

## Research Article

### Evaluation of the Anti-plasmodial Activity of the Methanolic Root Extracts of *Anthocleista nobilis* G. Don, *Nauclea latifolia* Smith and *Napoleona imperialis* P. Beauv

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**Abstract:** The emergence of resistant strains of the malaria parasite has necessitated the continued search for other effective, safe and cheap plant-based anti-malarial agents. This study was carried out to evaluate *in vivo* the anti-plasmodial effect of the extract of a combination of three plants as used in traditional medicine in South-east, Nigeria. Dried and ground roots of the three plants: *Anthocleista nobilis*, *Nauclea latifolia* and *Napoleona imperialis* were extracted in 70% methanol as a combination of equal weight and individually. The extracts were thereafter administered singly and in combination to albino mice of both sexes. From the result, *N. latifolia* extract exhibited the highest potency, in the curative test, with  $78.7 \pm 0.7$  mean parasite inhibition ( $p < 0.05$ ). This was followed by the compound extract, with mean parasite inhibition of  $70.4 \pm 0.4$ , whereas Chloroquine (standard control) caused mean parasite inhibition of  $91.6 \pm 0.2$  ( $p < 0.05$ ). The study also shows that *N. imperialis* extract has a significant anti-pyretic activity, similar to that of the control ( $p < 0.05$ ). The results suggest that the individual plants have anti-plasmodial effect in varying degrees; however when used in combination, it gives improved symptomatic relief from malaria and extended the mean survival time of the treated mice.

**Keywords:** Anti-plasmodial, curative, parasitemia, passaging, *Plasmodium berghei*, prophylactic, suppressive

## INTRODUCTION

Malaria is a very important parasitic infectious disease of humans (WHO, 2013a). Chloroquine has been the most widely used anti-malaria, however, the spread of resistance to the drug led to its withdrawal from use in most countries in sub-Saharan Africa in the 1990s (Mwai *et al.*, 2009). The resistance of malaria parasite to Chloroquine and other anti-malaria has driven scientists into an intensive search for more effective agents against the scourge. Currently, there are reports of parasite resistance to the newly developed Artemisinin in some regions (WHO, 2013b). Thus, there is an urgent need for increased efforts in anti-malarial drug discovery especially in Africa (Fidock *et al.*, 2004). In recent times, natural products of plant sources have been the centre of focus as the main source of new, safer and more effective bioactive compounds with medicinal properties (Dike *et al.*, 2012). An appreciable level of studies has been done on African traditional medicinal plants, ranging from ethno botanical surveys, to the actual extraction of the active ingredients in the plants (Ibe and Nwufu, 2005; Udeinya *et al.*, 2006). It has been noted that in traditional practice, several plants are often used in combination (Rasoanaivo *et al.*, 2011). Therefore,

evaluation of possible synergistic interaction between compounds present in herbal mixtures is vital.

An ethnobotanical survey of plants used in the treatment of various ailments in South Eastern, Nigeria herbal markets and the treatment practices of the herbalists reveals that one of the concoctions sold for malaria therapy is made of an alcoholic extract of three plant roots namely; *Anthocleista nobilis*, *Nauclea latifolia* and *Napoleona imperialis*. Therefore, the aim of the study was to investigate the *in vivo* anti-plasmodial activity of the three plants used to prepare one of the anti-malaria concoctions.

The study would offer a scientific backing in the use of traditional herbs either singly or in combination against malaria.

*Nauclea latifolia* is a tree of the family, Rubiaceae. Extracts from both its roots and stem have been shown to have anti-helminthic, anti-diabetic, blood pressure lowering effect and analgesic properties (Onyeyili *et al.*, 2001; Gidado *et al.*, 2005; Nworgu *et al.*, 2008; Abbah *et al.*, 2005).

*Anthocleista nobilis* is a plant of family-Loganiaceae. The tree has light grey bark and produces oblong, dark green leaves with white, fleshy flowers. Its fruit consists of an oblong berry with numerous brown seeds. It is found in northwestern forested areas of Africa. *A. nobilis* contains the alkaloids brucine and

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loganine and has been noted to be antispasmodic and neurotropic, having a marked hypotensive effect (Abel and Busia, 2005; Madubunyi and Asuzu, 1996). It has also been shown to have relaxant property and anti-viral properties (Madubunyi and Asuzu, 1996; Ayodele *et al.*, 2012).

