ANTI-PLASMODIAL ACTIVITY OF ASTEMIZOLE-METHYLENE BLUE COMBINATION THERAPY AGAINST CHLOROQUINE SENSITIVE AND RESISTANT *PLASMODIUM FALCIPARUM* AND THEIR SAFETY IN BALB/C MICE

JOYCE NYIRONGO (B.Sc.)

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF MASTER OF SCIENCE (APPLIED PARASITOLOGY) IN THE SCHOOL OF PURE AND APPLIED SCIENCES OF KENYATTA UNIVERSITY

JANUARY, 2020

DECLARATION

I hereby declare that this thesis is my original work and has not been presented for a degree or other awards in other University.

Signature: _____ Date: _____

Joyce Nyirongo

I56F/33764/2015

Department of Zoological Sciences

Supervisors' Approval

We confirm that the work reported in this thesis was carried out by the candidate under our supervision.

Dr. Jemimah Simbauni

School of Pure and Applied Sciences

Department of Zoological Sciences

Kenyatta University

Signature: _____ Date: _____

Dr. Lucy Kamau

School of Agriculture and Enterprise Development

Department of Animal Sciences

Kenyatta University

Signature: _____ Date: _____

Dr. Faith Onditi

Department of Tropical and Infectious Diseases

Institute of Primate Research

Signature: _____ Date: _____

DEDICATION

I dedicate this thesis to my parents Dr. Neil Douglas Nyirongo and Mrs. Irene Taona Nyirongo and my siblings.

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I would like to thank God for being with me throughout this journey. Indeed, I saw the work of His hands as He moved things and repositioned them in my favor. I cannot thank my parents enough for their sacrifice to see me through my studies. Their love and kindness will forever remain steadfast in my heart.

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ABBREVIATIONS AND ACRONYMS

µg/ml	Microgram per milllitre
µl	Microliter
nM	Nano molar
AAALAC	Association of assessment and accreditation of laboratory animal care
ACT	Artemisinin-based Combination Therapy
AS01	Adjuvant system
AST	Astemizole
AST-MB	Astemizole-methylene blue
AS	Artesunate
AQ	Amodiaquine
AQ-AS	Amodiaquine-artesunate
CDC	Center for disease control
DNA	Deoxyribonucleic acid
Eag1 K+ channel	Ether-a go-go-1 potassium ion channel
ECG	Electrocardiogram
EDTA	Ethylenediaminetetraacetic acid
FIC	Fractional Inhibitory Concentration
g/dL	Grams per decilitre
HEK	Human Embryonic Kidney cells
HERG	Human Ether-a-go-go-Related Gene

HTMV	High Titer Measles Vaccine
IC_{50}	Inhibitory Concentration 50
ISERC	Institutional scientific ethical review committee
IRS	Insecticide Residual Spray
ITNs	Insecticide Treated Nets
IPR	Institute of Primate Research
IPTp-SP	Intermittent Preventive Treatment with Sulfadoxine-pyrimethamine
mg/ml	Milligram per milliliter
MB	Methylene Blue
MB-AS	Methylene-blue- artesunate
MB-SQ	Methylene blue-amodiaquine
MCH	Mean Corpuscular Hemoglobin
NACOSTI	National Commission for Science, Technology and Innovation
$NaHCO_3$	Sodium hydrogen carbonate
PCV	Packed cell volume
pg	Picograms
rpm	Revolutions per minute
RPMI 1640	Rosewell Park Memorial Institute 1640
RTS,S	R-central repeat region of the *Plasmodium falciparum* circumsporozoite, T- cell epitopes of circumsporozoite, S-Hepatitis B surface antigen, S- surface antigen proteins of yeast cells

SSA	Sub-Saharan Africa
SP	Sulfadoxine-pyrimethamine
TID	Tropical Infectious Diseases
U/L	Units per litre
WHO	World Health Organization

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ABSTRACT

Plasmodium is protozoa from the *Apicomplexa* phylum which causes malaria. In the tropics and sub-tropics, approximately 3.3 billion people are at risk. Artemisinin Combination Therapy (ACT), the current prime treatment, has been reported to have a possible emergence of resistance. This is a major obstacle that contributes to high mortality. Drug repurposing offers an appealing alternative to *de novo* drug development. Although astemizole and methylene blue have been reported to have anti-malarial properties, their safety when used in combination has not been explored. This study aimed at evaluating the optimum growth of *Plasmodium falciparum 3D7* and *W2*, efficacy of astemizole-methylene blue combination therapy these strains and the safety of the combination therapy.To establish this, the growth potential of *Plasmodium falciparum 3D7* and *W2* was assessed by maintaining a continuous culture. Afterwhich, eight concentrations of astemizole-methyelene (1:1,3:1 and 3:1) using drug concentrations of range 7.81 µg/ml to 1 mg/ml, were assessed in triplicate against the *Plasmodium* strains. The parasites were cultured in complete media containing human erythrocytes in 96 well plates at 36.8^0C. Parasitemia was determined by microscopy and non-linear regression was used to determine the interactions of the drugs. Combinations that had high efficacy AST-MB 3:1 and 1:3 were administered in Balb/c mice (N=25) intraperitoneally. Clinical symptoms, hematology, biochemistry, and gross pathology were assessed and results presented as mean ± standard error of mean. ANOVA was used to analyze the results. Differences were considered significant if *P* values were less than 0.05*($p< 0.05$), F* values were more than 2.78. Astemizole-methylene blue 3:1 (31.25 µg/ml and IC_{50} of 22.28±0.24 µg/ml) was the most efficacious drug combinations against *P. falciparum 3D7* (F= 8.439, p=0.017). Whereas astemizole-methylene blue 1:3 (31.25 µg/ml and IC_{50} of 15.07±0.60 µg/ml was the most efficacious drug combinations against *P. falciparum W2* (F= 5.428, p=0.0035). In spite of this, all astemizole-methylene blue drug combinations showed antagonism (FIC>2). Also, astemizole was found to be less efficacious against both parasite strains in comparison to methylene blue. Frome the toxicity study, astemizole-methylene blue 3:1 drug combinations was associated with lower weight of the heart (F= 8.967, df=4, p=0.007) and liver (F= 4.339, p=0.0001) compared to the negative controls. This indicates abnormalities in these organs. Astemizole-methylene blue 3:1 reduced the platelet levels to undetectable amounts (F= 27.40, df=4, p=0.005). Both *Plasmodium falciparum 3D7* and *W2* had good and similar growth potentials, astemizole-methylene blue combinations were efficacious against both parasite strains and astemizole-methyelene blue 3:1 adversely affected Balb/c mice. This study recommends evaluating methylene blue and astemizole combinations with lower concentrations of astemizole to counter the effects.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Malaria is a disease caused by protozoan parasites of the genus *Plasmodium* that continues to claim many lives. As of year 2017, 219 million cases of malaria were recorded globally (WHO, 2018). At present, chemotherapy is the major approach for both malaria prevention and remedy (Agnandji *et al*., 2011). However, some patients living in endemic areas continue to harbor high parasitemia levels even after being treated for the disease (Schneider *et al.,* 2008 ; Kakolwa *et al.,* 2018).

Owing to widespread anti-malarial drug resistance, monotherapies have proven to be less effective (Matthews *et al.,* 2013). *Plasmodium* resistance to chloroquine had a negative impact on malaria treatment (Wellems, 2001; Chinappi *et al.,* 2010). This prompted the search for new therapies. As such, the use of combination therapies with artemisinin was aimed at combating parasite resistance against monotherapies that had been reported in many laboratory settings (WHO, 2010). In view of this, the traditional methods of drug development are not only time-consuming but also costly. Furthermore, 90% of drug trials fail at the developmental stage (Mathews *et al.,* 2013). Therefore drug repurposing offers a faster approach to drug development.

Astemizole (AST) with the trade names, Histmanol, Cilergil, and Almizol, is an artificial piperidinyl-benzimidazol derivative which has anti-allergic properties. Initially, it was used to treat seasonal allergic rhinitis in humans (Karapetyan *et al.,* 2013). It has been used

to treat several diseases including cancer and malaria. In Breast cancer, Eag1 K^+ channels are important for proliferation. Astemizole inhibits Eag1 currents by selectively binding to open channels. It thus reduces proliferation (García-Quiroz *et al.,* 2012). Astemizole was identified as a prospective antimalarial during a drug screening process. At sub-micromolar levels, it inhibits the multiplication of *Plasmodium falciparum* with different sensitivities to chloroquine (Chong *et al.,* 2006 ; Kumar *et al.,* 2018).

Methylene blue (MB) was first synthesized in 1876 as an aniline-based dye for the textile industry. It did not perform well in the textile industry. Hence, its use was ceased. In a quest to find ways of staining cells, methylene blue was used and found to be efficient. Methylene blue was the first synthetic compound that was ever used as an antiseptic in medical settings (Oz *et al.,* 2011). It has been used to treat several conditions and diseases such as septic shock, hepatopulmonary syndrome, malaria and methemoglobinemia (a complication of anemia caused by malaria) (Ginimuge and Jyothi, 2010). In 1891, it was adopted as the first synthetic anti-malarial (Suwanarusk *et al*., 2015). Therefore, this made it the first synthetic remedy that was used in humans to treat malaria (Lu *et al.,* 2018). However, its use as an anti-malarial drug was discontinued when chloroquine was introduced into the market (Lu *et al.,* 2018). This was because chloroquine served as a better remedy against malaria.

1.2 Statement of the Problem

Malaria is a public health problem with a high burden in sub-Saharan Africa. In 2017, 219 million cases and 435,000 deaths worldwide, 93% of these were in Africa (WHO, 2018). The emergence of *Plasmodium* resistance to artemisinin-based combination therapy

(ACT), and the current drug of choice against malaria, is worrisome and poses to threaten all efforts to control malaria. The parasite resistance to drugs results from their resistant genetic profiles and occurrence of mutations, interfere with efforts to reduce mortality (Mwangi *et al.,* 2016). For example, in Cambodia Asia, resistance to ACT was reported in 2006 and in Africa, cases of delayed clearance of the parasite after the administration of ACT, have also been reported (WHO, 2017). Similarly, the emergence of resistance against other anti-malarial drugs has been reported (Ramakrishnan *et al.,* 2017). To date, there is currently no licensed and available vaccine against malaria (WHO, 2018). Therefore there is an urgent need to come up with better drug interventions as soon as possible. Drug repurposing offers this alternative by reducing the time and resources employed in coming up with drug interventions.

1.3 Justification of the Study

Drug repurposing provides a cheaper and faster approach in treating and preventing malaria compared to *de novo* drug development. For example, methylene blue has been proven to be safe and efficacious against malaria (Bountogo *et al.,* 2010). Astemizole was reported to be potent against *P. falciparum* (Kumar *et al.,* 2018). Astemizole and methylene blue have mechanisms of action that are similar to chloroquine, the anti-malarial drug that took decades before resistance was documented (Chinappi *et al.,* 2010). However, investigations have not been done to determine the outcome of the combination of AST and MB against malaria parasites. Combining drugs reduces chances of the parasites evading drug effects. Information regarding their formulations and mechanisms of action are available. This, therefore, made it easier to investigate these drug candidates. In view

of this, an *in vitro* study was done to assess the efficacy of the drugs when used in combination againt *Plasmodium falciparum W2* (chloroquine-resistant) and *P. falciparum 3D7* (chloroquine-sensitive) strains.

The drugs were assessed against *Plasmodium falciparum W2* (chloroquine-resistant) and *P. falciparum 3D7* (chloroquine-sensitive) strains owing the the fact that astemizole and methylene blue have similar modes of action to chloroquine. After assessing the growth potential of the parasites, the efficacy and interactions of the drug combinations were assessed *in vitro* and subsequently safety was evaluated in Balb/c mice with the aim of characterizing the possible side effects of the drug combinations. Lorke's toxicity test was used because it uses fewer animals, it is simple and requires less time while Balb/c mice were used as they are highly susceptible to malaria parasites (Li *et al.*, 2017). Overall, this study provided information regarding the growth potential of *Plasmodium falciparum* chloroquine-sensitive *3D7* and chloroquine-resistant *W2* strains, the efficacy of AST-MB combination therapy against these strains and the drug combination safety *in vivo*.

1.4 Research Questions

(i) What is the growth potential of chloroquine-sensitive *3D7* in comparison to chloroquine-resistant W2 *P. falciparum* strains *in vitro*?

(ii) What is the anti-plasmodial efficacy of astemizole-methylene blue combination against chloroquine-sensitive *3D7* and chloroquine-resistant *W2 P. falciparum* strains *in vitro*?

(iii) What is the toxicity of astemizole-methylene blue combination *in vivo* in Balb/c

mice?

1.5 Null Hypotheses

(i) The growth potential of chloroquine-sensitive *3D7* is not similar to that of chloroquine-resistant W2 *P. falciparum* strains.

(ii) Astemizole and methylene blue combination therapy is not efficacious against the chloroquine-sensitive *3D7* and chloroquine-resistant W2 *P. falciparum* strains *in vitro*.

(iii) Astemizole and methylene blue combination therapy is not toxic *in vivo* in Balb/c mice.

1.6 Objectives of the Study

1.6.1 General Objective

To investigate the anti-plasmodial activity of astemizole and methylene blue combination therapy against *P. falciparum* strains and its toxicity *in vivo*.

1.6.2 Specific Objectives

(i) To determine the growth potential of chloroquine-sensitive *3D7* and chloroquine-resistant *W2 P. falciparum* strains *in vitro.*

(ii) To determine the anti-plasmodial efficacy of astemizole, methylene blue and their combination therapy against chloroquine-sensitive *3D7* and chloroquine-resistant W2 *P. falciparum* strains *in vitro*.

(iii) To evaluate the toxicity of astemizole and methylene blue combination therapy *in vivo* in Balb/c mice.

1.7 Significance of the Study

This study sought to provide knowledge on the growth potential of *Plasmodium falciparum 3D7* and *W2* strains in drug susceptibility assays. Both strains were found to have high growth potentials and therefore ideal for use in drug studies as the parasites growth limits the period for which the drug assays can be conducted. It also provided vital information on the safety and efficacy of the drug combination therapy and insight into phenothiazine-antihistamine interaction. Additionally, it provided information on the antagonistic interaction of astemizole-methylene blue combination against *falciparum* parasites. The toxicity assessment of the drugs provided information on the effects of the drugs on Balb/c mice; their behavior, biochemical and hematological profiles and organ changes. Drug repurposing approach employed in this study, is cheaper and faster as compared to *de novo* drug development. This study provided vital information on astemizole and methylene blue combination therapy against *Plasmodium falciparum* parasites which can form baseline information for their use in drug repurposing for malaria.

1.8 Limitations of the Study

Stool samples from Balb/c mice treated with methylene blue and drug combinations were not analyzed to determine the effect of the drugs on the digestive system, although the colour blue was consistent in the stools.

CHAPTER TWO

LITERATURE REVIEW

2.1 Overview of malaria parasites and treatment

Malaria is an infectious disease caused by *Plasmodium* parasites which are spread when an infected female *Anopheles* mosquito takes a blood meal (WHO, 2018**)**. There are many species of *Plasmodium* but only five infect and cause malaria in human beings. These species are found in specific areas. They include: *P. falciparum* found in the tropical and sub-tropical areas, *P. vivax* found in Asia and Latin America, *P. ovale* found in Africa and the Pacific islands, *P. malariae* that is globally distributed and *P. knowlesi* found throughout Southeast Asia (Autino *et al.*, 2012). This wide distribution of species poses challenges in disease control. Although chemotherapy remains the major strategy for malaria control (Tcherniuk *et al.,* 2015). Artemisinin and its derivatives are the current drugs for treatment against malaria. Administering drugs in monotherapies increases the risk of early drug resistance. Therefore, drug combination therapy was introduced to reduce the chances of drug resistance. Artemisinin combination therapy (ACT) was adopted to prevent the parasite resistance to artemisinin (Tse *et al.,* 2019). However, an account of the possible emergence of resistance to ACT was given from Southeast Asia (Dondorp *et al*., 2010).

2.2 Life cycle of *Plasmodium*

The life cycle of *Plasmodium* starts when an infected female *Anopheles* mosquito takes a blood meal (Fig 2.1). In this process, sporozoites are inoculated into the human host. With the aid of the blood stream, the sporozoites move to the liver and mature into schizonts

which upon rupturing, release merozoites into the blood. Trophozoites are formed and eventually mature into schizonts. Schizonts rupture and release merozoites. Differentiation of some parasites into gametocytes occurs. These are ingested by the mosquito when it bites an infected human host. Fertilization happens in the mosquito's gut and zygotes are formed. The zygotes then become motile and elongated ookinetes. The ookinetes develop into oocytes in the mid gut. The oocytes rupture releasing sporozoites which make their way to the salivary glands and the cycle begins again (CDC, 2017).

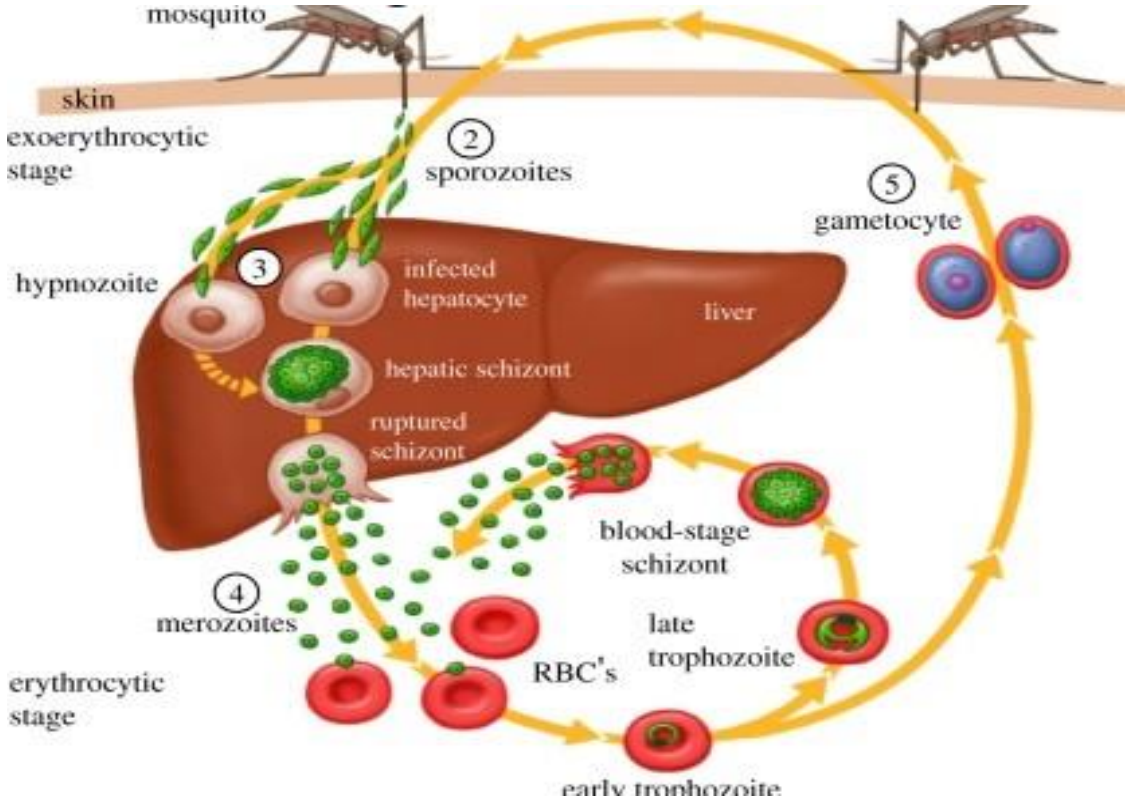


Figure 2.1: Life cycle of *Plasmodium* parasites (Hill, 2011)

2.3 Current efforts to control malaria

A number of approaches have been taken to control malaria, a disease which poses as one

of the major health threats in sub-Saharan Africa (SSA). These include vector control and chemotherapy (WHO, 2018).

