ANTIMALARIAL EFFICACY AND SAFETY OF *Senna occidentalis* (L.) LINK ROOTS EXTRACT

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Antimalarial Efficacy and Safety of *Senna occidentalis* (L.) Link Roots Extract

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A Thesis Submitted in Fulfilment of the Requirements for the Degree of Doctor of Philosophy in Zoology (Animal Parasitology) of the Jomo Kenyatta University of Agriculture and Technology

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DECLARATION

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

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DEDICATION

To my loving wife Sheila Wankwe for your unwavering support and encouragement throughout the study period and to my lovely children Skylar and Scardwielle; you all endured many days of my absence. To Dr. Hezekiel Oira, Ph.D (Mount Kenya University, Kenya) for believing in my academic potential and giving me a learning opportunity during my high school years.

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ACRONYMS AND ABREVIATIONS

ACT	Artemisinin Combination Therapy
ALP	Alkaline Phosphatase
APAD	3-Acetyl Pyridine Adenine Dinucleotide
APADH	3-Acetyl Pyridine Adenine Dinucleotide, reduced
ARRIVE	Animal Research: Reporting of *In Vivo* Experiments
ART	Artemisinin
ALT	Alanine transaminase
AST	Aspartate Aminotrasferase
ANOVA	Analysis of variance
ATP	Adenosine Triphosphate
ATQ	Atovaquone
CQ	Chloroquine
Cyt b	Cytochrome b
DHFR	Dihydrofolate Reductase
DHP	Dihydropteroate synthase
DMSO	Dimethylsulphoxide
DPX	Dibutylphthalate Polystyrene Xylene

EDTA	Ethylenediamine Tetraacetic Acid
GRAN	Granulocytes
HCT	Hematocrit
HMG	Hemoglobin
IC_{50}	50% Inhibitory Concentration
ISERC	Institutional Scientific and Ethics Review Committee
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KIPRE	Kenya Institute of Primate Research
KEMRI	Kenya Medical Research Institute
LDH	Lactate dehydrogenase
PLDH	Plasmodium Lactate Dehydrogenase
LMF	Lumefantrine
LYP	Lymphocytes
MCH	Mean Corpuscular Hemoglobin
MCV	Mean Corpuscular Volume
MEM	Minimum Essential Medium
MFQ	Mefloquine
MPV	Mean Platelet Volume

MST	Mean Survival Time
mtETC	Mitochondria Electron Transport Chain
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide
NAD	Nicotinamide Adenine Dinucleotide
NMK	National Museums of Kenya
NMR	Nuclear Magnetic Resonance
NPPs	New Permeation Pathways
PBS	Phosphate Buffered Saline
PCV	Packed Cell Volume
PDW	Platelet Distribution Width
PfCRT	*Plasmodium falciparum* Chloroquine Resistance Transporter
PfLDH	*Plasmodium falciparum* Lactate Dehydrogenase
PfMDR1	*Plasmodium falciparum* Multidrug Resistance-1
PLT	Platelets
PPQ	Piperaquine
RBCs	Red Blood Cells
RDW-SD	Red Blood Cell Distribution Width – Standard Deviation
RPM	Revolutions per Minute

RPMI	Roswell Park Memorial Institute
SAJOREC	Sino Africa Joint Research centre
SEM	Standard Error of the Mean
SP	Sulfadoxine-Pyrimethamine
TID	Tropical and Infectious Diseases
WBCs	White Blood Cells
WHO	World Health Organization

ABSTRACT

Senna occidentalis (L.) Link has been used worldwide in traditional treatment of many diseases and conditions. In Kenya, a decoction from the plant roots taken orally is used as a cure for malaria. Several studies have demonstrated that extracts from the plant possess antiplasmodial activity, *in vitro*. However, the safety and curative potency of the plant root against established malaria infection is yet to be scientifically validated, *in vivo*. On the other hand, there are reports on variation in bioactivity of extracts obtained from this plant species, depending on the plant part used and place of origin among other factors. In this study, the antimalarial activity and safety of *Senna occidentalis* roots extract was demonstrated *in vitro*, and in mice. Methanol, ethyl acetate, chloroform, hexane and water extracts of *S. occidentalis* root were tested for *in vitro* antiplasmodial activity against *Plsmodium falciparum*. The most active extracts were evaluated for cytotoxicity using 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. The extracts were then evaluated for curative potency and safety in *Plasmodium berghei* infected mice by monitoring parasite suppression and survival rates of the animals. In addition, whole blood and serum were collected from the animals and examined for changes in hematological and biochemical parameters respectively, using automated analyzers. Kidney, liver, lung, spleen and brain tissues were harvested from mice and examined for changes in organ architecture. Lastly, the bioactive extract was partitioned by column chromatography and the resultant fractions evaluated for antiplasmodial potency, *in vitro*. All of the solvent extracts tested in this study inhibited the propagation of *P. falciparum*, strain 3D7, *in vitro*, with polar extracts being more active than non-polar ones. Methanolic extracts had the highest activity (IC_{50} =1.76) while hexane extract displayed the lowest activity (IC_{50} =18.47). At the tested concentrations, methanolic and aqueous extracts exhibited high selectivity index against *P. falciparum* strain 3D7 (SI > 10) in the cytotoxicity assay. Further, the extracts significantly suppressed the propagation of *P. berghei* parasites ($P < 0.05$) in mice and increased the survival time of the infected animals ($P < 0.0001$). Infected mice that were treated with the extract depicted a significantly low level of total leucocytes ($p < 0.01$), red blood cell distribution width ($p < 0.01$) and a significantly high hemoglobin concentration ($p < 0.001$) compared to the infected animals that were administered with the vehicle only. The infected animals that were treated with the extract exhibited a significantly low level of urea, creatinine, bilirubin, and alkaline phosphatase ($p < 0.05$), compared to infected animals that were given the vehicle only. The level of sodium, potassium and chloride ions, lymphocytes, granulocytes, hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration, total protein, albumin, aspartate aminotransferase (AST), alanine transaminase (ALT), alkaline phosphatase (ALP), total platelets, mean platelet volume (MPV) and platelet distribution width of the infected animals treated with the extract was not significantly affected, compared with those of the infected animals that were given the vehicle only ($p > 0.05$). The extract alleviated organ pathological changes in the infected mice. The extract did not induce any remarkable adverse effect on the growth, hematological and biochemical parameters of uninfected animals ($p > 0.05$). In addition, administration of the extract did not alter the gross appearance and histological

architecture of the organs, implying that the extract was well tolerated in mice. All the fractions exhibited antiplasmodial activity against *P. falciparum*, *in vitro*, with one of the them depicting better activity when compared to the parent extract. In conclusion, *Senna occidentalis* (L.) Link root extract exhibits good antimalarial activity against *P. falciparum* and *P. berghei*, and may be safe in mice. The plant root could, therefore, be developed further into an antimalarial therapy to help in the fight against malaria.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

In the tropical and subtropical regions the world, protozoan diseases constitute the greatest health burden particularly in developing countries (Jumba *et al*., 2015). Among the most important diseases that fall under this category is malaria (Chandel *et al*., 2015, Satish *et al*., 2017, Somsak *et al*., 2016). Five *Plasmodium* species have been identified as the causative agents for malaria in humans; *Plasmodium falciparum, Plasmodium ovale, Plasmodium malariae, Plasmodium vivax* and *Plasmodium Knowlesi* (Ekasari *et al*., 2018). Malaria parasite transmission is through bites of female anopheles mosquitoes (Ekasari *et al*., 2018). More than 40% of the world's population is at risk of contracting the disease (Otegbade *et al*., 2017, Somsak *et al*., 2016). There were 247 million cases of malaria and 619,000 deaths, globally in 2021 (WHO, 2022).

Successful malaria control is based on chemoprophylaxis, early diagnosis and prompt treatment accompanied with vector control (Simwela and Waters, 2022, Tisnerat *et al*., 2022). These control programs have however been hindered by mainly drug resistance to antimalarial drugs, and the insecticides used for vector control. Most of the available antimalarial drugs are increasingly becoming ineffective (Chaniad *et al*., 2019, Cimanga *et al*., 2019, Omeiza *et al*., 2020). In addition, some of the available antimalarial drugs have been linked to adverse side effects such as headache, diarrhea, nausea, pruritus, anxiety, seizures and hair discoloration (Lozano-Cruz *et al*., 2021). Long-term use of the drugs may lead to heart, eye and neuron defects (Lozano-Cruz *et al*., 2021). Furthermore, there is no highly effective antimalarial vaccine (Simwela and Waters, 2022). To curb the spread of antimalarial drug resistance, WHO has recommended the use of combination therapy (Nondo *et al*., 2017). Use of Artemisinin combination therapy (ACT) has led to great achievements in the control and management of *P. falciparum* malaria across several regions of the world (Nondo *et al*., 2017). On other hand, there are reports of *Plasmodium*

resistance to artemisinin in some parts of South East Asia and Equatorial Guinea (Haidara *et al*., 2018, Nondo *et al*., 2017). Though costly, artemisinin and its derivatives, are the only remaining first line antimalarial drug that are effective against multi-drug resistant strains of malaria and the severe forms of malaria (LaCrue *et al*., 2011). Resistance to artemisinin is, therefore, a serious threat to malaria control programs (Otegbade *et al*., 2017). This is indicative of a need for continued search for novel antimalarial drugs.

Traditional medicine has shown great potential for the management of various health conditions (Ugwah-Oguejiofor *et al*., 2019) and has remained the most accessible and affordable means of treatment in Sub-Saharan Africa (Ferrer *et al*., 2016, Haidara *et al*., 2018, Nigatu *et al*., 2017). Natural products constitute an important resource in both traditional and conventional medical systems, throughout the world. Approximately 40% of approved drugs available in the market are either natural products or their derivatives (Aljawdah *et al*., 2022, Nigatu *et al*., 2017). Medicinal plants are a potential source of the much needed novel antimalarial drugs (Ishaku *et al*., 2016, Kaushik *et al*., 2015) and have been used traditionally to treat malaria or manage conditions associated with it in various parts of the world (Ekasari *et al*., 2018, Otegbade *et al*., 2017). Scientific validation of antimalarial activity of such plants could pave way for discovery of new antimalarial drugs (Rasoanaivo *et al*., 2011). Through such studies, important new drug targets and antimalarial compounds could be identified. More still, the identified compounds could be used as templates for synthesis of new synthetic antimalarial drugs.