*Napoleona imperialis* (P. Beavr) is an evergreen plant that grows abundantly in bush fallows, secondary bushes and marginal lands, in most of the tropical humid zone of West Africa and is of the family *lecythidaceae* (Kopel, 1990). It has been reported to have wound healing and anti-hypertensive property (Esimone *et al.*, 2005; Omale *et al.*, 2011).

## METHODOLOGY

**Experimental design:** Herbalists from randomly selected shops in the herbal market, South-eastern Nigeria were interviewed on their methods of treatment of malaria. All of the herbalists were unanimous in their use of anti-malaria herbs mostly in combination for enhanced efficacy and in their mode of preparation of the herbs (boiling in water or soaking in alcohol). Out of the 7 concoctions sold for malaria therapy in the market, one was chosen for this study and it was said to contain a mixture of three roots namely, *Anthocleista nobilis* (A), *Nauclea latifolia* (B) and *Napoleona imperialis* (C).

The animals were grouped for prophylactic, suppressive and curative studies and were treated with the methanolic extract of the plant's roots in varied doses (125, 250 and 500 mg/kg), singly and in combination, following inoculation with the plasmodium parasite.

**Plant material:** Whole plant parts of A, B and C were bought from one of the herb sellers in the herbal market and was authenticated by a botanist in the department of Plant Science and Biotechnology, University of Port Harcourt. A voucher specimen of their roots was deposited at the department's herbarium. The roots were washed, sun dried (30°C±0.5) to constant weight, chopped into bits and milled into coarse powder by a mechanical grinder.

**Equipments and Reagents:** Photo Microscope (Olympus, Japan), Syringes (2 mL, 5 mL), oral cannula, Cotton wool, Chloroquine phosphate syrups (Emzor Pharmaceuticals), EDTA bottles, Microscopic slides (Olympus, China), Hand gloves, Methylated spirit, Giemsa stain (Sigma), 70% Methanol (Sigma), Rotary evaporator, Distilled water, Dissecting kit and board, Weighing balance (Mettler AL 204), Urethane and 10% formalin.

**Preparation of extracts:** Two hundred and forty grams each of the pulverized herbs was measured using Mettler AL 204 analytical weighing balance machine

and transferred into three conical flasks. Also, eighty grams each of the three herbs was weighed and mixed together to give two hundred and forty grams of the combined herbs and transferred into the fourth flask. The respective herbs and their combination were extracted for 72 h using 1.5 L of 70% methanol. The herb-methanol mixtures were shaken daily to ensure proper extraction. Thereafter, it was filtered and the residue (marc) was stored in a dessicator to remove all traces of the solvent and stored for subsequent use. The filtrate was subjected to solvent recovery using a rotary evaporator 60°C leaving behind a semi solid extract. The extract was poured in warm into a pre-weighed beaker and allowed to cool in a fume cupboard. The beaker and its content were subsequently weighed and the weight of the dried extract was deduced as follows:

$$\% \text{ yield was calculated as: } \frac{\text{weight of dried extract obtained}}{\text{Weight of plant material used}} \times 100\%$$

Prior to use, 1 g/mL of each extract was prepared in sterile water was added to give a stock of 1000 mg in 5 mL, and subsequently, the varied doses were prepared using distilled water. These extraction procedures were carried out in the Malaria Research Laboratory, University of Port Harcourt, Port Harcourt, Nigeria.

**Experimental animals:** Albino mice of both sexes, average weight of 23 g and free from infection was obtained from the animal house facility of the Department of Pharmacology, Faculty of Basic Medical Sciences University of Port Harcourt, Choba, Rivers State, Nigeria. They were acclimatized for a week and were maintained in a well ventilated room, with temperature of 25°C±1 and fed on TopFeed finisher and water ad libitum for the entire duration of the study. Good hygiene was maintained by constant cleaning of the cages and replacement of their beddings.

