

Enhancement of Antimalaria Effect of Mefloquine by Chlorpheniramine

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Abstract Antimalarial drug resistance has emerged as one of the greatest challenges facing malarial control. The aim of this investigation is to enhance the antimalarial effect of mefloquine (MQ) by combining it with chlorpheniramine (CPM) using *Plasmodium berghei*-infected wistar rat model. *P. berghei* parasites (10⁶) were administered intraperitoneally into albino rats weighing 118-208g. 20% parasitaemia was taken and treatment commenced after day 4. The haematological, biochemical and pharmacokinetic investigations of malarial infected rats were carried out followed by the histopathological changes in the liver. The albino rats (35) were randomized into 7 groups of 5 animals each. Treatment of infected rats with drug combination was as follows: Group I: neither infected nor treated; group II: mefloquine alone at dose 1250mg/70kg man weight; group III: chlorpheniramine alone at dose 4mg/70kg man weight; group IV: mefloquine at dose 1250mg/70kg man and chlorpheniramine (4mg/70kg man) together; group V: chlorpheniramine (4mg/70kg man) followed by mefloquine (1250mg/70kg man) 30 mins after; group VI: mefloquine followed by chlorpheniramine 30 mins after; group VII: infected and untreated serving as control. The baseline parasitaemia before treatment were 37.6%, 32.4%, 32.4%, 34.6%, 38.4% and 51.2% for MQ alone, CPM alone, MQ + CPM, CPM + MQ 30, MQ + CPM 30 and Negative control respectively. Administration of the various drug combinations resulted in significant ($p > 0.05$) percent parasite reductions of 7.4%, 26.5%, 89.2%, 22%, 76% and -104% on day 1, and 86.2%, 71.6%, 92.8%, 59.5%, 82.8% and -140.6% on day 3 respectively. The ranking of the percent parasite reductions on day 3 was MQ + CPM > CPM alone > MQ + CPM 30 > MQ alone > CPM + MQ 30. The RBC count, haemoglobin and PCV showed no significant difference ($p > 0.05$) in all the groups. ALT showed a significant ($p < 0.05$) increase in blood of infected and untreated group compared to all the other groups. AST was significant ($p < 0.05$) in infected and untreated rats compared to all of the other groups. The results showed that chlorpheniramine enhances the antiplasmodial activity of mefloquine and the combination gives an affordable and highly effective antimalarial.

Keywords *P. falciparum*, Drug resistance, Drug combination, Antimalarials, Mefloquine, Chlorpheniramine

1. Introduction

Malaria is an acute and chronic mosquito-borne ancient disease of man characterized by chills, fever, anemia, splenomegaly and damage to other organs such as the liver and brain [1]. Young children, pregnant women and non-immune visitors to malarious areas are at greatest risk of severe or fatal illness. According to Centre for Disease Control (CDC), nearly 85% of malaria cases in Africa are caused by *P. falciparum*, the remaining cases being caused by the other three strains. *P. vivax* is now the most geographically widespread of the human malaria, occurring in much of Asia, Central and South America, the Middle East, where 70-90% of the malaria burden is of this species and the

rest due to *P. falciparum* [2]. *P. Malaria* causes sporadic infections in Africa, Parts of India, Western Pacific and South America, whereas *P. ovale* is restricted to tropical Africa, New Guinea and Philippines [3, 4]. *P. knowlesi* has been reported in South East Asian countries such as Malaysia, Thailand, Vietnam, Myanmar and Philippines [5].

Anti-malarial drug resistance has emerged as one of the greatest challenges facing malaria control today leading to malaria morbidity and mortality [5, 6]. Drug resistance has been implicated in the spread of malaria to new areas and re-emergence of malaria in areas where the disease had been eradicated. Drug resistance has also played a significant role in the occurrence and severity of epidemics in some parts of the world. Population movement has introduced resistant parasites to areas previously free of drug resistance. *P. falciparum* has also developed resistance to nearly all of the other currently available antimalarial drugs such as sulfadoxine/pyrimethamine, mefloquine, halofantrine and quinine. Although resistance to these drugs tends to be much

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less widespread geographically, in some areas of the world, the impact of multi-drug resistant malaria can be extensive. Most recently, a low-grade resistance to artemisinin-based drugs has emerged in parts of Southeast Asia. Parasite resistance to antimalarials has been documented in three of the five malaria species known to affect humans; *P. falciparum*, *P. vivax* and *P. malariae* [7].

However, most of these drugs still have a place and their life-span could be prolonged if better deployed and used, and also by rationally combining them based on pharmacodynamics and pharmacokinetics properties with other drugs. Pyronaridine, a Chinese drug was combined with pyrimethamine [8]; artesunate is used in combination with mefloquine or tetracycline in Southeast Asia [9-11]. Chloroquine resistance can be reversed by combining chloroquine with calcium channel blockers, verapamil or chlorpromazine [6]. Antihistamine compounds cyproheptadine, ketotifen reversed chloroquine resistance in *P. yoelii* mice [12, 13].

Mefloquine, a 4-quinolinemethanol derivative (Fig. 1) and 2-aryl substituted chemical structural analog of quinine was invented in 1970 at Walter Reed Army Institute of Research (WRAIR) [14]. Mefloquine was first used to treat chloroquine-resistant *P. falciparum* malaria in Thailand where it was formulated with pyrimethamine-sulfadoxine (fansimef[®]) to delay development of drug resistant parasites. This strategy failed largely because slow elimination of mefloquine fostered the selection of resistant parasites at sub-therapeutic drug concentration [15].

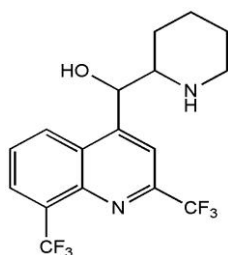


Figure 1. Chemical Structure of Mefloquine

Chlorpheniramine maleate (Fig. 2) a synthetic alkylamine derivative and salt of chlorpheniramine is prepared by chlorination of pheniramine in the *para* position of the phenyl ring. This chlorination at the *para* position of the phenyl ring increases the potency by almost 10-fold with no appreciable change in toxicity. Most of the antihistamine activity resides with the dextro isomer [16]. Studies show that chlorpheniramine maleate can reverse chloroquine resistant *Plasmodium falciparum* [17-19].

Higher dosage of chlorpheniramine enhances the efficacy of chloroquine in treating acute and uncomplicated *P. falciparum* in children from an endemic area of Nigeria [20]. Data on potentiation of antimalarial effect of mefloquine by chlorpheniramine in animals are lacking. Previous work by Nakorchai and Konthiang [21] was on *in vitro* studies and did not consider *in vivo* studies like pharmacokinetics of the combination, the effect of the combination on

haematological and biochemical parameters as well as the effect on the liver.

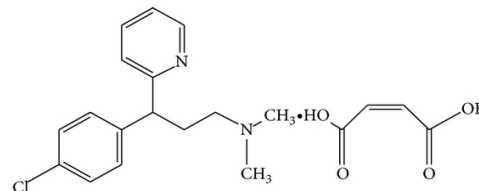


Figure 2. Chemical Structure of Chlorpheniramine maleate

The present study investigates the ability of chlorpheniramine to enhance or potentiate the therapeutic efficacy of mefloquine *in vivo* against *P. berghei* malaria in rat by assessing the percentage reduction in parasitaemia; changes in haematological and biochemical parameter; determination of histopathological changes in the liver; pharmacokinetic studies of mefloquine/chlorpheniramine combination in rat.

2. Materials and Methods

2.1. Materials

2.1.1. Drugs Used

Mefloquine hydrochloride tablets (Occulus Pharma Nigeria) and chlorpheniramine maleate tablets (Evans Medicals Nigeria). Tablets were dissolved in water for injection and concentration made to 7.49mg/ml for mefloquine and 0.024mg/ml for chlorpheniramine.

2.1.2. Animals

In-breed male and female (non-pregnant) albino rats weighing between 118g and 208g (mean weight 169.63) were obtained from the animal house of the Department of Veterinary Medicine, University of Nigeria Nsukka. The animals were observed under 12hours light/dark cycles in metabolic cages in a well ventilated rodent cubicle. They were fed on a rat pellet diet (Ladokun Farms, Nigeria) with water *ad libitum*. The use of animals in this study was in accordance with the guidelines approved by the Animal Ethical Committee, Nnamdi Azikiwe University Awka. The animals were acclimatized for 7 days prior to their randomization into various experimental groups.

2.1.3. Parasite

The *P. berghei* parasites used in this study was obtained from the Department of Pharmaceutics, University of Nigeria Nsukka. It was maintained by serial passage in mice which served as donor mice in this study.

2.1.4. Chemicals and Reagents

Alanine aminotransferase and Aspartate aminotransferase (Randox Laboratory, UK), Chloroform (Sigma Aldrich, USA), Drabkin's solution (Organo Biotech Laboratories, India), EDTA (Fushun Shunnum Chemical, China), Paraffin

wax (Green Mountain, China), Xylene (Shiyan Industries, India), Haematoxylin (Abbey Colour, Philadelphia), RBC diluting fluid (Organo Biotech Laboratories, India), WBC diluting fluid (Alpha Chemicals, India), Formal saline (Vet Way Industries, UK), Phosphate buffer (Vet Way Industries, UK), HPLC Grade Acetonitrile (Sigma – Aldrich Germany), HPLC grade Methanol (Sigma- Aldrich Germany), HPLC grade formic acid (Sigma -Aldrich Germany), HPLC grade water (double distilled water), mefloquine HCL USP analytical grade (Sigma-Aldrich Germany), chlorpheniramine maleate USP analytical grade (Sigma –Aldrich Germany).

All other reagents are of analytical grade.

2.2. Methods

2.2.1. Determination of Parasite Density and Inoculum Preparation

A standard inoculum was prepared from a donor rat with *P. berghei* parasitized erythrocytes. Infected blood from donor rat was obtained by cardiac puncture after anesthesia with chloroform. Each rat was infected with a standard inoculum of the 10^6 parasitized erythrocyte suspension in phosphate buffered saline (0.2ml) from a donor rat that was prepared based on percentage parasitaemia and number of erythrocytes counted per μL of blood using an improved haemocytometer.