Given the long history of use by humans, herbal medicine is assumed to be safe (Ibrahim *et al*., 2016). In contrast, emerging evidence reveals safety concerns regarding the use of some of these medicinal products (Koduru *et al*., 2006, Ugwah-Oguejiofor *et al*., 2019). Therefore, there is need to scientifically ascertain the efficacy and safety of these therapies.

Plant species belonging to genus *Senna* have been shown to possess antiplasmodial activity.For instance, piperidine alkaloids from *S. spectabilis* have been found to be active against *P. falciparum* (Pivatto *et al*., 2014). In addition, *S. singueana* leaf extract has been

shown to suppress *P. berghei* parasitemia in mice (Hiben *et al*., 2016). An ethnobotanical survey of medicinal plants used by the Digo and Duruma communities of Kwale County, Kenya, showed that a root decoction of *S. occidentalis i*s used as a cure for malaria. The decoction is taken orally thrice a day for 3 to 4 days (Muthaura *et al*., 2015, Nguta *et al*., 2010). The plant is similarly used to treat malaria in Congo (Tona *et al*., 2004) and Nigeria (Daskum *et al*., 2019). Leaf extracts from the plant inhibit *P. falciparum* growth, *in vitro* (Daskum *et al*., 2019, El Tahir *et al*., 1999, Murugan *et al*., 2015, Tona *et al*., 2004). The current study sought to investigate further the antimalarial potency of the plant extract against *P. falciparum, in vitro* and *Plasmodium berghei, in vivo*. It also sought to assess the safety of the plant root extract to facilitate its development into an antimalarial product. It is expected that the resultant therapy will greatly contribute in the fight against malaria especially in Sub-Saharan Africa, where malaria seems to impact the most due to limited availability of resources.

1.2 Statement of the Problem

Drug resistance is continually rendering most of the available antimalarial drugs ineffective. Consequently, malaria continues to be a major public health problem in Kenya and Africa as a whole. This has necessitated urgent and continued search for new antimalarial drugs. Natural products as guided by traditional antimalarial remedies offer potential sources for the much needed antimalarial drugs. As such, plants with claimed antimalarial activity are actively being investigated as sources of antimalarial medicines. Some of them have shown promising antimalarial potential, in *in vitro* and *in vivo* experimental models. However, most of these investigations have been limited to screening for antiplasmodial activity of the plant extracts with little or no emphasis on evaluation of their efficacy and safety with the aim of developing them into antimalarial products.

1.3 Justification of the Study

Malaria continues to be a major public health problem especially in resource constrained Sub-Saharan Africa. Artemisinin and its derivatives are the only remaining first line antimalarial drugs that are effective against multi-drug resistant strains of malaria, and the severe forms of the disease. On the other hand, there are emerging reports on malaria parasites resistance to artemisinin. As such, there is need for the search and development of new antimalarial drugs.

Natural products, especially medicinal plants, are a potential source of novel antimalarial products. They have been used for millennia to treat and or manage health disorders and to prevent diseases, malaria inclusive. Indeed, the most successful antimalarial drugs that are in use today, Artemisinin and Quinine, are plant derivatives. Plants, being natural products can be easily sourced and some of them are known to contain substances that can inhibit multidrug resistance which is the greatest challenge witnessed so far in the fight against malaria.

Some plant species belonging to genus Senna, (*S. petersiana* and *S. spectabilis*), have been reported to possess antiplasmodial activity. Similarly, there have been ethnomedical reports on the use of a root decoction of *S. occidentalis* as a cure for malaria among the Digo and Duruma communities of Kenya. Leaf extracts from the plant have been shown to suppress the growth of *Plasmodium* parasites *in vitro.* The current study seeks to assess further the antimalarial potential of the plant by evaluating the curative potency of the plant root extract. It also seeks to assess the safety of the plant root extract. The resultant information will guide further development of the plant root into the much needed new antimalarial remedy.

1.4 Hypotheses

1. *Senna occidentalis* (L.) Link root extract is not effective in inhibiting growth of malaria parasites

2. *Senna occidentalis* (L.) Link roots extract is not safe for use in treatment of *Plasmodium berghei* infected BALB/c mice

1.5 General Objective

To determine the antimalarial efficacy and safety of *Senna occidentalis* (L.) Link roots extract *in vitro* and in a mouse model

1.5.1 Specific Objectives

1. To determine the in *vitro* antiplasmodial activity of *Senna occidentalis* (L.) Link roots extract against *Plasmodium falciparum*
2. To evaluate the effect of *Senna occidentalis* (L.) Link roots extract on parasitemia and survival time of *Plasmodium berghe*i infected BALB/c mice
3. To assess the effect of *Senna occidentalis* (L.) Link roots extract on hematological, biochemical and histological parameters of *Plasmodium berghei* infected BALB/c mice
4. To evaluate *Senna occidentalis* (L.) Link roots extract fractions for antiplasmodial activity against *Plasmodium falciparum, in vitro*

CHAPTER TWO

LITERATURE REVIEW

2.1 The Malaria Burden

Malaria, a controllable and curable parasitic disease, continues to pose significant challenges in public health, especially in resource-constrained Sub-Saharan African regions (Baah *et al*., 2020, Oliveira-Lima *et al*., 2019, Omeiza *et al*., 2020). There were approximately 247 million malaria cases and 619,000 deaths, globally in 2021 (WHO, 2022). The disease causes significant morbidity and mortality especially in children less than 5 years of age and also in pregnant women (Inoue *et al*., 2013). This has been attributed to reduced immunity in the two groups of people (WHO, 2013). Regions of the world worst hit by Malaria include countries of the Sub-Saharan Africa, Latin America and some parts of Asia (Inoue *et al*., 2013, WHO, 2017, Sosovele *et al*., 2013). Figure 2.1 shows the global distribution of malaria cases for the year 2021.

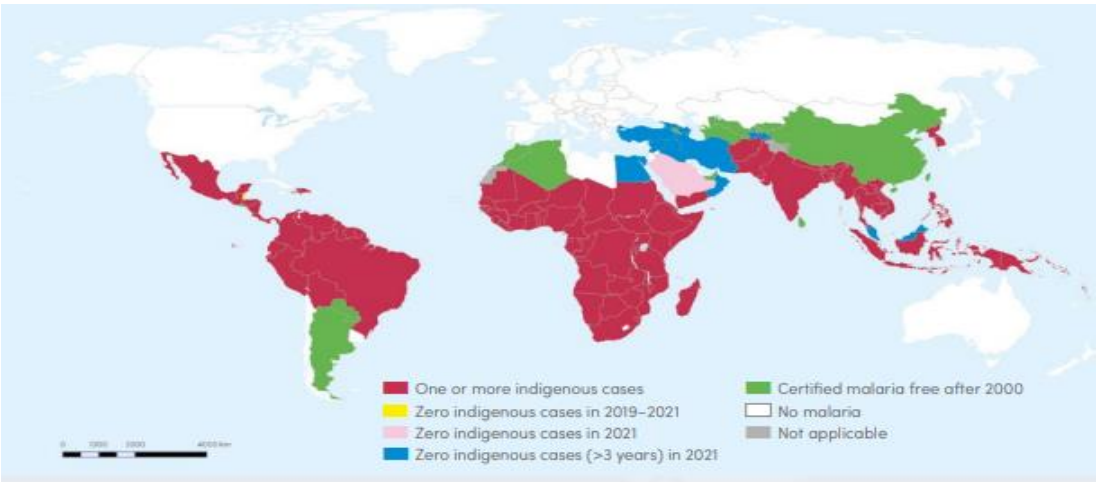


Figure 2.1: Global Distribution of Malaria Cases for the Year 2021

Source: (WHO, 2022)

Africa is worst hit by the malaria epidemic due to presence of (i) a very efficient vector (*Anopheles gambiae*) (ii) *P. falciparum* parasite which causes a severe form of malaria (iii) a conducive weather condition and lastly, (iv) scarcity of resources coupled with socio-economic instability (WHO, 2012). The disease imposes a considerable barrier to economic development especially in developing countries. It imposes economic costs cutting across individuals and governments. The costs at individual level include purchase of drugs for treating malaria at home, travel expenses to and from health facilities in search for medication, lost days of work, absence from school, expenses for preventive measures as well as burial expenses in case of deaths. At government level, the costs incurred include costs for maintenance of health facilities, purchase of drugs and supplies, public health interventions against malaria such as insecticide spraying and distribution of insecticide-treated bed nets and loss of income due to lost days of work (Hailu *et al*., 2017, Tefera *et al*., 2020).

About 93% all malaria deaths occur in Africa (Tefera *et al*., 2020). In Kenya, malaria is a major public health problem. It is the leading cause of morbidity and mortality, with 70% of the population being at risk. In Kenya, there occurs approximately 3.4 million clinical cases and 12,000 deaths annually (Lacey *et al*., 2023). About 90% of the malaria cases in Kenya are due to *P. falciparum* infection (Sultana *et al*., 2017). The disease is endemic in the coastal belt and regions in the Lake Victoria basin with epidemics occurring in areas bordering the endemic areas (Muthaura *et al*., 2011).

2.2 Antimalarial Drugs

Antimalarial drugs play a significant role in the control and prevention of malaria. The drugs are used for chemoprophylaxis, intermittent preventive therapies during pregnancy and in the prevention of progression of disease to severe states. The preferred drug for severe malaria is intravenous artesunate injection, with quinine as an alternative (Conrad and Rosenthal, 2019). Antimalarial drugs can be categorized into 4-aminiquinolones, 8-aminoquinolones, endoperoxides, antifolates, Naphthoquinones, Arly aminoalcohols and antibiotics (Ross and Fidock, 2019, Tibon *et al*., 2020). The various classes of

antimalarials target different parasite metabolic pathways to achieve the desired effects (Ross and Fidock, 2019). Figure 2.2 shows the targets for various classes of antimalarials.