2.3.1 Vector control

Vector control comprises of all the methods that are put in place to prevent the transmission of the *Plasmodium* parasites from the vector (mosquitoes) to the hosts which include humans (SmithGueye *et al.,* 2016). Indoor Residual Spraying (IRS) and Insecticide Treated bed Nets (ITNs) are the most commonly used methods to control disease transmission from vectors. However, between 2000 to 2015, *Anopheles* resistance to the known insecticides used in these methods has been reported in many of the malaria-endemic countries including Burkina Fuso and Ghana (Sougoufara *et al.,* 2017). Long-lasting insecticide treated nets have been distributed in many countries in SSA. The insecticide in these bed nets lasts up to a period of 3 years. Targeted parties were young children and pregnant women (Ranasinghe *et al*., 2015). Countries that have benefited from this include Rwanda and Kenya. From the 663 million malaria cases that have been reported to have reduced in SSA over the last 15 years, 73% is owed to the distribution of the bed nets (Tizifa *et al*., 2018).

However, it was noted that effective distribution did not necessarily mean that the recipients of the bed nets used them, approximately 47% of individuals did not sleep under a treated net in 2016 (Tizifa *et al*., 2018). Furthermore, the resistance to pyrethroids, the insecticide that is used to treat bed nets, retrograded the efforts in prevention through treated bed nets usage (Sougoufara *et al.,* 2017).

Indoor residual spraying (IRS) is used in conjunction with ITNs. A study conducted in Bioko Island showed that IRS was effective if there was a community coverage of up to 80%, coverage of less than 20% did not show much impact of the IRS (Larsen *et al*, 2017). It was shown that children living in areas which were sprayed with IRS had lower anemia and parasite loads as compared to those who lived in areas which were not sprayed (Steinhardt *et al*., 2013). Initially, IRS was recommended only for use in areas with low transmission rates (Fullman *et al.,* 2013). However, the use of IRS has been shown to be equally as effective as in endemic areas (Steinhardt *et al*., 2013).

2.3.2 Malaria vaccines and chemotherapy

At present, there is only one *P. falciparum* malaria vaccine candidate, Mosquirix (RTS, S/AS01). The RTS,S vaccine targets the circumsporozoite protein of *P. falciparum* and is given with an adjuvant system AS01 (Agnandji *et al.,* 2011). The effectiveness of RTS, S/AS01 malaria vaccine has been assessed in young children in clinical trials. In phase II clinical trials, upper respiratory infections and diaper rushes were frequently noted in vaccinated children. However, none of these occurred in subsequent phase III trials (Asante *et al.,* 2016)

Overall, the vaccine was reported to be safe and efficacious against malaria (18-36%) (Asante *et al.,* 2016). However, WHO reported its inability to reduce mortality in children of all age groups (Klein *et al.,* 2016). For example, the vaccine efficacy was lower in children between 6 to 12 weeks in both clinical malaria cases (23-30%) and in severe malaria cases (15-38%) (Mahmoudi and Keshavarz, 2017). Furthermore, the risk of

acquiring a malaria infection after being vaccinated was higher in female children as compared to male children. It was speculated that mortality is usually higher in female children who receive vaccines. An example of this was when the High-Titer Measles Vaccine (HTMV) was administered to children in the 1890s (Klein *et al.*, 2016). As the vaccine candidate, Mosquirix, is still undergoing trials, chemotherapy remains the main therapy against malaria.

The main drug of choice against malaria is Artemisinin combination therapy (ACT) (WHO, 2018). Artemisinin derivatives that are used in the combination therapies are artesunate, artemether and dihydroartemisinin (Okebe *et al.*, 2014). ACT acts against rings, trophozoites, schizonts and immature gametocyte stages of *Plasmodium*. When these parasite stages digest hemoglobin, the iron released causes the breakdown of the endoperoxide moiety of artemisinin. This then results in the formation of reactive oxygen species that aim for the nucleophilic groups in parasite proteins and lipids. This then kills the parasites (Fairhurst and Dondorp, 2016).

Artemisin combination therapy (ACT) drug resistance was first noted in Cambodia in 2009. The parasite clearance rate decreased by 100-fold. Not long after, delayed clearance of parasites was also noted in countries such as Thailand, Vietnam, and China in Southeast Asia (Fairhurst and Dondorp, 2016). Also, in sub-Saharan Africa, delayed clearance of parasites after taking ACT has also be noted, this could be due to the emergence of the mutant gene that was found in parasites in Southeast Asia (Slater *et al.*, 2016).

Other anti-malarial drugs have been used to treat malaria. An example of this is

chloroquine, a 4-aminoquinoline compound (Shujatullah *et al.*, 2012). It acts against the trophozoite stage of the *Plasmodium* parasite by preventing the detoxification hematin, a by-product of the digestion of hemoglobin. In a normal scenario, hematin is polymerized to hemozoin, which does not intoxicate the parasite. Chloroquine prevents this process so leading to the intoxication of the parasites (Parhizgar and Tahghighi, 2017).

Following drug resistance, chloroquine is no longer used as a first line anti-malarial drug. Malaŵi was the first African nation to cease the use of chloroquine as the first line drug due to drug resistance. Malaŵi adopted sulfadoxine-pyrimethamine (SP) as the first line drug in 1996 (Takala-Harrison and Laufer, 2015). Not very long after in 1996, drug resistance was reported in Ethiopia and the nation followed suit and replaced chloroquine with sulfadoxine-pyrimethamine as the first line therapy (Mekonnen *et al*., 2014).

At present, sulfadoxine-pyrimethamine is mostly used as a preventive treatment in pregnant woman against malaria. In a study conducted in Ghana, only 15% of pregnant women who used IPTp-SP (Intermittent Preventive Treatment with sulfadoxine-pyrimethamine) tested positive for malaria. Those who did not use IPTp-SP (45%) tested positive for malaria. However, drug resistance against sulfadoxine-pyrimethamine has been observed (Ceesay *et al.*, 2011). Regardless, IPTp-SP still remains to be effective in combating malaria in pregnant women (Nwaefuna *et al.*, 2015).

2.4 Drug repurposing

Drug repurposing, otherwise known as drug repositioning or re-profiling, refers to the use

of known drugs to treat diseases for which they were not primarily intended (Amantea *et al.,* 2015). Ordinarily, the *de novo* approach to drug development is costly and consumes too much time and there is a high chance of drug failure. Furthermore, the process from malaria drug discovery to producing a clinical candidate drug takes longer than 5 years and the lack of adequate funding tends to further prolong this process (Lotharius *et al*., 2014). On the contrary, drug repurposing reduces the time spent on developing new drugs, decreases the costs involved and the efforts required (Corsello *et al*., 2017). Moreover, the drugs that are used in drug repurposing, have already been approved. Hence, information on their pharmacology, formulations, and toxicity is readily available. Therefore, the new candidate drugs could quickly be ready for clinical trials and incorporated into health care (Amantea *et al.*,2015).

Studies have indicated the potency of broad-spectrum antibiotics against malaria. For example, doxycycline, a synthetically derived tetracycline, has been employed in treating malaria. Doxycycline's clinical usage was approved in 1967 as an anti-biotic. However, it is partially efficacious against *Plasmodium* parasites in the hepatic stage of the life cycle and has schizonticidal activities. In spite of this, its efficiency is increased when used in combination with other known anti-malarial drugs such as quinine (Tan *et al.,* 2011).

Also, dapsone, a sulfone antibiotic, that was initially used to treat leprosy and *dermatitis herpetiformis*, a chronic skin inflammation and it has anti-malarial activity (Wozel and Blasum, 2014). Under its marketed name, LapDap™, it was used in combination with chlorproguanil to treat malaria (Winstanley, 2001; Dunyo *et al.,* 2011). Overall, the

combination was efficacious. In spite of this, dapsone was reported to induce hemolytic anemia, a side effect, experienced in 20% of the treated patients. Consequently, it was retracted from the market in 2008 (Andrews *et al.,* 2014).

2.5 Astemizole as an anti-malarial drug

Astemizole (Fig 2.2), is a non-sedating subsequent generation antihistamine with a biological half-life of 24 hours which acts by competing with histamine at the H_1-receptor sites in the uterus, gastrointestinal tract, and bronchial muscles. In high doses, astemizole causes cardiac arrhythmia. Thus, it was withdrawn from the United States and Canadian markets by its manufacturing company in 1999 (Lyu *et al.,* 2018). However, it is currently sold as a non-proprietary drug in over 30 countries including, Cambodia, where malaria prevalence is high (Chong *et al.,* 2006).

It has been observed that during the intra-erythrocytic stage, *P. falciparum* crystallizes heme released from the disintegration of hemoglobin within the food vacuole to enhance their survival. Astemizole inhibits this process of crystallization (Kumar *et al.,* 2018). In a study involving a four-day suppression test, mice were infected with a chloroquine sensitive *P. vinckei* and then treated with desmethylastemizole which was administered intraperitoneally. Mice that were treated with astemizole and desmethylastemizole had an 80% and 81% reduction in parasitemia respectively. However, recrudescence occurred after day 4 post-treatment. The administering of astemizole for 4 days cured the infections. This demonstrated that astemizole is potent against malaria parasites and has the potential to be used in drug repurposing as an anti-malarial drug (Nzila *et al.,* 2011).

However, astemizole interferes with the human heart by causing long QT syndrome (a disorder of the heart rhythm). Q and T are amongst five of the distinct electrical waves that are mapped by ECG (electrocardiogram). The Q represents the depolarization of the ventricles and the T symbolizes the repolarization of the ventricles. Normally, the QT interval is a third of each heartbeat. In the long QT syndrome, the interval lasts longer, causing the abnormal beating of the heart which could result in sudden death (Wiśniowska *et al.,* 2016).

In an experiment to determine the electro-physiological effect of astemizole metabolites (desmethylastemizole and norastemizole), human ether-a-go-go-related gene (HERG) channels were expressed in embryonic kidney cell lines (HEK 293) and then studied using the patch clamp technique. It was reported that both astemizole and desmethylastemizole block the HERG current at similar concentrations. However, norastemizole caused incomplete blocking of HERG channels and a high drug concentration of 27 nM was required (Zhou *et al.,* 1999). The desmethylastemizole concentration in serum surpassed that of astemizole by 30-fold. This led to the conclusion that desmethylastemizole is the main culprit of long QT syndrome (Zhou *et al.,* 1999). However, in a survey conducted in 17 countries over a decade, results showed only one case of cardiac arrhythmia out of 8 million doses of astemizole (Chong *et al.,* 2006).

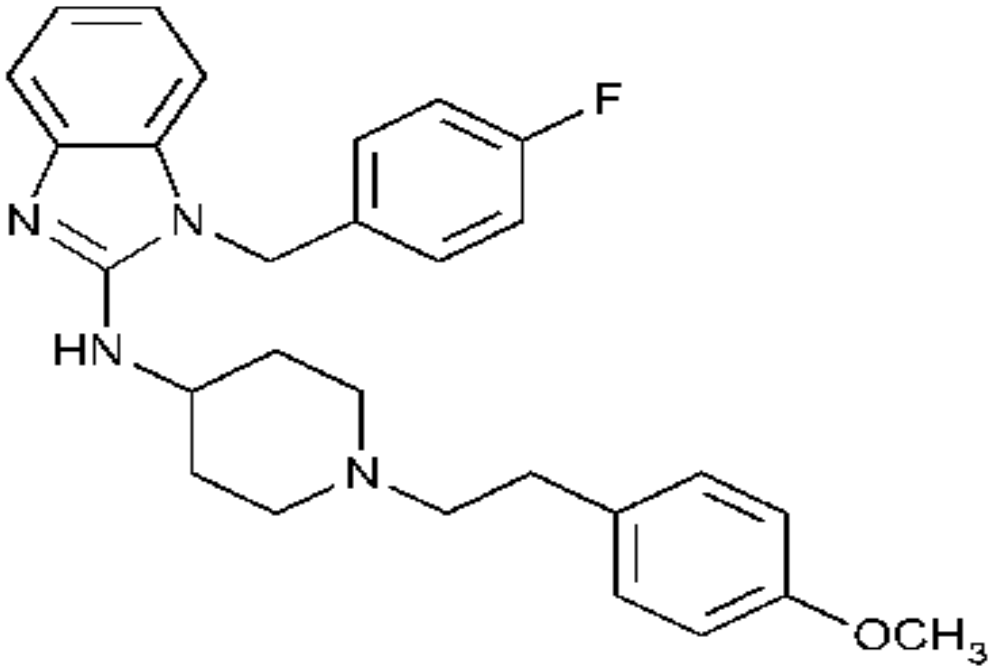


Figure 2.2: Chemical structure of astemizole (Chong *et. al,* 2006)

2.6 Methylene blue as an anti-malarial drug

Methylene blue, a phenothiazinium salt (Fig 2.3), has a half-life of 5-6 hours (Suwanarusk *et al.,* 2015). It has a mode of action similar to chloroquine in which it inhibits the *P. falciparum* glutathione reductase (Meissner *et al*., 2006; Ginimuge and Jyothi, 2010). In the trophozoite stage, *P. falciparum* metabolizes glutathione at an intense rate. Glutathione helps in anti-oxidative protection and in its reduced state, it supports cell growth by providing electrons for the deoxyribonucleic acid (DNA) synthesis and aids in detoxifying heme, a product of hemoglobin digestion (Becker *et al.,* 2003; Pastrana-Mena *et al., 2*010). Methylene blue may possibly reverse parasite resistance to chloroquine leading to the parasite's increased sensitivity to chloroquine (Schirmer *et al.,* 2003). It also prevents the polymerization of heme into hemozoin (van Schalkwyk *et al.,* 2016). Cardiac arrhythmias

and interference with mesenteric flow are some of the effects of toxicity from the drug. It has also been known to turn urine blue. In spite of this, toxicity has only been noted in doses higher than 2 mg/kg in humans (Ginimuge and Jyothi, 2010).

Methylene blue has been reported to have gametocytocidal properties. In a trial in Burkina Faso, methylene blue was used in combination with artesunate and amodiaquine. There was a decrease in gametocytes when all drug combinations were administered. However, methylene blue-amodiaquine (MB-AQ) and methylene blue-artesunate (MB-AS) combination proved to be more effective than artesunate-amodiaquine (AS-AQ) combination. The methylene blue based treatments rreduced *P. falciparum* gametocytemia by 18% whereas AQ-AS, only reduced the prevalence by 7% (Coulibaly *et al.,* 2009; Jorge *et al.,* 2019)

Another study conducted in Burkina Fuso, a combination of methylene blue and chloroquine was used to uncomplicated *falciparum* malaria. The results showed that methylene blue serves as an effective treatment against uncomplicated cases of *falciparum* malaria (Suwanarusk *et al.,* 2015). Although methylene blue alone was effective against malaria in sub-Saharan Africa, its combination with chloroquine was not effective. The combination therapy of chloroquine and methylene blue proved to be antagonistic against *P. falciparum* (Suwanarusk *et al.,* 2015).

Methylene blue has also been found to be potent against *P. vivax* parasites. The results from a study conducted by Suwanarusk *et al.* (2015), showed that methylene blue had

comparable efficacy to artesunate; it was also able to inhibit schizont maturation. Also, in a study by Mwangi *et al.,* (2016), the results showed that methylene blue was effective against *Plasmodium berghei* by reducing parasitemia to below detectable levels by day 5 post-treatment. This also demonstrated the use of methylene blue in the drug repurposing approach for malaria treatment. However, recrudescence was noted on day 6 after the treatment had ended.

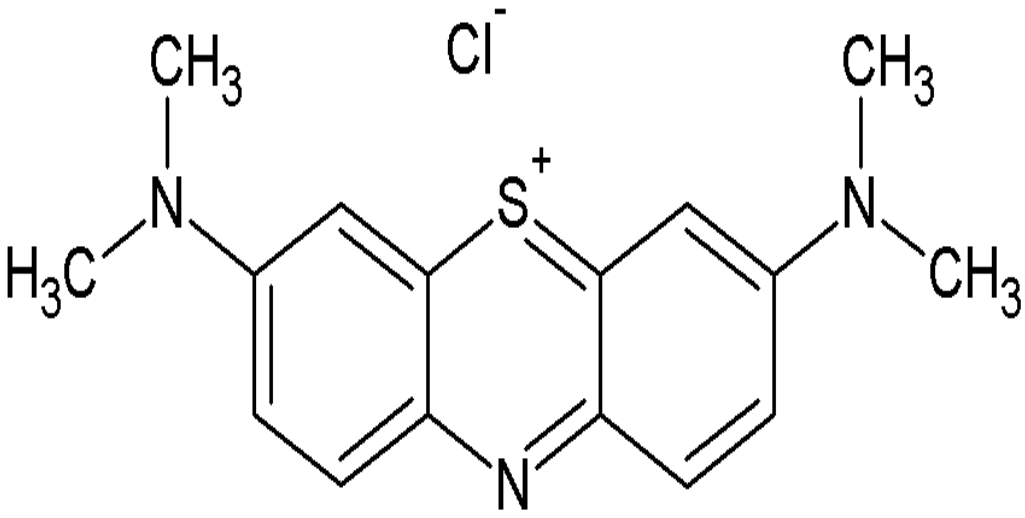


Figure 2.3: Chemical structure of methylene blue (Lau *et al.,* 2015)

In summary, astemizole and methylene blue are both safe and potent against malaria when used as monotherapies. The efficacy of methylene blue is increased when administered in combination with other drugs (Lu *et al.,* 2018) . Both drugs interfere with the blood stages of the parasites, the trophozoite stage, in which there is a high metabolism of glutathione and polymerization of heme into hemozoin (Becker *et al*., 2004; Pastrana-Mena *et al.,* 2010). Hence, their individual efficacy was expected to be amplified in combination (offer

a synergistic response). Moreover, the mechanism of action of methylene blue is similar to that of chloroquine, the drug which took decades to develop resistance.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study area

The study was conducted under the malaria program, Department of Tropical and Infectious Diseases (TID) at the Institute of Primate Research (IPR) in Karen, Nairobi County, Kenya (Fig 3.1).