**Preparation of inoculum:** The rodent malaria parasite, *P. berghei* ANKA strain (Chloroquine sensitive) was obtained from the Nigerian Institute of Medical Research (NIMR), Yaba, Lagos. The parasite was maintained in the Malarial Research Laboratory of University of Port Harcourt by serial blood passage from donor mouse to normal mice through Intraperitoneal (IP) inoculation. Parasitemia was assessed by thin blood films made by collecting blood from the cut tip of the tail, stained with Giemsa stain and viewed under a photo microscope. For passaging the parasite into the test animals, 0.2 mL of blood was collected from the auxiliary plexus of veins of one of the donor rat (parasitemia>35%). The blood was diluted with 5 mL of Phosphate Buffered Solution (PBS) pH 7.1, to give 2×10<sup>7</sup> parasitized red blood cells (PRBC) in an injection volume of 0.2 mL (IP) (Peter *et al.*, 1975; Fidock *et al.*, 2004).

**Acute Toxicity Test/LD 50 determination:** Albino mice (20-25 g) of either sex were used. The median lethal dose (LD 50) was determined for each of the extracts using the method of Lorke (1983). In the first phase, the mice were divided into four groups with five mice in each group and each group were administered with the methanol root extract at doses of 10, 100 and 1000 mg/kg body weight and distilled water respectively. Administration of extract was done orally with a cannula attached to a graduated syringe. The general behavior of the individual animals was observed continuously for 30 min after the treatment and then periodically for 4 h and thereafter over a period of 24 h, for signs of toxicity. In the second phase, groups of one mouse each were treated with more specific doses of the extract, respectively depending on the result obtained from the first phase. The mice were also observed for signs of toxicity (OECD). The mice were further observed for up to 14 days following treatment for any signs of toxicity and death. Any adverse effects, such as hypoactivity, piloerection, salivation, weight loss and syncope, in the treated groups were noted. The final LD 50 was calculated as the geometric mean of the lowest dose that caused death and the highest dose for which the animal survived.  $LD\ 50 = \sqrt{\text{mean of the lowest dose that caused death and the highest dose for which the animal survived}}$ . At the end of the acute toxicity period, entire livers and spleens were removed post mortem from mice after induction of terminal general anesthesia by inhalation of urethane and 10% formalin. Organ wet weights were measured and compared with controls as indices for degree of hepatomegaly and splenomegaly. Body weight was measured initially and at the end of the experiment to assess weight loss or gain.

Deriving from the outcome of the acute toxicity studies, dosage ranging from 125-500 mg/kg body weight for all extracts was chosen for the study.

#### **Anti-plasmodial study:**

**Prophylactic (repository test):** The method of Peter *et al.* (1975) was adopted in the evaluation of the prophylactic potential of the extracts. The mice were randomly divided into 6 groups and were treated with varied doses of extract A, B, C and A+B+C, with 5 mice in each group. Negative control group were given an equivalent volume of Phosphate Buffered Saline (PBS) kg<sup>-1</sup> body weight (b.wt) while the positive (standard control) was given 25 mg Chloroquine per kg body weight orally. All treatments were initiated on day 0 and continued until day 4; the mice were all infected with the parasite. Blood smears were then made from each mouse 72 h after treatment. The smears were placed on microscopic slides, fixed with methanol and stained with 10% Giemsa at pH 7.2 for 15 min and examined under the microscope at X100 magnification to assess the level of parasitemia. Percentage

parasitemia was calculated according to the method outlined by Iwalewa *et al.* (1997) as percentage parasitemia = (No. of parasites in treated/No. of parasites in control × 100).

**Curative (Established infection or rane test):** The curative potential of the extracts was done employing the method described by Ryley and Peters (1970). The mice were injected intraperitoneally with standard inoculum of 10<sup>7</sup> *P. berghei* NK 65 infected erythrocytes on the first day (day 0). After 72 hours and following confirmation of parasitemia, the mice were divided into 6 groups of six mice per group. These groups were treated with the prepared leaf extract of A, B, C and A+B+C (125, 250 and 500 mg/kg/day), Chloroquine (25 mg/kg/day) was given to the positive control and an equal volume of PBS was given to the negative control group. The treatment lasted for 4 days at a single dose per day after which blood smears were collected and examined microscopically to monitor the parasitemia level.

**Suppressive:** Peter's 4 day suppressive test (Peter *et al.*, 1975) was followed to evaluate the blood schizontocidal action against *P. berghei*. Donor albino mice previously infected with Chloroquine-sensitive *P. berghei* and with a rising parasitemia of >35% as determined using thin blood film, were sacrificed and the blood collected using EDTA bottle. On day 0, experimental as well as control groups of animals were inoculated with 10<sup>7</sup> *P. berghei*-infected red blood cells. The mice were then randomly divided into groups of five per cage and groups of experimental animals were given different doses of extracts (125, 250 and 500 mg/kg) of the plant extract per day, orally, consecutively from day 0 to day 3. The initial treatment was given two hours after infection. The other two groups received either 25 mg/kg Chloroquine per day (positive control), or 0.2 mL of PBS (negative control).

