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

*Enegide Chinedu1,2, Akah Peter A1, Ofili Charles C1, Agatemor Uzuazokaro M1, Ameh Solomon F4, Jacob Dabum L5, Onah Ifeoma A6*

1Department of Pharmacology, Novena University, Ogume, Delta State, Nigeria
2Department of Pharmacology & Toxicology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Nigeria
3Department of Public and Community Health, Novena University, Ogume, Delta State, Nigeria
4Department of Pharmacology & Toxicology, National Institute for Pharmaceutical Research and Development, Idu, Abuja, Nigeria
5Department of Human Physiology, Faculty of Medical Sciences, University of Jos, Jos, Nigeria
6Department of Pharmaceutical Microbiology & Biotechnology, Faculty of Pharmaceutical Sciences, University of Nigeria, Nsukka, Nigeria

**ABSTRACT**

**Background:** *Combretum nigricans* (Combretaceae) is a small, smooth bark tree used in traditional medicine to treat prurigo, dysentery, fever, and other symptoms of acute malaria in North-Central Nigeria. **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 artemunate 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 LD50 of the extract was >5000 mg/kg b.w. p.o. in mice. **Conclusion:** The result from the study indicates that *C. nigicans* crude leaf extract is safe orally and possesses significant antimalarial activity.

**KEYWORDS:** *Combretum nigricans*; Antimalarial; In vivo; *Plasmodium berghei*

**INTRODUCTION**

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

Medical chronicles have shown that plants are a vital source for developing new drugs, including antimalarials.[11, 12] As a matter of fact, some of the currently used antimalarial agents were derived from plant sources; these include the premiere antimalarial agent, quinine (derived from the stem-bark of cinchona plant), and the current standard antimalarial drug, artemisinin (derived from Artemisia annua). [13] These successes, coupled with the fact that several other plants are currently being used traditionally in various parts of the world to manage malaria, have actuated more research into medicinal plants to develop new antimalarial agents. About 80% of the African population still depends on herbs to manage several ailments, including malaria.[14, 15] Combretum nigricans is one such plant; it is a small tree with smooth bark found in North-Central Nigeria and is traditionally used to treat malaria fever. The seed has been reported to possess hypotensive and central nervous system depressant activities,[16] while the antileishmaniasis activity has also been reported. [17] The plant has also been reported to possess antifungal activity [16] as well as cytotoxic effect. [18] 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.

**Material and Methodology**

**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. In addition, 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 a fine powder using a plant milling machine. Using standard procedure as illustrated by Handa et al.,[19], 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.

**Phytochemical analysis:** The crude extract was subjected to phytochemical analysis for constituent identification using standard procedures illustrated by Harborne 20] and Evans [21] to test for the presence of alkaloids, saponin, glycosides, carbohydrates, flavonoids, tannins, terpenoids, resins, proteins, reducing sugar, quinines, 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 following the NIH guidelines for laboratory animal care and use (Revised 1985) [22] and the University of Nigeria regulations for laboratory animal use.

**Acute toxicity test (LD<sub>50</sub>):** The *C. nigricans* crude extract’s acute oral toxicity was carried out on Swiss albino mice using Lorke’s method. [23] A total of 12 mice of either sex were fasted overnight before 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:

\[ \text{LD}_{50} = \sqrt{\text{highest non-lethal dose} \times \text{least lethal dose}} \]

**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 the serial intraperitoneal 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 antimalarial studies**

**Parasite inoculation:** Standard inoculum consisting of about 1 × 10<sup>7</sup> 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. On the first day (D0), twenty-five Swiss albino mice of both sexes were inoculated intraperitoneally (IP) with 0.2 ml of standard inoculum of parasitized erythrocyte as described above. The mice were divided into five groups of five mice each (i.e., 5 mice per group) and treated for four consecutive days (D0 - D3). Group 1 mice (control) were administered 10 ml distilled water/kg body weight daily. Groups 2, 3, and 4 were administered with 200, 400, and 800 mg extract/kg body weight daily, respectively. The administration was done orally using an intragastric cannula. Group 5 mice were treated with artesunate 10 mg/kg body weight daily. On the fourth day of study (D4), blood from the tail of each mouse was smeared on a 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. to determine parasitaemia and percentage suppression.

Percentage suppression = \( \frac{A - B}{A} \times 100 \)

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. Twenty-five Swiss albino mice of both sexes were weighed and grouped into five groups of five mice each. The animals were inoculated intraperitoneally (IP) on the first day (D0) with 0.2 ml of standard inoculum of parasitized erythrocyte and left untreated until the fourth day (D3) of study. On the fourth day (D3), blood from the tail of each mouse was smeared on a microscope slide to make a thin film. The blood films were stained with Giemsa stain and examined under a light microscope to determine pre-treatment parasitemia. The animals were treated for four days (D3 - D6). Group 1 mice (control) were administered with 10 ml distilled water/kg body weight per oral. Groups 2 - 4 mice were administered with 200, 400, and 800 mg extract/kg body weight orally. At the same time, Group 5 mice were administered with artemesunate 10 mg/kg body weight orally. Blood was collected from each mouse by tail bleeding on D7, and thin blood film was made on a 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:

Parasite clearance (%) = \( \frac{X - Y}{X} \times 100 \)

Where; \( X \) = pre-treatment (D3) mean parasitaemia

\( Y \) = post-treatment (D7) 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 (D0); 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.

