IN VIVO AND TOXICITY ACTIVITY OF ETHANOL EXTRACT OF NUACLEA DIDERICHI

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ABSTRACT

The study shows that plants of the Nauclea family are a good source of phytomedicine for the treatment of malaria. The in vivo anti-malaria activity of the ethanol stem bark extract of Nauclea diderichii was evaluated in plasmodium berghei berghei infected mice. Oral acute infection, prophylactic effect against residual infection and the mean survival time using chloroquine sensitive Plasmodium berghei berghei NK65 infected mice was carried out. The Oral median lethal dose of the extract in mice was determined to be about 3800mg kg⁻¹ body weight. The extract at doses of (100, 200 and 400 mg kg⁻¹ b.w.) produced significant (P< 0.05; P< 0.01), dose dependent activity against the parasites in the suppressive, curative and prophylactic tests. The results suggest that the ethanol stem bark fraction of N. diderichii may possess antiplasmodial activity and thus lends credence to its ethno medical, and its indigenous usage for the traditional preparation of anti-malarial remedies.

Key words: Nauclea diderichii, Plasmodium berghei berghei, In vivo, Prophylactic, Toxicity, Malaria

INTRODUCTION

Malaria is one of the planet’s deadliest diseases and one of the leading causes of sickness and death in the world. It kills 2,800 children per day, 117 children per hour, 2 children per minute and 1 child per-thirty second - each year in Africa alone (Snow et al 2005). According to the World Health Organization, there are 300 to 500 million clinical cases of malaria each year resulting in 150 to 200 million deaths. It kills more than Aids and Cholera combined (W.H.O: Malaria report 2011). Quoting from the words of the UN millennium Economist, “Every month, as many children die of malaria in Africa as did in the tsunami; about 150,000” (WHO, 2008) The situation is further complicated by the worldwide emergence and rapid spread of resistance to several existing anti-malarial by Plasmodium falciparum that threatens to increase the above annual death toll (Mitsuyama et al., 1987; Gutmann et al., 1988; Mathias et al., 2000; Ganguly et al., 2001; Martino et al., 2002; Meshnick, 2002).

There is an urgent need for new anti malaria drugs and /or fine turned mode of actions of the existing ones. For as long as malaria has not been eradicated on the surface of the earth, research on the malaria parasites continues
Biomolecules of plant origin appear to serve as alternatives for the control of these human pathogens, more so, with a scientific back-up. There is now growing evidence that indicates a strong relationship between ethnic knowledge and sustainable use of biodiversity (Sullivan and Shealy 1997). The time-tested ethnic knowledge when supplemented with the latest scientific insights can offer new models of economic development, that are both eco-friendly and socially acceptable (Fauci, 1998). For instance, the tropical African chooses faith healing first, traditional herbal / orthodox medicine next and modern medicine only when the first two have failed (Shariff, 2001; Sudhakar, 2007). More so, due to the limited availability and / or affordability of pharmaceuticals and the effect of fake medical in the tropical countries, majority of the populations depend on traditional medical remedies (WHO, 2002; Zirrihi et al., 2005). The medicinal value of these metabolites is due mainly to the presence of chemicals substances that produce definite physiological action on the human body. Some of the valuable ones include: alkaloids, glucosides, steroid, flavonoids, terpenoids, fatty oils, resins, mucilages, tannins and gum.

In view of the above, this research delves into sourcing extracts from medicinal plants with known ethnomedical history. These are plants whose pure secondary metabolites could provide the expected chemotherapy for MDR-Malaria and also provides a “lead” whose chemotherapeutic index equals or even exceeds that of the exiting pharmaceuticals.

