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ORIGINAL ARTICLE

# *Azadirachta indica* extract–artesunic acid combination produces an increased cure rate of *Plasmodium berghei*-infected mice

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**Abstract**

*Context:* Available artemisinin-combination therapies (ACTs) are expensive. Various traditional herbal remedies for malaria involve plants, proven scientifically to have antiplasmodial effects, e.g., *Azadirachta indica* A. Juss. (Meliaceae).
*Objective:* Combination of an artemisinin-based compound and a medicinal herb extract will provide an indigenous alternative/herb-based ACT.
*Materials and methods:* The *in vivo* schizontocidal activity of the crude aqueous extract of 100, 500, and 1000 mg/kg *of A. indica* fresh leaves (NCE) and 6, 15, and 20 mg/kg of artesunic acid were determined, alone and in combination, while keeping the dose of artesunic acid constant at 15 mg/kg, using the Peter's 4-day suppressive test and Swiss albino mice. The $ED_{50}$ was calculated from the dose-response relationships. Percentage survival and cure were also determined.
*Results:* The average yield of two extractions of NCE was $8.33 \pm 1.67\%$. Combination of 1000 mg/kg of NCE and 15 mg/kg of artesunic acid, produced a significant reduction of parasitemia (96.87%), compared to 20 mg/kg of artesunic acid alone (68.14%). The combination had an $ED_{50}$ of 0.58 mg/kg while that of artesunic acid alone was 8.814 mg/kg. The combinations of NCE with artesunic acid produced a cure, although the artesunic acid did not produce a cure in 30 d.
*Discussion:* NCE increased the activity of artesunic acid in terms of reduction in parasitemia, and increased survival time and cure rate.
*Conclusion:* The combination of an artemisinin and aqueous extract of neem leaf is possible, providing a potentiated reduction of parasitemia, and increased cure rate.

**Keywords**

Artemisinin-based combination therapy, drug resistance, malaria parasites

**History**

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## Introduction

Due to resistance of the plasmodium parasite to single component antimalarials, drug therapy for malaria now involves the use of combination therapy. Artemisinins are almost always a constituent of such combinations because they are now the most active single component antimalarial but will produce fast recrudescence when used alone. They are used in combinations known as artemisinin-combination therapies (ACTs).

Various traditional herbal remedies for malaria contain plant extracts with scientifically proven antiplasmodial activity, for example, *Azadirachta indica* A. Juss. (Meliaceae). These extracts are a mixture of various antimalarial substances, thus making it difficult for the parasite to develop resistance against a single component. The leaf extract of *A. indica* has been prescribed orally for the treatment of malaria by Indian ayurvedic practitioners for centuries (Kausik et al., 2002). Dried neem leaf tea is used in Nigeria and Haiti to treat malaria (Abatan & Makinde, 1986; Ekanem, 1978). The leaf extract contains sterols, limonoids, flavonoids, glycosides, and coumarins (Mohammed, 2005). The mechanism of action of neem extract is believed to be redox perturbation that imposes substantial oxidant stress on the parasite (Iwu et al., 1986). The leaf extract has schizontocidal and gametocytocidal effects (Udeinya et al., 2008). Thus, the combination of two gametocytocidal agents, i.e., artesunic acid and neem extract would be expected to further decrease transmission of the malaria plasmodium.

The combination proposed in this study provides an indigenous cost-effective ACT and an alternative combination therapy.

## Materials and methods

### Experimental animals

Non-pregnant female Swiss albino mice, 8–14 weeks of age, weighing $25 \pm 2$ g, bred in the Faculty of Veterinary Medicine, University of Nigeria, Nsukka (UNN) were used

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in this study. They were kept in the Experimental Animal House of the Department of Biochemistry, for acclimatization prior to the study, where they were given standard animal feed, supplemented with *p*-aminobenzoic acid (45 mg/kg), and water *ad libitum*. The animals were handled according to the guidelines for laboratory animal use, approved by the laboratory animal use committee of UNN. *p*-Aminobenzoic acid (PABA) was given to the mice to supplement that produced by the parasite so as to ensure its survival. Mammals do not make PABA, but the parasite does. However, the amount they produce is not sufficient to ensure their survival. PABA is present in various food sources except breast milk (Kicska et al., 2003).

