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Full Length Research Paper

Antimalarial effects of Anthraquinone, Sodium nitroprusside, ^{NG}-nitro-l-arginine methyl ester, and their combinations on *Plasmodium berghei* in Mice

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Drug combinations represent current treatment strategies in the management of malaria. Nitric oxide (NO) has been proposed to inhibit *Plasmodium* growth due to its parasite-scavenging activity thus the effects of various combinations of an NO inducer - anthraquinone (25-100 mg/kg, ANT), an NO donor - sodium nitroprusside (0.5 mg/kg, SNP) and NO inhibitor -nitro-L-arginine methyl ester (40 mg/kg, L-NAME) and their combination drugs were evaluated using the antimalarial curative model in *Plasmodium berghei*-infected mice. Chloroquine (10 mg/kg, CQ) and distilled water were employed as the positive and negative controls, respectively. The plasma concentrations of nitric oxide in infected mice treated with ANT, SNP and L-NAME were determined spectrophotometrically. A significant ($P < 0.05$) decrease in the parasitaemia of ANT-treated groups at 50 and 100 mg/kg as well as L-NAME were observed. The result of this study showed the combinations of SNP and ANT (100 mg/kg), L-NAME and ANT, CQ and its combination with SNP and L-NAME were effective as they exhibited significant ($p < 0.05$) decreases in parasitaemia throughout the period of treatment justifying their antimalarial activity. However, SNP, the combinations of SNP plus ANT (25 and 50 mg/kg), SNP plus L-NAME were not effective as significant increases ($P<0.05$) in parasitaemia were observed. ANT and CQ demonstrated antiparasitic effect, which may be attributable to nitric oxide. Combinations of the ANT, L-NAME and CQ elicited increases in nitric oxide release, reduced parasitaemia while SNP released no significant amount of nitric oxide.

Key words: Nitric oxide, chloroquine, Plasmodium berghei, curative, drug combinations.

INTRODUCTION

Antimalarial drug combinations increase efficacy, shorten duration of treatment, enhance compliance and decrease the risk of resistant parasites arising through mutation during therapy (Kremner and Krishna, 2004). The role of nitric oxide (NO) in malaria is not well-understood though many reports have suggested its protective effect during the initial stages of malarial infection (Dascombe and Nahrevanian, 2003; Nahrevanian and Dascombe, 2003).

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Effective chemotherapy of malaria aims at reducing morbidity and mortality especially since a vaccine is unlikely to emerge soon. Multidrug resistance has been reported from most parts of the tropical/subtropical countries of the world making monotherapy and some of the available antimalarial combination chemotherapies ineffective (Kremner and Krishna, 2004). Therefore, newer antimalarial combination regimens which are likely to increase efficacy, shorten duration of treatment (and hence increase compliance), and decrease the risk of resistant parasites arising through mutation, are advocated (WHO, 2008).

Anthraquinones occur naturally in some plants such as

Aloe ferox, Cassia podocarpa and *Rhamnus purshianus* as well as fungi, lichens, and insects, where they serve as a basic skeleton for their pigments. Natural anthraquinone derivatives tend to have laxative effects (Muller-Lissner, 1993; Derksen *et al*., 1998). Fotie (2006) reported that atovaquone and hydroxy-naphtoquinone derivatives are effective against the multidrug resistant *Plasmodium* parasite. A remarkable synergistic antimalarial interaction between two structurally similar compounds, rufigallol, an anthraquinone derivative and exifone, a benzophenone derivative (*in vitro*), as well as that between exifone and vitamin C has been reported (Winter *et al*., 1995; 1996; 1997). However, anthraquinones are known to induce nitric oxide (NO) production from macrophages using lipo-polysaccharides (Al-Adhroey *et al*., 2011). In line with this, a possible protective role has been suggested for nitric oxide during malarial infection (Jones *et al*., 1996; Nahrevanian, 2004). High concentrations of nitric oxide in combination with sub-optimal doses of chloroquine suppressed the parasitaemia in chloroquine resistant malarial infection (Awasthi *et al.,* 2005). It is established that the host immune response to malaria involves phagocytosis as well as the production of nitric oxide and oxygen radicals that form part of the host defense system. In the same vein, haemoglobin degradation by the malaria parasite produces the redox active by-products, free haem and H_2O_2, conferring oxidative insult on the host cell (Becker *et al*., 2004). Malaria parasites are therefore highly susceptible to alterations in the reduction-oxidation (redox) equilibrium and a definitive effect of NO in malarial infection is desirable. The effects of the combinations of ANT, SNP and L-NAME(an inducer, donor and inhibitor, respectively) on the antimalarial activity of Plasmodium berghei-infected mice.