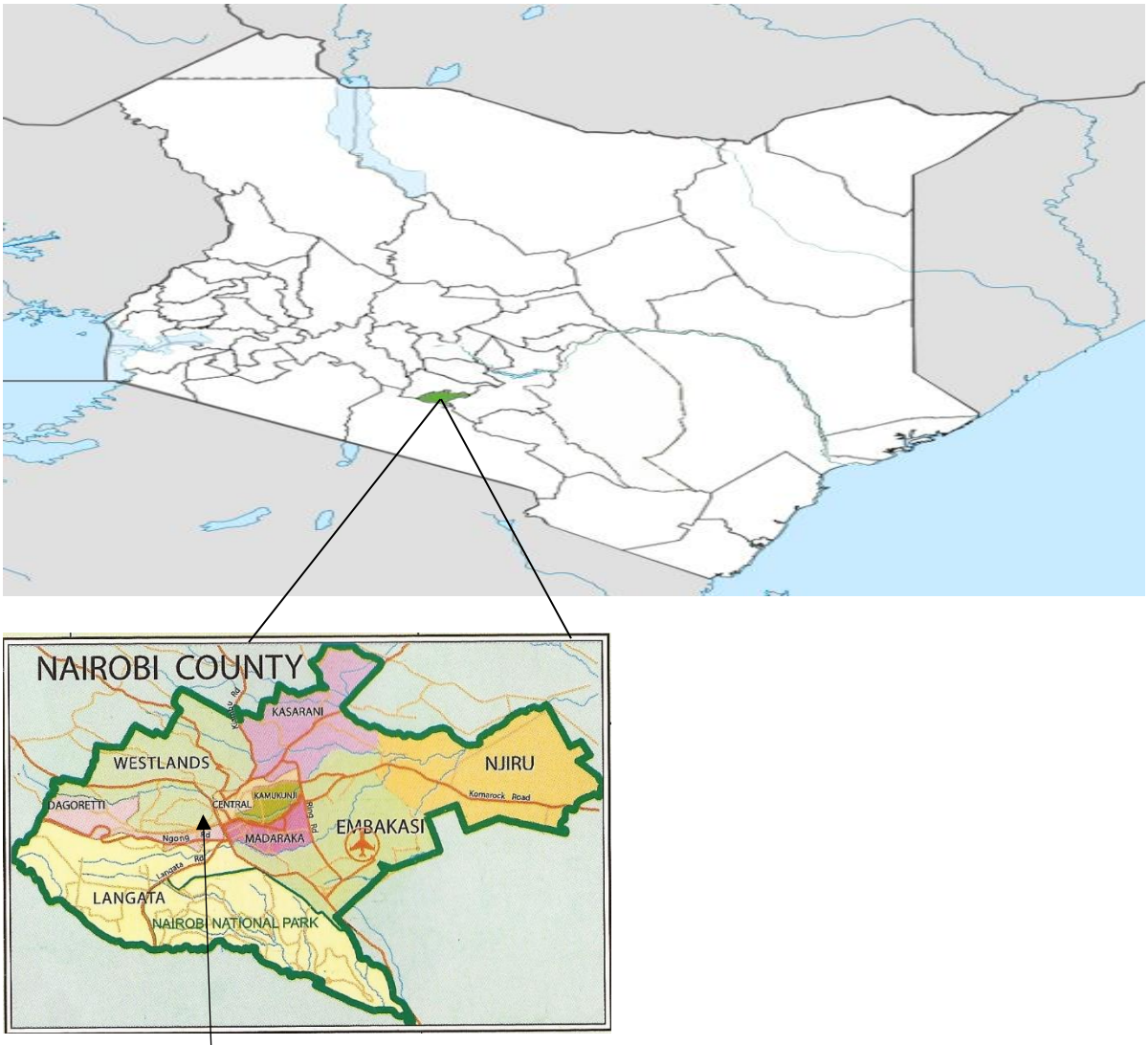


Figure 3.1: Map of Kenya showing the location of the study site

3.2 Study design

The growth potential of the parasites was determined through continuous culture. A randomized block study to determine the anti-plasmodial activity of AST-MB combination therapy *in vitro* and their safety *in vivo* (Fig 3.2 and Fig 3.3). In the anti-plasmodial activity assessment, a drug assay was set up in which test drugs (methylene blue, astemizole and their various combinations in ratios of 1:1, 1:3 and 3:1) were assessed against *Plasmodium falciparum 3D7* and *W2* parasites. Each drug was assessed in triplicates in serial dilutions ranging from 7.81 µg/ml to 1 mg/ml. Similarly, artemether-lumefantrine (Coartem™), positive control and negative control (no drug) wells were assessed against the *Plasmodium* parasites as shown in the layout (Fig 3.2). To distribute equal amounts of the *Plasmodium falciparum* parasites in the wells, parasite pellets were diluted with complete media and equal amounts were added to each well.

Plasmodium falciparum

Drug conc. µg/ml	AST alone			MB alone			Coartem			No drug		
A 1mg/ml	1	2	3	4	5	6	7	8	9	10	11	12
B 500												
C 250												
D 125												
E 62.5												
F 31.25												
G 15.63												
H 7.81												

A

Plasmodium falciparum

Drug conc. µg/ml	AST-MB (1:1)			AST-MB (3:1)			AST-MB (1:3)			No drug		
A 1	1	2	3	4	5	6	7	8	9	10	11	12
B 500												
C 250												
D 125												
E 62.5												
F 31.25												
G 15.63												
H 7.81												

B

Figure 3.2: An illustration of *In vitro* drug susceptibility set up (A) individual drugs, positive and negative controls (B) test drug combinations and negative control

The safety assessment *in vitro* was performed using 25 Balb/c mice (15 male and 10 female) assigned randomly to 5 groups of 5 mice each. Each group was put in distinct cages that were marked. Toxicity was assessed using modified Lorke's method (Fig 3.3).

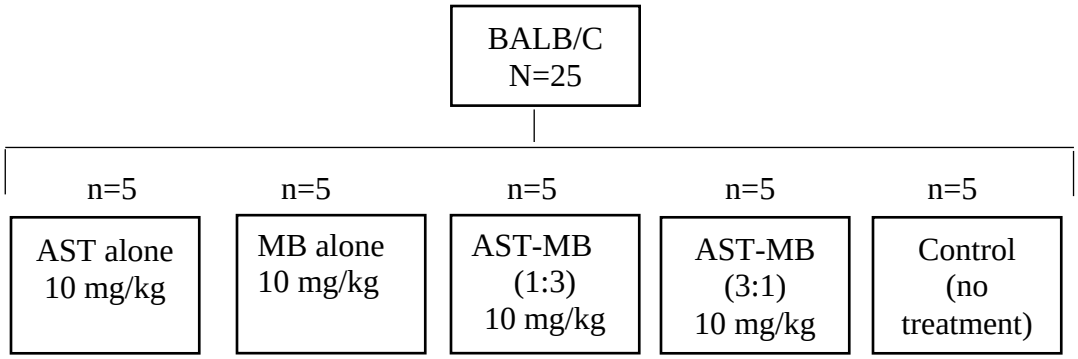


Figure 3.3: Lorke's toxicity method set up

3.3 Preparation of drug stock and working solutions

Anhydrous methylene blue (sourced from Sigma-Aldrich Laborchemikalien GmbH, Seelze, Germany), was weighed (100 mg) and dissolved in distilled water to produce 100 ml of 1 mg/ml stock solution (Mwangi *et al*., 2016). Astemizole (sourced from University of Cape Town – Department of Chemistry) (100 mg) was dissolved in absolute ethanol to produce 100 ml of 1 mg/ml stock solution. The solutions were filter sterilized through a 0.22 µm membrane filter (Sartorius Stedim Biotech, USA, and Ministart ®) and stored at 4^0C until required for use (Zhou *et al*., 1999). Similarly, 100 mg of Coartem™, was dissolved in absolute ethanol to produce 100 ml of 1 mg/ml stock solution.

According to Fivelman *et al.* (2004); Abiodun *et al.,* (2013), the drugs were combined in ratios of 1:1,1:3 and 3:1 and were prepared into final volumes of 1 ml for each drug combination. The first ratio, 1:1, was prepared by adding equal volumes of 500 µl (500 µg) of AST and MB (500 µg). Next, 750 µl (750 µg) of AST was added to 250 µl (250 µg) of MB to prepare 1 ml of 3:1 drug combination ratio. Lastly, 250 µl (250 µg) of AST was added to 750 µl (750 µg) of MB and to prepare 1 ml of the 3:1 drug combination ratio. The drugs were stored at 4^0C until required for use.

3.4.1 Preparation of incomplete culture medium

Rosewell Park Memorial Institute (RPMI) 1640 (GIBCO) media containing L-glutamine buffer was weighed to 10.43 g and placed in a one liter conical flask. To this, 38 ml of filter sterilized 5% $NaHCO_3$, 10 ml of filter sterilized 20% D-Glucose, 38 ml of HEPES (1M) and Gentamicin (500 µl from 50 mg/ml stock) were added. Distilled water (900 ml) was

then added. The pH of the solution was adjusted to 7.14 using 1M hydrochloric acid. Distilled water (100 ml) was added to fill up the solution to the mark. The solution was then filter-sterilized through a 0.22 μm membrane filter (Sartorius Stedim Biotech, USA, Ministart ®) and stored at 4 °C until required for use in the assay (Moll *et al.*, 2013).

3.4.2 Processing of blood serum

Blood from three different donors of different blood groups (A, B and O) who were previously screened for malaria, was collected in blood bags and left to stand for 24 hours at room temperature to facilitate coagulation. The blood was then kept at 4^{o}C before separating out the serum. The serum (250 ml) was transferred into labeled 50 ml centrifuge tubes in a sterile biosafety hood (Steag laminarflow-GmbH). The serum was then centrifuged at 2000 rpm for 10 minutes at room temperature (Hettich rotanta 460 centrifuge). The supernatant was transferred into other labeled 50 ml centrifuge tubes. The serum was heat inactivated by placing it in a water bath at 56^{o}C for 1 hour. It was then allowed to cool at room temperature before storing it at -20^{0}C until required (Lee *et al.*, 2010).

3.4.3 Preparation of complete culture medium

Complete culture medium (10%) was prepared by adding 1 part volume of sterile heat-inactivated human serum (mixed equal volumes of blood groups A, B, and O) to 9 parts volume of the previously prepared incomplete media (Section 3.4.1) in a sterile biosafety hood (Steag laminarflow-GmbH). This was then stored at 4^{o}C until required for use in maintaining the parasite culture medium.

3.4.4 Preparation of red blood cells for culture

Blood group O+ (positive) blood (50 ml) was collected in an anti-coagulant centrifuge tube (Becton Dickinson) and transferred into 50 ml centrifuge tubes in a sterile biosafety hood (Steag laminarflow-GmbH). The tubes were then centrifuged at 1500 rpm for a period of 10 minutes at room temperature. The red blood cells were washed three times with washing media (RPMI 1640 with $NaHCO_3$) (Sigma, Germany) by centrifuging at 1500 rpm for 10 minutes and the supernatant discarded. After the final wash, an equal amount of complete media was added to the blood making a 50% packed cell volume (PCV). It was then stored at 4°C until required for use (Moll *et al.,* 2013; Trager and Jensen, 1976).

3.5 Retrieval and *in vitro* culturing of malaria *P. falciparum* parasites

Three sterile salt solutions were prepared prior to retrieving the parasites (12% NaCl, 1.6% NaCl, and 0.9% NaCl) (Appendix III). The solutions were filter sterilized through a 0.22 µm membrane filter (Sartorius Stedim Biotech, USA, and Ministart ®). Using the NaCl gradient methodology as described by Sharrock *et al.* (2008); vials containing *P. falciparum* (*W2* and *3D7*) parasites were retrieved from the liquid nitrogen storage tank, quickly thawed in a water bath at 37ºC and moved to the sterile biosafety hood (Steag laminarflow-GmbH). Using a 1 ml sterile pipette, the contents of the vial were transferred into sterile 50 ml centrifuge tubes. Next, 12% NaCl solution was added a drop at a time while constantly mixing (1:5 ratio NaCl: cell mixture). This was left to stand for 3 minutes. Thereafter, 10 ml of the 1.6% NaCl solution was added to every 1 ml of original thawed blood drop by drop while constantly mixing. It was then left to stand for 5 minutes. Subsequently, 10 ml of 0.9% NaCl solution per 1 ml of the original volume was added drop

by drop while mixing. The contents were centrifuged at 1500 rpm for 10 minutes at room temperature once in a centrifuge (Hettich rotanta 460). The supernatant was aspirated off before re-suspending the pellet obtained with 10 ml of the pre-warmed complete medium (added drop by drop and gently swirled inside the tube). The suspension was centrifuged again at 1500 rpm for 10 minutes at room temperature.

To culture the parasites, the pellet was re-suspended in 5 ml of complete media and transferred to T25 and T75 culture flasks. Next, 200 µl of the washed O+ red blood cells was added into the culture. The culture was aerated with a special gas mixture (5% O_2, 5% CO_2 and 90% N_2 from BOC gases) for 30 seconds to 1 minute before tightly capping the flask (Plate 3.1). The culture was placed in an incubator (New Brunswick Scientific, United Kingdom) at 36.8 °C for 48 hours and the media was replenished after this incubation period elapsed.

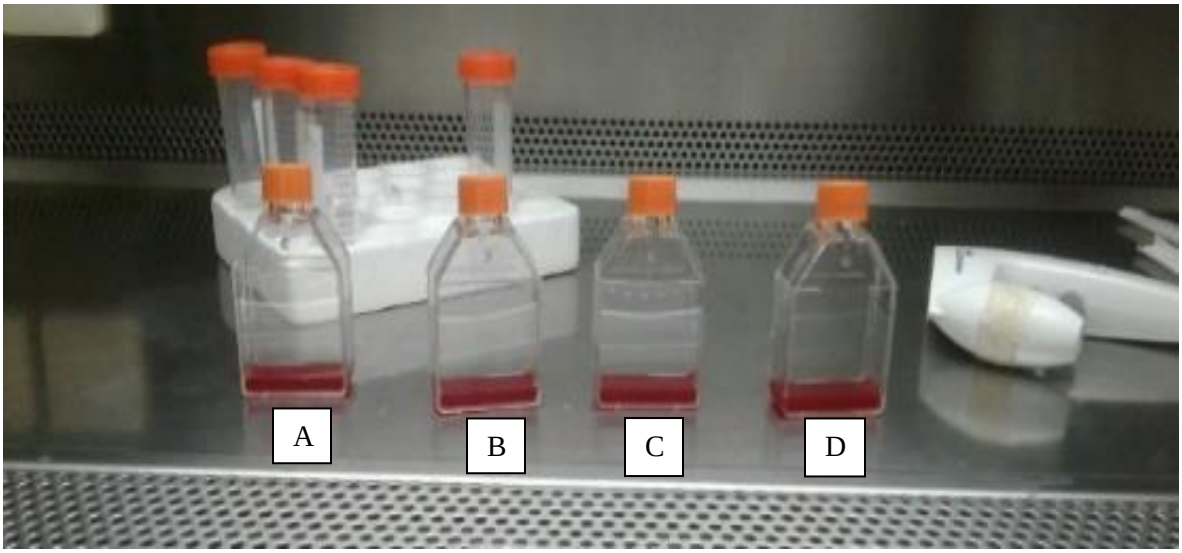


Plate 3.1 : *Plasmodium falciparum 3D7* (A and B) and *W2* (C and D) parasite cultures in blood

3.5.1 Maintaining Continuous *P. falciparum* Cultures

After every 48 hours, 1 ml serological pipette was used to transfer 0.2 ml of the contents of the culture flasks into 2 ml Eppendorf tubes (Fisherbrand™). Using an Eppendorf Microfuge centrifuge 5414 (Brinkmann), the tubes were centrifuged at 2000 rpm for 10 seconds. The supernatant was discarded and the pellet was used to prepare thin blood smears. The smears were left to air dry and fixed in absolute methanol for 10 seconds and then left to air dry again. They were then stained with 10% Giemsa solution for 25 minutes. The stain was washed off with running tap water until no traces of the stain were visibly present. The slides were then blotted with some tissue paper and left to air dry. Following this, the slides were observed under a microscope (Zeiss standard 20, Germany) at x 100 objective and oil immersion. A cell counter was used to count infected red blood cells relative to the total red blood cells and the parasitemia was determined using the following equation (WHO, 2016).

$$\text{Number of parasites per } \mu l = \frac{\text{Number of infected red blood cells}}{\text{Number of total red blood cells}} \times 5 \times 10^6$$

After determining the parasitemia, the culture media was replenished with fresh media. To do this, 10 ml sterile serological pipettes were used to transfer the remaining parasite culture from the culture flasks into 5 ml centrifuge tubes in the sterile biosafety hood (Steag laminarflow-GmbH). The tubes were centrifuged (Hettich rotanta 460 centrifuge) at 1500 rpm for 10 minutes. In the hood, the supernatant from the tubes was removed and the pellet was re-suspended in 200 µl human O+ blood and 5 ml of pre-warmed RPMI 1640 complete media to maintain the culture at 1.5% hematocrit and 1% parasitemia. The contents of the

centrifuge were transferred into culture flasks. This was followed by aeration of the parasite culture with a mixture of gases (5% O_2, 5% CO_2, and 90% N_2 from BOC gases) for 30 seconds to 1 minute. The culture flasks were tightly capped and placed in the incubator (New Brunswick Scientific, United Kingdom) for 48 hours.

3.6 Cryopreservation of *Plasmodium falciparum* Parasites

Prior to cryopreserving the parasites, a freeze mix was prepared by mixing 28 ml glycerol and 4.2% sorbitol in normal saline. The freeze mix was filter sterilized through a 0.22 µm filter unit. After this, cultures with ring forms of more than 5% were chosen. The parasite cultures were transferred from culture flasks into 50 ml centrifuge tubes in a sterile hood. The cultures were centrifuged once at 1500 rpm for 10 minutes after which, the supernatant was discarded. The packed cell volume (200 µl) was measured with a pipette and an equal volume of the freeze mix was added. This mixture was then aliquoted into 1.8 ml cryovials (Nalgene™) in a volume of less than 0.5 ml per vial. The vials were kept at 4^0C for 5 minutes, then transferred to -20^0C until they solidified and finally, they were plunged into liquid nitrogen (-80^0C) until they were required for use.

3.7 *In vitro* anti-plasmodial efficacy of *P. falciparum*

In the *in vitro* drug test, previously prepared astemizole, methylene blue drugs and their combinations in ratios of 1:1, 1:3 and 3:1 (Section 3.3) were assessed. In the sterile biosafety hood (Steag laminarflow-GmbH), a multi-channel pipette was used to transfer 50 µl of complete culture medium to each well of a 96 well plate. Next, 50 µl, of each drug was added and serially diluted in triplicate from row A to H. Finally, 50 µl of diluted

pellets (5.5×10^4 parasites per µl of blood) of *Plasmodium falciparum* parasites (acquired from Institute of Primate Research and Kenya Medical Institute bio-repository), were pipetted into each well plate and mixed 10 times with the multi-channel pipette. The well plates were covered with a lid and sealed with parafilm strips.

A glass desiccator jar was wiped with 70% ethanol. Paper towels moistened with distilled water were placed inside the jar at its base, to provide humidity which prevented evaporation of the culture media. The 96 well plates with the drugs and parasites were placed on top of the moistened paper towels. Then, a candle was lit and placed beside the plates to provide Carbon dioxide (CO_2) which in turn maintained the culture pH. The jar was then placed inside the incubator (New Brunswick Scientific, United Kingdom) set at 36.8^0C for 48 hours. After the incubation period, the parasitemia was determined as previously discussed (Section 3.5.1).

The parasitemia determined for each well plate was used to calculate the percentage suppression (Kabiru *et al.*, 2013), inhibitory concentration 50% (IC_{50}) and the Fractional inhibitory concentration (FIC) of the drugs. This provided information on the efficacy and interaction of the test drugs. Drug suppression was calculated as described by (Adetutu *et al.*, 2016)

$$\text{Suppression}(\%) = \frac{\text{Mean parasitemia in negative control - mean parasitemia in treatment}}{\text{Mean parasitemia in negative control}} \times 100$$

The Fraction inhibitory concentration **(**FIC) values were calculated using the IC_{50} values of the drugs as described in the formula by Meletiadis *et al.,* (2010)

$$FIC = \frac{\text{Fraction of drug concentration required to produce } IC_{50} \text{ when used in combination}}{\text{Fraction of drug concentration required to produce } IC_{50} \text{ when used alone}}$$

3.8 *In vivo* safety assessment using Lorke's method

The sample size of mice per group was determined using a formula by Arifin and Zahiruddin, 2017.

$n = DF/k + 1$

where:

n- number of animals per group

DF- Maximum degree of freedom for maximum one-way ANOVA designs

k- number of groups

$n = 20/5 + 1 = 5$

Therefore, 5 animals per group were used.