Table 1. Effect of artesunic acid and/or NCE on the growth of *P. berghei* in mice on days 4 and 7.

| | Reduction in parasitemia (%) ± SD | |
|---|---|---|
| Doses | Day 4 | Day 7 |
| Arta 6 mg/kg | 63.7 ± 17.0* | 37.3 ± 18.8 |
| Arta 15 mg/kg | 62.8 ± 17.3* | 70.3 ± 11.7* |
| Arta 20 mg/kg | 68.1 ± 8.5* | 72.1 ± 13.6* |
| NCE 100 mg/kg | 66.4 ± 11.5* | 37.3 ± 21.6 |
| NCE 500 mg/kg | 36.3 ± 32.1 | 52.6 ± 21.4 |
| NCE 1000 mg/kg | 40.7 ± 23.5 | 72.1 ± 13.2* |
| Arta 15 mg/kg + NCE 100 mg/kg | 79.2 ± 20.5* | 77.3 ± 14.7* |
| Arta 15 mg/kg + NCE 500 mg/kg | 83.3 ± 11.9* | 84.1 ± 5.7* |
| Arta 15 mg/kg + NCE 1000 mg/kg | 96.9 ± 2.9** | 89.8 ± 4.0* |

Arta, artesunic acid. *Significantly greater than the control. **Significantly greater than the control and Arta 20 mg, all at $p < 0.05$.

## The *Plasmodium* parasite

The parasite used was a chloroquine-sensitive strain of *Plasmodium berghei* NK 65 (Plasmodiidae), maintained in mice, from the National Institute of Pharmaceutical Research and Development (NIPRD), Abuja, Nigeria. The parasite was then subsequently passaged into fresh mice, which served as donor mice in this study.

## Herbal extract and drug

The *A. indica* plant was collected in March, 2010, from the botanical garden in Faculty of Pharmaceutical Sciences, UNN, and authenticated by Mr. A. Ozioko, International Centre for Ethnomedicine and Drug Development (INTERCEDD), Nigeria, formally Bio-resources