MATERIALS AND METHODS

Drugs, Reagents and Solvents

Chloroquine Phosphate injection, Anthraquinone (CAS:84-65, $C_{14}H_8O_2$, P Code:23109116 A90004-50G), Sodium Nitroprusside(CAS13755-38-9; $C_5FeN_6Na_2O.2H_2O$, Pcode: 33009203 71778-25G) were obtained from Sigma-Aldrich, Poole, UK and N^G-nitro-L-arginine methyl ester (L-NAME) from Fluka, Switzerland, Ethanol (Analar grade), Sodium nitrite (May and Baker, England), N-1-naphthylethylenediamine dihydrochloride (NED Solution) (BDH, England), Sulfanilamide (BDH, England), Phosphoric acid (Riedel-de Haen, Sigma), Giemsa (CAS No: 51811-82-6, $C_{14}H_{14}ClN_3S$, Wuhan Chemicals Co. Ltd., China).

Experimental Animals

Adult Swiss albino mice (Vom strain) of either sex, weighing 18-24 g were used. The animals were maintained at 25 ± 1 °C under natural 12 h daylight/ night conditions for 2 weeks before the experiment. All the animals had access to water and were fed with standard diet in the Animal House of the Department of Pharmacology, Obafemi Awolowo University, and the "Principle of Laboratory Animal Care" (NIH publication No. 85-23) guidelines and procedures were followed.

Malaria Parasite

The chloroquine-sensitive strain of *Plasmodium berghei* (NK 65) donated by MR4-Malaria Research and Reference Reagent Resource Center, (MR4 / ATCC, 10801 University Boulevard Manassas, VA 20110-2209 USA) and obtained from Prof. O. G. Ademowo of the Malaria Research Laboratories, Institute for Advanced Medical Research and Training, College of Medicine, University of Ibadan, Nigeria, was used. Parasites were maintained through weekly blood passaging in mice.

Antimalarial (Curative) Test

This was carried out according to Peters and Robinson (1999). Adult Swiss albino mice (75) weighing 18-24 g were randomly divided into fifteen groups of five animals each. Blood from pre-infected donor mouse was diluted with normal saline to obtain a standard inoculum of 1 x 10^7 infected erythrocytes and inoculated intra-peritoneally (i.p.) with 200 µl of the inoculum from the donor mouse. The mice were all allowed to stay for three days post-inoculation for the infection to be established and this was confirmed by parasite identification in the thin blood smear of the experimental mice. Treatment started on Day 3 (D 3) of infection and was for 5 days. Group I received distilled water (0.3 ml), serving as the negative control while the positive control groups received 0.5 mg/kg sodium nitroprusside (SNP), 40 mg/kg L-NAME, or 10 mg/kg chloroquine phosphate (Groups II, III and IV, respectively). Three of the groups (V, VI, and VII) were treated with 25, 50 and 100 mg/kg anthraquinones orally. Three more groups (VIII, IX, X) were given combinations of 0.5 mg/kg SNP and 25, 50 and 100 mg/kg anthraquinones, respectively while another set of three groups (XI, XII, and XIII) were given 40 mg/kg L-NAME in addition to the 25, 50 and 100 mg/kg anthraquinones. Groups XIV and XV were given 10mg/kg chloroquine with 0.5 mg/kg SNP, and 40 mg/kg L-NAME, respectively. On each day blood was taken from the tail of each mouse and a thin smear made on a microscope slide. The smears were fixed with methanol and stained with 10 % giemsa solution.