3.8.1 Experimental animals

Rules and regulations established by the Animal Science Department of Institute of Primate regarding the handling of mice were followed as also described by Hedrich and Bullock (2004). Balb/c mice aged 6 weeks (15 male and 10 female), weighing between 20 ± 2 g were randomly divided into 5 groups of 5 mice each (Section 3.8.1). Each group was kept in labeled standard Macrolon type II cages at 22°C and 60% -70% relative humidity. The mice were fed on commercial rodent pellets (Unga Farm Care (EA) Limited) and were

given free access to a constant supply of water.

3.9 Toxicity Assessment

Drug toxicity tests were conducted with the astemizole-methylene blue drug combinations in the ratios 1:3 and 3:1. However, AST-MB 1:1 showed the least favorable anti-plasmodial activity, therefore, it was not included in the subsequent toxicity assessment. A modified version of Lorke's acute toxicity method was employed (Fig 3.2). In this method, various doses of test drugs were administered to the Balb/c mice. Methylene blue was administered at 10 mg/kg, a dose which suppressed *Plasmodium berghei* by 80% (Dormoi and Pradines, 2013). Astemizole was also administered at 10 mg/kg, a safe dose which suppressed *Plasmodium* by 80% (Jong-Hwa *et al.,* 2008; Chong *et al.,* 2006). Further, the drug combinations were administered at a dose of 10 mg/kg, the initial dose in Lorke's toxicity test (Chinedu *et al.,* 2013). The drugs were administered via the intraperitoneal route on the body cavity (Plate 3.2).

Plate 3.2: Intraperitoneal administration of test drugs on body of Balb/c mouse

The toxicity test was carried out for 48 hours using Lorke's method (Chinedu *et al.*, 2013). During this period, the clinical symptoms, as well as the behavior patterns of the mice, were recorded. Parameters such as body weight of mice, the amount of food and water consumed and the colors of fur, eyes, ears, skin, and tails were recorded (Appendix V and VI).

After 48 hours, the mice were euthanized using CO_2. To do this, each mouse was placed in a CO_2 chamber until it became immobile. The mice were then moved to the operating tray where they were dissected. Cardiac puncture was done to collect whole blood from the mice using 25 G needle and 1 ml syringes. The whole blood was then transferred into

EDTA (BD) vacutainer tubes and 2 ml Eppendorf tubes. Blood in the Eppendorf tubes was left to stand for 30 minutes. Applicator sticks were used to dislodge the pellet of coagulated blood to maximize the serum to be collected. The Eppendorf tubes were allowed to stand for another 10 minutes, after which, the serum was transferred into other labeled Eppendorf tubes. The serum was then stored at -20^0C until required for use in biochemical analysis. The organs (heart, lungs, spleen, liver, kidneys, and brain) were harvested. Gross pathology of each animal was conducted in which the organs were weighed and their appearance was observed and recorded (Appendix VII).

3.10.1 Hematological tests

Prior to analyzing the blood, it was ensured that the blood mixed well with the EDTA by placing the collections tubes on a roller mixer (MS113). The blood while still in the tubes, was loaded into a hematology analyzer (Sysmex XE-5000, Kobe, Japan). The machine provided an analysis of the blood profile to show if the test drugs had any impact on the blood. Hence, it provided information on the safety of the test drugs.

3.10.2 Biochemical tests

The collected serum was thawed in a water bath set at 37^0C and analyzed using a chemical analyzer (Humalyzer 2000). From the serum, aspartate aminotransferase, alanine aminotransferase and total protein activity tests were conducted to provide information on the biochemical profiles which in turn provided information on the safety and toxicity of the test drugs.

3.10.2.1 Assay of aspartate aminotransferase activity

To perform this test, a GOT (ASAT) IFCC Liquiuv humazym test kit (Human diagnostics worldwide) was used. The substrate from this kit (2 ml) was added to 8 ml of the buffer and mixed thoroughly. From this solution, 1 ml was transferred into a 14 ml tube. Pre-thawed sample serum (100 µl) was added to the 14 ml tube. Each sample was analyzed separately through the chemical analyzer (Humalyzer 2000).

3.10.2.2 Assay of alanine aminotransferase activity

The alanine aminotransferase assay was conducted using a GPT (ALAT) IFCC modified Liquiuv humanzym test kit (Human diagnostics worldwide). From the test kit, 2 ml of the substrate was added to the entire bottle of the buffer solution (8 ml) and mixed thoroughly. Next, 1 ml of this solution was transferred to a 14 ml tube. The test serum added to the solution. This was then loaded in the chemical analyzer (Humalyzer 2000). Each sample was analyzed separately.

3.10.2.3 Assay of Total Protein Activity

To analyze the amount of total protein in the mice serum, a liquicolour photometric colorimetric test kit (Human diagnostics worldwide) was used. From the kit, 20 µl of the standard solution was pipetted into a 14 ml tube. The test serum (20 µl) was transferred into a separate 14 ml tube. To both tubes, 1000 µl of the reagent was added. The tubes were left to stand at room temperature for 10 minutes. Meanwhile, the absorbance of the blank (tap water) was measured using the chemical analyzer (Humalyzer 2000). After, the

standard solution was loaded into the machine and its absorbance measured. In a similar manner, the absorbance of the serum samples was measured. The absorbance values were an indicator of the amount of albumin and globulin in the serum. Any anomaly in the values was attributed to kidney and liver disorders. This provided information on the safety of the test drugs.

3.11 Data analysis

Non-linear regression from Graph Pad Prism 7 (San Diego, CA) software, was used to determine the Inhibitory concentration (IC_{50}) of the drugs activity *in vitro*. Subsequently, the IC_{50} values were used to calculate Fractional Inhibitory Concentration (FIC) values which in turn, provided information on the drug interactions. Additionally, isobolograms of the test drugs against the *Plasmodium falciparum* strains were plotted using the fixed dose ratio method in Microsoft Excel 2013.

Comparison of means of the measured values in treatment and the control groups in the *in vivo* toxicity assays were analyzed by ANOVA and adjusted with a Tukey Post Hoc test (SPSS 20). Differences were considered significant if the *P* values were less than 0.05 ($p< 0.05$) and the F values were greater than 2.70. All measurements were expressed as mean ± standard error of mean.

3.12 Ethical consideration

Ethical clearance was sought from the Institutional Scientific Ethical Review Committee (ISERC) at IPR (ISERC/09/2017), which reviews all research protocols carried out in the institute. This committee is mandated by the National government through the National

Commission of Science and Technology (NACOSTI) to review all scientific protocols involving animals for research. The Institute of Primate Research is an Association of Assessment and Accreditation of Laboratory Animal Care (AAALAC) accredited center (Appendix I). Authorization to conduct the research was also sought from The Graduate School, Kenyatta University (Appendix II).

CHAPTER FOUR

RESULTS

4.1 Optimization of Culturing of *Plasmodium falciparum* strains

Both *Plasmodium falciparum 3D7* and *W2* parasites exhibited an increase in the level of parasitemia (parasites/µl blood) from day 0 of culturing to day 11. The optimum growth was observed on day 11 for both *Plasmodium* strains (9.20 x 10^5 per µl blood and 6.96 x 10^5 per µl blood for *P. falciparum 3D7* and *W2* respectively). After day 11 post culture, the parasitemia of both strains dropped drastically to zero by day 18 (Fig 4.1). Furthermore, *Plasmodium falciparum 3D7* had a growth rate of 5.2-fold and *Plasmodium falciparum W2* had growth rate of 5.0-fold from day 0 to day 11 post culture.

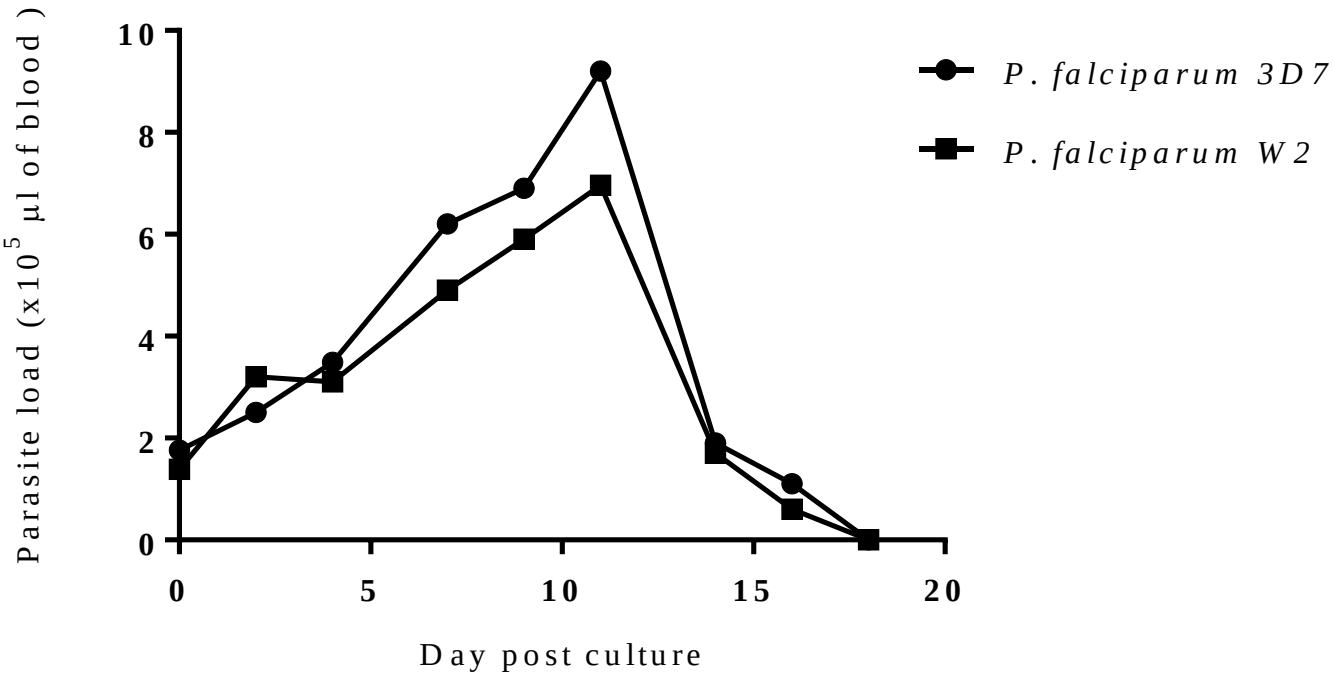


Figure 4.1: Parasitemia at optimum growth for *3D7* and *W2 Plasmodium falciparum* strains.

4.2 Anti-plasmodial efficacy of astemizole and methylene blue combination therapy drug against *P. falciparum 3D7* and *P. falciparum W2*

The results of assays of astemizole, methylene blue and their various combinations against *Plasmodium falciparum 3D7* strain showed that all drugs had anti-plasmodial activity. In comparison with the negative control at 125 µg/ml, all the test drugs produced lower parasitemia but MB alone caused the highest reduction in parasitemia (6.90 x 10^4 per µl blood) and AST caused the least reduction (9.2 x 10^4 per µl blood). At 62.5 µg/ml, AST-MB 1:1 caused the least reduction in parasitemia (4.10 x 10^4 per µl blood) and AST caused the least (9.5 x 10^4 per µl blood). At 31.25 µg/ml, AST-MB 3:1 caused the highest reduction in parasitemia (4.10 x 10^4 per µl blood) and AST-MB 1:1 caused the least (11.2 x10 x 10^4 per µl blood).

Further, at the concentration of 31.25 µg/ml, it was observed that the parasitemia of AST-MB 1:1 against *P. falciparum 3D7* was 1.1-fold that of the negative control and this made it the least efficacious test drug combination. At 15.63 µg/ml, AST-MB 3:1 caused the highest reduction in parasitemia (3.0 x 10^4 per µl blood) and MB alone caused the least (8.9 x 10^4 per µl blood). At 7.81 µg/ml, AST-MB 3:1 caused the most reduction in parasitemia (4.0 x 10^4 per µl blood) and AST-MB 1:1 caused the least (8.1 x 10^4 per µl blood), (Fig 4.2).

In spite of this, noteworthy differences were only noted for MB alone treatment (7.81 µg/ml) ($F= 8.439$, $df=2$, $p=0.0012$), AST-MB 3:1 treatment (31.25 µg/ml) ($F= 8.439$, $df=2$, $p=0.0017$) and AST-MB 1:1 (31.25 µg/ml) ($F= 8.439$, $df=2$, $p=0.0013$).

Overall, the most efficacious drug combination against *Plasmodium falciparum 3D7* was AST-MB 3:1 at 31.25 µg/ml. At this concentration, there was 51% parasite suppression.

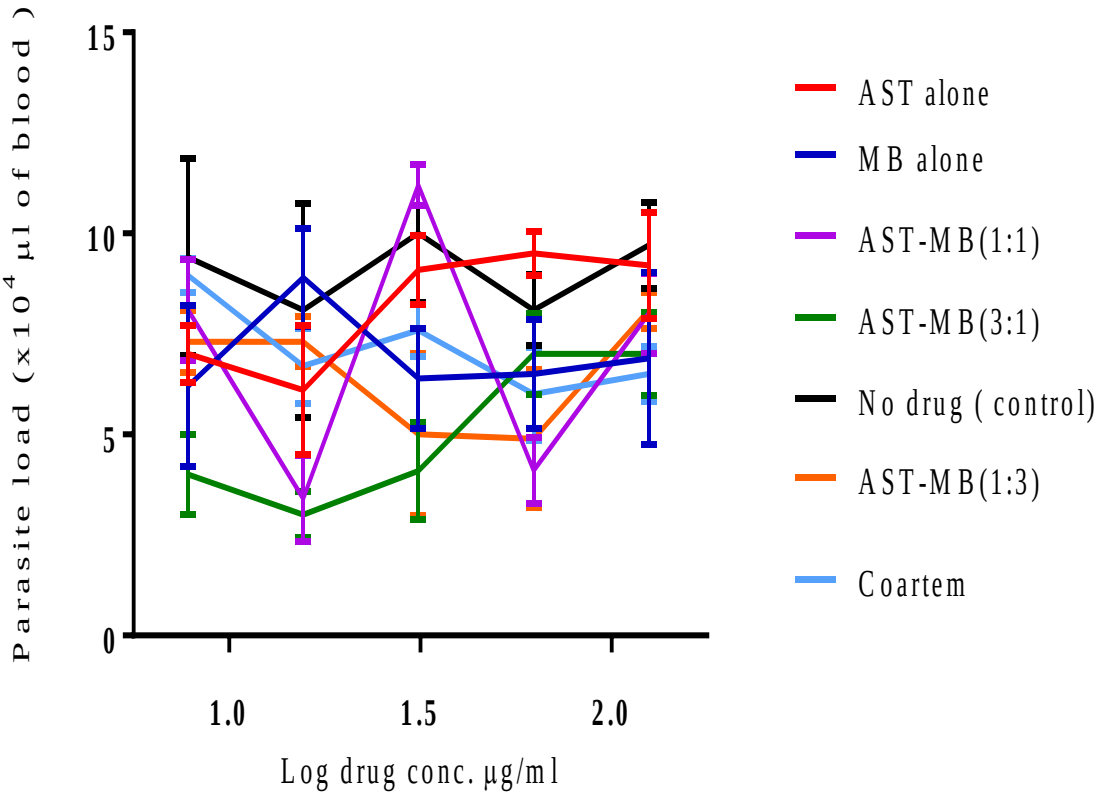


Figure 4.2: Parasitemia of astemizole-methylene blue drug combinations against *Plasmodium falciparum 3D7* strain

Similarly, all the test drugs showed anti-plasmodial activity against *Plasmodium falciparum W2* strain (Fig 4.3). At various concentrations, the test drugs showed variations in efficacy in comparison to the controls. At 125 µg/ml, AST-MB 1:3 and 3:1 caused the highest reduction in parasitemia (both 2.0 x 10^4 per µl blood) and the least reduction was

caused by AST-MB 1:1 (6.10 x 10^4 per µl blood). At 62.5 µg/ml, AST-MB 3:1 caused the highest reduction in parasitemia (2.9 x 10^4 per µl blood) and the least was observed in the AST-MB 1:3 plate. At 31.25 µg/ml, AST-MB 1:3 caused the highest reduction in parasitemia (1.7 x 10^4 per µl blood) and the least was observed in the AST-MB 1:1 plate (4.9 x 10^4 per µl blood). At 15.63 µg/ml, AST alone caused the highest reduction in parasitemia (4.9 x 10^4 per µl blood) and the least was noted in the AST-MB 1:1 plate. It was also observed that AST-MB 1:1 had a parasitemia that was 2-fold higher than that of the negative control. At 7.81 µg/ml, AST-MB 1:1 treatment showed the greatest reduction in parasitemia (5.5 x 10^4 per µl blood) and the least reduction was MB alone (8.4 x 10^4 per µl blood).

However, significant differences were observed with MB alone treatment (31.25 µg/ml) ($F=5.428$, $df=2$, $p=0.0029$), AST-MB 3:1 treatment (62.5 µg/ml) ($F=5.428$, $df=2$, $p=0.0029$), AST-MB 1:1 treatment (15.63 µg/ml) ($F=5.428$, $df=2$, $p=0.0012$) and AST-MB 1:3 treatment (31.35 µg/ml) ($F=5.428$, $df=2$, $p=0.0035$) in relation to the negative control.

Overall, the most efficacious treatment against *Plasmodium falciparum W2* strain was AST-MB 1:3 at 31.35 µg/ml. At this concentration, AST-MB 1:3 suppressed the parasite load by 53%.

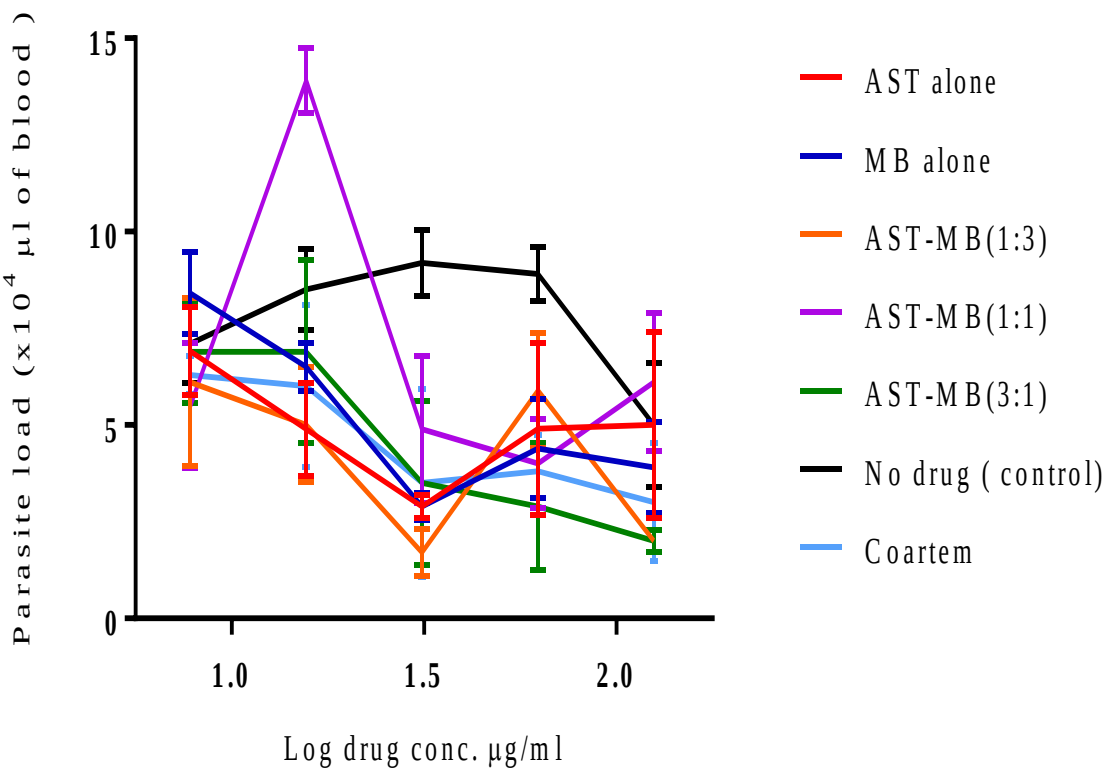


Figure 4.3: Parasitemia of astemizole-methylene blue drug combinations against *Plasmodium falciparum W2*

4.2.1 Inhibitory concentration 50 (IC_{50}) for drugs against *P. falciparum 3D7* and *W2* strains

The IC_{50} values of AST-MB combinations against *P. falciparum 3D7* were higher than the IC_{50} of methylene blue alone and Coartem (Table 4.1). A significant difference was observed between methylene blue alone and AST-MB 1:3 ($F=9.036$, $df=2$, $p=0.030$). In comparison to the IC_{50} value of astemizole alone, AST-MB 1:1 and 3:1 drug combinations had higher IC_{50} value than that of astemizole alone, however, these differences were statistically insignificant ($F=9.036$, $df=2$, $p=1.000$, and $p=1.000$ respectively). Astemizole-

methylene blue 1:3 had a lower IC_{50} value than astemizole but this was statistically insignificant (F=9.036, df=2, p=0.099). All the test drugs; astemizole alone, methylene blue alone and the drug combinations had IC_{50} values that were higher than those of Coartem against *P. falciparum 3D7*. Significant differences were observed in astemizole alone, AST-MB 1:1 and AST-MB 1:3 (F=9.036,df=2, p=0.033, p=0.031 and p=0.001 respectively). The results also showed that AST-MB 3:1, at a concentration of 31.25µg/ml, was the most efficacious drug combination against *P. falciparum 3D7* strain in comparison to Coartem. This difference was statistically significant (F=9.036, df=2, p=0.045).