2.2.2. Parasitaemia Determination

Blood samples were collected by bleeding via the tail vein of *P. berghei* infected rat and thin blood smears were made on microscope slides fixed with methanol and stained with 10% Giemsa stain for 30 min and then rinsed with tap water. Parasitaemia was determined by counting the number of PRBC for ten different fields. Parasitaemia was monitored daily using X100 objective of light microscope. Treatments commenced when about 20% parasitaemia were established in infected rat.

Curative treatments in the set of 35 albino rats randomized into 7 groups of 5 animals per group were introduced intra-peritoneally infected with 10^6 parasitized erythrocyte. Oral treatment commenced on day 4 post infection.

Group I (Uninfected and Untreated). Animals in this group were not infected and untreated.

Group II (mefloquine alone). Animals in this group were infected with *P. berghei* and treated on day 4 post infection with mefloquine alone at the dose of 1250 mg/70kg man.

Group III (chlorpheniramine alone). Animals in this group were infected with *P. berghei* and treated on day 4 post infection with chlorpheniramine alone at the dose of 4mg/70kg man.

Group IV (mefloquine + chlorpheniramine together). Animal in this group were infected with *P. berghei* and treated on day 4 post infected with mefloquine + chlorpheniramine together at the doses of 1250mg/70kg man and 4mg/70kg man for mefloquine and chlorpheniramine

respectively.

Group V (chlorpheniramine first followed by mefloquine 30mins later).

Animals in this group were infected with *P. berghei* and treated on day 4 post infection with chlorpheniramine given first at the dose of 4mg/70kg man and followed by mefloquine at the dose of 1250mg/70kg man 30 mins after.

Group VI (mefloquine first then followed by chlorpheniramine 30mins after) Animals in this group were infected with *P. berghei* and treated on day 4 post infection with mefloquine given first at the dose of 1250mg/70kg man, then followed by chlorpheniramine at the dose of 4mg/70kg man 30 mins later.

Group VII (infected and untreated)

Animals in this group were infected and untreated serving as control.

2.2.3. Haematological Parameters

The haematological parameters determined are percentage cell volume (% PCV), haemoglobin, erythrocytes and leukocyte counts.

2.2.4. Determination of Packed Cell Volume (PCV)

PCV is used to measure the amount of cells in the blood; blood is made up of cells and plasma. The amount of cells on the blood is expressed as a percentage of the total volume of blood.

Blood sample was collected from the ocular orbit of each mouse with a heparinized capillary tube by capillary action. The tube was sealed with plasticine at one end. The sealed capillary tubes were then arranged on the hematocrit centrifuge and spun at 10,000 rpm for five minutes to separate the blood into plasma and packed cells. The % PCV was read on the hematocrit reader and recorded. The PCV of each rat was measured before infection and on day 4 after infection using the formula:

$$\text{PCV} = \frac{\text{Volume of Erythrocytes in a given volume of blood}}{\text{Total blood Volume}} \times 100$$

2.2.5. Haemoglobin

Using the micropipette, 20 μL of blood was taken and mixed with 4ml of Drabkin solution in a test tube. The blood mixture was allowed to stand for 5mins and then read with a colorimeter at 540nm wavelength. The haemoglobin was recorded.

2.2.6. Determination of Total Leukocyte (White Blood Cell) Count

Total leukocyte is the total number of white blood cell in the blood. This experiment was carried out using a haemocytometer: a device used for counting red blood cells and white blood cells in a sample of blood. The blood was collected from the rat in an EDTA bottle to prevent clotting of the blood. Using the micropipette in the

haematocytometer, 20 μ L of blood was taken and mixed with 380 μ L of Gower solution. Using micropipette, the blood mixture was placed on a haemocytometer slide and viewed on a microscope with magnification of 40X. The White Blood Cell count was noted and recorded.

2.2.7. Determination of Total Erythrocyte (Red Blood Cell) Count

Total erythrocyte count is the total number of red blood cell in the blood. This experiment was carried out using a haemocytometer: a device used for counting red blood cells and white blood cells in the blood. The blood was collected from the rat in an EDTA bottle to prevent clotting of the blood. Using the micropipette, 20 μ L of blood was taken and mixed with 4 ml of RBC diluting fluid in a test tube. The blood mixture was allowed for 10 min, and then placed on a haemocytometer slide to be viewed on a microscope with magnification of 40X. The red blood cell count was noted and recorded.

2.2.8. Biochemical Parameters

Blood was collected by cardiac puncture and transferred into plan bottles. It was allowed to clot and centrifuged at 3500 rpm for 10mins and the clear sera collected by aspiration and store at -4°C . The sera were later thawed to room temperature and assayed for biochemical parameters namely: alanine aminotransferase (ALT), aspartate aminotransferase (AST)

2.2.9. Determination of Aspartate Amino Transferases (AST) Activity

The method of Reitman and Frankel [22] was used as outline in Randox kit as follows: 0.1ml of serum with 0.5ml of substrate solution (aspartate and 2 – ketoglutarate, in phosphate buffer (PH. 7.4) was incubated at 37°C for 30min. After incubation, 0.5ml of 2, 4, dinitrophenylhydrazine (DNPH) solution was added to arrest the reaction and kept for 20mins at room temperature. To this, 5.0ml of 0.4N sodium hydroxide was added and absorbance was read at 510nm wavelength. Activities were expressed as IU/L.

2.2.10. Determination of Alanine Aminotranferase (ALT) Activity

The activity of alanine aminotransferase was also assayed by the methods of Reitman and Frank [22] as outlined in Randox kits. Serum (0.1mL) with substrate solution (alanine and 2-keto glutarate, in phosphate buffer PH 7.4) (0.5mL) was incubated at 37°C for 30min. After incubation 2, 4 dinitrophenyl hydrazine (DPH) solution (0.5) was added to arrest the reaction and kept for 20min at room temperature. 0.4N sodium hydroxide (5.0mL) was added and absorbance was read at 510nm wavelength. Activities were expressed as IU/L.

2.2.11. Histopathology

Histopathological changes in the liver were assessed at the

end of the haematological and biochemical analysis. Tissue sections of the liver from experimental animal were fixed in 0.9% sodium chloride (normal saline) at room temperature for 72h and dehydrated in ascending grades of ethanol. Then after that, the tissue were stored in chloroform overnight, infiltrated and embedded in molten paraffin wax. The blocks were later trimmed and sectioned at 5-6 microns. The sections were de-paraffinized in xylene, then taken to water and subsequently stained with haematoxylin and Eosin (H and E).

2.2.12. Pharmacokinetics Studies Using HPLC

Inbred male and non-pregnant female albino rats (15) of 10 weeks old weighing between 90.2g and 134.1g were grouped as follows: Group A (Mefloquine alone); Group B (Chlorpheniramine alone); Group C (Mefloquine + chlorpheniramine together); Group D (Chlorpheniramine first + mefloquine 30 mins later); Group E (Mefloquine first + chlorpheniramine 30 mins later).

2.2.13. Preparation of the Standard Solution of Mefloquine

Mefloquine reference standard (25mg) was weighed (Weighing balance Sartorius TE-2145) accurately and transferred to 25ml volumetric flask and dissolved with 10ml of the mobile phase (methanol + acetonitrile, 75:25, v/v) and then made up to 25ml with the same mobile phase to get 1mg/ml. This was sonicated for 10mins and then diluted to get six different concentrations. All solutions were freshly prepared.

2.2.14. Construction of the Calibration Graph for Mefloquine

According to USP 2015 specification, the recommended concentration of the standard solution of mefloquine HCL was 0.05mg/ml. Aliquot portions (0.5-5ml) of 1mg/ml mefloquine standard solution was transferred to a series of 25ml measuring flask and completed to volume with the mobile phase (methanol + Acetonitrile, 75:25, v/v). The dilutions gave six different concentrations from 0.02mg/ml to 0.2mg /ml (20 μ g/ml to 200 μ g/ml) 10 μ L of the solution was injected from each flask and the chromatograms recorded, maintaining the flow rate at 1.0ml/min and wavelength at 222nm. The ratio of peak areas were measured corresponding to concentration of each. Calibration graph was constructed representing the relation between concentration and peak area. Concentrations of unknown samples were derived from the calibration graph.

2.2.15. Preparation of Standard Solution for Chlorpheniramine

25mg of Chlorpheniramine maleate reference standard was weighed accurately and transferred to 25ml volumetric flask. This was dissolved with 10ml of the mobile phase (methanol + acetonitrile, 75:25, v/v) and made up to 25ml with the same mobile phase to obtain 1mg/ml. It was then sonicated for 10 minutes.

2.2.16. Construction of the Calibration Graph for Chlorpheniramine

According to USP specification, the concentration of the standard solution is 8µg/ml.

To obtain different concentrations of the standard solution, the following dilutions were made:

2.5 ml of the standard solution (1.0mg/ml) was taken and enough volume of mobile phase added to make up to 25 ml (0.1mg/ml). Then aliquot portions (0.5ml, 1.0ml, 2.0ml, 3.0ml, 4.0ml, and 5.0 ml) of this solution (0.1mg/ml) were withdrawn and placed in different 25ml measuring flasks. Enough quantity of the mobile phase was added to each of the flasks to make up the volume to 25ml. The dilutions were done to yield six different concentrations ranging from 2µg/ml to 20µg/ml). All the solutions were freshly prepared. Then 10µL of the solution from each flask was injected into the HPLC machine (HPLC System Hitachi, Japan, equipped with auto sampler L-220 with capacity of 200 samples. pump – L- 2130, Oven L. – 2350, UV – Vis detector L2420FL, detector L 2485, Reservoir for mobile phase, micron filter (Biofil syringe filter PTFE Membrane) max pressure 4.5 bars (67.5psi) and pore size 0.45µm C18 column: 4.6nm×10cm; 5µ packing) to obtain the chromatograms. The flow rate is maintained at 1.0ml/min and the UV wavelength at 214nm. The peak areas were measured corresponding to the different concentrations. The data was used to construct a calibration graph depicting the relationship between the concentrations and the peak areas. The concentrations of the test samples were then derived from the calibration graph.