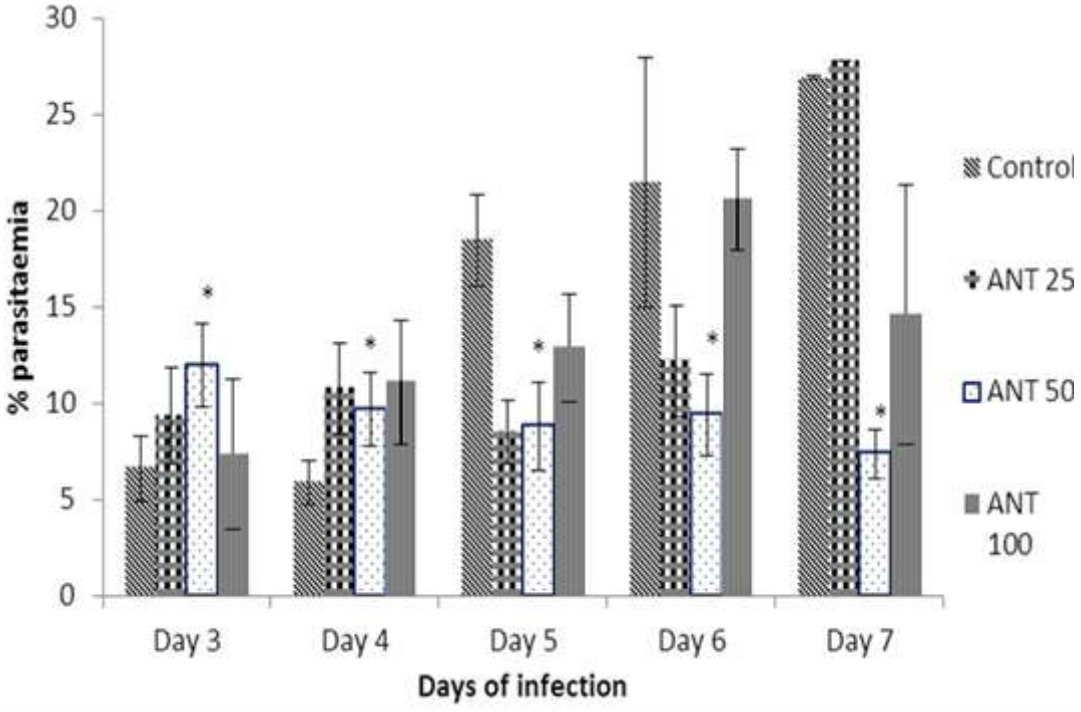

Figure 1. Effect of Anthraquinones (ANT) at different doses on parasitaemia level of *P. berghei*. Control, ANT 25mg/kg, ANT 50mg/kg, ANT 100mg/kg, Values are Mean ± S.E.M, n=6.

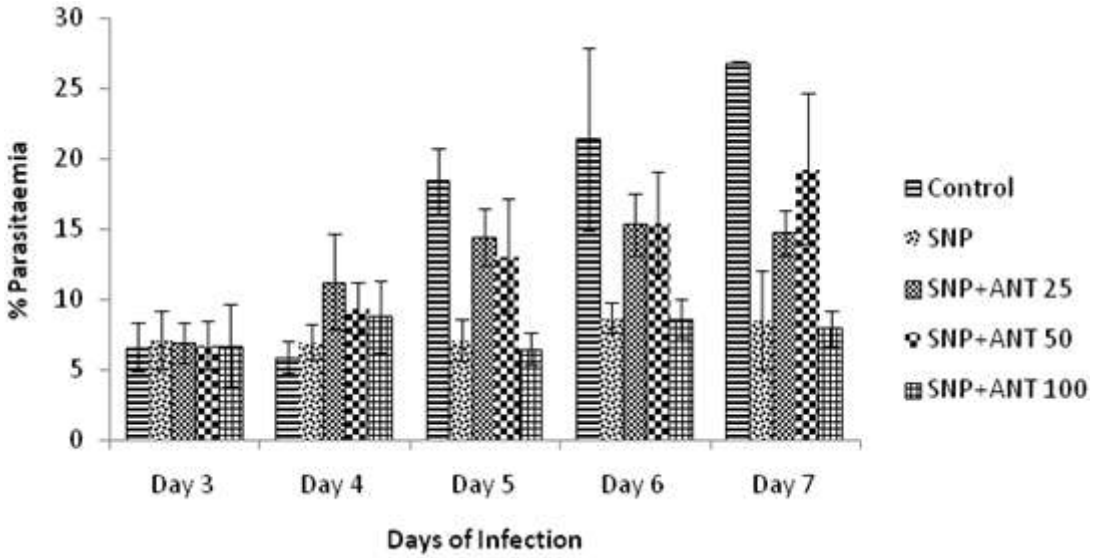

Figure 2. Effect of anthraquinone and Sodium Nitroprusside SNP alone and their combinations against *P. berghei* (Control – 0 mg/kg, SNP, 0.5mg/kg SNP 0.5mg/kg + ANT 25mg/kg, SNP 0.5mg/kg + ANT 50mg/kg, SNP 0.5mg/kg + ANT 100mg/kg. Values are Mean + S.E.M, n=6).

Evaluation of Parasitaemia

Each giemsa-stained smear was viewed under the microscope (Celestron, California, US) using a magnification of 1000. The parasitized and total red Blood cells were counted in ten fields of view. Percentage parasitaemia