In the drug assay against *P. falciparum W2* strain, the IC_{50} values of AST-MB combinations were higher than the IC_{50} of methylene blue alone (Table 4.1). The IC_{50} values of AST-MB drug combinations were all higher than those of astemizole alone except AST-MB 1:3 which had a lower but it was statistically insignificant IC_{50} value (F= 27.478, df=2, p=0.200). AST-MB 1:1, AST-MB 3:1 had higher IC_{50} values than the positive control and the differences were statistically significant (F= 27.478, df=2, p= 0.001 and p=0.008 respectively). AST-MB 1:3, at a concentration of 31.25 µg/ml, was the most efficacious drug combination against *P. falciparum W2* strain in comparison to Coartem. However, this difference was statistically insignificant

(F= 27.478, df=2, p=0.980).

Table 4.1: Drug IC_{50} values

Drug	Inhibitory concentration 50 (IC_{50}) (µg/ml) *P. falciparum 3D7*	Inhibitory concentration 50 (IC_{50}) (µg/ml) *P. falciparum W2*
Methylene blue	17.96±0.22	7.69±0.43
Astemizole	23.12±0.30	23.55±0.26
Astemizole-methylene blue 1:1	23.25±0.66	29.23±0.84
Astemizole-methylene blue 1:3	34.16±0.37	15.07±0.60
Astemizole-methylene blue 3:1	22.28±0.24	26.14±0.46
Coartem	7.15±0.24	16.56±0.30

4.2.2 Fraction inhibitory concentration (FIC) of astemizole-methylene blue against *P. falciparum 3D7* and *W2*

The FIC values show the interaction between the drug combinations (Appendix IV) and (Table 4.2). From the results obtained, the level of antagonism varied between combinations in the drug assays. In the assays against *Plasmodium falciparum 3D7,* AST-MB 1:3 combination had the highest FIC value (3.4) and AST-MB 3:1 had the least (2.2). Therefore, AST-MB 1:3 exhibited the highest antagonism in comparison to AST-MB 3:1. For *Plasmodium falciparum* W2 strain, AST-MB 1:1 drug combination had the highest FIC value (5.0) while AST-MB 1:3 had the least (2.6). Therefore, AST-MB 1:1 showed the highest antagonism compared to AST-MB 1:3.

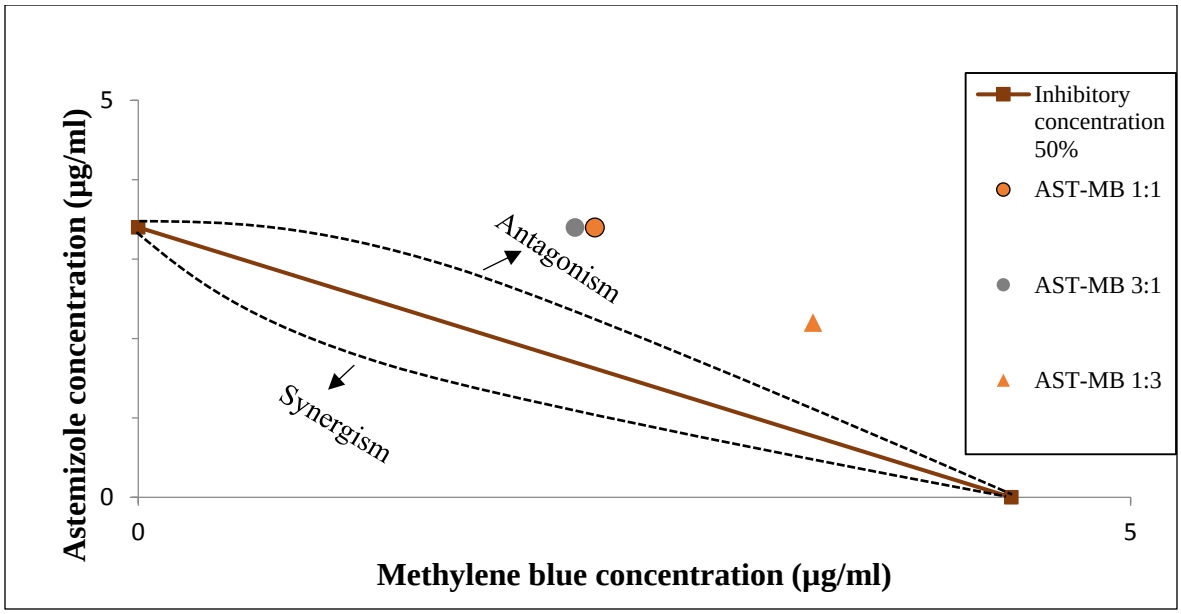
In summary, in drug assays of astemizole-methylene blue drug combinations against *Plasmodium falciparum 3D7* and *W2* strains, the least antagonistic combinations were AST-MB 3:1 and AST-MB 1:3 respectively.

Table 4.2: The fractional inhibitory concentrations (FIC) of the drugs

Drug	**FIC value (µg/ml)**	**Interaction**	**FIC value (µg/ml)**	**Interaction**
	P. falciparum 3D7		***P. falciparum W2***	
Astemizole-methylene blue 1:1	2.3	Slightly antagonistic	5.0	Antagonistic
Astemizole-methylene blue 1:3	3.4	Slightly antagonistic	2.6	Slightly antagonistic
Astemizole-methylene blue 3:1	2.2	Slightly antagonistic	4.5	Antagonistic

According to Te Dorsthorst *et al.,* (2002), the cut-offs for drug interactions are, if values are <1 synergistic, 1≥ and <2 additive, 2≥ and <4 slightly antagonistic, ≥ 4 antagonistic.

To illustrate these interactions, isobolograms (diagrams showing the drug combination interactions against individual drugs), were plotted to show the FIC values of the combinations against the FIC values of the individual drugs. The isobologram of the drug combinations against *Plasmodium falciparum 3D7* shows that all the drug combinations fall within the antagonism region (Fig 4.4 A). Similarly, the isobologram of the drug combinations against *Plasmodium falciparum W2* showed that all the drug combinations were antagonistic (Fig 4.4 B).



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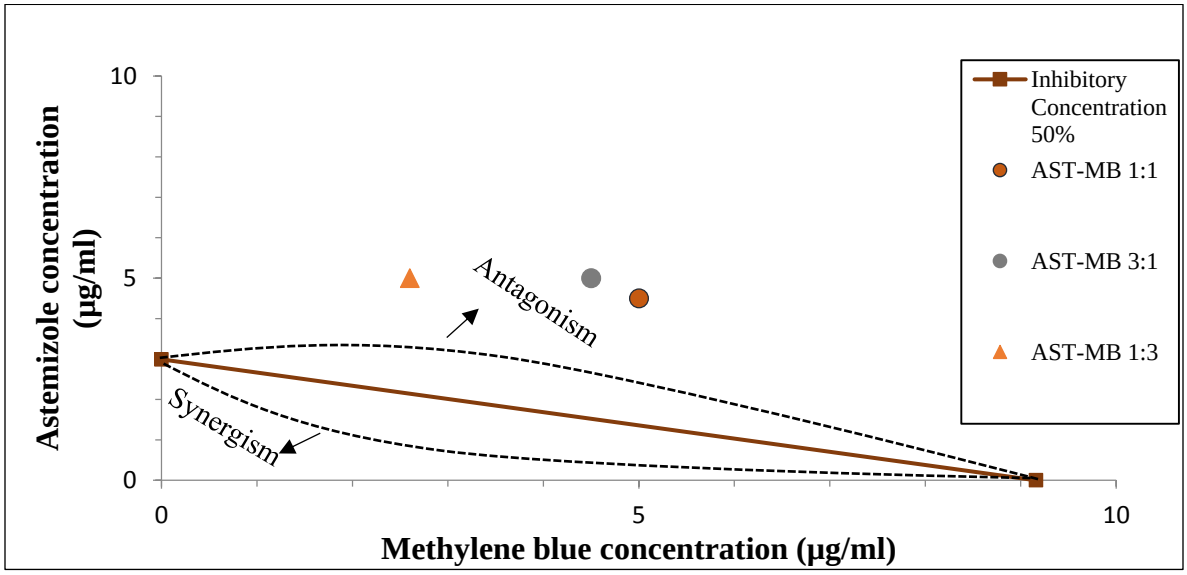


B

Figure 4.4: Isobolograms of the drug combinations interactions against *Plasmodium falciparum 3D7* (A) and *Plasmodium falciparum W2* (B)

4.3 Acute Toxicity Assessment

Throughout the acute toxicity assessment over 48 hours, no mortality was observed.

4.3.1 Observation of Clinical Symptoms

Recording of clinical symptoms was essential in drug safety assessments as they showed variations of the treated Balb/c mice and the non-treated (control). As such, abnormalities were attributed to drug effects (Apppendix V).

Between 0-2 hours, mice from all the groups decreased in body weight (Fig 4.5). The least change in body weight was recorded in the MB alone group in which there was no change and the greatest change was recorded in the AST alone group. However, these changes in body weight were less than those recorded in the control group. Between 2-26 hours, mice from all the groups increased in body weight except the AST alone treated group which showed a decrease in body weight. Between 26-28 hours, the body weights of all the treatment groups decreased. The body weights of MB alone and AST-MB 3:1 treatment groups increased between 28 and 48 hours whereas the weights in the AST alone and AST-MB 1:3 decreased (Appendix VI). However, this increase in weight was more than that in the control group. Statistically, there was a noteworthy difference in the MB alone treated group ($F= 3.533$, $df=4$, $p=0.013$).

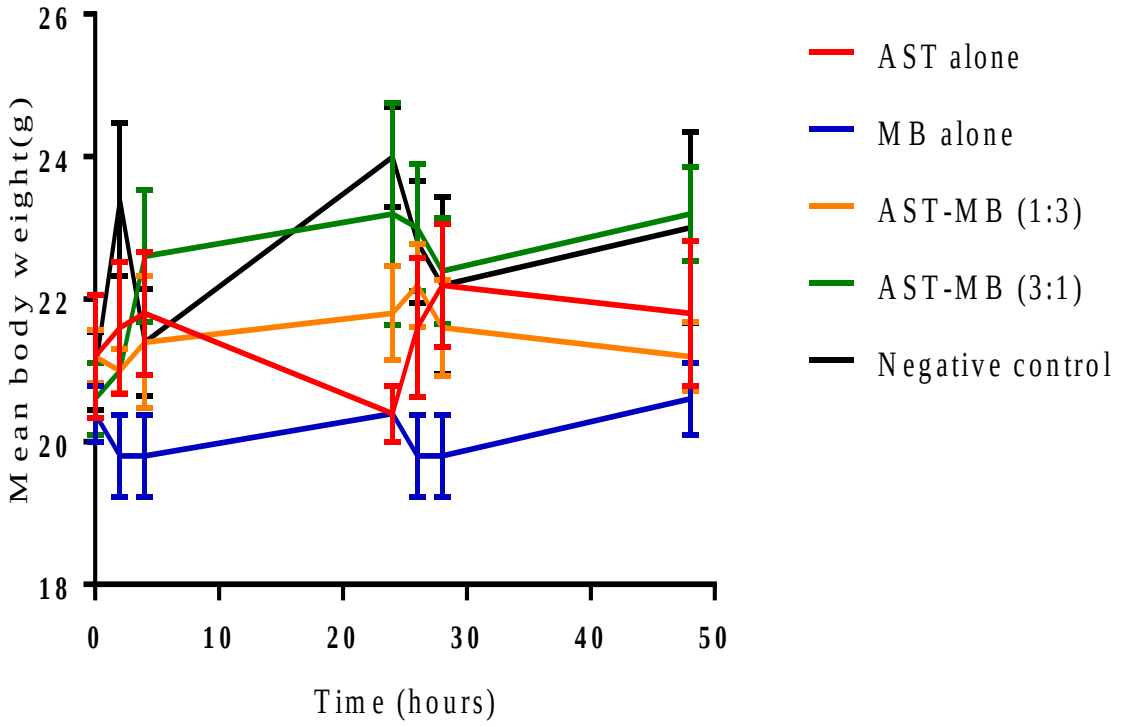


Figure 4.5: Changes in body weight (g) of Balb/c mice 48 hours post drug administration

The results also showed that the amount of pellets left after consumption decreased between 0-48 hours post drug administration (Fig 4.6). The highest decrease in food consumed was observed in the AST-MB 3:1 treatment groups and the least was in the MB only group. Between 0-4 hours, mice from all the treatment groups did not consume many pellets. A dramatic decrease in pellets left after consumption was observed between 4-24 hours in the treatment groups. The difference in food left after consumption within each treatment group was low between 24-28 hours. Another dramatic decrease in food left after consumption was observed between 28-48 hours (Appendix VI). However, these differences were not statistically significant ($F= 1.059$, $df=4$, $p=0.464$).

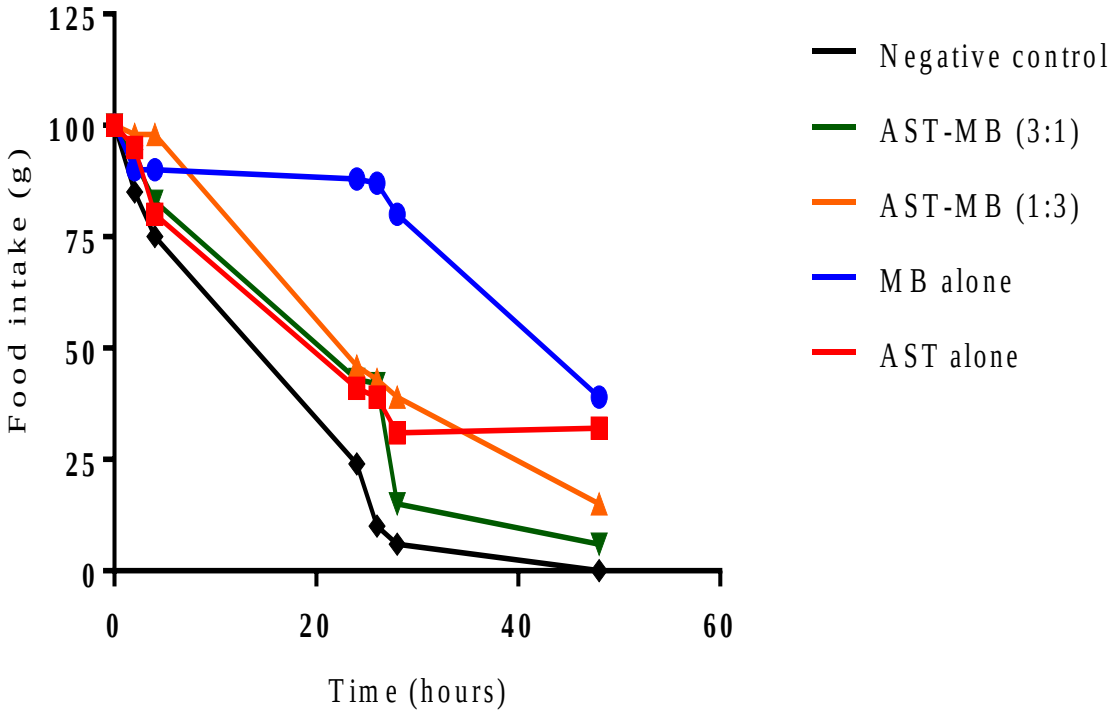


Figure 4.6: Weight of pellets left after consumption by Balb/c mice between 0-48 hours post drug administration

Also, the results showed that the amount of water consumed by each group decreased from 0-48 hours post drug administration (Fig 4.7). The highest decrease in water consumed was in the AST-MB 3:1 (Appendix VI) and the least was in the MB alone treatment groups. However, these differences were not statistically significant (F= 0.077, df=4, p=0.997).