2.3. Pharmacokinetics Studies

The animals were acclimatized under 12 hours light /dark cycles in metabolic cages in a well ventilated rodent cubicle. They were fed on a rat pellet diet (Ladokun Farms, Nigeria) with water *ad libitum*. The use of animals in this study was in accordance with the guidelines approved by the animal ethical committee, Nnamdi Azikiwe University, Awka. The animals were acclimatized for 7days and randomly selected into the various experimental groups.

The animals were fasted over night before the administration of the drugs. The drugs were administered to the rats according to their body weights: mefloquine 1250mg /70kg man/day) and chlorpheniramine (4mg/70kgman/day). 0.5ml of the blood samples were withdrawn at specific time intervals of 30mins, 1hr, 2hr, 4hrs 6hrs and 8hrs.

The blood samples were centrifuged at 3000rpm for 15mins using the auto refrigerated centrifuge (TGL20M China) and the supernatant transferred into a fresh tube and the cells washed with distilled water. Then equal volume of methanol was added to the cells and centrifuged at 15000rpm (TGL20M China) for 10mins and the supernatant transferred into a clean fresh tube.

The supernatant was labeled accordingly A and B (A, supernatant from centrifuged whole blood; B, supernatant

from washed and centrifuged cells). These were filtered using 0.45micron syringe filters and 10µL injected into the chromatographic system using the auto sampler and eluted by isocratic method using the mobile phase; methanol + acetonitrile mixture (75%_{v/v}: 25%_{v/v}) as organic phase and 0.1% formic as the aqueous phase and the stationary phase is C18 column and flow rate maintained at the rate of 1.0ml/min. It was detected by UV-Visible at 222nm for mefloquine and 214 nm for chlorpheniramine.

2.4. Statistical Analysis

The results were expressed as mean ± standard deviation (SD). Parameters in the groups were compared by one way (ANOVA) using the computer software statistical package for social sciences (SPSS) version 16. All data were analyzed at a 95% confidence interval and values were considered statistical significance ($p < 0.05$). The research statistic employed for the comparison of levels of parasite in the various groups was ANOVA at 95% confidence. Multiple comparisons using post hoc Dunnett test (with negative control group as reference) and Turkey test (excluding the negative control) were conducted on the data to ascertain the degree of significance in percent parasite reduction among the various experimental groups.

3. Results

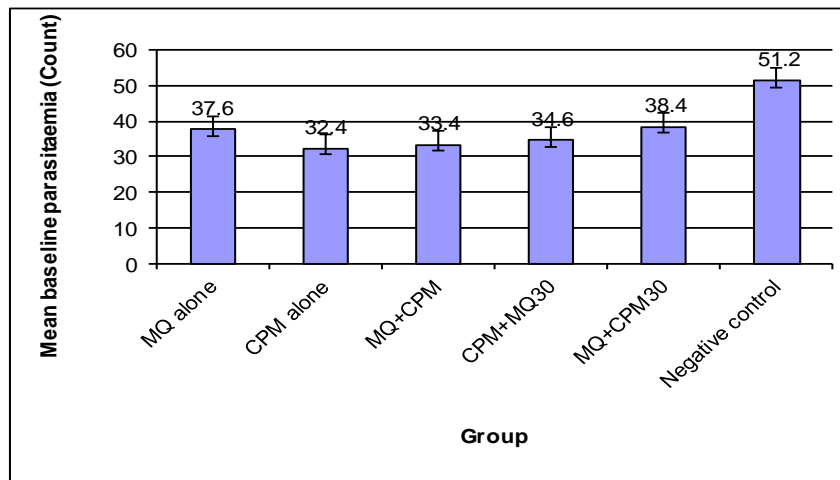
3.1. Antiplasmodial Testing

The results of antiplasmodial testing are shown in Figures 3 and 4.

3.2. Haematology

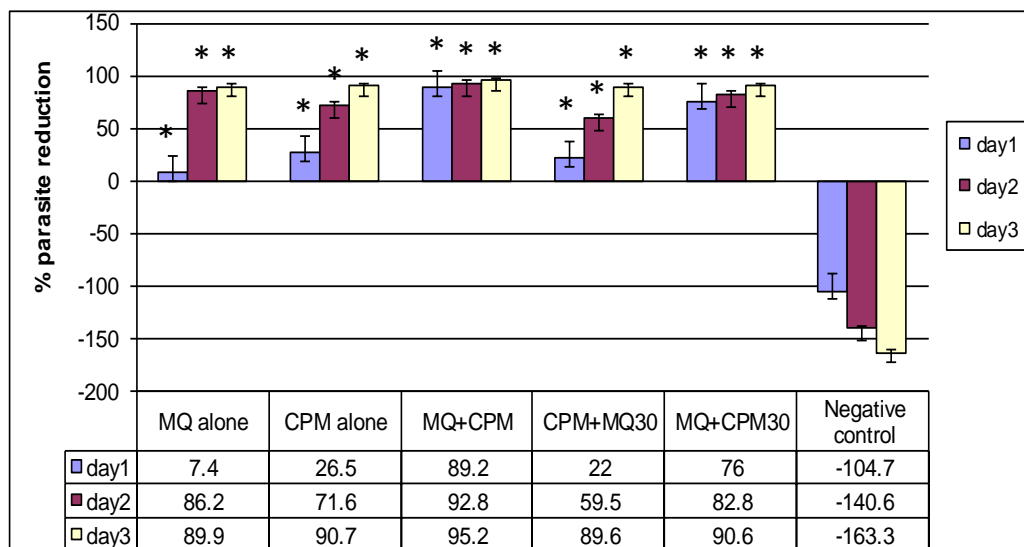
Figures 5 to 8 show the effect of treatment on haematological parameters;

Haemoglobin is found on the surface of the red blood cell as oxygen carrying pigment. As the malaria parasite attacks the red blood cells causing its haemolysis, there is reduction in the haemoglobin concentration. The growing malaria parasite also consumes and degrades haemoglobin [26]. This leads to further decrease in haemoglobin concentration of the red blood cells. Group VII (infected untreated) showed significantly ($p < 0.05$) lower haemoglobin when compared to groups II, III, IV, V and VI (mefloquin alone, chlorpheniramine alone, mefloquine + chlorpheniramine together, chlorpheniramine first + mefloquine 30mins later and mefloquine first + chlorpheniramine 30mins later respectively. This is as a result of the administration of treatment to the animals which killed the parasite and prevented further digestion of haemoglobin. But there was no significant difference ($p < 0.05$) between groups II, III, IV, V and VI. This indicates that mefloquine + chlorpheniramine combination cannot induce haemoglobin reduction in any way.



Baseline parasitaemia of treatment groups including negative control indifferent ($p > 0.05$)

Figure 3. Baseline parasitaemia of experimental animals before treatment



*significant difference at $p < 0.05$ when compared to negative control group (infected and untreated)

Figure 4. Percent parasite reductions of experimental groups from day 1-3

3.3. Packed Cell Volume

The results of the packed cell volume is shown in Figure 7 shows that the percentage PCV of group VII rat (infected and untreated) decreased significantly ($p < 0.05$) compared to all the other groups. PCV drop is as a result of the parasite attacking the red blood cells and causing haemolysis. A significant decrease in PCV of group VII (infected and untreated compared to group II, III, IV, V and VI treated with mefloquine alone, chlorpheniramine alone, mefloquine + chlorpheniramine together, chlorpheniramine first + mefloquine 30mins later and mefloquine first + chlorpheniramine 30mins later respectively was observed.

3.4. White Blood Cell

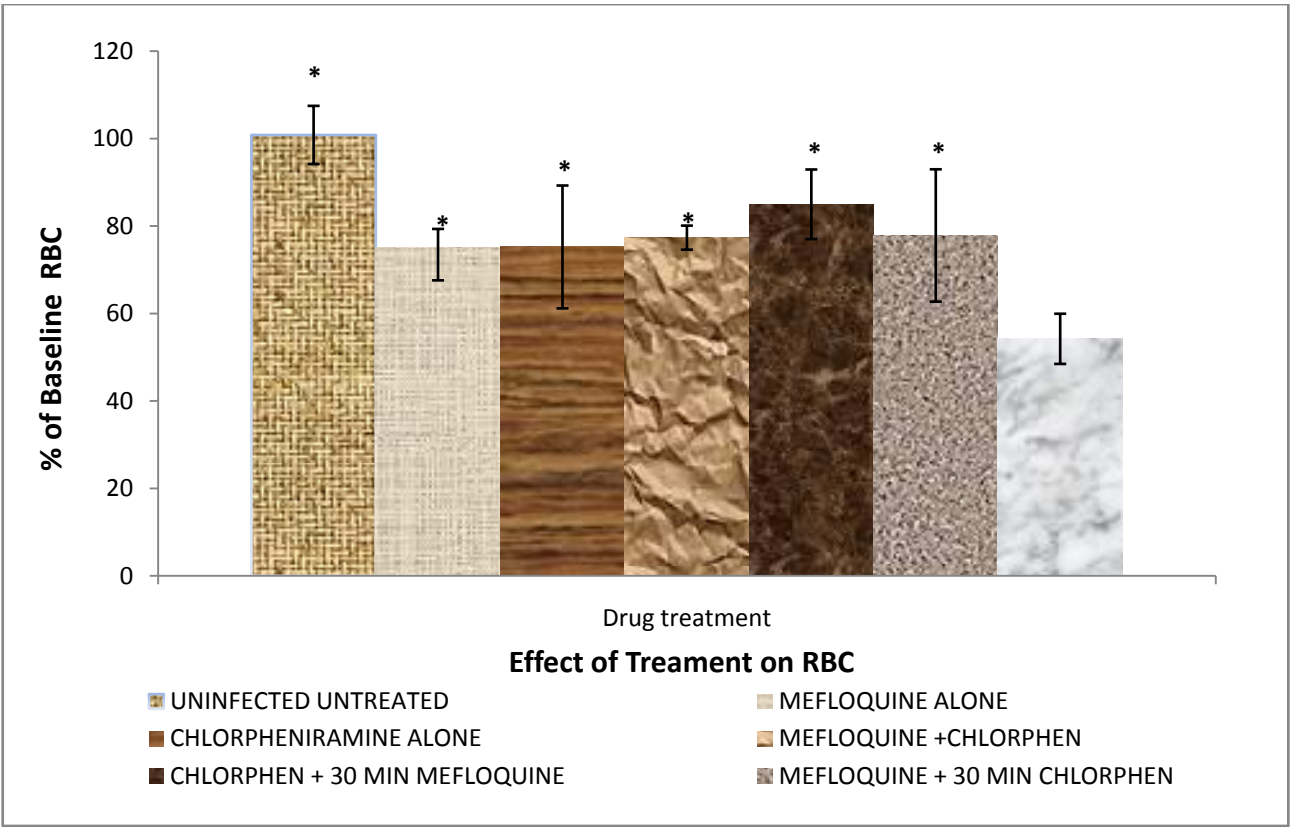
The results of white blood cell are shown in Figure 8.

