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In-vivo Antiplasmodial Impact of Dihydroartemisinin-Piperaquine-Clindamycin on *Plasmodium berghei*-Infected Mice

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Abstract

Objective: The concurrent use of antibiotics and antimalarial drugs may increase Plasmodium susceptibility. Clindamycin (C) is an antibiotic with potential antiplasmodial activity. Dihydroartemisinin-piperaquine (D-P) is an effective antimalarial drug. This study examined the antiplasmodial effect of dihydroartemisinin-piperaquine-clindamycin (D-P-C) on mice infected with *Plasmodium berghei*.

Methods: Adult Swiss albino mice (25-30g) of n=6/group were used. Using the curative, suppressive and prophylactic tests, mice were infected with *Plasmodium. berghei* and orally treated per day with D-P (1.71/13.7mg/kg), C (10mg/kg) and D-P-C, respectively. The positive control was orally treated per day with chloroquine (CQ) (10 mg/kg) whereas the normal and the negative controls were orally treated per day with normal saline (0.2ml).

Results: In the curative, suppressive and prophylactic tests, D-P-C decreased percentage parasitemia levels with significant difference observed at $p<0.05$ when compared to individual doses of C, D-P and CQ. D-P-C significantly prolonged mean survival time with difference observed at $p< 0.05$ when compared to individual doses of D and D-P. The anti-anemic effect of D-P-C was characterized by increased hemoglobin, red blood cells, packed cell volume and decreased white blood cells with significant difference observed at $p<0.05$ when compared to individual doses of C, D-P and CQ. Treatment with D-P-C eradicated liver *Plasmodium*.

Conclusion: D-P-C showed promising antiplasmodial activity. It may be used for the treatment of malaria.

Keywords: Artemisinin, Piperaquine, Clindamycin, Plasmodium, Mice

1. Introduction

Malaria is a major global health challenge, which affects 300 to 500 million people annually. It is one of the primary causes of disease and death in tropical regions, with most impact on children under 5 years of age. In humans malaria is caused by five parasite species with *Plasmodium vivax* (*P. vivax*) and *P. falciparum*, posing the greatest threat being responsible for the most reported malaria cases worldwide [1]. Effective and efficient interventions are being used for malaria control such as early treatment of malaria with artemisinin combination therapies (ACTs), intermittent preventive treatment for pregnant women, and reduction of human-vector contact [2]. The intervention with ACTs as first-line treatment for malaria especially in the tropics has immensely reduced the menace of malaria [3].

Dihydroartemisinin-piperaquine (D-P), one of the frequently used ACTs that is very effective against multidrug resistant *P. falciparum*, was adopted as the first-line antimalarial treatment in Cambodia in 2008 [4]. It has an excellent safety and tolerability profile with an efficacy of 96 – 98% [5]. The use of D-P is uniquely advantageous due to the fast parasite clearance activity of D and the prolong effect of P in eradicating residual parasites [4]. It also has a post-treatment prophylactic effect which prevents re-infections that is longer than other ACTs [6]. However, D/P has been associated with some cases of treatment failures and decreased parasite clearance, which suggests emerging parasite resistance [7]. *Plasmodium* parasite resistance occurred mostly toward artemisinins, thereby placing the high burden of parasite eradication on partner drugs [5].

Clindamycin (C), which is a lincosamide antibiotic was developed in 1966 through the modification of naturally occurring lincomycin. It acts by inhibiting bacterial protein synthesis at the level of the 50S ribosome. *In-vitro* studies showed it has a wide spectrum of activity against *staphylococci*, *pneumococci*, *streptococci* and most anaerobic bacteria [8] and exerts a prolonged post-antibiotic effect. It decreases toxin production and increases microbial opsonization and phagocytosis at sub-inhibitory concentration [8]. In addition to its antibacterial activity, it has promising antiplasmodial activity. Available scientific information showed that C monotherapy has an efficacy of 98% against *P. falciparum* malaria [9] [10]. In semi-immune adults with malaria, C in combination with chloroquie (CQ) produced 97% cure rate [11]. Also, the combination of C with quinine or quinidine was reported as an efficient and practicable option for malaria treatment [11]. However, there is a paucity of clinical studies on it combination with artemisinin derivatives and some antimalarial drugs [12]. The combination of D-P with C may be an effective anti-malarial drug combination due to their different antiplasmodial modes of action. The fast-action of D, the long term antiplasmodial activity of P [13] and the post-antibiotic effect of C [8] could be uniquely advantageous.

