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In-vivo Antiplasmodial Effect of Dihydroartemisinin-Piperaquine-Nitrofurantoin on *Plasmodium berghei*-Infected Mice

Udeme O. Georgewill [a], Festus Azibanigha Joseph [a*] and Elias Adikwu [b]

[a] Department of Pharmacology, Faculty of Basic Clinical Sciences, University of Port Harcourt, Port Harcourt, Nigeria.
[b] Department of Pharmacology /Toxicology, Faculty of Pharmacy, Niger Delta University, Bayelsa State, Nigeria.

Authors' contributions

This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.

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ABSTRACT

Nitrofurantoin (NT) used for the treatment of urinary tract infections may have antiplasmodial activity. Dihydroartemisinin-piperaquine (DP) is an artemisinin based combination therapy used for the treatment of malaria. This study evaluated the antiplasmodial effect of dihydroartemisinin-piperaquine-nitrofurantoin (DP-NT) on mice infected with *Plasmodium berghei.* Adult Swiss albino mice (30-35 g) of both sexes were used. The mice were randomly grouped, inoculated with *Plasmodium berghei,* and treated orally with DP (1.7/13.7 mg/kg), NT (57.1 mg/kg) and DP-NT (1.71/13.7/ 57.1 mg/kg), respectively using curative, prophylactic and suppressive tests. The negative control was orally treated with normal saline (0.3 mL), while the positive control was orally treated with chloroquine CQ (10mg/kg). After treatment, blood samples were collected and evaluated for percentage parasitemia, inhibitions and hematological parameters. Liver samples were evaluated for histological changes. The mice were observed for mean survival time (MST). Treatment with DP-NT decreased parasitemia levels when compared to individual doses of DP and NT with significant difference observed at $p<0.05$. DP-NT prolonged MST when compared to

**Corresponding author: E-mail: festusazibanigha@gmail.com;*

individual doses of DP and NT with significant difference observed at p<0.05. The decrease in packed cell volume, red blood cells, hemoglobin and increase in white blood cells in parasitized mice were significantly restored by DP-NT when compared to individual doses of DP and NT with difference observed at p<0.05. DP-NT eradicated liver *Plasmodium* parasite. NT remarkably increased the antiplasmodial activity of DP. DP-NT may be used for the treatment of malaria.

Keywords: Nitrofurantoin; dihydroartemisinin; piperaquine; Plasmodium; mice.

1. INTRODUCTION

Malaria is a major health challenge in tropical Africa despite the various antimalarial programs available. Malaria is a protozoan blood infection caused by mosquito borne *Plasmodium* parasite transmitted mostly by female anopheles mosquito [1]. The incidence of malaria echoes globally with a burden of 229 million cases in 2019 out of which 409, 000 deaths were recorded [2]. About 67% of malaria deaths in the world occurred in children under 5 years, which formed the most vulnerable population. African continent has the highest amount of global malaria burden with 94% cases of malaria and deaths [3]. Malaria has detrimental impact on the economy of most African countries. The economic impact of malaria in the world was estimated to be 3 billion US Dollars in 2019 [2].

Artemisinin-based combination therapies (ACTs) are the mainstay for malaria treatment. The use of ACTs, which combines artemisinin derivatives with partner drugs has largely caused significant reduction in malaria-related mortality in endemic regions. Dihydroartemisinin–piperaquine (DP) is one of the ACTs that is effective against *Plasmodium* falciparum. It was adopted as the first-line antimalarial treatment in Cambodia in 2008 [4,5]. ACTs including DP have played remarkable functions in reducing the incidence of malaria between 2010 and 2016 [6]. However, the remarkable progress achieved with the use of DP and other ACTs is seriously haunted by decreased efficacy characterized by delayed parasite clearance and high recrudescence rates as reported in Western Cambodia [7]. *Plasmodium* parasite resistance to ACTs has spread to some countries in Southeast Asia. A primary concern is the spread of *Plasmodium* parasite resistance to Sub-Saharan Africa a malaria endemic region [8].

