EVALUATING ANTI-PLASMODIAL ACTIVITIES OF SOYBEAN (*Glycine max*) SEED EXTRACTS

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(Medical Laboratory Science)

JOMO KENYATTA UNIVERSITY

OF

AGRICULTURE AND TECHNOLOGY

2022

Evaluating Anti-Plasmodial Activities of Soybean (*Glycine Max*) Seed Extracts

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A Thesis Submitted in Partial Fulfilment of the Requirements for the Degree of Master of Science in Medical Laboratory Sciences of the Jomo Kenyatta University of Agriculture and Technology

2022

DECLARATION

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

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The thesis has been submitted for examination with our approval as University supervisors

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DEDICATION

I dedicate this thesis to my parents, Mr. Hezron Nyandwaro and Mrs. Racheal Moraa and my siblings, Emily Nyandwaro and Mercy Nyandwaro for their patience, financial and moral support in the course of this study.

ACKNOWLEDGEMENT

I thank the most supreme God for the care, good health and support during the research journey. I am deeply indebted to acknowledge my supervisors and mentors Dr. Amos Mbugua and Dr. Francis Kimani for their selfless support and encouragement. For sure, it was unachievable without their input. I thank the KEMRI fraternity for the space and equipment provided. Specifically, KEMRI malaria culture laboratory; Mr. Njoka Muturi, animal house; Lucy and Job Oyweri for the endless provision of animals and help in laboratory animal handling respectively. I appreciate all the essential criticism injected by my course mates. Finally, I thank my family for the financial and moral support.

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ABBREVIATIONS AND ACRONYMS

ACT	Artemisinin based Combination Therapy
ANKA	A strain of *plasmodium berghei* parasite
ANOVA	Analysis of Variance
CO_2	Carbon dioxide
CQ	Chloroquine
CPM	Counts per minute
DDAW	Double distilled Water
DMSO	Dimethyl sulfoxide
FMOH	Federal Ministry of Health
G-3H	Hypoxanthine
IC_{50}	Inhibition Concentration
IRS	Indoor Residual Spraying
ITNs	Insecticide Treated Nets
IP	Intraperitoneal
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenya Medical Research Institute
LA	Lactic acid

LD 50	Lethal dosage that kills 50% of subject under study
$NaHCO_3$	Sodium hydrogen carbonate
NO_2	Nitrogen oxide
O_2	Oxygen
PBS	Phosphate buffered saline
PS	Parasitaemia suppression
RPMI	Roswell park memorial institute
SE	Soy bean Extract
WHO	World Health Organization

ABSTRACT

Plasmodium parasite resistance to *Artemisinin Combination Therapy* (ACT) in Thailand-Cambodia border, lately in Kigali Rwanda and Uganda, calls for development of new, affordable, safe and effective anti-malarial drugs. Studies done previously on soybean have established that it possesses anti-microbial, anti-inflammatory, anti-cancerous and anti-oxidant properties. The aim of this study was to conduct an *in vitro* evaluation of the anti-plasmodial activity of Soybean extracts using *Plasmodium falciparum* cultures, followed by *in vivo* evaluation of anti-malarial activity and safety of the extracts in *Plasmodium berghei* strain ANKA infected mice. Aqueous, methanol and peptide extracts of soy bean seeds were prepared. *In vitro* evaluation of the extracts for anti-plasmodial activity was carried out using two *P. falciparum* strains: D6, a chloroquine sensitive Sierra Leone 1 strain and W2, a chloroquine-resistant Indochina 1 strain obtained from KEMRI, Nairobi. Following the *in vitro* assessment, extracts showing the best activity (peptide and methanol) were selected for *in vivo* assay with mice infected with *P. berghei* ANKA strain. *Plasmodium berghei* ANKA is a surrogate for malaria. The two extracts were tested for their therapeutic potential (Curative test). The peptide extract was further assessed to determine whether it could prevent the establishment of a *P. berghei* infection. For the curative tests, methanol and peptide extracts were separately administered orally to three groups of five *P. berghei* infected Swiss albino mice for four days, at three dosage levels: 800, 400 and 200 mg/kg/day. The dosages were selected based on existing literature of animal studies, that at 1000mg/kg, the extract is considered lethal and as such, 800mg/kg was ideal. The study followed a dose dependent model. In the prophylactic test, the similar dosage regimen was applied at baseline to 3 groups of uninfected mice using the peptide extract which was administered orally for 4 days as a standard procedure. From the *in vitro* assay, peptide and methanol extracts showed good activity with IC_{50} of 19.97±2.57 µg/ml and 10.14±9.04 µg/ml, respectively against the D6 strain. The IC_{50} values for the peptide and methanol extracts were 28.61±1.32 µg/ml and 14.87±3.43 µg/ml, respectively against the W2 strain. On *in vivo* assay, methanol and peptide extracts exhibited high parasite suppressive activity of 72.9% and 71.9%, respectively using the 800 mg/kg dose. In prophylactic test, the peptide extracts group suppression of parasitemia was 64.66%, 57.12% and 43.14% for doses of 800, 400 and 200 mg/kg. Notably, there was significant decrease ($p<0.001$) in suppression with lower doses. The extract showed no mortality in mice up to a dose of 5000mg/kg within initial 24h and subsequent 14 days. This is an indication that the plant is safer to use with the recorded antimalarial activity. However, more research needs to be done on this plant to possibly establish lead compounds.

CHAPTER ONE

INTRODUCTION

1.1 Background information

Malaria caused by *Plasmodium falciparum* is the most worrisome recording the highest morbidity and mortality in sub–Saharan Africa. The population most affected is pregnant women and children below 5 years (Aminake and Pradel, 2013). Global malaria cases are approximately 300 million yearly with roughly one million deaths (Bundy *et al*., 2018). Anti-malarial drugs such as Artemisinin-based combination therapies (ACTs) face the growing threat of drug resistance (Recht *et al*., 2017). Drug resistance parasites develop following prolonged exposure to antimalarial drugs. This drug pressure facilitates emergence of parasite strains that possess molecular mechanisms that compromise or circumvent drug efficacy. Such is the case with ACT resistant *P. falciparum* parasites that delay parasite clearance and have been identified in South-East Asia. The growing threat of drug resistance to ACTs and other currently administered drugs necessitates the development of novel products for malaria treatment (van der Pluijm *et al*., 2021).

About 94 species of plants have produced 122 drugs through discovery of ethno botanical leads (Kinghorn *et al*., 2011). Majority of traditional medicinal plants are thought to be safe due to ethno medical knowledge available. Drugs have been generated from plants because of their availability, efficacy and their mode of action (Ridley, 2002). However, more studies are necessary to evaluate and validate new drugs from traditional medicine (Ginsburg and Deharo, 2011).

Soybeans (*Glycine max*) belong to the large botanical family Leguminosae and they grow in tropical, subtropical and temperate climatic regions. Their seeds measure 8 to 10 mm in diameter and grow within a pod similar to that of peas (Smỳkal *et al*., 2015).

They contain 20% oil content and are rich in phytochemicals which have health benefits hence making them neutroceuticals or functional food crops. (El Sohaimy, 2012).

Traditionally, *Glycine max* leaves, roots and seeds are used for treatment of wide range of ailments including malaria (Okeke *et al*., 2008). The plant has been demonstrated to possess significant biological activities such as antioxidant (Malenčić *et al*., 2007), anti-inflammatory (Kim *et al*., 2017), anti-diabetic (Zang *et al*., 2011), anti-depressants (Núñez *et al*., 2000), and anti-microbial (Arora *et al*., 2013). Soybeans have a healing potential against atherosclerosis, osteoporosis and various types of cancers including those affecting the prostate, breast and uterus (Paliyath *et al*., 2011). They also contain isoflavones which are thought to be a major contributor to the ant-oxidative activity of this legume (Pushpangadan *et al*., 2014).

Previous study on anti-malarial properties of Soy bean fat emulsions showed that *Glycine max* exhibited a promising inhibitory activity against 3D7 strain of *P. falciparum* with IC_{50} values ranging from 8.07±2.13 to 13.02±2.35 µg/ml (Deharo *et al*., 1995). The soy bean lipid extracts used in this study by were Intralipid® and Ivelip® which are commercial products used for intravenous lipid supplementation. The study demonstrated the antimalarial potential of soy beans even though radical cure could not be established. The current study set out to assess anti-plasmodial and anti-malarial activities of Soy beans seed extracts besides the lipid extracts. Additionally, toxicity study was conducted to determine the lethal dose of the extracts.

1.2 Statement of the problem

Antimalarials such as chloroquine, mefloquine, sulphadoxine and pyrimethamine have gained considerable drug resistance against *P. falciparum*. Likewise, insecticide resistance in Anopheline mosquito vectors are on the rise (Brouqui et al., 2012). Artemisia, the latest plant for malaria treatment is currently facing challenges of resistance against *plasmodium* parasite (Wongsrichanalai *et al*., 2002). This lead to

development of combination therapy of ACTs by WHO as first line malaria drugs. ACTs have been seen as a great achievement until recent studies which indicated decline of efficacy against ACTs in Thai-Cambodia border, region historically known for emergence of antimalarial drug resistance. Artemisinin is now established in Western Cambodia, Vietnam, Thailand, Eastern Myanmar and Northern Cambodia (Wangdi *et al*., 2015). Therefore, is a matter of time before the spread of resistant *P. falciparum* strain is eminent to endemic areas. Resistance of malarial drugs possess a threat to the control, treatment and management of malaria. There is need to research and generate new knowledge towards malaria control, so that to control the rise in drug resistant parasites of malaria (Alonso *et al*., 2011). Therefore, there is urge and need to develop better platforms for control measures and techniques from natural products. Most people in Kenya are unable to afford pharmaceutical drugs so they rely on traditional remedies which are sold cheaply in streets and not registered (Patwardhan and Partwardhan, 2005). This study investigated anti-malarial activities and safety properties of Soy bean in order to determine its potential as a preliminary step for establishment of novel and cheaper anti-malarial agent.