Therefore, the current study assessed the antiplasmodial action of D-P-C on mice infected with *P. berghei*

2. Materials and Methods

2.1 Animals

Swiss albino mice of both sexes (25-30g) used for this study were purchased from the animal handling unit of Department of Pharmacology, Faculty of Basic Clinical Sciences, University of Port Harcourt, Rivers State, Nigeria. The mice were housed in standard cages with access to standard pellet diet and water ad *libitum*. The mice were acclimated for 2 weeks before the commencement of the study. The mice were maintained under standard laboratory conditions in accordance with the National Institute of Health (NIH) guideline for the care and use of Laboratory animals [14].

2.2 Malaria parasite

P. berghei (NK65) (CQ sensitive) was obtained from the Nigerian Institute of Medical Research, Yaba, Lagos State. *P. berghei* was supplied in donor mice and kept alive by continuous intraperitoneal (ip) passage in mice after every five days. A standard inoculum containing P. *berghei* (1×10^7) of parasitized erythrocytes from a donor mouse in 0.2 ml was used to infect the experimental mice ip.

2.3 Drugs

Dihydroartemisinin-piperaquine (D-P), manufactured by Artepharm Co., Ltd., China, Clindamycin (C) manufactured by Mediplantex National Pharmaaceutical, Viet Nam and Chloroquine (CQ) manufactured by Evans Pharm, Nigeria were used. The following doses were used: D-P (1.71/13.7mg/kg) [15], CQ (10mg/kg) [16] and C (10 mg/kg) [11].

2.4 Antiplasmodial test

2.4.1 Curative test

The method explained by Ryley and Peters [17] was used for the curative test. Thirty six Swiss albino mice (n=6/group) were randomly grouped and used. A group was used as normal control (non-parasitized) while other groups were inoculated with *P. berghei* (1×10^7) ip and allowed for 3 days before treatment. The mice were treated orally with D-P (1.71/13.7mg/kg), C (10 mg/kg) and D-P-C daily for 4 days, respectively. The normal control (non-parasitized) was treated orally with normal saline (0.2mL) daily for 4 days. The negative and positive controls were treated orally with normal saline (0.2mL) and CQ daily for 4 days, respectively. After treatment, on day 5, blood samples were collected from the tail of the mice on slides. After fixation with alcohol and staining with 10% Giemsa stain at pH 7.2, the slides were rinsed with distilled water and air dried at room temperature. Parasitized erythrocytes were counted with the aid of neubauer in a light microscope. Percentage parasitemia and inhibitions were calculated using the formula below.

$$\% \text{ Parasitemia} = \frac{P}{N} \times 100\%$$

Where P=Number of parasitized red blood cells (RBCs)

N=Total number of RBCs count

$$\% \text{ Inhibition} = \frac{T}{N} \times 100\%$$

Where T=(% Parasitemia of negative control −% Parasitemia of treated group)

N=% Parasitemia of negative control

2.4.2 Suppressive test

The method described by Knight and Peters [18] was used for the suppressive test. Thirty Swiss albino mice (n=6/group) were randomly grouped and inoculated with P. berghei (1×10^7) ip and allowed for 2 hours before treatment. The negative and positive controls were treated orally with normal saline (0.2mL) and CQ (10mg/kg) daily for 4 days, respectively. The mice in the experimental groups were treated orally with D-P (1.71/13.7mg/kg), C (10 mg/kg) and D-P-C daily for 4 days, respectively. After treatment, on day 5, blood samples were collected from the tail of the mice on slides and stained with 10% Giemsa stain. Percentage parasitemia and inhibitions were calculated using the formula above.

2.5 Prophylactic test

The method described by Peters [19] was used for prophylactic test. Thirty Swiss albino mice (n=6/group) used were randomly grouped. The negative and positive controls were treated orally with normal saline (0.2mL) and CQ (10mg/kg) daily for 4 days, respectively. The experimental mice were treated orally with D-P (1.71/13.7mg/kg), C (10 mg/kg) and D-P-C daily for 4 days, respectively. On the 5th day, the mice were inoculated with *P. berghei* (1×10^7) ip. After 72 hours, blood samples were collected from the tail of the mice on slides and stained with 10% Giemsa stain. Percentage parasitemia and inhibitions were calculated using the formula above.