Plant parts of the nauclea family (Rubiaceae) are used in forms of decoctions and concoctions for the treatment of common tropical diseases like malaria fever, diarrhea, dysentery, stomach upset, jaundice dizziness etc (Ijomah et al., 1997, Idu and Onyibe 2007). Their active ingredients, secondary metabolites (Ghani, 1990; Dobelis, 1993; Fatope, 2001; and Kubmarawa et al., 2007), are a source of human sustenance. Nauclea diderichii is an evergreen tree that reaches a height of 30-40 m and a diameter of 0.9-1.5 m;bole cylindrical, slender, straight and branchless, rising to 20-30 m and a broad spherical crown with thick foliage. It is reputed for its numerous medicinal uses as a tonic and fever medicine, chewing stick, toothaches; dental caries, septic mouth, malaria, diarrhea and dysentery cure (Lamidi 1995, Chidambara, et al; 2003). Nauclea diderichii (De Wild & Dur) Meril (Rubiaceae). (Commercial name: bilinga) Common name; African peach

**MATERIALS AND METHODS**

**Collection of plant materials:**

Fresh samples of the stem bark of the African peach were collected in Gembu LGA, Taraba State Nigeria, and were identified in the Biological Sciences Department of Adamawa State University, Mubi. The FHI number is 0194 and a specimen of the plant was deposited in the herbarium. The sample (5.4kg) was air dried in the laboratory before pounding to a fine powder using pestle and mortar to about 60 mesh sizes and then stored in a dry container.

**EXTRACTION**

1.0kg of the powdered stem bark was accurately weighed and percolated with 7.5L of distilled ethanol for 72hrs. After which there was decantation, filtration, and concentration using rotary evaporator (R110) at 35°C to obtain ethanol soluble fractions, (F.E01). labeled, F.E.S. [230.5g], and kept in the refrigerator @ 4°C for further work.

**In Vivo Anti- Malarial Activity**

The in vivo anti- malarial activity of the ethanol leaf extract was evaluated in infected mice with the chloroquine sensitive plasmodium berghei berghei NK65 strain. Oral acute toxicity of the methanol leaf extract with modified Lorke’s method was evaluated against early infection, curative effect against established infection, prophylactic effect against residual infection and the mean survival period. Tests for the extracts were carried out in National Institute for Pharmaceutical Research and Development (NIPRD) Idu Abuja, Nigeria.

**Animals**

Four (4) weeks old-albino mice weighing 18 – 22g, obtained from the National Veterinary Research Institute (NVRI) Vom Jos Plateau State, Nigeria were used for the study. Test animals were received and fed with water ad libitum in accordance with NIH guide for the care and use of laboratory animals NIH Publication (No. 83 -23) revised (1985).

**Acute toxicity test (LD50)**

The oral acute toxicity of Nauclea diderichii ethanol stem bark extract was carried out on mice using modified Lorke’s assay (1983). This is in order to determine dose- and time-response relationships in order to estimate a no-observed adverse-effect level , which can be used to establish safety criteria for the chemical, (Frederich, et al 2008).

The study was performed in two phases. In phase one, nine mice were randomized into three groups of three mice each and administered 10, 100 and 1000 mg kg⁻¹ body weight (b. wt) of the extract orally.

The mice were observed for paw licking, salivation, stretching of the entire body, weakness, sleep, respiratory distress, coma and death in the first four (4) hrs and subsequently daily for seven (7) days. In phase two, another fresh set of nine mice were randomized in to three groups of three mice each and administered 1600, 2900 and 5000mg kg⁻¹ b. wt of the extract orally, based on the result of the first phase.
Test animals were observed for signs of toxicity and mortality for the first four critical hrs and thereafter, daily for 7 days. The LD$_{50}$ was calculated as the square root of the product of the lowest lethal dose and highest non-lethal dose. The geometric mean of the consecutive doses for which 0 % and 100% survival rates were recorded in the second phase, the oral median lethal dose was calculated using the formula:

$$LD_{50} = \sqrt{\text{Minimum toxic dose} \times \text{maximum toxic dose}}.$$

The rodent parasites Plasmodium berghei berghei NK 65 were obtained from the National Institute for Medical Research (NIMR) Lagos, Nigeria. The parasites were sustained by continuous intraperitoneal passage in mice (Adzu and Haruna, 2007) every 24 days. The infected mice were used for the study. Prior to the beginning of the study, one of the infected mice was kept and observed to reproduce disease symptoms similar to human infection (English, 1996).