Plasmodium parasite resistance to ACTs can be curtailed by the use of triple antimalarial regimen [9, 10]. Drugs such as antibiotics with potential antiplasmodial activity can be repurposed as partner drugs with artemisinin derivatives forming triple regimen [11]. Nitrofurantoin (NT) is a synthetic antibiotic derivative of imidazodinedione. It is a broad spectrum antibiotic used for treatment of urinary tract infections. It inhibits both gram-positive and gram-negative bacteria. It acts by inhibiting bacteria DNA, RNA and cell wall protein syntheses. NT is converted by bacterial reductases to more electrophillic residues, which are irreversible inhibitors of citric acid cycle, DNA, RNA and protein syntheses [12]. It also acts by activating bacterial flavor proteins to intermediates, which inhibit bacterial ribosomal proteins [13]. Interestingly, in addition to the antibiotic effect of NT, it has potential antiplasmodial activity. NT and some nitroaromatic compounds are speculated to exhibit antiplasmodial activity, by inhibiting glutathione reductase and electron transport chain in *Plasmodium* parasite [14-16]. NT has also shown potential therapeutic benefit in the treatment of *Toxoplasma gondaii* [17]. This study assessed if NT can increase the antiplasmodial activity of DP in *Plasmodium berghei*-infected mice.

2. MATERIALS AND METHODS

2.1 Drugs and Experimental Animals

Dihydroartemisinin-piperaquine (DP) (Bliss GVS Pharma. Ltd), nitrofurantoin (NT) (De-Shawn Pharm, Lab. Ltd) and chloroquine phosphate (CQ) (Emzor Pharm, Ltd) were used. Adult Swiss albino mice (30-35g) of both sexes purchased from the animal house of the Department of Pharmacology, Faculty of Basic Clinical Sciences, University of Port Harcourt, Rivers State, Nigeria were used. The mice were kept in cages under normal environmental conditions and were acclimated for 2 weeks with free access to feeds and water. The following doses of drugs were used: CQ (10 mg/kg) [18], NT (57.1 mg/kg) [19] and DP (1.71/13.7mg/kg) [20].

2.2 Parasite Inoculation

Chloroquine-sensitive *Plasmodium berghei* (*P. berghei*) (NK65) in donor mice obtained from the

Nigerian Institute for Malaria Research, Yaba Lagos, was used. The parasites were maintained weekly by blood transfer from the *P. berghei*-infected mice to healthy mice through intraperitoneal (i.p) route.

2.3 Evaluation of Antiplasmodial Activity

2.3.1 Curative antiplasmodial activity

It was evaluated as described by Ryley and Peters (1970) [21]. Adult Swiss albino mice inoculated with *P. berghei* ($1x10^7$) (i.p) were randomly grouped into 5 of 5 mice each. After 3 days, the mice were orally treated daily for 4 days as follows: The negative and positive controls were treated with normal saline (0.3 mL) and CQ (10 mg/kg), respectively. Other groups were treated with NT (57.1 mg/kg), DP (1.71/13.7 mg/kg), and DP-NT (1.71/13.7/57.1 mg/kg), respectively. On day 5, tail blood samples were collected and thin blood films were prepared on slides. The slides were fixed with methanol and stained with Giemsa stain and examined using a light microscope. Percentage parasitemia and inhibitions were calculated using the formula below

$$\% \text{ Parasitemia} = \frac{NR}{TR} \times 100$$

NR: Number of parasitized RBCs
TR: Total number of RBCs count

$$\% \text{ Inhibition} = \frac{NC - NY}{NC}$$

NC: % Parasitemia of negative control
NY: % Parasitemia of treated group

2.3.2 Suppressive antiplasmodial activity

It was evaluated using the procedure reported by Knight and Peters (1980) [22]. Adult Swiss albino mice were inoculated with *P. berghei* ($1x10^7$) and randomized into 5 groups of 5 mice/group. After 3 hours, the mice were orally treated daily for 4 days as follows: The negative and positive controls were treated with normal saline (0.3 mL) and CQ (10 mg/kg), respectively. The mice in the other groups were treated with NT (57.1 mg/kg), DP (1.71/13.7mg/kg), and DP-NT (1.71/13.7/57.1 mg/kg), respectively. On day 5, tail blood samples were collected and thin blood films were prepared on slides and processed as explained above. Percentage parasitemia and inhibitions were calculated using the above formula.