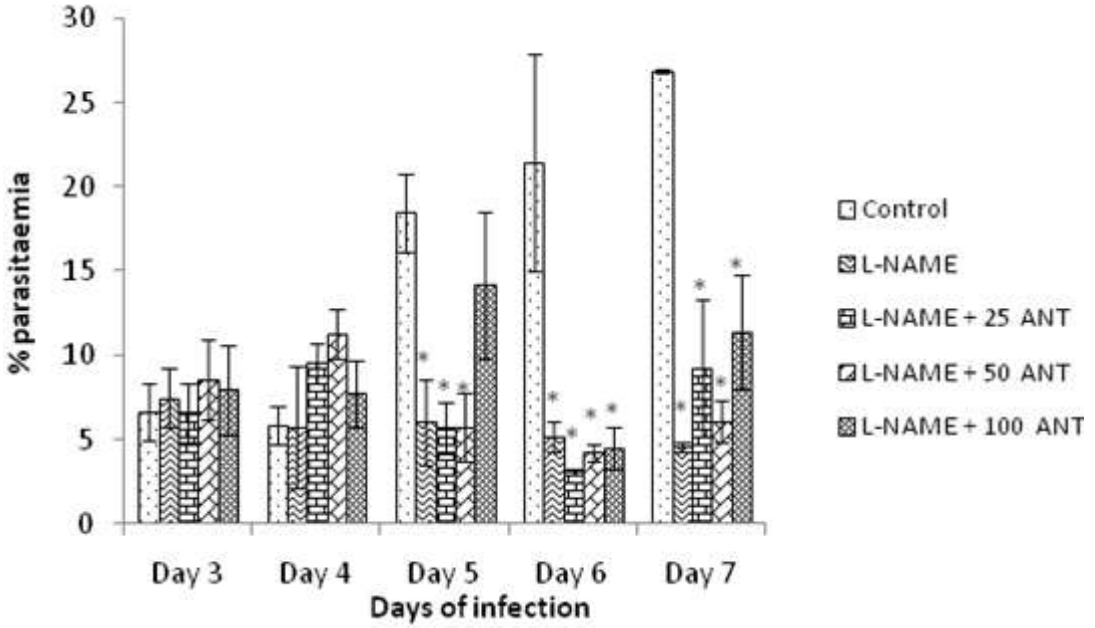

Figure 3. Effects of L-NAME(40 mg/kg) and L-NAME plus ANT (25-100 mg/kg) Control (L-NAME 40mg/kg), L-NAME 40mg/kg + ANT 25mg/kg , L-NAME 40mg/kg + ANT 50mg/kg , L-NAME 40mg/kg + ANT100mg/kg .Values are Mean ± S.E.M, n=6.

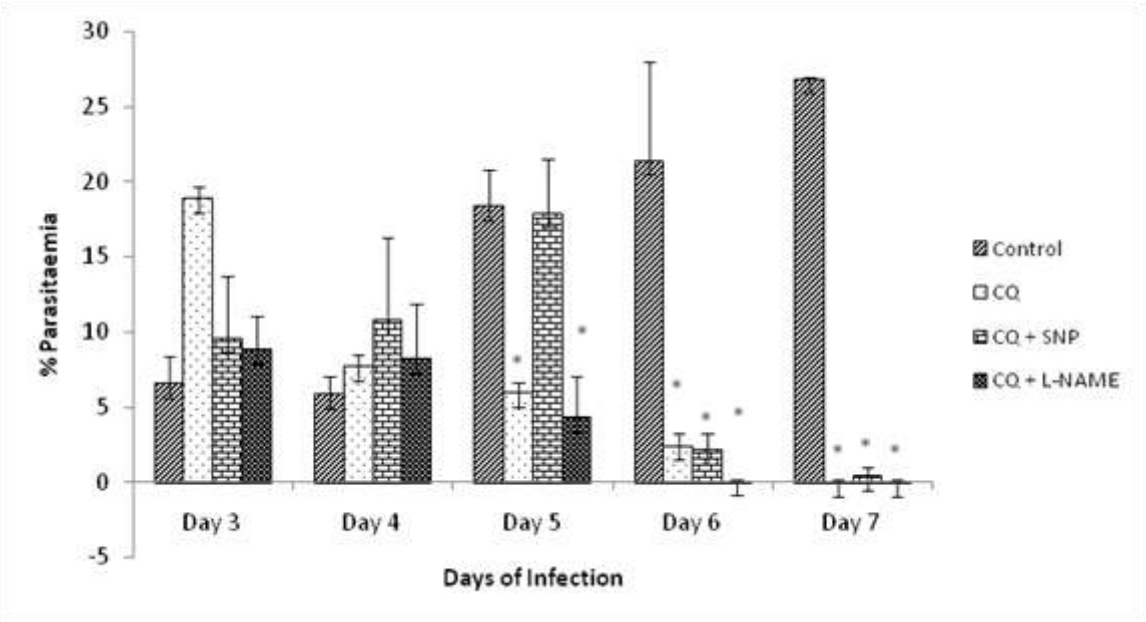

Figure 4. Effects of Chloroquine (10 mg/kg), chloroquine plus SNP and chloroquine plus L-NAME (CQ 10mg/kg + SNP 0.5mg/kg ,CQ 10mg/kg + L-NAME 40mg/kg . Values are Mean ± S.E.M, n=6).