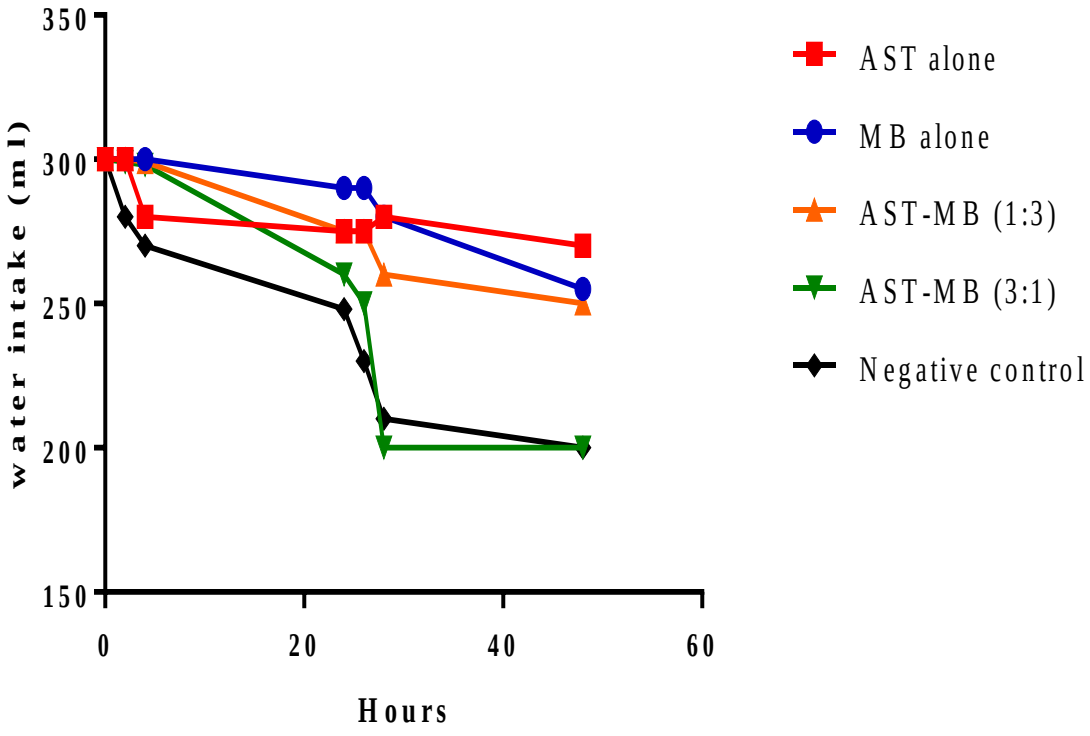


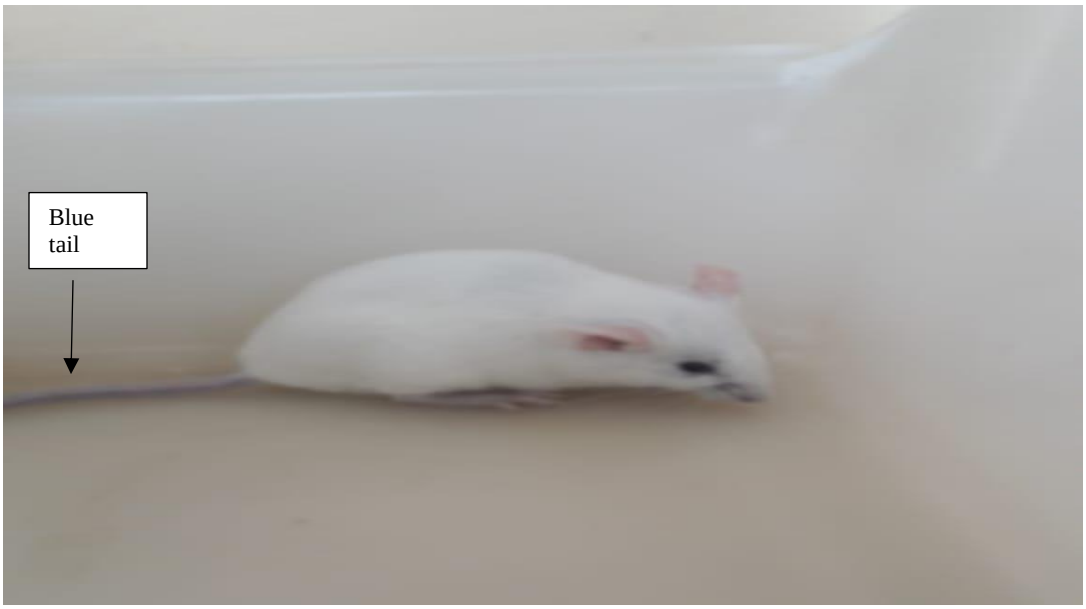
Figure 4.7: Remaining Water after Consumption (ml) by Balb/c Mice between 0-48 hours

The results showed that Balb/c mice from all the treated groups clustered to one end of the cage between 0-24 hours post drug administration. Furthermore, the mice in the AST alone were observed to have minor tremors. However, between 24-48 hours, the mice in the treated groups began to exhibit normal behavior.

Change in urine color and pH were also observed. In the MB alone group, the urine color was blue and in the drug combination groups, it was blue-green between 0-24 hours. The urine color in both the MB alone group changed from blue to blue-green and that in the combination group changed from blue-green to green, between 24-48 hours. However, in the AST only group, the urine color was normal (umber) both between 0-24 hours and 24-

48 hours. Despite the differences in color, the pH of the urine in all the groups ranged from 5 to 8. Furthermore, blue color was observed in the eyes, ears, skin and tails and mouths of the mice in the MB alone and the drug combination groups between 0-24 hours post drug administration (Plate 4.1). However, the eyes, ears, skin, and tails in these groups regained their normal color between 24-48 hours post drug administration (Plate 4.2). The eyes, ears, skin and tails and mouths of mice in the AST alone group retained their normal color both between 0-24 hours and 24-48 hours. The fur remained normal throughout the 48 hours in all the treated groups. The stool was normal and formed except in the AST-MB 3:1 treatment group after 26 hours, loose stool with mucus was observed in 2 mice. Also after 26 hours, formed blue stool was observed in 2 mice in the AST-MB 1:3 treatment group.

Plate 4.1: Photograph of blue eyes observed in Balb/c mice from MB alone and drug combination groups between 0-24 hours post drug administration



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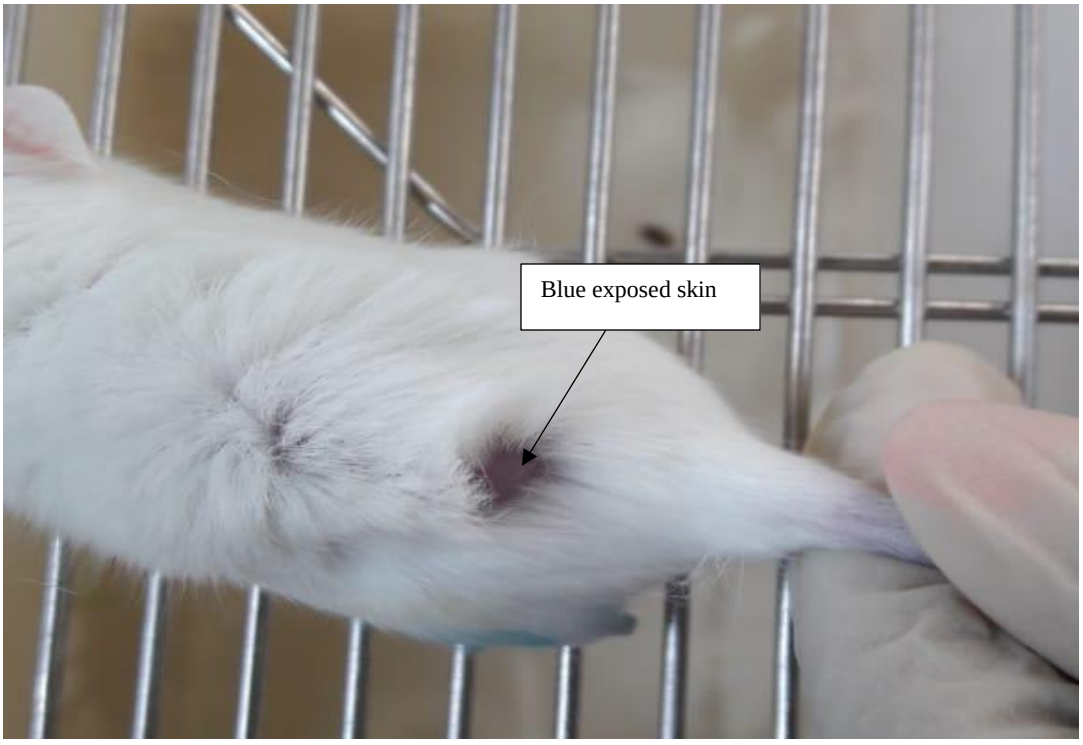


B

Plate 4.2: Photograph of the tail (A) and skin exposed by shaving off fur (B) observed in Balb/c mice 0-24 hours post drug administration

4.3.2 Biochemical analysis

This analysis provided information on the biochemical functions (liver, kidney, and heart)

of Balb/c mice from treated groups after 48 hours post drug administration.

The levels of alanine aminotransferase enzyme in the serum of the Balb/c mice from all the treated groups (67.6 U/L for AST, 86.8 U/L for MB, 84.5 U/L for AST-MB 1:3 and 43.4 U/L for AST-MB 3:1) were less than that in the negative control group (124.2 U/L) (Fig 4.8). Among the treatment groups, the highest level of alanine aminotransferase was in the MB alone group (86.8 U/L) and the least was in the AST-MB 3:1 group (43.4 U/L). In spite of these variations in measurements, significant differences were only noted in the AST-MB 3:1 group (F= 4.476, df=4, p=0.046).

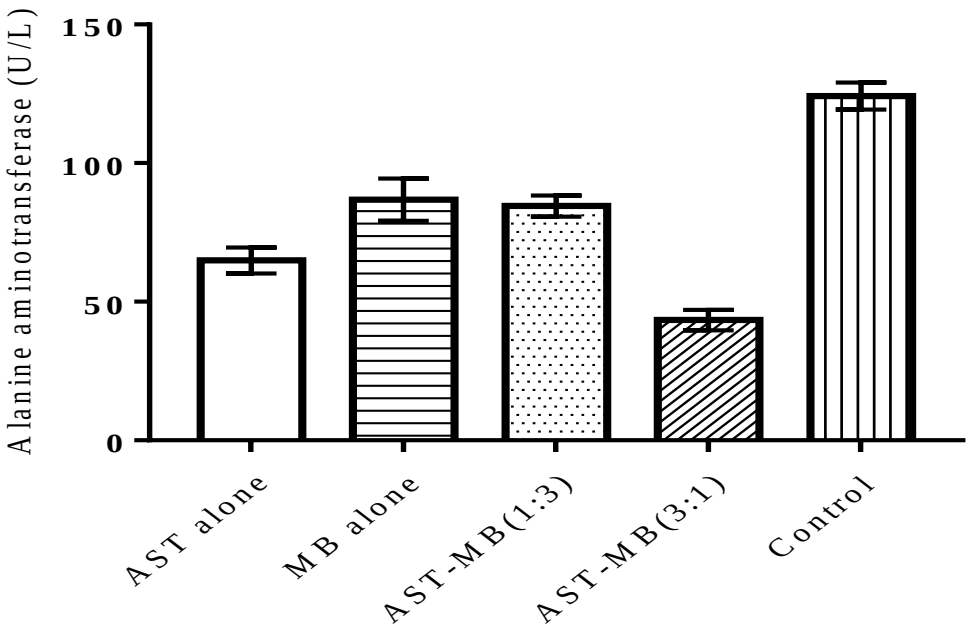


Figure 4.8: Mean alanine aminotransferase levels (U/L) of treated and control groups after 48 hours

The results also showed that aspartate aminotransferase levels in serum of mice in the treatment groups were lower (113.9 U/L for AST, 276.4 U/L for MB, 142.0 U/L for AST-

MB 1:3 and 165.6 U/L for AST-MB 3:1) as compared to the negative control group (289.8 U/L) (Fig 4.9). Among the treatment groups, the highest aspartate aminotransferase level was in the MB alone group (276.4 U/L) and the least in the AST group (113.9 U/L). However, these differences were not significant (F= 1.762, df=4, p = 0.273).

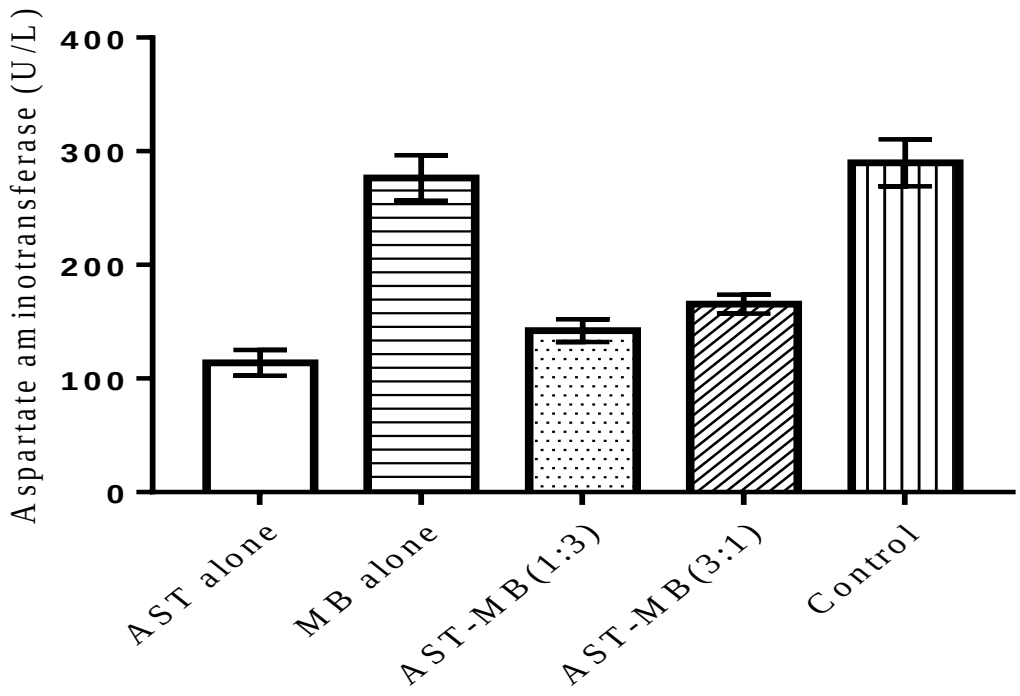


Figure 4.9: Mean aspartate aminotransferase levels (U/L) of treated and control groups after 48 hours

The total protein levels in the serum from all the treatment groups were higher than in the negative control group (15.4 g/dL for AST, 17.7 g/dL for MB, 16.6 g/dL for AST-MB 1:3 and 14.4 g/dL for AST-MB 3:1) and 12.9 g/dL in the control groups (Fig 4.10). In the treatment groups, the highest total protein level was in the MB alone group (17.7 g/dL) and the lowest level was in the AST-MB 3:1 treatment group (14.4 g/dL). However, these differences were not significant (F= 0.282, df=4, p= 0.878).

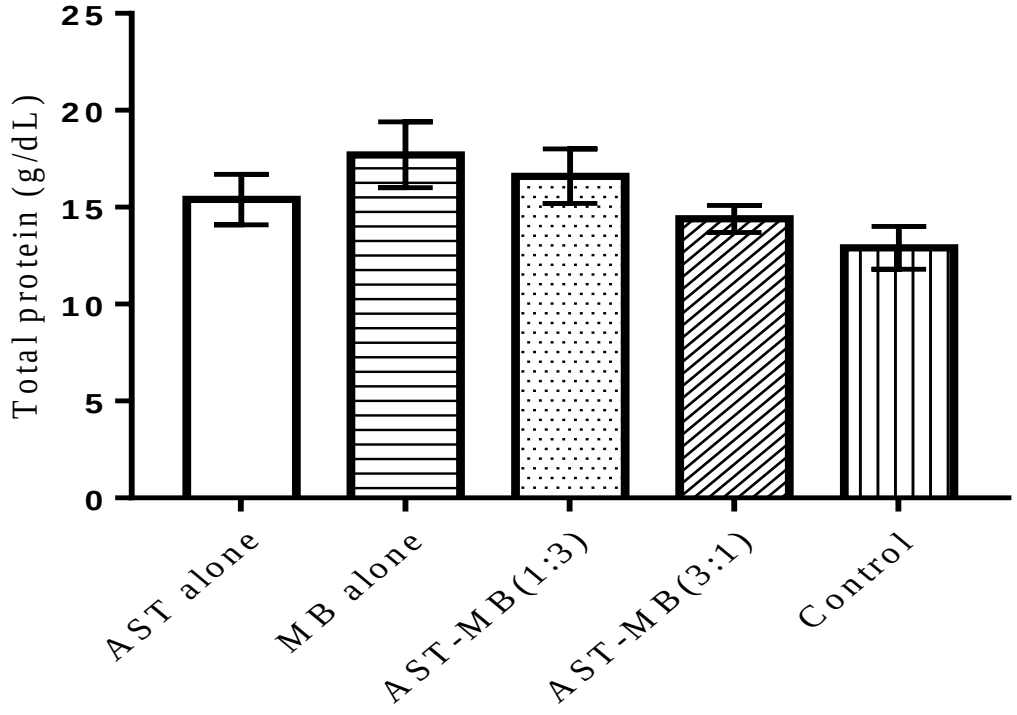


Figure 4.10: Mean total protein levels (g/dL) of serum in treated and control groups after 48 hours

4.3.3 Assessment of hematological parameters

Hematological parameters provided information on the overall profile of the blood with regard to each blood component 48 hours post drug administration. A decrease in white blood cell counts, mean corpuscular hemoglobin and platelet levels was observed to in the treatment groups in comparison to the negative control.

The white blood cell count showed that all the treated groups had lower counts (3.2×10^3/µl for AST, 3.4×10^3/µl for MB, 4.9×10^3/µl for AST-MB 1:3 and 4.9×10^3/µl for AST-MB 3:1) in comparison to the count in the control group (5.5×10^3/µl) (Fig 4.11). Between

the treated groups, AST-MB 1:3 and AST-MB 3:1 groups had equal as well as the highest white blood cell counts (4.9 x 10^3/µl) and AST alone group had the lowest white blood cell counts (3.2x10^3/µl). However, these differences were not statistically significant (F=, df=4, p=0.600).

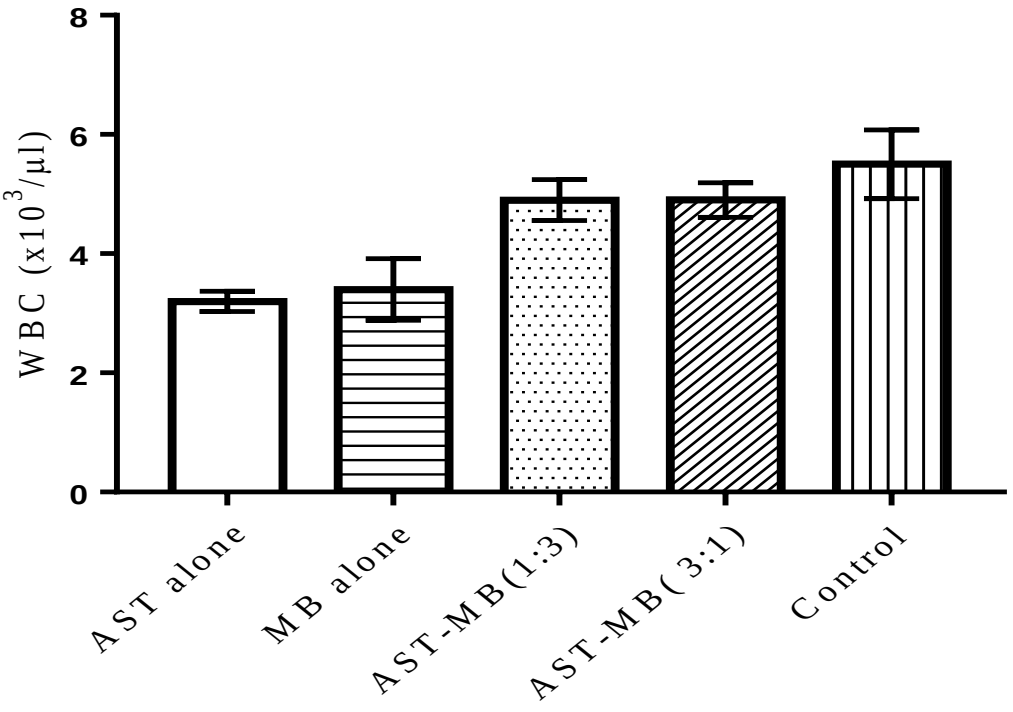


Figure 4.11: Mean white blood cell count (x 10^3/µl) of treated and control groups after 48 hours

The mean platelet volume of the mice from all the treated groups treated group were lower (569 x 10^3/µl for MB, 906 x 10^3/µl for AST-MB 1:3 and undetectable for AST-MB 3:1) as compared to the value in the control group (1099 x 10^3/µl). However, the platelet volume in the AST alone group was higher (1517 x 10^3/µl) than that in the control group (Fig 4.12). In spite of this, significant differences were only notable in the AST-MB 3:1 treated group (F= 27.40, df=4, p=0.005).