2.3.3 Prophylactic antiplasmodial activity

It was evaluated using the method explained by Peters (1967) [23]. Adult Swiss albino mice were randomized into 5 groups of 5 mice/group. The mice were orally treated daily for 4 days as follows: The negative and positive controls were treated with normal saline (0.3mL) and CQ (10 mg/kg), respectively. Other groups were treated with NT (57.1mg/kg), DP (1.71/13.7mg/kg), and DP-NT ((1.71/13.7/57.1 mg/kg), respectively. On day 5, the mice were inoculated with *P. berghei* ($1x10^7$) (i.p). After 3 days, blood samples were collected from the tail of the mice. Thereafter, thin blood films were prepared on slides and processed as explained above. Percentage parasitemia and inhibitions were calculated using the formula above.

2.3.4 Determination of mean survival time

The mice in the control and treated groups were routinely observed for mortality and expressed in days. Mortality expressed as mean survival time (MST) was calculated as shown below

$$\text{MST: } \frac{\text{Sum of survival time of the mice in the group (Days)}}{\text{Total number of mice}}$$

2.3.5 Evaluation of hematological parameters

Blood specimen from the curative group were collected and evaluated for red blood cells (RBCs), packed cell volume (PCV), white blood cells (WBCs) and hemoglobin (Hb).

2.3.6 Histology of the liver

Liver tissues were sliced and fixed in 10% formalin for 24hr and dehydrated in alcohol of ascending concentrations. Liver tissues were embedded in paraffin and sectioned (3µm thickness) using a microtome. Liver tissues were stained with Hematoxylin and Eosin on slides, examined using a light microscope and relevant sections photographed.

2.4 Statistical Analysis

Results as mean $\pm$ standard error of mean (SEM). Variations between groups were evaluated using one-way analysis of variance (ANOVA) followed by Tukey's *post-hoc* test. Significance was considered at $p < 0.05$.

3. RESULTS

3.1 Curative Activity of Dihydroartemisinin-piperaquine-nitrofurantoin on *Plasmodium berghei*-Infected Mice

Treatment with DP-NT significantly decreased percentage parasitemia when compared to treatment with individual doses of N and DP at p<0.05. Treatment with NT, DP, and DP-NT produced 56.10%, 75.62%, and 91.41%, parasitemia inhibitions, respectively when compared to 85.62% inhibition produced by CQ (Table 1). MST was significantly prolonged by DP-NT when compared to treatment with individual doses of NT and DP at p<0.05 (Table 1).

3.2 Suppressive Activity of Dihydroartemisinin-piperaquine-nitrofurantion on *Plasmodium berghei*-Infected Mice

Treatment with DP-NT produced significant decreases in percentage parasitemia when compared to treatment with individual doses of NT and DP at p<0.05. Treatment with NT, DP, and DP-NT produced the following inhibitions; 66.10%, 80.47% and 93.94%, respectively while CQ produced 87.45% (Table 2). Treatment with DP-NT significantly prolonged MST when compared to treatment with individual doses of NT and DP at p<0.05 (Table 2).

3.3 Prophylactic Activity of Dihydroartemisinin-piperaquine-nitrofurantion on *Plasmodium berghei*-Infected Mice

Percentage parasitemia was significantly decreased in mice treated with DP-NT when compared to treatment with individual doses of NT, and DP at p<0.05. NT, DP and DP-NT produced 70.22%, 87.76% and 97.40% parasitemia inhibitions, respectively whereas CQ produced 97.40% parasitemia inhibition (Table 3). Treatment with DP-NT significantly prolonged MST when compared individual doses of NT and DP at p<0.05 (Table 3).