**ANTI-PLASMODIAL STUDIES**

**Suppressive test**

The Peter's 4days suppressive test against chloroquine sensitive plasmodium berghei berghei NK 65 infection in mice was employed (Peter 1967). Adult Swiss albino mice weighing 18 -22 g were incubated by intraperitoneal (IP) injection with standard inoculums of the plasmodium berghei berghei with $1 \times 10^7$ infected erythrocytes. The mice were randomly divided into five (5) groups of six (6) mice per group and treated for 4 consecutive days with 100, 200 and 400 mg extract kg$^{-1}$ b. wt orally daily.

Two control groups were used: Positive control was treated daily with 5 mg chloroquine kg$^{-1}$ b. wt while the negative control was given 5 mL kg$^{-1}$ normal saline. On day 5 of the experiment, blood was collected from the tail of each mouse and smear on to a microscope slide to make a film (Saidu et al., 2000). The blood films were fixed with methanol, stained with 10 % Giemsa at pH 7.2 for 10min and parasitaemia examined microscopically.

The percentage suppression of parasitaemia was calculated for each dose level by comparing the parasitaemia in infected control with those of treated mice. I.E

$$\text{Average % suppression} = \frac{A - B}{A}$$

Where A = Average percentage parasitaemia in negative control group, and B = Average percentage parasitaemia in test group.

**EVALUATION OF SCHIZONTOCIDAL ACTIVITY OF NAUCELA DIDERICHII ON ESTABLISHED INFECTION (CURATIVE OR RANE TEST)**

Evaluation of the potential of the ethanol fraction of Nauclea diderichii stem bark was carried out according to the method described by Ryley and Peters (1970). The mice were infected intraperitoneally with standard inoculums of $1 \times 10^7$ Plasmodium berghei berghei NK 65 infected erythrocytes on the first day (day 0). Seventy-two hours later, the mice were divided into 5 groups of six mice each.

The groups were orally treated with stem bark of Nauclea diderichii extract at 100, 200 and 400 mg kg$^{-1}$ 'day' chloroquine (5 mg kg$^{-1}$ 'day') was administered to the positive control and an equal volume of distilled water was given to the negative control group. The treatment was carried out once daily for 5 days and blood smears were collected and examined microscopically to monitor the parasitaemia level.

**EVALUATION OF THE PROPHYLACTIC ACTIVITY OF NAUCLEA DIDERICHII (REPOSITORY TEST)**

Evaluation of the prophylactic potential of the N. diderichii stem bark extract was carried out according to Peters (1967).

Adult mice were randomized into 5 groups of six mice each. Group 1 was given 10 mL distilled water kg$^{-1}$ b. wt. orally. Group 2, 3 and 4 were given 100, 200, and 400 mg extract kg$^{-1}$ b. wt and group 5 was given 5 mg chloroquine kg$^{-1}$ b. wt intraperitoneally. Treatment was initiated on day 0 and continued till day 4 when, the mice were all infected with $1 \times 10^7$ infected erythrocytes. Blood smears were then made from each mouse 72 h after treatment (Abatan and Makinde 1986) and increase or decrease in parasiteamia determined using the formula above.

**STATISTICAL ANALYSIS**

One way ANOVA test was used to analyze and compare the results at a 95% confident level, values of $p \geq 0.05$; $P \geq 0.01$, were considered significant, results were expressed as Mean ± SE of mean.

**RESULTS**

**Anti-plasmodium investigations:**

The anti-plasmodium activity of N. diderichii fraction against Plasmodium berghei berghei, the Acute toxicity, the suppressive, the curative, the prophylactic and mean survival time, are shown in Tables 1 and 2.
Table 1: Acute toxicity test of the ethanol extract of the stem bark of *Nauclea diderichii*