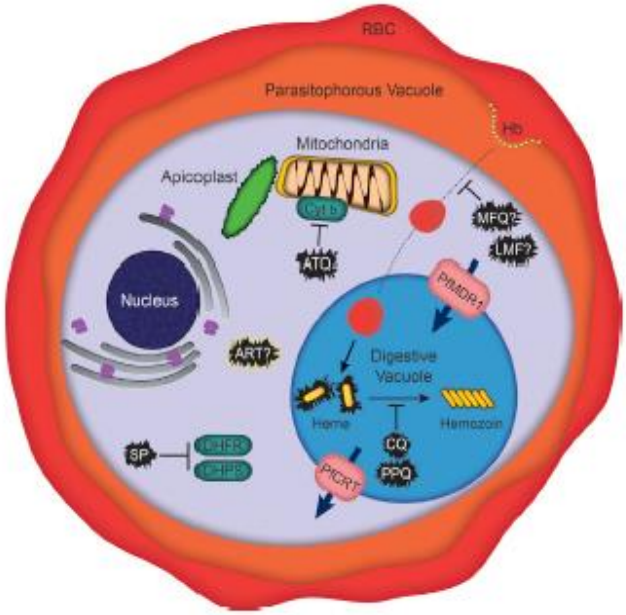


Figure 2.2: Schematic Representation of *P. falciparum* Trophozoite Showing Targets of the Various Antimalarial Classes

Key: ART, artemisinin; ATQ, atovaquone; CQ, chloroquine; Cyt b, cytochrome b; DHFR, dihydrofolate reductase; DHPS, dihydropteroate synthase; HGB, hemoglobin; LMF, lumefantrine; MFQ, mefloquine; PfCRT, *P. falciparum* chloroquine resistance transporter; PfMDR1, *P. falciparum* multidrug resistance-1; PPQ, piperaquine; RBC, red blood cell; SP, sulfadoxine pyrimethamine (Ross and Fidock, 2019).

2.2.1 4-Aminoquinolones (Chloroquine, Amodaquine and Piperaquine)

Chloroquine is used for the treatment of uncomplicated cases of *P. falciparum* and *P. ovale* (Tisnerat *et al*., 2022). Amodaquine is used in combination therapy with artemisinins and chloroquine for management of severe and uncomplicated cases of *P. falciparum* and *P. ovale* (Ross and Fidock, 2019). Piperaquine is used in combination with

dihydroartemisinin for the management of uncomplicated *P. falciparum* malaria (Ross and Fidock, 2019). The mode of action of this class of compounds is thought to be heme intoxication. The drugs prevent heme from polymerization (crystallization). Free heme is toxic to malaria parasites (Combrinck *et al*., 2013, Ross and Fidock, 2019).

2.2.2 8-Aminoquinolones (Primaquine)

Primaquine is used to treat uncomplicated *P. falciparum* malaria in combination with chloroquine sulphodoxine-pyrimethamine or artemisinins (Tisnerat *et al*., 2022). It is also used in the treatment of uncomplicated *P. vivax and P. ovale* malaria, achieving radical cure (Belete, 2020, Ross and Fidock, 2019, Tisnerat *et al*., 2022). Its mode of action is not well understood. However, the interruption of the parasite mitochondria membrane has been proposed (Talapko *et al*., 2019).

2.2.3 Endoperoxides (Artemisinin, Dihydroartemisinin, Artesunate and Artemether)

They are used in the management of both severe and uncomplicated *P. falciparum* malaria, in combination with other antimalarial drugs (Belete, 2020). It is also used in the treatment of chloroquine resistant malaria. The mode of action of these drugs is not well known (Tisnerat *et al*., 2022, Tse *et al*., 2019). However, production of free radicals has been suggested as their mode of action (Ross and Fidock, 2019). Oxidative stress has been associated with promotion of malaria parasites killing (Percário *et al*., 2012, Tse *et al*., 2019).

2.2.4 Antifolates (Sulfadoxine-Pyrimethamine and Proguanil)

Pyrimethamine in combination with sulfadoxine is the choice medication for intermittent malaria preventive therapy during pregnancy (Ross and Fidock, 2019, Tisnerat *et al*., 2022). Proguanil is used in in chemoprophylaxis (Talapko *et al*., 2019). It is also used in combination with atovaquone to manage *P. falciparum* malaria (Belete, 2020, Tse *et al*., 2019). Pyrimethamine and proguanil inhibit folate metabolism by acting on two enzymes; dihydroforate reductase (dhfr) and dihydropteroate synthatase (dhpts) (Ross and Fidock,

2019, Talapko *et al*., 2019, Tisnerat *et al*., 2022). Folate is an important cofactor in amino acids and nucleotides synthesis in *Plasmodium* species, without which the parasites die (Tisnerat *et al*., 2022).

2.2.5 Naphthoquinone (Atovaquone)

Atovaquone is used in combination with proguanil to manage *P. falciparum* malaria (Belete, 2020, Tse *et al*., 2019). It is known to block the mitochondria electron transport chain by acting as a cytochrome bc-1 complex inhibitor (Tse *et al*., 2019). More often, asexual blood stage malaria parasites scavenge host glucose for their glycolytic pathway. The parasites avoid oxidative phosphorylation and convert the glucose to lactate that in turn supplies their ATPs. Regardless, some mitochondrial electron transport activity is crucial for regeneration of ubiquinone, an acceptor for dihydroorotate dehydrogenase, an essential enzyme for pyrimidine biosynthesis (Sturm *et al*., 2015). Pyrimidines are essential for parasite synthesis of nucleic acids (DNA and RNA), phospholipids and glycoproteins (Stocks *et al*., 2014). Atovaquone competitively inhibits ubiquinone, thus leading to parasites death (Sturm *et al*., 2015). Malaria parasites resistance to atovaquone develops very fast (Blasco *et al*., 2017, Lunev *et al*., 2016, Nixon *et al*., 2013). It has been demonstrated that atovaquone is a cytostatic drug (Painter *et al*., 2010). One of the key concerns regarding cytostatic drugs as opposed to cytotoxic drugs is the ease with which the parasites develop resistance against them (Painter *et al*., 2010).

2.2.6 Aryl Aminoalcohols (Quinine, Mefloquine, Lumefantrine)

Quinine is used in the management of severe malaria. Its mode of action is thought to be the inhibition of hem detoxification (Tisnerat *et al*., 2022). Mefloquine is used to cure uncomplicated and severe *P. falciparum* and *P. vivax* malaria (WHO, 2016). The mode of action is proposed to be by inhibition of parasite hem detoxification and the inhibition of parasite ribosomes. Lumefantrine is used in combination with artemether as a cure for *P. falciparum* malaria (Tisnerat *et al*., 2022). The mode of action of lumefantrine is not well

understood but is thought to be via heme polymerization in the food vacuole (Combrinck *et al*., 2013).

2.2.7 Antibiotics (Tetracycline, Doxycycline and Clindamycin)

Tetracycline and doxycycline are used for chemoprophylaxis of uncomplicated malaria, and for the treatment of severe malaria in combination with quinine (Belete, 2020, Tisnerat *et al*., 2022). Clindamycin is used in the management of uncomplicated and severe malaria in combination with quinine (WHO, 2016). These antibiotics inhibit DNA replication, transcription and translation at the parasite apicoplast, thereby leading to parasites death (Gaillard *et al*., 2016, Tisnerat *et al*., 2022). Apicoplasts take part in the biosynthesis of fatty acids, heme and isoprenoids (Gaillard *et al*., 2016).

2.3 Resistance to Antimalarial Drugs

Drug resistance refers to a situation where a cell or organism is able to survive in drug concentrations that would normally destroy or inhibit multiplication of such cells or organisms belonging to the same species (Arrey Tarkang *et al*., 2014, Cowell and Winzeler, 2019). Successful malaria control is based on early diagnosis and prompt treatment accompanied with vector control (Simwela and Waters, 2022). Access to both prevention and treatment services have been on the increase over time (Simwela and Waters, 2022, WHO, 2012). The preventive strategies include; sleeping under insecticide treated bed nets (INTs), indoor residual sprays (IRS) and intermittent preventive treatment in pregnancy (IPTp), (Nondo *et al*., 2017, WHO, 2012). However, malaria control strategies are faced by a myriad of challenges among them, lack of an effective vaccine (Otegbade *et al*., 2017) and existence of resistance to both insecticides and antimalarial drugs in some areas such as Latin America, South East Asia and Sub-Saharan Africa (Haidara *et al*., 2018, Nondo *et al*., 2017). In addition, the preventive tools are of limited access in developing countries especially in Sub-Saharan Africa due to financial constraints (WHO, 2012). On the other hand, there has been a decrease in the number of people applying IRS as a preventive strategy because of a shift from the usage of

pyrethroids to more expensive non-pyrethroid insecticides as a result of emergence of pyrethroid resistance (WHO, 2013).

The use of antimalarial drugs for prevention or cure is currently under threat due to emergence of malaria parasite strains resistant to antimalarials (Ross and Fidock, 2019). There has occurred resistance to almost all available first line antimalarial drugs (Chaniad *et al*., 2019, Cimanga *et al*., 2019, Omeiza *et al*., 2020). It is several decades now since the emergency of antimalarial resistance to aminoquinolones and antifolates (Conrad and Rosenthal, 2019). Resistance to newer antimalarial drugs such as artemisinin combination therapies (ACTs) is yet to spread widely, and such therapies are still effective in most parts of Africa (Conrad and Rosenthal, 2019). The cases of resistance to artemisinin reported so far are from South East Asia (Myanmar, Cambodia, Laos, Vietnam, Thailand and Southern China), with the African parasite populations still being very sensitive to artemisinin (Conrad and Rosenthal, 2019, Haidara *et al*., 2018, Nondo *et al*., 2017). Resistance to artemisinin presents a serious threat to malaria control programs (Otegbade *et al*., 2017). It is feared that artemisinin resistance may spread to Sub-Saharan Africa, an area of high transmission rates of *P. falciparum*, the most severe form of malaria and revert the gains made so far. This is indicative of a need for continued search for novel antimalarial drugs.

