American Journal of
Biomedical Science & Research

www.biomedgrid.com
ISSN: 2642-1747

Research Article

Antimalarial Assessment of Artesunate/Ketoconazole in a Mouse Model

Gift Crucifix Pender[1], Nwakaego Omonigho Ebong[2], Liverpool Ebiladei[3] and Elias Adikwu[4]*

[1]*Department of Pharmacology and Toxicology, University of Rwanda, Rwanda*

[2]*Department of Pharmacology and Toxicology, Madonna University, Nigeria*

[3]*Department of Pharmacology, University of PortHarcourt, Nigeria*

[4]*Department of Pharmacology and Toxicology, Niger Delta University, Nigeria*

***Corresponding author:** Elias Adikwu, Department of Pharmacology and Toxicology, Faculty of Pharmacy, Niger Delta University, Wilberforce Island, Bayelsa State, Nigeria.

To Cite This Article: *Gift Crucifix Pender, Nwakaego Omonigho Ebong, Liverpool Ebiladei and Elias Adikwu. Antimalarial Assessment of Artesunate/Ketoconazole in a Mouse Model. Am J Biomed Sci & Res. 2023 19(4) AJBSR.MS.ID.002601,* DOI: 10.34297/AJBSR.2023.19.002601

Received: June 06, 2023; **Published:** July 12, 2023

Abstract

Malaria is a common cause of death in the tropics. Drug repurposing is a quick and effective modality for the discovery of antimalarial drugs. Artesunate (AS) is combined with other antimalarial drugs for malaria treatment. Ketoconazole (KT) is an antifungal drug with possible antiplasmodial activity. This study evaluated the antiplasmodial effect of AS/KT on a mouse model infected with *Plasmodium berghei*. Sixty Swiss albino mice of both sexes weighing 30-35g randomly grouped into n=5/ group were used. The mice were inoculated with *Plasmodium berghei* ($1x10^7$) intraperitoneally. Thereafter, using the curative and suppressive protocols, the mice were orally treated with AS (12mg/kg/day), KT (7mg/kg/day) and AS/KT, respectively. Chloroquine (CQ) (10mg/kg/day) served as the standard control. After treatment, blood samples were evaluated for percentage parasitemia, hematological and liver function parameters. The mice were observed for mean survival time and liver tissues were histologically assessed for changes. In the curative, and suppressive tests, AS/KT significantly ($p<0.05$) decreased percentage parasitemia when compared to AS or KT. In the curative test, AS, KT and AS/KT produced 83.21%, 71. 84% and 92.11% parasitemia inhibitions, respectively while CQ produced 90.63% parasitemia inhibition. AS/KT significantly ($p<0.05$) prolonged mean survival time in the curative test when compared to AS or KT. AS/KT significantly ($p<0.05$) restored hematological parameters (hemoglobin, red blood cells, packed cell volume and white blood cells) when compared to AS or KT. AS/KT restored liver histology in the parasitized mice. AS/KT shows promising antiplasmodial activity.

Keywords: Artesunate, Ketoconazole, Combination, Malaria, Mice

Introduction

About 3.2 billion people globally are at risk of malaria with greater than 200 million cases of malaria infection reported yearly. According to the World Health Organization (WHO) in 2015, there were 214 million cases of malaria and 438,000 malaria-related mortality, primarily in sub-Saharan Africa [1]. Vector control programme and antimalarial drug use have been the main approaches for the control of malaria infection [2]. The major set-backs to the aforementioned approaches are cost and the development of resistance by malaria parasites [3]. Hence the urgent need to develop novel and cost-effective antimalarial drugs as well as synergistic partners for artemisinins cannot be overemphasized. The reliance on the traditional drug development methods to deliver on this goal have been associated with significant implications on both cost and time.

Drug repurposing or repositioning connotes the use of an existing drug in diseases other than those it was originally used for. It offers a route to significantly shorten the traditional drug development pipelines [4]. It affords attractive, alternate and valid paradigm for drug discovery [5,6]. For diseases like malaria, drug repositioning may not only deliver novel candidates, but also pro-

vide partner drugs for combinatorial regimens with artemisinins, thereby increasing longevity of these highly effective and affordable frontline drugs [7,8].