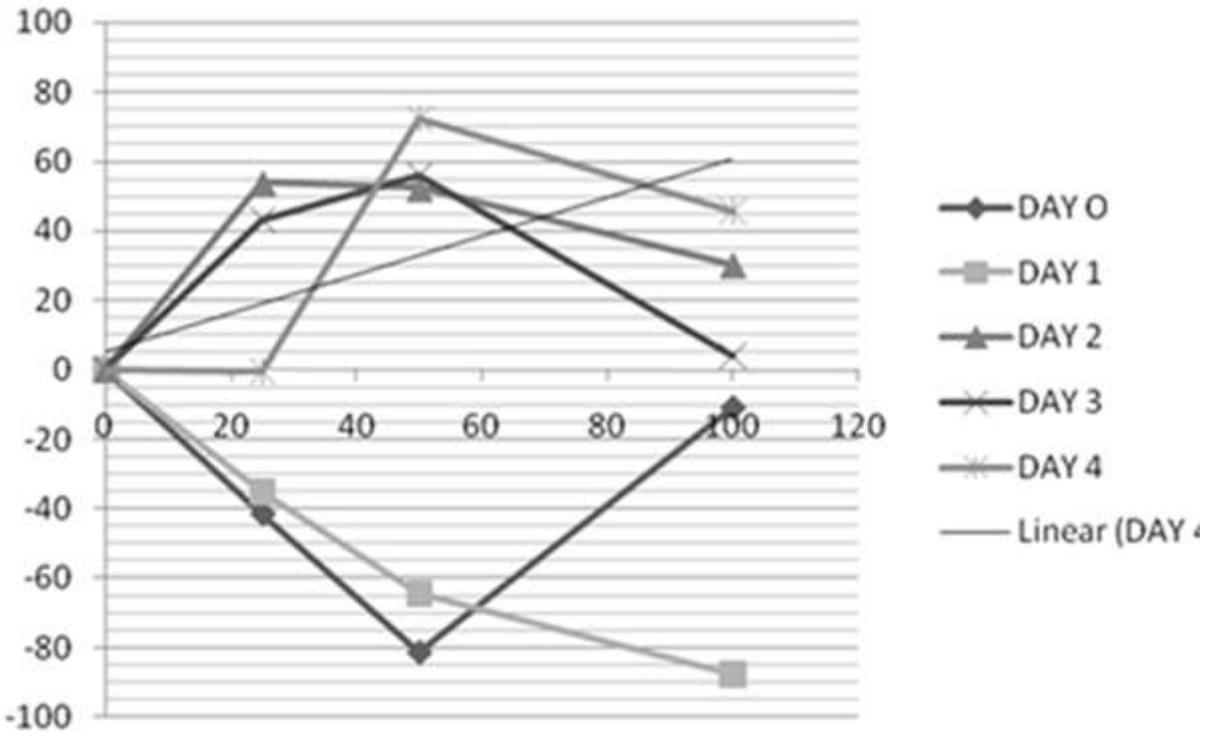

Figure 5. The ED_{50} value of anthraquinones (ANT) from 25, 50 and 100 mg/kg doses as obtained from the percentage clearance of parasitaemia (% chemosuppression) against *P. berghei* infection in mice. (ED_{50} = 80 mg/kg).