The time taken for drugs to achieve parasite clearance is an important marker of resistance (Cowell and Winzeler, 2019). In this era of combination therapy for curing malaria, this marker may not be as important (Cowell and Winzeler, 2019). This is because treatment failure may not be observed in case where the parasites develop resistance to only one of the drugs used in the combination therapy (Cowell and Winzeler, 2019). Development of resistance begin with genetic mutation, which is normally spontaneous and rare (White, 2004). The mutation is followed by selection for the mutant parasites which is often influenced by factors such as high parasite density, low drug concentrations in serum and immune status of the host (Cowell and Winzeler, 2019, Hastings *et al*., 2002). Level of malaria transmission may influence selection for resistance (White, 2004). In areas of low transmission rates, individuals are likely to be less immune to malaria and therefore get

symptomatic disease and seek treatment (Babiker *et al*., 2013). In high transmission areas, a single infection may involve several genotypes. Therefore, resistant genotypes compete with wild-type parasite strains (Cowell and Winzeler, 2019). On the other hand, asymptomatic cases may act as a reservoir for sensitive parasites (Babiker *et al*., 2013). The spread of resistance is also dependent on the transmissibility of the allele. Alleles conferring resistance to atovaquone have been shown to cause parasites death in the mosquito, thus preventing spread to the vertebrate host (Cowell and Winzeler, 2019). Similarly, the half-life of drugs has been shown to influence selection of the mutant parasites. Drugs such as chloroquine, mefloquine and piperaquine (have long half-life), tend to select for the resistant parasite strains (Cowell and Winzeler, 2019, Goodman *et al*., 2016).

More often, antimalarial resistance seem to originate from outside of Africa (Conrad and Rosenthal, 2019, Ross and Fidock, 2019). This may be explained by among other factors, an elevated immunity among African populations (Conrad and Rosenthal, 2019). On the other hand, increased instances of malaria parasites resistance to antimalarial drugs in the Southeast Asia region may be due to by low transmission rates, which allow spread of unfit mutations. Such unfit mutations result from possible lack of competition among the within human asexual parasites or other parasite genomes that would take part in recombination within the sexual stages in the mosquitoes (Carneiro *et al*., 2010). The spread of unfit mutations have been associated with age (older age) of the infected populations in the region. This age group is more reluctant in seeking medical intervention. Similarly, low rates of infection have led to less robust acquired immunity leading to over-reliance on antimalarial drugs, hence increased selection pressures (Ross and Fidock, 2019). On the other hand, the malaria parasites in this region constitute a strong population structure, with distinct allele frequencies among the sub-populations. The resultant of this is small effective population sizes. Resistance stem quickly from such population structures (Takala-Harrison and Laufer, 2015).

2.4 Plants as Potential Source of Antimalarial Drugs

Natural products, especially medicinal plants, have been fronted by the scientific community as a potential source of the much needed novel antimalarial drugs (Kaushik *et al*., 2015). For millennia, medicinal plants have been used to treat malaria or manage conditions associated with it in various parts of the world (Ekasari *et al*., 2018, Otegbade *et al*., 2017). Either the roots, leaves, stem bark or at times the whole plant is boiled, soaked in water or crushed then soaked and the extract drunk alone or mixed with food or palatable drinks such as soup or milk (Muthaura *et al*., 2011). The extract could be from a single plant or a combination of several plants. The long use of medicinal plants as a cure for malaria imply that such plants potentially contain antimalarial bioactive principles. This underscores the need to subject the plants traditionally used to treat malaria to laboratory investigations to validate their usage. Through such studies, important new antimalarial drugs and targets can be identified. More still, the identified compounds can be used as templates for synthesis of new novel synthetic antimalarial drugs (Rasoanaivo *et al*., 2011).

The much needed antimalarial drug should be simple to use, inexpensive and easily accessible to the poor who are most vulnerable to malaria (Muthaura *et al*., 2011). Phytomedicines have been shown to be relatively cost effective, easily accessible and of fewer side effects compared to synthetic drugs. In addition, plants contain substances that may inhibit multidrug resistance; the greatest challenge so far in the fight against malaria infection. More still, they contain compounds that induce immunostimulation as well as modulation of adverse effects of diseases (Ginsburg and Deharo, 2011, Rasoanaivo *et al*., 2011). Noteworthy, the most successful antimalarial drugs that are in use today, Artemisinin and Quinine, are plant derivatives (Rasoanaivo *et al*., 2011).

The first antimalarial drug was quinine which was isolated in 1820 from a plant; the bark of Cinchona species. The isolated compound was put to use till 1940 when synthetic quinine was produced using the plant isolate as a template (Tisnerat *et al*., 2022). Since then, a lot of effort was put in research aimed at discovery of new antimalarial drugs to no

success until 1976 when a new compound (artemisinin) was identified; This was a plant derivative of *Artemisia annua* (Tisnerat *et al*., 2022, Yuan *et al*., 2016). From artemisinin, synthetics such as arteether, artemether and sodium artesunate have been made and are increasingly in use today (Tisnerat *et al*., 2022). More recently, atovaquone was isolated from *Tabenua* species, a plant, and shown to be effective in combination with proguanil (Arrey Tarkang *et al*., 2014). However, the production costs have hindered its usage in a wider scale (Arrey Tarkang *et al*., 2014). Artemisinin and quinine have therefore remained as two of the most important antimalarials drugs that are in use today (Haidara *et al*., 2018, Otegbade *et al*., 2017). In view of the successes of Artemisinin and Quinine, both of which are plant derivatives, the next novel antimalarial drug may as well be a plant derivative.

Several natural products, especially medicinal plants, have been investigated for antimalarial activities leading to identification of many antiplasmodial compounds. Such plants include *Artemisia annua, Vernonia amygdalina, Bidens pilosa, Asparagus africanas, Azadirachta indica, Argemone Mexicana, Gossypium spp, Uvaria leptocladon and Ajuga remota* among others as reviewed extensively (Habibi *et al*., 2022). Triterpines, quinines, quassinoids, lignans, limnoids, coumarins and alkaloids constitute some of the identified antimalarial compounds (Adebayo and Krettli, 2011, Alebie *et al*., 2017). Development of such bioactive compounds alone or in combination with established affordable antimalarial pharmacological agents to which *Plasmodium* parasites have exhibited resistance would ensure availability of the much desired novel efficacious and affordable antimalarial agents.

2.5 Ethnopharmacology and Antimalarial Drug Discovery and Development

Traditional medicine (TM) is the use of indigenous medicinal and aromatic plants, animal parts or organic and inorganic materials for preventive and therapeutic purposes (Kamala *et al*., 2018). In some countries, TM is regarded as complementary or alternative medicine. Medicinal plants form an important component of traditional medicine. The plants have been used for very many years to treat health disorders and to prevent diseases including epidemics (Archana *et al*., 2010, Obioma *et al*., 2014). Currently, about 80% of healthcare

amongst world population is achieved through traditional medicine. This is due to either inaccessibility or unaffordability of conventional medicine (Ekasari *et al*., 2018, Okon *et al*., 2014).

Drug development process is very costly. It may take 12 to 15 years and in excess of $1 billion for a drug to be available for use (Hughes *et al*., 2011, Sinha *et al*., 2017). On the other hand, funding for antimalarial drug development is limited (Willcox *et al*., 2011). Because the impact of malaria is felt most by people living in resource-constrained countries of the world, the available market for antimalarial drugs is limited. Consequently, financial investment for antimalarial drug development is also inadequate. For instance, atavaquone which is an antimalarial drug derived from lapachol (plant isolate) obtained from *Tabeuia* species has limited usage in malaria endemic areas due to high cost of production (Oliveira *et al*., 2009). Therefore, it would be ideal to have access to affordable antimalarial drugs in these resource-constrained populations. This can only be achieved by reducing the cost of drug development pipeline.

Ethnopharmacology and mass screening constitute the common approaches to drug discovery and development from plants (Ezenyi and Salawu, 2016, Pan *et al*., 2013). Ethnopharmacology has been associated with higher success rates of discovering active compounds from plants (Adebayo and Krettli, 2011, Ezenyi and Salawu, 2016). The current and most successful antimalarial drug, artemisinin and, quinine whose derivatives are still in use today were discovered through this approach. The approach has been associated with reduced toxicity of resultant products. It is also time and cost effective compared to mass screening of plants for specific biological activities (Kigen *et al*., 2013, Pan *et al*., 2013).

Ethnomedical assessment of plant species is regarded as the first step in discovery of drugs from medicinal plants (Vitalini *et al*., 2013). This is followed by isolation of the bioactive principles and pharmacological screening leading to drug development through two operational modes as shown in Figure 2.3 (Pieters and Vlietinck, 2005). In one of the approaches, a standardized extract is developed as a drug. In the second approach, a pure

isolate of the bioactive compound is developed as a drug. Ethnopharmacology has been associated with higher success rates of discovering active compounds (Ezenyi and Salawu, 2016, Pan *et al*., 2013).