Artesunate is an antimalarial drug that is a semi-synthetic, and water-soluble, artemisinin derivative. It displays high and fast antiplasmodial activity with a spectrum of specific action on the various stages of malaria parasites. It vividly and rapidly kills circulating ring-stage parasites and prevents their maturation and sequestration in organs such as the brain and liver [9]. It has been used in combination with other antimalarial drugs such as amodiaquine, mefloquine, sulfadoxine/pyrimethamine and pyronaridine for the treatment of malaria with outstanding results [10]. It is imperative for its combination with new antimalarial drugs to be explored.

The effectiveness of some antifungal drugs against malaria parasites have been documented in some studies [11]. Ketoconazole, an antifungal that inhibits ergosterol formation has been reported to have notable antimalarial activity [12]. *In-vitro* studies reported that ketoconazole in combination with α/β arteether showed increased antimalarial activity against chloroquine-sensitive and chloroquine-resistant *Plasmodium falciparum* strains as well as with the multidrug-resistant *Plasmodium yoelii nigeriensis* [13]. In the absence of *in-vivo* studies, this study investigated the potential of repurposing ketoconazole in combination with artesunate as an antimalarial drug in *Plasmodium berghei*-infected mice.

Methods

Animals

Sixty (60) adult Swiss albino mice (30-35g) of both sexes were obtained from the animal house of the Department of Pharmacology, Faculty of Basic Clinical Sciences, University of Port Harcourt, Rivers State, Nigeria. The mice were kept under standard conditions (temperature 24˚C± 1˚C; 12:12 days/night; relative humidity of 60%±5%) and were housed in wood-shaving-bedded standard plastic cages. The study was performed in the Department of Pharmacology/Toxicology, Faculty of Pharmacy, Niger Delta University, Nigeria.

Ethical Considerations and Approval

Ethical approval was granted by the Research Ethics Committee of the Department of Pharmacology/Toxicology, Faculty of Pharmacy, Niger Delta University, Nigeria. The mice were handled using the guidelines of the National Research Council [14].

Drugs and Chemicals

Chloroquine (Evans, Nigeria), artesunate (Mekophar, Vietnam), ketoconazole (Medreich, India), and methanol (JHD Sci-Tech. Co. Ltd, China) were used. Artesunate (12 mg/kg) and KT (7 mg/kg) used were clinical doses. Chloroquine (10 mg/kg) was used as the standard [15]

Plasmodium Parasite

Chloroquine (CQ) sensitive *Plasmodium berghei* sourced from the National Institute of Medical Research, Yaba, Lagos State, Nigeria was used. It was preserved by the inoculation of a fresh mouse from a donor mouse intraperitoneally every four days.

Inoculation of Mice with Parasite

Mouse infected with *Plasmodium berghei* with a parasitemia level of 35% served as the donor. Blood sample was collected using an ethylene diamine tetra acetic acid bottle, retro-orbitally, and percentage parasitemia and red blood cells (RBCs) count were determined. Preparation of inoculum was done by diluting the blood with normal saline which was administered intraperitoneally. Approximately 1×10^7 parasitized RBCs were present in 0.2mL of the blood solution.

Evaluation of Antiplasmodial Activity

Curative Test

The method explained by Adikwu, *et al.,* 2022 [15] was used. The mice were infected with RBCs containing *Plamodium berghei* (1×10^7) intraperitoneally. The mice were grouped into 6 of n=5/group and allowed for 3 days. Thereafter, the mice were orally treated with AS (12mg/kg/day), KT (7mg/kg/day), and AS/KT respectively for 4 days. The standard control was orally treated with CQ (10mg/kg/day) while the parasitized and normal controls were orally treated daily with normal saline. Tail blood samples were obtained and thin blood films were produced on microscope slides. The slides were fixed in methanol allowed to dry and stained with 10% Giemsa stain. The slides were viewed with the aid of a microscope and the percentage parasitemia and inhibitions were calculated as shown below.