2.6 Determination of mean survival time

Mean survival time (MST) was observed in both controls and treated groups and expressed in days using the formula below.

$$\text{MST} = \frac{T}{N}$$

Where T=Sum of survival time of all the mice in the group in days

N=Total number of mice in that group

2.7 Assessment of hematological indices

Blood samples of the mice used for the curative test were evaluated for red blood cells (RBCs), packed cell volume (PCV), hemoglobin (Hb) and white blood cells (WBCs), with the aid of an autoanalyzer (Cell-Dyn Model 331 430).

2.8 Histology of the liver

Liver tissues were dissected, immersed in 10% formalin saline for 24hr and dehydrated in graded alcohol concentrations. Liver tissues were processed and embedded in paraffin block. Liver tissues were sectioned (3µm each) and stained with Haematoxylin and Eosin. Stained portioned were examined on slides with the aid of a light microscope for histological changes.

2.9 Statistical analysis

Results for (n=6/group) obtained were presented as mean ± S.E.M (SEM: Standard error of mean). The results were analyzed using one was analysis of variance (ANOVA) with the aid of Graph pad Prism. $P < 0.05$ was set as significance.

3. Results

3.1 Curative antiplasmodial effect of dihydroartemisinin-piperaquine-clindamycin on mice infected with *Plasmodium berghei*

Percentage parasitemia decreased in mice treated with D-P-C with significant difference observed at $p<0.05$ when compared to individual doses of C, D-P and CQ (Table 1). Percentage inhibitions of 71.2%, 60.6%, 92.7% and 80.0% were observed in mice treated with D-P, C, D-P-C and CQ, respectively (Table 1). MST was prolonged in mice treated with D-P-C with significant difference at $p<0.05$ when compared to individual doses of C, D-P and CQ (Table 1).

3.2 Suppressive antiplasmodial effect of dihydroartemisinin-piperaquine-clindamycin on mice infected with *Plasmodium berghei*

Mice treated with D-P-C showed significant decreases in percentage parasitemia at $p<0.05$ when compared to individual doses of C, D-P and CQ (Table 2). The observed decreases in parasitemia translated to 76.9%, 65.3%, 96.7% and 83.5% inhibitions in mice treated with D-P, C, D-P-C and CQ, respectively (Table 2). Significant prolongation of MST occurred in mice treated with D-P-C which differ at $p<0.05$ when compared to individual doses of C, D-P and CQ (Table 2).

3.3 Prophylactic antiplasmodial effect of dihydroartemisinin-piperaquine-clindamycin on mice Infected with *Plasmodium berghei*

Mice treated with D-P-C showed decreased percentage parasitemia with significant difference observed at $p<0.05$ when compared to individual doses of C, D-P and CQ (Table 3). Percentage inhibitions in mice treated with D-P, C, D-P-C and CQ represent 76.6%, 69.1%, 97.8%, and 87.1%, respectively (Table 3). MST was significantly prolonged in mice treated with D-P-C with difference observed at $p<0.05$ when compared to individual doses of C, D-P and CQ (Table 3).

3.4 Effect of dihydroartemisinin-piperaquine-clindamycin on hematological indices of mice infected with *Plasmodium berghei*

P. berghei infected mice showed decreases in RBCs, Hb, and PCV levels with increases in WBCs when compared to normal control. However, mice treated with D-P-C showed increases in RBCs, Hb, and PCV levels and decreases in WBCs with difference observed at $p<0.05$ when compared to individual doses of C, D-P and CQ (Table 4).

3.5 Antiplasmodialeffect of dihydroartemisinin-piperaquine-clindamycin on the liver of mice infected with *Plasmodium berghei*

The liver micrographs of the control and treated mice are presented in figures 1-6. Figure 1: Liver of normal control showed normal hepatocyte and central vein. Figures 2 and 3: liver of negative control showed merozites, fatty change and central vein congestion. Figure 4: The liver of mice treated with D-P showed normal hepatocyte and central vein congestion. 5: Liver of mice treated with C showed normal hepatocyte and merozites. Figure 6: liver of mice treated with D-P-C showed normal hepatocytes and central vein congestion x400

Table 1: Curative antiplasmodial effect of dihydroartemisinin-piperaquine-clindamycin on mice infected with *Plasmodium berghei*