### Temperature determination:

The rectal temperature of each mouse in all groups was taken before infection (D0), during treatment (D3 - D6), and after treatment (D7). 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 were expressed as the mean, standard error of the mean (Mean ± 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, tanins, 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.

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

<table>
<thead>
<tr>
<th>Phase</th>
<th>Extract dose (mg/kg)</th>
<th>No. of mortality after 24 hours</th>
<th>No. of mortality after 14 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phase 1</td>
<td>10</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>100</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>Phase 2</td>
<td>1600</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>2900</td>
<td>0/1</td>
<td>0/1</td>
</tr>
<tr>
<td></td>
<td>5000</td>
<td>0/1</td>
<td>0/1</td>
</tr>
</tbody>
</table>

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].

Table 2. Chemosuppressive activity of *C. nigricans* crude leaf extract in *P. berghei* infected mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Mean parasite count</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10 ml/kg</td>
<td>21.2 ± 0.80</td>
</tr>
<tr>
<td><em>C. nigricans</em></td>
<td>200</td>
<td>10.8 ± 1.02*</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>9.0 ± 1.10*</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>5.2 ± 0.37*</td>
</tr>
<tr>
<td>Artesunate</td>
<td>10</td>
<td>4.6 ± 0.24*</td>
</tr>
</tbody>
</table>

Values expressed as Mean ± SEM, where n=5, *significant at P<0.05

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].

Table 3. Curative activity of *C. nigricans* crude leaf extract in *P. berghei* infected mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose (mg/kg)</th>
<th>Day 3</th>
<th>Day 7</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>10 ml/kg</td>
<td>28± 1.3</td>
<td>35.9±1.78</td>
</tr>
<tr>
<td><em>C. nigricans</em></td>
<td>200</td>
<td>25.2±0.6</td>
<td>14.8± 1.9*</td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>26.8± 1.7</td>
<td>8.2± 1.07*</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>26.4±1.2</td>
<td>9.3± 0.3*</td>
</tr>
<tr>
<td>Artesunate</td>
<td>10</td>
<td>26±3.5</td>
<td>8± 0.58*</td>
</tr>
</tbody>
</table>

Values expressed as Mean ± 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 the established infection (Rane test)
The crude extract exhibited a significant (P<0.05) curative effect in established infection [Table 3].

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 a progressive decrease in mice body temperature of the control from study D3 through D7. This observed decrease in body temperature was as low as 32.6 °C on D7. *C. nigricans* extract substantially prevented the decrease of temperature in a dose-related manner, with *C. nigricans* crude leaf extract.
There was a prolongation in the mean survival time of the extract-treated groups, and the artemesunate 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 artemesunate had the longest survival time, 21.3 days [Figure 4].

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. The extract gave a significant (P<0.05) chemosuppressive effect against *P. berghei* in a dose-related fashion in the suppressive study. 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 artemesunate (78.3% chemosuppression), the standard antimalarial drug used for the study.

The extract also demonstrated significant (P<0.05) erythrocyte clearance of the *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 D7; this decrease in body temperature was less in the extract-treated groups and artemesunate group. Though fever (pyrexia) is one of the major manifestations of malaria in humans [33], it is different in the rodent model of malaria, which is usually characterized by hypothermia rather than pyrexia.[34] 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. [35, 36] This sustained hypothermia in *P. berghei* infected mice may be attributed to the general debilitating effects of malaria on the host, resulting in excessive body heat loss and ultimately leading to death.[36] The treatment of *P. berghei* infected mice with a potent antimalarial agent will prevent this phenomenon (i.e., hypothermia); this is because the body temperature of *P. berghei* infected mice usually decreases as the parasite level increases; hence agents that prevent parasite increase will also prevent the significant decrease of body temperature. Hence the effect of the extract on the 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 eighth day (D7), maybe due to a 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 a 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.[37] A candidate antimalarial agent is considered
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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.[38 - 41] Terpenoids are known to possess oxidant generating potential, which elicits antiplasmodial and cytotoxic effects. The cytotoxic effect of pentacyclic triterpenes (a class of terpenoids) isolated from *C. nigricans* has previously been reported by Simon et al.[44] Similarly, the antiplasmodial activity of triterpenes isolated from the methanolic leaf extract of *Combretum racemosum* (a plant from the Combretum genus also) has also been reported.[45] The mechanism of action of artemisinin (a terpenoid isolated from *Atermesia annua*) and its derivatives (including artesunate) against *Plasmodium* parasites is based on their oxidant producing activity and has also been reported to possess cytotoxic activity against invasive breast ductal carcinoma.[46] This evidence suggests 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 effects.[39, 40, 47][42, 43]

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

**CONCLUSION**

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

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