Suppressive Test

The protocol explained by Adikwu, *et al.,* 2022 [15] was used. The mice were intraperitoneally infected with RBCs containing *Plasmodium berghei* (1×10^7). The mice were randomly grouped into 6 of 5 mice /group. The mice were allowed for 2 hours, thereafter, they were orally treated with AS (12mg/kg/day), KT (7mg/kg/day), and AS/KT respectively for 4 days. The standard control was orally treated with CQ (10mg/kg/day) while the parasitized and normal controls were treated daily with normal saline. Tail blood samples were obtained, and thin blood films were prepared and processed as explained above. The percentage parasitemia and inhibitions were calculated as shown below.

$$\%\text{Parasitemia} = \frac{\text{Number of parasitized red blood cells(RBCs)} \times 100}{\text{Total number of RBCs count}}$$

$$\%\ \text{Inhibition} = \frac{(\%\text{Parasitemia of negative control} - \%\text{Parasitemia of treated group}) \times 100}{\%\text{Parasitemia of negative control}}$$

Evaluation of Mean Survival Time

The mice in the curative group were observed for mortality in days which was calculated as mean survival time (MST) using the formula below

$$MST = \frac{\text{Sum of survival time of all mice in a group (days)}}{\text{Total number of mice in that group}}$$

Analyses of Hematological and Liver Biochemical Parameters

On the final day of treatment, blood samples were collected in heparinized sample containers from the mice used for the curative study. The samples were assessed for Alanine Amino Transferase (ALT), Aspartate Amino Transferase (AST), Alkaline Phosphatase (ALP), Red Blood Cells (RBCs), Hemoglobin (Hb), White Blood Cells (WBCs) and Packed Cell Volume (PCV) using an auto analyzer.

Liver Histology

After the observation of mean survival time, liver samples were obtained from the mice used for curative study and stored in formalin saline for 24hr. There after, the samples were processed and sectioned (3µcm thick) using a microtome. The sectioned liver tissues were stained on slides with hematoxylin and eosin and viewed with the aid of a microscope. The relevant sections were photographed.

Data Analysis

Data was analyzed using the One-way Analysis of Variance (ANOVA) followed by Turkey's post hoc test. Data was presented as mean± standard error of mean (mean± SEM). A $p< 0.05$ was considered significant.

Results

Curative Effect of Artesunate/Ketoconazole on *Plasmodium berghei*-Infected Mice

Treatment with AS/KT produced significant daily parasitemia reductions ($p<0.05$) when compared to treatment with AS and KT individually. On day 4, AS/KT produced 3.51±0.38 % parasitemia, while AS, KT and CQ produced 7.47±0.22%, 12.53±0.47% and, 4.71±0.34% parasitemia, respectively. AS/KT produced 92.11 % inhibition whereas AS, KT and CQ produced 83.21% 71.84% and 90.63% inhibitions, respectively. It was observed that AS/KT increased MST significantly ($p<0.05$) when compared to AS and KT, respectively (Table 1).

Table 1: Curative effect of artesunate/ketoconazole on *Plasmodium berghei*-infected mice.

Treatment	%Parasitemia Day 1	%Parasitemia Day 2	%Parasitemia Day 4	% Inhibition Day 4	MST (Days)
PC	30.12±2.22^a	37.47±3.50^a	44.50±4.51^a	-	9.01±0.21^a
CQ	17.23±1.50^b	8.13±0.43^b	4.17±0.34^b	90.63	27.22±2.00^b
AS	18.50±1.21^c	11.20±0.61^c	7.47±0.22^c	83.21	21.64±0.45^c
KT	22.57±0.50^d	16.51±0.27^d	12.53 ±0.47^d	71.84	15.27±2.47^d
AS/KT	13.27±1.71^e	7.45±0.61^d	3.51±0.38^b	92.11	29.44±0.38^b

Note*: Data expressed as mean ± standard error of mean, n=5, PC: Parasitized Control, CQ: Chloroquine (Standard), AS: Artesunate, KT: Ketoconazole. MST: Mean Survival Time, Values with different superscripts down the column differ significantly at $p<0.05$ (ANOVA).

Suppressive Effect of Artesunate/Ketoconazole on *Plasmodium berghei*-Infected Mice

It was observed that in the suppressive study, treatment with AS/KT significantly ($p<0.05$) decreased percentage parasitemia when compared to AS and KT respectively. Treatment with AS/KT produced 1.12±0.01% parasitemia when compared to 3.00±0.50%, 5.01±0.23% and 1.33±0.07% parasitemia produced by AS, KT and CQ, respectively. AS/KT produced inhibition which represents 94.45% whereas inhibitions which represent 85.13%, 75. 16% and 93.41% were produced by AS, KT and CQ respectively (Table 2).

Table 2: Suppressive effect of artesunate/ketoconazole on *Plasmodium berghei*-infected mice.