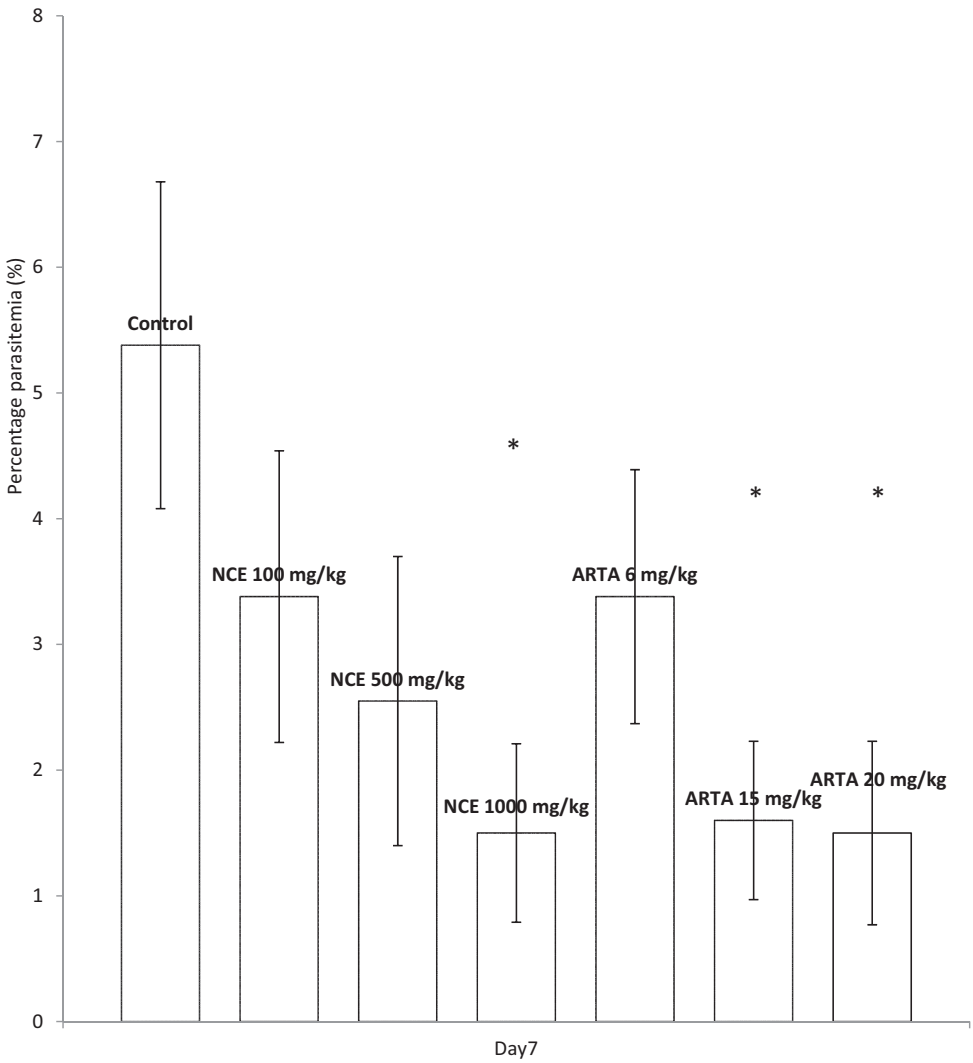


Figure 1. Percentage parasitemia in infected mice treated with single doses of NCE or Arta. *Parasitemia in treated group is significantly lower than that of control group at $p < 0.05$.

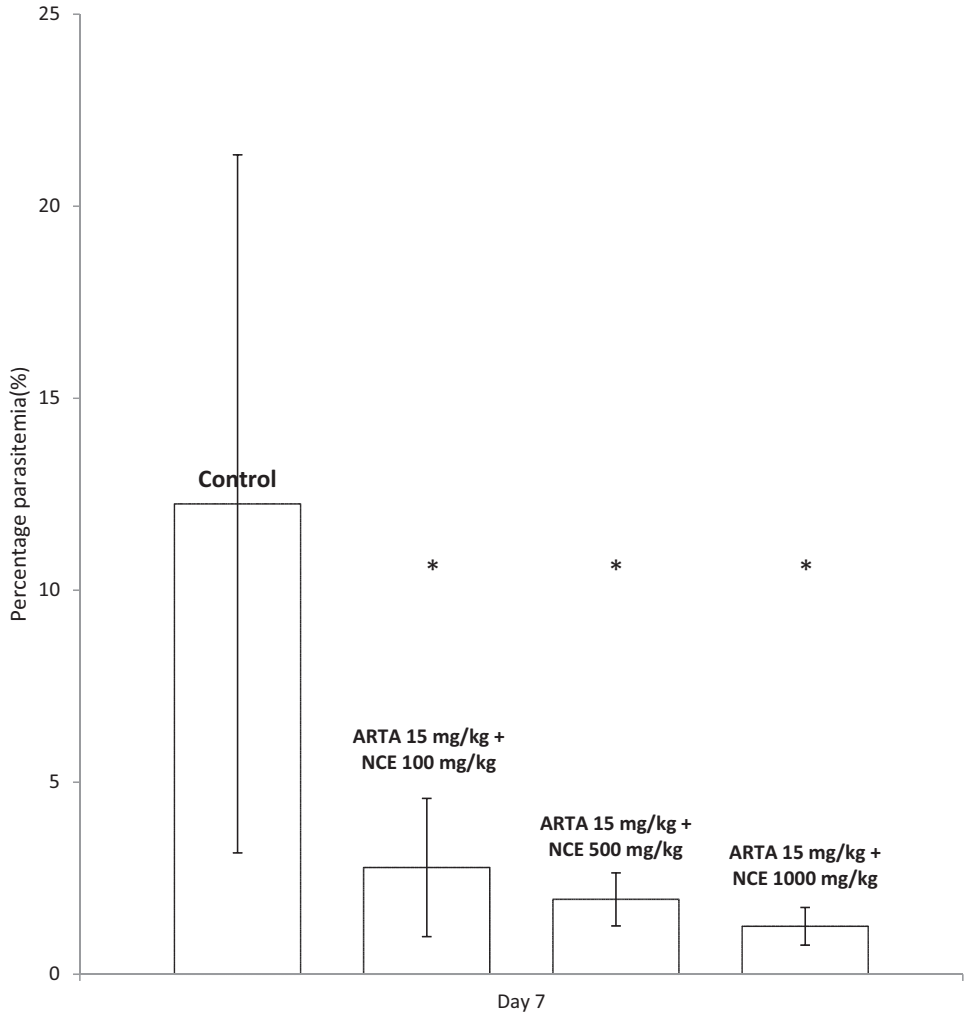


Figure 2. Percentage parasitemia in infected mice given combination treatment. *Parasitemia in treated group is significantly lower than that of the control at $p < 0.05$.