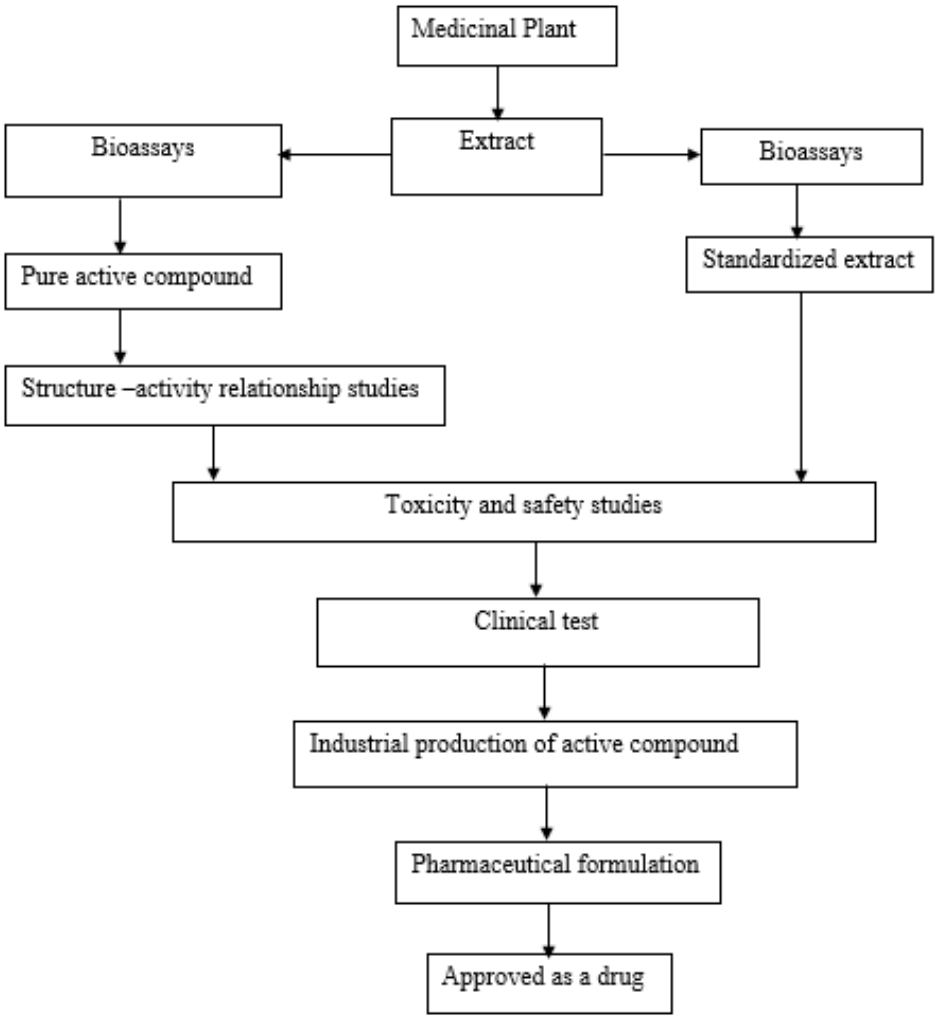


Figure 2.3: Schematic Representation of Approached For Development of Drugs from Medicinal Plants

Source: (Pieters and Vlietinck, 2005).

2.6 Challenges Facing Antimalarial Drug Discovery and Development from Medicinal Plants

Drug discovery and development from medicinal plants has faced by a myriad of challenges. The greatest challenge has been collection of genuine and accurate ethnomedical information on the medicinal plants. In addition, there has been deforestation, disclosure of information by the Traditional Medical Practitioners (TMPs), presence of many quacks masquerading as TMPs, lack of clear intellectual property guidelines, mistrust between researchers and herbalists as well as socio-cultural issues (Kigen *et al*., 2013). It is highly probable that medically important knowledge is being lost because some of the TMPs may be dying without having shared the knowledge (Ginsburg and Deharo, 2011, Kigen *et al*., 2013). Given these limitations, most plants of medicinal importance are yet to be explored. It has been noted that the medicinal potential of about 90% of the flora and fauna species are yet to be explored (Ginsburg and Deharo, 2011). Indeed, it has been observed previously that only a small portion of plants with claimed antimalarial activity has been investigated (Muthaura *et al*., 2011).

In some instances, research on antimalarial drug discovery and development from plants sources has been in-exhaustive. Most of the investigations are limited to general screening for antiplasmodial effects of crude extracts (Oliveira *et al*., 2009) and have rarely considered efficacy and safety evaluations of the extracts. Emphasis has been on publication output as the end-point (Addae-Mensah *et al*., 2011), rather than the development of utilizable antimalarial products. The studies are purely academic, probably due to, limited funding (Kigen *et al*., 2013). On the other hand, the investigations that have advanced beyond general screening for antimalarial activity tend to focus on isolation and identification of pure compounds as opposed to whole extracts (Deharo and Ginsburg, 2011).

In some cases, the investigators have made use of therapeutically undesirable doses (Heinrich *et al*., 2020). This has limited the translation of pre-clinical studies to clinical phases. Similarly, clinical investigation on efficacy and safety of the lead extracts and or

isolates is largely lacking (Ezenyi and Salawu, 2016, Muthaura *et al*., 2011, Oliveira *et al*., 2009).

More often, plant extracts or isolates that fail to exhibit antimalarial activity in *ex vivo* models are excluded from further investigations. However, it is noteworthy that some of these products may be active only upon metabolism by the host system (Mesia *et al*., 2010, Wells, 2011). For instance, 8-aminoquinolines have been shown to be active against *Plasmodium* but only upon metabolism by the liver. Similarly, *Nauclea pobeguinii* extracts have been shown to be active against *Plasmodium* parasites *in vivo* but not *ex vivo* (Mesia *et al*., 2010, Wells, 2011). These observations indicate the need for researchers to be extra cautious when screening products for antiplasmodial activity using *in vitro* assays. They also emphasize the role that reverse pharmacology could play in antimalarial drug discovery and development from medicinal plants.

To improve on drug discovery and development through ethnopharmacology, it would be appropriate to mimic as much as possible the drug preparation procedures being used by TMP (Adebayo and Krettli, 2011). This includes proper recognition of plant species, their classification, the preferred part of the plant, the method of extract preparation, the preferred route of administration and other plant combinations where applicable (Ginsburg and Deharo, 2011, Pan *et al*., 2013). This will ensure preservation of pharmacodynamic synergy or additivity, multidrug resistant inhibitory compounds as well as complementary killing mechanisms like immunostimulation and modulation of adverse effects (Ginsburg and Deharo, 2011). This will eventually translate into enhanced efficacy and safety of the resultant antimalarial drug candidates.

Drug preparation procedures play an important role in the efficacy and safety of phytomedicines. For instance, in a sub-chronic toxicity investigation of a beverage made from *Cassia occidentalis* seeds, it was observed that a beverage made from raw seeds induced toxicity while the roasted form of the seeds had no significant toxic effect. The roasting and extraction process was a key aspect of detoxification in this case (Essa'a and Medoua, 2013). Similarly, during the screening phase for anti-plasmodial activity of

Artemisinin (Qinghaosu) that led to the discovery of current cherished antimalarial drug, artemisinin, extraction at high temperature rendered the extracts inactive against the *Plasmodium* parasites (Miller and Su, 2011). However, on extraction at reduced temperature, the extracts gave promising results rekindling the hopes of its development into an antimalarial drug. The decision to consider the latter approach was arrived at upon considering the ethnomedical preparation procedure (Liao, 2009, Miller and Su, 2011).

In order to limit the loss of biodiversity of the highly utilized antimalarial medicinal plants, their domestication using modern farming techniques such as plant tissue culture technology is highly recommended. In addition to ensuring a reliable supply of these products, this would go a long way in ensuring reproducibility of the active ingredients, hence standardization of the products. More still, farming on these plants can act as a source of income to the people involved, thus contributing to improved livelihoods (Muriuki *et al*., 2012, Oliveira *et al*., 2009).

2.7 Standardization of Antimalarial Phytomedicines

In ethnopharmacology, pharmacological screening and drug development involve two operational approaches (Pieters and Vlietinck, 2005). Either, a standardized extract or a pure isolate of the bioactive material is developed into a drug. In either of these approaches, measures are put into place to in order ensure that each package of medicine contains the correct amount of therapeutic ingredients (Sagar *et al*., 2005), a process commonly referred to as product standardization. This process is much easier and cheaper to carry out when the therapeutic outcome of interest is exerted by either one or few compounds of the extract. However, standardization of the phytochemical content of crude or semi-purified plant extracts is very complex, expensive and time consuming (Aarland *et al*., 2014, Kunle *et al*., 2012). This explains, partly, the preference by some researchers to adopt the pure isolate approach in the process of drug discovery and development.

Noteworthy, the therapeutic property of medicinal plants is generally achieved by several active compounds working additively or synergistically, and at times, with inert compounds (Ginsburg and Deharo, 2011). Such inert compounds may serve to improve the stability and bioavailability of the active ingredients. As such, preservation of these properties is highly recommended during drug development from medicinal plants (Ginsburg and Deharo, 2011). Importantly, the phytochemical content of medicinal plants is influenced by factors such as the geographical location, the part of the plant used, the season of the year when plant material was collected as well as the methods of preparation and storage (Ginsburg and Deharo, 2011, Oliveira *et al*., 2009, Vijayalakshmi *et al*., 2013). These factors can be well defined and consolidated to ensure reproducibility, in terms of presence and quantity, of the bioactive ingredients in the manufacturing process (Kunle *et al*., 2012, Vaidya and Devasagayam, 2007). This would guarantee therapeutic consistency of phytomedicines. The approach to achieving this, perhaps, would be the development of innovative methods of standardization for such multi-component therapeutic products. Current technologies in Nuclear Magnetic Resonance (NMR) spectroscopy can analyze the phytochemical content and identify new chemical entities in crude plant extracts without chromatographic isolations (Bilia, 2014, Deborde *et al*., 2019). As such, NMR has shown great potential as a tool for standardization of crude or semi-purified medicinal plant products (Bilia, 2014, Booker *et al*., 2014). Similarly, plants that have been confirmed to contain antimalarial agents can be propagated using modern farming methods like tissue culture technology to ensure qualitative and quantitative reproducibility of the bioactive therapeutic ingredients (Ginsburg and Deharo, 2011, Kigen *et al*., 2013, Muriuki *et al*., 2012).