On day four of the test, thin blood smears were prepared using well-labeled and properly cleaned slides. Blood was collected from the tail vein of each animal and dry blood films were fixed with methanol and subsequently, stained with Giemsa for 25 min. They were then washed with phosphate buffer, pH 7.2 and allowed to dry. To ensure optimal film quality, slides were made in duplicates. The slides were then microscopically examined using ×100 magnification in oil immersion (Model Olympus photo microscope). The percentage suppression of parasitemia was calculated for each dose level by comparing the parasitemia in infected controls with those of treated mice. Average percentage suppression of parasitemia was calculated using the formula:

$$A = \frac{[(B-C)]}{B} \times 100$$

where,

- A = Average percentage suppression
- B = Average percentage parasitemia in the control group
- C = Average percentage parasitemia in the test group

Student's t-test was used to compare the differences in the results between the groups. The mice were then monitored for 30 days.

**Evaluation of antipyretic activities of the extract:** The basal temperature of the test animals in the suppressive study group were measured by introducing 1.5 cm of digital thermometer into their rectum. Rise in body temperature of at least 1°C in the parasitized mice being treated with the various extract of differing doses will be monitored after inoculation with *P. berghei* parasite and subsequently at time intervals (30 min-4 h) following establishment of pyrexia.

**Determination of the effect of extract on mean survival time of treated animals:** The mean survival time for each group was determined arithmetically by finding the average survival time (days) of the mice (post-inoculation) in each group over a period of 28 days (D0-D27). Any death that occurred during this period was recorded.

**Data analysis:** The data was analyzed using student t-test and differences between mean were considered significant of (p<0.05) level was used. All results are expressed as mean±SEM (standard error of the means).

## RESULTS AND DISCUSSION

**The percentage yield of extracts:** The plants *A. nobilis*, *N. latifolia* and *N. imperialis* yielded 10.41 g w/w, 7.63 g w/w and 10.0 g w/w of extracts, respectively while the plant combination yielded 9.63g w/w of extract (Table 1).

**Determination of median lethal dose (LD 50):** There were no deaths or any signs of acute toxicity observed after oral administration of single doses up to 1000 mg/kg of the decoction of the roots of *A. nobilis*, *N. latifolia* and *N. imperialis*. These data indicate that the medium lethal dose (LD<sub>50</sub>) should be higher than 1,000 mg/kg for test animals. There were no significant changes observed in the behavior, cutaneous effects and breathing of the male and female mice. However, administration with *N. imperialis* produced salivation in the mice immediately after administration which ceases within 5-10 min, while lethargy and watery stool were observed in the animals within the first six hours of administration of the extracts combinations, for doses greater than 500 mg/kg. Post mortem evaluation of liver and spleen size did not show any significant changes when compared to the control. Weight gain pattern was similar for all test animals.

### Evaluation of Anti-plasmodial Activity:

**Prophylactic study:** The methanol root extracts caused a moderately low (p<0.05) and dose dependent decrease in parasite counts, unlike the Chloroquine- treated group, while the control group showed a daily

Table 1: Yield of the methanolic root extracts

Method of extraction	Percentage (%) yield of extracts			
	<i>A. nobilis</i>	<i>N. latifolia</i>	<i>N. imperialis</i>	Extract combination
Cold maceration process	10.41g w/w	7.63g w/w	10.0 g w/w	9.63 g w/w