3.5. Biochemical Parameters

The result of the effect of treatment on liver enzyme, alanine aminotransferase are shown in Figure 9 while Figure 10 shows the effect of treatment on liver enzyme: Aspartate aminotransferase. In Figure 9, there was a significant ($p < 0.05$) increase of alanine aminotransferase (ALT) in the blood of group VII (infected untreated) compared to all the other groups. This is as a result of malaria parasite infection which caused injury to the liver leading to the release of the enzyme in the blood stream.

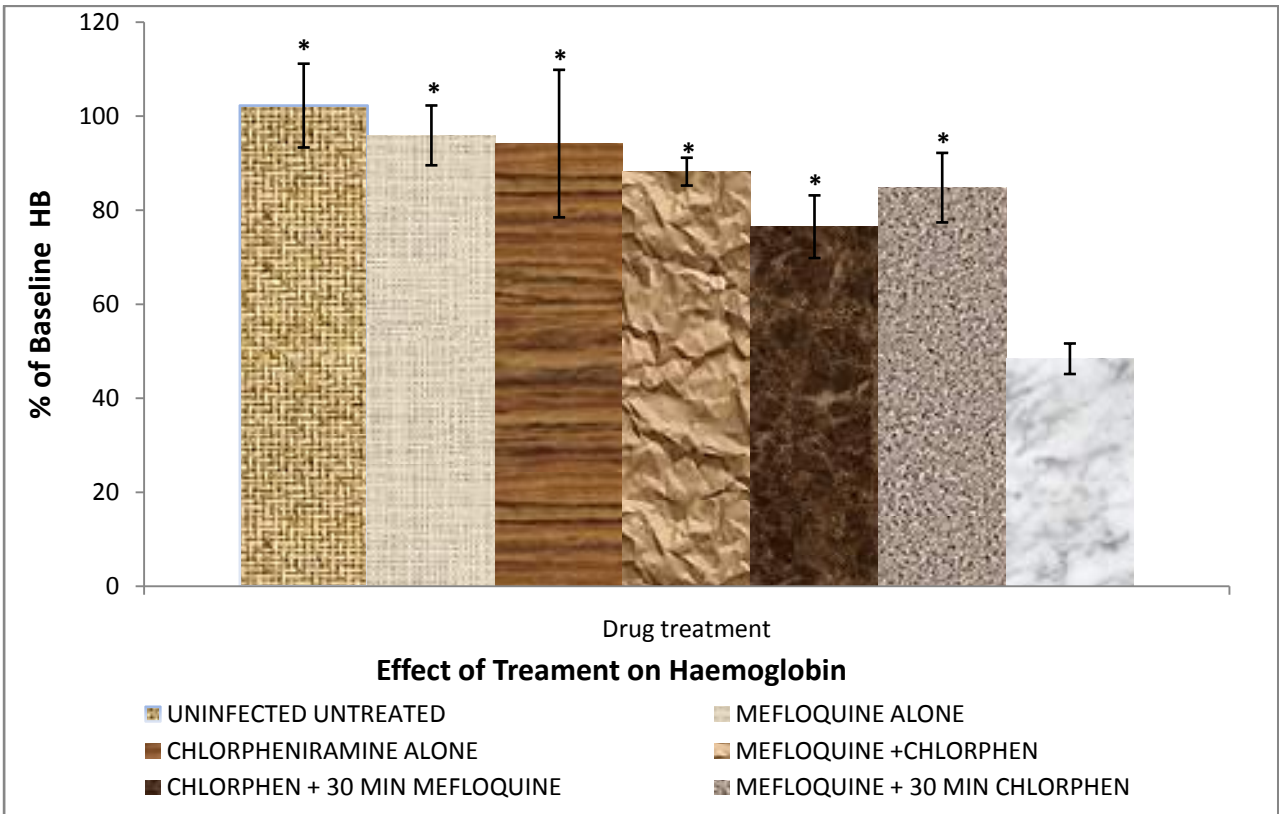
3.6. Result of AST

The results of AST on the effect of treatment on liver enzyme are shown in Figure 10.