Treatment	% Parasitemia	% Inhibition	MST (Days)
NC	34.60±0.08	0.0	9.11±0.67
CQ	6.92±0.16[a]	80.0	37.00±2.90[a]
C	13.60±0.83[b]	60.6	27.25±4.12[b]
D-P	9.95±0.45[c]	71.2	30.89±2.14[c]
D-P-C	2.53±0.06[d]	92.7	38.15±3.70[a]

NC: Negative control, CQ: Chloroquine, C; Clindamycin, D-P: dihydroartemisinin-piperaquine. MST: Mean Survival time; n=6, Values expressed as mean±SEM, SEM: Standard error of mean. Values with difference superscripts (a, b, c and d) down the column significantly differ at p<0.05 (ANOVA)

Table 2: Suppressive antiplasmodial effect of dihydroartemisinin-piperaquine-clindamycin on mice infected with *Plasmodium berghei*

Treatment	% Parasitemia	% Inhibition	MST (Days)
NC	29.30±0.15	0.0	9.33±1.43
CQ	4.84±0.46[a]	83.5	40.07±1.27[a]
C	10.20±0.03[b]	65.3	26.17±2.14[b]
D-P	6.78±0.02[c]	76.9	32.10±4.10[c]
D-P-C	0.97±0.00[d]	96.7	41.24±5.70[a]

NC: Negative control, CQ: Chloroquine, C: Clindamycin, D-P: dihydroartemisinin-piperaquine. MST: Mean Survival time; n=6, Values expressed as mean±SEM, SEM: Standard error of mean. Values with difference superscripts (a, b, c and d) down the column significantly differ at p<0.05 (ANOVA)

Table 3: Prophylactic antiplasmodial effect of dihydroartemisinin-piperaquine-clindamycin on mice infected with *Plasmodium berghei*

Treatment	% Parasitemia	% Inhibition	MST (Days)
NC	24.10±0.11	0.0	6.38±0.86
CQ	3.11±0.15[a]	87.1	43.11±1.90[a]
C	7.46±0.99[b]	69.1	30.40±5.12[b]
D-P	5.65±0.07[c]	76.6	33.82±6.21[b]
D-P-C	0.53±0.21[d]	97.8	46.01±7.10[a]

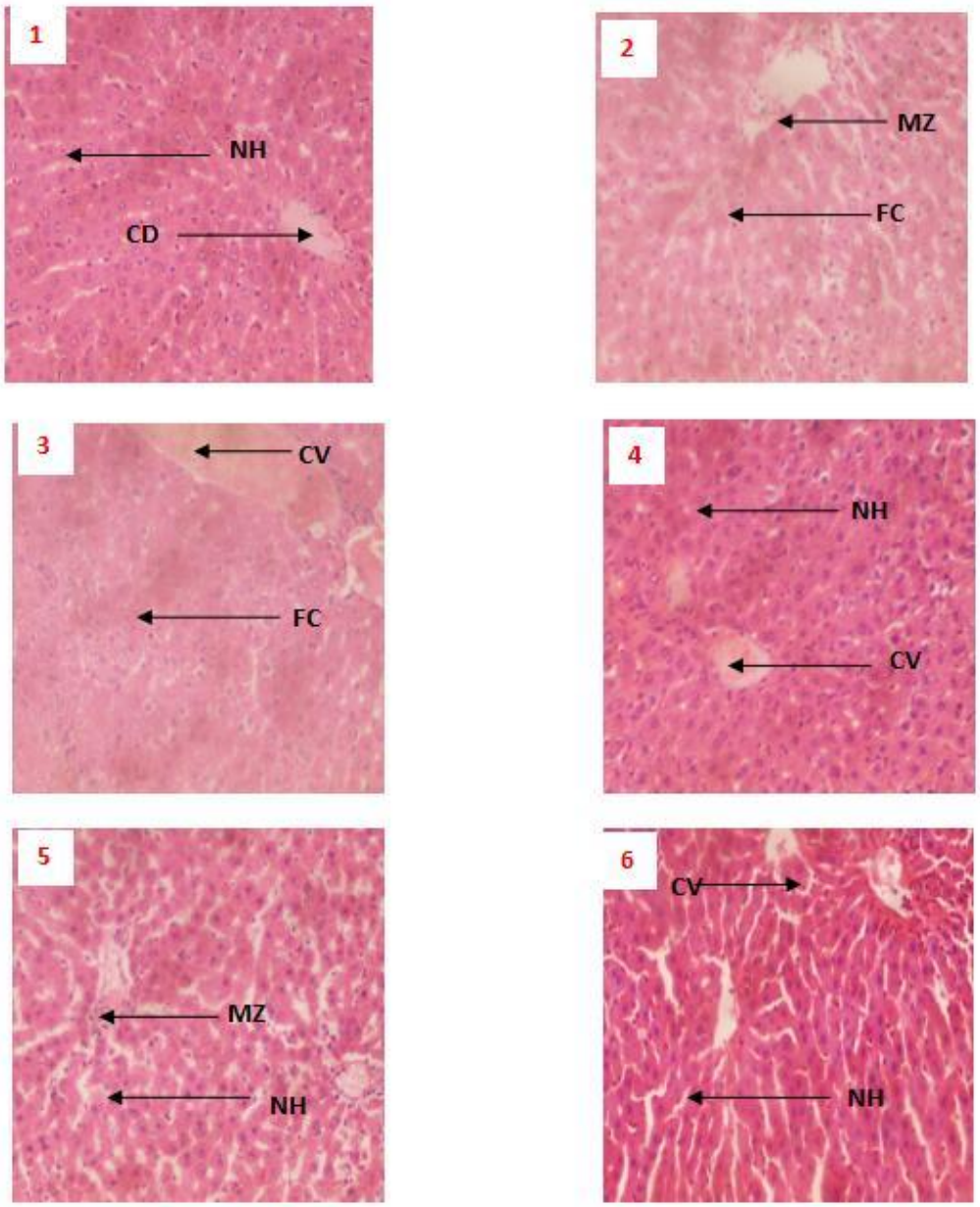
NC: Negative control, CQ: Chloroquine, C: Clindamycin, D-P: dihydroartemisinin-piperaquine. MST: Mean Survival time; n=6, Values expressed as mean±SEM, SEM: Standard error of mean. Values with difference superscripts (a, b, c and d) down the column significantly differ at p<0.05 (ANOVA)