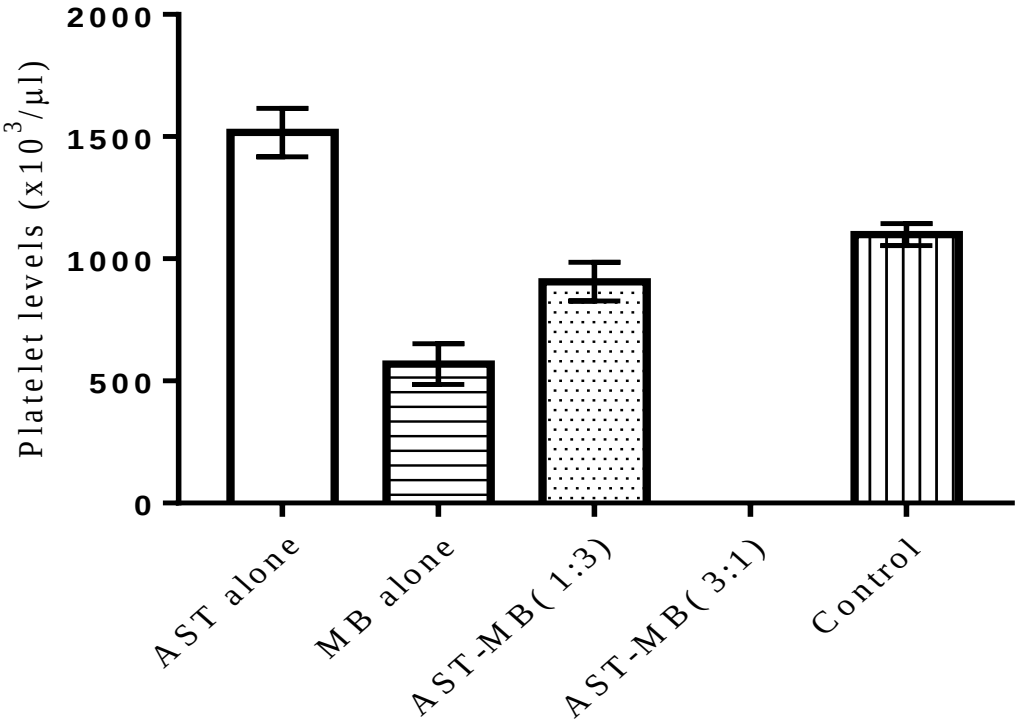


Figure 4.12: Mean platelet volume (x 10^3/µl) of mice from treated and control groups

The mean corpuscular hemoglobin (MCH) levels were lower (16.95 pg for AST, 23.86 pg for AST-MB 1:3 and 17.21 pg for AST-MB 3:1) in all the treatment groups as compared to the control (25.82 pg) except in the MB alone group in which the level was higher (31.61 pg) (Fig 4.13). Among the treatment groups, AST-MB 3:1 group, had the lowest mean corpuscular hemoglobin (16.95 pg). However, the differences were not statistically significant (F= 10.69, df=4, p=0.083).

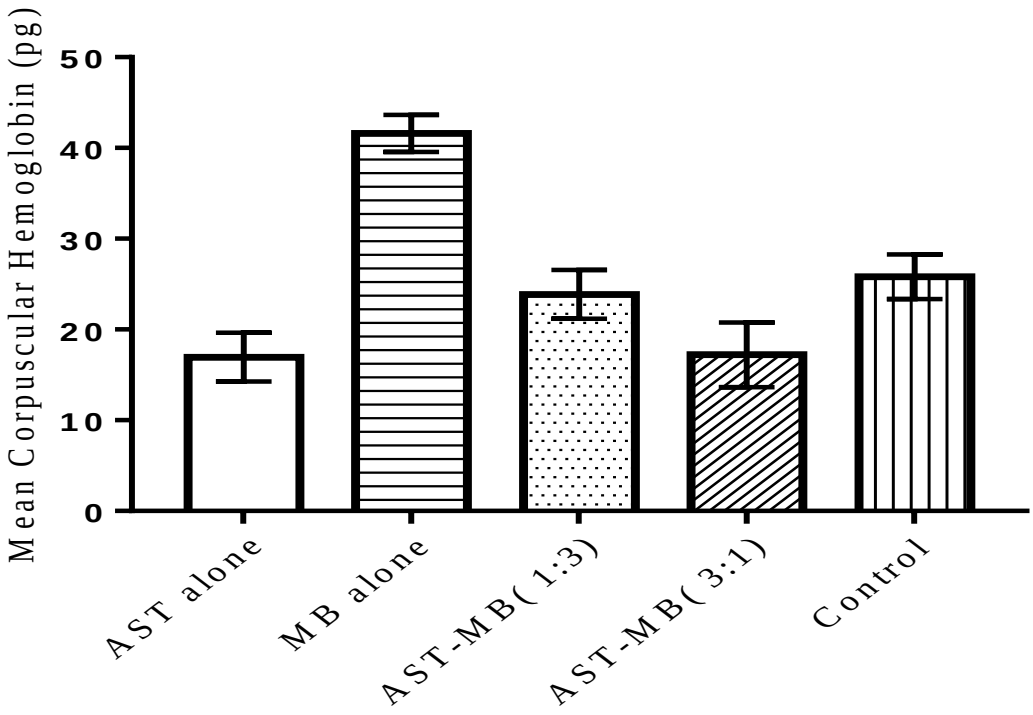


Figure 4.13: Mean corpuscular hemoglobin (pg) of mice from treated groups

It was also observed that the red blood cell counts, hemoglobin levels and hematocrit levels were high in the mice to which the test drugs had been administered as compared to the control group.

The results showed that the number of red blood cells from all the treated groups were higher (7.92 x 10^6/µl for AST, 7.07 x 10^6/µl for AST-MB 1:3 and 82 x 10^6/µl for AST-MB 3:1) than those in the control group (5.09 x 10^6/µl). However, the red blood cell count in the MB alone group was lower than that in the control group (4.18 x 10^6/µl) (Fig 4.14). In the treated groups, the highest red blood cell count was in the AST-MB 3:1 group and the least was observed in the MB only group. In spite of this, the differences were not

statistically significant (F= 2.528, df=4, p=0.168).

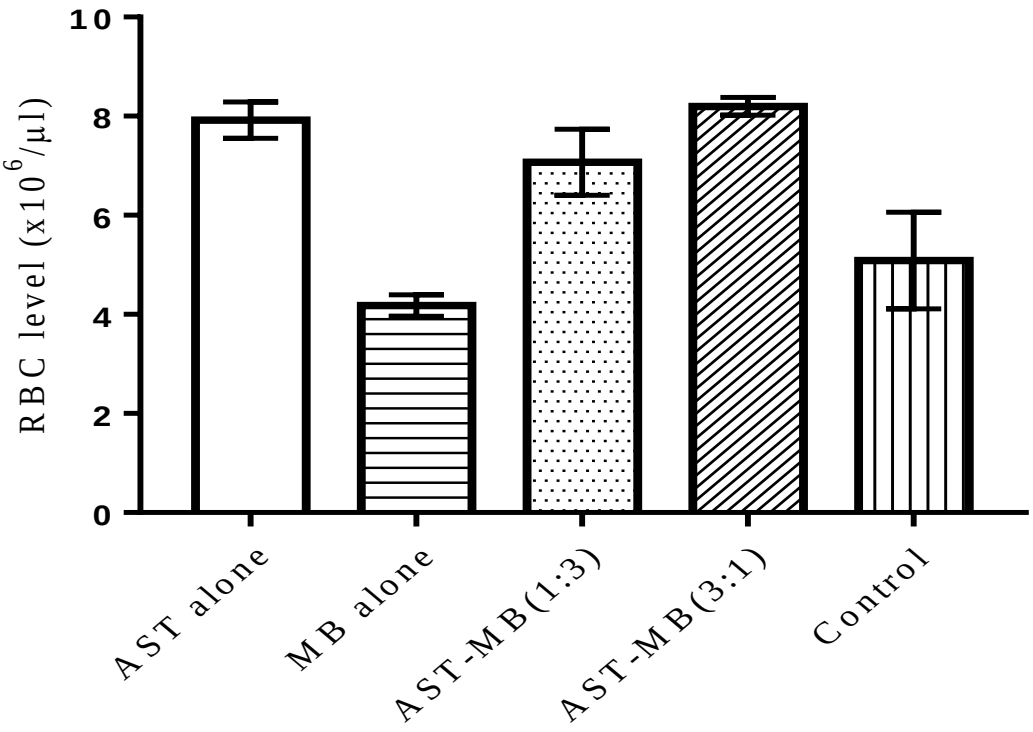


Figure 4.14: Mean red blood cell count (x 10^6/µl) of treated and control groups after 48 hours

The hematocrit levels in the treatment groups were higher (36. 56 % in AST, 31.13% for AST-MB1:3 and 35.42% in the AST-MB 3:1) than those in the control group (22.95%). However, the hematocrit levels in the MB alone group were less than those in the control group (20.10%) (Fig 4.15). The highest hematocrit level was in the AST-MB 3:1 treated group and the least was in the MB alone group. The differences between the treatment groups and control groups were statistically insignificant (F= 1.435, df=4, p=0.345).

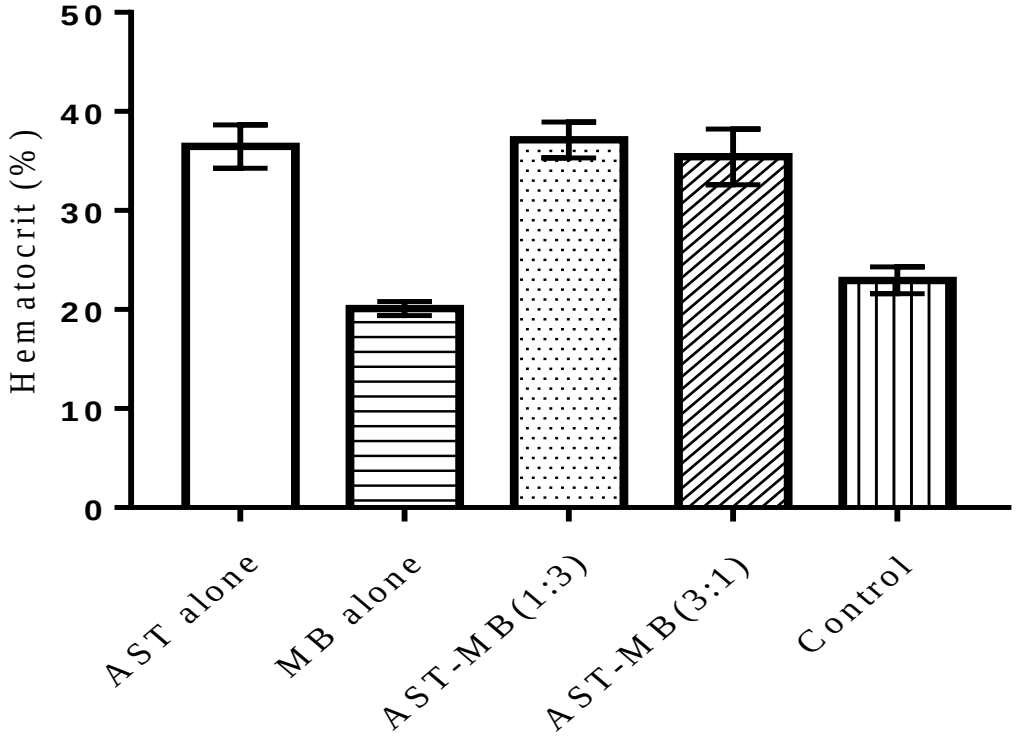


Figure 4.15: Mean hematocrit (%) of treated and control groups after 48 hours

The results showed that the hemoglobin concentration in all the treatment groups was higher (13.4 g/dL for AST, 16.7 g/dL for MB, 16.8 g/dL for AST-MB 1:3 and 14.1 g/dL for AST-MB 3:1) than that in the control group (13.1 g/dL) (Fig 4.16). Between the treatment groups, the AST-MB 1:3 group showed highest hemoglobin levels (16.8 g/dL) and the least was in the AST alone group (13.4 g/dL. The differences were not significant ($F= 1.121$, $df=4$, $p=0.440$).

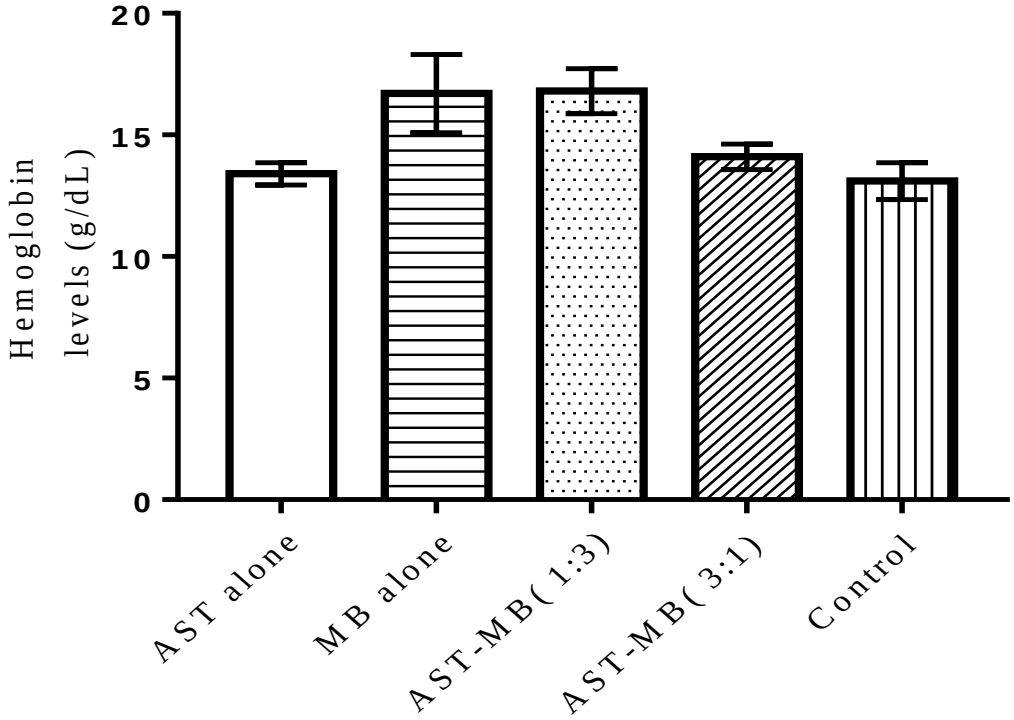


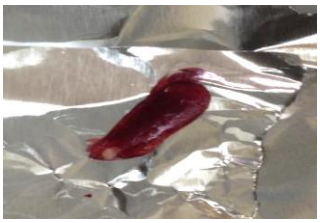
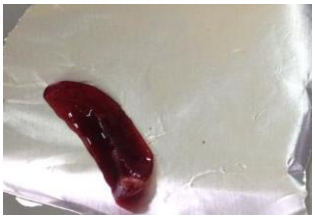
Figure 4.16: Mean hemoglobin concentration (g/dL) of treated and control groups after 48 hours

4.3.4 Gross pathology of Balb/c mice harvested organs

Gross pathology involved the macroscopic observation of Balb/c mice and their organs (Appendix VII). It provided a general overview of the drug effects on the organs. The color, shape, and weight of the organs were recorded. The harvested organs included the heart, liver, kidneys, spleen, lungs, and brain.

There was no difference in the color and shape of the harvested organs from the treatment groups and those from the control groups (Plate 4.3).

Treatment groups **Control group**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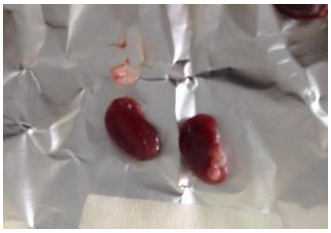
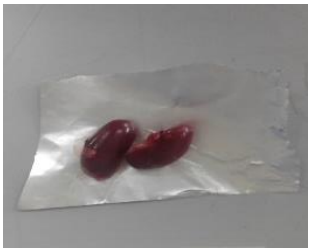
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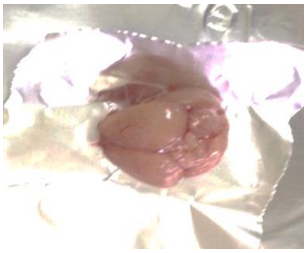
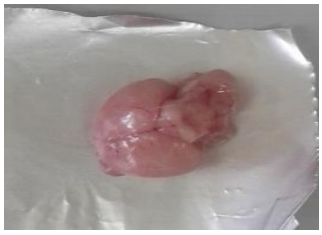
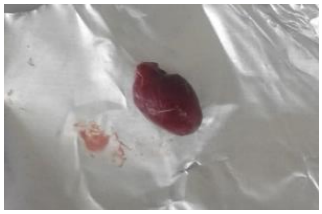
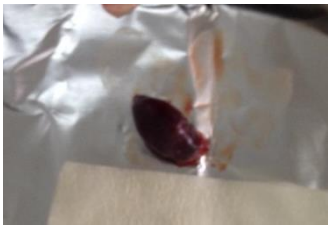


C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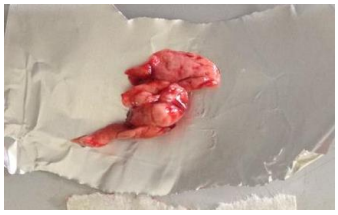
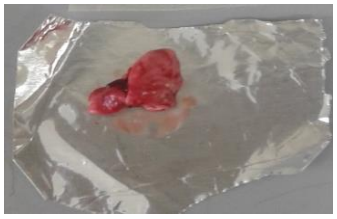
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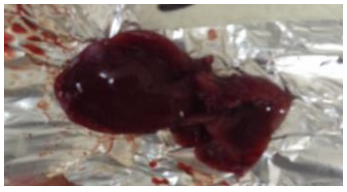
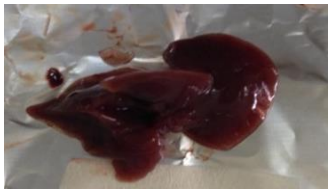
F

Plate 4.3: Harvested organs from treatment and control groups. A Spleen, B-Kidneys, C-Brain, D- Heart, E- Lungs, F- Liver

However, the skin of the Balb/c mice was stained blue from the methylene blue alone and the drug combinations. The blue color was more concentrated at the point of drug administration (Plate 4.4). The intestines were also noted to have a blue color. However, all organs were intact and in their appropriate positions.

Plate 4.4: Photograph of sacrificed Balb/c mouse showing the blue discoloration of skin at the point of drug administration

Further, the weights of the harvested organs of mice from treated and control groups were taken after 48 hours (Table 4.3). The mean weights of the all the organs were less than those in the control group except the mean spleen weight of the AST alone treated group ($F= 8.967$, $df=4$, $p=0.999$), mean weight of lungs of the AST alone and AST-MB 3:1

treated groups (F= 1.239 df=4, p=0.999 and F= 1.239 p=1.000 respectively), and the mean weights of the brain of the AST alone treated groups (F= 1.372, df=4,p=0.826). These differences were not statistically significant.

The mean heart weight of the AST-MB 3:1 group (0.100 ± 0.008 g) was less that in the control group (0.136 ± 0.008g) and also the AST-MB 1:3 combination and the AST alone and MB alone groups. This was statistically significant (F= 8.967, df=4, p=0.007). Also, the mean weight of the liver in the AST-MB 3:1 treated group was less (1.002 ± 0.075 g) than that of the control group (1.296 ± 0.075 g) and also the AST-MB 1:3 combination and the AST alone and MB alone groups and this was significantly different (F= 4.339, df=4, p=0.001).