2.8 Government Health Policies on Phytomedicines

Formulation and adoption of policies relating to herbal medicine use by governments of respective countries can play a major role in availing the standardized antimalarial products. Such policies include, for instance, incorporating herbal medicine into mainstream healthcare provision (Kigen *et al*., 2013, Muriuki *et al*., 2012). This can in turn contribute significantly in the fight against malaria by availing such medications

through enhanced research and conservation efforts. Countries that have adopted these policies for treatment of various ailments have recorded some degree of success (Kigen *et al*., 2013). Formulation of such policies have been previously attempted in Kenya but with little success (Kigen *et al*., 2013, Mwangi, 2004). Several other African countries have not been successful in formulating such policy guidelines either (Madiba, 2010). Nevertheless, the promulgation of the new Kenyan constitution in the year, 2010 rekindled the hopes of a success in such regulations and guidelines. There is progress towards formulation of such policies in Kenya as demonstrated by passage of the Health Act, 2017 by parliament (Harrington, 2018). This has opened room for integration of traditional and alternative medicine into the mainstream healthcare. In the Act, parliament was required to constitute a body whose mandate is to regulate traditional and alternative medical practice in the country. Similarly, the ministry of health was tasked with formulating policies to guide cross referral of patients between TMPs and conventional health workers. Once fully implemented, the act is likely to create room for standardized clinical trials for the lead phytomedicines and antimalarial products. Such policy guidelines are likely to enable the utilization of reverse pharmacology, a potentially powerful approach, in drug discovery and development especially from medicinal plants (Surh, 2011, Willcox *et al*., 2011).

In Kenya, the Pharmacy and Poisons Board (PPB) is responsible for the registration of commercially prepared medicinal products. This regulatory body has registered several imported herbal products that are used in the management of various diseases, but not malaria. Perhaps, the lack of a registered antimalarial herbal product in the Kenyan market is because such products have not been listed as essential drugs in accordance with the Kenyan guidelines on malaria treatment (Ndwigah *et al*., 2018). Elsewhere, herbal medicines form part of approved remedies for various ailments (Kigen *et al*., 2013, Wells, 2011). Such countries have achieved improved health care for its citizens. This is perhaps indicative of the need for health policy makers of malaria endemic countries to fast track the process of formulating policy guidelines aimed at incorporating herbal medicine into mainstream health services delivery.

2.9 Toxicity and Safety of Phytomedicines

In modern drug discovery and development, safety of the drug is paramount. It is regarded as being important than even the efficacy of the product. In traditional medicine, there has been a presumption that the products are safe (Ibrahim *et al*., 2016, Manaharan *et al*., 2014). However, in more recent times, product safety in traditional medicine is becoming a great concern (Koduru *et al*., 2006, Ugwah-Oguejiofor *et al*., 2019). This is because there are many documented reports on harmful effects resulting from improper use of medicinal plants (Manaharan *et al*., 2014). These reports have been associated with lack of understanding on the preparation procedure and improper use of the phytomedicines (WHO, 2002). The toxicity has also been associated with microbial contamination resulting from poor sanitation in the preparation, handling and storage of the phytomedicines (Ezekwesili-Ofili *et al*., 2014), herbal drug interactions in case of co-administration of orthodox medicines or supplements and herbal medicines (WHO, 2002, Phua *et al*., 2009). Similarly, toxicity has been associated with improper identification of the medicinal plant species (Gebrelibanos *et al*., 2014). Therefore, it is necessary that the safety of plant products as medicine get to be elucidated before the products can be used in primary health care provision. If found to be toxic, detoxification procedures could be suggested.

2.10 Phytomedicines as Modulators of Malaria Pathogenesis

Mechanism of action for most natural antimalarial products is largely unknown (Mojab, 2012). It could be that most of the products selectively acts on the biochemical pathways only unique to the parasites of interest and thus directly killing them (Wright, 2009). On the other hand, overall cure or management of the disease by the antimalarial natural remedies may be achieved or enhanced by, for instance, by complementary mechanisms such as immunostimulation and modulation of the adverse effects (Rasoanaivo *et al*., 2011).

Host immune responses play a very key role in pathogen suppression and elimination (Chia *et al*., 2014). It is documented that antimalarial chemotherapy coupled with immunomodulatory agents enhance resolution of the disease (Pradhan and Ghosh, 2013, Vathsala *et al*., 2012). Plant extracts can stimulate the host innate and or adaptive immunity contributing to malaria prophylaxis and cure (Rasoanaivo *et al*., 2011). In a study aimed at investigating the effects of Picroliv (a standardized fraction isolate from *Picrorhiza kurroa* ethanol root extract) and its potential in treatment against resistant *Plasmodium yoelii* infection in mice, the extract fraction was found to stimulate the immune system. The efficacy of the drug to which the parasites were resistant (chloroquine) was also enhanced by Picroliv (Dwivedi *et al*., 2008). In another study that was aimed at determining the effects of Juzen-taiho-to, a Chinese Oriental Herbal Medicine, on Non-lethal rodent malaria, the herbal medicine was found to suppress the disease by host immunostimulation (Yamaura *et al*., 1996).

Fever, recurrent chills, intense headaches, vomiting, joint pains, fatigue, hepatomegaly, splenomegaly, hypoglycaemia, acidosis, hemolytic anaemia and glomerulonephritis constitute some of the adverse effects associated with *Plasmodium* infection (Arrey Tarkang *et al*., 2014, Milner *et al*., 2012). Modulation of the mechanisms that lead to development of some of these adverse clinical symptoms could be key for enhanced resolution of the disease during herbal therapy (Rasoanaivo *et al*., 2011). For instance, one of the clinical symptoms of malaria is vomiting and, ginger, a plant of established anti-emetic effect in expectant mothers, though not yet tested in the context of malaria, is widely used in preparation of traditional antimalarial medicine (Rasoanaivo *et al*., 2011). In another study, reviewed elsewhere (Rasoanaivo *et al*., 2011), several compounds of known immunological effects were tested against a murine model of cerebral malaria, a disease whose pathology is mediated by immune responses. The researchers concluded that in order to reduce the severity of the disease, immunomodulators capable of preventing cerebral malaria should be used together with the antimalarial drugs during therapy.

Most of the investigations on plant products as antimalarial agents tend to consider only the direct parasite killing mechanism of the extracts and rarely investigate other mechanisms. Consequently, there is very little documented information on the role of complementary mechanisms in malarial disease resolution during antimalarial therapies (Rasoanaivo *et al*., 2011). Thus, there is need for researchers to carry out more studies aimed at elucidating the role of these complementary mechanisms in malaria cure and management.

2.11 *Senna occidentalis* (L.) Link

The *Senna* genera is widespread and very diverse, especially in the tropics where it is used as an ornamental plant, food and shade. Some of the plant species from this genus are an important source of traditional and modern medicines (Gebrelibanos *et al*., 2014). *Senna spectabilis* (Pivatto *et al*., 2014) and *S. petersiana* (Hiben *et al*., 2016), have been shown to possess antiplasmodial activity. *Senna occidentalis (*L).Link (Plate 2.1) is frequently used in traditional medicine (Manikandaselvi *et al*., 2016). This is a shrub that belongs to Leguminosae family and was formally known as *Cassia occidentalis* (Ishaku *et al*., 2016). The plant parts have been used to treat ringworms, scabies, abscesses, fevers, bone fractures, sore eyes, throat infections, hematuria, rheumatism, asthma, tuberculosis, diabetes, constipation, typhoid fever, hemoglobin disorders and liver disorders. It has also used in the treatment of hepatitis, edema, snake and insect bites, chicken pox and guinea worm disease (Manikandaselvi *et al*., 2016, Onakpa and Owoleke, 2010, Veerachari and Bopaiah, 2012). In Kenyan traditional medicine, a root decoction of *S. occidentalis* is used by the Digo, Duruma and Luhya communities in the treatment of malaria (Mukungu *et al*., 2016, Muthaura *et al*., 2015, Nguta *et al*., 2010).

Plate 2.1: *Senna occidentalis* (L.) Link (a) *Senna occidentalis* (L.) Link Roots (b)

2.12 Phytochemical Composition of *S. occidentalis*

The phytochemicals present in *S. occidentalis* have been documented. The chemical groups so far identified include sugars, cardiac glycosides, amino acids, saponins, resins, terpenes, alkaloids, phenolics, anthroquinones, balsams, phlobatannins, steroids, flavonoids and steroids (Issa *et al*., 2020, Nde *et al*., 2022). Many compounds have been isolated from the plant extracts. They include emodin, epigenin, aurantiobtusin, cassiollin, cassiaoccidentalin A, B and C, aloe-amine, isorhein, achrosine, xanthorin, sitosterol, mannitol, mannopyranosyl, obtusifolin, obtusin, oleic acid, quercetin, rhein, physcion, rubrofusarin, kaempferol, lignoceric acid, matteucinol, chrysarobin, chrysophanol, galactopyranosil among others (Nde *et al*., 2022, Singh *et al*., 2017).

The secondary metabolites present in plants are responsible for their biological activities (Abubakar and Haque, 2020). For instance, the flavonoids and resins could account for the anti-inflammatory activities, alkaloids have antimalarial activity, tannins are anti-parasitic and facilitate wound healing, terpenes are anti-tumor and anti-viral, saponins are anti-viral, anti-tumor, antioxidant and anti-inflammatory (Enechi *et al*., 2019, Okokon *et*

al., 2017, Uzor, 2020, Vijayalakshmi *et al*., 2013). Chrysophanone, emodin and anthraquinones have been shown to possess wound healing properties (Vijayalakshmi *et al*., 2013).

The phytochemical composition of plants is dependent on the geographical origin of the plant, based on the climate and soil conditions (Oliveira *et al*., 2009, Vijayalakshmi *et al*., 2013). For instance, in a study conducted in Africa, *S. occidentalis* extracts of stems, roots, bark and leaves collected from Ivory Coast had no alkaloids while the same samples collected from Ethiopia had a lot of alkaloids (Manikandaselvi *et al*., 2016). In addition, different plant parts (roots, stems and leaves) could have differences in phytochemical composition in terms of the amount and or constituent (Silva *et al*., 2011). For instance, lower amounts of anthraquinones have been observed in leaf and stem extracts of *S. occidentalis* compared to the seed extracts of the same plant species (Silva *et al*., 2011). This is suggestive of possible variations in bioactivity for plant extracts depending on where it is collected from, the plant parts used, age of the plant and even the season of the year when it is collected.