Table 4: Effect of dihydroartemisinin-piperaquine-clindamycin on hematological indices of mice infected with *Plasmodium berghei*

Treatment	RBC (x106)	WBC (cells/L)	PCV (%)	HB (g/dL)
AC	7.04±0.22	5.15±0.70	56.52±1.08	17.23±0.38
NC	1.21±0.86[a]	12.8±0.14[a]	21.53±3.10[a]	6.42±1.76[a]
CQ	5.00±0.33[b]	6.13±0.35[b]	44.81±2.35[b]	13.54±0.41[b]
C	2.22±0.17[c]	9.62±0.52[c]	30.10±1.85[c]	10.41±0.44[c]
D-P	4.01±0.57[d]	7.40±0.36[d]	40.74±0.98[b]	13.23±0.17[b]
D-P-C	6.55±0.26[e]	5.25±0.30[e]	51.02±1.13[d]	16.70±0.83[d]

AC: Normal Control, NC: Negative control, CQ: Chloroquine, C: Clindamycin, D-P: dihydroartemisinin-piperaquine. RBC: Red blood cells, WBC: White blood cells, PCV: Packed cell volume, Hb: Haemoglobin; n=6,

Values expressed as mean±SEM, SEM: Standard error of mean. Values with difference superscripts (a, b, c, d and e) down the column significantly differ at p<0.05 (ANOVA)



The liver micrographs of the control and treated mice are presented in figures 1-6. Figure 1: Liver of normal control showed normal hepatocyte (NH) and central vein (CD). Figure 2 and 3: Liver of negative control showed central vein congestion (CV), merozoites (MZ) and fatty change (FC). Figure 4: Liver of mice treated with D-P showed normal hepatocyte (NH) and central vein congestion (CV). 5: Liver of mice treated with C showed normal hepatocyte (NH) and merozoites (MZ). Figure 6: Liver of mice treated with D-P-C showed normal hepatocytes (NH) and central vein congestion (CV) (F) x400.