1.3 Justification

Natural plant extracts have been a source of new drugs. However, there is limited scientific knowledge and information available to assess the efficacy and safety of these herbal remedies. Natural products could provide a platform for drug discovery. In folk medicine, people use decoctions, concoctions and infusions from various plants to treat diseases they encounter. A variety of plants have been used in ethno medicine by different communities to treat malaria. The plant parts used include leaves, fruits, roots, stem bark as well as whole plant particularly when herbs are used. These parts are prepared by boiling, soaking in cold water, crushing of plant parts followed by soaking. The extracts can either be drunk alone, mixed with food, soup or even milk to make them palatable. Sometimes a decoction from single plant is administered alone or infusion containing extracts from two or three plants is given to the patient (Kokwaro,

2009). Studies done previously on soybean have established that indeed it has anti-microbial, anti-inflammatory, anti-cancerous and anti-oxidant properties. This provides a platform for investigation of anti-plasmodial using the soybean. In malaria treatment, safe and cheap development and potent anti-malarial plant extracts may solve the drug resistance challenge of malaria. Natural plants and seeds could provide starting points in drug discovery. From the past, traditional herbal medicine has provided a good basis for anti-malarial lead discovery and drug development. Quinine is an example of anti-malarial drug of plant origin isolated from bark of Cinchona tree in 1820 (Greenwood, 1992).

1.4 Objectives

1.4.1 General objectives

To evaluate antimalarial activity of different extracts of soy bean.

1.4.2 Specific objectives

1. To determine *in vitro* activity of Soybean extract against *Plasmodium falciparum* cultures.
2. To evaluate activity of Soybean extracts against *Plasmodium berghei* in mice.
3. To determine acute toxicity of Soybean extracts in mice
4. To investigate phytochemicals, present in Soybean extracts.

1.5 Null Hypothesis

Soybean Extract has no anti-plasmodial/antimalarial activity.

1.6 Limitations

The pure chemical compounds responsible for the anti-plasmodial and/ or antimalarial effects were not isolated in the study.

CHAPTER TWO

LITERATURE REVIEW

2.1 Introduction to malaria

Transmission of malaria is by mosquito and considered life threatening parasitic disease. Globally, malaria leads in cases of morbidity and mortality in children according to World Health Organization's in 2018. Statistically, malaria is responsible for roughly 2.3 and 9% disease burden worldwide and in Africa congruently. At least, 219 million occurrences of malaria were determined in 2017 globally as compared to 239 million incidences in 2010 and 217 million cases in 2016 according to the World Health organization report of 2018. Breaking it further, approximately, in 2017, 435 000 malaria deaths were recorded which is a drop from 451 000 and 607 000 estimated deaths in 2016 and in 2010 respectively (WHO, 2018). In Kenya, the western part is the most prevalent where children below 5 years of age and pregnant women are the most struck (Kepha et al., 2016a). Among the patients seeking treatment from ailments in malaria Kenyan-endemic regions, about 21% suffer from malaria. There has been shown a decrease in malaria morbidity and mortality due to increased malaria interventions (Mogeni et al., 2016). However, the disease remains a public health threat (Kepha et al., 2016b).

2.1.1 Life cycle of malaria parasite

Malaria develops in two phases: an exoerythrocytic and an erythrocytic phase. The exoerythrocytic phase involves infection of hepatic system, whereas erythrocytic phase involves infection of erythrocytes. When infected mosquito comes into contact with person's skin to feed on a meal, saliva which contain sporozoites enter blood stream and move into the liver. In the period of 30 minutes after being introduced into human body, sporozoites invade and infect hepatocytes multiplying asymptomatically and asexually for 6-15 days. Organism differentiate once in the liver to produce a thousand of

merozoites, which after rapture of cells, migrate to blood cells, thus forming the erythrocytes stage (Bledsoe, 2005). The parasite further multiplies asexually into ring forms then into trophozoites (feeding stage) and finally schizonts (a reproductive stage) and back to merozoites. Gametocytes (sexually form) are produced which infect the insect and continue the life cycle and finally invade fresh RBCs when they break out of their hosts. This simultaneous wave of merozoites escaping and infecting the RBCs causes classical description of waves of fever. The parasite is relatively protected from attack by the body's immune system because for most of human life cycle it resides within the liver and blood cells and is relatively invisible to immune surveillance. However, circulating blood cells (infected) are destroyed in the spleen. To prevent this cause, the *P. falciparum* parasite portrays adhesive proteins on the surface of infected blood cells, causing the blood cells to bind to the walls of small blood vessels, thereby sequestering the parasite from passage through the general circulation of the spleen (Rasti *et al*., 2004). The blockage of these vessels causes symptoms such as placental and cerebral malaria. In cerebral malaria, it can cause a coma due to the sequestrated RBCs which can breach the blood brain barrier (MacPherson *et al*., 1985). Mosquitoes are attracted to pregnant women (Lindsay *et al*., 2000) and malaria in pregnant women causes, infant mortality, low birth weight and stillbirths (Van Geertruyden *et al*., 2004) particularly in infection of *P. falciparum* but also in other species infection, such as *P. vivax* (Rodriguez-Morales *et al*., 2006).

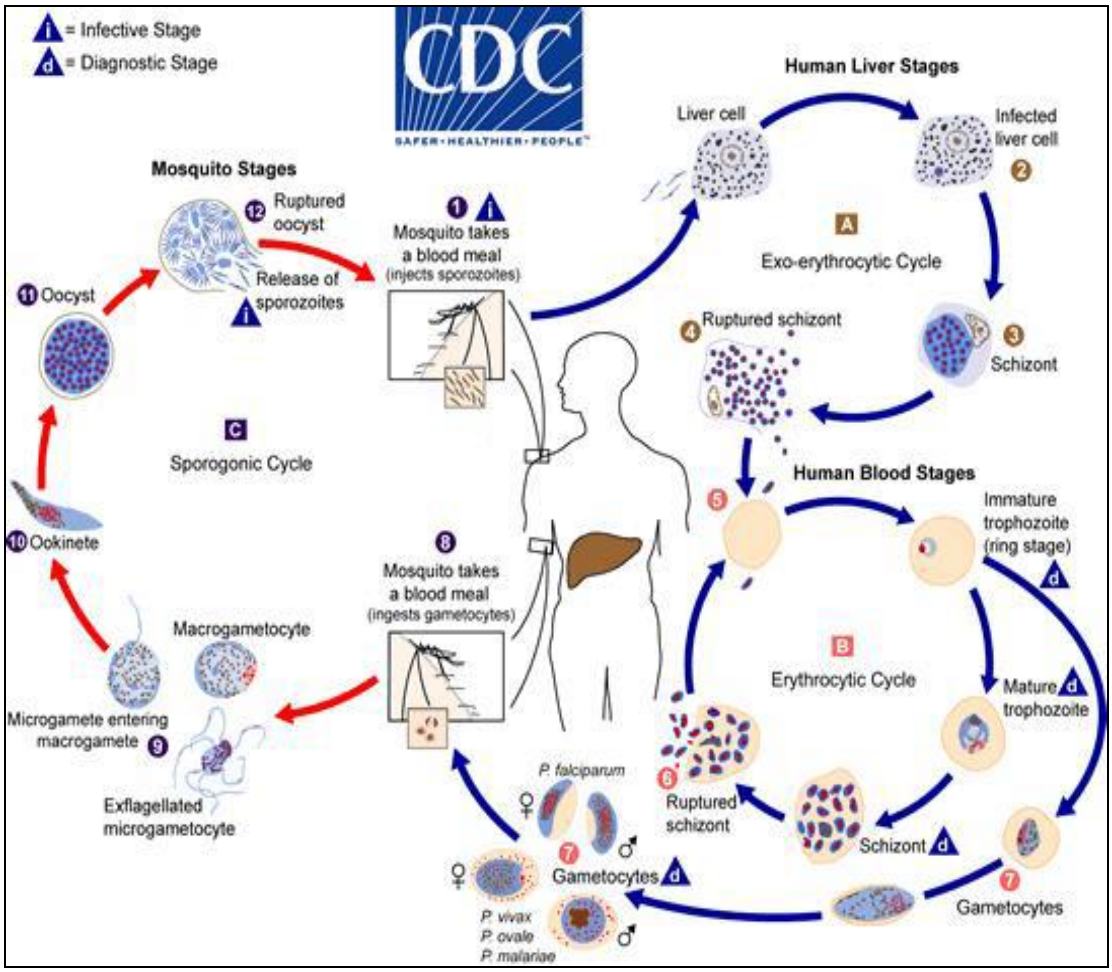

Figure 2.1: Lifecycle of malaria parasite

Cited fromhttps://www.ncbi.nlm.nih.gov/books/NBK5951/figure/malaria_LifeCycle/

2.1.2 Human *Plasmodium* Species

Four plasmodium species that causes malaria include *P. falciparum*, *P. ovale*, *P. malariae* and *P. vivax*. Others like *P. knowlesi* have been found in research to contribute to human malaria burden. (Cox-Singh *et al*., 2008). Other species such as rodents, birds, reptiles, monkeys and chimpanzees can be infected by the plasmodium species mentioned above (Escalante and Sastre, 1994).

Plasmodium falciparum is deadly with 90% of mortality cases of malaria. *Plasmodium vivax* is second and is viable in body for longer period than *P. falciparum* leading body deterioration and damage. *P. malariae* cause malaria of third common. Its progression is not faster compared to other *P. ovale* and is of less significance to the body and less pathogenic in humans (Breman *et al*., 2006). Transmission of malaria is through a bite of anopheles mosquito (infected) during feeding of blood from human body (Dieme *et al*., 2015). Another species *Plasmodium knowlesi* causing zoonotic malaria in humans which naturally infects Macaque monkey has been recently recognized (Antinori *et al*., 2013).