Treatment	% Parasitemia	% Inhibition
PC	20.17±2.00^a	-
CQ	1.33±0.07^b	93.41
AS	3.00±0.50^c	85.13
KT	5.01±0.23^d	75.16
AS/KT	1.12±0.01^b	94.45

Note*: Data expressed as mean ± standard error of mean, n=5, PC: Parasitized Control, CQ: Chloroquine (Standard), AS: Artesunate, KT: Ketoconazole. MST: Mean Survival Time, Values with different superscripts down the column differ significantly at $p<0.05$ (ANOVA).

Effect of Artesunate/Ketoconazole on Hematological and Liver Parameters of *Plasmodium berghei*-Infected Mice

Significantly ($p<0.05$) decreased RBCs, PCV and Hb and significantly ($p<0.05$) increased WBCs were observed in *Plasmodium berghei*-infected mice when compared to the normal control. On the other hand, AS/KT significantly increased RBCs, PCV and Hb and significantly decreased WBCs when compared to AS or KT at $p<0.05$. The effects of AS/KT on RBCs, PCV, Hb and WBCs were not statistically ($p>0.05$) different from CQ. Furthermore, AS/KT had no significant ($p>0.05$) effects on serum ALT, AST and ALP levels when compared to AS or KT (Tables 3,4).

Table 3: Effect of artesunate/ketoconazole on hematological parameters on *Plasmodium berghei*-infected mice.

Treatment	PCV (%)	Hb (g/dL)	RBCs (X 10^6)	WBCs (cells/L)
NC	44.50± 3.50	14.85±0.15	6.65±0.05	7.50±0.30
PC	20.50± 2.50[a]	4.77±0.43[a]	2.40±0.30[a]	24.65±1.85[a]
CQ	41.00± 4.50[b]	14.45±0.55[b]	6.25±0.25[b]	7.75±0.45[b]
AS	31.00± 2.00[c]	11.00±1.00[c]	4.40±0.10[c]	14.35±0.85[c]
KT	26.50± 2.50[d]	9.50±0.20[d]	3.70±0.14[d]	18.00±0.50[d]
AS/KT	41.50± 2.00[b]	14.60±0.40[b]	6.29±0.30[b]	7.25±0.45[b]

Note*: Data expressed as mean ± standard error of mean, n=5, NC: Normal Control, PC: Parasitized Control, CQ: Chloroquine (Standard), AS: Artesunate, KT: Ketoconazole. RBCs: Red Blood Cells, WBCs: White Blood Cells, PCV: Packed Cell Volume, Hb: Hemoglobin; Values with different superscripts down the column differ significantly at $p<0.05$ (ANOVA).

Table 4: Effect of artesunate/ketoconazole on liver biochemical parameters of parasitized mice.

Treatment	AST (IU/L)	ALT (IU/L)	ALP (IU/L)
NC	34.00±1.33	23.00±3.00	30.50±2.52
PC	37.50±3.50	25.50±3.50	33.50±3.51
CQ	44.00±2.45	26.50±2.62	32.50±2.63
AS	34.50±2.50	25.23±2.22	33.00±3.41
KT	34.00±3.41	24.00±3.34	31.50±2.50
AS/KT	36.50±4.50	26.14±2.51	34.50±4.32

Note*: Data expressed as mean ± standard error of mean, n=5, NC: Normal Control, PC: Parasitized Control, CQ: Chloroquine (Standard), AS: Artesunate, KT: Ketoconazole. AST: Aspartate Aminotransferase, ALT: Alanine Aminotransferase, ALP: Alkaline Phosphatase.

Effect of Artesunate/Ketoconazole on Liver Histology

The liver of the control mice showed normal hepatocytes, sinusoids and central vein (Figure A), while the liver of the parasitized control mice showed steatosis, inflammatory cells, and parasitized red blood cells (Figure B). The liver of parasitized mice treated with chloroquine showed normal sinusoids, hepatocytes and congested central vein (Figure C). The liver of parasitized mice treated with artesunate (Figure D) and ketoconazole (Figure E) showed parasitized red blood cells, inflammatory cells, normal hepatocytes and sinusoids whereas the liver of parasitized mice treated with artesunate/ketoconazole showed normal hepatocytes, sinusoids and central vein congestion (Figure F).