4. Discussion

Malaria is a parasitic disease, which affects about 219 million people causing about 435,000 deaths in the world in 2017 [3, 20]. Malaria associated mortality in the world ranges from 0.3-2.2% with most deaths in the tropics where there are frequent cases of severe malaria [21]. Substantial progress has been made in the fight against malaria associated mortality and morbidity especially in Sub-Sahara

Africa and Asian countries. In the fight against malaria, the combination of artemisinin derivatives with partner drugs has decreased malaria burden in the world however, with some resistant challenges posed by *Plasmodium* parasites [22]. Some antibiotics including C have shown promising antiplasmodial activities [23] and could serve as partner drugs when combined with artemisinin derivatives. Therefore, this study examined the antiplasmodial activity of D-P-C on mice infected with *P. berghei*. The rodent parasite, *P. berghei* has been used in studying the effect of potential antimalarial drugs on mice [23]. Rodent models of antimalarial studies have been validated via the discovery of many conventional antimalarial drugs such as quinine and more recently artemisinin derivatives [25]. One of the advantages of the mouse model is the detection of compounds, which require bioactivation and/or which have immunomodulatory effect [26]. This study used a 4-day suppressive model and the Reye's test (curative test), which are widely used for the assessments of antimalarial activities of natural or synthetic products on early and established infections, respectively [26-27]. CQ was used as the standard in this study [28]. In this study, in the curative and suppressive tests, D-P-C significantly decreased percentage parasitemia in treated mice. It also produced percentage inhibitions, which were better than the standard (CQ). In the prophylactic test, treatment with D-P-C decreased percentage parasitemia in treated mice, which was also better than CQ. Studies have shown that decrease in MST is an integral feature of a mouse model of *P. berghei* infection and is used as one of the indices for experimental malarial studies. An effective antimalarial drug is expected to prolong MST with relation to the standard [15]. In the curative, suppressive and prophylactic studies, treatment with D-P-C prolonged MST with similar effect as CQ.

Anemia develops rapidly with severe malaria infection. Parasitized RBCs are destroyed as parasites matured into schizonts and subsequent rupture from RBCs. The parasitized RBCs are also destroyed by the mononuclear phagocyte system in the spleen, which recognizes parasites as antigens on the surface of the parasitized RBCs [29]. This study observed notable features of anemia in *P. berghei* infected mice marked by decreased RBCs, Hb, and PCV with increased WBCs, which were previously reported [15]. Interestingly, decreased anemia was noted in mice treated with D-P-C, which was marked by increased RBCs, Hb, and PCV with decreased WBCs. In the present study, histological assessment of the liver of *P. berghei* -infected mice showed fatty change, merozoites, and vascular congestion, which support previous reports [30]. The aforementioned changes were conspicuously absent in the liver of mice treated with D-P-C. This indicates that the clinical use of D-P-C may clear *Plasmodium* parasite harbored in the blood and the liver.

Dihydroartemisinin-piperaquine (D-P) is a fixed dose co-formulated ACT used increasingly for the treatment of malaria. D and P have different antiplasmodial modes of action. P is said to have a similar mechanism of action with CQ, which accumulates in parasite food vacuole. In the food vacuole, CQ inhibits the detoxification of heme therefore causing heme build up and the formation of CQ-heme complex. CQ-heme complex is highly toxic to parasites and disrupts membrane function [31]. D is the active metabolite of artesunate and artemether, which antiplasmodial action occurs via a two-step mechanism. It is first activated by intraparasitic heme-iron, which catalyzes the cleavage of its endoperoxide. This activation causes the release of free radical intermediate which kills parasites by alkylating one or more essential plasmodial protein(s) [32]. C is a lincosamide antibiotic with excellent activity against Gram-positive or -negative anaerobes and Gram-positive cocci. It inhibits bacterial protein synthesis via impact on the 50S ribosome. The antiplasmodial activity of C has not been elucidated, but speculatively, it has been attributed to its effect on *Plasmodium* apicoplast. It acts slowly and accumulates in parasites [12, 33]. The combined antiplasmodial activity observed in the current study may be due to the effects of the constituent drugs on different target sites.

5. Conclusions

Based on the findings in this study, C seems effective as a partner drug with D-P for the treatment of malaria.

Ethical approval

Ethical approval (NDU/PHARM/PCO/AEC/066A) was obtained from Research Ethics Committee of the Department of Pharmacology/Toxicology, Faculty of Pharmacy, Niger Delta University, Bayelsa State on 13 July 2021.

Conflict of Interests

The authors declare that there are no conflicts of interest related to this article

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Authors' contributions

EA was involved in the conceptualization, design, execution, supervision, sourcing of materials, statistical analysis, draft and review of the manuscript; SAI participated in the sourcing of material, design, execution, statistical analysis, drafting and review of manuscript while NOE was involved in the design, execution, statistical analysis, review and editing of the manuscript. EA, SAI and NOE read and approved the final manuscript.

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