2.1.3 Rodents *Plasmodium* species

Rodents are widely used in research studies because their genetic composition resembles that of humans. The main used rodents used for research purposes in laboratory models include *P. chabaudi*, *P. berghei*, *P. yoelii*, and *P. vinckei* (Fidock *et al*., 2004). In 1948 Vincke and Lips discovered *Plasmodium berghei* and from there isolation of five strains namely LUKA, ANKA, NK65, SP11 and K173. Rodents such as rats, hamsters and mice are infected by *Plasmodium berghei* (Muthui, 2013). Comparable sensitivity is shown to all isolates and there is no significance change in iso-enzymes and anti-plasmodia remedies (Alves, 2016).

2.2 Pathogenicity of malaria

Infected erythrocytes ruptures releasing metabolites, parasitic material, cellular debris and hemozoin (i.e. malaria pigment) (Mawson, 2013). After that, the activity of reticuloendothelial increases and is defined by macrophages which digest hemozoin, normal and infected erythrocytes. The liver and spleen in particular are enlarged during the process. The pathology tends to benign with little mortality in malaria except *P. falciparum* (Sinard, 1996). The proinflammatory cytokines are believed to play a role in disease manifestations and especially tumor necrosis factor alpha which are higher levels of tumor factor alpha and other proinflammatory cytokines which are also associated

with cerebral malaria, anemia, and respiratory discomfort or distress (Law *et al*., 1996). However, the role inflammatory immune response plays in the resolution of the disease and its pathogenesis is not well defined. Pathology of severe *falciparum* malaria is believed to be contributed by cytoadherance and sequestistration. Cytoadherance of trophozoite and infected schizont erythrocytes to endothelial cells of deep vascular beds in vital organs, especially lung, heart, gut, placenta and brain is referred to as Sequestration (De Souza and Riley, 2002).

2.2.1 Malaria symptoms

Malaria manifestation in the body includes symptoms such as vomiting, fever, joint pain, shivering, anemia (caused by hemolysis), hemoglobinuria and retinal damage (Gul *et al*., 2016). Malaria is an acute illness. After the infective mosquito bite in an immunocompromised individual, symptoms appear after 7 days or more (usually 10-15 days). At first, symptoms includes headache, fever, chills and vomiting which makes it difficult to recognize as malaria (Hawker *et al*., 2008). The progression of *P. falciparum* malaria is severe and often leads to death if not treated quickly or within 24 hrs. In children with severe malaria the following symptoms develops; severe anemia, respiratory distress in relation to metabolic acidosis, or cerebral malaria. Multi organ involvement is also frequent in adults (Dondorp *et al*., 2008). There is a probability of people developing partial immunity in malaria endemic areas allowing asymptomatic infections to occur. During a period of rapid brain development, it leads to anemia and also direct brain damage. Children and pregnant women are more vulnerable to cerebral malaria as a result of damage of neurological function (Olness, 2003).

2.3 Malaria control and prevention

2.3.1 Vector control and intervention

The World Health Organization defines malaria control as the reduction in mortality and morbidity levels or rates through deliberate efforts of preventive and curative tools

available (Organization, 2002). Alternatives that target vector and parasite can be used to manage malaria associated worldwide. Strategies that rely on management and preventive are used to control malaria according to (Wilke and Marrelli, 2015). The following are some of the measures used; indoor residual spraying (IRS), long lasting insecticidal nets (LLINS), intermittent preventive treatment for pregnant women (IPTP), malaria vaccine and antimalarial drugs (White *et al*., 2011). There is need for integrated approach against malaria infection such as vector control, use of insecticides and chemotherapeutic, chemoprophylaxis and case management (Guerin *et al*., 2002). Various factors to consider in malaria management and control include (i) the anthropological, biological culture and social characteristics of the population; (ii) periodicity and intensity of malaria transmission; (iii) malaria parasite species and their antimicrobial drugs sensitivity; (iv) the mosquito vector species, and their susceptibility to insecticides and behaviors, each species of mosquito is thought to transmit malaria with its own behavior characteristics and ecological requirement; (v) change in social and ecological environment ; (vi) existing health services characteristics (Phillips, 2001).

Programs aimed by FMOH in current malaria program include; patient's malaria, individuals exposed and the mosquito vector. Also through diagnosis of parasite and prompt treatment, using Artemisinin based combination therapy. Lately prophylaxis exposure with nets treated and lasting insecticides and vector control by indoor residual spraying respectively (Gari *et al*., 2016).

2.3.1.1 Indoor residual spraying (IRS).

One of the standardized control method widely used in the world. It kills mosquitoes anytime they enter the house for feeding, which often does 2-3 days and this makes few of them to survive. IRS has been widely used all over the world and successful programs are underway in parts of Africa (Griffin *et al*., 2010). The effectiveness of IRS relies on the adherence to application procedures, efficacy of the insecticide, public acceptance of

spraying, and availability of well-maintained equipment, adequately trained spraying personnel, efficient supervision and strong financial support (Sherrard-Smith et al., 2018). Classes of insecticides widely used include organochlorines, organophosphates, carbamates and pyrethroids (Büyüksönmez *et al*., 1999). Selections of this depend on cost, efficacy and availability. Several insecticides can be used as water based compounds. Seasonality of malaria transmission and insecticide residual effect depends on the frequency of spraying. The selection of IRS as a control strategy needs to be based on local assessment of malaria risk and programme capacity (World Health Organization, 2019) . DDT which was the insecticide of choice for IRS during the Global Malaria Eradication Campaign (1955-1969) did not achieve the set objectives, it managed to eliminate malaria cases and reducing the disease burden (Oxborough, 2016). But in recent studies in South Africa, DDT has reduced malaria cases by 80% which has revived interest in insecticides (Cohen *et al*., 2012).

2.3.1.2 Insecticide treated nets (ITNs)

Nets are treated as insecticides designed to kill or prevent mosquitoes go past the net to search or feed for blood and are considered to be not a perfect barrier. ITNs are estimated to be twice effective compared to untreated nets (Breman *et al*., 2006). It has been established that ITN reduce child mortality in endemic areas of Africa (Binka and Akweongo, 2006). Such nets provide partial protection against biting and reduce the number of effective malaria parasite in mosquitoes but management of disease depends heavily on chemotherapy (Phillips, 2001).

2.3.1.3 Attraction and repellants

Mosquitoes are attracted to a number of chemical compounds that they can detect from 50 yards away (Gillies and Wilkes, 1969). The females' mosquitoes thirsty for blood for the purpose of production of their eggs especially the iron and protein human blood. Scientific knowledge suggests that mosquitoes are attracted to the following parameters.

Bacteria; about one trillion microbes live in the human skin and create body odor. This microbes enables the body to attract mosquitoes (Tyagi, 2004). Chemical compounds; the human body contains different chemical components which attract mosquitoes. They include lactic acid, ammonia, carboxylic acid and octanol (Mathew *et al*., 2013). The mosquitoes are attracted more to carbon dioxide. This means the more you emit carbon dioxide the more you attract the mosquitoes (Costantini *et al*., 1996). Older people produce more carbon dioxide compared to smaller people hence they attract more bites from mosquitoes (Takken and Verhulst, 2013). Movement and heat; this also contribute to attraction of mosquitoes. By somebody exercising on a warm summer evening is likely to be a perfect target (Spielman, 2001). Repellants such as DEET (N, N-diethyl-meta-toulamide) are widely used in hundred products and different concentrations. However, many studies suggest DEET cause potentially harmful effects such as brain damage. A variety of suggestions are provided on how to make your own natural mosquito repellant, as well as safe and soothing remedies for bites (Lo et al., 2018).

2.3.2 Diagnostic

Severe malaria can be misdiagnosed easily with diseases like dengue fever and therefore leading to wrong medication. This will result on severe life threatening conditions (Tangpukdee *et al*., 2009). Study investigations suggest that malaria retinopathy is better (collective sensitivity of 90%) than any other clinical or laboratory feature in differentiating malaria from non-malaria coma (USSAR, 1990).

2.3.2.1 Microscopy

Thin and thick films are used in identification of malaria parasites. Thick film enables screening of larger volume of blood and is more reliable and sensitive than thin film therefore, picking levels of infection which are low (World Health Organization, 1991). Thin films allow species identification because the parasite is best preserved in this form. Appearance of parasite is much more distorted and therefore distinguishing

between different species in thin film can be much more difficult. Diagnosis using this method is limited because it needs experienced microscopist (Hommel, 2002). To beat the limitations of microscopy, molecular tools have been used with appropriate markers (Zheng & Cheng, 2017).

2.3.2.2 Rapid diagnostic tests

In areas where laboratory staff is not experienced or microscope is not available, immunochromatographic tests (Malaria Rapid Diagnostic Tests, Antigen-capture Assays or 'Dipsticks have been developed (Moody, 2002). The test uses the *P. falciparum* lactate dehydrogenase, (pLDH), a 33kDa oxidoreductase. It is the last enzyme of the glycolytic pathway, essential for ATP generation and one of the most abundant enzymes expressed by *P. falciparum* (Wariso & Nwauche, 2011). pLDH does not persist in the blood but clears about the same time as the parasite following successful treatment (Hopkins *et al*., 2007). More accurate molecular methods have been established to differentiate parasite nucleic acid using polymerase chain reaction (PCR) (Singh, 1997).

2.4 Treatment of malaria

2.4.1 Antimalarial drugs

Plasmodium parasites are killed by at both sexual and asexual stages by antimalarial drugs. Drugs manufactured from Ethno medicinal plants include Quinine which produces mefloquine and chloroquine drugs (Gurib-Fakim, 2006). Quinine drug is isolated from plants of *cinchona Species* (Andrade-Neto *et al*., 2003). Another drug component obtained is Artemisinin which is used in development and production of drugs such as artemether and artesunate (Mutabingwa, 2005).