*significant difference at $p < 0.05$ when compared to infected and untreated

Figure 5. Effect of treatment on red blood cell count



*significance difference $p < 0.05$ when compared to infected and untreated

Figure 6. Effect of treatment on serum haemoglobin

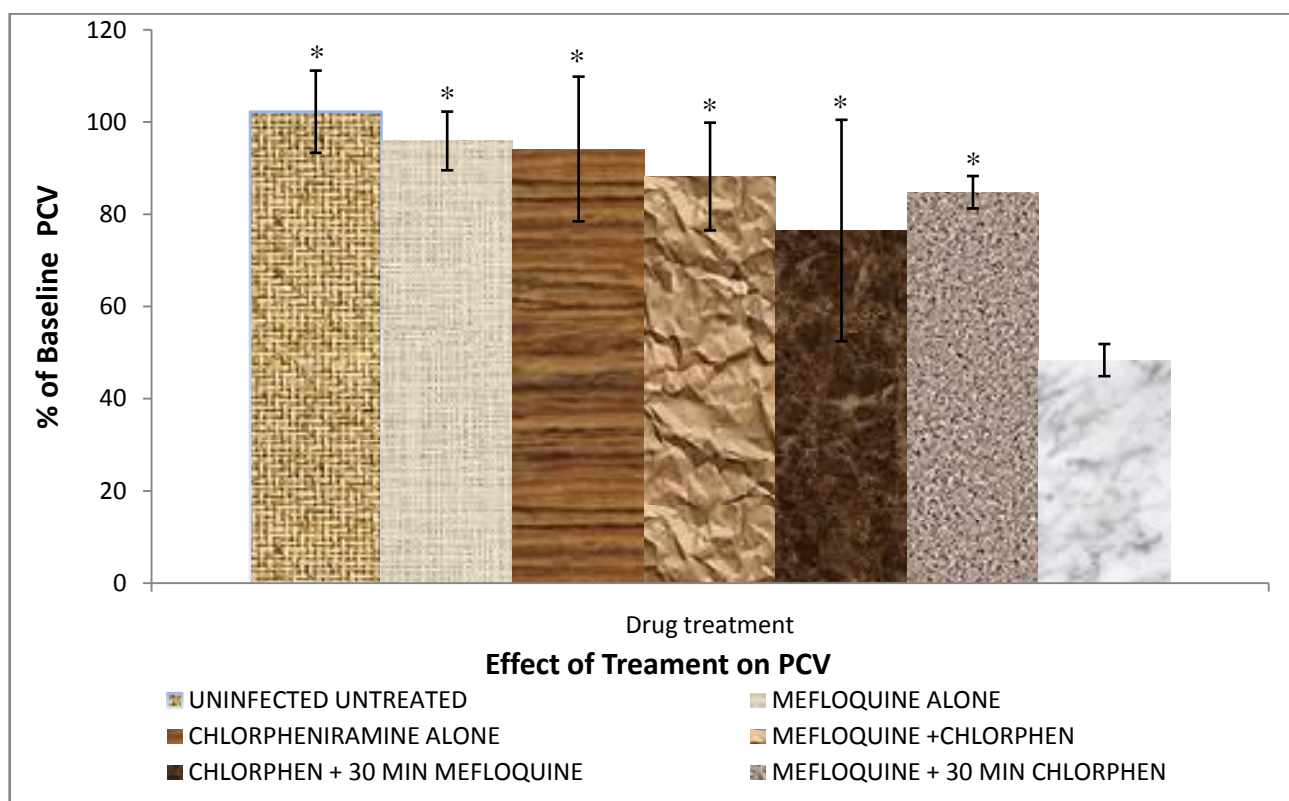


Figure 7. Effect of treatment on packed cell volume

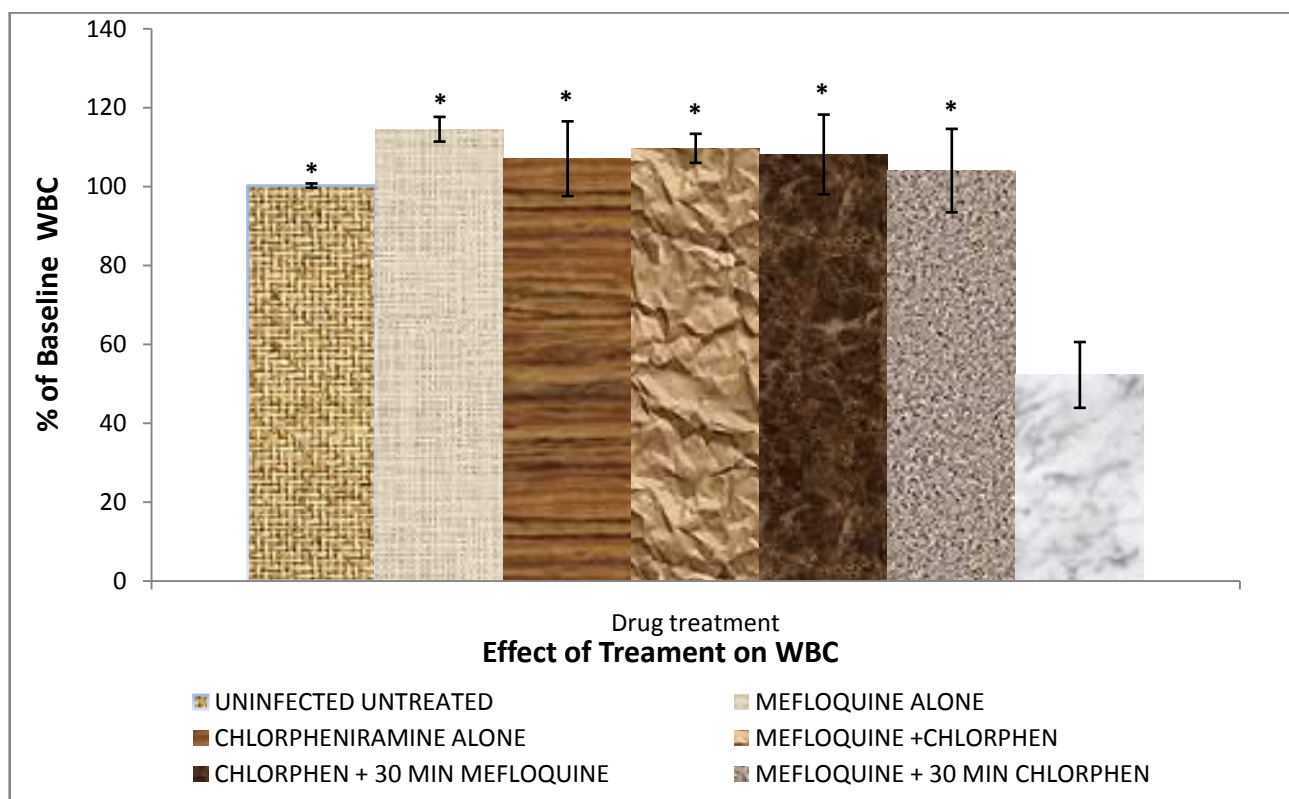
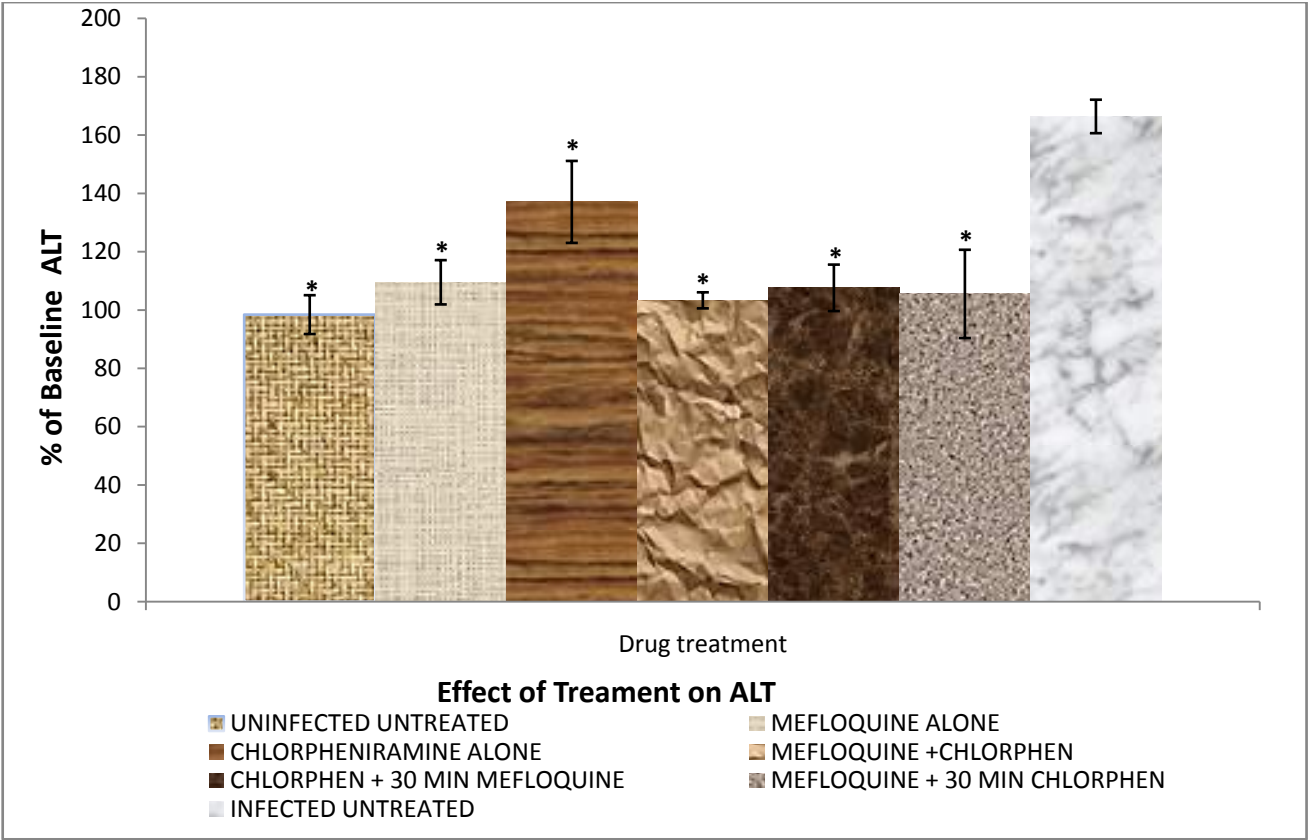
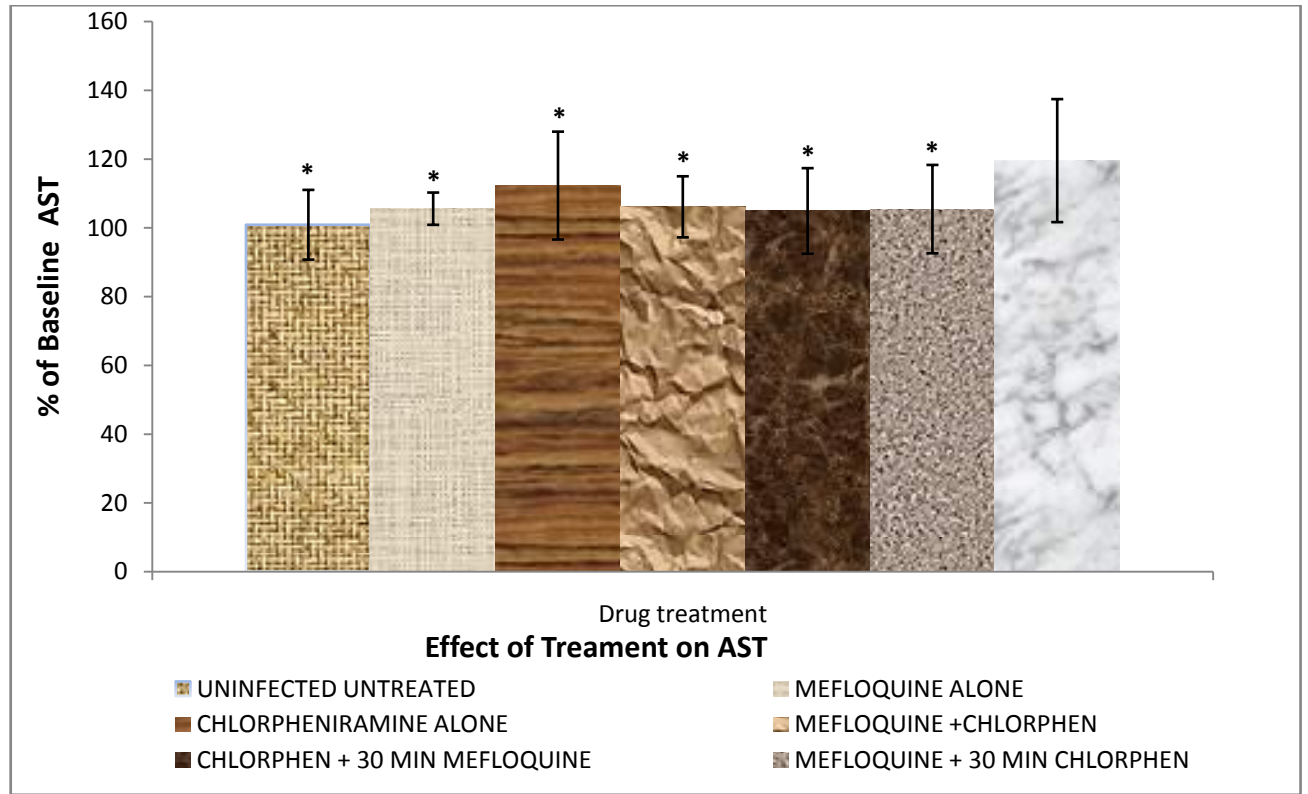


Figure 8. Effect of treatment on white blood cell count



*significant difference at $p < 0.05$ when compared to infected and untreated

Figure 9. Effect of treatment on serum alanine aminotransferase



*significant difference at $p < 0.05$ when compared to infected and untreated

Figure 10. Effect of treatment on serum aspartate aminotransferase (AST) level

3.7. Histopathological Effects on the Liver

The results of the histopathological effects on the liver are shown in Figures 11-16. Figs. 14, 15 and 16 Photomicrographs of liver section from experimental animal groups 4, 5 and 6 (MQ + CPM together), (CPM + MQ 30mins) and (MQ + CPM 30mins) respectively showing red pulp, white pulp and the central arteriole portal arrow (PA) H and E (x 40).