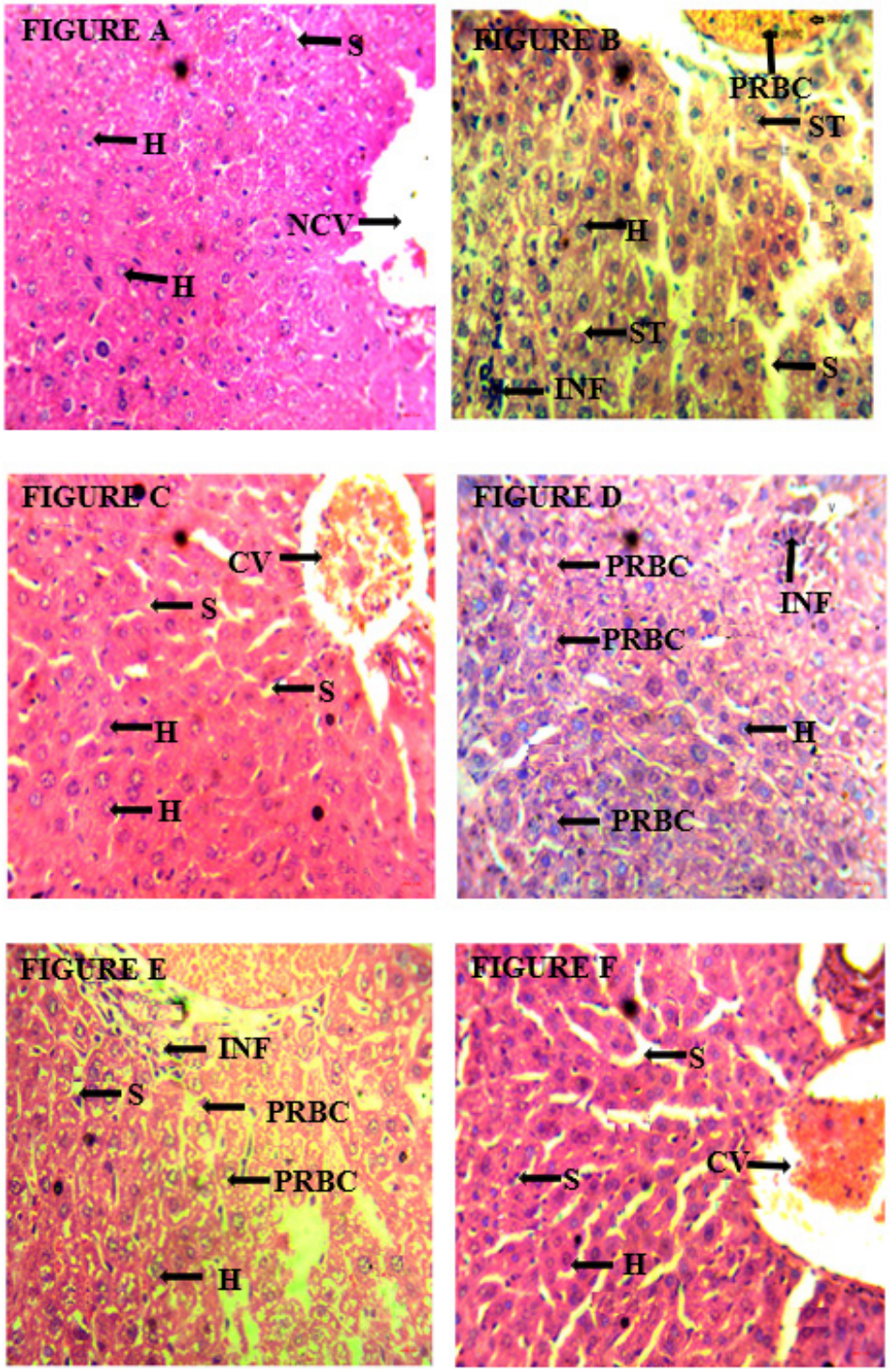

Figure A: Liver of the control mice, **Figure B**: Liver of parasitized control mice, **Figure C:** Liver of parasitized mice treated with chloroquine, **Figure D**: Liver of parasitized mice treated with artesunate, **Figure E**: Liver of parasitized mice treated with ketoconazole, **Figure F**: Liver of parasitized treated with artesunate/ketoconazole.
Note*: H: Hepatocytes, S: Sinusoids, NCV: Normal Central Vein, CV: Central Vein Congestion, INF: Inflammatory Cells, ST: Steatosis, PRBC: Parasitized Red Blood Cells.