Antimalarial drugs classification is based on therapeutic response to malaria parasite and core functional chemical structures. Therapeutic response to malaria drugs include

quinine, halofantrine, mefloquine, chloroquine, amodiaquine, atovaquone, sulfadoxine and Artemisinin. (Wongsrichanalai *et al*., 2002). Amodiaquine, chloroquine, quinine and Artemisinin are anti-gametocyte drugs. Primaquine, lumefantrine and pyrimethamine are drugs that target liver schizont (Saif, 2017).

2.4.2 Mechanism of action of known antimalarial drugs

2.4.2.1Aminoquinolines

Aminoquinolines include drugs such as chloroquine, amodiaquine, quinine and mefloquine (White, 2007). They work by concentrating inside the acidic digestive vacuole, where they bind haem and interfere with haem detoxification. As a result, parasites dies by blocking the polymerization of the toxic pigments called haemozoin of malaria pigment, leading to cell lysis and parasite cells auto digestion (Monti *et al*., 1999). The quinolone antimalarial work by accumulating in acidic food vacuole of the malaria parasites due to their basicity (Roux and Biot, 2012)

2.4.2.2 Sesquiterpene endoperoxide

Artemisinin's mechanism of action has been a subject of extensive research work by several groups but remain open for more development (Foley and Tilley, 1998). Artemisinin's mechanism of action involves activation of the endoperoxide moiety by haem (ferriprotoporphyrin ix), which is liberated in the infected erythrocytes during haemoglobin digestion. The cleavages of the Endoperoxide Bridge hemolytically by heme (ferriprotoporphyrin ix), a ubiquitous cellular component of *Plasmodium falciparum*, give rise to reactive free radial intermediates that allegate vital parasite protein molecules (Y. Wang, 2009). Therefore, artemisinin anti-malarial activity is mediated by free radicals. Haem accumulates in the parasite as a result of hemoglobin digestion and haem (ferriprotoporphyrin ix) formed then reacts with artemisinin to generate free radicals by hemolysis of the endoperoxide. Interaction between haem and

Artemisinin results in membrane damage of parasite (Hoppe *et al*., 2004). Blood stages of parasite are killed rapidly by artemisinin which leads clearance rate of anti-malaria to be short.

2.5 Drug resistance in Malaria

The definition of anti-malaria resistance is ability of a parasite strain to survive and/or multiply despite the administration and absorption of a drug given in doses equal to or higher than those usually recommended, but within tolerance subjects by WHO (Power, 2017). *Plasmodium falciparum* and *Plasmodium vivax* are commonly affected by resistance of anti-malarial drugs. *Plasmodium falciparum* being the affected most to nearly all anti-malaria in current use, although distribution of resistance to any single anti-malarial drug varies greatly geographically (Bloland & Organization, 2001). *Plasmodium vivax* has been shown to be resistant in some areas to chloroquine (Marques *et al*., 2014). ACTs which are best treatment of *Plasmodium falciparum* and have energized worldwide programs in management of malaria (Krisher *et al*., 2016). Drugs such artemisinin and its derivatives are facing resistant from malaria parasites worldwide which is a concern to strategy control of malaria (White, 2004). Current drugs of anti-malaria act on a limited number of biological targets hence there is need to develop drugs that will attack novel molecular targets.

When adequate blood concentration of drug is not able to kill susceptible parasite, resistance occurs. Treatment failure results from anti-malarial drug resistance in an individual patient, by increasing the number of malarial infections in the body (White *et al*., 2009). In many cases, drug resistance does not contribute to treatment failure in all circumstances. Factors such as dosing, noncompliance with duration of drug regimen, poor or erratic absorption and misdiagnosis lead to treatment failures. The above factors cause drug resistance by increasing chances of exposure of parasite to suboptimal drug levels (Bloland and Organization, 2001). The resistance profile of existing antimalarial drugs is shown in table 1.

Table 2.1: Existing Antimalarial drugs, uses and resistance profile (Amadi et al., 2019)

Common name	Clinical use	Resistance
Artemisinin	In Artemisinin-based combination therapies	Possibly emerging
Lumefantrine	Most common first line antimalarial therapy in Africa.	No evidence of high level resistance.
Amodiaquine	In combination with artesunate in parts of Africa.	Limited cross resistance to chloroquine.
Quinine/ quinidine	Mainly for treating severe malaria, often with antibiotics	Exists at low level.
Chloroquine	Former first line treatment for uncomplicated malaria	Widespread
Pyrimethamine	For intermittent preventive treatment combined with sulphadoxine	Widespread
Primaquine	For eliminating liver stage parasites, including dormant forms of plasmodium vivax	Widespread

2.6 Malaria and natural products development

In the modern world, natural products have been the key pillar to the development of drug for a prolonged time and continues to provide new platform of discovery of drugs (Koehn and Carter, 2005). Natural products and plants remain the primary source of many important orthodox medicines currently in the drug market. This implies that many drugs are developed from plants and natural products. A good example includes artemisinin an anti-malarial drug and anti-cancer agent, taxol from *taxus beviforia* both are developed from plants. 39% of all new approved drugs in 1983-1984 were developed from natural products or derived from natural products (Wang *et al*., 2007). Also in a survey it was established 60% -80% of antibacterial and anticancer drugs were from natural products (Simmons *et al*., 2005). Quinine and artemisinin drugs which are widely used for treatment of malaria were all obtained from plants and are used for treatment of malaria (Greenwood, 1992). With increased spread of resistance of anti-

malarial standard drugs like chloroquine and emergence of artemisinin-resistant parasites, new ideas for development of malaria remedies are urgently needed.

2.6.1 African traditional medicine plants

Plants are involved in a major system of indigenous (medicinal plants) system widely used in Africa (Fennell *et al*., 2004). They are widely used in management of malaria and related symptoms. Treatment of a variety of parasitic diseases in Africa is done by indigenous plants (Ssegawa and Kasenene, 2007), because of affordability and accessibility, traditional plants have been primarily used for treatment in healthcare system where local system and society uses them as a source of treatment. Drugs developed from plants provide an outstanding achievement to modern therapy (Balunas and Kinghorn, 2005). Approximately 25% of modern medicine was developed from indirectly or directly from plants of medicinal origins and application of both traditional and modern technology (Fowler, 2006). Drugs like digoxin and diyitoxin are developed from *digitalis* leaves, quinine from *cinchona* bark, and morphine from *papaver somniferum* and cocaine from *Erythroxylum coca* (Kong *et al.,* 2003). This implies that development is useful and of great importance. About 122 drugs have been developed from 94 species of plants through ethno botanical research leads (Heinrich, 2000).

2.7 Soybeans

Soybean (*Glycine max*) belongs to a large botanical family Leguminosae growing in tropical, subtropical and temperate climatic regions. Soy bean seeds contain 20% oil content and are 8 to 10 mm in diameter and grow within a pod similar to that of peas (Smỳkal *et al*., 2015). Vegetable Soybean contain rich phytochemicals which are beneficial to the human being hence considered a neutroceuticals or a functional food crop. One of the importance of soy beans is reducing the risk of a wide range of hazardous diseases like atherosclerosis, osteoporosis, types of cancer (prostate, breast and uterus,) hence attracting people's attention across the world (Gogus and Smith,

2010). It also contains isoflavones which are thought to be a major breakthrough responsible for ant-oxidative activity (Ponnusha *et al*., 2011). Glycine max seeds contain different proteins which have anti-nutritional and/or toxic effects, such as soybean agglutinin (an N-acetylgalactosamine-specific lectin). Lectin isolated from Glycine max is a carbohydrate which binds to protein highly specific to terminal non-reducing N-acetyl-D-galactosamine but less to D-galactose (Bhol, 2012). Presence of galactose during biofilm formation had various effects in the presence or absence of Soybean Agglutinin (SBL) (Pérez-Giménez *et al*., 2012). Lectin isolated from soybean has a vital role in the initial recognition of *Rhizobium japonicumby* the plant, which leads to a strain-specific, nitrogen-fixing symbiosis (van Rhijn and Vanderleyden, 1995). Supplementation of soybean lectin (SBL) in diets resulted in a decrease in the activity of trypsin while protein levels and amylase activity increased in the pancreatic juice (Bhol, 2012). Consumption of Soy beans and soy products has been involved with reducing the risks of various cancers such as prostate and other chronic inflammatory diseases. Epidemiological evidence and studies in cancer models suggest that isoflavones play an important role in cancer prevention. For example, the consumption of soy products, which contain a mixture of soybean isoflavones, may contribute to the relatively lower rates of breast, colon, and prostate cancer in countries such as Japan and China (Sarkar and Li, 2003). However, the exact biological mechanisms underlying this effect remain to be fully elucidated. The most tenable mechanism being postulated for soybean isoflavones. Cancer preventive function is that soybean isoflavones have strong antioxidant capacity. The two pharmacophores that account for soybean isoflavones antioxidant activity are the 4 hydroxyl group on the B-ring and the hydroxyl groups on the AC-ring. All of them could be hydrogen/electron donators (Hassan, 2006). Some positives results about antioxidant and antibacterial activity of soybean seeds have been established (Malenčić *et al*., 2007). Soybean seeds are rich in proteins, isoflavones and phytoestrogens, while Ginestein a soy isoflavones, has been reported to possess anti-cancerous (Ponnusha *et al*., 2011).