3.4 Effect of Dihdroartemisinin-piperaquine-nitrofurantoin on Hematological Parameters of *Plasmodium berghe*i-infected Mice

Significant (p<0.05) decreases in RBCs, Hb, and PCV levels with significant (p<0.05) increases in WBCs levels occurred in *P. berghei*-infected mice when compared to non-parasitized mice (MC) (Table 4). On the other hand, treatment with DP-NT significantly increased RBCs, Hb and PCV levels and significantly decreased WBCs levels at p<0.05 when compared to treatment with individual doses of NT and DP (Table 4).

3.5 Effect of dihydroartemisinin-piperaquine-nitrofurantoin on Liver Histopathology of *Plasmodium berghei*-infected Mice

The liver of the control mice showed normal hepatocytes, central vein, and Sinusoids (Fig. A). Liver of parasitized mice showed merozoites, congested central vein, steatosis, normal hepatocytes and Sinusoids (Fig. B). Liver of parasitized mice treated with CQ showed normal hepatocytes, central vein, and Sinusoids (Fig. C). Liver of parasitized mice treated with NT showed normal hepatocytes, Sinusoids and congested central vein (Fig. D). Liver of mice treated with individual doses of DP and DP-NT showed absence of merozoites, normal hepatocytes, and Sinusoids (Figs E and F).

Table 1. Curative activity of dihydroartemisinin-piperaquine-nitrofurantoin on *Plasmodium berghei*-infected mice

Treatment	% Parasitemia	% Inhibition	MST (Days)
NC	36.22±2.11	0.00	9.14±1.10
CQ	5.39±0.68 [a]	85.12	26.56±3.24 [a]
NT	15.90±1.16 [b]	56.10	20.19±3.21 [b]
DP	8.83±1.02 [c]	75.62	25.87±3.07 [a]
DP-NT	3.11±0.37 [d]	91.41	31.78±4.15 [c]

NC: Negative control, CQ: Chloroquine, NT: Nitrofurantoin, DP: Dihydroartemisinin- piperaquine, MST: Mean survival time; n= 5, Data expressed as mean+ SEM, SEM: Standard error of mean. Values with different superscripts down the column differ at p<0.05.

Table 2. Suppressive activity of dihydroartemisinin-piperaquine-nitrofurantoin on *Plasmodium berghei*-infected mice

Treatment	% Parasitemia	% Inhibition	MST (Days)
NC	30.21±1.20	0.00	9.22±1.27
CQ	3.79±0.06 [a]	87.45	28.1±1.44 [a]
NT	10.24±0.64 [b]	66.10	22.7±2.40 [b]
DP	5.90±0.83 [c]	80.47	27.0±2.11 [a]
DP-NT	1.83±0.77 [d]	93.94	33.0±3.03 [c]

NC: Negative control, CQ: Chloroquine, NT: Nitrofurantoin, DP: Dihydroartemisinin- piperaquine, MST: Mean survival time; n= 5, Data as mean+ SEM, SEM: Standard error of mean. Values with different superscripts down the column differ at p<0.05.

Table 3. Prophylactic activity of dihydroartemisinin-piperaquine-nitrofurantoin on *Plasmodium berghei*-infected mice