Table 2. Effect of single treatment (NCE or artesunic acid) on the survival and cure of infected mice.

| Doses | Mean survival time (days) ± SD | Percentage survival (%) | Percentage cure (%) |
|---|---|---|---|
| Control group | 19.80 ± 4.44 | 0.00 | 0.00 |
| Arta 6 mg/kg | 21.00 ± 3.74 | 0.00 | 0.00 |
| Arta 15 mg/kg | 21.80 ± 3.34* | 0.00 | 0.00 |
| Arta 20 mg/kg | 22.80 ± 2.28* | 0.00 | 0.00 |
| NCE 100 mg/kg | 11.20 ± 8.29 | 0.00 | 0.00 |
| NCE 500 mg/kg | 18.20 ± 4.32 | 0.00 | 0.00 |
| NCE 1000 mg/kg | 23.80 ± 4.76* | 20.00 | 0.00 |

*Significant when compared to neem 100 mg/kg at $p < 0.05$.

Table 3. Effect of combination treatment (NCE and artesunic acid) on the survival and cure of infected mice.

| Doses | Mean survival time (days) ± SD | Percentage survival (%) | Percentage cure (%) |
|---|---|---|---|
| Control group | 11.2 ± 4.6 | 0.00 | 0.00 |
| Arta 15 mg + NCE 100 mg | 28.2 ± 4.0* | 80.00 | 40.00 |
| Arta 15 mg + NCE 500 mg | 28.2 ± 4.0* | 80.00 | 40.00 |
| Arta 15 mg + NCE 1000 mg | 28.2 ± 4.0* | 80.00 | 60.00 |

*Significant when compared to control at $p < 0.05$.

Table 4. $ED_{50}$, $ED_{90}$, and correlation coefficient ($R^2$) of the artesunic acid, NCE, and NCE combined with artesunic acid.

| Treatment | $ED_{50}$ | $R^2$ |
|---|---|---|
| Arta | 8.91 | 0.969 |
| NCE | 275.42 | 0.915 |
| NCE + Arta (15 mg/kg) | 0.58 | 0.969 |

Arta, artesunic acids.

Development Centre, BDC, and a voucher specimen, INTERCEDD 917 was deposited there. The fresh leaves of *A. indica* (60 g) were rinsed twice in clean water and allowed to drip dry. The leaves were grounded and then macerated in cold distilled water for 24 h. The fluid was recovered by passing through a fine mesh of muslin cloth and allowed to sediment. The supernatant, called neem crude extract (NCE), was stored in a refrigerator at 2–4 °C. A measured aliquot of NCE was evaporated to dryness in order to determine the concentration. NCE was diluted with a mixture of Tween 80 and ethanol in sterile distilled water, so as to enable the administration of doses of 100, 500, and 1000 mg/kg of the extract. The selected doses were based on studies on the antimalarial activity of aqueous neem leaf extract (Abatan & Makinde, 1986).