CHAPTER THREE

MATERIALS AND METHODOLOGY

3.1 Study design

The study was a laboratory based experimental investigation of the potential anti-plasmodial and anti-malarial activity of Soybean (*Glycine max*) extract (SE). The anti-plasmodial activity of crude SE was accomplished through evaluation of anti-plasmodial activity of crude SE (*in vitro*) on *P. falciparum* cultures and a *Plasmodium berghei* mouse malaria model (*in vivo)* evaluation. The study was carried out at the Centre for Biotechnology Research and development (CBRD) and Animal House at the Kenya Medical Research Institute (KEMRI) respectively.

3.2 Sourcing of Glycine max seeds

Soybean seeds variety SB 19 were collected and packed in plastic paper bags from Kenya Agriculture and Livestock Research Organization (KALRO) in Nairobi Kenya in October 2018. The seeds were air dried for 3 weeks at room temperature and pulverized using laboratory mills.

3.3 Preparation of Soy bean extracts

Three extracts (water, methanol and peptide) were prepared from the milled Soybean seeds.

3.3.1 Aqueous and methanol extract preparation

100g of Soybean powder was macerated in 1 litre of respective solvents (water or methanol). After dissolution, the solutions of samples were shaken in an incubator shaker at moderate temperature of 37°C±2 for 7 days. The samples were taken out of

shaker afterwards and filtered initially with muslin cloth and then with Whatman filter paper #1 so that a transparent solution was obtained. The aqueous and methanol filtrates were next placed in an open water bath for the solvent to evaporate at 35°C±2 for 6-7 hours daily until dried extracts were obtained. The dried extracts were transferred into vials and stored at -20°C for preservation until further processing.

3.3.2 Peptide extract preparation

To prepare the peptide extract, 10g of Soybean powder was added to a 100 ml cold extraction buffer (10mM Na_2HPO_4, 15mM NaH_2PO_4, 100Mm KCl, 1.5% EDTA) PH 5. The solution was incubated for 3 hours at 4°C. The precipitate was obtained by saturation and centrifuged at 2500 revolution per minute. The crude peptide pellets were air dried and stored at - 20°C for further analysis. A single extract was prepared for use in both in vitro and in vivo experiments. Despite prolonged usage of Glycine max extract for months, it still demonstrated continued activity and therefore possible long shelf life.

3.3 Determination of extract toxicity (LD_{50})

The median lethal dose (LD_{50}) was determined for estimating acute toxicity of Soybean extract in Swiss albino mice model using the method of Lorke, (1983). The extracts used were methanolic and peptide extracts. The method involved administration of different (1000, 3000 and 5000mg/kg) doses of SE orally to the three groups of mice. Mortality was monitored within the initial 24h and successive behavioral changes were observed for the next 14 days. Physical observations monitored were as follows: decreased motor activity, decreased body/limb tone, writhing, respiration and death.

3.4 Phytochemical screening

Screening for phytochemicals of Soy bean extracts was carried to reveal the presence of chemicals such as alkaloids, terpenoids, saponins, glycosides, tannins, flavanoids,

phenols and sterols as illustrated by (Sheel *et al*., 2014; Tiwari *et al*., 2011). The experiments were run as follows:

Detection of alkaloids: An extract of 1 gm, received an addition of three drops of HCL acid. Later a test of Wagner (Iodine in potassium iodide) was used as follows; three drops of Wagner's reagent followed the sample. Establishment of reddish-brown precipitate indicated presence of alkaloids.

Detection of Terpenoids: Use of Salkowski test; a mix of 1 gm of the extract and 2ml of chloroform was done. Conc. Sulphuric acid of 3ml, was added with care forming a layer. Development of a reddish-brown boundary is an indication of terpenoids.

Detection of saponins: Foam Test; to 1.5 grams of the extract, 2 ml of water was added and shaken well. Presence of saponins is indicated by the formation of persistent foam.

Detection of glycosides: To 1 gram of extract, 1 ml of water was added and mixed well by shaking. An aqueous solution of 2 ml of sodium hydroxide was added. Yellow colouration confirmed glycosides presence.

Detection of tannins: The determination was via Gelatin Test. Briefly, 1.2 grams of extract, 3ml of 1% gelatin solution containing sodium chloride was added. A white precipitate formation showed the presence of tannins.

Detection of flavonoids: Alkaline Reagent Test: 1.3 gm of extract was subjected to 5 drops of sodium hydroxide solution. An intense yellow colouration turning colourless on exposure to 3 drops of dilute HCL means flavonoids were present.

Detection of phenols: Ferric Chloride Test; 4 drops of ferric chloride solution were added to 1.5 grams of extract. Development of black colour confirmed phenols were present.

Detection of sterols: Salkowski's Test; A mixture of 2ml chloroform and 2 ml of concentrated sulphuric acid were added to 1.5 grams of extract. Formation of yellow fluorescence and subsequent red layer of chloroform and acid is as a result of the availability of sterols.

3.5 *In vitro* evaluation of *anti-plasmodial* activity

3.5.1 *In vitro* culturing of *P. falciparum*

The procedures for the *in vitro* culture studies were conducted in accordance to Trager and Jensen, (2005) research. Chloroquine (CQ)-sensitive (D6) and CQ-resistant (W2) *P. falciparum* strains were used to assess the anti-plasmodial activity of Soybean extract and fraction of erythrocytic stages *in vitro.* The cultures were maintained at the Centre for Biotechnology Research and Development (CBRD) at the Kenya Medical Research Institute (KEMRI). The *P. falciparum* cultures were maintained according to the method described by (Trager & Jensen, 1976) with minor modifications. *P. falciparum* (D6 and W2) cultures were maintained in fresh human erythrocytes of O+ suspended at 4% hematocrit in RPMI 1640 (sigma) containing 0.2% sodium bicarbonate, 0.5% albumax, and 45µg/L hypoxanthine and 50 µg/L gentamicin and incubated at 37°C under a gas mixture of 5% oxygen, 5% carbon dioxide and 90% nitrogen. Infected erythrocytes were transferred into fresh complete medium to propagate the culture daily. In *P. falciparum* (INDO strain) culture medium, albumax was replaced by 10% pooled human serum. Parasitemia was determined qualitatively and quantitatively by light microscopy using Giemsa staining. A thin blood smear was done on a glass slide and fixed in methanol. The fixed slide was stained with 10% Giemsa solution for 10 minutes and microscopically read under oil immersion.

3.5.2 *In vitro* anti-plasmodial assays

To assess the anti-plasmodial activity of the SE extracts, an *in vitro* serial micro-dilution assay technique that measures the ability of extracts to inhibit the incorporation of [G3-H] hypoxanthine by *P. falciparum* was used (Sixsmith et al., 1984). The experiments were conducted on 96-well plates. First, 25µl of culture medium was added to all the wells of a 96-well flat-bottomed microtiter plate except those on the 2nd row. 50 µl aliquots of the three extract solutions (aqueous, methanol and peptide) were added, in duplicate, to the wells of the second row. A Titertek motorized hand diluter (Flow laboratories, Uxbridge UK) was used to make two-fold serial dilution of each Soybean extract over a 64-fold concentrations range. The stock solutions (100 µg/ml) of the three Soybean extracts were diluted two-fold down until a concentration of 1.56 µg/ml was reached. The susceptibility test was performed using an initial parasitemia of 0.4% by adding 200 µl of 1.5% hematocrit *P. falciparum* culture to each well on the 96-well plate, with the exception of the last 4 wells of the first row. Parasitized and non-parasitized erythrocytes were added into all test wells in such a way that the 0.4% parasitemia and 1.5% hematocrit was maintained. Before each anti-plasmodial test, parasite cultures were synchronized in a ring stage which were obtained following serial treatment with chloroquine. The first row acted as positive control and was therefore not treated. The first four wells served as negative control for normal growth and contained 200 µl of *Plasmodium* blank red blood cells. Chloroquine substituted the extract as reference drugs (positive controls) for standardizing the experiment. After the plates were incubated at 37°C (3% CO_2, 5% O_2 and 92% NO_2) for 48hrs, radio-labelled hypoxanthine was then added to each well (0.5 µl in 25 µl of culture medium). The plates were further incubated for 18 hours to allow its uptake by surviving parasites. After the second incubation, the plates were frozen overnight at -20°C to stop the growth. The plates were thawed at room temperature for 1.5 hours before harvesting. Each well was harvested using Betaplate TM cell harvester (Wallace, Zurich Switzerland), which transferred the red blood cells onto a glass fibre filter where they

were washed with distilled water. The dried filters were then inserted into a plastic foil with 10ml of scintillation fluid and counted in a Betaplate TM liquid scintillation counter (Wallac, micro beta Trilux). The results were recorded as counts per minute (cpm) per well at each concentration. Data was transferred to MS Excel software and expressed as a percentage of the untreated controls. The drug concentration capable of inhibiting 50% of the *P. falciparum* (IC_{50}) was determined by logarithmic transformation of drug concentration and radioactive counts per minutes (cpm) using the formula;

IC_{50}= antilog [logX1+ (log Y_{50}-log Y1) (log X2-logX1)/ (log Y2-log Y1)]

where Y_{50}, was the cpm value midway between parasitized and non-parasitized control cultures and X1, X2, were the concentrations and Y1 and Y2 , the cpm values, respectively, for the data points above and below the cpm midpoints (Tona et al., 2001). The determined SE IC_{50} values were classified in Table 1 as per criteria provided by (Ager, 1984).