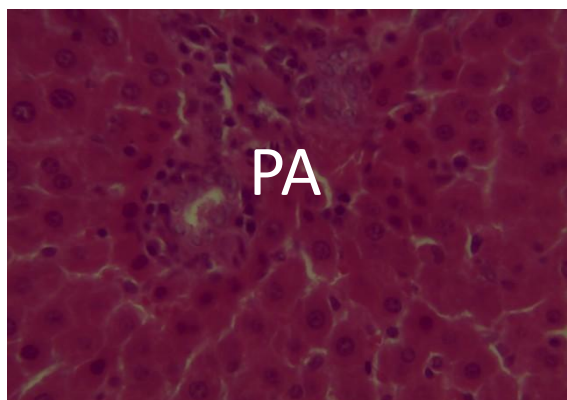


Figure 11. A photomicrograph of liver Section from experimental animal Group 1 uninfected showing the portal area (PA) H and E (x 40)

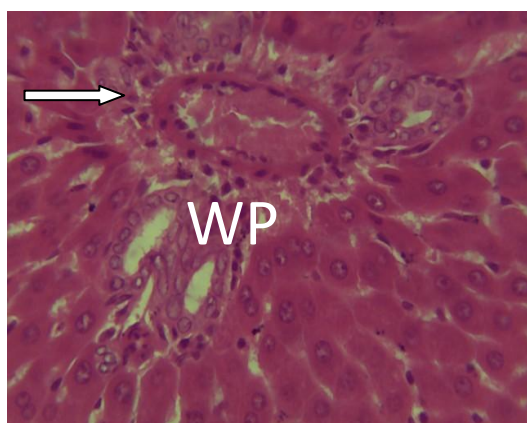


Figure 12. Photomicrograph of liver section from experimental animal group 2 (MQ) alone showing white pulp and the central arteriole. H and E (x 40)

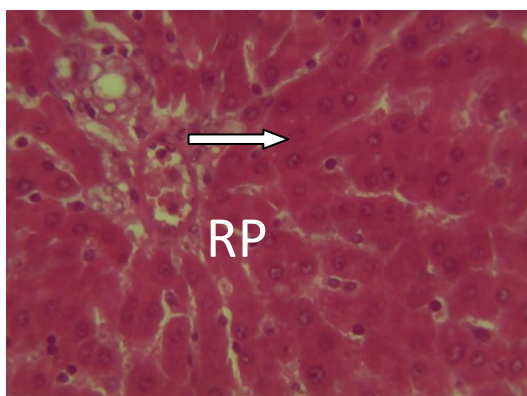


Figure 13. Photomicrograph of liver section from experimental animal group 3 (CPM) alone showing red pulp (RP). H and E(x 40)

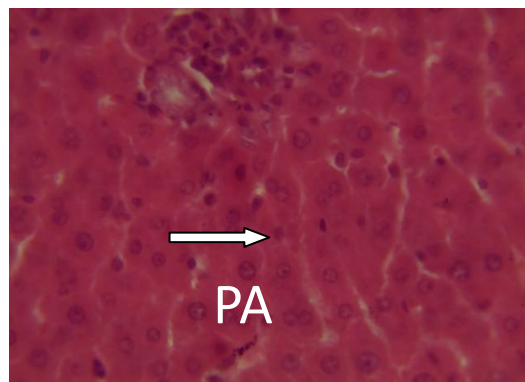


Figure 14. Photomicrographs of liver section from experimental animal groups 4 i. e. (MQ + CPM together) showing red pulp, white pulp and the central arteriole portal arrow (PA) H and E (x 40)

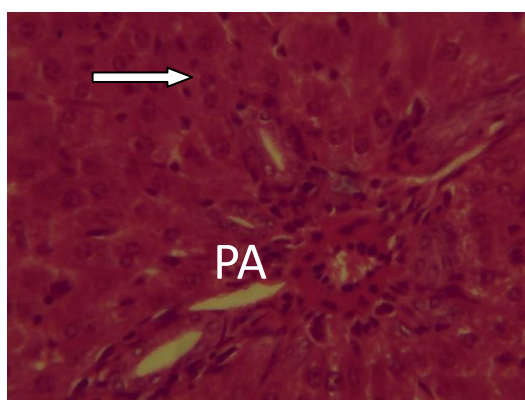


Figure 15. A Photomicrograph of liver section from experimental animal group 7 infected untreated showing portal area damaged (PA) H and E (x 40)

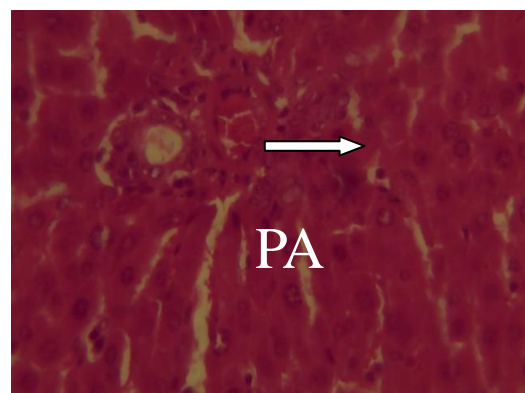


Figure 16. Photomicrographs of liver section from experimental animal groups 6 (MQ + CPM 30mins) showing red pulp, white pulp and the central arteriole portal arrow (PA) H and E (x 40)

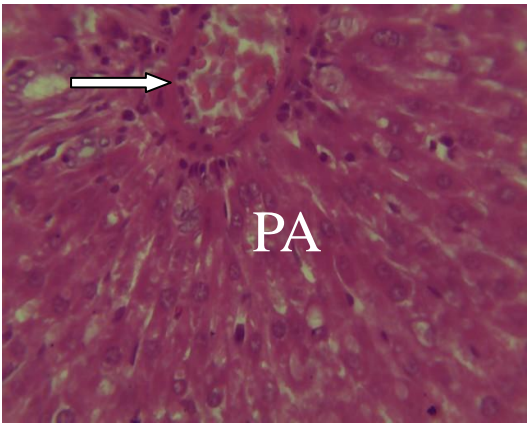


Figure 17. A Photomicrograph of liver section from experimental animal group 7 infected untreated showing portal area damaged (PA) H and E (x 40)

3.8. Pharmacokinetics

The results of the pharmacokinetic profile are shown in Figures 18-23.

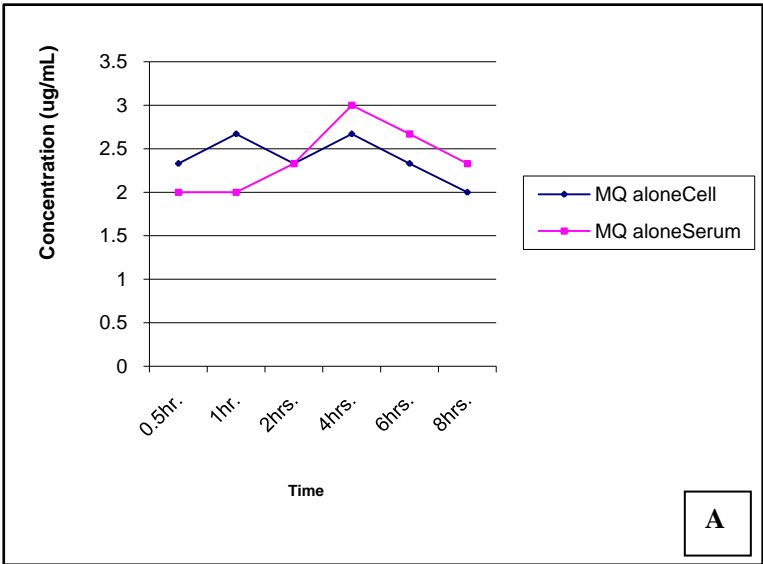


Figure 18. Trends of Mefloquine (MQ) concentrations in cell and serum when administered alone

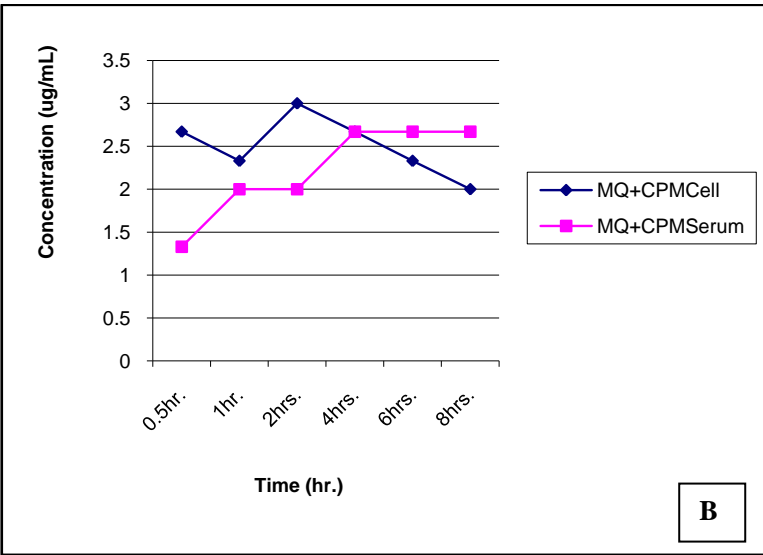


Figure 19. Trends of Mefloquine (MQ) concentrations in cell and serum when administered concomitantly with Chlorpheniramine (CPM)

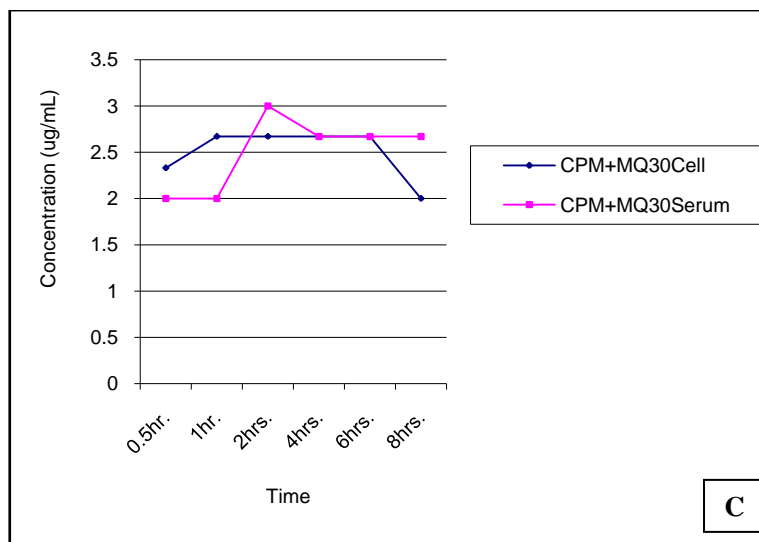


Figure 20. Trends of Mefloquine (MQ) concentrations in cell and serum when administered 30min. after Chlorpheniramine (CPM)