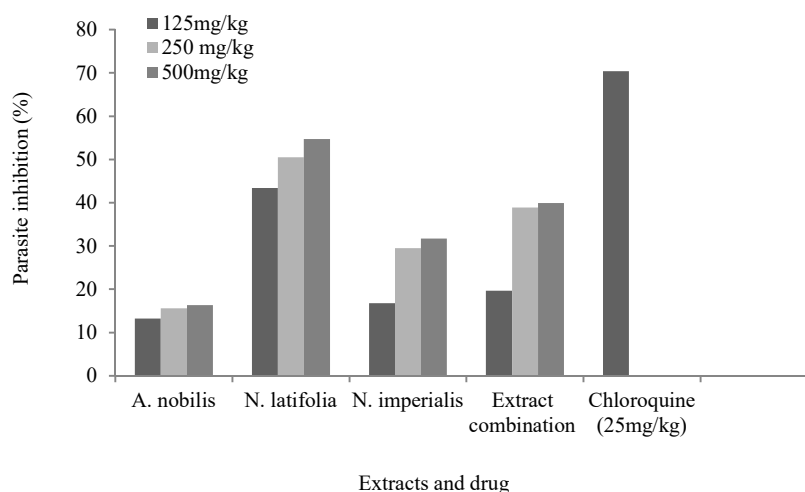


Fig. 1: Percentage inhibitory effect of the extracts on parasitemia in mice-prophylactic study

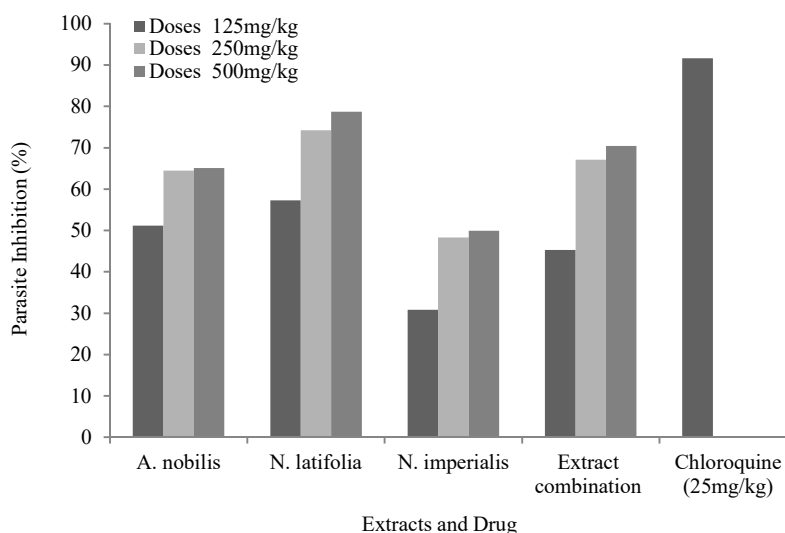


Fig. 2: Percentage inhibitory effect of the extracts on parasitemia in mice -curative study

increase in parasitemia. The percentage parasite inhibitory effect produced by extract A were  $13.2\% \pm 0.2$ ,  $15.6\% \pm 0.6$  and  $16.3\% \pm 1.1$ ; extract B,  $43.4\% \pm 0.4$ ,  $50.5\% \pm 0.1$  and  $54.7\% \pm 0.2$ ; extract C,  $16.8\% \pm 0.2$ ,  $29.5\% \pm 0.7$  and  $31.7\% \pm 0.5$  and extract A+B+C,  $19.7\% \pm 1.2$ ,  $38.9\% \pm 0.3$  and  $39.9\% \pm 0.1$  at 125, 250 and 500 mg/kg b.wt, respectively. At 25 mg/kg, Chloroquine produced  $70.4\% \pm 1.4$  chemosuppression (Fig. 1).

**Curative study:** The methanol root extracts caused a very high ( $p < 0.05$ ) and dose dependent parasite inhibition, while the negative control group showed absence of parasite inhibitory agent. The percentage parasite inhibitory effect produced by extract A were  $51.2\% \pm 0.6$ ,  $64.5\% \pm 0.4$  and  $65.1\% \pm 0.3$ ; extract B,  $57.3\% \pm 1.3$ ,  $74.2\% \pm 0.2$  and  $78.7\% \pm 0.7$ ; extract C,  $30.8\% \pm 0.3$ ,  $48.3\% \pm 0.8$  and  $49.9\% \pm 0.2$  and extract A+B+C,  $45.3\% \pm 0.5$ ,  $67.1\% \pm 0.4$  and  $70.4\% \pm 0.5$  at 125, 250 and 500 mg/kg body weight, respectively, while Chloroquine at 25 mg/kg, showed a percentage parasite inhibition of  $91.6 \pm 0.2$  (Fig. 2).