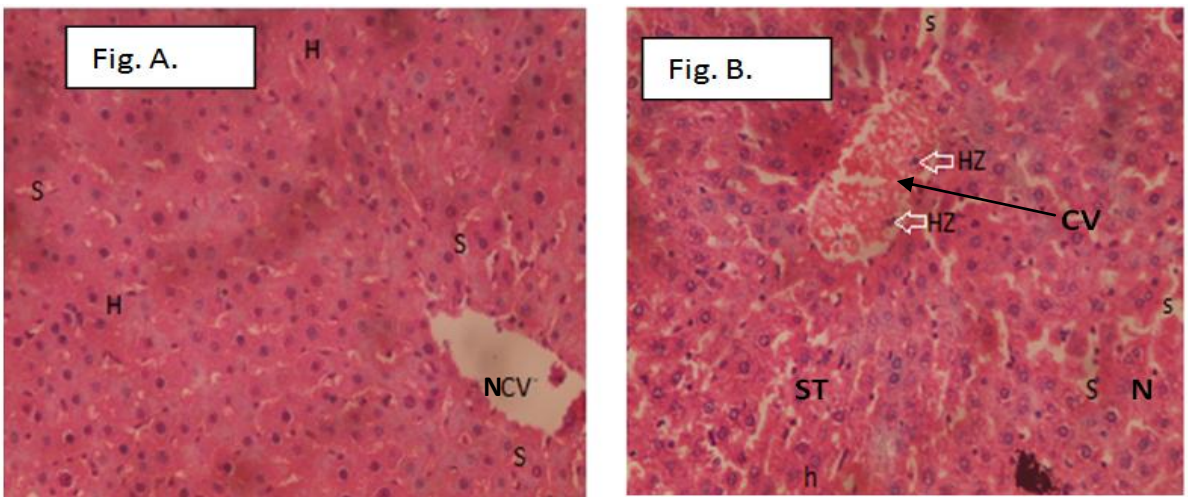
Treatment	% Parasitemia	% Inhibition	MST (Days)
NC	20.01±2.20	0.00	9.34±1.01
CQ	2.10±0.27 [a]	89.51	30.52±2.12 [a]
NT	5.96±0.11 [b]	70.22	23.84±3.21 [b]
DP	2.45±0.08 [a]	87.76	28.54±2.10 [a]
DP-NT	0.52±0.16 [c]	97.40	35.34±3.33 [c]

NC: Negative control, CQ: Chloroquine, NT: Nitrofurantoin, DP: Dihydroartemisinin- piperaquine, MST: Mean survival time; n= 5, Data as mean + SEM, SEM: Standard error of mean, Values with different superscript down the column differ at p<0.05.

Table 4. Effect of dihydroartemisinin-piperaquine-nitrofurantoin on hematological indices of *Plasmodium berghei*-infected mice

Treatment	PCV %	HB g/dL	RBCs x10^6	WBCs (cells/L)
MC	57.50±6.50	16.01±0.01	6.88±0.13	4.27±0.01
NC	29.00±2.00	7.04±0.26	2.11±0.02	13.13±0.20
CQ	47.11±3.00 [a]	12.46±0.21 [a]	5.15±0.15 [a]	6.56±0.05 [a]
NT	37.50±4.50 [b]	10.06±0.11 [b]	3.29±0.02 [b]	9.00±0.30 [b]
DP	45.10±5.00 [a]	12.40±0.05 [a]	5.10±0.09 [a]	6.98±0.12 [a]
DP-NT	55.50±6.50 [c]	15.83±0.05 [c]	6.60±0.05 [c]	4.46±0.10 [c]

MC: Normal control, NC: Negative control, CQ: Chloroquine, NT: Nitrofurantoin, DP: Dihydroartemisinin – piperaquine, RBCs: Red blood cells, WBCs: White blood cells, PCV: packed cell volume, Hb: Hemoglobin, n = 5, Data as mean + SEM, SEM: Standard error of mean, Values with different superscript down the column differ at p<0.05.