<table>
<thead>
<tr>
<th>Vol. (mL)</th>
<th>Signs of toxicity</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>10mg / kg (1mg/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18g</td>
<td>0.18</td>
<td>x</td>
</tr>
<tr>
<td>20g</td>
<td>0.20</td>
<td>x</td>
</tr>
<tr>
<td>21g</td>
<td>0.21</td>
<td>x</td>
</tr>
<tr>
<td>100mg /kg (10mg/mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20g</td>
<td>0.20</td>
<td>x</td>
</tr>
<tr>
<td>22g</td>
<td>0.22</td>
<td>x</td>
</tr>
<tr>
<td>18g</td>
<td>0.18</td>
<td>x</td>
</tr>
<tr>
<td>1000mg /Kg (100mg /mL)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>21g</td>
<td>0.21</td>
<td>Paw licking</td>
</tr>
<tr>
<td>19g</td>
<td>0.19</td>
<td>Erect fur</td>
</tr>
<tr>
<td>18g</td>
<td>0.18</td>
<td>salivation</td>
</tr>
</tbody>
</table>

Table (1b) Phase II: Ethanol extract of the stem bark of *Nauclea diderichii*  
(Concentrations based on phase I)

<table>
<thead>
<tr>
<th>Vol.,(mL)</th>
<th>Signs of toxicity</th>
<th>Survival</th>
</tr>
</thead>
<tbody>
<tr>
<td>1600mg /kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>20g</td>
<td>0.20</td>
<td>salivation</td>
</tr>
<tr>
<td>18g</td>
<td>0.18</td>
<td>Paw licking</td>
</tr>
<tr>
<td>21g</td>
<td>0.21</td>
<td>Stretching /writing</td>
</tr>
<tr>
<td>2900mg /kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>22g</td>
<td>0.22</td>
<td>Erect fur</td>
</tr>
<tr>
<td>19g</td>
<td>0.19</td>
<td>calmness</td>
</tr>
<tr>
<td>20g</td>
<td>0.20</td>
<td>Reduced locomotion</td>
</tr>
<tr>
<td>5000mg / kg</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19g</td>
<td>0.19</td>
<td>Erect fur</td>
</tr>
<tr>
<td>18g</td>
<td>0.18</td>
<td>weakness</td>
</tr>
<tr>
<td>21g</td>
<td>0.21</td>
<td>Writing, comatose, convulsion, death</td>
</tr>
</tbody>
</table>

DISCUSSION

Acute toxicity

The mice were treated intraperitoneally with single dose of 10 – 5000 mg kg⁻¹ b. wt. of *N. diderichii* stem bark extract after being starved for 24h. The extract at 10 – 100 mg kg⁻¹ (phase 1) produced no physical signs of toxicity in the mice 24h after administration. But from 1000 to 5000 mg kg⁻¹ (phase 1& 2) there were some physical signs: salivation, paw licking, stretching / writing, calmness etc, within the first minutes of administration.

There was however no mortality at all dose levels used. The median lethal dose LD₅₀ was estimated to be ≥ 3800mg kg⁻¹ b. wt. However, the observed reduced activity of the treated mice showed that the extract possessed central depressant effect.

The absence of death following oral administration of the extract, at below 5000mg extract kg⁻¹ b. wt. suggest that the extracts were non acutely toxic (Salawu et al., 2009). This high safety profile may have been responsible for the wide spread use of this indigenous medicinal plants in different ethno-therapeutic interventions. Although,
primate models provide a better prediction of anti-malaria
efficacy in human than in rodent models, the later have
also been validated through the identification of several
conventional anti-malaria, drugs such as chloroquine,
halofantrine, mefloquine, maldox and more

Table 1: Phase Analysis of Acute toxicity test of the ethanol extract of the stem bark of *Nauclea diderichii*