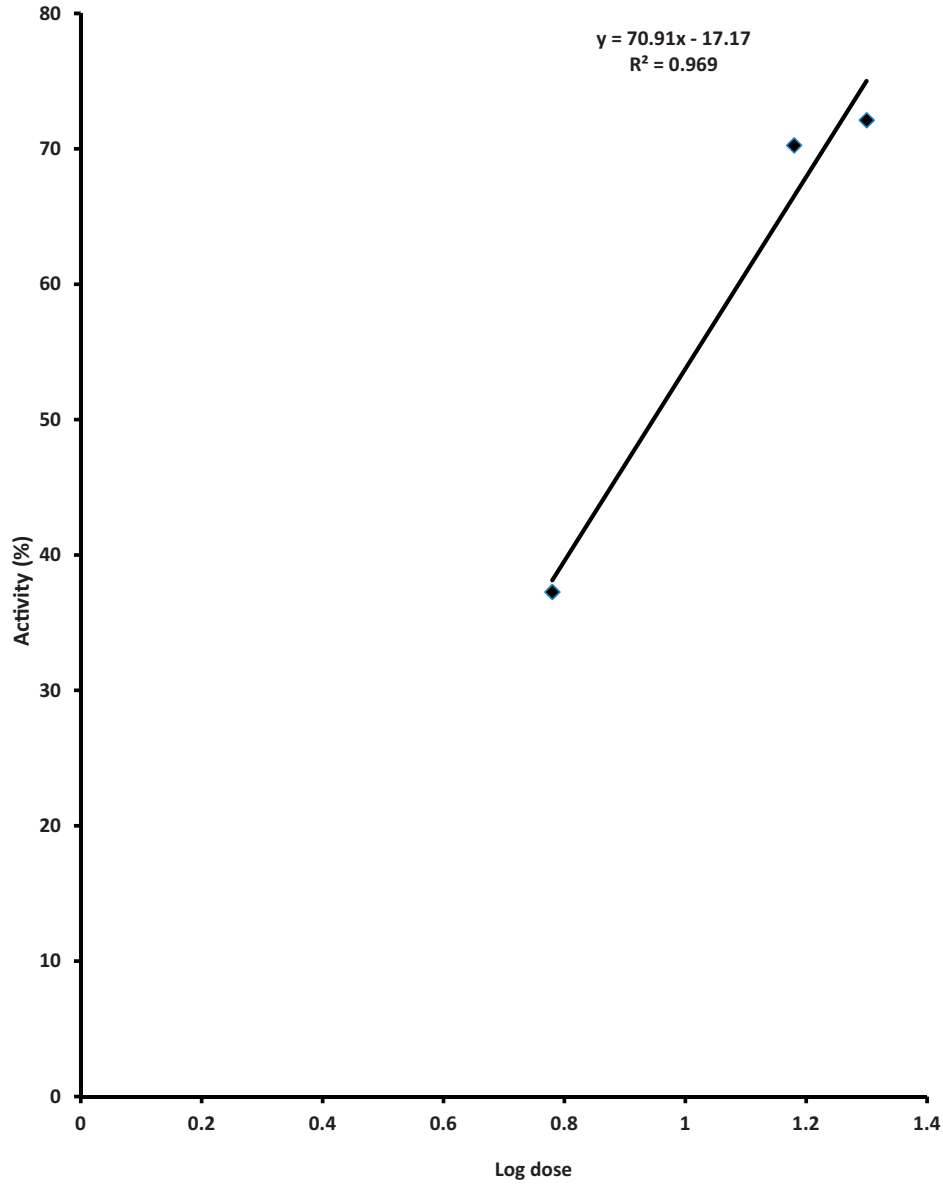


Figure 3. Percentage reduction of parasitemia versus log dose of artesunic acid.

The pure artesunic acid powder (Arta) used in this study was a generous gift from Emzor Pharmaceutical Industries, Lagos, Nigeria. It was dissolved in a mixture of 7% Tween 80 and 3% ethanol to provide doses of 6, 15, and 20 mg/kg. These doses were based on the $ED_{50}$ of artesunate on *P. berghei* infected mice (Malik et al., 2008).

### *In vivo* schizontocidal activity

This was carried out using the Peter's 4-day suppressive test (Kalra et al., 2006). On day 0, the parasitemia and red blood cell count of the donor mice was determined by using a Giemsa-stained thin blood smear and an improved Neubauer counting chamber, respectively. The blood was then collected by cardiac puncture and from the retro-orbital plexus vein, and diluted with physiological saline to give a concentration of $10^8$ parasitized erythrocytes per milliliter. About 0.2 ml of the cell suspension was injected intraperitoneally into each experimental mouse. The mice were randomly placed into seven groups of five mice each. The negative control group was given 7% Tween 80 and 3% ethanol in sterile distilled water. Equal volumes of the drug, extract, and placebo were at administered orally at 4, 24, 48, and 72 h post infection. On days 4 and 7 post inoculation, thin blood smears of the test mice stained with 10% Giemsa solution were used to determine the percentage parasitemia microscopically, by counting four fields of approximately 100 erythrocytes per field. The antimalarial activity was determined by using the equation;

$$\text{Activity} = 100 - \left[\left(\frac{\text{mean parasitemia in treated group}}{\text{mean parasitemia in control group}}\right) \times 100\right].$$

The mice were monitored for 30 d post infection and time of death (days) were recorded. Each mouse still alive on day 30 was checked for parasitemia. The lowest dose of artesunic acid that gave a significant reduction in parasitemia, both on days 4 and 7, i.e., 15 mg/kg, was chosen as the dose of artesunic acid to be combined with different doses of NCE.