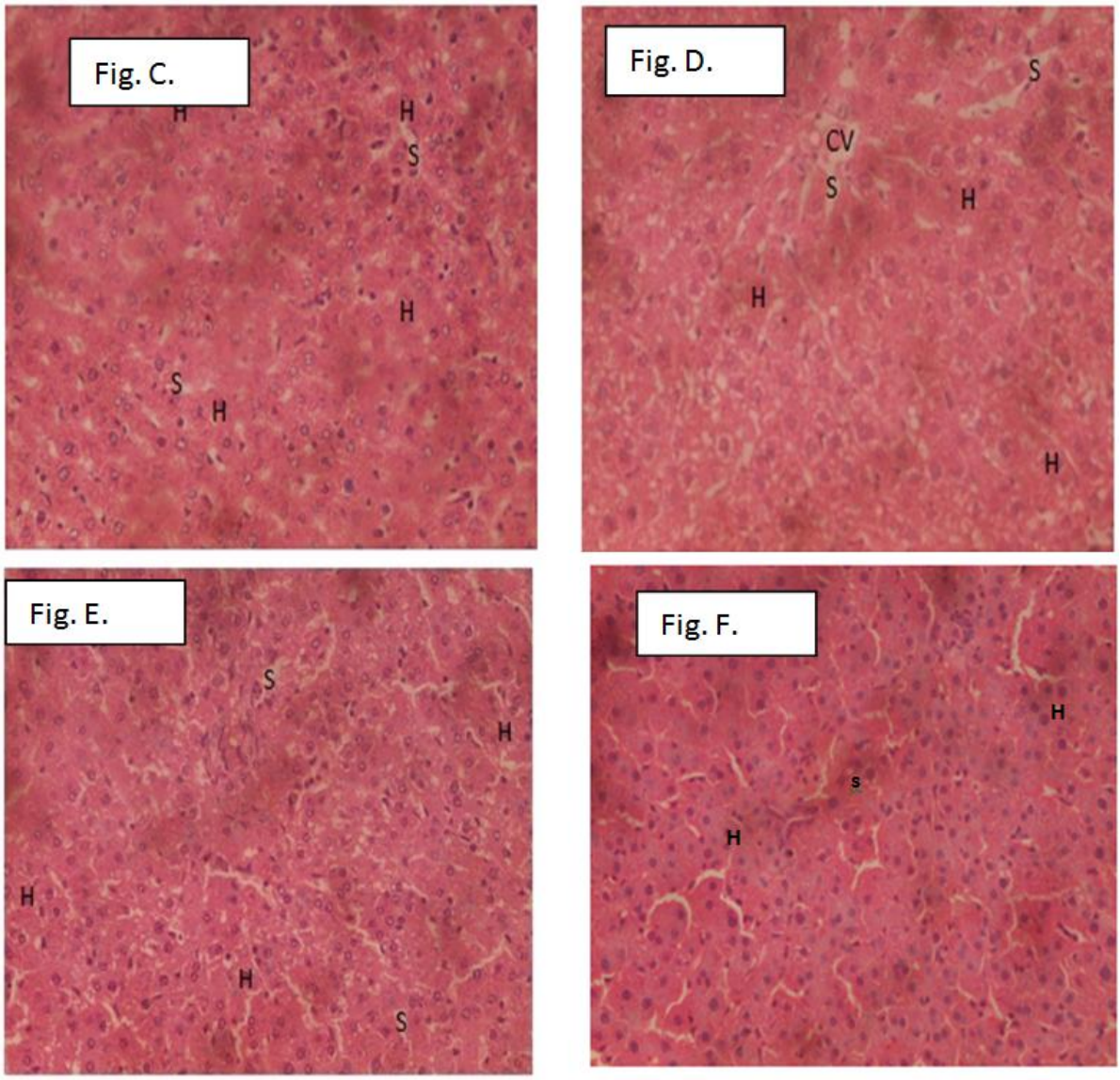


Fig A: Liver of the control mice. Fig B: Liver of parasitized mice. Fig C: Liver of parasitized mice treated with CQ (10mg/kg). Fig. D: Liver of parasitized mice treated with NT (57.1 mg/kg). Fig E: Liver of parasitized mice treated with DP (1.71/13.7mg/kg). Fig F: Liver of parasitized mice treated with DP-NT. HZ: Merozoites, NCV: Normal central vein, CV: Congested central vein H: Normal hepatocytes. ST: Steatosis, S: Sinusoids with Kupffer cells X400