Discussion

The vacuum in the market for new antimalarial drugs and the paucity of affordable alternatives in the developmental pipeline, make it imperative that very fast drug developmental methods are urgently established to avoid the imminent consequences of drug failure [4]. The aforementioned can be achieved through the repur-

posing of existing clinically used drugs by taking advantage of their combinations with artemisinins. This work provides an in-depth assessment of the antimalarial activity of KT in partnership with AS in parasitized mice. The current study used the most common mouse models of malaria which employ rodent-specific parasite species such as *Plasmodium berghei* that cause immune responses and distinct pathologies and model different manifestations of human diseases including malaria [16]. This study used the two *in-vivo* models frequently employed for the antimalarial screening of new compounds which are 4 days suppressive and curative tests that evaluate the suppressive and curative capabilities of candidate drugs on early and established infections respectively [17]. The two animal models were also used, because they permit possible pro-drug effect and the activity of the host defense system in infection eradication [18]. The observation in the current study showed notable curative antiplasmodial activity of AS/KT characterized by daily reductions in percentage parasitemia. The observed curative antiplasmodial activity of AS/KT was at par with the standard control. Also, AS/KT produced very visible suppressive antiplasmodial activity marked by reduced percentage parasitemia, which was the same with the effect produced by the standard control.

Severe malaria manifests a variety of clinical syndromes which depends on the properties of the host and the parasite. There is now budding evidence, from both human and mouse studies of malaria, which showed that anemia is not only related to hemolysis of infected and clearance of uninfected RBCs, but also due to inadequate erythroid response by the infected host [19]. Rodent malaria species infected with *Plasmodium species* have been used to investigate the contribution of various aspects of anemia in malaria. Studies used hematological markers, which include PCV, Hb and RBCs for assessing malaria-related anemia while WBCs, total and differential counts are determinants of the severity of infections [20]. In this study, AS/KT remarkably alleviates anemia in the parasitized mice marked by conspicuous increased RBCs, PCV, and Hb. It also alleviates the severity of malaria infection as characterized by decreased WBCs. Interestingly, the propensity of AS/KT to alleviate anemia is comparable to the standard control. Malaria is a significant cause of mortality worldwide. One of the significant goals of antimalarial drug use is the prevention or reduction of malaria related death. Experimentally, in malaria studies, MST in measured to ascertain the proficiencies of drug candidates to reduced or prevent malaria-related death [21]. The present study observed that AS/KT remarkably prolonged MST in the treated mice. Its impact on MST was comparable to the standard control. At present, monitoring of malaria-associated liver injury is heterogeneous, with the measurement of biochemical liver function tests which involves liver enzymes (AST and ALT) being the primary determinant for liver injury [22]. Also, the measurement of the aforementioned indices is imperative due to the potential of some antimalarial drugs to cause liver injury [23,24]. The current study observed that treatment with AS/KT had no deleterious effects on serum AST, ALT and ALP levels.

Liver involvement in severe malaria infection especially in *Plasmodium falciparum* infection is a significant cause of mortality in humans characterized by deleterious liver morphological changes [25]. After mortality, this study histologically examined the liver of mice in the control and treated groups. Steatosis, inflammatory cell infiltrations, and PRBCs were conspicuous in the liver of the death parasitized control. The observation correlates with similar changes in the liver of patients who died of malaria reported by Viriyavejakul*, et al.,* 2014 [25]. Interestingly, the afore mentioned changes were absent in the liver of AS/KT treated mice except for the observed central vein congestion. The study shows that KT augmented the antiplasmodial activity of AS. The mechanism of the antiplasmodial action of KT is not known, but its antifungal effect involves the inhibition of the formation of ergosterol in cell membrane. Studies have shown that KT is a primary inhibitor of liver CYP 3A4. It may have increased the antiplasmodial activity of AS by inhibiting CYP 3A4, which is responsible for the metabolism of AS thereby prolonging the plasma concentration of AS [13]. Conclusion: This study showed that AS/KT exhibited remarkable suppressive and curative antiplasmodial activities. It is suggested that KT can be repurposed in combination with AS for the treatment of malaria.

Funding

None.

Acknowledgement

None.

Conflicts of Interest

None.

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