<table>
<thead>
<tr>
<th>Dose (mg / kg b.w)</th>
<th>Dose (mg kg⁻¹ b.w)</th>
<th>Vol. (mL)</th>
<th>Toxicity Sign</th>
<th>Death / Alive</th>
</tr>
</thead>
<tbody>
<tr>
<td>10mg / kg</td>
<td>18g</td>
<td>0.18</td>
<td>-</td>
<td>Alive</td>
</tr>
<tr>
<td></td>
<td>20g</td>
<td>0.20</td>
<td>-</td>
<td>Alive</td>
</tr>
<tr>
<td></td>
<td>21g</td>
<td>0.21</td>
<td>-</td>
<td>Alive</td>
</tr>
<tr>
<td>100mg /kg</td>
<td>20g</td>
<td>0.20</td>
<td>-</td>
<td>Alive</td>
</tr>
<tr>
<td></td>
<td>22g</td>
<td>0.22</td>
<td>-</td>
<td>Alive</td>
</tr>
<tr>
<td></td>
<td>18g</td>
<td>0.18</td>
<td>-</td>
<td>Alive</td>
</tr>
<tr>
<td>Phase I</td>
<td>1000mg /Kg</td>
<td>21g</td>
<td>0.21</td>
<td>Paw licking</td>
</tr>
<tr>
<td></td>
<td></td>
<td>19g</td>
<td>0.19</td>
<td>Erect fur</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18g</td>
<td>0.18</td>
<td>salivation</td>
</tr>
<tr>
<td>1600mg /kg</td>
<td>20g</td>
<td>0.20</td>
<td>salivation</td>
<td>Alive</td>
</tr>
<tr>
<td></td>
<td>18g</td>
<td>0.18</td>
<td>Paw licking</td>
<td>Alive</td>
</tr>
<tr>
<td></td>
<td>21g</td>
<td>0.21</td>
<td>Stretching/writing</td>
<td>Alive</td>
</tr>
<tr>
<td>2900mg /kg</td>
<td>22g</td>
<td>0.22</td>
<td>Erect fur</td>
<td>Alive</td>
</tr>
<tr>
<td></td>
<td>19g</td>
<td>0.19</td>
<td>calmness</td>
<td>Alive</td>
</tr>
<tr>
<td></td>
<td>20g</td>
<td>0.20</td>
<td>Decreased mobility</td>
<td>Alive</td>
</tr>
<tr>
<td>Phase II</td>
<td>5000mg / kg</td>
<td>19g</td>
<td>0.19</td>
<td>Erect fur</td>
</tr>
<tr>
<td></td>
<td></td>
<td>18g</td>
<td>0.18</td>
<td>weakness</td>
</tr>
<tr>
<td></td>
<td></td>
<td>21g</td>
<td>0.21</td>
<td>Writing, comatose, convulsion, death</td>
</tr>
</tbody>
</table>

Key: absent (-) present (+)

Table 2: Suppressive, Curative, Prophylatic effects and Mean Survival rate of *N. diderichii* ethanol stem bark extracts and chloroquine against *P. berghei berghei* infection in Swiss Albino Mice

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Parasite</th>
<th>Chemo-suppression</th>
<th>Mean Survival time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Suppressive</td>
<td>Curative</td>
<td>Prophylatic</td>
</tr>
<tr>
<td>Normal saline 5 mL kg⁻¹ (control)</td>
<td>5.72 ± 1.31</td>
<td>48.8 ± 2.22</td>
<td>7.89 ± 1.41</td>
</tr>
<tr>
<td>Extract 100mg kg⁻¹</td>
<td>3.42 ± 1.12*</td>
<td>16.8 ± 2.12*</td>
<td>4.52 ± 1.32*</td>
</tr>
<tr>
<td>Extract 200mg kg⁻¹</td>
<td>2.65 ± 0.98*</td>
<td>6.00 ± 0.82*</td>
<td>2.84 ± 0.88*</td>
</tr>
<tr>
<td>Extract 400mg kg⁻¹</td>
<td>1.51 ± 0.86**</td>
<td>0.40 ± 0.16**</td>
<td>1.81 ± 0.96**</td>
</tr>
<tr>
<td>CQ 5 mg kg⁻¹</td>
<td>0.44 ± 0.29**</td>
<td>0.21 ± 0.14**</td>
<td>0.64 ± 0.31**</td>
</tr>
</tbody>
</table>

*Significant different from control at P≤ 0.05 and **at P≤ 0.01
The recent artemisinin, amalar plus, derivatives (Ryley and Peters, 1970). The research consolidates that of (Dolabela et al., 2008 and Molecules, 2009).