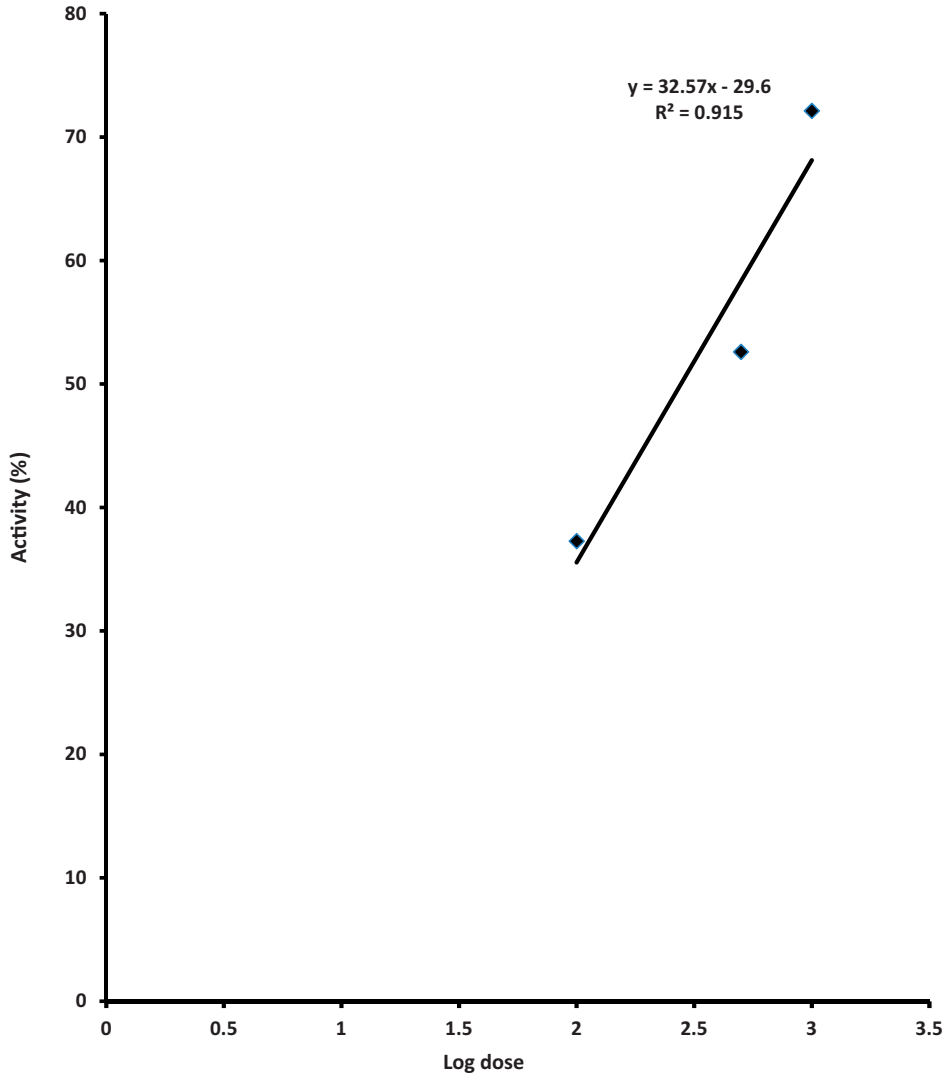


Figure 4. Percentage reduction of parasitemia versus log dose of NCE.

The antimalarial activity of these combinations was also determined using the above method.

### Determination of $ED_{50}$

The linear equations of the dose–response relationship of the crude drug and/or artesunic acid were used to calculate their $ED_{50}$ on day 7.

### Data analysis

All the results were analyzed by using ANOVA with multiple comparison tests (Games–Howell's and Tukey HSD tests).

## Results

### *In vivo* schizontocidal activity

Table 1 shows the antimalarial activity, i.e., the mean percentage reduction in parasitemia of the drugs alone and in combination, compared to the control, on days 4 and 7. The mean parasitemia values on days 4 and 7 are shown in Figures 1 and 2.

### Survival time and percentage cure

Tables 2 and 3 show the survival time based on a 30 d observation period, percentage survival at the end of this period and percentage of the infected mice that are cured, i.e., do not have any parasite in their blood at day 30.

### $ED_{50}$ of the crude extract and drug

Due to the plasmodiastatic effect brought about by *Eperthrozoon coccoides*, the $ED_{50}$ of artesunic acid and NCE, alone and in combination was calculated based on day 7 not day 4 as shown in Table 4. The $ED_{50}$ of the drugs was calculated by using the linear equation of the dose–response relationship of the drugs, Figures 3–5. The $ED_{50}$ of artesunic acid and NCE were 8.814 and 277.95 mg/kg, respectively. While the $ED_{50}$ of the combination was 0.58 mg/kg.