**Suppressive study:** The methanol root extracts caused a moderately high ( $p < 0.05$ ) and dose dependent parasite inhibition, while the negative control group showed increases in the parasite count in the test animals. The percentage parasite inhibitory effect produced by extract A were  $49.1\% \pm 0.7$ ,  $53.7\% \pm 0.2$  and  $54.4\% \pm 1.2$ ; extract B,  $55.8\% \pm 0.8$ ,  $70.5\% \pm 0.1$  and  $74.9\% \pm 0.5$ ; extract C,  $28.8\% \pm 0.3$ ,  $48.1\% \pm 0.7$  and  $49.0\% \pm 1.1$  and extract A+B+C,  $44.0\% \pm 0.3$ ,  $61.1\% \pm 0.5$  and  $68.9\% \pm 0.6$  at 125, 250 and 500 mg/kg body weight, respectively while Chloroquine at 25 mg/kg, showed a percentage parasite inhibition of  $90.3 \pm 0.2$  (Fig. 3).

**Determination of anti-pyretic activity of extracts:** Increase in the rectal body temperature of the studied

mice was observed first, 18 h following the inoculation of *P. berghei*. Treatment with the extract B, C and A+B+C at 500 mg/kg dose, decreased the rectal temperature of the mice in a dose dependent manner. In the presence of Chloroquine, 25 mg/kg, the increase in body temperature following *P. berghei* inoculation did not peak as high as it did with the extracts; its maximum temperature was at  $37.9^{\circ}\text{C}$ , by the 2<sup>nd</sup> h from the time of body temperature change. Extract A did not show anti-pyretic activity, the body temperature of the animals in this group rose steadily to approx.  $40.0^{\circ}\text{C}$ . On the other hand, body temperature of the animals undergoing treatment with extract B increased up to  $39.3^{\circ}\text{C}$ , only to drop afterwards at the third hour to  $37.8^{\circ}\text{C}$ . The extract combination was also found to show a significant anti-pyretic activity ( $p < 0.05$ ). Extract C showed the highest anti-pyretic activity, result of which is similar to that of the control-Chloroquine ( $p < 0.05$ ) (Fig. 4).

**Effect of the extract on the mean survival time of the animals:** Chloroquine at 25 mg/kg gave a mean survival time of  $28.0 \pm 0.0$  days as compared to  $25.0 \pm 1.2$ ,  $27.2 \pm 0.4$ ,  $26.1 \pm 0.1$ ,  $27.6 \pm 0.5$  and  $7.2 \pm 0.3$  for groups treated with extract A, B, C, A+B+C, 500 mg/kg and PBS (negative control), respectively. The results from this study showed that the extract combination extended the mean survival time of treated mice beyond that of the individual extracts and in a fashion comparable to that of the standard control (Fig. 5).

In summary, the present study shows that the crude extracts of the combination of the three plants have intrinsic anti-malarial properties, which are dose dependent. The methanolic root extract of *N. latifolia* displayed the highest level of inhibition of parasitemia followed by the extract combination, both at 500

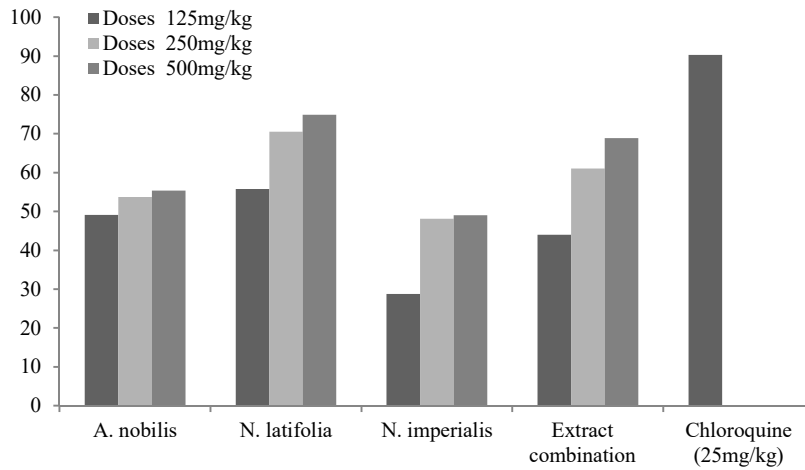


Fig. 3: Percentage inhibitory effect of the extracts on parasitemia in mice-suppressive study