2.13 Bioactivity of *S. occidentalis*

Senna occidentalis extracts have been shown to possess antimicrobial activity against various microbes (Daniyan *et al*., 2011, Sadiq *et al*., 2012, Yeragamreddy *et al*., 2013). In a study aimed at evaluating the effects of *S. occidentalis* extract on mycoses, the extracts were found to be effective against the investigated fungi. In some instances, they had better activity when compared to some standard antifungal drugs (Davariya and Vala, 2011).

In studies aimed at determining the larvicidal potential of *S. occidentalis*, the extracts were found to be effective against *Anopheles stephense larvae* (Dhandapani and Kadarkarai, 2011), *Callosobruchus maculatus* (Lienard *et al*., 1993) and mosquito larvae (Venkateswarlu *et al*., 2012). In yet another study, the extract was observed to cause

mortality in termite workers (Isoptera) thereby protecting wood against damage by the termites (Abdullahi *et al*., 2012).

Senna occidentalis has been observed to possess anti-parasite ability. The anthelminthic potential of the plant extract was evaluated using adult earthworms (*Pheritima posthuma*). The extract was observed to cause worm mortality in a dose dependent manner (Sayyad *et al*., 2014). In a study meant to assess the effects of the plant extract against *Haemonchus contortus* egg-hatching and the development of the larva, the extract exhibited remarkable inhibition of egg-hatching and larval development (Eguale *et al*., 2011).

Senna occidentalis has also been shown to possess many more other biological activities. These include antioxidant, nephroprotective and hepatoprotective activity, immunomodulatory activity, antiflammatory activity, antidiabetic activity, analgesic and antipyretic activity, antianxiety and antidepressant activity as well as antimutagenic activity (Silva *et al*., 2011, Singh *et al*., 2017, Vijayalakshmi *et al*., 2013). The many biological activities of *S. occidentalis* as described herein demonstrates that *S. occidentalis* is a potential source of medicine against various diseases and conditions.

Various *in vitro* antiplasmodial studies have shown that extracts from *S. occidentalis* inhibit *P. falciparum* growth. In one of the investigations, the plant exhibited half-maximal inhibitory concentration (IC_{50}) values of 65µg/ml and 110µg/ml against the chloroquine sensitive (strain 3D7) and the resistant (strain Dd2) of *P. falciparum*, respectively (El Tahir *et al*., 1999). In another study, the chloroquine sensitive (3D7) and resistant (INDO) strains of *P. falciparum,* parasite growth suppression was similarly observed, with IC_{50} values of 48.80µg/ml and 54.28 µg/ml respectively (Murugan *et al*., 2015). In yet another study, an extract from the plant demonstrated high suppressive activity (IC_{50} < 3µg/ml)against a chloroquine sensitive *P. falciparum* strain (Tona *et al*., 2004). Further, an extract from the plant species was shown to exhibit a similarly high antiplasmodial activity against *P. falciparum* (Daskum *et al*., 2019). Despite the *in vitro* demonstration of *S. occidentalis* (L.) Link as a potential source of the much needed antimalarial medicine, information on its *in vivo* antimalarial efficacy is largely lacking.

2.14 Toxicity and Safety of *S. occidentalis*

Senna occidentalis has been documented as a potential source of effective medicine against various infections and diseases (Yeragamreddy *et al*., 2013). Most of the studies that are proponents of the usage of *S. occidentalis* as possible therapy against various infections rarely report or give caution on the safety or toxicity of the plant extracts. However, there are three notable studies that have conclusively suggested that leaf extracts of the plant are safe. In one of the studies aimed at determining the toxicity of the plant in rats, oral administration of the ethanol leaf extracts did not induce any adverse toxic effects in rats (Tanimu and Wudil, 2012). The second study aimed at investigating the effects of oral administration of *S. occidentalis* during pregnancy in wistar rats. Comparison of the test and control groups indicated no significant effect on the parameters of interest (Aragão *et al*., 2009). In yet another study, acute and subacute administration of the hydroalcoholic leaf and stem extracts of *S. occidentalis* was found to be safe in rats (Silva *et al*., 2011).

On the other hand, there are a lot of reports on the potential toxicity of *S. occidentalis* extracts (Vijayalakshmi *et al*., 2013). In a study that was investigating the effect of the leaf extract *S. occidentalis* on typhoid fever in rats, it was suggested that the leaf extract is possibly toxic to the animals (Saidu *et al*., 2011). In a study aimed at investigating the effects of *C. occidentalis (S. occidentalis)* on biochemical markers of tissue damage in rats, there were significant changes in the biochemical markers which were indicative of toxicity of the extract (Nuhu and Aliyu, 2008). In a sub-chronic toxicity investigation of the effect of a beverage made from *C. occidentalis* seeds, it was observed that the beverage made from raw seeds induced toxicity while the roasted seeds had no significant toxic effects (Essa'a and Medoua, 2013).

Therefore, it is evident that the question on whether *S. occidentalis* is safe as medicines is not well understood. The disease model, the plant part used, the method of preparation of the extract, the quantity of dosage and lengthy of exposure to the extract could explain the variations on the data on safety of the *S. occidentalis* extracts. Documented information

suggest that use of seeds, especially raw seeds (Essa'a and Medoua, 2013), chronic administration of the extract (Barbosa-Ferreira *et al*., 2005), very high dosage (Jafri *et al*., 1999) and aqueous extraction (Essa'a and Medoua, 2013) is more likely to be associated with toxicity of *S. occidentalis*. It has been suggested that anthraquinones and their derivatives such as toxalbumins and emodin glycosides are responsible for *S. occidentalis* toxicity (Hatano *et al*., 1999).

Senna occidentalis data on human toxicity is very limited despite the widespread usage of the plant in ethnomedicine (Vashishtha *et al*., 2009). In the only notable study involving humans, poisoning with *Cassia occidentalis (Syn: S. occidentalis)* beans was significantly associated with hepatomyoencephalopathy in young children in India (Vashishtha *et al*., 2007). Given the great therapeutic potential of *S. occidentalis* and contradictory toxicity reports in different disease models, the safety and toxicity of this plant species continues to be a great subject for scientific investigation and debate.

2.15 Extraction Techniques for Bioactive Plant Materials

The first step in separation of desired natural products from plant materials is the extraction step (Zhang *et al*., 2018). Extraction aims at separating the compounds of interest (secondary metabolites) from other plant artefacts such as the insoluble matrix, chlorophyll, salts, glycoproteins and starch (Abubakar and Haque, 2020). The secondary metabolites make up about 10% of the total weight of the plant and do account for bioactivity (Okokon *et al*., 2017, Pandey and Tripathi, 2014). Good extraction procedures should be simple, rapid, and of good yields that are free from impurities. Where solvents are used as extractants, the solvents are used sequentially with increasing polarity so as to enhance efficiency (Pandey and Tripathi, 2014). Solid-liquid extraction is the most widely used procedure in the extraction of bioactive components from medicinal plant materials (Abubakar and Haque, 2020).

The stages in solid-liquid extraction include; solvent infiltration into a solid cellular matrix, dissolution of solutes into the solvents, diffusions of the solutes out of the solid

matrix and collection of the solutes (Zhang *et al*., 2018). The extraction techniques include soxhlet extraction, maceration, infusion, decoction, sonication and percolation among others (Pandey and Tripathi, 2014, Zhang *et al*., 2018). Of these, Soxhlet extraction and maceration constitute the classical techniques.

Soxhlet extraction is appropriate when the solubility of desired compound is limited in a solvent. The same batch of the solvent is repeatedly passed (solvent recycling) through the sample (plant material) to maximize extraction. The procedure is not good for thermolabile compounds since such compounds may degrade due to prolonged heating (Abubakar and Haque, 2020, Pandey and Tripathi, 2014, Zhang *et al*., 2018). In maceration, a powdered plant material is placed in a stoppered container with an appropriate solvent, for a defined period with frequent mixing to enhance dissolution. The method is recommended when the desired compounds are thermolabile since prolonged heating may lead to degradation of the compounds of interest (Abubakar and Haque, 2020). Decoction method is suitable for extraction of heat stable water-soluble constituents of the plant. The plant material is boiled for 15 minutes in water, allowed to cool, strained and cold passed through it so as to produce the desired volume (Abubakar and Haque, 2020, Pandey and Tripathi, 2014). Sonication procedure involves the use of ultrasound with frequencies, which enhance permeability of cell walls, and produces cavitation. It is costly and at times, the high ultrasound energy induces undesirable changes to the bioactive molecules hence its use is a bit limited (Abubakar and Haque, 2020, Pandey and Tripathi, 2014). Percolation makes use of an apparatus called a percolator. A percolator is a, cone-shaped vessel that is narrow, and open at both ends. The plant material (solid) is appropriately moistened with menstruum, allowed to stand for some hours, packed and the top of the percolator closed. More menstruum is layered above the mass and the mixture allowed to macerate. Twenty-four hours later, the percolator outlet is opened to collect the extract (Abubakar and Haque, 2020, Pandey and Tripathi, 2014).