**Suppressive test**

N. diderichii stem bark extract exerted dose dependent chemo-suppressive effect against Plasmodium berghei berghei NK 65 malaria parasites. The extract caused a significant (P<0.05; P<0.01) chemo-suppression of 59.20, 67.62 & 77 when compared to both controls. The standard drug chloroquine caused chemo-suppressions of 96.01 (Table 2) which were higher than the groups treated with the plant extract. The observed higher efficacy of chloroquine may in part be due to non selectivity of the extract or slow absorption and poor bioavailability of the crude extract.

This is common with medicinal plants extracts as confirmed by, (Adzu and Haruna, 2007). The significant chemo-suppression produced by the extract on day 4 is consistent with the traditional use of the plant as an herbal medicament against malaria in tropical Africa.

**Curative effect**

Stem bark of N. diderichii ethanol extract produced significant (P< 0.05; P<0.01) dose dependent reduction in parasitaemia levels in the extract treated groups of Plasmodium berghei berghei NK 65 malaria parasite with a similar reduction in the chloroquine treated group (positive control). The average percentage parasitaemia reduction of the extract treated groups on day 7 were 65.57, 87.70, 99.18 for the 100, 200 and 400mg /kg /day respectively. Chloroquine 5mg /kg b. wt exerted 99.59% reduction of the parasite (Table2).

While there was a daily increase in the parasitaemia in the negative control group, the average percentage parasitaemia decreases in the extract and the positive control.

This is in consonance with the earlier reports (Tantchou et al., 1986, Adjanohoun et al., 1996, Odeku et al., 2008. Titanji et al., 2008 and Idowu et al., 2010) using the plant Alstonia boonei and (Agbedahunsi et al., 1998) using Khaya senegalensis. This is consistent with natural products of plant origin due to the crude nature of the extract.

**Prophylactic effect**

The ethanol extract of stem bark of N. diderichii produced significant (P< 0.05; P<0.05) dose dependent reduction in parasitaemia levels in the extract treated groups of Plasmodium berghei berghei NK 65 malaria of 42.24, 47.28 & 59.08 while 5mg chloroquine/kg b. wt. caused 89.40% reduction in parasite count (Table 2). The results indicated that the stem bark extract of N. diderichii possesses blood schizontcidal activity as evident from the chemo-suppression obtained during the four day early infection test and the 30 days curative / established infection which is comparable to the standard drug chloroquine, 5 mg / kg / day. This is in line with the work of Hassan et al., 2004, Faruq et al., 2004, Olafimihan 2004), which show that there are secondary metabolites in plants, used to suppress these schizones.

**Survival period**

From (Table 2), the extract appears to be highly effective against the species of Plasmodium berghei berghei (NK 65). The mean survival period of the Swiss albino mice treated with the extract in established infection during a period of one month showed that as the dose increases, the survival time increases. Mice treated with chloroquine 5mg / kg b. wt. per day survived for 30 days. Those treated with the extract at 100mg, 200mg and 400mg / kg b. wt. per day survived for 23, 26 & 28 days. The animals in the negative control group, which were treated with distilled water /normal saline, were found to have a mean survival period of 9 days. The mean survival time is 19 ±1 days, but the extract even at 100mg /kg b. wt is 23days.

Thus if purified, this extract could be a good source of sustenance showing the efficacy of the extract compared with the standard drug.

This confirms literature as these secondary metabolites are used to treat malaria, asthma, dysentery / diarrhea and other hay fevers (Gill 1992, Burkil 1994, Rahila et al., 1994).

The results of the above phytochemistry confirms with literature (Hassan et al., 2004, Faruq et al., 2004, Olafimihan 2004).

Plasmodium berghei berghei parasite is used in predicting the treatment outcomes of any suspected anti-malaria agent due to its high sensitivity to chloroquine making it the most appropriate parasite for this research (peter and Anatoli, 1998a).

However, present findings seem to deviate from the previous study by (Oze et al., 2007), who demonstrated that some plants may be nephrotoxic when administered in at higher doses. Currently, no single drug is effective for the treatment of multidrug resistant (MDR) malaria and combination therapy includes artemisinin derivatives such as artesunate (David et al., 2004) or combinations with older...
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