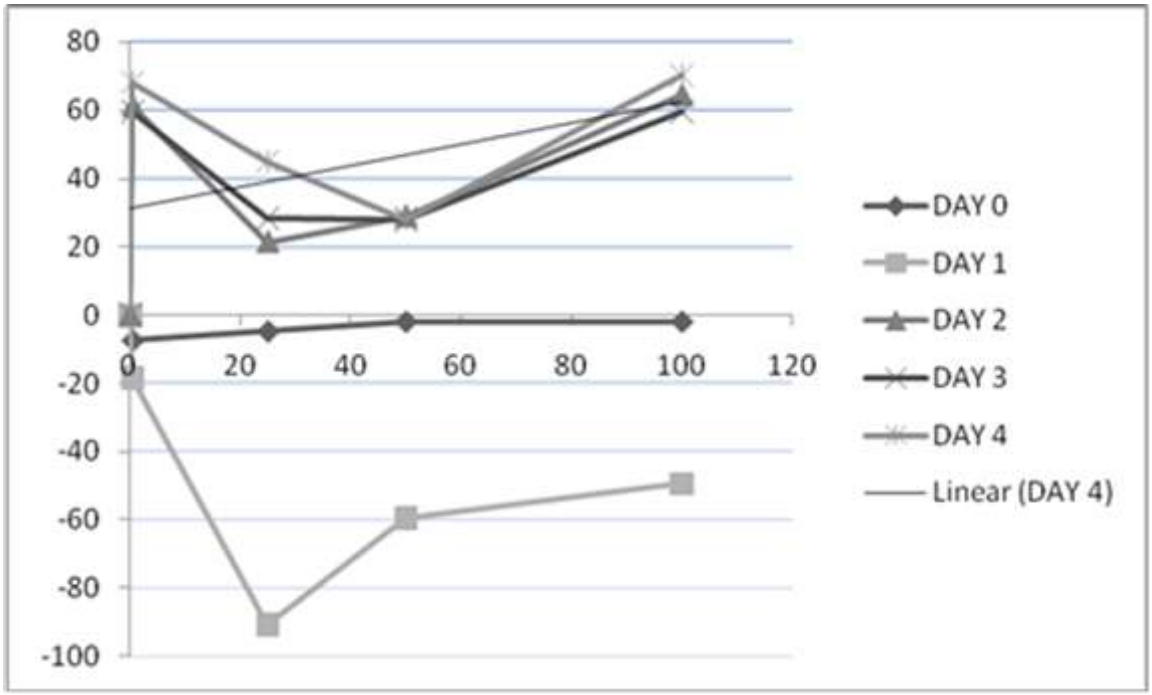

Figure 6. The ED_{50} value of anthraquinones (ANT) in the presence of 0.5 mg/kg SNP with 25, 50 and 100 mg/kg doses as obtained from the percentage clearance of parasitaemia (% chemosuppression) against *P. berghei* infection in mice. (ED_{50} = 47mg/kg).

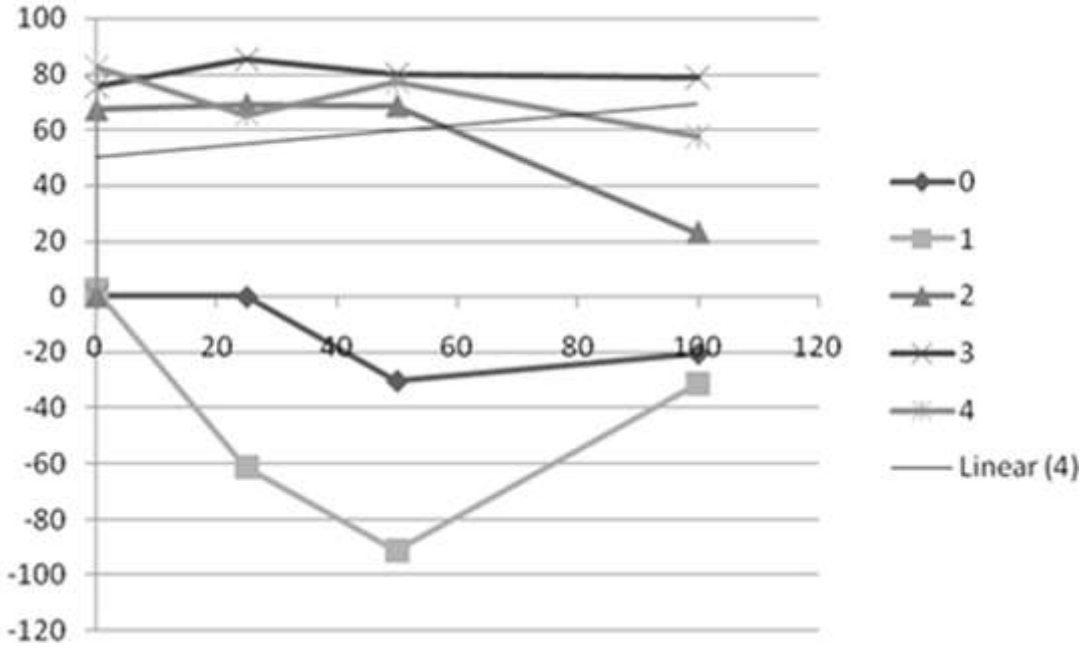

Figure 7. The ED_{50} value of anthraquinones (ANT) in the presence of 40 mg/kg L-NAME with 25, 50 and 100 mg/kg doses as obtained from the percentage clearance of parasitaemia (% chemosuppression) against *P. berghei* infection in mice. (ED_{50} = > 0.1 mg/kg).

Table 1. Determination of the nitric oxide concentration in plasma obtained from *P. berghei*-infected mice treated with anthraquinone, SNP, L-NAME and their various combinations (In curative (established) malaria infection).