Table 3.1: Adopted classification of antiplasmodial activity (Muthaura *et al*., 2007)

IC_{50} value (µg/ml)	**Category of activity**
>100	Inactive
50 -100	Low
10 -50	Moderate
$\leq$ 10	High

3.6 Animals, handling and parasites used

Female Swiss albino mice aging 6-8 weeks' old were randomly selected from KEMRI animal facility Nairobi. They were weighed to achieve 20$\pm$2 g and housed in well labeled standard microlon type II cages in the experimental rooms. The relative humidity in the rooms was maintained at 60-70%. Additionally, appropriate ventilation and room temperature were observed. The animals were fed with viable rodent feed and provided

with water *ad libitum*. Each cage accommodated five mice per test sample. *Plasmodium berghei* ANKA parasites were the candidates for evaluation of parasite reduction in mice (Ager, 1984). The parasites were obtained from Kenya Medical Research Institute (KEMRI) Nairobi and maintained by sub passage in mice. Blood infected with *P. berghei* strain ANKA was harvested from donor mice by heart puncture into 15 ml centrifuge tubes containing 1% (w/v) heparin. The parasitized blood was diluted to attain roughly 10^8 parasitized red blood cells (RBCs) per ml. The experimental animals were intraperitoneally infected with 0.2 ml ($2x10^7$parasitized RBCs) and randomly grouped.

3.7 Extracts and administration

The SE extracts and chloroquine were dissolved in 3% dimethyl sulfoxide and 10% tween 80 in PBS. The Soybean extracts (200, 400, 800 mg/kg), vehicle (3% dimethyl sulfoxide, 10% tween 80 in PBS) and chloroquine (5mg/kg) were administered orally to the test groups, negative group and the positive groups respectively. A stainless metallic feeding cannula was used during administration (Peters, 1965).

3.8 Assessment of prophylactic activity of Soy bean extract

The prophylactic activity of SE and chloroquine were assessed by using the method described by (Ryley & Peters, 1970). The mice were randomly divided into 5 groups of 5 mice each in a cage. Group 1 to 3 were orally administered with 200, 400, 800 mg/kg/day of SE respectively. Group 4 mice were administered with 5mg/kg/day of chloroquine (positive control) while group 5 mice received a placebo consisting of 3% dimethyl sulfoxide and 10% tween 80 in PBS (negative control). Administration of the SE continued consecutively for 3 days i.e., from day 0 to day 3. On the fourth day, the mice were inoculated with *P. berghei* (ANKA). The parasitemia level of each mouse was assessed by blood smear after 72 hours. Survival rate was monitored on a daily basis in all groups for 28 days' post-inoculation. Giemsa-stained thin blood smears were prepared from the tail of each animal to determine parasitemia and percentage inhibition.

3.9 Assessment of curative effect of Soy bean extract

The method to evaluate the schizonticide activity of SE in established infection as described by (Tona et al., 2001) was used. 0.2ml of *P. berghei* ANKA ($2x10^7$parasitized RBCs) was injected intraperitoneally into 25 mice on the first day. Two hours later, the mice were divided randomly into five groups of 5 mice each. The experimental groups were treated orally with a single 0.2 ml dose of SE at one of the three dosage levels of 200, 400, 800 mg/kg in groups 1 to 3, respectively. For the positive control group (group 4), 5ml/kg/day of chloroquine was administered. Group 5, which were the negative control, received the placebo (vehicle; 3% dimethyl sulfoxide, 10% tween 80 in PBS). The SE and drugs were administered daily for 3 (day 0 to 3) days. Thin smears of Giemsa were prepared from blood obtained from the tail collected on each day of treatment for purpose of monitoring the infection. Parasitemia was determined after 4 days (24hrs after last treatment) by microscopic examination by counting parasites in 4 fields of covering 100 erythrocytes per view of thin blood film sampled from the tail of experiment mouse stained with 10% Giemsa solution. The difference between the mean number of parasites per view in negative control group (100%) and those of experimental groups was calculated and expressed as percentage parasitemia suppression (chemo suppression) for each group, according to the formula below:

Parasitemia suppression (PS) = [(A-B)/A] ×100 according to (Tona et al., 2001).

Where A =Mean parasitemia in negative control on day 4.

B= Corresponding parasitemia in test group.

Percentage parasitemia was calculated as parasitized number of erythrocytes per 100 erythrocytes while chemo suppression percentage was taken as inhibition of parasite multiplication relative to the control expressed in percentage. Survival rate was monitored on a daily basis in all groups for 28 days' post-inoculation.

3.10 Data and statistical analysis

Analyzed data was presented as mean ± standard deviation. *In vitro* tests were done in duplicate and data evaluated by Microsoft Excel 2016 with aid of nonlinear regression analysis to determine IC_{50}. Analysis for the *in vivo* work was achieved using SPSS Version 25. Within groups, statistical significance for contrast of parasitemia reduction and survival time was attained using one-way analysis of variance (ANOVA) and Tukey's Honest Significant Difference post-hoc test.

3.11 Ethical consideration

Permission to carry out the study was obtained from Scientific and Ethics Review Unit (SERU), (Study SERU No. 3795). The experiments were carried out in compliance with Animal Care and Use Committee (ACUC) Regulations of KEMRI. In addition; the internationally established values for laboratory animal use and care as per WHO recommendations were followed. Intraperitoneal injection was conducted using gauge 23 needles. Death as a means of experimental end, was done in a humane manner whereby animals were sacrificed in carbon (iv) oxide gas chamber. The dead animals were correctly bagged in well labelled plastic bags and incinerated.

CHAPTER FOUR

RESULTS

4.1 Phytochemical analysis

Eight qualitative chemical tests were performed on the three Soybean extracts to determine the presence of phenols, flavonoids, tannins, steroids, alkaloids, saponins, glycosides and terpenoids. The results showed presence of steroids, alkaloids and saponins in aqueous extract and methanol extract (Table 3). Flavanoids, steroids, glycosides and tannins were present in peptide extract.

Table 4.1: Results of the phytochemical screening of the three extracts of Soybeans Extract

Phytochemicals	Aqueous Extract	Methanol Extract	Peptide Extract
Phenols	–	–	–
Flavanoids	–	+	+
Tannins	–	–	+
Steroids	+	+	+
Alkaloids	+	+	+
Saponins	+	+	–
Glycosides	–	+	+
Terpenoids	–	–	–

*Phytochemicals are indicated as present (+) or absent (–)

4.2 Acute toxicity study

The results of acute toxicity showed that the extract caused no mortality within the dose range of 1500-5000mg/Kg within the initial 24 hours as well as within the successive 14 days. This was a clear indication that doses used are safe. No physical and behavioral signs of toxicity such as decreased motor activity, decreased body/limb tone, writhing, respiration and death amongst others were observed. This suggests that the LD_{50} of the peptide and methanolic extracts is greater than 5000mg/Kg orally.

4.3 *In vitro* assessment of SE anti-plasmodium activity

The activities of the three different extracts of *Glycine max* against two strains of *P. falciparum* ranged from inactive for the aqueous extract, to moderately active for both the methanol and peptide extracts (Table 4). Methanol and Peptide extracts showed good activity against D6 and W2 strains. Methanol extract showed the best activity followed by peptide extract. Water extract had IC_{50}>200 µg/ml thus no activity against the two strains of *P. falciparum*.

Table 4.2: In *vitro* ant-plasmodial activity of three extracts of *Glycine max*

Drug/Extract	Extract yield (%)	D6 - IC_{50} (Mean±SD) (µg/ml)	W2 - IC_{50} (Mean±SD) (µg/ml)
H_2O	9.90	>200	>200
Methanol	7.40	10.142±9.043	14.867±3.439
Peptide	57.50	19.967±2.517	28.613±1.324
Control (CQ)		0.011 ± 3.120	0.091 ± 0.031

* The results are presented as mean ± SD of mean of IC_{50}s

4.4 *In vivo* anti-malarial curative activity of methanol and peptide extracts of *Glycine max*

Two Soybean extracts that displayed activity (methanol and peptide) in the *in vitro* studies were next tested for anti-malarial activity against *P. berghei* ANKA infected Swiss albino mice. This was accomplished using 4-day suppressive method for methanol and peptide extracts. The results are presented in Table 5 below. Chemosuppresion was established in a dose dependent manner after four days of antimalarial treatment using SE extracts (200-800 mg/kg). The mean parasitemia in groups treated with methanol ranged from 3.48±0.37 to 5.99±0.18 while that of animals treated with peptide extract varied from 3.61±0.27 to 5.87±0.25. The mean parasitemia in the negative control group was 12.87±0.26. There was a significant percentage parasitemia difference between the

test groups when compared with the untreated control group ($P < 0.001$). At 800 mg/Kg, the extracts demonstrated the highest chemosuppresion. Remarkably, there was a slight difference in chemosuppresion between the positive control and the two extracts. The extracts were able to prolong survival of the animals after treatment termination in comparison to the negative control.

Table 4.3: In *vivo* anti-malarial activities of crude extract of Glycine max in Curative test.

Drug/ Extract	Dose mg/Kg	Mean ± SD parasitemia (%)	% suppression of parasite	Mean survival time (days)
Methanol	200	5.99±0.18	53.45	10.50±0.58
	400	4.54±0.22	64.67	11.25±0.96
	800	3.48±0.37	72.93	16.25±0.96
Peptide	200	5.87±0.25	54.39	11.00±0.82
	400	4.52±0.13	64.89	12.40±1.14
	800	3.61±0.27	71.90	15.60±1.52
CQ		1.19±0.36	90.72	28.25±1.50
vehicle (3% dimethyl sulfoxide, 10% tween 80 in PBS)		12.87±0.26		5.00±0.82

*The results are expressed as mean ± SD.

4.5 *In vivo* anti-malarial prophylactic activity of peptide extract of *Glycine max*

In prophylactic assay, the parasitemia in the negative control group was significantly higher than in any of the test group ($P < 0.001$). All the animals in the positive group displayed suppression of parasitemia of 83.09%. The peptide extracts group suppression of parasitemia was 64.66%, 57.12% and 43.14% for doses of 800, 400 and 200 mg/kg. The mean parasitemia in groups treated with peptide ranged from 3.94+0.46 to 6.35+0.22. The results are presented in Table 6 below. The peptide extract was able to prolong survival of the animals after treatment termination in comparison to the negative control.