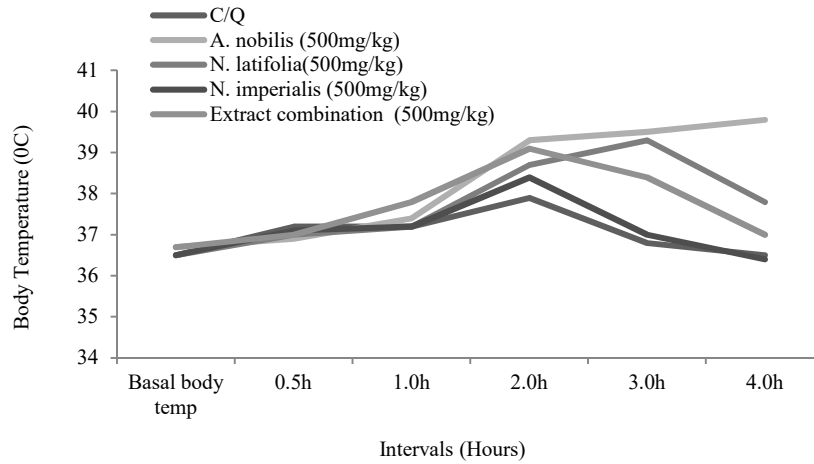


Fig. 4: Effect of extracts on rectal body temperature of mice

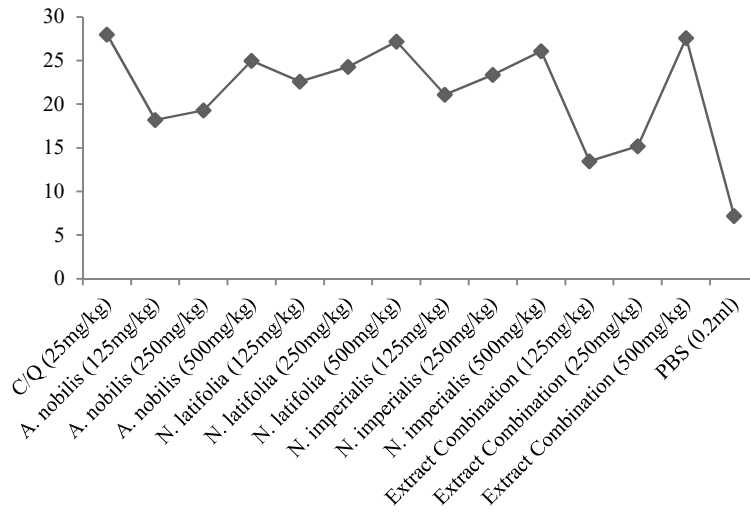


Fig. 5: Mean survival time of mice treated with extracts versus Chloroquine and drug vehicle

mg/kg. The anti-malaria and anti-pyretic effect, *N. latifolia* exhibited agrees with previous findings in this regard (Abbah *et al.*, 2005; Taiwe *et al.*, 2011).

*Napoleona imperialis* is noted for its anti-microbial and wound healing property (Esimone *et al.*, 2005); however, for the first time, this study has shown that it has a significant anti-pyretic activity similar to that of the control ( $p < 0.05$ ) and a mild inhibitory effect on *P. berghei*.

*P. berghei*, a rodent malaria parasite though, not able to infect man and other primates has been used because of its sensitivity to Chloroquine and the result confirmed that the *P. berghei* used in the study is Chloroquine sensitive as it exhibited mean parasite inhibition of  $91.6 \pm 0.2$  ( $p < 0.05$ ) in the curative study.

### CONCLUSION

Our present studies are directed towards identifying potent compounds from plants indigenous to Nigerians, so that more effective, affordable and available drugs can be developed. It is always interesting to see if the medicinal use of a particular plant or plants in ethnobotanical setting can be validated through scientific studies.

From this study, it can be concluded that the methanolic extracts of the roots of *A. nobilis*, *N. latifolia* and *N. imperialis* have shown anti-plasmodial effects on *P. berghei*-infected albino mice in varying degrees and in a dose-dependent manner. The extract combination demonstrated synergistic inhibitory effect on the *in vivo* plasmodial development. The sum total of the individual pharmacological properties of the herbal mixture resulted in an observable general improvement of symptoms in test animals and an extension of their mean survival time.

This result therefore, offers a scientific basis for the traditional use of these herbs in combination for the treatment of malaria. However, further characterization of the plants extracts and in-depth pharmacokinetic and pharmacodynamic studies are recommended, so as to elucidate on their mechanism of action.

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