Test agents (mg/kg)	Concentration (µM)	Chemosuppression (%)
Negative Control	4.48±0.60	0
ANT 25	13.63±0.99[a]	0
ANT 50	15.89±0.40[a]	72.5
ANT 100	12.04±0.45[a]	45.7
SNP 0.5	6.67±0.90	68.2
SNP 0.5+ANT 25	4.90±0.29	44.9
SNP 0.5 + ANT 50	3.22±0.11	28.3
SNP 0.5+ANT 100	6.23±0.54	70.3
L-NAME 40	18.00±2.27[a]	82.9
L-NAME 40+ANT 25	16.69±0.20[a]	65.6
L-NAME 40+ANT 50	18.24±0.24[a]	77.4
L-NAME 40 + ANT 100	10.44±0.19[a]	57.7
CQ 10	43.15±0.37[a]	99.7
CQ 10 +SNP 0.5	50.05±0.52[a,b]	98.3
CQ 10 +L-NAME 40	46.64±0.67[a,b]	99.7

a* = p < 0.05 between control and test doses, b*= p < 0.05 between chloroquine and chloroquine combinations.

was calculated as follows:

$$\%\,\text{parasitaemia} = \frac{\text{Total number of parasitized red blood cell}}{\text{Total number of red blood cell}} \times 100$$

% Chemosuppression which is the % clearance of the parasite by each agent, was calculated as :

Control parasitaemia – Test group parasitaemia

Control group parasitaemia

Nitric Oxide Assay

The method of Green *et al*. (1982) was adopted in the estimation of nitric oxide level in plasma. Blood was obtained via cardiac puncture from anaesthetized mice at day 7 post inoculation. The two-step assay was based on the fact that the addition of sulfanilamide and N-(1-naphthyl) ethylenediamine generates a product that can be monitored spectrophotometrically. First, deproteinized 30% zinc sulphate with 1ml of the plasma (1:8) was centrifuged at 5000 revolutions per minute for 5 minutes. Second, about 1.5 ml of Griess reagent (1% sulphanilamide and 0.1% naphthylethelene diamine dihydrochloride in 2.5% phosphoric acid) was added to 1ml of the clear supernatant of the plasma. Absorbance was measured using a spectrophotometer (Camspec M107, China) at 530 nm after 20 minutes.

Statistical Analysis

Values were expressed as mean ± S.E.M. Statistical significance was determined using the student's t-test. The results obtained were compared using Analysis of variance (ANOVA) followed by Student-Newman-Keul's test. Values with $p < 0.05$ were considered significant

RESULTS

The result of this study showed that parasitaemia of *Plasmodium berghei*-infected mice was established as indicated by the rise of parasitaemia in the untreated group from days 0-5. There was a significant ($P<0.05$) decrease in the parasitaemia of anthraquinone-treated groups at 50 and 100 mg/kg (Figure. 1) while a significant increase ($P<0.05$) in parasitaemia was observed in groups treated with SNP and L-NAME alone. However, on day 5 post-treatment, only L-NAME reduced parasitaemia significantly ($P<0.05$). The observed increase in parasitaemia level indicated that the combinations of SNP plus anthraquinones (25 and 50 mg/kg) were not effective, while the combination of SNP plus ANT (100 mg/kg) was effective as it exhibited a decrease in parasitaemia (Figure 2). The combinations of L-NAME with ANT separately gave maximum parasitaemia reduction at day 6 (Figure 3). Similarly, the results from the groups treated with CQ and its combination with SNP and L-NAME (Figure 4), showed significant ($p < 0.05$) decrease in parasitaemia throughout the period of treatment.

The percentage chemo-suppression was determined as the percentage of the average parasitaemia relative to the negative control. The percentage chemo-suppression for individual doses of ANT was 0, 72.5, and 45.7% at 25, 50 and 100mg/kg respectively. SNP alone gave chemo-suppression of 68.2% and its combination with ANT 25, 50 and 100mg/kg gave chemo-suppression of 44.9, 28.3 and 70.3%, respectively. In addition, L-NAME gave chemo-suppression of 82.9% while its combination with ANT 25, 50 and 100mg/kg gave chemo-suppression of 65.6, 77.4 and 57.7%, respectively. The CQ-treated group, CQ plus SNP and CQ plus L-NAME gave percentage chemo-suppression values of 99.7, 98.3 and 99.7%, respectively (Table 1).

The ED_{50} of ANT, ANT plus SNP and ANT plus L-NAME was determined by the extrapolation of doses of each agent that caused 50 % chemo-suppression of parasitaemia on Day 7. The ED_{50} for ANT alone was 80 mg/kg (Figure 5). The SNP plus ANT combination gave an ED_{50} was 47.0 mg/kg (Figure. 6) which showed an enhanced activity (Lower ED_{50} value). However, L-NAME (40mg/kg) added in combination with the same graded doses (0, 25, 50 and 100mg/kg) of ANT gave ED_{50} value of less than 0.1 mg/kg and could not be determined from the plot (Figure. 7).

The production of NO measured from the plasma of mice in all the groups showed that the negative control group gave 4.48 µM. ANT (25, 50, 100 mg/kg) also gave 12.04-15.89 µM which were significant ($p < 0.05$) compared to the control. The groups treated with SNP and SNP plus ANT gave 6.67 µM and 3.22-6.23 µM, respectively, which were not significantly different from the control ($p > 0.05$). Furthermore, L-NAME and L-NAME plus ANT gave 10.44-18.24 µM while CQ, CQ plus SNP, CQ plus L-NAME exhibited 43.15 - 50.05 µM which were highly significant compared to the control ($p < 0.05$) (Table 1).