Table 4.4: In vivo anti-malarial activities of crude extract of Glycine max in Prophylactic test.

Extract	Dose mg/Kg	Mean ± SD parasitemia (%)	% suppression of parasite	Mean survival time (days)
Peptide	200	6.35±0.22	43.14	7.50±0.58
	400	4.78±0.30	57.12	9.25±0.96
	800	3.94±0.46	64.66	10.25±0.96
CQ		1.89±0.16	83.09	28.25±1.50
vehicle (3% dimethyl sulfoxide, 10% tween 80 in PBS)		11.16±1.15		5.00±0.82

*The results are expressed as mean ± SD.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

5.1.1 Existing knowledge in regard to the study

Many antimalarial drugs currently available on the market have been developed from plants and natural products (Heinrich, 2000). *Plasmodium falciparum* resistance to the existing antimalarial calls for improved interventions (Mueller *et al*., 2000). The best solution to this challenge remains to be probably therapeutic plants (Gasquet *et al*., 1993; Wright & Phillipson, 1990). Artemisinin derivatives have been used to manage malaria for long (Zhang *et al*., 2009). Quinine which is widely used for treatment of malaria was obtained from *Cinchona ofcinalis* plants (Saxena *et al*., 2003). Most people in Africa have greatly relied on traditional remedies due to affordability (Cunningham, 1993). This study investigated anti-malarial activities and safety properties of Soybean (*Glycine max*) in order to determine their potential as a source of a novel and cheaper anti-malarial agent.

The study clearly demonstrates the potential activity of the *Glycine max* seeds extract against malaria.

5.1.2 Phytochemical study

Phytochemical analysis of methanol and peptide extract revealed presence of flavonoids, alkaloids, steroids and glycosides unlike in aqueous extract which had only steroids and alkaloids in Table 3. It has been reported that oral administration of phytochemicals like saponins, tannins and phenols possess ability to suppress cellular immunity (Nafiu *et al*., 2013). Previous research has shown that the amount of secondary metabolites available in the plants under examination play a greater role in drug research. Formerly, alkaloids,

saponins, flavonoids and tannins have been demonstrated to aid antimicrobial and antimalarial activities of selected medicinal plants (Ghosh *et al*., 2010). Notably, the study results were in conformity with those of Arora *et al*., (2013) who reported presence of alkaloids, flavonoids, tannins and saponins when determining antimicrobial effects of *Glycine max*. Therefore, the results of this study may have been influenced by single or combination of the mentioned phytochemical ingredients in the crude extracts in exerting activity against malaria.

5.1.3 Toxicity assay

An ideal antimalarial should be safe without any adverse effects. Toxicity study was done to evaluate the suitability of the use of this plant extract. The outcomes indicated that the concentrations of the extracts applied on the *in vivo* experiments were harmless with no animal death noted within the initial 24 hours and successive 14 days at 1500-5000 mg/kg dosage. Importantly, animals stayed alive for the entire four days of the experiments indicating safety as suggested by (Satayavivad *et al*., 1998) in their work.

5.1.4 *In vitro* and *in vivo* findings

Study findings agree with those of Deharo et al. who carried out the evaluation of antiplasmodial and antimalarial properties of soy bean fat emulsions. They recorded antiplasmodial activity of IC_{50} of 13.02 ± 2.35 $mg.ml^{-1}$ when they used Ivelip test sample indicating quick parasite inhibition (Deharo *et al*., 1995). In this study, *in vitro* assays of peptide and methanol extracts showed activity with IC_{50} of 19.97±2.57 µg/ml and 10.14±9.04 µg/ml against D6 strain and 28.61±1.32 µg/ml and 14.87±3.43 µg/ml against W2 strain respectively as shown in Table 4. The use of D6 and W2 strains provokes the model behind acquisition of drug resistance. Elaborately, in this study, W2 strain which is chloroquine resistant, tries to outcompete the inhibitory activity of peptide extract exhibiting 28.61±1.32 µg/ml in comparison to the methanol extract showing 14.87±3.43 µg/ml. On the other hand, D6 strain is left vulnerable because of its chloroquine

susceptibility, whereby the peptide at IC_{50} of 19.97±2.57 µg/ml and 10.14±9.04 µg/ml is a true reflection of the same. Therefore, this may be seen as a preliminary step towards drug resistance establishment under continuous drug pressure. However, the study differed on the *in vivo* assay whereby they reported lower percentage parasite inhibition upon use of Ivelip test sample i.e., at 3.2 g. kg^{-1}, an inhibition of 35±26 was recorded. The difference in the results may be possibly due to difference of the samples used. Nevertheless, both studies provide a clear picture on the potential use of this plant for malaria management. In this study, the *in vivo* assay showed good activity of significant reduction in percentage parasitemia on the test groups compared to negative control group ($P<0.001$). *In vivo* antiplasmodial activity falls into 3 categories of classification; moderate, good and very good if the extract displayed parasitemia suppression percentage equal to or greater than 50% (Tarkang *et al*., 2014). In the present study, very good results were obtained in the curative test with methanol and peptide extracts exhibiting over 50% chemosuppression. Methanol and peptide extracts exhibited high suppressive activity of 72.9% and 71.9% using 800 mg/kg dose respectively. Notably, there was significant decrease ($p<0.001$) in activity with lower doses to 64.7% and 64.9% at 400mg/kg and 53.4% and 54.4% at 200 mg/kg respectively in curative test as indicated in Table 5. A maximum parasite suppression of 72.9% and 71.9% was produced by methanol and peptide in the highest dose of 800mg/kg and longest survival time compared to other doses. This might be due to the fact that active compounds responsible for the antimalarial activity mostly occur in low levels in natural products and activity may not be detected in lower doses (Batista et al., 2009). Likewise, in prophylactic test, peptide extract exhibited suppressive activity of 64.7% at 800mg/kg and 57.1% at 400mg/kg and 43.1% at 200mg/kg as shown in Table 6. Studies done previously on *Glycine max* established in deed that it has antioxidant properties (Malenčić *et al*., 2007). It has been reported that antioxidant activity can inhibit heme polymerization as heme as to be oxidized before polymerization, and the unpolymerized heme is very toxic to malaria (Monti *et al*., 1999). This can also be assumed to be one of the factor that lead to presence of antimalarial in *Glycine max*. The chemosuppresion

data showed that parasite clearance was much more pronounced on the fourth day. This may be attributed to high drug concentration in the blood due to repeated dosing. Interestingly, the parasite reduction activity exhibited on the fourth day suggests that the bioavailability of the chemical components present in the crude extracts are not possibly affected by biotransformation and physiological factors. The *in vivo* antiplasmodial activity of plant extract is a better screening method compared to *in vitro* method because some drugs act as prodrugs, febrifuges or immune modulators.

5.1.5 Potential mechanism of action of *Glycine max*

The mechanism of action of *Glycine max* is not yet known. However, existing literature have shown that some plants and seeds exhibit antiplasmodial activity either by causing red blood cells oxidation (Salvagno *et al*., 2015) or by inhibitory protein synthesis (Baragaña *et al*., 2015) depending on their phytochemical constituents. Flavonoids are known to exert antiplasmodial activity by chelating with nucleic acid base pairing of the parasite (Okokon *et al*., 2017). Therefore, it's possible that the antiplasmodial activity exhibited by *Glycine max* could have been as a result of the above-mentioned ways or by yet a different unknown mechanism. The limitation of the study is that identification of specific compounds responsible for antimalarial activity was not done. Therefore, we recommend that further analysis should be done on *Glycine max* seeds to identify the specific antimalarial compounds present.

5.2 Conclusion

Glycine max extracts demonstrated significant ($P < 0.05$) parasitemia reduction activity in all the antimalarial evaluations. Therefore, these results provide room for scientific exploitation of the plant to manage malaria. The study justifies the use of soy bean plant to traditionally heal malaria among the Igbo people in Nigeria.

5.3 Recommendations

1. The possibility of *Glycine max* incorporated in food substances should be considered in a wake to determine if the body can develop immune to malaria.
2. The use of *Glycine max* can be exploited scientifically in combination with other plants for effective treatment of malaria.
3. The active compounds in the peptide and methanolic extracts of soy bean need to be isolated.

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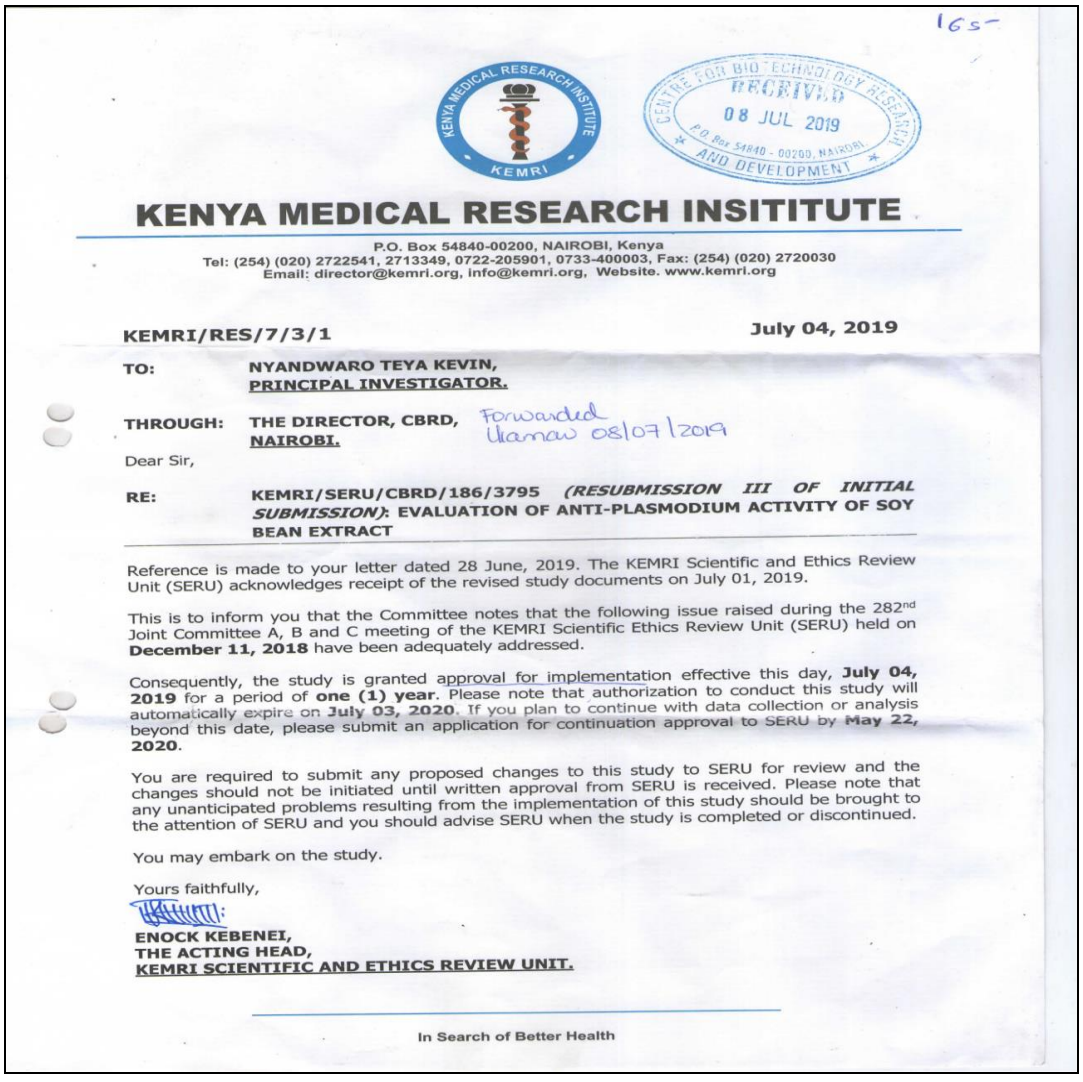
APPENDICES

Appendix I: Research Ethical Approvals

(i) Scientific and Ethics Review Unit (KEMRI) Approval



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KENYA MEDICAL RESEARCH INSTITUTE

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KENYA MEDICAL RESEARCH INSITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
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Email: director@kemri.org, info@kemri.org, Website. www.kemri.org

KEMRI/RES/7/3/1 July 04, 2019

TO: NYANDWARO TEYA KEVIN,
PRINCIPAL INVESTIGATOR.

THROUGH: THE DIRECTOR, CBRD, Forwarded Ukamaw 08/07/2019
NAIROBI.

Dear Sir,

RE: KEMRI/SERU/CBRD/186/3795 *(RESUBMISSION III OF INITIAL SUBMISSION)*: EVALUATION OF ANTI-PLASMODIUM ACTIVITY OF SOY BEAN EXTRACT

Reference is made to your letter dated 28 June, 2019. The KEMRI Scientific and Ethics Review Unit (SERU) acknowledges receipt of the revised study documents on July 01, 2019.

This is to inform you that the Committee notes that the following issue raised during the 282nd Joint Committee A, B and C meeting of the KEMRI Scientific Ethics Review Unit (SERU) held on **December 11, 2018** have been adequately addressed.

Consequently, the study is granted approval for implementation effective this day, **July 04, 2019** for a period of **one (1) year**. Please note that authorization to conduct this study will automatically expire on **July 03, 2020**. If you plan to continue with data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **May 22, 2020**.

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received. Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

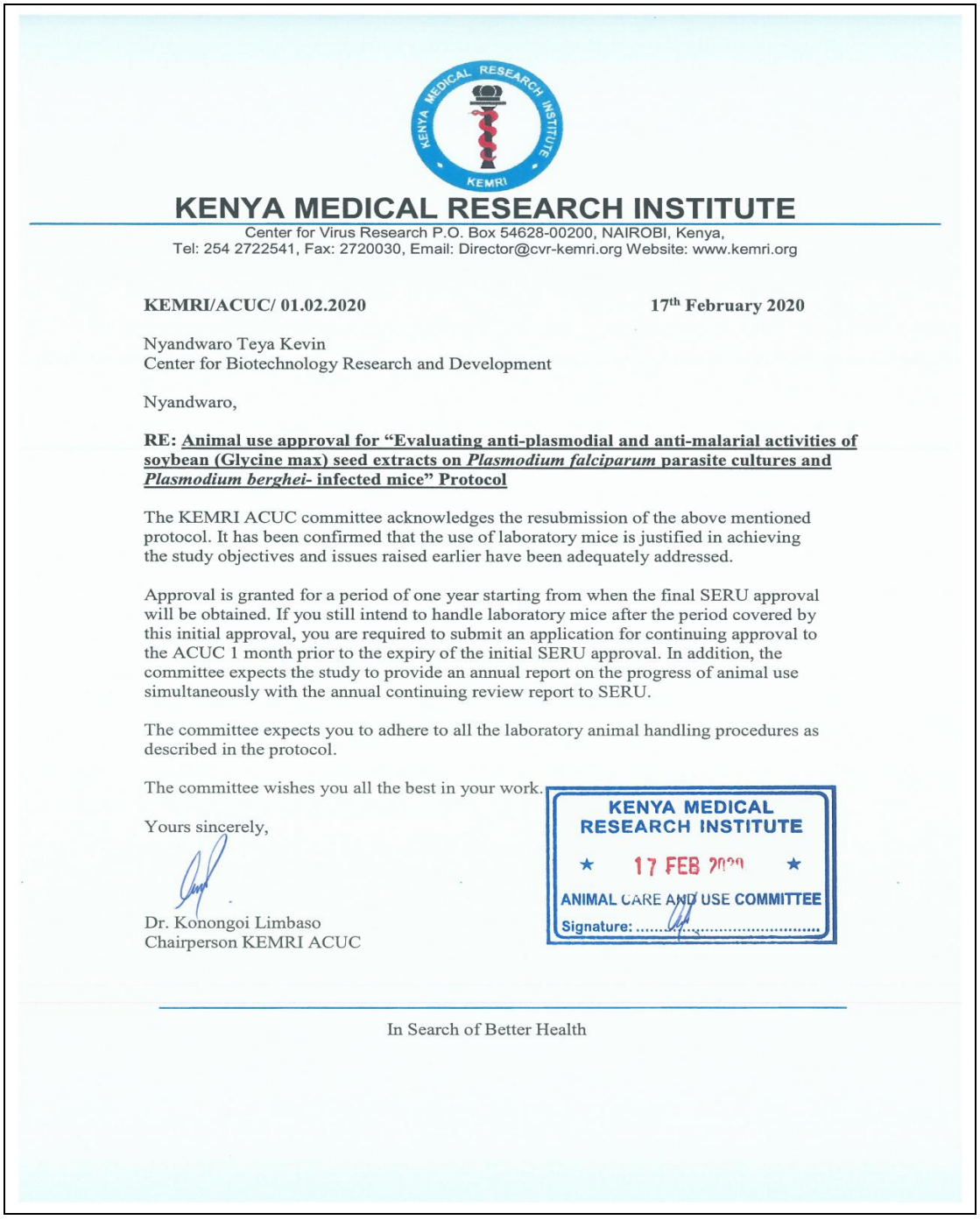
You may embark on the study.

Yours faithfully,

ENOCK KEBENEI,
THE ACTING HEAD,
KEMRI SCIENTIFIC AND ETHICS REVIEW UNIT.

In Search of Better Health

(ii) **KEMRI Animal Care Unit Committee Approval (ACUC)**



KENYA MEDICAL RESEARCH INSTITUTE

Center for Virus Research P.O. Box 54628-00200, NAIROBI, Kenya,
Tel: 254 2722541, Fax: 2720030, Email: Director@cvr-kemri.org Website: www.kemri.org

KEMRI/ACUC/ 01.02.2020 **17th February 2020**

Nyandwaro Teya Kevin
Center for Biotechnology Research and Development

Nyandwaro,

RE: Animal use approval for "Evaluating anti-plasmodial and anti-malarial activities of soybean (Glycine max) seed extracts on *Plasmodium falciparum* parasite cultures and *Plasmodium berghei*- infected mice" Protocol

The KEMRI ACUC committee acknowledges the resubmission of the above mentioned protocol. It has been confirmed that the use of laboratory mice is justified in achieving the study objectives and issues raised earlier have been adequately addressed.

Approval is granted for a period of one year starting from when the final SERU approval will be obtained. If you still intend to handle laboratory mice after the period covered by this initial approval, you are required to submit an application for continuing approval to the ACUC 1 month prior to the expiry of the initial SERU approval. In addition, the committee expects the study to provide an annual report on the progress of animal use simultaneously with the annual continuing review report to SERU.

The committee expects you to adhere to all the laboratory animal handling procedures as described in the protocol.

The committee wishes you all the best in your work.

Yours sincerely,

Dr. Konongoi Limbaso
Chairperson KEMRI ACUC

KENYA MEDICAL RESEARCH INSTITUTE
★ 17 FEB 2020 ★
ANIMAL CARE AND USE COMMITTEE
Signature: .....

In Search of Better Health

Appendix II: Paper publication

Research Article

Evaluating Antiplasmodial and Antimalarial Activities of Soybean (*Glycine max*) Seed Extracts on *P. falciparum* Parasite Cultures and *P. berghei*-Infected Mice

Kevin Nyandwaro[1], **Job Oyweri**[2], **Francis Kimani**[3] **and Amos Mbugua**[1]

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Received 14 November 2019; Revised 12 January 2020; Accepted 24 January 2020; Published 18 February 2020

Academic Editor: Patrizia Messi