4. DISCUSSION

The development of *Plasmodium* parasite resistance to antimalarial drugs is a major barrier to successful malaria treatment in malaria-endemic regions. It has contributed to the resurgence of malaria infection and increase in malaria associated death in recent years [24]. Factors such as cost and length of clinical trials have reduced the pace for new drug discovery and development. This has encouraged the use of non-conventional methods including drug repurposing to fast track the discovery of new antimalarial drugs [25]. This study examined the antiplasmodial activity of NT in combination with DP in mice parasitized with *P. berghei*. Curative, suppressive and curative tests used for the antiplasmodial assessments of potential drugs were used for this study [26]. Mice model was used for this study because, it allows for detailed assessment of multiple and specific pathophysiological processes caused by malaria infection, which is not possible in humans [27]. Due to possible prodrug effect and the

involvement of the immune system in the eradication of infection, an *in-vivo* model was used [28].

In this study, in the curative, suppressive and prophylactic tests, treatment with DP-NT decreased percentage parasitemia levels and increased percentage inhibitions.The assessment of MST is imperative in antiplasmodial studies. A drug candidate that significantly prolongs MST may be a potential antimalarial drug [26]. Treatment with DP-NT caused notable prolongation of MST in the curative, suppressive and prophylactic tests. Severe anemia caused by malaria is a contributing factor to malaria associated morbidity in humans and is an important pathological feature of rodent model of malaria infections. During malaria infection, a number of factors including the destruction of RBCs due to parasite replication contributes to observed anemia [29]. A mouse model of malaria induced anemia is often marked by decreased PCV, Hb and RBCs levels [26], which is consistent with the observation in the current study. It is interesting that treatment with DP-NT caused notable reduction in anemia marked by elevated Hb, RBCs and PCV levels with decreased WBCs levels.

The liver remains a significant hibernating ground for malaria parasites. The pathogenesis of liver impairment in malaria is complex and not well understood. Findings including vascular congestion, swollen hepatocytes, Kupffer cell hyperplasia, and steatosis were reported in malaria associated liver dysfunction [30]. In this study, the liver of parasitized mice showed vascular congestion, merozoites, and steatosis. However, the aforementioned liver changes were absent in the liver of DP-NT-treated mice. This observation showed that DP-NT has potential to prevent recrudescence that can occur due to uneradicated *Plasmodium* parasite hibernating in the liver. The antiplasmodial activity of DP-NT may be connected to the abilities of the constituent drugs to attack parasites at different sites. Dihydroartemisinin inhibits *Plasmodium* parasite through the cleavage of the endoperoxide bridge and the generation of free radicals [31]. Piperaquine is said to have similar antiplasmodial mechanism with CQ. CQ forms CQ-haematin complex and hemoglobin in *Plasmodium* parasite food vacuole, which disrupt enzymatic processes [32]. The mechanisms for the antiplasmodial activity of NT are not well understood. Its antibacterial activity involves the inhibition of bacterial DNA, RNA, and cell wall protein syntheses. It also acts by activating bacterial flavor proteins to intermediates, which inhibit bacterial ribosomal proteins [33]. Some studies speculated that NT produces free radicals [34] and inhibits glutathione reductase an antioxidant defense in *Plasmodium* parasite [35]. NT can also act through redox cycling, oxidation of oxyhemoglobin, and the inhibition of electron transport chain in *Plasmodium* parasite [16].

5. CONCLUSION

In this study, NT remarkably increased the antiplasmodial activity of DP in *P. berghei*-infected mice. DP-NT may serve as an effective antimalarial drug.

DISCLAIMER

The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation, but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.

CONSENT

It is not applicable.

ETHICAL APPROVAL

This study was approved by the Research Ethics Committees of the University of Port Harcourt.

COMPETING INTERESTS

Authors have declared that no competing interests exist.

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