DISCUSSION

This study aimed to investigate the relationship between the production of NO and chemosuppressive antimalarial activity in ANT (an NO inducer), SNP (an NO donor) and L-NAME (an NO inhibitor) as well as their combinations. ANT at 50 mg/kg exhibited a daily reduction in parasitaemia with corresponding increase in chemosuppression, indicative of antimalarial activity (Okokon *et al.,* 2006) while SNP, L-NAME and their respective combinations showed increases in percentage parasitaemia with corresponding low chemosuppression, indicative of no effect on antimalarial activity (Elufioye and Agbedahunsi, 2004). As expected, chloroquine alone exhibited a significant reduction in percentage parasitaemia while its combination with SNP and L- NAME gave decreasing percentage parasitaemia implying their

antimalarial activities. Chloroquine accumulates in the intraparasitic food vacuole and inhibits the formation of non-toxic haemozoin thus leaving heme which is toxic to the parasite (Sullivan *et al.,* 1996; Vippagunta, *et al.,* 1999; Hempelmann, 2007). CQ is able to stimulate the enzyme, NO synthase (NOS) activity in murine, porcine, and human endothelial cells *in vitro* via an impairment of iron metabolism (Ghigo *et al.,* 1998; Chen *et al.,* 2005). Apart from this, antimalarial effect of CQ is expressed through the production of heme to generate RNOS which leads to the production of nitric oxide via iNOS enzyme (Young *et al.,* 1999; Chen *et al.,* 2005). The effectiveness of CQ with or without SNP or L-NAME combinations therefore could be an indication of NO generation.

In this study, the production of NO was determined in *P. berghei*-infected mice after five days of treatment (D7) with the different agents and their respective combinations. The negative control group had the lowest concentration of NO which is an indication that very little amount of NO could be released in *Plasmodium*-infected mice as parasites always mop up generated superoxide anion and RNOS (Nahrevanian, 2004). Unexpectedly, low concentrations of NO were obtained in groups treated with SNP (an NO donor) and SNP plus ANT. This finding is consistent with the report from Dascombe and Nahrevanian (2003), who reported that NO donors such as S-nitroso glutathione and S-nitroso-N-acetylpenicillamine did not generate NO in *P. berghei* infection. This observation was ascribed to the chemical instability and short duration of action of SNP. Nitric oxide (NO) is an unstable radical produced from the oxidative deamination of L-arginine to L-citrulline with the reaction catalyzed by nitric oxide synthase (NOS). It can also be generated non-enzymatically from a group of compounds called NO donors, such as sodium nitroprusside (Diejomaoh *et al.* 2003). It has been shown that NO generated or induced in a biological system can either produce direct or indirect effects (Wink *et al*., 1996a; 1996b). Bates *et al.* (1991) showed that nitroprusside does not release NO spontaneously except in the presence of vascular tissue because at therapeutic dose levels, its distribution is probably mainly extracellular. Furthermore, Morikawa *et al.* (1995) expressed that SNP (below 10 µM) alone cannot induce NO_2 generation. L-NAME, an inhibitor of NO synthesis, was expected to elicit an increase in parasitaemia; but the reverse was the case in this study. Significant amounts of NO were produced in the groups treated with CQ, CQ plus SNP and CQ plus L-NAME plus various doses of ANT with consequent parasite suppression. L-NAME has been proven to specifically inhibit endothelial NOS (eNOS) and brain NOS (bNOS) more than the inducible NOS (iNOS). iNOS is expressed in higher amounts in *Plasmodium*-infected cells compared to endothelium and brain cells. As a result, L-NAME may not be able to exert its inhibitory effect on NO synthesis in malaria or inflammatory conditions (Southan and Szabó, 1996). Two NOS inhibitors, aminoguanidine hydrochloride and N^G-monomethyl-L-arginine have also been reported to be without effect on malaria parasites in mice (Jones *et al.,* 1996; Hirunpetcharat *et al.,* 1999; Favre *et al.,* 1999). Quite unexpectedly, L-NAME being a NO inhibitor generated more NO than SNP at all the concentrations used in this study. This may be because it cannot inhibit the iNOS induced in malaria infection. It is worthy to note that NO concentrations showed some correlation with the percentage chemosuppression obtained. It shows therefore that the amount of NO produced is directly proportional to the chemotherapeutic efficacies of agents used especially in ANT and CQ groups thereby supporting the beneficial effect of NO in *Plasmodium berghei* infection.

CONCLUSION

In conclusion, ANT possesses antimalarial activity which could be due to the nitric oxide generation. SNP and L-NAME did not significantly improve the curative antimalarial activity of ANT when combined.

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