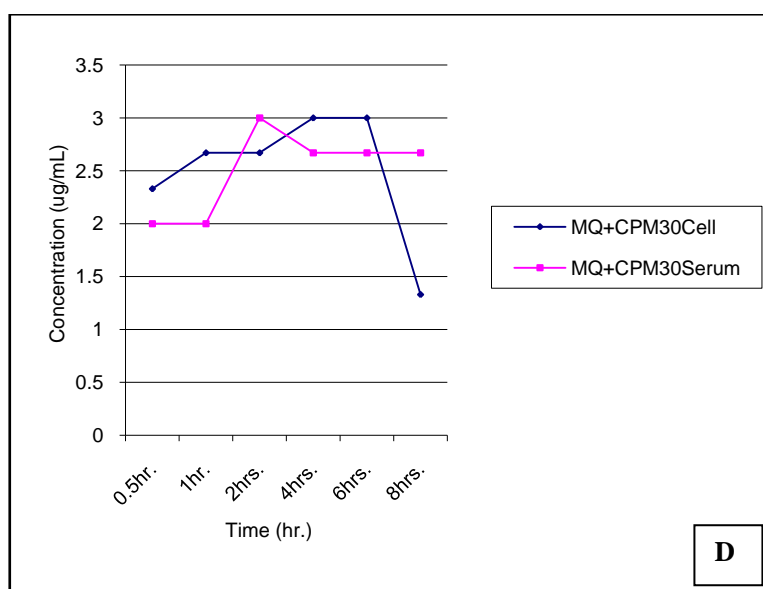


Figure 21. Trends of Mefloquine (MQ) concentrations in cell and serum when administered 30 min. before Chlorpheniramine (CPM)

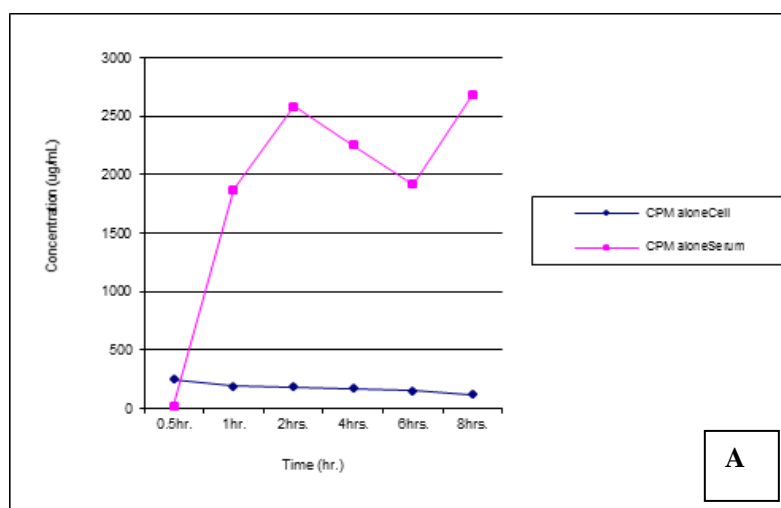


Figure 22. Trends of Chlorpheniramine (CPM) concentrations in cell and serum when administered alone

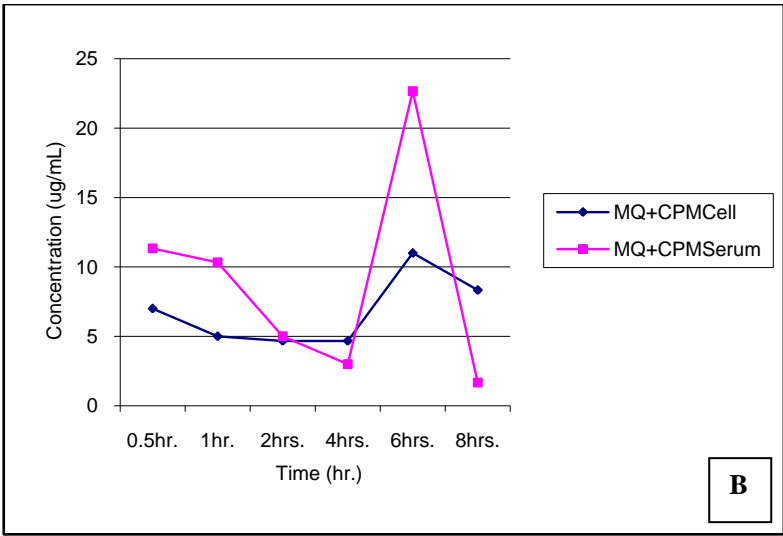


Figure 23. Trends of Chlorpheniramine (CPM) concentrations in cell and serum when administered concomitantly with Mefloquine (MQ)

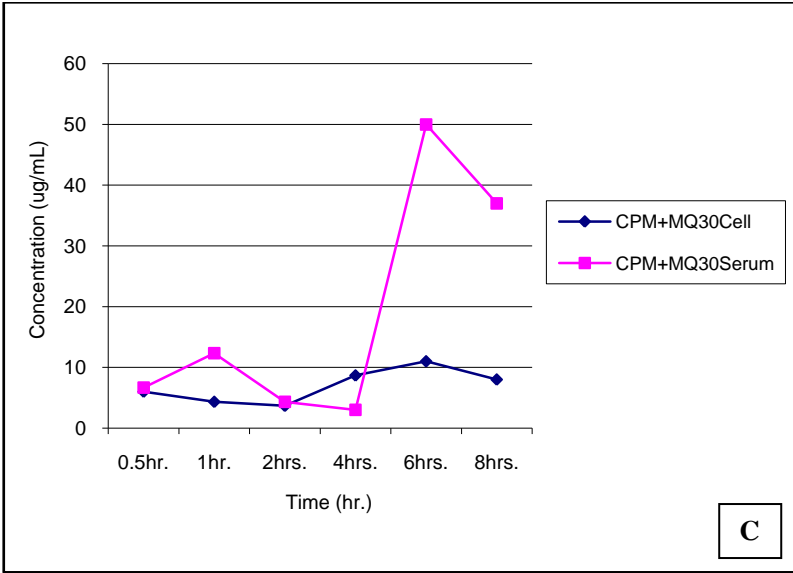


Figure 24. Trends of Chlorpheniramine (CPM) concentrations in cell and serum when administered 30 min. before Mefloquine (MQ)

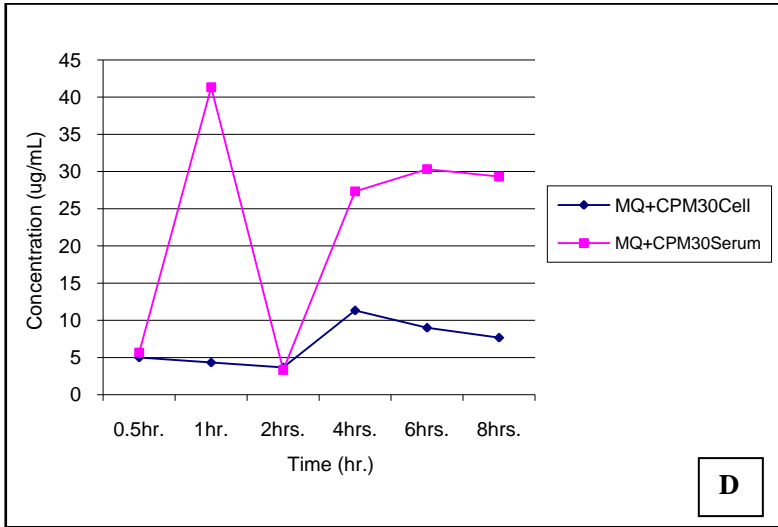


Figure 25. Trends of Chlorpheniramine (CPM) concentrations in cell and serum when administered 30 min. after Mefloquine (MQ)

4. Discussion

4.1. Antiplasmodial Testing

Figure 3 shows the baseline parasitaemia in experimental treatment groups, including the negative control, prior to treatment. The various groups before the commencement of treatment had comparable baseline levels of parasite count ($p > 0.05$) as shown in Figure 3. Figure 4 depicts the overall trend in percent parasite reduction from baseline parasitaemia for the various treatment groups, including the control, from Day 1 to Day 3.

The Dunnett post hoc test on the treatment groups against the negative control group revealed statistically significant reduction in the levels of parasite in the treatment groups from Day 1 to Day 3 ($p < 0.05$). When post hoc Tukey test was conducted on the treatment groups, excluding the negative control group, the results revealed that on Day 1, experimental group to which mefloquine was administered concomitantly with chlorpheniramine demonstrated the most significant parasite reduction from baseline parasitaemia compared with the group to which mefloquine was administered alone ($p=0.000$), CPM administered alone ($p=0.006$) and when mefloquine administered 30 min later than chlorpheniramine ($p=0.001$). But the percent parasite reduction in the group to which chlorpheniramine 30 min later than mefloquine was found to be statistically insignificantly different ($p = 0.804$).

On Day 2, the residual parasite count in the group to which mefloquine and chlorpheniramine were administered concomitantly also demonstrated significantly the highest reduction in parasite levels from baseline parasitaemia compared with the group to which chlorpheniramine was administered alone ($p = 0.033$) and that to which mefloquine was administered 30 min later than chlorpheniramine ($p=0.000$). Compared with the group to which mefloquine was administered alone and that to which chlorpheniramine was administered 30 min later, the percent parasite reductions were insignificantly different ($p > 0.05$). By Day 3, the results revealed that the percent reduction of parasites in all treatment group were averagely 90%, which were statistically ($p > 0.05$) the same when compared.

4.2. Haematology

Merck manual defines haematology as the study of the numbers and morphology of the cellular elements of the blood. The red cells (erythrocytes) white cells (leucocytes) and the platelets (thrombocytes) and the use of these result in the diagnosis and monitoring of disease. Haematological parameters are indicators of the physiological status of animals acting as a pathological reflector of the status of exposed animals to toxicant disease and other conditions [23]. The data on percentage red blood cell, haemoglobin and packed cell volume before treatment shows no significant difference ($p > 0.05$) in all the groups of the animals. This means that all the animals were of the same health status before treatment.