Table 4.3: Weights (Mean ± SEM g) of harvested organs from treated mice

Organ	MB alone	AST alone	AST-MB (1:3)	AST-MB (3:1)	Control	P value
	MEAN±SEM, n=5					
Heart	0.128±0.008	0.132±0.008	0.141±0.008	**0.100±0.008***	0.136±0.008	0.007
Liver	1.144±0.075	1.114±0.075	1.217±0.075	**1.002±0.075***	1.296±0.075	0.001
Kidney	0.344±0.034	0.358±0.034	0.343±0.034	0.372±0.034	0.372±0.034	0.854
Spleen	0.723±0.016	0.099±0.016	0.090±0.016	0.106±0.016	0.095±0.016	0.298
Lung	0.154±0.016	0.179±0.016	0.166±0.016	0.170±0.016	0.167±0.016	0.326
Brain	0.376±0.037	0.458±0.037	0.397±0.037	0.404±0.037	0.419±0.037	0.279

Statistically significant * (ANOVA, df=4)

CHAPTER FIVE

DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Optimization of culturing conditions of *Plasmodium falciparum of 3D7* and *W2*

Plasmodium falciparum 3D7 had a growth rate of 5.2-fold and *Plasmodium falciparum W2* had growth rate of 5.0-fold from day 0 to day 11 post culture. The parasites grew in culture at different rates despite both being cultured under the same conditions. These conditions were: parasites were cultured with O+ human red blood cells, supplemented with 10% human blood serum and RPMI 1640 and aerated with a special gas mixture of 5% O_2, 5% CO_2 and 90% N_2 and incubated at 36.8 ^{0}C for 48 hours. These results concur with findings of a study by Murray *et al*. (2017) in which it was observed that multiplication rates differ among *P. falciparum* parasite strains. In spite of this, similar growth traits were observed in which both strains showed an increase in parasitemia from day 0 to day 11 post culture.

During this culturing period, it was observed that the parasite cultures had a greater percentage of rings as compared to other stages after every 48 hours. This meant that the parasite cycle had begun again and so the growth pattern was normal. After day 11 post culture, the parasitemia in both strains began to decrease and eventually, the parasites ceased to grow after day 18 post culture. During this period, it was observed that the majority of the parasites had not penetrated the red blood cells. This thus interfered with the growth rate. This was similar to previous observations by Chotivanich *et al.* (2000) whereby the findings showed that multiplication rates are dependent on factors such as intra-erythrocyte cycle and erythrocyte invasion.

5.1.2 Anti-plasmodial efficacy of treatment drugs against *Plasmodium falciparum 3D7* and *W2*

Astemizole (AST) alone showed potency against the parasites and this concurred with findings by (Nzila *et al.,* 2011). Methylene blue (MB) alone also had anti-plasmodial activity against both *Plasmodium* strains as previously observed by Suwanarusk *et al.* (2015). The anti-plasmodial activity was demonstrated by the parasitemia of the parasites of astemizole alone and methylene blue alone being lower than those in the negative control. This implied that astemizole alone and methylene blue alone suppressed the growth of the parasites. Also, the drug combinations , astemizole-methylene blue 1:1,1:3 and 3:1 had parasitemia that was lower than that in the negative control against both parasite strains and so, also showed anti-plasmodial efficacy.

However, the inhibitory concentration 50% (IC_{50}) values of the drug combinations against *Plasmodium falciparum 3D7,* were higher than that of Coartem, the current drug of choice against malaria, implying that the drug combinations were less effective than the Coartem (Pinheiro *et al.,* 2018). Furthermore, the results of the fractional inhibitory concentration (FIC), showed that the drug combination interactions were antagonistic.

Likewise, the IC_{50} values of the drug combinations except AST-MB 1:3 (15.07±0.60 µg/ml) were higher than that of Coartem (16.56±0.30 µg/ml) against *Plasmodium falciparum W2* strain. This therefore revealed that AST-MB 1:1 and AST-MB 3:1 were less effective than Coartem. The FIC values of the drug combinations also the drugs interacted antagonistically. In spite of AST-MB 1:3 having a lower IC_{50} value than

Coartem, the difference was insignificant. Although this was the case, the drug combinations, including AST-MB 1:3 were still antagonistic. Therefore, the drug combinations cannot be used to treat malaria caused by *Plasmodium falciparum W2* strain. It was observed that astemizole, had the least efficacy against both parasite strains in comparison to methylene blue. This suggests that astemizole may have suppressed methylene blue *in vitro.* The drug combinations would therefore not be ideal for treating malaria caused by *Plasmodium falciparum 3D7* strain.

5.1.3 Acute toxicity assessment of astemizole-methylene blue combination in Balb/c mice

The results showed that all the drugs, except MB alone, did not affect the body weight of the mice. In the MB alone group, the weight of the mice decreased. The weight of the remaining pellets after 48 hours, was the highest in the MB alone group. This suggests that the decrease in body weight was a result of anorexia. Variations in body weight are attributed to food (appetite) and water consumption as well as behavior (Ellacott *et al.,* 2010). Throughout the 48 hours, the mice in the treated groups clustered together on one end of the cage and showed signs of lethargy. As a result, they did not consume much water and food. The group to which astemizole alone was administered, minor tremors were noted, concurring with a previous observation by Riordan *et al*., (2002) in which tremors were observed in mice where astemizole had been administered. Astemizole causes long QT syndrome (Wiśniowska *et al.,* 2016). Tremors are one of the symptoms of long QT syndrome (Sadrnia *et al.,* 2013). But between 24-28 hours, the mice in the treatment groups all began to exhibit normal behavior. This was because the drugs may

have worn off (half-life of MB 5-6 hours and AST 24 hours)

The skin, snout, and tail became blue of the methylene blue alone and drug combination groups. The urine and stool of the mice in the drug combination groups was blue-green. In the methylene blue alone group, the urine was blue, similar to what was observed by Prakash *et al.* (2017). The urine of the in the drug combination groups was blue-green, a lighter shade of the blue observed in the MB alone group. However, the blue and blue-green colours are self-limiting and are not harmful (Oz *et al.*, 2011). AST in the drug combinations may have decreased the intensity of the characteristic blue color of methylene blue. This reduction in the blue color intensity was a result of a reduction-oxidation reaction. As was illustrated in previous studies, in the presence of glucose, methylene blue is colorless (reduced form) and is blue in its oxidized form (Azmat *et al.*, 2011). Further, astemizole acts as an anti-oxidant (Zhang *et al.*, 2017). This means that astemizole would favor the formation of the colorless form of methylene blue. However, achieving the colorless form is highly dependent on the concentration. In this study, however, only a reduction in color intensity was observed.

The alanine aminotransferase level was low in the AST-MB 3:1 group. Alanine aminotransferase is an enzyme that catalyzes the amino acid to produce oxaloacetate which plays an important role in energy generation. It is mostly found in the liver but considerable concentrations can be found in the kidneys, heart and skeletal muscles (Kim *et al*., 2008). The low level of this enzyme in the AST-MB 3:1 group, indicates that there was a liver, abnormality. However, the level of aspartate aminotransferase, an enzyme that aids in gluconeogenesis and amino acid metabolism by catalyzing the transfer of amino groups

(Kunutsor *et al*., 2014), in was normal in all groups. The enzyme is predominately found in the heart and liver. This therefore shows that the liver could be the organ that was affected by the AST-MB 3:1 combination.

Total protein, is a measure of the amount of albumin and globulin. The results showed that the groups had normal total protein levels. Therefore, the amount of albumin and globin in the mice was normal. The levels of alanine aminotransferase, aspartate aminotransferase and total protein indicate that the drugs except AST-MB 3:1, did not have an impact on the biochemistry of the mice. The AST-MB 3:1 treatment reduced the amount of alanine aminotransferase in the mice. This revealed that AST-MB 3:1 drug combination may have caused liver abnormality.

The AST-MB 3:1 treatment also caused platelets to decrease to below detectable levels in the mice. This suggested that AST-MB 3:1, induced thrombocytopenia in the mice. This observation concurs with findings by Visentin and Liu (2007) who observed very low platelet counts and attributed this to drugs administered. Antihistamines, astemizole inclusive, interfere with the structural components of plasma (Petríková *et al.,* 2006).

Gross pathology of the harvested organs (spleen, liver, lungs, heart, kidneys and the brain) from all treatment groups showed that all organs had normal color. Some differences were noted in the heart and liver weights. The AST-MB 3:1 treatment was associated with lower mean heart and liver weights of the mice. Lower weight of the liver could occur as a result of toxicological changes within the organ (Jong-Hwa *et al.,* 2008). This notion is supported

by the value of alanine aminotransferase which was less than that of aspartate aminotransferase. This concurs with observations by Kim *et al*. (2008) in which aspartate aminotransferase levels that were higher than alanine aminotransferase are attributed to liver abnormalities. Furthermore, in the AST-MB 3:1 group, the mean heart weight was lower than that in the control group. Astemizole causes heart and liver disorders as previously observed in a study by Jong-Hwa *et al.* (2008). This, therefore, suggests that astemizole in the AST-MB 3:1 drug combination was the main cause of the heart and liver anomalies in this study.

5.2 Conclusions

The following conclusions were drawn from the study results:

(i) *Plasmodium falciparum 3D7* and *P. falciparum W2* strains without being cultured with the drugs both had good growth potential under standard conditions. However, *Plasmodium falciparum 3D7* grew slightly faster than *P. falciparum W2* in culture. Both parasites achieved optimum growth potential at 11 day post culture. The parasites were cultured in human blood supplemented by RPMI 1640 media, 10% human serum and erythrocytes, aerated with 5% O_2, 5% CO_2 and 90% N_2 gas mixture and incubated at 36.8^0C).

(ii) All the test drugs (AST alone, MB alone, AS-MB 1:1, AS-MB 1:3 and AST-MB 3:1 showed anti-plasmodial activity against both *Plasmodium falciparum 3D7* and *W2* strains *in vitro*. Furthermore, AST-MB 3:1 was the most efficacious drug combination against *P. falciparum 3D7* strain at 31.25 µg/ml

(51% parasite suppression) whereas AST-MB 1:3 was the most efficacious drug combination against *P. falciparum W2* at 31.25 µg/ml (53% parasite suppression).

(iii) Acute toxicity tests showed that AST alone, MB alone and AST-MB 1:3 groups did not adversely affect the Balb/c mice. However, AST-MB 3:1 drug combination caused abnormalities in the liver and heart.

5.3 Recommendations

The study recommends the following:

(i) Researchers should look into optimizing *Plasmodium falciparum 3D7* and *P. falciparum W2* cultures before carrying out drug susceptibility tests to determine the optimum growth period.

(ii) Astemizole and methylene blue drug combinations showed anti-plasmodial activity but were antagonistic. Therefore, further evaluation could be done using the same combinations a lower concentrations of astemizole to counter the effects.

(iii) Astemizole and methylene blue 3:1 had some toxic effects to Balb/c mice *in vivo.* Therefore, researchers should use combinations in other ratios other than AST-MB 3:1.

5.4 Recommendations for further research

(i) Evaluate the effects of drug combinations with astemizole metabolites (desmethylastemizole and norastemizole) against *Plasmodium falciparum* strains.

(ii) Evaluation of astemizole-mehylene blue combination therapy at lower astemizole concetrations.

(iii) Histological examination of the harvested Balb/c mice organs from the astemizole-methylene blue drug combination treated experimental animals.

(iv) Analysis of liver and heart to determine the cause of the reduction of the organ weights.

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APPENDICES

APPENDIX I- IPR Ethical review approval certificate

This was part of a study scheduled to run for 2 years

Institute of Primate Research
Address: P. O. Box 24481-00502 Karen Nairobi Kenya | Tel: +254 02 2606235 | Fax: +254 02 2606231
URL: www.primateresearch.org | Email: directoripr@primateresearch.org

INSTITUTIONAL REVIEW COMMITTEE (IRC)

FINAL PROPOSAL APPROVAL FORM

Our ref: **ISERC/09/2017**

Dear **Dr. Faith Onditi,**

It is my pleasure to inform you that your proposal entitled **"Assessment of AST-MB combination therapy on selected malaria parasites."** has been reviewed by the Institutional Review Committee (IRC) at a meeting of 20th February 2018.

The proposal was reviewed on the scientific merit and ethical considerations on the use of animals for research purposes.

The committee is guided by the Institutional guidelines as well as International regulations, including those of WHO, NIH, PVEN and Helsinki Convention on the humane treatment of animals for scientific purposes and GLP.

This proposal has been approved and you are bound by the IPR Intellectual Property Policy.

Signed.....Chairman IRC: Dr. NGALLA JILLANI

Signed .....Secretary IRC: Dr. MERCY AKINYI

Date

INSTITUTE OF PRIMATE RESEARCH
INSTITUTIONAL REVIEW COMMITTEE
P. O. Box 24481-00502 KAREN
NAIROBI - KENYA
APPROVED 5th APRIL 2018

APPENDIX II- Authorization letter from Kenyatta University graduate school for data collection

KENYATTA UNIVERSITY
GRADUATE SCHOOL

E-mail: dean-graduate@ku.ac.ke
Website: www.ku.ac.ke

P.O. Box 43844, 00100
NAIROBI, KENYA
Tel. 020-8704150

Internal Memo

FROM: Dean, Graduate School

DATE: 29th October, 2018

TO: Ms. Joyce Nyirongo
C/o Department of Zoological Sciences

REF: I56F/33764/2015

SUBJECT: APPROVAL OF RESEARCH PROPOSAL

We acknowledge receipt of your Research Proposal after fulfilling recommendations raised by the Graduate School Board of 19th September, 2018.

You may now proceed with your Data collection, subject to clearance with the Director General, National Commission for Science, Technology & Innovation.

As you embark on your data collection, please note that you will be required to submit to Graduate School completed Supervision Tracking Forms per semester. The form has been developed to replace the Progress Report Forms. The Supervision Tracking Forms are available at the University's Website under Graduate School webpage downloads.

Thank you.

JULIA GITU
FOR: DEAN, GRADUATE SCHOOL

CC. Chairman, Department of Zoological Sciences
Supervisors:

1. Dr. Jemimah Simbauni
C/o Department of Zoological Sciences
Kenyatta University

2. Dr. Lucy Kamau
C/o Department of Animal Sciences
Kenyatta University

3. Dr. Faith Onditi
Department of Tropical and Infectious Diseases
Institute of Primate Research
C/o Department of Zoological Science
Kenyatta University

APPENDIX III- Preparation of Reagents

Reagents

Sodium chloride (NaCl)

NaCl 1.6% - Weigh 1.6g of NaCl and dissolve in 100ml distilled water

NaCl 0.9%- Weight 0.9g of NaCl and dissolve in 100ml distilled water

NaCl 12%- Weigh 12g of NaCl and dissolve in 100ml distilled water.

APPENDIX IV: Inhibitory Concentration (50%) and Fractional Inhibitory Concentration

Inhibitory Concentration (50%)

Model Used by the Graph Pad Prism software to determine the inhibitory concentration (50%) was done as follows:

$$Y = \text{Bottom} + \frac{(\text{Top-Bottom})}{1+10^{(\text{LogIC}_{50}\text{-X})\text{-Hill Slope}}}$$

Where Top is the Y value of the Top Plateau and Bottom is the Y value of the Bottom plateau and IC_{50} is the 50% inhibitory concentration.

Fractional inhibitory concentration calculations (FIC)

$$\text{FIC} = \frac{\text{Fraction of drug concentration required to produce } IC_{50} \text{ when used in combination}}{\text{Fraction of drug concentration required to produce } IC_{50} \text{ when used alone.}}$$

$$\text{FIC (AST-MB 1:1)} = \frac{IC_{50} \text{ of (AST-MB 1:1)}}{IC_{50} \text{ of AST}} + \frac{IC_{50} \text{ of (AST-MB 1:1)}}{IC_{50} \text{ of MB}}$$

$$\text{FIC (AST-MB 3:1)} = \frac{IC_{50} \text{ of (AST-MB 3:1)}}{IC_{50} \text{ of AST}} + \frac{IC_{50} \text{ of (AST-MB 3:1)}}{IC_{50} \text{ of MB}}$$

$$\text{FIC (AST-MB 1:3)} = \text{IC50} \frac{\text{ of (AST-MB 1:3)}}{IC_{50} \text{ of AST}} + \frac{IC_{50} \text{ of (AST-MB 1:3)}}{IC_{50} \text{ of MB}}$$

Calculations for FIC values for *Plasmodium falciparum 3D7:*

FIC (AST-MB 1:1) = 23.24/23.12 + 23.24/17.96 = 2.3
FIC (AST-MB 3:1) = 22.28 /23.12 + 22.28/17.96= 2.2
FIC (AST-MB 1:3) = 34.16/23.12 + 34.16/17.96= 3.4

Calculations for FIC values for *Plasmodium falciparum W2:*

FIC (AST-MB 1:1) = 29.23/23.55 + 29.23/ 7.69= 5.0
FIC (AST-MB 3:1) = 26.14/23.55 + 26.14/ 7.69= 4.5
FIC (AST-MB 1:3) = 15.07/23.55 + 15.07/7.69= 2.6

APPENDIX V- Recording of clinical symptoms data sheet

DATE:

Toxicity Assay Cage Number:									
Time									
Animal number									
Gender									
Body weight									
Weight of cubes									
Water									
Characteristics of stool									
Body fur									
Urine color, pH									
Behavior patterns									
Eyes									
Ears									
Mouth									
Tail									
Others									

APPENDIX VI- Mice body weights, food and water consumed

MEAN BODY WEIGHTS OF MICE (g)

Time	MB alone	AST alone	AST-MB (1:3)	AST-MB (3:1)	Control
	MEAN ± SEM				
0 hours	20.4±1.38	21.2±1.38	22.8±1.38	20.6±1.38	22.8±1.38
2 hours	19.8±1.10	21.6±1.10	22.0±1.10	21.0±1.10	23.8±1.10
4 hours	19.8±1.16	21.8±1.16	21.4±1.16	22.6±1.16	21.4±1.16
24 hours	20.4±1.57	20.4±1.57	21.8±1.57	20.4±1.57	21.2±1.57
26 hours	19.8±1.12	21.6±1.12	22.2±1.12	23.0±1.12	22.8±1.12
28 hours	19.8±1.21	22.2±1.21	21.6±1.21	22.4±1.21	22.4±1.21
48 hours	20.6±1.27	21.8±1.27	21.2±1.27	21.6±1.27	23.0±1.27

WEIGHT OF FOOD (g) IN EACH GROUP OF MICE

Time	MB alone	AST alone	AST-MB (1:3)	AST-MB (3:1)	Control
	MEAN PELLETS PER CAGE				
0 hours	100	100	100	100	100
2 hours	90	95	98	92	85
4 hours	90	80	98	83	75
24 hours	88	41	46	43	15
26 hours	87	39	43	42	10
28 hours	80	32	39	15	6
48 hours	39	31	15	6	0

WATER (ml) IN EACH GROUP OF MICE

Time	MB only	AST only	AST-MB (1:3)	AST-MB(3:1)	Control
			MEAN WATER PER CAGE		
0 hours	300	300	300	300	300
2 hours	300	300	300	299	280
4 hours	300	280	299	298	270
24 hours	290	275	275	260	248
26 hours	290	275	275	250	230
28 hours	280	280	260	200	210
48 hours	255	270	250	200	200

APPENDIX VII: Gross pathology recording data sheet

Toxicity assay of astemizole methylene blue combination therapy in Balb/c mice (Gross pathology)

Date and time:						
Group mouse number	Heart	liver	kidney	spleen	lungs	Brain
	Color: Shape: Weight: Others:	Color: Shape: Weight: Others:	Color: Shape: Weight: Others:	Color: Shape: Weight: Others:	Color: Shape: Weight: Others:	Color: Shape: Weight: Others:
	Color: Shape: Weight: Others:	Color: Shape: Weight: Others:	Color: Shape: Weight: Others:	Color: Shape: Weight: Others:	Color: Shape: Weight: Others:	Color: Shape: Weight: Others: