ANTI-PLASMODIAL AND IMMUNOMODULATORY EFFECTS OF COMBINED *AJUGA REMOTA* AND *CAESALPINIA VOLKENSII* LEAF EXTRACTS DURING MALARIA INFECTION IN BALB/C MICE

KAMAU LUCY WANJERI

MASTER OF SCIENCE

(Zoology)

JOMO KENYATTA UNIVERSITY OF

AGRICULTURE AND TECHNOLOGY

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Anti-plasmodial and Immunomodulatory Effects of Combined *Ajuga remota* and *Caesalpinia volkensii* Leaf Extracts during Malaria Infection in Balb/c Mice

Kamau Lucy Wanjeri

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DECLARATION

This research thesis is my original work and not been presented for a degree at any other university.

Signature…………………………… Date…………………………………….

Kamau Lucy Wanjeri

This thesis has been submitted for examination with our approval as university supervisors.

Signature……………………………… Date……………………………………

Prof. Rebecca Waihenya

JKUAT, Kenya

Signature……………………………… Date……………………………………

Dr. Lucy Ochola

IPR, Kenya

DEDICATION

This project is dedicated to my classmates who have taken up the challenge to participate in conducting a project as well. It is also dedicated to my husband and relatives who have been very supportive by all means. Finally, it is dedicated to my fellow scientists who are dealing with natural products since it poses a promising research area in drug development.

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The Institute of Primate Research staff cannot go unnoticed for every support availed by them. Special thanks to the National Museums of Kenya for identifying the two plants used for the study. JKUAT chemistry department also allowed me to extract the leaves, which was highly appreciated. I appreciate the Institute of Primate Research ethics committee for reviewing my work and giving it an ethical clearance.

I managed to attend conferences here in Kenya and one in South Africa. I appreciate South African Association for Laboratory Animal Science for giving me an opportunity to attend their conference in Stellenbosch, South Africa. 3rd Africa International Biotechnology and Biomedical Conference held at Nairobi, Kenya also gave me an opportunity to present my work. Finally, 8th KEMRI Annual Scientific and Health (KASH) Conference, Nairobi, Kenya gave me an opportunity to give an oral presentation.

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LIST OF ABREVIATIONS

AD	Deionized water
ANOVA	Analysis of variance
AT-rich	Adenine thymine rich
Ar	*Ajuga remota*
BSA	Bovine serum albumin
CM	Cerebral malaria
CO_2	Carbon dioxide
CSF	Cerebrospinal fluid
Cv	*Caesalpinia volkensii*
DC	Dendritic cells
DDT	Dichlorodiphenyltrichloroethane
DHA	Dihydroartemisinin
DOMC	Division of malaria control
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FOXP3	Fork-head box p3

HEPES	4-(2-hydroxyethyl)-piperazineethanesulfonic acid
HIS	Heat inactivated serum
HRP	Horseradish peroxidase
IFN-γ/α	Interferon-gamma/alpha
IgG	Immunoglobulin G
IL	Interleukin
iNOS	Inducible nitric oxide synthase
I.P.R	Institute of Primate Research
ITNs	Insecticide treated nets
J.K.U.A.T	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenya Medical Research Institute
LPS	Lipopolysaccharide
MHC	Major Histocompatibility Complex
N_2	Nitrogen
NaOH	Sodium hydroxide
NIOS	National Institute of Open Schooling
NO	Nitric oxide
O_2	Oxygen
PAMPs	Pathogen associated molecular patterns
P.	*Plasmodium*

PBS Phosphate-buffered saline

PI Principal Investigator

PRBC Parasitized red blood cells

RBC Red blood cells

ROS Reactive oxygen species

RNA Ribonucleic acid

TGF-β Transforming growth factor beta

Th cells Thymus helper cells

TLRs Toll like receptors

TMB Tetramethylbenzidine

TNF-α/β Tumor necrosis factor-alpha/beta

T-regs Regulatory thymus cells

UV- Ultra-violet

WHO World health organization

ABSTRACT

Malaria remains one of the most devastating infectious diseases leading to approximately 216 million infections and 445,000 deaths each year. Artemisinin combined therapy remains the first line treatment for malaria for over a decade, however there are concerns about emerging resistance especially after recrudesce was observed in South East Asia. This, therefore, stresses the need to search for alternative effective antimalarial treatment remedies. Several other plants apart from *Artemisia annua* have been found to have comparable anti-malarial effects either as a single preparation or a combination of different plants. This study evaluated the anti-malarial and immunomodulatory activities of *Ajuga remota* and *Caesalpinia volkensii* leaf extracts in *Plasmodium berghei* infected Balb/c mice as single and combined therapies. *Ajuga remota* and *Caesalpinia volkensii* leaves were extracted using ethanol and petroleum ether respectively. To assess the antimalarial activity, *in vitro* based inhibition assays were performed using *P. falciparum* 3D7. The individual extracts were at 2000 µg/ml while the combined extract had eleven combinations from 0:100 to 100:0 for *A. remota* + *C volkensii*. Chloroquine was used as a control drug at 500 µg/ml. The impact of individual and combinations of these plant extracts on the immune response was evaluated in Balb/c mice infected with *P. berghei*. To evaluate the impact of individual and a combination of the two plant extracts, five groups of mice (n=15 each) were inoculated with 200µl of 10^7 *P. berghei* parasites. At 2, 24, 48 and 72 hours post infection; group 1 was not treated; the second group received dihydroartemisinin (DHA); the third received *A. remota* extract; the fourth *C. volkensii* while the last group received the combined extract in the ratio of 1:1. Animals were monitored for 10 days where parasitaemia was recorded on day 2, 4, 5 and 6. At days 0, 6, 8 and day 10 post-infection three mice per group were euthanized, serum and spleens samples were obtained. Levels of immunoglobulin G (IgG) and interferon-γ (IFN-γ) were determined using enzyme-linked immunosorbent assays (ELISAs). Analysis of variance and Kruskal-Wallis non-parametric test were then done. Percentage growth inhibitions obtained were, *Ajuga remota* 44.9%; *Caesalpinia volkensii* 57.9% while the highest combined concentration gave 59.12% inhibition *in vitro*. *In vivo* antimalarial activity assessed using the 4-day suppressive assay, showed that *P. berghei* growth in mice was suppressed by 83.66% using a combination of *A. remota* and *C. volkensii* plant extracts, while each of the extracts suppressed growth of parasites by -23.31% and 4.34% respectively and DHA by 54.2%. IgG and IFN-γ levels in the treated groups were compared to the negative control group. IgG and IFN-γ levels ($p = 0.0052$ and $p = 0.0331$ respectively) varied during different time points showing significant difference in various groups. In conclusion, individual plant extracts though inhibited *P. falciparum in vitro*, they were not effective in suppressing *P. berghei* infection in mice. A combination of *A. remota* and *C. volkensii* plant extracts was effective at suppressing parasite growth *in vivo* just as the conventional drug DHA. Administration of the extracts affected the IgG and IFN-γ levels as compared to the baseline levels. *A. remota* herbs and *C. volkensii* trees should be safeguard as this study provides that they are a source of an alternative malaria treatment remedy.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Malaria is a vector-borne disease caused by *Plasmodium* species, which include *P. falciparum, P, vivax*, *P. malariae*, *P. ovale* and *P. knowlesi* (Sato, 2021). Most malaria-induced mortality is caused by *P. falciparum* transmitted by female *Anopheles* mosquitoes from one host to another (Good & Doolan, 2010). Globally 3.3 billion people are at risk of malaria infection and development of the disease and in Africa, there are 278 million people exposed to the parasite due to lack of proper control measures (WHO, 2014).

There were 229 million malaria cases in 2019 compared to 228 million cases in 2018 (WHO, 2021). The estimated number of malaria deaths was at 409 000 in 2019, compared with 411 000 deaths in 2018 (WHO, 2021). Pregnant women, Children under 5 years and HIV patients still remain at risk of getting malaria (WHO, 2021). Africa's burden was about 94% of all malaria deaths with the following countries accounting for half of all the deaths: Nigeria (23%), the Democratic Republic of the Congo (11%), United Republic of Tanzania (5%), Burkina Faso (4%), Mozambique (4%) and Niger (4%) (WHO, 2021). In Africa, there are 274000-child deaths in 2019 accounting for 67% of all malaria deaths globally (WHO, 2021).

Several efforts have been made to control malaria using vector control strategies and chemotherapy. Malaria vector control measures that include insecticides such as DDT, lindane, and dieldrin were used extensively during the mid-1900s until 1970 and then stopped due to environmental concerns (Gillen, 2007; Corbel & Guessan, 2013). In India

attempts to control vectors led to infections vanishing in the 1960s and recurring in 1976 (Sharma *et al*., 2011). In Kenya, under the Division of Malaria Control, the following strategies have been undertaken: use of insecticide-treated nets (ITNs); case management, improving malaria preparedness and response (Hay *et al*., 2003; National Malaria Control Programme, 2019). Following the increased ownership of ITNs from 50% in 2010 to 80% in 2016, led to 54% of the people at risk being protected (WHO, 2017).

Chemotherapy remains the most effective way of fighting the disease. A number of drugs such as chloroquine, pyrimethamine, quinine, artemisinin have been used to treat malaria. Failure to control the disease has been due to the emergence of resistance. A number of these drugs are derived from plants such as quinine and artemisinin that have high potency (Woodrow, *et al*., 2005; Uzor, 2020). Artemisinin combination therapy is currently used as the first-line antimalarial drugs (WHO, 2014). Intermittent preventive treatment in pregnancy with sulfadoxine-pyrimethamine has assisted in protecting women while 15 million children have been protected through seasonal malaria chemoprevention programs in Africa (WHO, 2017).

Cases of reduced sensitivity of the parasite to artemisinin were reported in Greater Mekong sub-region (WHO, 2014). With this trend, it is highly likely that the parasite will eventually develop resistance to any drug that is used widely. If artemisinin resistance occurs, then, controlling malaria may become a challenge. This highlights the urgent need to conduct tests and come up with a new alternative treatment to counteract any resistant to artemisinin.

Herbal drugs are safe and efficient in treating a number of ailments; however they require further scientific verification (Obidike, *et al*., 2015). It is, therefore, necessary to find out if plants used traditionally to cure malaria are effective or even better in malaria

treatment (Ginsburg & Deharo, 2011). Plants that are used traditionally as antimalarial agents, offer a valuable or alternative source of drugs. In Kenya, a study carried out in the Central region, identified 58 species in 54 genera and 33 families were identified as anti-malarial herbal remedies (Njoroge & Bussmann, 2006). The most common species are *C. volkensii*, *Strychno shenningsii*, *A. remota*, *Warburgia ugandensis* and *Olea europaea*. *Ajuga remota* is also called bulge weed, round pine or carpet bulge while *Caesalpinia volkensii* also known as harms and both plants are used in Embu County to treat malaria (Kareru *et al*., 2007). The two plants are also among the commonly used species for curing malaria especially within Central Kenya (Njoroge & Bussmann, 2006).

Gitua and others (2012) assessed *A. remota* for *in vivo* antimalarial activity using *P. berghei* in mice and found that the leaves extracts were the most effective in suppressing the parasite levels. In comparison, Kuria and colleagues (2001) concluded that although *C. volkensii* extracts are effective as antimalarials against chloroquine sensitive strain than the resistant strain of *P. falciparum*, it is not as effective as *A. remota*. Both plants elicit a concentration-dependent growth inhibition of the malaria parasite (Kuria *et al*., 2001; Cocquyt, *et al*., 2011).

During malaria infection Th1, Th2, B cells and macrophages are rapidly activated (Perez-Mazliah & Langhorne, 2015). IFN-γ contributes to the immunity against blood stage infection rendering protective immune responses (Gun, *et al*., 2014). IFN-γ contributes to the immunity against blood stage infection rendering protective immune responses and further induces IgG blood stage specific antibodies (Gun, *et al*., 2014). A study conducted by Ma and colleagues 2007 showed that administration of artesunate and chloroquine never had any impact on the immune system compared to the untreated

group. Although plants are able to exhibit strong antimalarial activities, very few studies have evaluated the effects of plant extracts on immunomodulation.

Immunomodulation comes as a result of antibody and cell mediated responses such as cytokines and antibody production during an infection. Thus these immune molecules with an introduction of an extract would have an impact on the immune responses (Namrata, *et al*., 2016). This study, therefore, analyzed the efficacy of a combination of crude extracts of *A. remota* and *C. volkensii* and the impact on IgG and IFN-γ responses in *P. berghei* infected mice.

1.2 Statement of the problem

Malaria is endemic in some parts of Kenya as well as other parts of the world and require more interventions. The emergence of *Plasmodium* resistance to available drugs still remains a threat. More so given that the first line drug available is artemisinin-based combination therapy that continues to benefit many. However, health officials are concerned about its ongoing effectiveness and are struggling to implement policies to slow the spread of artemisinin resistance.

Due to these concerns, scientists continue to search for new approaches, sparking collaborations and spurring people to take action to fight the emergence of further resistance. Alternative approaches include searching for new or modifying available drugs or utilizing plants known to exhibit antimalarial activity. *C. volkensii* and *A. remota* plant extracts *in vitro* tests have shown anti-plasmodial activities against chloroquine sensitive and resistant strains. However, the effect on the immune system during infections with *P. berghei* and administration of the two combined plant extracts has not been evaluated.

1.3 Justification

Malaria is one of the major infectious diseases in the world where 3.3 billion people are at risk of getting malaria while in Africa 278 million people are exposed to the parasite. In as much as there are a number of control measures in place to prevent malaria, lack of control leads to 170 million working days lost. Although control measures are in place, epidemics are possible in areas where mosquitoes persist. A number of drugs have been used to cure the disease but they are faced with resistance hence bringing about the search for new drugs. Artemisinin combined therapy is the remedy left as the first line treatment. However, there is reduced sensitivity in some areas of the world. This, however, charges an urgent need to conduct tests to come up with new alternative drugs.

Traditional plants have been investigated and have shown positive results against *Plasmodium* parasites and currently offer an alternative source of antimalarial drugs. From the many plants known in Kenya, *A. remota* and *C. volkensii* exhibit antiplasmodial activity but there are no studies comprising the two combined. The impact of plants activity on the immune system is still not well defined. The Kenya Food and Drugs Authority bill recommend that immunomodulation and autoimmunity study be conducted on all investigational drugs. Therefore, it is important to establish the effects of combined *A. remota* and *C. volkensii* leaf extracts on the immune system. This research will, therefore, investigate the effects of combined *A. remota* and *C. volkensii* extracts therapy on *P. berghei* ANKA infected Balb/c mice.

1.4 Hypothesis

A combination of *Ajuga remota* and *Caesalpinia volkensii* (*Ar* + *Cv*) plant extracts does not suppress *Plasmodium berghei* growth or alter IgG and IFN-γ levels during malaria infection in mice.

1.5 General objective

To evaluate antiplasmodial and the modulation of the immune system by combined leaf extracts of *Ajuga remota and Caesalpinia volkensii* during malaria infection in BALB/c mice.

1.6 Specific objectives

1. To evaluate the effect of combined ethanol *A. remota* and petroleum ether *C. volkensii* extracts on *P. falciparum in vitro* and *P. berghei in vivo*.

2. To determine the changes in total IgG levels upon administration of a combination of *Ajuga remota* and *Caesalpinia volkensii* extracts in *P. berghei* infected BALB/c mice.

3. To determine the changes in IFN-γ levels upon administration a combination of *Ajuga remota* and *Caesalpinia volkensii* extracts in *P. berghei* infected BALB/c mice.

CHAPTER TWO

LITERATURE REVIEW

2.1 Malaria

2.1.1 Etiology

Malaria is a mosquito-transmitted parasitic disease caused by *Plasmodium* species. *Plasmodium* parasites are microscopic protozoa that belong to phylum Apicomplexa (Sato, 2021). The parasite is in the order Sporozoa and genus *Plasmodium*. *Plasmodium* species include *P. falciparum, P. vivax*, *P. malariae*, *P. ovale* and *P. knowlesi* (Wiser, 2019). Female *Anopheles* mosquito transmits the parasite. The disease characterization is by chills, intense fever, and sweating and often includes other symptoms such as headache, malaise, fatigue, body aches, nausea, and vomiting (Marrelli & Brotto, 2016).

2.1.2 Life cycle of *Plasmodium*

The infection starts when a *Plasmodium*-infected female mosquito takes a blood meal from the human or animal host. The mosquito deposits the sporozoites present in the salivary gland into the skin through its proboscis. The sporozoites can remain in the skin for at least six hours after inoculation (Yamauchi *et al*., 2007; Sinnis & Zavala, 2012). Once in the circulation, they get into the liver where the infected hepatocytes undergo exo-erythrocytic schizogony and yield pre-erythrocytic schizonts that contain 10,000 to 30,000 merozoites (Nureye & Assefa, 2020). Some species have a dormant stage called hypnozoite. They remain for weeks to years in the liver; before the development of pre-erythrocytic schizogony.

The erythrocytic stage starts in the blood. A single merozoite enters an erythrocyte and replicates to about 16 merozoites before their rapture (Good & Doolan, 2010). Within the erythrocyte, the merozoites develop into a ring, trophozoite and schizont stages (Nureye & Assefa, 2020). Some of the merozoites differentiate into sexual forms, macrogamete (female) and microgamete (male) (Nureye & Assefa, 2020).

The mosquito, therefore, takes in the two gametes and the gametogenesis proceeds (Nureye & Assefa, 2020). The microgamete fertilizes the macrogamete forming a zygote. The zygote develops into ookinete that penetrates the mid-gut epithelial cells and differentiates into an oocyst. Oocysts undergo asexual replication and produce sporozoites (Gillen, 2007; Bennink, *et al.*, 2016). The sporozoites are deposited into hemocoel that later invades the salivary gland (Perlmann & Troye-Blomberg, 2002; Sato, *et al.*, 2014). When the mosquito takes another blood meal from a susceptible vertebrate, the cycle begins again as shown in figure 2.1 below (Granberg, 2010).

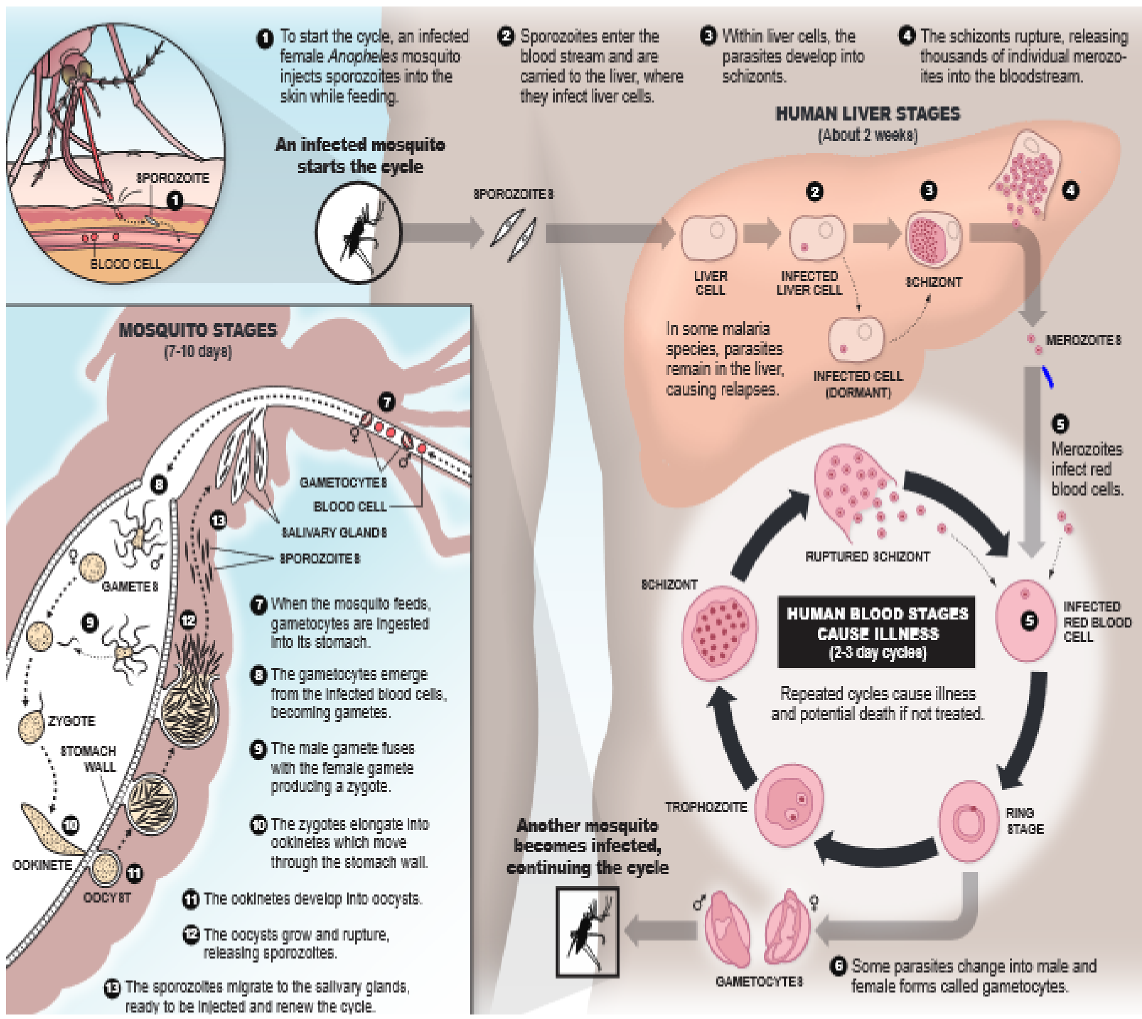

Figure 2.1: The life cycle of malaria (Granberg, 2010)

2.1.3 Immunology in the *Anopheles* mosquito

The first line of defense consists of the structural barriers of the surface exoskeleton (cuticle) and the peritrophic matrix of the mid-gut (Kumar, *et al.*, 2018). Mechanisms of insect immunity include cellular phagocytosis and melanization, serine protease cascades leading to coagulation and humoral melanization, and transient production of antimicrobial peptides by the fat body, hemocytes, and epithelial tissues (Rosales, 2017).

A number of potential pattern recognition receptors have been characterized which include proteins that can bind LPS, peptidoglycan, β-1,3-glucan, and chitin (Rosales, 2017).

Molecular incompatibilities may prevent essential interactions between parasite–mosquitoes, like in *Anopheles freeborni* and *Plasmodium knowlesi* combination where the parasite does not invade the salivary glands (Perlmann & Troye-Blomberg, 2002). These incompatibilities caused by the mosquito's innate defense reactions against the parasite have also been documented (Ghosh, *et al*., 2000). One of the mosquito defense mechanism against macroparasites is melanotic encapsulation, which leads to the deposition of electron-dense melanin-containing material that immobilizes and kills the parasite on the basal side of the mid-gut epithelium (Boissière, *et al*., 2012). Another refractory mechanism is the lysis of ookinetes in the mid-gut epithelium of a selected *A. gambiae* strain (Perlmann & Troye-Blomberg, 2002).

2.1.4 Immunity to *Plasmodium*

Immunity to *Plasmodium* infection starts from innate to adaptive (Good & Doolan, 2010). Innate immunity is triggered when pathogen-associated molecular patterns (PAMPs) are recognized by Toll-like receptors (TLRs) which lead to secretion of pro-inflammatory cytokines, chemokines, and interferons (Kinyanjui, 2012). TLR2 and TLR4 recognize the malaria parasite glycophosphoinositol (Franklin *et al*., 2009). Hemozoin, a product of hemoglobin when digested by *Plasmodium*, is recognized by TLR9 (Franklin, *et al*., 2009). However, it is possible that innate immunity induced by non-TLR receptors and AT-rich parasite DNA fragments exist (Sharma *et al*., 2011).

The dendritic cells (DC) recognize the sporozoites in the skin. Some of the sporozoites leave the skin and get to the lymph nodes where the DCs internalize them (Amino *et al*.,

2006; Yap, *et al*., 2019). In the liver, the infected cells are processed and presented on the surface by MHC class 1 or class 11 molecules recognized by $CD8^+$ or $CD4^+$ T cells, respectively (Good & Doolan, 2010). The activated $CD8^+$ T cells get into the liver and eliminate the infected cells (Chakravarty *et al*., 2007). The $CD8^+$ T cells kill them directly by various cytotoxicity mechanisms (Kinyanjui, 2012).

The release of cytokines occurs in both innate and adaptive responses. Early interferon gamma (IFN-γ) prevents the progress of the disease (Perlaza *et al*., 2011). Natural killer cells are a source of early pro-inflammatory responses like IFN-γ and TNF-α against *Plasmodium* (Korbel *et al*., 2005; Burrack, *et al*., 2019). IFN-α/γ, TNF-β and interleukin-12 (IL-12) are produced by T helper cell when $CD4^+$ cell differentiate to Th1 while Th2 produces IL-4, 5, 6, 9, 10 and 13 associated with the production of antibodies during type 1 and type 2 responses respectively (Kinyanjui, 2012). Type 1 responses bring about parasite killing mediated by IFN-γ, TNF-α and nitric oxide secreted by Th1, macrophages and NK cells (Kinyanjui, 2012). The characterization of type 11 responses shows less symptomatic chronic infections (Frimpong, *et al*., 2018). It also inhibits pro-inflammatory cytokines and stimulates B-cells to secrete antibodies IgG and IgM (Ly & Hansen, 2019).

The pro-inflammatory cytokines, e.g. TNF-α, IFN-γ, IL-1B, and IL-10, were thought to be responsible for cerebral symptoms, whilst anemia was associated with low levels of IL-12 and transforming growth factor-β (Perkins, *et al*., 2000). *P. falciparum*-infected erythrocytes produce pyrogenic material which triggers the release of TNF (and other cytokines) from host mononuclear cells (Dunst, *et al*., 2017).

Elevations of TNF result in pyrexia in both *falciparum* and *vivax* malaria (Perlmann & Troye-Blomberg, 2002). Furthermore, elevations in TNF can exert antiparasitic effects by inhibiting parasite multiplication and synergizing with other factors to produce

gametocidal effects (Perlmann & Troye-Blomberg, 2002). While TNF is the best-researched inflammatory mediator in malaria, there is also evidence of increased IL-10 production in severe malaria, associated with elevations in TNF levels that may contribute to severe disease (Wilson, *et al*., 2010). Thus increased production of TNF early in malarial infection may be protective, whereas prolonged, high TNF levels may be harmful.

Regulatory T-cells (T-regs) express CD25 and FOXP3 markers that enable them to mediate their actions through IL-10 and transforming growth factor-β (TGF-β) (Kinyanjui, 2012). These cytokines reduce immunopathology by suppressing the pro-inflammatory cytokines (Nie *et al*., 2007). However, when anti-inflammatory cytokines are induced too early, they can suppress the pro-inflammatory cytokines hence allowing high parasitemia levels (Walther *et al*., 2005; Kumar, *et al*., 2019). Transforming growth factor-β appears to be associated with increased risk of clinical disease and high parasite growth (Walker, *et al*., 2014). There is a need for a balance to ensure cure as shown in the figure below (Good & Doolan, 2010).

In response to parasite ligands recognized by pattern-recognition receptors, such as TLRs and CD36, or inflammatory cytokines, such as IFN-γ, DCs mature and migrate to the spleen. The maturation of DCs has associated with the up-regulation of expression of MHC class II molecules, CD40, CD80, CD86, and adhesion molecules and the production of cytokines including IL-12. IL-12 activates NK cells to produce IFN-γ and induces the differentiation of Th1cells (Good & Doolan, 2010). Figure 2.2 below shows possible regulation of adaptive immunity to blood-stage malaria by cytokines produced by cells of the innate immune response

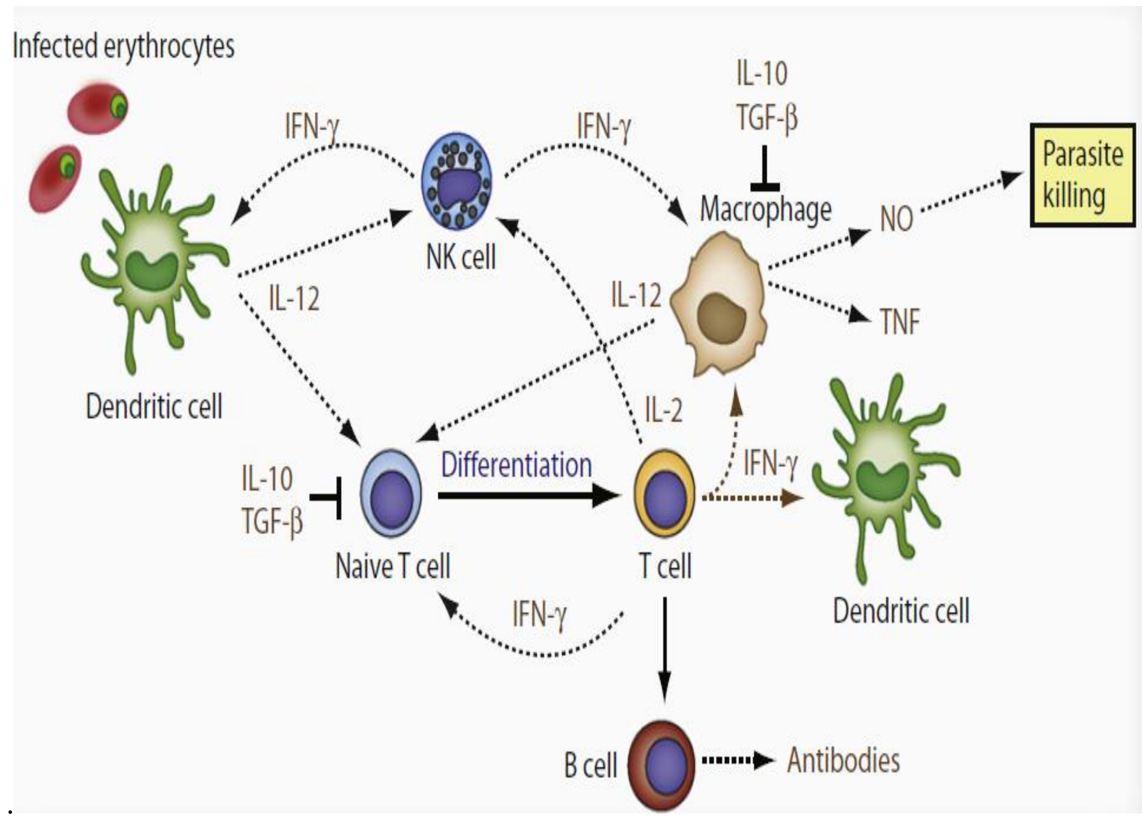

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Figure 2.2: Linking innate and adaptive immunity to blood-stage malaria (Good & Doolan, 2010)

Therefore, the production of cytokines, particularly IFN-γ, by NK cells results in DC maturation and enhances the effect of parasite-derived maturation stimuli, facilitating the clonal expansion of antigen-specific naive $CD4^+$ T cells. IL-2 produced by antigen-specific Th1 cells further activates NK cells to produce IFN-γ, which induces DC maturation and activates macrophages, further amplifying the adaptive immune response (Good & Doolan, 2010). Cytokines such as IL-10 and TGF-β negatively regulate both innate and adaptive responses (Good & Doolan, 2010).

2.1.5 Antimalarial drugs

Among the treatment drugs used include: Artemisinin and Derivatives, Atovaquone, Proguanil, Pyrimethamine, Sulfadoxine, Chloroquine/Hdroxychloquine, Quinine/Quinidine, Mefloquine and Primaquine. Table 2.1 shows their mode of action and mechanisms of resistance.

Table 2.1: Antimalarial drug mechanisms of action and resistance (Basicmedical, 2016)

DRUG	MECHANISM OF ACTION	MECHANISM OF RESISTANCE
Artemisinin and Derivatives	Production of toxic heme-adducts	Not known at this time
Atovaquone	Inhibits mitochondrial electron transport in the cytochrome bc, complex	Nucleotide polymorphisms in the cytochrome b gene
Proguanil	Inhibits dihydrfolate reductase-thymidylate synthase	Mutations in the amino acid sequence near the dihydrofolate-reductase binding site
Pyrimethamine	Inhibits *Plasmodium* dihydrofolate-reductase	Mutation in dihydrofolate-reductase binding site
Sulfadoxine	Inhibits *Plasmodium* dihydropteroate synthase	Mutations in dihydropteroate synthase gene
Chloroquine/ Hdroxychloquine	Production of toxic heme adducts	Production of chloroquine efflux transporter
Quinine/Quinidine	Production of toxic heme adducts	Production of efflux transporter; amplification of *pfmdr* 1 gene
Mefloquine	Production of toxic heme adducts There is also a cytosolic mode of action	Amplification of *pfmdr* 1 gene that accumulates drug in digestive vacuole away from cytosolic site of action
Primaquine	Production of reactive oxygen species	Not known at this time

Various drugs have been used as emergency standby treatment remedies. The table 2.2 below shows how the medication is done during treatment.

Table 2.2: Emergency standby treatment (Chiodini, *et al*., 2015)

Situation	Medication	Adult Dosing Regimen	Remarks
	20 mg artemether + 120 mg lumefantrine (available as a combination preparation)	4 tablets initially, followed by 5 further doses of 4 tablets each given at 8, 24, 36, 48 and 60 hours (total of 24 tablets over 60 hours)	Take with food to enhance drug absorption
	250 mg atovaquone + 100 mg proguanil (available as a combination preparation)	4 tablets as a single dose daily for 3 consecutive days	NA
	300 mg quinine + 100 mg doxycycline	Quinine, 2 tablets 3 times a day for 3 days; doxycycline, 1 tablet twice a day for 7 days	NA
Pregnancy	300 mg quinine + 150 mg clindamycin	Quinine, 2 tablets 3 times a day for 5-7 days; clindamycin, 3 tablets 3 times a day for 5 days	Pregnant travelers should avoid malarious areas

Mg= miligram

2.1.6 Mouse models of blood stage malaria infections

An overall description of the major rodent parasites that have been used to investigate the mechanisms of immunity and pathogenesis is given in table 2.3. Some parasites give rise to lethal infections in all strains of mice, others are lethal only in some strains and

some parasites are nonlethal in all immunologically competent mice. Nonlethal infections are generally used to investigate mechanisms of immunity and immunoregulation. Several parasites, such as *P. berghei* (ANKA; PBA), *P. vinckei* and *P. chabaudi* have been used extensively for studying pathology (Perlmann & Troye-Blomberg, 2002).

Table 2.3: Experimental malaria infection in different mice strains (Perlmann & Troye-Blomberg, 2002 and Li, *et al.*, 2001).

Plasmodium	**Strain/clone**	**Mouse strain**	**Lethal infection**	**Experimental study**	**Pathology**
berghei	ANKA	CBA/T6 BALB/c C57BL/6	Yes Days 6-8	Pathogenesis of CM	CM, no anemia, no hypoglycemia
		DBA/21	Yes Days 15-22	Resolving CM model	Resolving CM anemia
	K173	CBA/T6 BALB/c DBA/21	Yes Days 15-22	Non CM Control for CM study	No cerebral involvement, anemia
		C57BL/6	Yes Days 6-8	Pathogenesis of CM	CM, no anemia
yoelii	17X	Swiss BALB/c	Yes Days 7-9	Pathogenesis of CM	CM with RBC sequestration, anemia and hypoglycemia
		CBA/Ca BALB/c C57BL/6	No	Immune mechanisms, pathogenesis	No CM, anemia, no hypoglycemia
	YM (lethal)	CBA/T6 BALB/c C57BL/6 DBA/21 Swiss	Yes Days 7-8	Vaccines	No cerebral involvement, hypoglycemia
chabaudi	AS	C57BL/6 CBA/Ca B10 series BALB/c	No Peak day 8-10	Immune mechanisms, pathogenesis	No cerebral involvement, anemia, hypoglycemia
		A/J DBA/21	Yes Day 9		
chabaudi adaml	556KA	BALB/c C3H C57BL/6	No Peak day 7-11	Immune mechanisms, pathogenesis	No cerebral involvement, anemia,
vincket		BALB/c	Yes Day 8	Chemotherapy, immune mechanisms, pathogenesis	No cerebral involvement, anemia, hypoglycemia
Vincket petterl	CR	C57BL/6 BALB/c	No Peak day 10	Chemotherapy, immune mechanisms	No cerebral involvement

Where CM = Cerebral Malaria; RBC = Red blood cells

2.2 Herbal medicine for malaria

Artemisinin and quinine derivatives are two main groups derived from traditional medicine (Merlin & Gerard, 2004). Traditional medicine is widely used to treat malaria since it is more available and affordable (Merlin & Gerard, 2004). However, there are few clinical data on safety and efficacy; the concentration of active ingredients in a plant species varies depending on several factors and there is no consensus on which plant, preparations, and dosages are the most effective (Merlin & Gerard, 2004).

2.3 Study plants

2.3.1 *Ajuga remota* (bugleweed, round pine or carpet bulge)

It is found in kingdom Plantae, phylum Tracheophyta, class Magnoliopsida, subclass Asterids, order Lamiales, family Lamiaceae, subfamily Teucrioideae, and tribe Ajugeae in genus *Ajuga* (Roskov, *et al*., 2014). Family Lamiaceae has about 236 genera and contains 6,900 to 7,200 species (Tamokou, *et al*., 2017). The genus of *Ajuga* contains at least 301 species (Cocquyt, *et al*., 2011). Its vernacular name in central Kenya is "Wanjiru wa rurii".

It grows at forest margins and drier bushland at an altitude of 1160-2800 meters above the sea level. Within East African Region, the plant is accessible in Kenya, Tanzania, and Uganda (http://tanzaniabiodiversity.com). The known isolated compounds include neoclerodane- diterpenes and diterpenoids, beta-sitosterol, hytoecdysteroids, gamma-sitosterol, triterpenes specific sterols, ceryl alcohol, quinols, withanolides, tannins, anthocyanidin-glucosides and iridoid glycosides, flavonoids, triglycerides and essential oils (Cocquyt, *et al*., 2011 and Pal & Pawar, 2011).

Ajuga remota is an important food for pigs and cattle (http://tanzaniabiodiversity.com). Almost all parts of this plant are bitter causing it to be inedible to most animals, insects, and birds (Kuria, *et al*., 2001). It is used to treat liver problems, pneumonia and in experimental hypertensive rats, it reduces pressure (Kuria, *et al*., 2001). Its leaves extract treat malaria, stomachache and other problems (Kuria, *et al*., 2001). In Ethiopian medicine, *A. remota* is widely used traditionally to treat stomach pain and high blood pressure, mainly in Bahirdar Zuria, Amhara region (Ragunathan & Abay, 2009).

2.3.2 Caesalpinia volkensii Harms

It is found in phylum Tracheophyta, class Magnoliopsida, order Fabales, family Fabaceae and from genus *Caesalpinia* (Roskov, *et al*., 2014). The genus contains about 200 species that are native to America. There are about 25 species indigenous, cultivated or naturalized in Africa. Its vernacular name in Central Kenya is mubuthi or mucuthi. There are other species apart from a *volkensii* like *C. truthae* Harms indigenous in Somali, Tanzania, Kenya, and Ethiopia, have medicinal uses (Schemelzer & Gurib-Fakin, 2008). *Caesalpinia volkensii* originates from Ethiopia, Kenya, Uganda, and Tanzania. It is found in forests and forest margins up to 2100 m altitude and near homesteads (Schemelzer & Gurib-Fakin, 2008).

Chemical assessment shows that it contains at least seven compounds namely: furanoditerpene; deoxycaesaldekarin D, voucapane, voucapan-1, 5-diol, caesaldekarin C, voucapan-5-ol, deoxycaesaldekarin C, 5-hydroxy vinhaticoic acid and three cinnamyl esters viz triacontanyl-(E)-ferulate, triacontanyl-(E)-caffaete and 30-hydroxytriacontanyl-(E)-ferulate (Ochieng, *et al*., 2011).

Caesalpinia volkensii extracts are important as they are used mostly in Kenya and Tanzania to treat malaria (Schemelzer & Gurib-Fakin, 2008). Its leaf decoction used to reduce pain during pregnancy (Schemelzer & Gurib-Fakin, 2008). Pregnant women take

its powdered pods to relieve stomachache when dissolved in water. Due to its roots having aphrodisiac properties, they are eaten, cooked or added to palm wine (Schemelzer & Gurib-Fakin, 2008). It has also been used to treat bilharzia and gonorrhea too (Schemelzer & Gurib-Fakin, 2008). The plant's seed treats stomach ulcers while crushed flower buds treat eye problems. In Tanzania, the roots make red dye. The fruits are poisonous though the leaves are good for feeding camels (Schemelzer & Gurib-Fakin, 2008).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study site

This study was conducted at the Institute of Primate Research – Karen and the plant leaves were obtained from Sagana in Kirinyaga County. Sagana is along the Nairobi-Nyeri highway, 100 km north of Nairobi (Global Positioning System, 2021). The latitude of Sagana is -0.669513, and the longitude is 37.206114. It is a city located at Kenya with the global positioning systems coordinates of 0° 40' 10.2468" S and 37° 12' 22.0104" E. The elevation of Sagana is 1197.985 m above sea level (Global Positioning System, 2021).

3.2 Plant leaves sampling design

A. remota plant leaves were randomly selected from various farms in Sagana town as they were readily available. *C. volkensii* plant leaves were obtained from only one tree which was locally available in January 2015 as most people had cut the trees down since they would occupy a large part of their farms.

3.3 Experimental design

Completely randomized block design which involves the principles of replication and randomization was used in the course of the experiment. This was used to assign animal subjects to the experimental treatments randomly. Randomization gave each group out of 75 mice, an equal opportunity of being assigned to either treatment 1, 2, 3, 4 or 5 where equal replications were applied. The treatments were as follows 1st group received

P. berghei only; 2nd received *P. berghei* and dihydroartemisinin (DHA); 3rd received *P. berghei* and plant extracts combination (*A. remota* + C. *volkensii*); 4th received *P. berghei* and *A. remota* while the 5th received *P. berghei* and *C. volkensii* (Figure 3.1). The mice were subjected to a 4 days suppressive test after which serum and spleen cells were obtained for immunological assays at days 6, 8 and 10. The design provided a maximum number of degrees of freedom to the error. (Kothari, 2004).

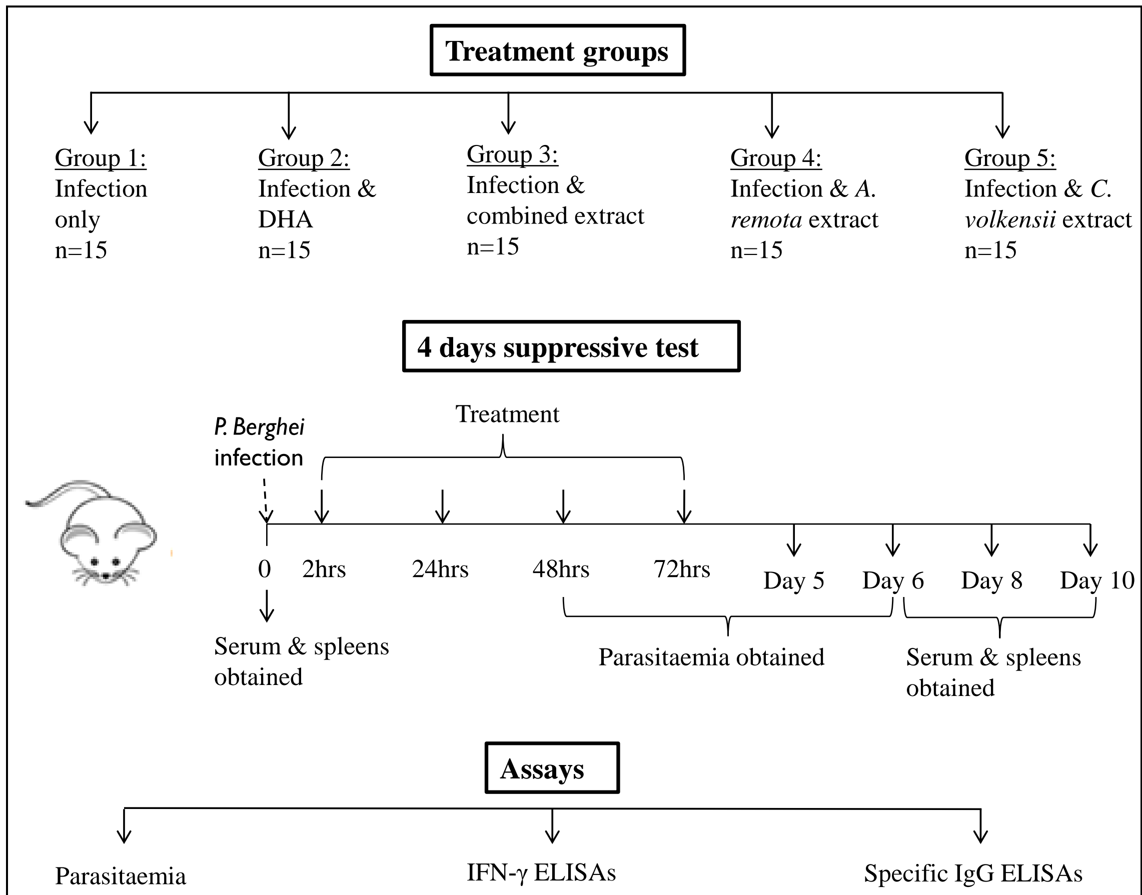


Figure 3.1: in vivo experimental design

Infection was done using *P. berghei* ANKA strain. DHA = Dihydroartemisinin was used as a positive control; the combined extract = *A. remota* ethanol extract + *C. volkensii*

petroleum ether extract; IFN-γ = interferon gamma; IgG = Immunoglobulin G and ELISAs = enzyme-linked immunosorbent assays. Treatment was done at 2 hrs, 24 hrs, 48 hrs and 72 hrs post infection during the 4-days suppressive test. Parasitaemia was obtained on days 2, 4, 5 and 6 post infection while serum and spleen cells for IgG and IFN-γ ELISAs were obtained on days 6, 8 and 10.

3.4 Study population and parasites

This study was carried out with Balb/c mice (males and females) aged 6-8 weeks acquired from the Institute of Primate Research (IPR), Kenya. The parasite strain was *P. berghei ANKA* (Fidock, *et al*., 2004) contributed by *Thomas McCuthchan*, MR4.

3.5 Sampling design and sample size determination

The mice were selected using simple random design. The sample size was 81 mice based on previous studies at IPR and published literature. The donor group had 6 mice. Seventy five mice were used for immunology tests. From the five groups, every group consisted of 15 mice where 3 mice were euthanized at days 0, 6, 8 and 10th. On day 0, only three naive mice were euthanized. Parasitaemia was monitored days 2, 4, 5 and 6.

3.6 Ethical approval

This study was approved by Institutional Science and Ethics Committee (IRC) of IPR; reference number: IRC/05/16 (appendix 1).

3.7 Laboratory procedures

3.7.1 Plant materials collection and preparation

Plant leaves are the most effective parts in parasite growth suppression. They were collected from Sagana, Kirinyaga and identified at the National Museum of Kenya (NMK) herbarium where voucher specimens were preserved. The leaves were then air dried under a shade at Jomo Kenyatta University of Agriculture and Technology (JKUAT). The plant materials were ground into a fine dark green powder using a hammer grinder and thereafter they were extracted in the JKUAT chemistry GK labs.

3.7.2 Extraction of plant leaves

The extracting solvents were selected from literature where *A. remota* was suitable while extracted using ethanol and *C. volkensii* with petroleum ether (Gitua, *et al.*, 2012). To 300 g of each of *A. remota* and *C. volkensii* powder was added to 1.5 liters of absolute ethanol (Sigma, US) and petroleum ether (Sigma, US) respectively. The mixture was allowed to settle for 48hrs, supernatant decanted and filtered through a whatman filter paper (Sigma, US). The extract was concentrated using a Büchi rotary evaporator R-900 and stored at 4°C (Ogila, 2011). Approximately 300 g of each *C. volkensii* and *A. remota* dried leaves powder, yielded 6.0 g of petroleum ether extract and 35.8 g of ethanol extract respectively.

3.7.3 Extracts assessment

3.7.3.1 Preparation of culture media

To prepare 500 ml of incomplete media, this was constituted with 18.75 ml of HEPES (Sigma Aldrich, USA), 5 ml of 20% glucose, 3 ml of 1M NaOH, 5 ml of 200 mm glutamine and 25 µl gentamycin in RPMI 1640 medium (Sigma Aldrich, UK). Complete

media was made by adding 10% heat-inactivated pooled human serum (Fidock, *et al*., 2004).

3.7.3.2 Preparation of human erythrocytes

Erythrocytes of O^+ blood group were obtained from KEMRI and cells washed three times with RPMI 1640 to separate the erythrocytes from the buffy coat and plasma. Cells were centrifuged at 1500 rpm for 10 minutes at room temperature. After the wash, the cells were supplemented with incomplete media and stored at 4ºC. The prepared cells were viable for 14 days to give an optimum parasite growth (Fidock, *et al*, 2004).

3.7.3.3 Extracts preparation for inhibition assay

The extracts were prepared by adding 100 mg of the extracts to 200 µl of ethanol and was allowed to dissolve. 10 mg/ml stock solution was made by topping it up to 10 ml with incomplete media for *in vitro* assays and filter sterilized using 0.45 µm syringe filter (Thermo Fisher Scientific, US). Out of the stock solution took 200 µl into 300 µL of complete me/dia to make 4 mg/ml.

3.7.3.4 Cultures preparation and maintenance

A cryovial containing *Plasmodium falciparum* 3D7 was thawed in a 37ºC water bath and in a sterile hood transferred the content into a sterile 50ml centrifuge tube. 12% NaCl solution was added drop-wise in the ratio of 1:5 to cell mixture while shaking and allowed to stand for 5 minutes. Ten volumes of 1.6% NaCl solution was added drop-wise while shaking. Another ten volumes of a 0.9% NaCl solution was added while shaking after which the parasites were centrifuged at 1500 rpm for 5 minutes at room temperature.

The pellet obtained was re-suspended by gently swirling the tube until most of it was suspended. Ten volumes of complete media were added while shaking and centrifuged at 1500 rpm for 5 minutes to aspirate the supernatant. To the pellet added 5 mls of complete media and transferred it to T25 culture flask. Uninfected RBCs were added and the flask was aerated with a gas mixture of 5% CO_2, 5% O_2 and 90% N_2. The cultures were maintained at 2-4% hematocrit and were incubated at 36-37ºC.

The medium was changed after every 48 hrs. The parasitemia was monitored by making a thin smear and giemsa staining was done for analysis under a microscope. To maintain the parasite some of the cultures were frozen using the heat-inactivated human serum and freeze medium. The vials were stored in Mr. frosty in -20ºC for 30 minutes then to -80ºC overnight and finally put in liquid nitrogen until when required.

3.7.3.5 *Plasmodium* inhibition assay

To test for anti-plasmodial activity, the reagents and the extracts needed were prepared. For every extract made 10 mg/ml in 4% ethanol and RPMI 1640. The stock solutions made were filter-sterilized and made into a concentration of 4mg/ml in complete media. Using normal human type O^+ RBC diluted parasitized red blood cells (PRBC) from *P. falciparum* 3D7 cultures infected with young trophozoites (ring stage) to a parasitemia of approximately 1% and adjusted the hematocrit to 2.5% in complete media. Duplicate tests in 96-well flat-bottomed tissue culture plates were set up and100µl of complete media were added to every well.

To start with 2000 µg/ml took out 200 µl of the 4 mg/ml extracts solution and added it to the first well in the 96 well culture plate. Combined *A. remota* and *C. volkensii* extract solutions was dispensed into the 96 well microtitre plates to give eleven combinations ratios of 100:0 (*A. remota* and *C. volkensii*), 10:90, 20:80, 30:70, 40:60, 50:50 and

0:100 (*A. remota* and *C. volkensii*) respectively (Kebenei, *et al*., 2012). The following controls were used chloroquine, ethanol, petroleum ether and RPMI 1640 in duplicates. 100 µl of 1.3% parasite culture at 2.5% hematocrit was added to the wells. The plates were incubated at 37°C for 48 hrs at a low O_2 gas. The supernatant was removed to get the pellet. Thin smears were made in duplicates and stained using Giemsa stain. The slides were stored at room temperature until they were analyzed under the UV-microscope (Zeizz, West Germany).

3.7.3.6 Study animals

This study was carried out with Balb/c mice (males and females) aged 6-8 weeks acquired from the Institute of Primate Research (IPR), Kenya. They were kept in an air-conditioned room at 22°C and 50-70% relative humidity and were fed with pellets (Unga farm care ltd, Nairobi, Kenya) and water *ad libitum*.

3.7.3.7 Preparation of plant extracts

The mice were weighed to get the average weight per group. Following the average weights obtained, the suitable extracts were measured ensuring that the groups received 80 mg/kg just like the artemisinin tablet. The extracts were mixed by adding 2.5% ethanol (Sigma, US) to every tube and mixed till dissolved. The ethanol was used for dissolving the extract as it was not soluble in 1X phosphate buffered saline (PBS). Finally, 1XPBS was added to make it to 8 ml. For the combined extract *A. remota* and *C. volkensii* was constituted as in the above step in the ratio of 1:1. DHA that was used as a +ve control drug was dissolved in PBS only to a final concentration of 10 mg/kg body weight.

3.7.3.8 Mice infection

A cryovial of parasites, *Plasmodium berghei ANKA,* stored in liquid nitrogen was thawed and washed with 0.9% NaCl followed by administration into naive recipient donor mice n=6 through intravenous route. The peripheral blood parasites were monitored from day 4. Blood from the donor mice was collected during ascending parasitaemia of 10% and above through cardiac puncture. The parasitized blood from the donor mice was diluted in sterile PBS to make 5 x 10^7 infected erythrocytes per milliliter. 200 µl of 10^7 infected erythrocytes were injected intraperitoneally into mice N=75 that were used in the test groups described above.

3.7.3.9 Four days suppressive test

The infected mice were randomly selected into five groups (n=15) and were marked. While group 1 was infected with malaria parasite only, groups 2, 3, 4 and 5 were subsequently subjected to a four day suppressive treatment regimen. Group 2 was treated with DHA; group 3 a combination of *A. remota* and C. *volkensii* (*Ar*E + *Cv*PE); group 4 *A. remota* (*Ar*E) and group 5 *C. volkensii* (*Cv*PE) as shown in figure 3.1.

The mice were treated after 2 hours post infection with a single dose of the test extracts orally followed by treatment after 24 hrs, 48 hrs, and 72 hrs post infection as per (Fidock, *et al*., 2004). Twenty four hours after the last treatment, tail blood smears from selected animals were prepared and stained with Giemsa in order to determine the parasitaemia levels under the microscope (Zeizz, West Germany).

The formula below was used to calculate the percentage parasite growth suppression (Moll, *et al*., 2008):

$$\frac{\text{(Percent parasitaemia in control – percent parasitaemia in sample)} \times 100}{\text{per cent parasitaemia in control}}$$

The mice blood smears for parasite monitoring were taken for the days 2, 4, 5 and 6. The mice were euthanized at days 0, 6, 8 and 10 where serum and spleen cells were obtained.

3.7.3.10 Serum collection

Three mice per group were euthanized and peripheral blood was collected by cardiac puncture at 0, 6, 8 and 10th-day post infection. The blood was allowed to coagulate at 4°C overnight. It was centrifuged for 10 min at 1500 rpm. The serum was stored at -20°C until use. Mouse IgG (Santa Cruz Biotech) was used to determine the *P. berghei* specific IgG levels.

3.7.3.11 Spleen cell preparation

Three mice per group were euthanized on days 0, 6, 8 and 10th post-infection and the spleens were removed under sterile conditions. The spleens were placed in 1ml of RPMI 1640 (Sigma Adrich, St Louis, MO) in a 15 ml conical tube on ice before processing them. The spleens were transferred into a cell strainer, the organ was broken with the help of a sterile plastic syringe piston, and the cells collected into 3 ml of wash medium in a sterile petri-dish. The cell suspension was transferred into a 15 ml conical tube.

The tissue debris was left to sediment for a few seconds and the supernatant was transferred to a fresh 15 ml conical tube. The supernatant was centrifuged at 1500 rpm for 10 min at 4ºC and washed it two more times. The pellet was suspended in 5 ml ACK lysis buffer (Gibco, USA) for 2 minutes at room temperature. The cell suspension was centrifuged for 10 minutes at 1500 rpm at 4ºC. The pellet obtained was suspended in 2 ml of PBS. The cells were counted and frozen in cryovials using freeze mix (90% HIS

FBS (Gibco, Canada) and 10% DMSO) starting at -20ºC for 30 min, then to -80ºC overnight and finally into liquid nitrogen tanks until when required.

3.7.4 *P. berghei* antigen preparation

Late blood stage parasites extract was prepared as per (Nyakundi, *et al*., 2016). The pellet obtained was re-suspended in 4X PBS that contained protease inhibitor cocktail. The suspension was sonicated at an output 6 for 10 seconds with 1-minute intervals in ice to lyse parasite cell wall. The bursts were repeated 6 times. All the parasite cells were confirmed microscopically that they were broken. The lysate was transferred into an eppendorf tube and centrifuged at 14000 rpm for 60 min at 4ºC. The supernatant was collected, which is the antigen, filter sterilized and its protein concentration determined.

Seven dilutions of Bovine Serum Albumin (BSA, Bio-Rad laboratories GmbH, München, Germany) as well as that of *P. berghei* antigen were prepared to be tested. 10 µl of each standard and antigen solution as put into separate micro titer plate wells. One part of the dye reagent (Bio-Rad laboratories GmbH, München, Germany) was diluted with four parts of distilled water and added 200 µl of the diluted dye into the micro titer wells mixing it thoroughly. The plate was incubated for 5 minutes and absorbance measured at 630 nm. The results obtained were analyzed using graph-pad prism. The standard concentration was used to generate the antigen concentration as 990.042 µg/ml. The antigen was stored at -20ºC until use.

3.7.5 Immunological tests

3.7.5.1 IgG ELISA

NUNC Maxisorp® 96-well ELISA plates were coated with mouse 1.25 µg/ml *P. berghei* antigen and incubated overnight at 4°C. The plates were washed twice with

PBS. The microtitre wells were blocked with PBS-Tween (0.05% Tween 20 (Fisher Biotech, USA) + 5% BSA) and incubated for two hours at 37ºC. The wells were washed 5 times with PBS-Tween and samples or standards diluted in blocking buffer at a ratio of 1:200.

The plates were incubated for two hours at 37ºC and washed 5 times with PBS-Tween. HRP conjugated goat anti-mouse IgG antibodies (Santa Cruz Biotech) diluted 1:1000 in blocking buffer was added into the wells and incubated for two hours at 37ºC. The plates were washed 5 times with PBS-Tween; 3,3',5,5'-Tetramethylbenzidine (TMB KPL, USA) substrate was added, and the optical density readings obtained using ELISA reader (Biotek Elx808) at a wavelength of 630 nm.

3.7.5.2 IFN-γ Cytokines assay

3.7.5.2.1 Spleen cells culture

Spleen cells from liquid nitrogen were removed and quickly thawed at 37ºC water bath and transferred into 5 ml 1X PBS 10% fetal bovine serum (FBS Gibco, Canada) this was followed by centrifugation at 1500 rpm for 10 minutes, aspirated the supernatant and dislodged pellet. Approximately 2 ml of complete media (RPMI-1640; Sigma-Aldrich, St. Louis, MO, 10%FBS; Gibco, Canada, 1%HEPES; Sigma-Aldrich, Dorset, UK, 1% Gentamycin; Sigma-Aldrich, Dorset, UK, 1% L-glutamin; Fisher Biotech, Wembley, Australia) was added to the tube. The cells were counted using a hemocytometer by mixing the 10 µl of cell suspension plus 10 µl trypan blue.

The cells were incubated for 2 hours at 37ºC in CO_2 incubator. The cells were centrifuged to obtain the pellet as described above and re-suspended in 100 µl of cold PBS per 1 million cells. The cells were re-suspended in complete media (1 ml for every million cells). Added 1 ml of cell suspension to 48 well plates (VWR, North America)

so that each well had 1 million cells. The stimulators (*P. berghei* antigen and Concanavalin A) were added too; collected the supernatants at 72 hrs and was stored at -20ºC (Mumo, 2013).

3.7.5.2.2 IFN-γ ELISA

NUNC Maxisorp® 96-well ELISA plates were coated with 1µg/ml of mAb AN18 (Mabtech) and incubated overnight at 4°C. The plates were washed twice with PBS and blocked with incubation buffer (PBS with 0.05% Tween 20 + 0.1% BSA) after which they were incubated for one hour at room temperature. The plates were washed 5 times with PBS-Tween. Samples or standards diluted in incubation buffer were added and incubated for two hours at room temperature.

The wells were washed 5 times with PBS-Tween; mAb R4-6A2-biotin (Mabtech) at 0.5 µg/ml in incubation buffer was added and incubated for one hour at room temperature. The wells were washed 5 times with PBS-Tween after which Streptavidin-HRP (Mabtech) diluted 1:1000 in incubation buffer was added and incubated for one hour at room temperature. The wells were washed 5 times in PBS-Tween; TMB substrate was added, and the optical density readings obtained using ELISA reader (Biotek Elx808) at a wavelength of 630 nm.

3.8 Data analysis

Graphs were obtained to present the means and the data analysis was done using Graph Pad Prism version 7.00 for windows. The parasite load statistical analysis was done using ANOVA for *in vitro* assays where the levels of significance were determined by Tukey's post-test and Kruskal-Wallis non-parametric test followed by uncorrected Dunn's multiple comparison post-hoc analysis for the *in vivo* experiment. Linear regression analysis was used to identify significant predictors of percentage parasitemia

levels with days post infection. The IFN-γ cytokine and IgG concentrations were determined by interpolating the optical density values with a standard curve. Differences with p values of <0.05 were considered significant.

CHAPTER FOUR

RESULTS

4.1 *P. falciparum* inhibition analysis

Concentrations of *Ar*E: *Cv*PE at 1600:400, 1200:800, 600:1400 and 200:1800 µg/ml had the parasitemia increase from the initial percentage parasitemia of 1.3% as shown in figure 4.1. The concentration that exhibited the highest inhibition is at 400: 1600 *Ar*E: *Cv*PE since it had a high reduction of parasites compared with the individual extract as shown below (figure 4.1). Using ANOVA a p-value = 0.0435 was obtained. Tukey's multiple comparisons test showed the tests having significance difference were: Ar/Cv400/1600 vs untreated (*p=0.0382), Cv 2000 vs untreated (*p=0.0440) and CQ vs untreated (*p=0.0367).

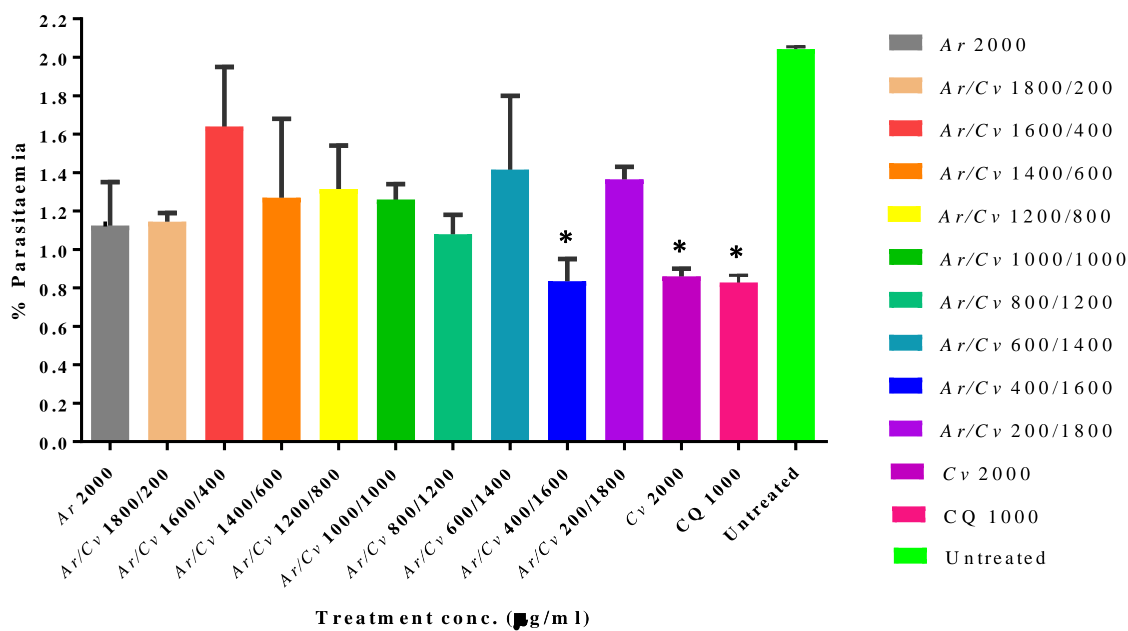


Figure 4.1: The reduction in parasitaemia of *P. falciparum* 3D7

Ar/*Cv* = *A. remota* ethanol extract/ *C. volkensii* petroleum ether extract. The concentrations for *Ar*E:*Cv*PE (µg/ml) were as follows 2000:0 (*Ar*E only), 1800:200, 1600:400, 1400:600, 1200:800, 1000:1000, 800:1200, 600:1400, 400:1600, 200:1800 and 0:2000 (*Cv*PE only). The control drug CQ = Chloroquine

The highest inhibition exhibited by the combined extract (*Ar*E + *Cv*PE), was 59.12% at a ratio of 400:1600 *in vitro*, while the individual extracts *Ar*E and *Cv*PE had 44.9% and 57.9% inhibition respectively as shown in figure 4.2. Chloroquine treated group had 68.1% inhibition. Using ANOVA the inhibition results showed that there was no significant difference between means (p-value = 0.1951).

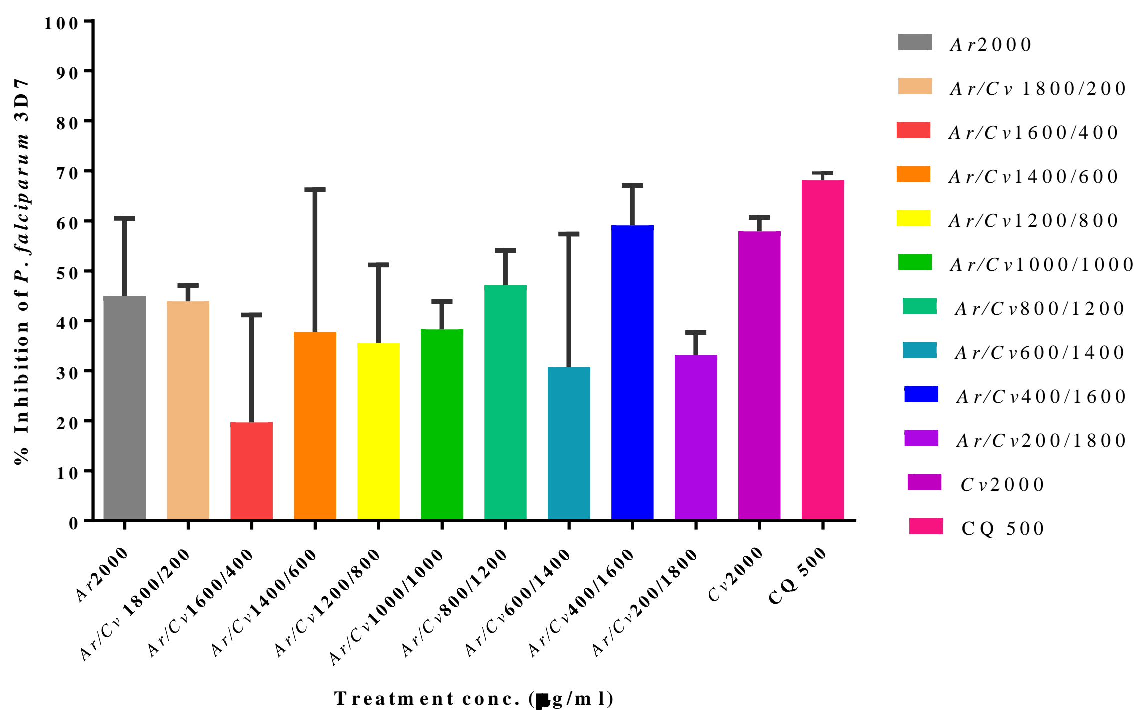


Figure 4.2: The percentage inhibition of *P. falciparum* 3D7

Ar/*Cv* = *A. remota* ethanol extract/ *C. volkensii* petroleum ether extract. The concentrations for *Ar*E:*Cv*PE (µg/ml) were as follows 2000:0 (*Ar*E only), 1800:200, 1600:400, 1400:600, 1200:800, 1000:1000, 800:1200, 600:1400, 400:1600, 200:1800 and 0:2000 (*Cv*PE only). The control drug: CQ = Chloroquine.

4.2 *P. berghei* suppression analysis

The mice parasitaemia was monitored during the four-day suppression test till day 6 as per (Fidock, *et al.*, 2004). During the four-day suppression test, *P. berghei* growth was suppressed by 83.7% on day five when a combination of plant extracts was used. Whereas, *Ar*E and *Cv*PE extracts suppressed growth by -23.3% and 4.3% respectively. The parasitaemia on day 5 was highest in the group treated with *Ar*E with a mean peak of 48.6±22.4ml^3 X10^6/ml (mean±SEM) of parasitized red blood cells (PRBCs) and

lowest in the group treated with the combined extract with a mean of 6.4±0.7ml^3 X10^6/ml (figure 4.3).

On day 6, *Ar*E suppressed *P. berghei* by -15.5%, *Cv*PE by -43.1%, DHA by 90.5%, and the combined extract by 66.7% respectively. This shows that the groups treated with the conventional drug and the combined extract, their suppression effect was seen even after 48 hrs post-treatment. The group that had the highest parasitaemia was the group treated with *Cv*PE with a mean of 90.3±23.2 ml^3 X 10^6/ml and the lowest was the group treated with DHA with a mean of 5.99±0.5 ml^3 X 10^6/ml while the one treated with the combined extract had 21.0±3.1 ml^3 X 10^6/ml (figure 4.3).

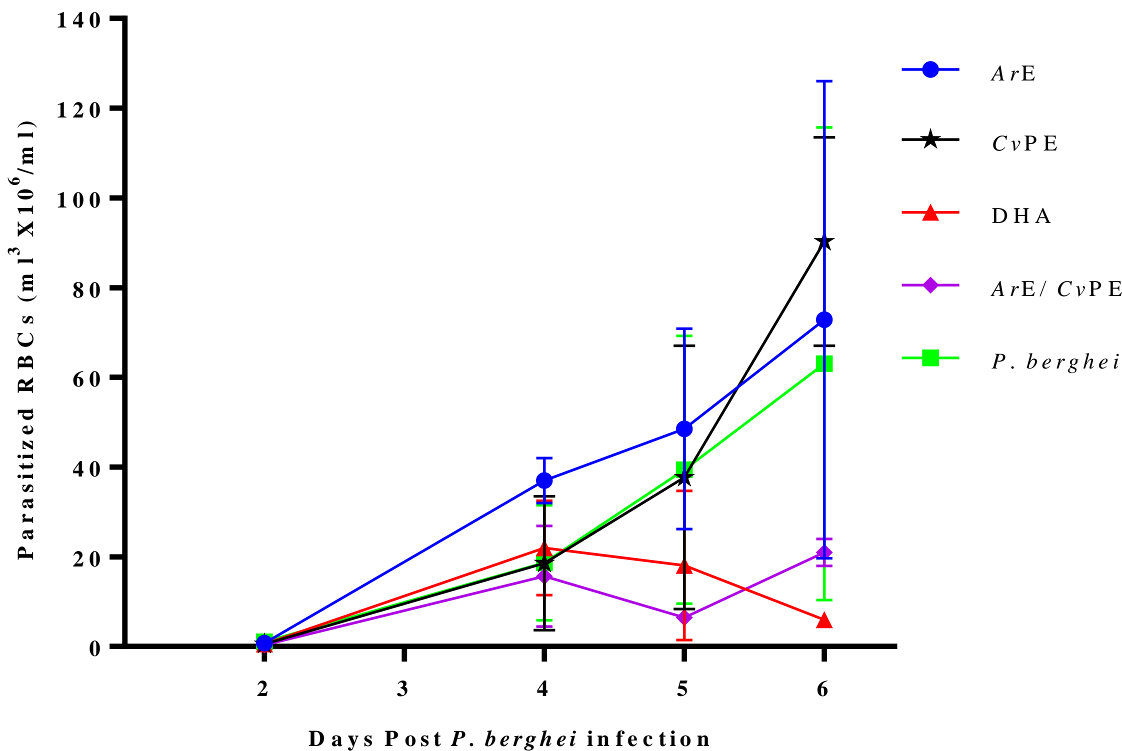


Figure 4.3: *P. berghei* mean parasitaemia in mice post-infection

Plant extracts used: *Ar*E = *A. remota*, ethanol extract and *Cv*PE = *C. volkensii*, petroleum ether extract. Positive control drug: DHA = dihydroartemisinin. Negative control: *P. berghei* = the infected untreated group**.**

The five slopes showed a significance difference of p value <0.05 (p = 0.0231). Linear regression showed that as the days increased the parasitemia increased for the group treated with individual extracts and in the one that received *P. berghei* only unlike in the groups treated with the combined extract and DHA. The p values obtained for *Ar*E, *Cv*PE, DHA, *Cv*PE/*Ar*E and *P. berghei* are as follows: 0.02*, 0.1591, 0.5693, 0.2194, and 0.0774 respectively showing a significance in *Ar*E treated group only. The medians were statistically significant (p = 0.0018) where post-hoc analysis showed significance among mean in various treatments. Various groups had adjustments p-values less than 0.05 hence indicating the areas of significance as shown in table 4.1. Day 2 had the lowest parasitaemia hence almost all the other results of day 4, 5 and 6 differed with its means. There are two groups that showed a significant difference at different time point other than with day 2 which are *Cv*PE day 6 vs. DHA day 5 (p = 0.0496) and DHA day 6 vs. *Cv*PE day 6 (p = 0.0496) indicating that *Cv*PE had a high parasitaemia as compared to DHA.

Table 4.1: Post-hoc adjustments p-values of parasitaemia means

4 days test (Days 2, 4, 5 & 6)	p-value	4 days test (Days 2, 4, 5 & 6)	p-value
*Ar*E 4 vs. *Ar*E 2	0.0132	*Cv*PE 6 vs. DHA 2	0.0008
*Ar*E 5 vs. *Ar*E 2	0.022	*Ar*E/ *Cv*PE 6 vs. DHA 2	0.0206
*Cv*PE 5 vs. *Ar*E 2	0.042	*P. berghei* 6 vs. DHA 2	0.0132
P. berghei 5 vs. *Ar*E 2	0.0234	*Ar*E 4 vs. *Ar*E/ *Cv*PE 2	0.0047
*Ar*E 6 vs. *Ar*E 2	0.0141	DHA 4 vs. *Ar*E/ *Cv*PE 2	0.0315
*Cv*PE 6 vs. *Ar*E 2	0.0017	*P. berghei* 4 vs. *Ar*E/ *Cv*PE 2	0.0469
*Ar*E/ *Cv*PE 6 vs. *Ar*E 2	0.0354	*Ar*E 5 vs. *Ar*E/ *Cv*PE 2	0.0083
P. berghei 6 vs. *Ar*E 2	0.0234	*Cv*PE 5 vs. *Ar*E/ *Cv*PE 2	0.0171
*Ar*E 4 vs. *Cv*PE 2	0.0062	*P. berghei* 5 vs. *Ar*E/ *Cv*PE 2	0.0088
DHA 4 vs. *Cv*PE 2	0.0397	*Ar*E 6 vs. *Ar*E/ *Cv*PE 2	0.005
*Ar*E 5 vs. *Cv*PE 2	0.0108	*Cv*PE 6 vs. *Ar*E/ *Cv*PE 2	0.0005
*Cv*PE 5 vs. *Cv*PE 2	0.022	*Ar*E/ *Cv*PE 6 vs. *Ar*E/ *Cv*PE 2	0.0141
P. berghei 5 vs. *Cv*PE 2	0.0116	*P. berghei* 6 vs. *Ar*E/ *Cv*PE 2	0.0088
*Ar*E 6 vs. *Cv*PE 2	0.0067	*Ar*E 4 vs. *P. berghei* 2	0.016
*Cv*PE 6 vs. *Cv*PE 2	0.0007	*Ar*E 5 vs. *P. berghei* 2	0.0264
*Ar*E/ *Cv*PE 6 vs. *Cv*PE 2	0.0182	*Cv*PE 5 vs. *P. berghei* 2	0.0496
P. berghei 6 vs. *Cv*PE 2	0.0116	*P. berghei* 5 vs. *P. berghei* 2	0.028
*Ar*E 4 vs. DHA 2	0.0072	*Ar*E 6 vs. *P. berghei* 2	0.0171
DHA 4 vs. DHA 2	0.0444	*Cv*PE 6 vs. *P. berghei* 2	0.0022
*Ar*E 5 vs. DHA 2	0.0124	*Ar*E/ *Cv*PE 6 vs. *P. berghei* 2	0.042
*Cv*PE 5 vs. DHA 2	0.0248	*P. berghei* 6 vs. *P. berghei* 2	0.028
P. berghei 5 vs. DHA 2	0.0132	*Cv*PE 6 vs. DHA 5	0.0496
*Ar*E 6 vs. DHA 2	0.0077	DHA 6 vs. *Cv*PE 6	0.0496

Where *Ar*E= *A. remota* ethanol extract, *Cv*PE= *C. volkensii* petroleum ether extract and DHA= dihydroartemisinin. 2, 4, 5 and 6 = days post-infection and vs = versus

4.3 Total IgG levels analysis

IgG levels were evaluated in mice infected with *P. berghei* and treated with plant extracts. The concentration of IgG had no difference with non-treated group when the

impact of plant extracts and DHA were evaluated. For *A. remota* the concentration reduced on day 6 then increased on day 8 while on day 10 the level reduced. For *C. volkensii* the concentration reduced on day 6 and increased gradually from day 8 and 10. A combination of plant extracts lead to a decrease on day 6 and a slight increase on day 10.

On day 6, the group that received *P. berghei* only had the highest concentration of IgG (24975±21549 pg/ml) compared to the rest of the groups (figure 4.4). The group that was treated with *A. remota* had the lowest IgG levels at 3206±1369 pg/ml. Control/naive mice had 2580±119.1 pg/ml; while the groups treated with: *C. volkensii, Ar/Cv* and DHA had 3455±615.5 pg/ml, 4673±2112 pg/ml and 4284±2639 pg/ml respectively.

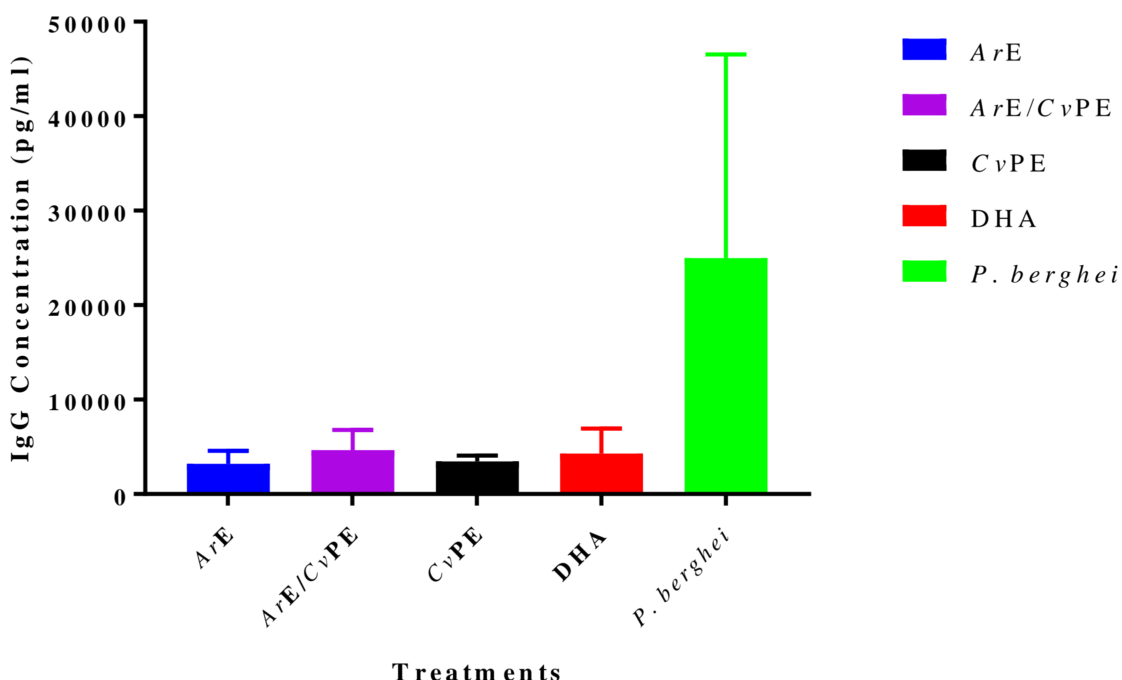


Figure 4.4: A bar graph showing the IgG concentrations for day 6 post infection

*Ar*E = *A. remota*, ethanol extract and *Cv*PE = *C. volkensii*, petroleum ether extract. Positive control drug: DHA = dihydroartemisinin. Negative control: *P. berghei* = the infected untreated group.

On day 8, the group treated with DHA had the highest concentration (41251±17276 pg/ml) compared to the rest of the groups. The control mice (naive mice) had 2580±119.1 pg/ml; *A. remota* 10168±5582 pg/ml; *C. volkensii* 8390±3506 pg/ml; *Ar/Cv* 3905±808.6 pg/ml which was the lowest concentration, while the negative control (*P. berghei* only) had 7803±2467 pg/ml as shown in figure 4.5.

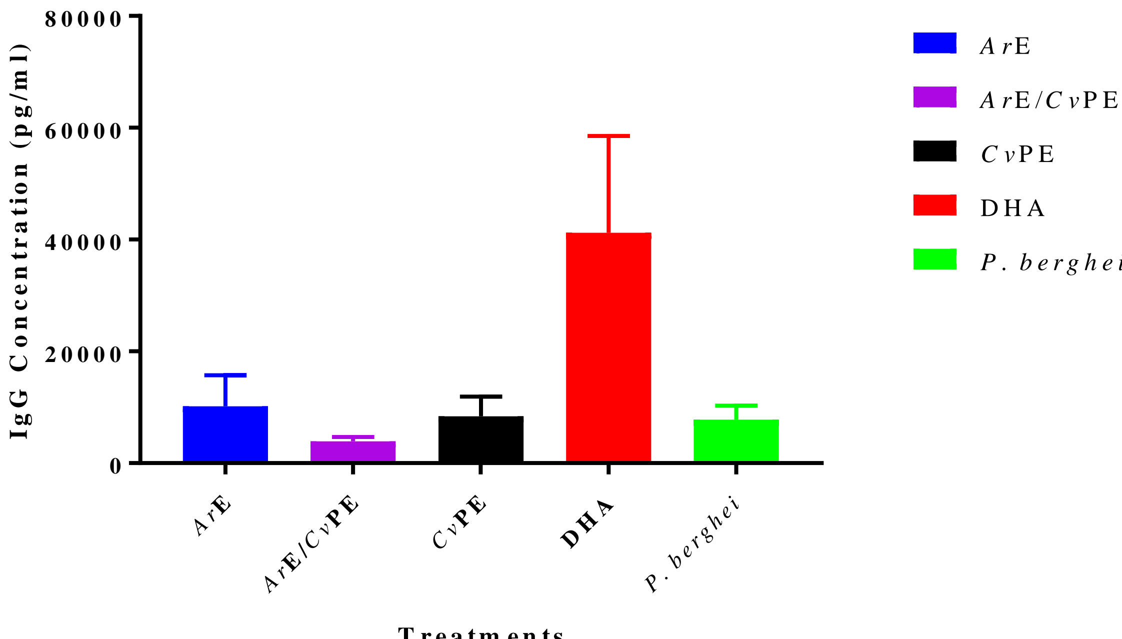


Figure 4.5: A bar graph showing the IgG concentrations for day 8 post-infection

*Ar*E = *A. remota*, ethanol extract and *Cv*PE = *C. volkensii*, petroleum ether extract. Positive control drug: DHA = dihydroartemisinin. Negative control: *P. berghei* = the infected untreated group.

At day 10, the untreated group had the highest concentration of 22236±6562 pg/ml compared to the rest of the groups. As in figure 4.6, control mice had 2580±119.1 pg/ml; *A. remota* 9317±2667 pg/ml; *C. volkensii* 21472±17198 pg/ml; *Ar/Cv* 7586±2327 pg/ml; DHA had 6916±932.8 pg/ml which was the lowest, while the negative control (*P. berghei* only) had 22236±6562 pg/ml.

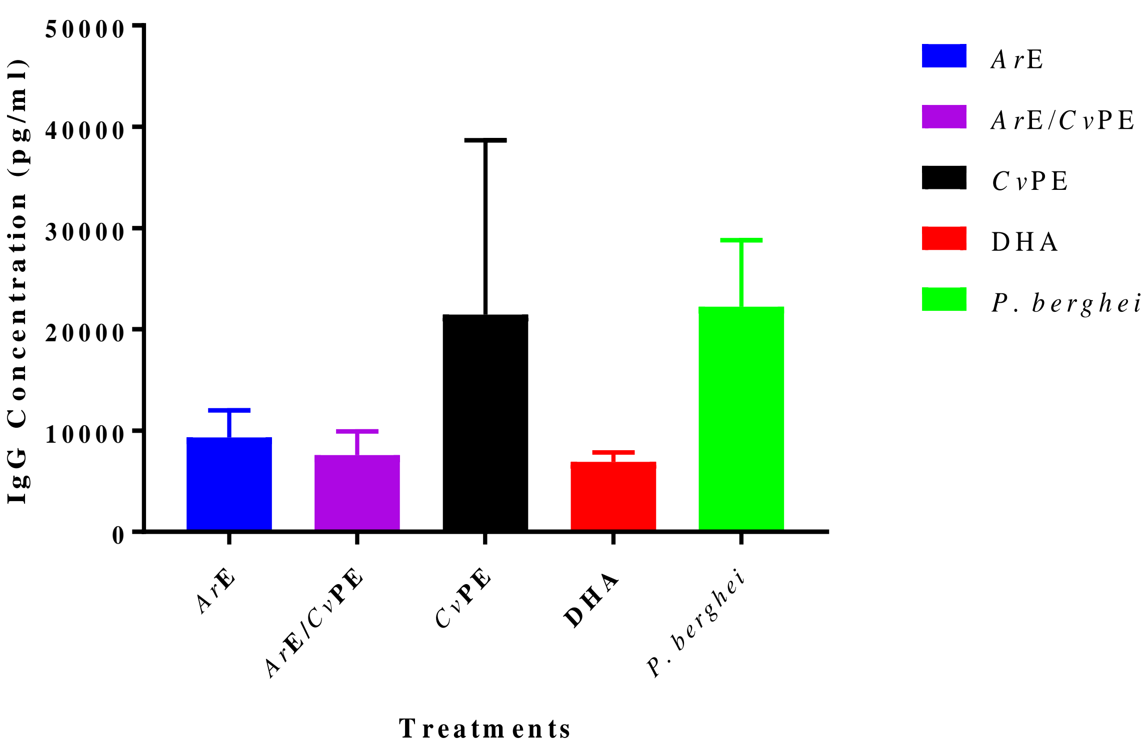


Figure 4.6: A bar graph showing the IgG concentrations for day 10 post-infection

*Ar*E = *A. remota*, ethanol extract and *Cv*PE = *C. volkensii*, petroleum ether extract. Positive control drug: DHA = dihydroartemisinin. Negative control: *P. berghei* = the infected untreated group**.**

The results of IgG concentrations post infection showed that there was a significant difference among the medians (p = 0.0052). Post hoc analysis showed the following points of significance as shown in table 4.2. The significance was between all the groups with the baseline samples while others included *Ar*E day 6 vs. DHA day 8 (p = 0.0145), *Ar*E day 6 vs. *P. berghei* (infected untreated group) day 10 (p = 0.0296), *Ar*E/*Cv*PE day 6 vs. DHA day 8 (p = 0.0145), *Ar*E/*Cv*PE day 6 vs. *P. berghei* only day 10 (p = 0.0296), *Cv*PE day 6 vs. DHA day 8 (p = 0.017), *Cv*PE day 6 vs. *P. berghei* only day 10 (p = 0.0342) and DHA day 6 vs. DHA day 8 (p = 0.0287).

Table 4.2: Post-hoc adjustments p-values of mice IgG means post-infection

IgG (days 6, 8 & 10)	p-value	IgG (days 6, 8 & 10)	p-value
*Ar*E 0 vs. *Ar*E 8	0.0418	DHA 0 vs. *Ar*E 8	0.0418
*Ar*E 0 vs. *Cv*PE 8	0.0418	DHA 0 vs. *Cv*PE 8	0.0418
*Ar*E 0 vs. DHA 8	0.0024	DHA 0 vs. DHA 8	0.0024
*Ar*E 0 vs. *P. berghei* 8	0.0494	DHA 0 vs. *P. berghei* 8	0.0494
*Ar*E 0 vs. *Ar*E 10	0.0247	DHA 0 vs. *Ar*E 10	0.0247
*Ar*E 0 vs. *Cv*PE 10	0.0467	DHA 0 vs. *Cv*PE 10	0.0467
*Ar*E 0 vs. DHA 10	0.0442	DHA 0 vs. DHA 10	0.0442
*Ar*E 0 vs. *P. berghei* 10	0.0058	DHA 0 vs. *P. berghei* 10	0.0058
*Ar*E/*Cv*PE 0 vs. *Ar*E 8	0.0418	*P. berghei* 0 vs. *Ar*E 8	0.0418
*Ar*E/*Cv*PE 0 vs. *Cv*PE 8	0.0418	*P. berghei* 0 vs. *Cv*PE 8	0.0418
*Ar*E/*Cv*PE 0 vs. DHA 8	0.0024	*P. berghei* 0 vs. DHA 8	0.0024
*Ar*E/*Cv*PE 0 vs. *P. berghei* 8	0.0494	*P. berghei* 0 vs. *P. berghei* 8	0.0494
*Ar*E/*Cv*PE 0 vs. *Ar*E 10	0.0247	*P. berghei* 0 vs. *Ar*E 10	0.0247
*Ar*E/*Cv*PE 0 vs. *Cv*PE 10	0.0467	*P. berghei* 0 vs. *Cv*PE 10	0.0467
*Ar*E/*Cv*PE 0 vs. DHA 10	0.0442	*P. berghei* 0 vs. DHA 10	0.0442
*Ar*E/*Cv*PE 0 vs. *P. berghei* 10	0.0058	*P. berghei* 0 vs. *P. berghei* 10	0.0058
*Cv*PE 0 vs. *Ar*E 8	0.0418	*Ar*E 6 vs. DHA 8	0.0145
*Cv*PE 0 vs. *Cv*PE 8	0.0418	*Ar*E 6 vs. *P. berghei* 10	0.0296
*Cv*PE 0 vs. DHA 8	0.0024	*Ar*E/*Cv*PE 6 vs. DHA 8	0.0145
*Cv*PE 0 vs. *P. berghei* 8	0.0494	*Ar*E/*Cv*PE 6 vs. *P. berghei* 10	0.0296
*Cv*PE 0 vs. *Ar*E 10	0.0247	*Cv*PE 6 vs. DHA 8	0.017
*Cv*PE 0 vs. *Cv*PE 10	0.0467	*Cv*PE 6 vs. *P. berghei* 10	0.0342
*Cv*PE 0 vs. DHA 10	0.0442	DHA 6 vs. DHA 8	0.0287
*Cv*PE 0 vs. *P. berghei* 10	0.0058		

Where *Ar*E= *A. remota* ethanol extract, *Cv*PE= *C. volkensii* petroleum ether extract and DHA= dihydroartemisinin. 0, 6, 8 and 10 = days and vs = versus

4.4 IFN-γ responses analysis

IFN-γ levels were evaluated in mice infected with *P. berghei* and treated with plant extracts. Similar to IgG, IFN-γ levels varied for each plant extract and DHA. The change in IFN-γ levels was assessed. The baseline mice had a concentration of 2601±2601 pg/ml. The negative control group had concentration of IFN-γ lower on day 6 (7116±3193 pg/ml) compared to day 8 (16291±10189 pg/ml) which decreased on day 10 (9544±341.4 pg/ml). The groups treated with *A. remota*, had the concentrations lower on day 8 (5610±108.6 pg/ml) compared to day 6 (6542±3272 pg/ml) and increased on day 10 (10121±691.3 pg/ml). *C. volkensii* had a concentration of 7306±1999 pg/ml on day 6, registered a slight decrease on day 8 (7257±602.9 pg/ml) and an increase on day 10 with 11756±1093 pg/ml. The groups treated with the combined extract (6792±1923 pg/ml, 7300±1673 pg/ml and 9957±257.7 pg/ml), and DHA (4712±2534 pg/ml, 7297±1639 pg/ml and 9585±198.5 pg/ml) had the concentration increasing from day 6 throughout till day 10 respectively.

The figure 4.7 below shows that the group treated with the combined extract had a concentration of 6792±1923 pg/ml; the group treated with *A. remota* extract had 6542±3272 pg/ml; the group that received *P. berghei* only had 7116±3193 pg/ml; *C. volkensii* had 7306±1999 pg/ml while the group that received DHA had 4712±2534 pg/ml on day 6.

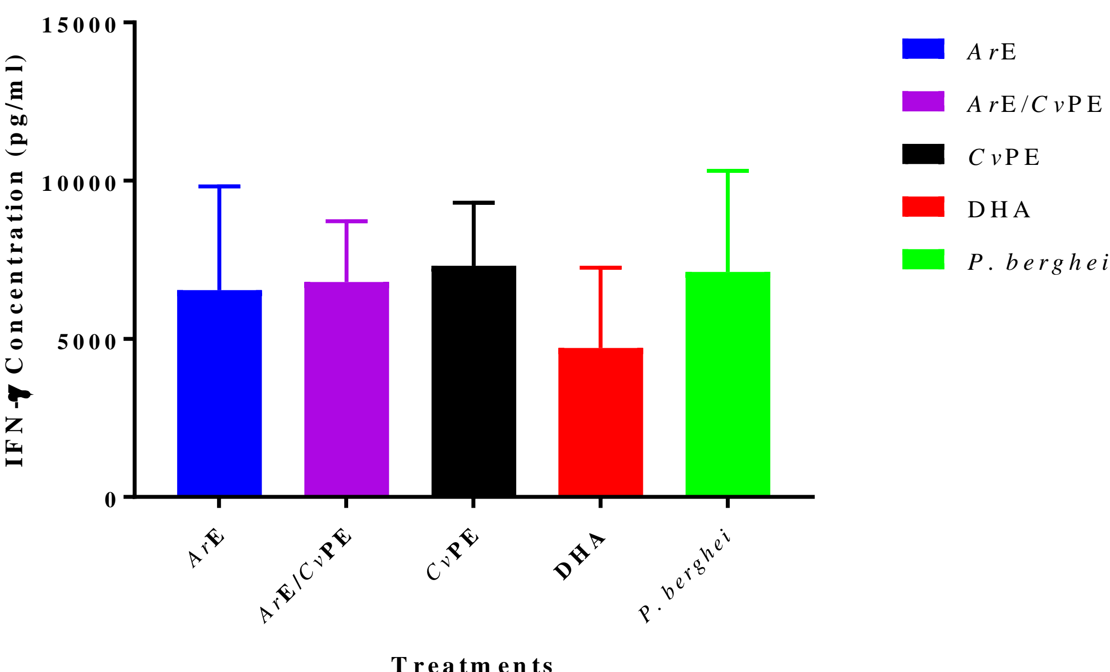


Figure 4.7: A bar graph showing the IFN-γ concentrations for day 6 post-infection

*Ar*E = *A. remota*, ethanol extract and *Cv*PE = *C. volkensii*, petroleum ether extract. Positive control drug: DHA = dihydroartemisinin. Negative control: *P. berghei* = the infected untreated group**.**

The untreated group had the highest concentration of 16291±10189 pg/ml. The group treated with the combined extract had 7300±1673 pg/ml; *A. remota* had 5610±108.6 pg/ml; *C. volkensii* had 7257±602.9 pg/ml while DHA had 7297±1639 pg/ml on day 8 as in figure 4.8.

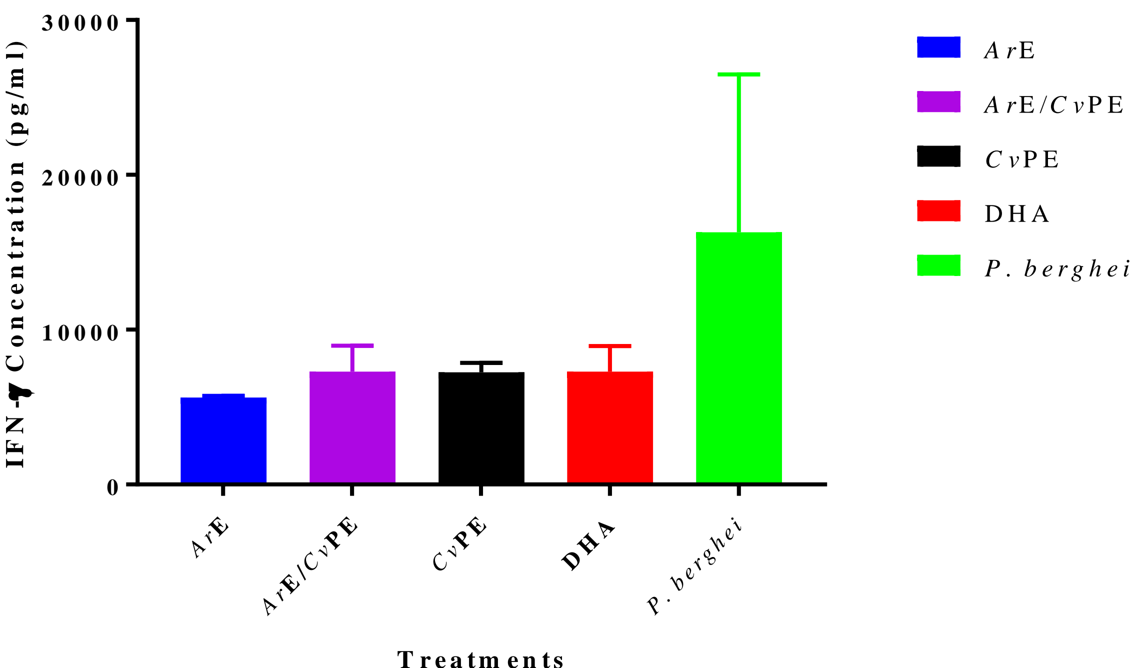


Figure 4.8: A bar graph showing the IFN-γ concentrations for day 8 post-infection

*Ar*E = *A. remota*, ethanol extract and *Cv*PE = *C. volkensii*, petroleum ether extract. Positive control drug: DHA = dihydroartemisinin. Negative control: *P. berghei* = the infected untreated group**.**

The group treated with the *C. volkensii* extract had the highest concentration of 11756±1093 pg/ml followed by the group treated with *A. remota* extract with 10121±691.3 pg/ml. The group that received *P. berghei* only had 9544±341.4 pg/ml; the combined extract had 9957±257.7 pg/ml while DHA had 9585±198.5 pg/ml on day 10 as shown in figure 4.9.

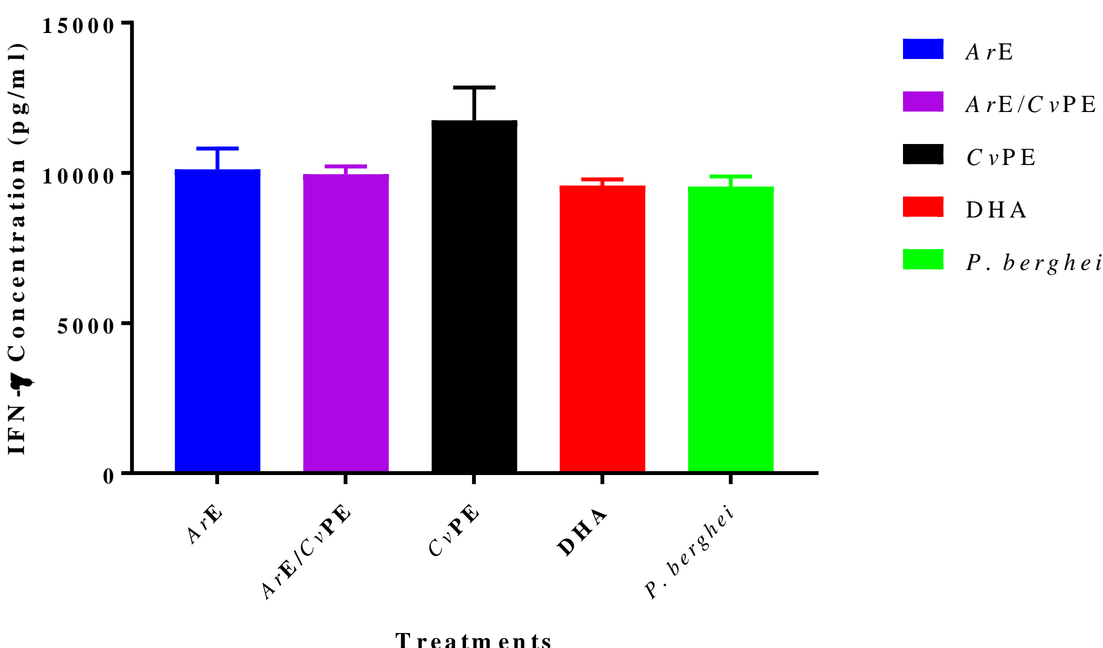


Figure 4.9: A bar graph showing the IFN-γ concentrations for day 10 post-infection

*Ar*E = *A. remota*, ethanol extract and *Cv*PE = *C. volkensii*, petroleum ether extract. Positive control drug: DHA = dihydroartemisinin. Negative control: *P. berghei* = the infected untreated group**.**

The INF-γ results showed that there was a significant difference among medians (p = 0.0331). Post-hoc analysis showed areas of significance as shown in table 4.3. There was a significancedifference between baseline and the other days 6, 8 and 10 samples as well as other time points as follows: DHA day 6 vs. *Ar*E day 10 (p = 0.0473), DHA day 6 vs. *Ar*E/*Cv*PE day 10 (p = 0.0435), DHA day 6 vs. *Cv*PE day 10 (p = 0.0172), *Ar*E day 8 vs. *Ar*E day10 (p = 0.0447), *Ar*E day 8 vs. *Ar*E/*Cv*PE day 10 (p = 0.0411) and *Ar*E day 8 vs. *Cv*PE day 10 (p = 0.0161).

Table 4.3: Post-hoc adjustments p-values in mice IFN-γ means post-infection

IFN-γ (days 6, 8 & 10)	p-value	IFN-γ (days 6, 8 & 10)	p-value
*Ar*E 0 vs. *Ar*E 10	0.0189	DHA 0 vs. *Ar*E/*Cv*PE 10	0.0172
*Ar*E 0 vs. *Ar*E/*Cv*PE 10	0.0172	DHA 0 vs. *Cv*PE 10	0.006
*Ar*E 0 vs. *Cv*PE 10	0.006	DHA 0 vs. DHA 10	0.0326
*Ar*E 0 vs. DHA 10	0.0326	DHA 0 vs. *P. berghei* 10	0.0388
*Ar*E 0 vs. *P. berghei* 10	0.0388	*P. berghei* 0 vs. *Ar*E 10	0.0189
*Ar*E/*Cv*PE 0 vs. *Ar*E 10	0.0189	*P. berghei* 0 vs. *Ar*E/*Cv*PE 10	0.0172
*Ar*E/*Cv*PE 0 vs. *Ar*E/*Cv*PE 10	0.0172	*P. berghei* 0 vs. *Cv*PE 10	0.006
*Ar*E/*Cv*PE 0 vs. *Cv*PE 10	0.006	*P. berghei* 0 vs. DHA 10	0.0326
*Ar*E/*Cv*PE 0 vs. DHA 10	0.0326	*P. berghei* 0 vs. *P. berghei* 10	0.0388
*Ar*E/*Cv*PE 0 vs. *P. berghei* 10	0.0388	DHA 6 vs. *Ar*E 10	0.0473
*Cv*PE 0 vs. *Ar*E 10	0.0189	DHA 6 vs. *Ar*E/*Cv*PE 10	0.0435
*Cv*PE 0 vs. *Ar*E/*Cv*PE 10	0.0172	DHA 6 vs. *Cv*PE 10	0.0172
*Cv*PE 0 vs. *Cv*PE 10	0.006	*Ar*E 8 vs. *Ar*E 10	0.0447
*Cv*PE 0 vs. DHA 10	0.0326	*Ar*E 8 vs. *Ar*E/*Cv*PE 10	0.0411
*Cv*PE 0 vs. *P. berghei* 10	0.0388	*Ar*E 8 vs. *Cv*PE 10	0.0161
DHA 0 vs. *Ar*E 10	0.0189		

Where *Ar*E = *A. remota* ethanol extract, *Cv*PE = *C. volkensii* petroleum ether extract and DHA = dihydroartemisinin. 0, 6, 8 and 10 = days and vs = versus

CHAPTER FIVE

DISCUSSION

5.0 Introduction

Antimalarial drug treatments do not always work due to the wrong diagnosis; incorrect choice of drugs; sub-optimal regimen, on-compliance; sub-optimal absorption; idiosyncratic pharmacokinetics; poor quality drugs and resistance of the pathogen to the drug (Sullivan, 2006). Traditional herbs and plants are a source of alternative drugs and *A. annua* has provided us with a potent antimalaria drug artemisinin used in the treatment of malaria. Due to concerns raised about emerging resistance in South East Asia, there is an urgent need to find alternative drugs (WHO, 2014).

Herbal preparations for *A. remota* and *C. volkensii* are widely used in ethnomedicine to manage malarial illnesses in Central Kenya (Kareru, *et al*., 2007). Experiments that consist of both *in vitro* and *in vivo* assays have shown that both plants exhibit anti-malaria properties (Cocquyt, *et al*., 2011 and Gitua, *et al*., 2012). However, there is no data available on its efficacy and impact of a combination of the two plants on the immune system during malaria infection. Combination therapy research in the search for an ideal combination of drugs that can act synergistically or additively is therefore of prime importance to prolong the development of resistance to antimalarial drugs, reduce the cost of treatment and stop recrudescence (Govindan, *et al*., 2016).

In this study, the combined extract of *A. remota* and *C. volkensii* extracted with ethanol and petroleum ether respectively was evaluated. This was done in a murine model *in vitro* in order to demonstrate how the combined extract affects the immune system

during *P. berghei* infection. Peripheral blood for serum and spleens were obtained in order to evaluate the immunoglobulin G and IFN-γ respectively.

5.1 *P. falciparum* 3D7 Inhibition

All the tested extracts of both plants (*A. remota* and *C. volkensii*) showed growth inhibition of the malarial parasite hence giving a clear indication that these plants extracts show anti-plasmodial effects. The individual plant leaves extract also had inhibitory effects on chloroquine sensitive *P. falciparum* 3D7 strain. This coincides with a study done in Cameroon that presented leaves of *D. principum*, and *T. catappa* as well as bark of *T. mantaly* having significant anti-plasmodial activities, with good selectivity against chloroquine-sensitive and -resistant strains of *P. falciparum* (Marie, *et al*., 2018).

When the combined concentrations were maintained at 2000 µg/ml with different ratios (100:0 - 0:100) of *Ar*E + *Cv*PE concentrations, showed *P. falciparum* 3D7 inhibition though there was no significant difference among the means of individual extracts and combined extracts *in vitro*. Low parasitaemia was seen with increase in *C. volkensii* concentrations especially in *A. remota*/*C. volkensii* 400:1600 µg/ml group as compared with the untreated group. These results corresponds to a study on *Carissa edulis* and *A. annua* which showed synergism in all the combination ratios of 90:10 – 10:90 which decreased with increase in the concentration of *A. annua* (Kebenei, *et al*., 2012). Though no significant difference was shown, there are some studies that showed effectiveness of combined therapy while others don't. Cinchona alkaloids have shown synergy with almost 30 alkaloids having been described active against *P. falciparum in vitro*, and some of which are not (Rasoanaivo, *et al*., 2011).

In this study one combination which is *A. remota*/*C. volkensii* 400:1600 µg/ml matched up with chloroquine 500 µg/ml parasitaemia levels showing significance while

compared with the untreated wells *p = 0.0382 and *p = 0.0367 respectively. These results are as documented by Rasoanaivo and colleagues (2011) that Kenyan anti-malarial plants combinations have also shown synergy where single plant extract would not match up with chloroquine but two combinations would. Another study indicated that the tested combinations of aqueous extracts of *A. cordifolia* and *M. indica* extracts exhibited synergistic effects *in vitro* against various *P. falciparum* strains (Jibira, *et al*., 2020)

5.2 Suppression of *P. berghei* infection in mice

A. remota and *C. volkensii* are commonly used traditionally in Kenya for malaria treatment. They are mostly boiled with water and administered orally. However, it has been documented that alcohol such as ethanol being more effective for extraction as more active substances will be extracted (Kuria, *et al*., 2001). This study therefore used *A. remota* ethanol extract and *C. volkensii* petroleum ether extract as they had been proved previously as most effective extracts in parasite inhibition. It had both single or individual extracts and a combined extract as treatments while dihydroartemisinin was used as the control drug. The mice were treated orally with a single dose for four days form 2 hours and after every 24 hours post-infection.

This study revealed that the combined extract was the most effective at suppressing *P. berghei* growth on day five post infection and after treatment with 83.66% suppression followed by DHA with 54.17% and *A. remota* was the poorest giving a negative suppression of -23.31% while *C. volkensii* suppressed the parasite by 4%. On day 6 the individual extracts showed no suppression with the parasitaemia higher than that of the untreated group (negative control group). DHA and the combined extract however had a suppression of 90.5%, and 66.7% respectively on day 6. This showed that individual

extracts were not active in parasite suppression as the combined. This concurs with a study that evaluated *Vernonia amygdalina* leaf extract that showed a higher efficacy in clearing *P. berghei* when combined with chloroquine unlike when used individually (Iwalokun, 2008).

Tarkang and colleagues (2014) found out that Nefang (combined aqueous extract of *Mangifera indica*, *Psidium guajava*, *Carica papaya*, *Cymbopogon citratus* and *Ocimum gratissimum*) exhibited excellent *in vivo* anti-malarial activities in rodents confirming its synergistic effects and it safe to use. Another study showed 5-Aminolevulinic acid and sodium ferrous citrate combination rescued more than 50% of mice from *P. yoelii* which showed safety since it was administered for over 30 days to cure the infected mice (Suzuki, *et al*., 2015). A combination therapy of arteether and garlic pearl oil gave 100% survival where if the concentration of garlic was increased, there was the complete protection of animals from malaria (Govindan, *et al*., 2016). Therefore, *A. remota* and *C. volkensii* extracts showed the ability to kill and inhibit parasite growth in mouse models when used in combination unlike when used individually.

5.3 Total IgG specific to *P. berghei* antigen

Medicinal plants are known to modulate the immune system (Singh, *et al*., 2017). All the animal groups that received plant extracts and DHA exhibited varying trends of *P. berghei* specific IgG. In this study, DHA showed the highest concentration of IgG (41251±17276 pg/ml) on day 8 while the combined extract showed the lowest concentration of 3905±808.6 pg/ml on the same day. Low levels of IgG shows that there is a high production of Th1 cytokines which are involved in parasite elimination.

The antibody secreted decreases parasitaemia by opsonizing infected red blood cells to facilitate active uptake by macrophages or by activating the complement cascade

(Nunes, *et al*., 2009). Studies in humans indicate an association between IgG1 and IgG3 levels hence protection against severe disease (Walker, *et al*., 2014). These two antibody isotypes have been revealed capable of facilitating opsonization (Leitner, *et al*., 2020).

In this study, high levels of total IgG antibody production in all groups were recorded throughout the study as compared to baseline samples. There were significant differences between the highest and lowest levels of protection. Based on these results, the role of IgG was evident in protecting the animals against high parasitaemia especially on day 8 and 10. The significant difference occurred between the individual plant extracts and the conventional drug or the combined extract which showed a tremendous suppression at the end of 4-days suppressive test. This coincides with a study done by Govindan and colleagues (2016) where arteether and garlic combination therapy resulted in a remarkable increase in anti-parasite antibody IgG contributing protective immunity during the recrudescence phase.

5.4 IFN-γ produced specifically for *P. berghei*

All the animal groups that received plant extracts and DHA exhibited varying trends of IFN-γ. IFN-γ production in the spleen of mice is triggered by *P. berghei* infection and that IFN-γ was released into the blood (Villegas-Mendez, *et al*., 2011). IFN-γ is an important macrophage activating factor (MAF) involved in the immune response to malaria (McCall & Sauerwein, 2010). It is produced by both CD8+ and CD4+ T lymphocytes in specific responses to antigen as well as by natural killer cells in a nonspecific manner (McCall & Sauerwein, 2010).

The *A. remota* and *C. volkensii* compounds have shown in combination to kill and inhibit the growth of parasites *in vitro* and in mouse models. The splenic T-lymphocytes proliferation modulated cytokine expression *in vitro*. IFN- γ production in the spleen of

mice is triggered by *P. berghei* infection and that IFN- γ is released into the blood, from where it is not rapidly eliminated because it cannot bind to its receptor (Villegas-Mendez, *et al*., 2011). The study revealed that between 6th to 10th days post infection IFN-γ was released indicating secretion at later days as well.

IFN-γ contributes to the resolution of primary infection by restricting parasite replication hence control parasitaemia and enhance host survival (He, *et al*., 2020). High parasite suppression in the group treated with a combined extract is attributed to the stimulation caused by the extract leading to IFN-γ production in mice during *P. berghei* infection. Zhu and colleagues (2012) established that innate immune responses contribute to the control of blood-stage malaria infection, decreases parasite load, and slows down the advancement to severe disease. The early inflammatory response is essential to control parasite replication in order to promote the clearance of infected erythrocytes (Garcia, *et al*., 2010).

Medicinal plants are known to modulate the immune system (Singh, *et al*., 2017). Results of this study concur with those of Dey and Chaudhuri (2014) where the Th1 cytokine (IFN-γ) expression on administration of *Dioscorea alata* was observed to increase in mice during *P. berghei* infection. In this study, the treatment of the infected mice with a combination of *A. remota* and *C. volkensii* might have alleviated the inflammatory effect of the *P. berghei* parasite by direct inhibitory effect on cytokines production (IFN-γ). The results of this study confirm that IFN-γ is significant in parasite clearance yet insufficient alone.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

In conclusion, individual plant extracts inhibited *P. falciparum in vitro*, though they were not effective in suppressing *P. berghei* infection in mice. Therefore, *A. remota* and *C. volkensii* extracts showed the ability to kill and suppress parasite growth in mouse models when used in combination unlike when used individually. A combination of *A. remota* and *C. volkensii* plant extracts proved more effective though it was not able to clear parasites completely just as the conventional drug DHA. This indicated a synergistic or additive effect of the combined extracts of *A. remota* and *C. volkensii* unlike that of the individual extracts. IgG and IFN-γ levels varied during different time points although showing significant difference. This is evidence that administration of the extracts affect the IgG and IFN-γ levels as compared to the baseline. This study therefore showed that *A. remota* and *C. volkensii* combined plant extract is a possible alternative for malaria remedy. *A. remota* herbs and *C. volkensii* trees should be safeguard as this study provides that they are a source of an alternative malaria treatment remedy.

6.2 Recommendations

- Further, isolation and characterization of molecules from these plants to identify the active molecules is suggested.

- Combining two plants showed a better antimalarial effect unlike the individual plants, therefore, more research on combination therapy using natural products should be done to come up with an alternative antimalarial drug.
- This study recommend that the evaluation of the combined extract (*Ar*E + *Cv*PE) IC50 to be done.
- The study recommend that further tests should be done on the remaining immunoglobulin as well as the pro-inflammatory and anti-inflammatory cytokines not tested such as IL-4, TNF-α, IL-10, etc.
- Further research should be done to obtain a dosage appropriate for the combined extract and determine toxicity which was a challenge to conduct.
- It is recommend that the combined extract should be tested on primates as well as determine any pathological symptoms in animals.

REFERENCES

Amino, R., Thiberge, S., Martin, B., Celli, S., Shorte, S., Frischknecht, F., & Menard, R. (2006). Quantitative imaging of *Plasmodium* transmission from mosquito to mammal. *Nature Medicine*(12), 220-224.

Basicmedical. (2016, September 3). *Basicmedical Ke: Fastest Biomedical Insight Engine*. Retrieved April 13, 2018, from Basicmedical Key Web site: http://basicmedicalkey.com/chemotherapy-of-malaria-3/

Bennink, S., Kiesow, M. J., & Pradel, G. (2016). The Development of Malaria Parasites in the Mosquito Midgut. *Cell Microbiol, 18*(7), 905-918.

Boissière, A., Tchioffo, M. T., Bachar, D., Abate, L., Marie, A., Nsango, S. E., . . . Morlais, I. (2012). Midgut Microbiota of the Malaria Mosquito Vector *Anopheles gambiae* and Interactions with *Plasmodium falciparum* Infection. *PLoS Pathog, 8*(5), 1-12.

Burrack, K. S., Hart, G. T., & Hamilton, S. E. (2019). Contributions of natural killer cells to the immune response against *Plasmodium*. *Malaria Journal, 18*, 321.

Chakravarty, S., Cockburn, I., Kuk, S., Overstreet, M., Sacci, J., & Zavala, F. (2007). CD8+ T lymphocytes protective against malaria liver stages are primed in skin-draining lymph nodes. *Nature Medicine*(13), 1035-1041.

Chiodini, P., Patel, D., Whitty, C., & Lalloo, D. (2015). Guidelines for malaria prevention in travellers from the United Kingdom. *Public Health England*.

Cocquyt, K., Cos, P., Herdewijn, P., Maes, L., Van den Steen, P., & Laekeman, G. (2011). *The Free Library*. Retrieved August 28, 2015, from The Free Library Web Site: http://the free library.com

Corbel, V., & Guessan, R. N. (2013). Distribution, Mechanisms, Impact and Management of Insecticide Resistance in Malaria Vectors: A Pragmatic Review. In S. Manguin, *Anopheles mosquitoes - New Insights Into Malaria Vectors.* IntechOpen. Retrieved from https://www.intechopen.com/chapters/43899

Dey, P., & Chaudhuri, T. K. (2014). *In vitro* modulation of TH1 and TH2 cytokine expression by edible tuber of *Dioscorea alata* and study of correlation patterns of the cytokine expression. *Food Science and Human Wellness*, 1-8.

Dunst, J., Kamena, F., & Matuschewski, K. (2017). Cytokines and Chemokines in Cerebral Malaria Pathogenesis. *Front Cell Infect Microbiol, 7*, 324-332. doi:10.3389/fcimb.2017.00324

Fidock, D., Rosenthal, P., Croft, S., Reto, B., & Nwaka, S. (2004). Antimalarial drug discovery: efficacy models for compound screening (supplementary document). *Nature Reviews Drug Discovery, 3*, 509-520.

Franklin, B., Parroche, P., Ataide, M., Lauw, F., Ropert, C., de Oliveira, R., . . . Gazzinelli, R. (2009). Malaria primes the innate immune response due to

interferon-gamma induced enhancement of toll-like receptor exression and function. *Proceedings of the National Academy of Sciences of the Unite States of America, 75*(4), 5789-5794.

Frimpong, A., Kusi, K., Tornyigah, B., Ofori, M. F., & Ndifon, W. (2018). Characterization of T Cell Activation and Regulation in Children with Asymptomatic *Plasmodium falciparum* Infection. *Malar J, 17*, 263. doi:10.1186/s12936-018-2410-6

Garcia, C., Guabiraba, R., Soriani, F., & Teixeira, M. (2010). The development of anti-inflammatory drugs for infectious diseases. *Discov. Med, 10*, 479-488.

Ghosh, A., Edwards, M., & Jacobs-Lorena, M. (2000). The journey of the malaria parasite in the mosquito: Hopes for the new century. *Parasitol Today*(16), 196-201.

Gillen, A. (2007). *The Genesis of Germs: The Origin of Diseases and the Coming Plagues.* United States of America: Master Books, Inc.

Ginsburg, H., & Deharo, E. (2011). A call for using natural compounds in the development of new antimalarial treatments - an introduction. *Malaria Journal, 10*(1).

Gitua, J., Muchiri, D., & Nguyen, X. (2012). In vivo antimalarial activity of *Ajuga remota* water extracts against *Plasmodium berghei* in mice. *Southeast Asian Journal of Tropical Medicine and Public Health, 43*(3), 545-548.

Global Positioning System. (2021). *Countrycoordinate*. Retrieved 9 2021, 22, from www.countrycoordinate.com: https://www.countrycoordinate.com/city-sagana-kenya/

Good, M., & Doolan, D. (2010). Malaria vaccine design: immunological considerations. *Immunity Review*.

Govindan, V., Panduranga, A., & Murthy, P. (2016). Assessment of *in vivo* antimalarial activity of arteether and garlic oil combination therapy. *Biochemistry and Biophysics Reports*, 359-364.

Granberg, A. (2010). *The Life Cycle of Malaria.* Retrieved June 3, 2015, from The Carter Center: http://www.cartercenter.org

Gun, S. Y., Claser, C., Tan, K. S., & Rénia, L. (2014). Interferons and Interferon Regulatory Factors in Malaria. *Mediators of Inflammation*, 1-21.

He, X., Xia, L., Tumas, K. C., Wu, J., & Su, X. Z. (2020). Type I Interferons and Malaria: A Double-Edge Sword Against a Complex Parasitic Disease. *Front. Cell. Infect. Microbiol, 10*, 1-13.

Iwalokun, B. (2008). Enhanced antimalarial effects of chloroquine by aqueous *Vernonia amygdalina* leaf extract in mice infected with chloroquine resistant and sensitive *Plasmodium berghei* strains. *African Health Sciences, 1*(8), 25-35.

Jibira, Y., Cudjoe, E., Tei-Maya, F. M., Ayensu, B., & Amoah, L. E. (2020). The Effectiveness of Varying Combination Ratios of *A. cordifolia* and *M. indica*

against Field and Laboratory Strains of *P. falciparum In Vitro*. *Journal of Parasitology Research*, 1-6.

Kareru, P., Kenji, G., Gachanja, A., Keriko, J., & Mungai, G. (2007). Traditional medicines among the Embu and Mbeere peoples of Kenya. *Afr J Tradit, Complement Altern Med, 4*(1), 75-86.

Kebenei, J., Ndalut, P., & Sabah, A. (2012). Anti-plasmodial Activity of Some Medicinal Plants used for Treatment of Malaria and Synergism of Methanolic Extracts of *Carissa edulis* and *Artemisia annua*. *Kabarack Journal of Research and Innovation, 1*(1), 84-93.

Kinyanjui, S. (2012). The immunology of malaria,. In O. Omolade (Ed.), *Malaria Parasites* (pp. 175-200). INTECH.

Korbel, D., Newman, K., Almeida, C., Davis, D., & Riley, E. (2005). Heterogeneous human NK cells responses to *Plasmodium falciparum*-infected erythrocytes. *Journal of Immunology, 175*(11), 7466-7473.

Kothari, C. (2004). *Research Methodology: Methods and Techniques* (second ed.). New Delhi: New Age International.

Kumar, A., Srivastava, P., Sirisena, P., Dubey, S. K., Kumar, R., Shrinet, J., & Sunil, S. (2018). Mosquito Innate Immunity. *Insects, 9*(3), 1-34.

Kumar, R., Ng, S., & Engwerda, C. (2019). The Role of IL-10 in Malaria: A Double Edged Sword. *Front Immunol, 10*, 229.

Kuria, K., De Coster, S., Muriuki, G., Masengo, W., Kibwage, I., Hoogmartens, J., & Laekeman, G. (2001). Antimalarial activity of *Ajuga remota* Benth (Labiatae) and *Caesalpinia volkensii* Harms (Caesalpiniaceae): *in vitro* confirmation of ethnopharmacological use. *Journal of Ethnopharmacology*(74), 141-148.

Leitner, W. W., Haraway, M., Pierson, T., & Bergmann-Leitner, E. S. (2020). Role of Opsonophagocytosis in Immune Protection against Malaria. *Vaccines, 8*, 1-15.

Li, C., Seixas, E., & Langhorne, J. (2001). Rodent malarias: The mouse as a model for understanding immune responses and pathology induced by the erythrocytic stages of the parasite. *Med Microbiol Immunol, 189*, 115-126.

Ly, A., & Hansen, D. S. (2019). Development of B Cell Memory in Malaria. *Front Immunol*, 1-11. doi:10.3389/fimmu.2019.00559

Ma, S., Zheng, L., Liu, Y., Guo, S., Feng, H., Chen, G., . . . Cao, Y. (2007). *Plasmodium yoelii*: Influence of antimalarial treatment on acquisition of immunity in BALB/c and DBA/2 mice. *Experimental Parasitology*(116), 266-272.

Marie, T. K., Mfouapon, H. M., Kemgne, E. A., Mbouna, C. D., Fokou, P. V., Sahal, D., & Boyom, F. F. (2018). Anti-*Plasmodium falciparum* Activity of Extracts from 10 Cameroonian Medicinal Plants. *Medicines, 5*(115), 1-14.

Marrelli, M. T., & Brotto, M. (2016). The Effect of Malaria and Anti-malarial Drugs on Skeletal and Cardiac Muscles. *Malaria Journal, 15*(524), 1-6. Retrieved from https://doi.org/10.1186/s12936-016-1577-y

McCall, M., & Sauerwein, R. (2010). Interferon-gamma--central mediator of protective immune responses against the pre-erythrocytic and blood stage of malaria. *J Leukoc Biol, 88*, 1131-1143.

Merlin, L. W., & Gerard, B. (2004). Traditional herbal medicines for malaria. *British Medical Journal , 329*, 1-4.

Moll, K., Ljungström, I., Perlmann, H., Scherf, A., & Wahlgren, M. (2008). *Mehods in Malaria Research* (5th ed.). Paris, France: BioMalPar.

Mumo, R. (2013). *Effect of Chemokine Adjuvants on Immunogenicity and Cross-protective Efficacy of Serine Repeat Antigen (SERA) DNA Vaccine Candidate Against Plasmodium berghei in Mice.* Nairobi: University of Nairobi.

Namrata, S., Mukul, T., & Mehta, S. (2016). A Review on Herbal Plants as Immunomodulators. *I. J. of Pharm. Sceince and Reserch*, 3602-10.

National Malaria Control Programme. (2019). *Kenya Malaria Programme Review 2018.* Nairobi: Ministry of Health.

Nie, C., Bernard, N., Schofield, L., & Hansen, D. (2007). CD4+ CD25+ regulatory T cells suppress CD4+ T-cell function and inhibit the development of *Plasmodium berghei* specific TH1 responses involved in cerebral malaria pathogenesis. *Infection and Immunity, 75*(5), 2275-2282.

Njoroge, G., & Bussmann, R. (2006). Diversity and utilization of antimalarial ethnophytotherapeutic remedies among the Kikuyus (Central Kenya). *Journal of Ethnobiology and Ethnomedicine, 2*(8).

Nunes, J., Starnbach, M., & Wirth, D. (2009). Secreted antibody is required for immunity to *Plasmodium berghei*. *Infection and Immunity, 77*(1), 414-418.

Nureye, D., & Assefa, S. (2020). Old and Recent Advances in Life Cycle, Pathogenesis, Diagnosis, Prevention, and Treatment of Malaria Including Perspectives in Ethiopia. *The Scientific World Journal*, 1-17.

Nyakundi, R. K., Nyamongo, O., Maamun, J., Akinyi, M., Mulei, I., Farah, I. O., . . . Kariuki, T. M. (2016). Protective Effect of Chronic Schistosomiasis in Baboons Coinfected with *Schistosoma mansoni* and *Plasmodium knowlesi*. *Infection and Immunity, 84*(5), 1320-1330.

Obidike, I., Amodu, B., & Emeje, M. (2015). Antimalarial properties of SAABMAL ®: an ethnomedicinal polyherbal formulation for the treatment of uncomplicated malaria infection in the tropics. *Indian Journal of Medical Research, 141*(2), 221-227.