Figure 5 shows that percentage red blood cells were significantly higher in group I (uninfected and untreated) compare to groups (II, III, IV, V, VI and VII). The high RBC in group I is a result of being free from malarial infections or complete absence of malaria parasite. It also shows in Fig 5 that group VII has a significant ($p < 0.05$) decrease in RBC, as a result of malarial parasite. This group was infected but not treated in other words this group is sick when compared to other groups. The observed decrease in RBC may be due to multiple causes of which repeated haemolysis of infected red cells may be one of them.

The haemolysis may be due to non immune destruction of parasitized red cells in case of high parasitaemia or immune mediated destruction of parasitized as well non parasitized red cells because the changes in the red cell antigen structure brought about by the parasitic invasion stimulate the production of antibodies against the red cell. This triggers immune mediated red cell lysis [24].

There was a significant ($p > 0.05$) increase in percentage red blood cell in groups (II, III, IV, V and VI) when compared to group VII (infected and untreated). The reason is that the antimalarial destroyed the parasite from circulation and the cells begin to gradually divided and replenish the blood.

It was also observed that groups (II, IV, V and VI) did not show significant ($p < 0.05$) difference in percentage red blood cell. This means that combination of mefloquine with chlorpheniramine.

Figure 6 shows that the percentage haemoglobin for rats in group VII (infected untreated) were significantly ($p < 0.05$) lower than that for group I (uninfected and untreated). This actually confirms the work of Quintero et al., [25] which states that malaria is associated with severe anemia especially in children and pregnant mothers.

4.3. Packed Cell Volume

Packed cell volume (PCV) is the hematocrit value expressed as the percentage of cellular elements with that of whole blood. This is the value of red cell packed at the bottom of a hematocrit tube when the blood is centrifuged, it is important in determining anemia, extent of dehydration and recovery from dehydration after treatment [27].

This means that the malaria parasite actually attacked the red blood cells and reduced their PCV but on treatment, blood cells regenerate leading to improved % PCV. There was no significant ($p < 0.05$) difference in PCV between groups II, IV, V, and VI treated with mefloquine alone, mefloquine + chlorpheniramine together, chlorpheniramine first + mefloquine 30mins later and mefloquine first + chlorpheniramine 30mins later respectively.

4.4. White Blood Cell

White blood cell (WBC) counts during malaria are generally characterized as being low to normal, a phenomenon that is widely thought to reflect localization of leukocytes away from the peripheral circulation or limb

where blood is usually taken from during the test to the spleen and other marginal pools rather than actual depletion or stasis [28].

Leukocytosis is typically reported in a fraction of cases and may be associated with concurrent infections and or poor prognosis [28].

Figure 8 shows that group VII (infected untreated) has the lowest white blood cell (WBC) count. This is so because some of the WBC must have migrated to the spleen and other organs to fight the infections thereby reducing the quantity of WBC in the limb or periphery where the blood sample is collected for analysis. Group I (uninfected untreated) has a significantly ($p < 0.05$) higher WBC which shows that there are enough WBC in the periphery. There was no significant difference between groups (II, IV, V and VI) because of the treatment given which reduced the parasite load and then encouraged the WBC in the periphery.

4.5. Biochemical Parameters

Alanine aminotransferase (ALT) is normally found in the blood, but when the liver is damaged or diseased, it releases ALT into the blood stream which makes ALT levels go up [29]. The observed increase in ALT in group VII is as a result of liver injury and altered hepatocyte integrity caused by the malaria parasite infection.

Group I (uninfected untreated) has a level of ALT which serves as a control. The level of ALT in groups (II, IV, V, and VI) shows no significance difference ($p < 0.05$) compared to group I. The reason is that the animals were infected but later treated thereby reducing the level of the ALT in their blood stream. This also shows that combination of mefloquine and chlorpheniramine does not induce a change in ALT level and it is safe for the liver.

In group III (chlorpheniramine alone) there was an elevated level of ALT but significantly lower ($p < 0.05$) than group VII (infected untreated). The reason could be that chlorpheniramine alone is not really a good antimalarial because of this it was unable to clear the liver of the disease and normalize the liver.

Aspartate aminotransferase (AST) is found in high concentration in the liver, heart, skeletal muscle and kidney, and in conjunction with ALT to detect liver disorders [30].

Figure 10 shows that group VII (infected untreated) is significantly higher than all other groups. This is as a result of untreated malaria infection which induces injury to the liver thereby causing increase in the level of AST. But groups II, IV, V, and VI treated with mefloquine alone, mefloquine + chlorpheniramine together, chlorpheniramine first + mefloquine 30mins later, mefloquine first + chlorpheniramine 30mins later respectively showed no significant difference ($P > 0.05$) in AST compared to group I (uninfected and untreated) it shows that the combination of mefloquine and chlorpheniramine does not induce further liver enzymes changes.

4.6. Histopathology

It was observed from photomicrograph that the group I

(uninfected and untreated) shows no sign of liver damage because the portal area is intact but when compared to group VII (infected and untreated) which shows clearly portal damage. This is as a result of the malaria parasite infections. Group II (mefloquine alone) shows white pulp and central arteriole and group III (chlorpheniramine alone) shows red pulp. Then group IV, V and VI shows red pulp, white pulp and central arteriole. There is no liver damage in these groups, the liver is intact. This shows that combination of mefloquine + chlorpheniramine has no damaging effect on the liver.

Red pulp is made up of venous sinuses (blood filled cavities) and splenic cords. The function is to filter the blood of antigens, defective or worn out red blood cells while the white pulp is lymphatic tissue (which is part of the immune system) mainly made up of white blood cells [31].

4.7. Pharmacokinetics Studies

All concentrations obtained from HPLC output in milligram (mg) were converted to microgram (μg), checked and entered into SPSS V. 20 spreadsheet for inferential statistics. Independent samples-t-test was conducted to see if the interactions between mefloquine and chlorpheniramine in cell and serum compartments were significantly different at 95% confidence. Four scenarios were considered for both mefloquine and chlorpheniramine: Administered alone, concomitantly; 30min before and 30min after.

4.7.1. Mefloquine (MQ)

At 30 mins post administration, the mean concentrations of mefloquine administered alone in cell and serum compartments were insignificantly different ($p = 0.374$). When administered concomitantly with chlorpheniramine, cell and serum concentrations were also found to be statistically insignificantly ($p = 0.148$) different even though there was an observable slight increase in the cell. When mefloquine was administered 30min succeeding chlorpheniramine, its concentration in the cell was slightly above that found in the serum but these concentrations were found to be statistically insignificantly different ($p = 0.374$). In the fourth scenario, mefloquine was administered 30min preceding chlorpheniramine and its concentration in the serum was insignificantly slightly above that in the cell ($p = 0.374$). At 1,2,4,6 and 8 hours post administration, mefloquine concentrations in the cell and serum compartments were found to be comparable ($p > 0.05$) (Figs 18-21). From the foregoing, chlorpheniramine interaction with mefloquine did not significantly affect mefloquine concentrations in cell and serum.

4.7.2. Chlorpheniramine (CPM)

Chlorpheniramine concentrations in cell and serum were significantly ($p < 0.05$) reduced in the presence of mefloquine 30min post administration, a higher concentration was found in cell when administered alone ($p = 0.001$). After 1,2,4,6 and 8 hours post administration, chlorpheniramine concentrations became significantly ($p <$

0.001) higher in serum than in cell (Figures 21 – 24).

Malaria is an acute and chronic mosquito-borne disease of man characterized by chills, fever, anaemia, splenomegaly and damage to other organs such as liver and brain. It is an ancient disease and it is still leading Africa's health problem. The emergence of multidrug resistant strain of *P. falciparum* has made the treatment of malaria difficult. *P. falciparum* is resistant to chloroquine, mefloquine, amodiaquine leading to introduction of artemisinin based combination therapy (ACT).

According to WHO report in 2015, artemisinin combination therapy resistance was observed mainly in Cambodia, attributing to the resistance of the malaria parasite to the partner drugs like piperazine and mefloquine leading to the failure of artemisinin. Mefloquine is a drug of choice in the treatment of malaria because of its long half life leading to short duration of the therapy. But increasing resistance to mefloquine has led to suspension of mefloquine as a drug of choice. Combination of chlorpheniramine with mefloquine has showed an improvement in the activity of mefloquine in treating chloroquine resistant *Plasmodium falciparum* malaria. This is believed to be as a result of chlorpheniramine reversing or inhibiting the efflux system in the plasmodium parasite. This work also confirms the work by Osonwa *et al.*, [32] 2015 in which they observed the increase in efficacy of chloroquine when combined with chlorpheniramine. The efficacy of mefloquine and chlorpheniramine combination was compared with that of mefloquine alone. The time of administration of the mefloquine like; mefloquine + chlorpheniramine together, chlorpheniramine given first + mefloquine 30mins later and mefloquine given first + chlorpheniramine 30mins later was also considered to ascertain the best time for the combination that will give the best result when compared to mefloquine alone. The effect of the combination on haematological, biochemical and histopathological changes in the liver was assessed in treated and untreated rats. The pharmacokinetics studies was also carried out to ascertain the bioavailability of the various combination in comparison to mefloquine alone.

5. Conclusions

The results showed that chlorpheniramine enhances the antiplasmodial activity of mefloquine. Chlorpheniramine is safe and affordable, combining chlorpheniramine with mefloquine will give an affordable and highly effective antimalarial combination.

6. Recommendations

More work needs to be done on this topic especially on the effect of the combination in a transgenic rat infected with *P. falciparum*. This will be followed by the formulation of the drug in a fixed dose form and also carry out the stability studies. The *in vitro* and *in vivo* studies of the formulated

drug should also be carried out to determine the efficacy, toxicity and pharmacokinetics of the formulation.

Abbreviations as Used in the Text

MQ: Mefloquine; CPM: Chlorpheniramine maleate; RBC: Red Blood Cells; PCV: Packed Cell Volume; ALT: Alanine aminotransferase; CDC: Centre for Disease Control; WRAIR: Walter Reed Army Institute of Research; PRBC: Parasitized Red Blood Cells; EDTA: Ethylene diamine tetraacetic acid; WBC: White Blood Cells; AST: Aspartate aminotransferase; USP: United States Pharmacopoeia.

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