## Discussion

The development of affordable herb-based ACTs as alternative cost-effective antimalarial medicines is imperative, in consideration of people living in low income communities.

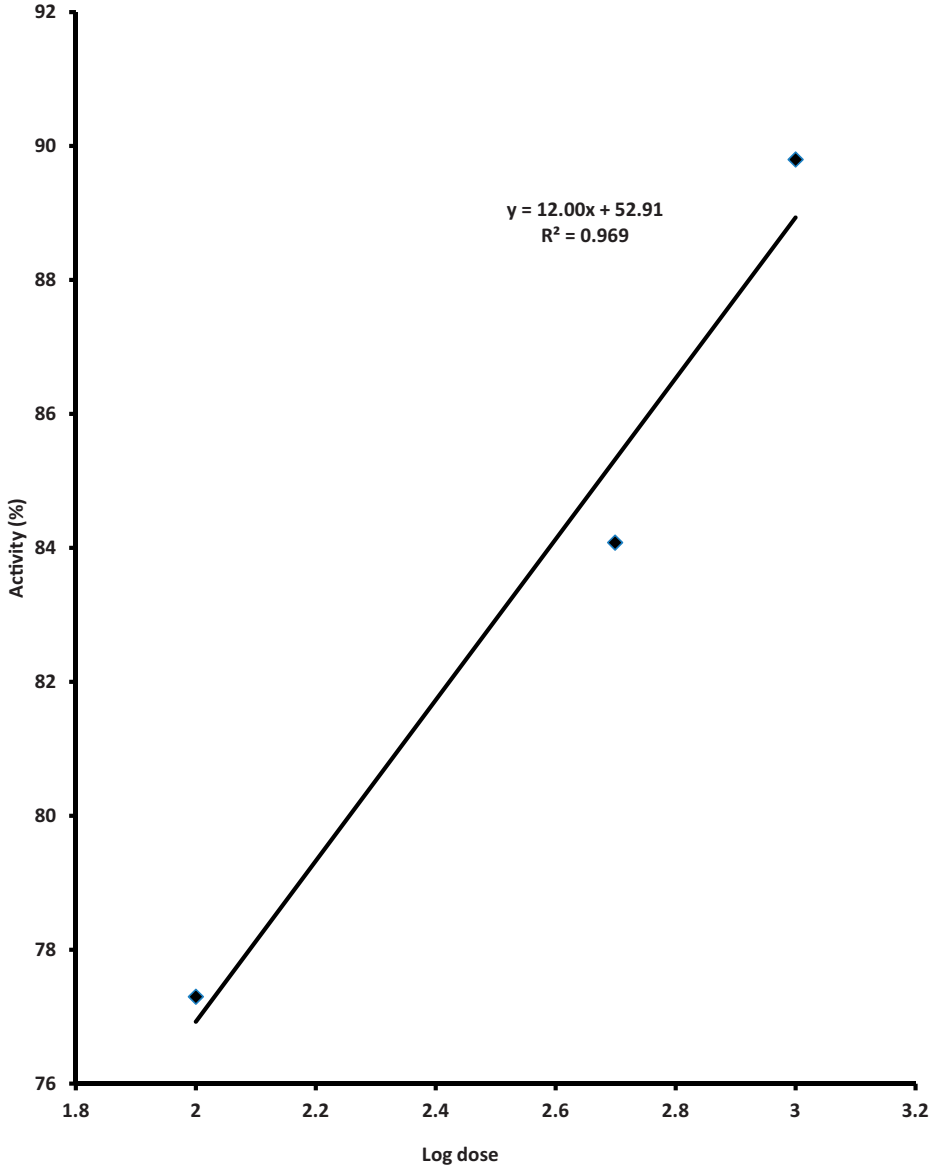


Figure 5. Percentage reduction of parasitemia versus log dose of NCE in combination with artesunic acid.

Combination therapy also provides an opportunity for dose reduction of the components, thus leading to reduced drug adverse reaction.

On the average, the yield of neem aqueous crude leaf extract (NCE) was $8.33 \pm 1.67\%$. At the initial stage of this study, a lag in the replication of the parasite was observed in the single mice treatment groups. Such plasmodiastatic effect has been attributed to possible blood contamination by *Eperythrozoon coccoides* (Neimark, 2005). *E. coccoides* inhibits the multiplication of the plasmodium, and enables the mice to survive longer after infection by *P. berghei*. It interferes with the course of the malaria (Peters, 1965). *E. coccoides* causes parasitemia that peaks on days 2–5 (acute infection stage) and subsequently declines rapidly (latent infection stage), such that by day 6 or 7, the number of organisms in the peripheral blood is very low, even beyond detection. So, for the first 5 d, the growth of *P. berghei* was inhibited by both the drugs and *E. coccoides*. Death from infections with *E. coccoides* is very rare (Neimark, 2005).

On day 7, the parasitemia levels of the groups treated with NCE decreased in a dose-dependent manner contrary to what was seen on day 4 when we had interference from the *E. coccoides.* This resulted in an anomaly where the group given 100 mg/kg of NCE had a significant reduction in parasitemia on day 4 but not on day 7. And, the parasitemia reduction in groups given 1000 mg/kg was significant on day 7 not on day 4. This may also indicate that NCE is a slow acting antiplasmodial agent.

The treatment of *P. berghei* infected mice with 6 mg/kg of artesunic acid produced a significant reduction in parasitemia compared to the control on day 4, but not on day 7, probably due to recrudescence as a result of *Eperythrozoon coccoides* plasmodiastatic effect. Higher doses of artesunic acid, 15 or 20 mg/kg equally, significantly suppressed the parasitemia of the infected mice on days 4 and 7.

The combination treatment yielded a significant reduction in parasitemia compared to the control on days 4 and 7. The combination of artesunic acid at 15 mg/kg and NCE at 1000 mg/kg produced a significant reduction in parasitemia than artesunic acid, 20 mg/kg, alone on day 4. Neem extract, thus, potentiated the antimalarial activity of artesunic acid through a pharmacodynamic interaction. Neem leaf extract

has been found to increase the serum concentrations of artesunate in our laboratory (Ezeora et al., unpublished project in our laboratory). This pharmacokinetic interaction is also important in the observed potentiation.

The survival of the treated mice revealed a dose-dependent pattern. The group treated with NCE 100 mg/kg survived for an average of 11.20 d, which was significantly lower than that of the groups given NCE 500 mg/kg, artesunic acid 15 or 20 mg/kg. There was a significant difference in survival time between groups receiving 1000 mg/kg of NCE and control group. At least one mouse in the group that received 1000 mg/kg of NCE survived up untill day 30, although the mouse was not cured of parasitemia. All the groups that received combination treatments survived significantly, compared to the control. Not less than two mice in all the combination treatment groups were cured, with cure rate ranging between 40 and 60%. Aqueous neem leaf extract thus has the capacity to prevent recrudescence that arise when artemisinins are administered alone. It will improve cure and survival of malaria infections.

Due to the plasmodiastatic effect brought about by *E. coccoides*, the $ED_{50}$ values of artesunic acid and NCE were calculated on day 7 and not day 4. The $ED_{50}$ of artesunic acid (8.8 mg/kg) agrees well with that of artesunate (8.0 mg/kg), which was obtained using the ANKA strain of *P. berghei* in infected mice (Malik, 2008). NCE with an $ED_{50}$ of 277.95 mg/kg has a moderate antimalarial activity (Malik, 2008). The $ED_{50}$ of the combination therapy (0.58 mg/kg) was lower than that of NCE or artesunic acid alone and this indicates that the dose of each component drug can be reduced to achieve the same effect.

## Conclusion

In this study, two substances (NCE and artesunic acid) both of which have transmission blocking activities against plasmodium parasites have been combined. NCE potentiated the antiplasmodial action of artesunic acid. Use of the combinations of artesunic acid and NCE prolonged the survival of the infected mice, when compared to artesunic acid alone. The combinations also produced a cure (at least 40%), while the doses of artesunic acid used in this study did not produce any cure at the end of the 30 d period of this study. Therefore, this study provides a basis for the development of an herb-based antimalarial combination therapy, as it may prevent fast recrudescence that comes from administering artemisinins alone. This study also provides the basis for developing a neem constituent-based combination with artemisinin compounds as ACTs for effective antimalarial therapy. The comparative toxicity of this combination will also have to be evaluated.

## Acknowledgements

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## Declaration of interest

The authors report no declaration of interest.

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