Ochieng, C., Owuor, P., Mang'uro, L., Akala, H., & Ishola, I. (2011). Antiplasmodial and antinocieceptive constituents from *Caesalpinia volkensii* Harms (Caesalpiniaceae). *The 14th NAPRECA symposium and AAMPS.* (pp. 270-273). Nairobi: Ethnoveterinary medicine symposium.

Ogila, K. (2011). Antimicrobial and immunomodulatory properties of extracts of *Asparagus setaceous* Kunth and *Caesalpinia volkensii* Harm. *Thesis*. Nairobi.

Pal, A., & Pawar, R. (2011). A study on *Ajuga bacteosa* wall ex. Benth for analgesic activity. *International Journal of Current Biomedical Science, 1*(2), 12-14.

Perez-Mazliah, D., & Langhorne, J. (2015). CD4T-cell Subsets in Malaria:TH1/TH2 revisited. *Frontiers In Immunology, 5*, 1-8.

Perkins, D., Weinberg, J., & Kremsner, P. (2000). Reduced interleukin-12 and transforming growth factorbeta 1 in severe childhood malaria: Relationship of cytokine balance with disease severity. *J Infect Dis, 182*, 988-992.

Perlaza, B., Sauzet, J., Brahimi, K., BenMohamed, L., & Druilhe, P. (2011). Inteferon gamma, a valuable surrogate marker of *Plasmodium falciparum* pre-erthrocytic stages protective immunity. *Malaria Journal, 10*(1), 27.

Perlmann, P., & Troye-Blomberg, M. (2002). *Malaria Immunology* (2nd ed., Vol. 80). Switzerland: S Karger Pub.

Ragunathan, M., & Abay, S. (2009). Ethnomedicinal survey of folk drugs used in Bahirdar Zuria. *Ethiopia IJTK, 8*(2), 281-284.

Rasoanaivo, P., Wright, C. W., Willcox, M. L., & Gilbert, B. (2011). Whole Plant Extracts Versus Single Compounds for the Treatment of Malaria: Synergy and Positive Interactions. *Malaria Journal, 10*(Suppl 1), 1-12.

Rosales, C. (2017). Cellular and Molecular Mechanisms of Insect Immunity. *Insect Physiology and Ecology*, 170-212.

Roskov, Y., Abucay, L., Orrell, T., Nicolson, D., Kunze, T, . . . De Wever, A. (2014). *Catalogue of Life: 2015 Annual Checklist*. (Integrated Taxonomic Information System) Retrieved 3 18, 2016, from Calogue of Life Web site: http://www.catalogueof life.org

Sato, S. (2021). Plasmodium—a Brief Introduction to the Parasites Causing Human Malaria and Their Basic Biology. *Sato Journal of Physiological Anthropology, 40*(1), 1-13.

Sato, Y., Montagna, G. N., & Matuschewski, K. (2014). *Plasmodium berghei* Sporozoites Acquire Virulence and Immunogenicity during Mosquito Hemocoel Transit. *Infect Immun, 83*(3), 1164-1172.

Schemelzer, G., & Gurib-Fakin, A. (Eds.). (2008). Medicinal Plant 1. 133.

Sharma, S., Deoliveira, R., Kalantari, P., Parroche, P., Goutagny, N., Jiang, Z., . . . Golenbock, D. (2011). Innate immune recognition of an AT-rich stem-loop DNA motif in the *Plasmodium falciparum* genome. *Immunity, 35*(2), 194-207.

Singh, N., Tailang, M., & Mehta, S. (2017). A review on herbal plants as immunomodulators. *International Journal of Pharmaceutical Science and Research, 6*, 3602-3610.

Sinnis, P., & Zavala, F. (2012). The Skin: Where Malaria Infection and the Host Immune Response Begin. *NIH Public Access, 34*(6), 787-792.

Sullivan, D. (2006). *Malaria Chemotherapy and Drug Resistance.* Johns Hopkins University- School of Public Health.

Suzuki, S., Hikosaka, K., Balogun, E., Komatsuya, K., Niikura, M., Fumie Kobayashi, E., . . . Kita, K. (2015). *In vivo* curative and protective potential of orally administered 5-aminolevulinic acid plus ferrous ion against malaria. *Antimicrobial Agents and Chemotherapy, 59*(11), 6960-6967.

Tamokou, J. D., Mbaveng, A. T., & Kuete, V. (2017). Chapter 8 - Antimicrobial Activities of African Medicinal Spices and Vegetables. In V. Kuete, *Medicinal Spices and Vegetables from Africa: Therapeutic Potential Against Metabolic, Inflammatory, Infectious and Systemic Diseases* (pp. 207-237). Cameroon: Academic Press.

Tanzania Biodiversity. (2015). Retrieved June 3, 2015, from Tanzania biodiversity web site: http://tanzaniabiodiversity.com

Tarkang, P., Okalebo, F., Ayong, L., Agbor, G., & Guantar, A. (2014). Anti-malarial activity of a polyherbal product Nefang) during early and established *Plasmodium* infection in redent models. *Malaria Journal, 13*(456).

Uzor, P. F. (2020). Alkaloids from Plants with Antimalarial Activity: A Review of Recent Studies. *Evidence-Based Complementary and Alternative Medicine, 2020*, 1-17.

Villegas-Mendez, A., Souza, J. B., Murungi, L., Hafalla, J. C., N., S. T., Greig, R., . . . Couper, K. N. (2011). Heterogeneous and Tissue Specific Regulation of Effector T cell Responses by IFN-γ During *Plasmodium berghei* ANKA Infection. *J Immunol, 187*(6), 2885-2897.

Walker, K. M., Okitsu, S., Porter, D. W., Duncan, C., Amacker, M., Pluschke, G., . . . Todryk, S. M. (2014). Antibody and T-cell Responses Associated With Experimental Human Malaria Infection or Vaccination Show Limited Relationships. *The Journal of Cell, Molecules, Systems and Technologies, 145*, 71-81.

Walther, M., Tongren, J., Andrews, L., Korbel, D., King, E., Fletcher, H., . . . Hill, A. (2005). Upregulation of TGF-beta, FOXP3, and CD4+CD25+ regulatory T cells correlates with more rapid parasite growth in human malaria infection. *Immunity, 23*(3), 287-296.

WHO. (2014). *World Health Organization.* Retrieved Jan 10, 2015, from https://www.who.int/malaria/publications/world_malaria_report_2014/wmr-2014-no-profiles.pdf

WHO. (2017, November 29). *World Health Organization.* Retrieved 4 4, 2018, from https://www.who.int/malaria/publications/world-malaria-report-2017/report/en/

WHO. (2021, April 1). *World Health Organization.* Retrieved 9 22, 2021, from https://www.who.int/news-room/fact-sheets/detail/malaria

Wilson, N. O., Bythwood, T., Solomon, W., Jolly, P., Yatich, N., Jiang, Y., . . . Stiles, J. K. (2010). Elevated Levels of IL-10 and G-CSF Associated with Asymptomatic Malaria in Pregnant Women. *Infectious Diseases in Obstetrics and Gynecology, 2010*, 1-7. doi:10.1155/2010/317430

Wiser, M. F. (2019, November 22). *Tulane Edu.* Retrieved from Tulane University Web site: https://www.tulane.edu/~wiser/protozoology/notes/pl_sp.html

Woodrow, C. J., Haynes, R. K., & Krishna, S. (2005). Artemisinins. *Postgrad Med J*, 71-78.

Yamauchi, L., Coppi, A., Snounou, G., & Sinnis, P. (2007). *Plasmodium* sporozoites trickle out of the injection site. *Cell. Microbiol*, 1215-1222.

Yap, X. Z., Lundie, R. J., Beeson, J. G., & Keeffe, M. O. (2019). Dendritic Cell Responses and Function in Malaria. *Front Immunol, 10*, 357-365.

Zhu, X., Pan, Y., Zheng, L., Cui, L., & Cao, Y. (2012). Polysaccharides from the Chinese medicinal herb *Achyranthes bidentata* enhance anti-malarial immunity during *Plasmodium yoelii* 17XL infection in mice. *Malaria Journal, 11*(49), 5-7.

APPENDICES

Appendix I: Ethical approval

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

P.O BOX 24481, KAREN, NAIROBI
TELEPHONE 254-20-882571/4
FAX: 254-20-882546
E-Mail: ircsecretary@primateresearch.org

INSTITUTIONAL REVIEW COMMITTEE (IRC)

FINAL PROPOSAL APPROVAL FORM

Our ref: **IRC/05/16**

Dear **Dr. Lucy Ochola,**

It is my pleasure to inform you that your proposal entitled ***Evaluation of Combined Ajuga remota and Caesalpinia volkensii Extracts as an Anti-Malarial Phytomedicine*** in collaboration with **Prof. Rebecca Waihenya** of Jommo Kenyatta University of Agriculture and Technology, has been reviewed by the Institutional Review Committee (IRC) at a meeting of 21st March 2016. 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.

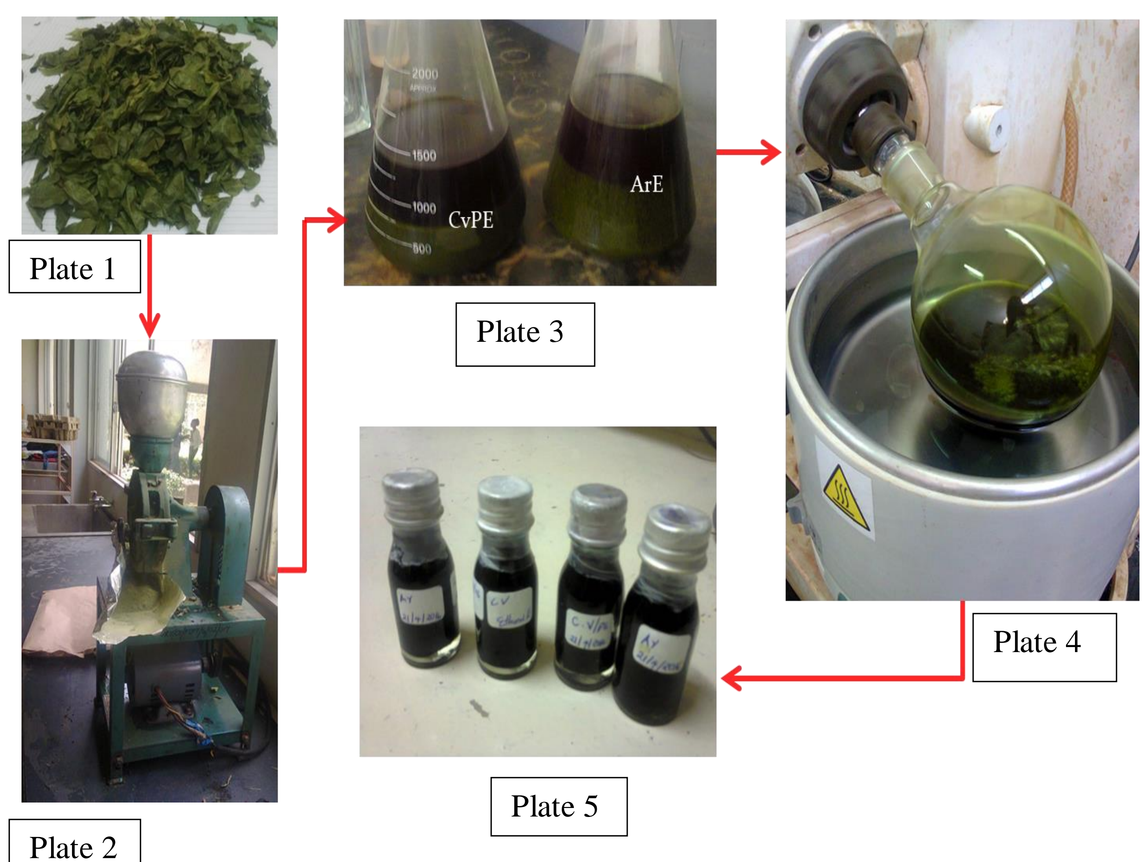
Signed...
Chairman IRC DR. I. O. FARAH

Signed........DR. NGALLA JILLANI..........
Secretary IRC

Date ...27TH APRIL 2016...........

INSTITUTE OF PRIMATE RESEARCH
INSTITUTIONAL REVIEW COMMITTEE
P. O. Box 24481-00502 KAREN
NAIROBI - KENYA
APPROVED..27/04/2016....

Appendix II: Extraction photos



<u>**Key**</u>

Plate 1: Air dried leaves

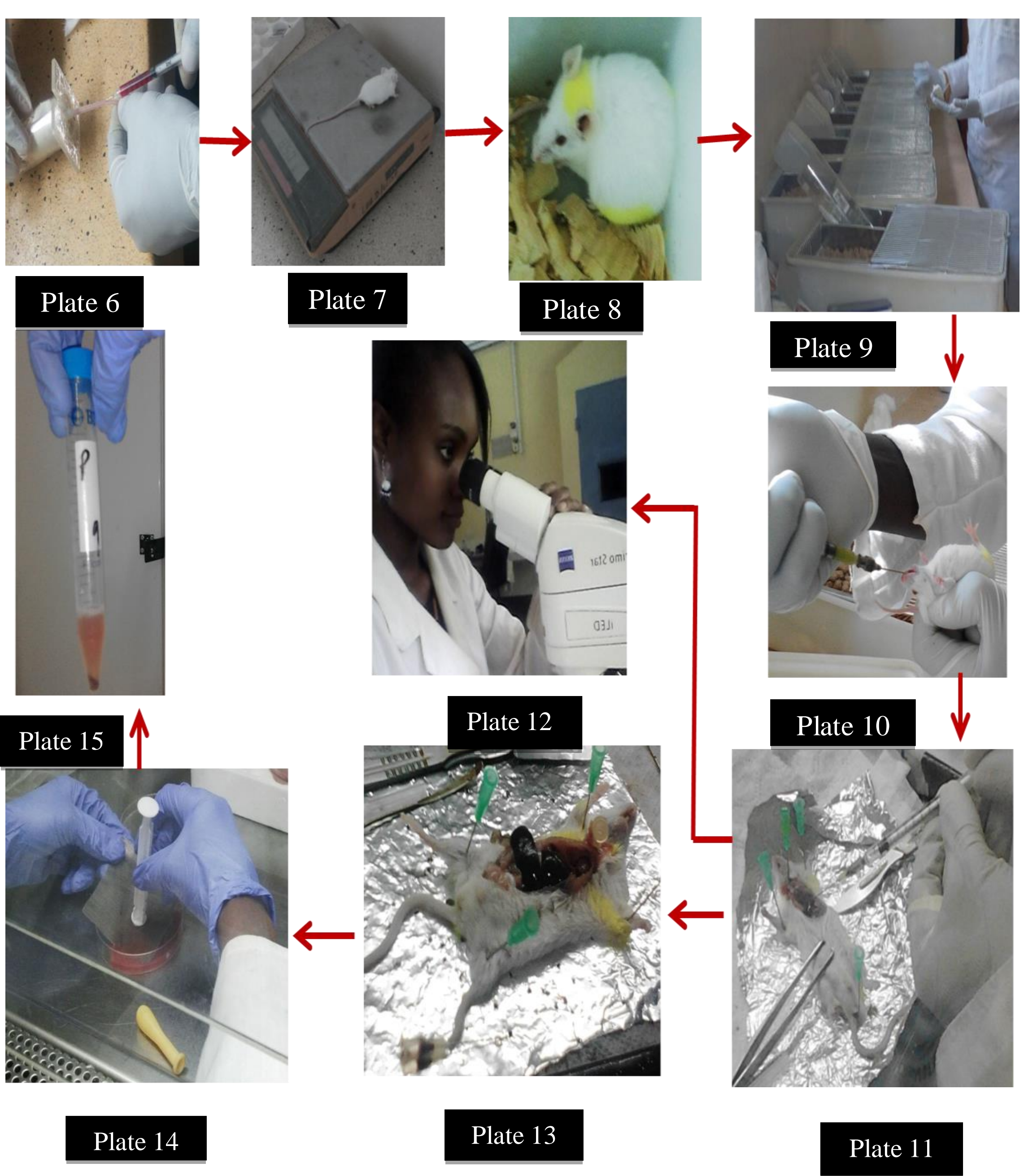
Plate 2: Grinding the leaves

Plate 3: Soaking the leaves powder with petroleum ether and ethanol solvents

Plate 4: Rotaeverpolating the extracts

Plate 5: Extracts obtained

Appendix III: *In vivo* photos



Key

Plate 6: Infecting donor mouse with *P. berghei*

Plate 7: Weighing a mouse on the weighing balance

Plate 8: Marking a mouse with picric acid

Plate 9: Grouping of the mice

Plate 10: Extract administration to a mouse

Plate 11: Mouse cardiac puncture

Plate 12: Determination parasitaemia under a microscope

Plate 13: Removing a spleen

Plate 14: Crushing a spleen

Plate 15: Spleen cells obtained

Appendix IV: Rodents experimental time flame

Student name ……………………………………………………………………………………….
Supervisor……………………………………………………………………………………..
Project number………………………………………………………………………………...
Department ………………………………………………………………………………………

Day	Date	Work	Samples
-	18th	Marking	
-	19th	Infection of the 4 donor mice	Thin smear
-	22nd -24th	Smear	Thin smear
0	25th	Infection of 105 mice + treatment of 4 groups after 2 hours + euthanize 2 mice + 4 smears (donor)	Blood (serum) + spleen + thin smears
1	26th	treatment with plant extracts	
2	27th	treatment + euthanize 15 mice	Blood (serum) + spleen + thin smears
3	28th	treatment with plant extracts	Thin smears
4	29th	treatment + euthanize 15 mice + 20 smear	Blood (serum) + spleen + thin smears
5	30th	20 smear	Thin smears
6	31st	euthanize 15 mice + 20 smear	Blood (serum) + spleen + thin smears
7	1st	20 Smears	Thin smear
8	2nd	euthanize 15 mice + 20 smear	Blood (serum) + spleen + thin smears
10	4th	euthanize 15 mice	Blood (serum) + spleen
12	6th	euthanize 15 mice	Blood (serum) + spleen
14	8th	euthanize 15 mice	Blood (serum) + spleen

Approved by: Signature:

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Appendix V: Sample collection table

Student name ………………………………………………………………………………..
Supervisor……………………………………………………………………………………
Project number……………………………………………………………………………….
Department ………………………………………………………………………………..

Date	Group	A. no.	Blood	Spleen	Comments

Approved by: Signature:

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