract were 13.5, 16.0 and 18.5 days for 200, 400 and 800 mg/kg respectively. The group administered artesunate had the longest survival time, 21.3 days [Figure 4].



**Figure 3.** Effect of *C. nigricans* crude leaf extract on the body temperature of mice infected with *P. berghei* (curative test). Art = artesunate, CNCE= *Combretum nigricans* crude leaf extract.





**Figure 4.** Effect of *C. nigricans* on the mean survival times of mice infected with *P. berghei* in curative test. Art = artesunate, CNCE = *Combretum nigricans* crude extract.

## DISCUSSION

In this study, the *in vivo* antimalarial activity of *Combretum nigricans*, a plant used in traditional medicine in North-Central Nigeria was evaluated on *Plasmodium berghei* infected mice using the suppressive and curative *in vivomodels*. *In vivomodels* were employed for this study because it takes into account possible pro-drug effect and possible involvement of immune system in extermination of the infection.<sup>[27, 12]</sup> Rodent Malaria models (especially *P. berghei*), are known to elicit disease features similar to those of human Plasmodial infection,<sup>[28 - 30]</sup> hence are used in preclinical research to predict possible clinical outcomes of prospective antimalarial agents. Several important antimalarial agents such as chloroquine, halofantrine, mefloquine and artemisinin were identified by rodent malaria model.<sup>[31]</sup> These facts therefore justify its application for the study.

The Peters' 4-day suppressive test on early malaria infection and Rane's curative test on established malaria infection is commonly employed for screening antimalarial agents. In both methods, the reduction of parasitemia in treated group to a level  $\leq 90\%$  of the level in the control (untreated) group indicates a potent antimalarial activity by the test agent.<sup>[32]</sup>

The result obtained from the study showed that all doses of the crude extract demonstrated significant ( $P < 0.05$ ) antimalarial activity in both test models. In the suppressive study, the extract gave significant ( $P < 0.05$ ) chemosuppressive effect against *P. berghei* in a dose-related fashion. The highest chemosuppressive activity by the extract (75.5% chemosuppression) was observed in the group treated with 800 mg/kg (highest test dose). This effect was closely related to that elicited by artesunate (78.3% chemosuppression), the standard antimalarial drug used for the study.

The extract also demonstrated significant ( $P < 0.05$ ) erythrocyte clearance of *Plasmodium* parasite in

the curative study. The highest extract activity was observed in the administered 400 mg extract/kg (69.4%); however, this was relatively close to the parasite clearance elicited by the highest test dose, 800 mg/kg (64.8%). This may serve as an indication that *C. nigricans* crude extract 400 mg/kg, may be the best therapeutic dose in mice. The control group showed a significant decrease in body temperature which progressed till D<sub>7</sub>, this decrease in body temperature was however less in the extract treated groups and artesunate group. Though fever (pyrexia) is one of the major manifestations of malaria in humans,<sup>[33]</sup> it is however different in rodent model of malaria which is usually characterized by hypothermia rather than pyrexia.<sup>[34]</sup> Previous studies have shown that the body temperature of mice infected with *P. berghei* will progressively decrease significantly from the fourth day of infection if left untreated.<sup>[35, 36]</sup> This sustained hypothermia in *P. berghei* infected mice may be attributed to the general debilitating effects of malaria on the host, which results in excessive loss of body heat and ultimately leads to death.<sup>[36]</sup> The treatment of *P. berghei* infected mice with a potent antimalarial agent will prevent this phenomenon (i.e., hypothermia); this is because body temperature of *P. berghei* infected mice usually decreases as the parasite level increases, hence agents that prevent parasite increase will also prevent significant decrease of body temperature. Hence the effect of the extract on body temperature of the treated mice can be attributed to its antiplasmodial activity, while the substantial decrease in the body temperature of the control group which progressed till the eight day (D<sub>7</sub>) may be due to progressive increase in the erythrocyte parasite level. The extract also prolonged the mean survival time in the curative test. This effect was significant in groups treated with extract doses of 400 and 800 mg/kg, which had mean survival time of 16.0 and 18.5 days respectively. Mean survival time is a useful parameter for evaluating the antimalarial efficacy of potential antimalarial agents.<sup>[37]</sup> A candidate antimalarial agent is considered to be potent if the mice in the treatment group survive for more than 12 days after infection. These observations have demonstrated that the *C. nigricans* crude extract is a potent antimalarial agent; however, the therapeutic effects of plant extracts are known to be elicited by the bioactive chemicals (phytochemicals) present in them. Hence, the extract's antimalarial activity may also be due to the phytochemicals present in it. The phytochemical assessment results revealed the presence of several important phytochemicals in the extract whose antimalarial activities have been documented. Among them are terpenoids, alkaloids, saponins and flavonoids.<sup>[38 - 41]</sup> Terpenoids are known to possess oxidant generating potential, which elicit antiplasmodial and cytotoxic effects. The cytotoxic effect of pentacyclic triterpenes (a class of terpenoids) isolated from *C. nigricans* have previously been reported by Simon *et al.*<sup>[44]</sup> Similarly, the antiplasmodial activity of triterpenes isolated from the methanolic leaf extract of *Combretum racemosum* (a plant from the Combretum genus also) have been reported.<sup>[45]</sup> The mechanism of action of artemisinin (a terpenoid isolated from *Asteresia annua*) and its derivatives (including artesunate) against Plasmodium parasites is based on their oxidant producing activity, and have also been reported to possess cytotoxic activity against invasive breast ductal carcinoma.<sup>[46]</sup> These evidences suggest that terpenoids present in *C. nigricans* are the culprit antiplasmodial principle in the plant, and may elicit their antiplasmodial effect through oxidant producing mechanism. Aside from antiplasmodial activity elicited by terpenoids, other bioactive components such as flavonoids, alkaloids and saponins present in the extract may have also elicited complementary antiplasmodial effect.<sup>[39, 40, 47][42, 43]</sup>

The acute toxicity test result showed that the oral median lethal dose of *C. nigricans* crude extract was greater than 5000 mg/kg in mice. According to previous studies, therapeutic agents with oral acute toxicity values greater than 5000 mg/kg are regarded as being safe or practically non-toxic.<sup>[48, 49]</sup> This therefore indicates that coupled with its antimalarial potency, the extract also has a wide therapeutic window in mice.

## CONCLUSION

The result from the study indicates that *C. nigricans* crude leaf extract possesses significant antimalarial activity, and this may serve as a scientific justification for its application as an antimalarial agent ethnomedicine.

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