Extraction efficiency is affected by factors such as properties of the extractant, particle size of the raw materials, solvent - solid matrix ratio, temperature for extraction and

duration of extraction (Zhang *et al*., 2018, Zhou *et al*., 2012). In solvent selection, the safety, cost, selectivity and solubility are considered. It is important to note that the law of similarity and intermiscibility (like dissolves like) plays a great role here. This is based on polarity values where extractants with polarity values closer the value of the solutes of interest are better extractants for that solute. In the interest of phytochemical investigations, alcohol solvents (methanol and ethanol) are preferred. The small the particle size, the better the extraction. Decreasing particle sizes enhances penetration of the solvents into the solid matrix and diffusion of the solutes out the matrix. Nevertheless, the particle size should not be too small because this will cause difficulties during filtration. The higher the temperature, the higher the solubility and diffusion of the solutes. However, too high temperatures may cause decomposition of thermolabile compounds and loss of solvents. Increase in extraction time leads to increased extraction efficiency but only to the equilibrium point of the solute inside and outside of the solid matrix. An increase in solvent-to-solid matrix ration leads to an increase in extraction efficiency (Zhang *et al*., 2018). In the extraction of bioactive compounds from medicinal plants, extraction with an appropriate range of solvents in solid-liquid phase is preferred. The procedure is touted as being viable, efficient and convenient (Abubakar and Haque, 2020).

2.16 Fractionation of Plant Materials

Fractionation is a separation technique in which a particular mixture of substances is divided over a phase transition into several smaller quantities based on a particular gradient (separation gradient). This implies that the resultant individual smaller quantities (fractions) are separated based on variations in a specific feature (Abubakar and Haque, 2020, Wright, 2016). In fractionation of bioactive plant materials, the constituents of the plant material are separated based on their polarity (Zhang *et al*., 2018). In order to isolate a pure bioactive chemical entity of natural origin from natural products, a systematic separation of the extracted material based on their physiological properties followed by analysis of its biological activity is preferred. This is referred to as bioactivity (bioassay) guided isolation (Abubakar and Haque, 2020, Wright, 2016). The rationale for isolation of pure bioactive compounds in the development of drugs from medicinal plants stem

from the fact that other than the bioactive molecules, the extracts may contain other constituents that may be harmful the host (Fabricant and Farnsworth, 2001). In addition, isolation of pure compounds can allow structural analysis and modifications that may enhance the efficacy and safety of the identified bioactive isolate (Fabricant and Farnsworth, 2001). For natural products research, the major limitation of the bioactivity guided fractionation approach is the isolation of previously identified compounds (Hostettmann *et al*., 2001).

2.17 Therapeutically Relevant Doses for *in vitro* and *in vivo* Bioactivity Assays

When carrying out bioactivity assays for extracts/compounds, it is important to consider pharmacologically relevant dose ranges. This would ensure applicability of the dosages in therapy. It is also important to clearly define the route and frequency of administration of the extract or pure compound. For medicinal plant research, it would be important to mimic the ethnomedical approach (Heinrich *et al*., 2020, Kigen *et al*., 2013). Toxicity studies may give an indication of if the selected upper limit dose is tolerable in the experimental model (Heinrich *et al*., 2020). For oral route administration of extracts, an upper limit dose range of 100-200mg/kg body weight is therapeutically desirable for *in vivo* studies. For pure compounds, 50mg/kg as upper limit dose is appropriate for *in vivo* oral route. For subcutaneous, intraperitoneal and intravenous routes, much lesser doses are desirable. For *in vitro* assays, the upper limit dose can be in the range of 100-200µg/ml for extracts and 30–50µM for pure compounds. Nevertheless, higher doses can be used in case of toxicological assessments (Heinrich *et al*., 2020).

2.18 *In vitro* Antiplasmodial Activity Assays

Given the great challenge posed by malaria to public health, antimalarial efficacy tests; both *in vitro* and *in vivo*, have been put forward to aid in the assessment and development of compounds that could be used in its management (Sinha *et al*., 2017). The *in vitro* methods are based on the ability to culture human malaria parasites in human erythrocytes (Maji, 2018). The *in vitro* methods include Giemsa stained slide method, Flow cytometry,

Micro-test (Mark III), Hypoxanthine uptake method and determination of lactate dehydrogenase activity, Enzyme Linked Immunosorbent Assay among others (Calderón *et al*., 2012, Maji, 2018, Sinha *et al*., 2017). The *in vitro* methods are considered to be time saving and efficient because they allow assessment for a large number of different compounds of interest at the same time (Calderón *et al*., 2012, Maji, 2018).

2.18.1 Giemsa Stained Slide Method (Light Microscopy)

The *in vitro* screening of compounds for antiplasmodial activity requires the ability to culture *P. falciparum* in human erythrocytes (Calderón *et al*., 2012). Here, a 1-2% parasite density is inoculated in a 5% human erythrocyte suspension. The test compound and standards are then added at different concentrations in a 96-well flat-bottomed plate and continuously and incubated at 37^{o}C for 72 hours. Culture smears are then made, Giemsa stained, parasitaemia determined and comparisons drawn between the test variables (Maji, 2018). This method is cost effective and reports a single concentration as the end point. This is the minimum inhibitory concentration (MIC). Though cost effective, the method is not good for testing a large library of compounds (Calderón *et al*., 2012).

2.18.2 Flow Cytometry

This is done as described (Kalra *et al*., 2006). The method is based on ability to distinguish cells containing Deoxyribonucleic acid (DNA) from the rest (Calderón *et al*., 2012). Human red blood cells (RBCs) lack DNA while *Plasmodium* infected RBCs will have DNA (the *Plasmodium* DNA). Uninfected RBCs and platelets do not synthesize nucleic acids in culture. Leukocytes are unable to multiply and lyse within a short time. As such only the *Plasmodium* parasites are actively dividing in culture (Sinha *et al*., 2017). The parasitized erythrocytes are incubated with the compound of interest and the standard drugs and then stained with hydroethidine or DAPI (4, 6-diamidino -2-phenylindole). Sybr Green I, YOYO-I and PicoGreen have also been developed and are used as DNA interacting dyes in flow cytometry. The stain is taken up by the parasite DNA. A flow cytometer is then used to give counts and different stages of the parasitized cells in the

test and control groups. The method is reliable, sensitive and relatively simple hence time saving (Calderón *et al*., 2012, Sinha *et al*., 2017). However, it is relatively expensive in terms of reagents, equipment acquisition and maintenance, and requires specialized expertise (Calderón *et al*., 2012, Maji, 2018, Sinha *et al*., 2017).

I2.18.3 Hypoxanthine Uptake Method

Red blood cells and platelets lack potential to synthesize nucleic acids (DNA and RNA), proteins and membranes during *in vitro* culture. Similarly, leukocytes do not multiply, and as such, they get depleted within a few days. Therefore, the only dividing cells are the malaria parasites (Maji, 2018). Hypoxanthine is required by *P. falciparum* for purine salvage and DNA synthesis (Fidock *et al*., 2004). Hypoxanthine uptake method is used to estimate the parasite growth inhibition rate. To reflect the rate of parasite growth, Hypoxanthine is radio-labelled and incorporated into *Plasmodium* cultures containing different concentrations of the test compounds and standards, incubated for some time and radioactivity determined. The parasites are able to incorporate into their DNA, radioactive material added into the culture media. The incorporated radioactive material can be measured to indirectly determine the rate of multiplication of the parasites (Fidock *et al*., 2004, Maji, 2018). The acceptable mean count per minute (cpm) for hypoxanthine uptake is 10000. Lesser counts are indicative of reduced growth and multiplication (Fidock *et al*., 2004).

Another popular radioactive material that can be used instead of (3H) hypoxanthine is (3H) ethanolamine. The incorporation of the isotopes is directly proportional to increase in parasitemia. Quantification of the incorporated radioactive isotopes is done with the help of a liquid scintillation counter (Sinha *et al*., 2017). The assay has been a reference method for drug sensitivity assays for quite some time, especially in developed countries. The assay requires well-equipped laboratories and highly expensive equipment. It also requires stringent regulations regarding the handling and disposal of radioactive material. These requirements have limited its application in field settings and in low-income countries (Calderón *et al*., 2012, Maji, 2018, Sinha *et al*., 2017).

2.18.4 Parasite Lactate Dehydrogenase Method

This is an enzyme based assay (Sinha *et al*., 2017). Lactate dehydrogenase (LDH) enzyme is a terminal product in glycolysis of *Plasmodium* species. *Plasmodium* parasites make use of anaerobic glycolysis. As such, they need regeneration of nicotinamide adenine dinucleotide (NAD) to ensure a continuous supply of glucose. *Plasmodium* LDH structurally different from that of the host, in terms of amino acid composition (Maji, 2018). Therefore, the production and accumulation of PLDH is used as indicators of parasite viability (metabolic activity) in drug sensitivity assays. The activity of PLDH is differentiated from that of the host (human) LDH by use of APAD, instead of NAD for human LDH (Maji, 2018). In such assays, the ability of PLDH enzyme to quickly use of 3-acetyl pyridine adenine dinucleotide (APAD) in a reaction that converts lactate to pyruvate is measured (Sinha *et al*., 2017). Host RBCs LDH carry out a similar process but at a very slowly in the presence of APAD (Sinha *et al*., 2017). Therefore, the activity of pLDH is distinguished from the activity of host LDH by use of APAD, instead of NAD by host RBCs LDH. In the reaction of lactate to pyruvate in the presence of LDH and APAD, APAD is reduced to APADH. The APADH, in turn reduces tetrazolium to formazan, a blue product which is measured by spectroscopy (Maji, 2018). There is a direct relationship between PLDH activity and parasitemia level. The method is highly sensitive and reliable, automated and fast enabling High throughput screening (HTS). On the other hand, the assay is quite expensive in terms of reagents and equipment (Maji, 2018, Sinha *et al*., 2017).

2.18.5 Histidine Rich Protein II Method

Histidine rich protein II (HRP II) occur in the cytoplasm and other cellular compartments of the malaria parasites (Sinha *et al*., 2017). This assay measures the HRP II levels. The enzyme levels are directly proportional to the parasite density (Maji, 2018). The assay is reliable, highly sensitive and fast. The assay is limited by longer culture time (72 hours), though this allows for determination of drug sensitivity for slow acting antimalarial medicines (Maji, 2018, Sinha *et al*., 2017).

2.19 Limitations of *In vitro* Antiplasmodial Assays

In vitro assays are commonly used for screening and development of antimalarial drugs. These assays are perceived to be time and cost effective especially because they allow for the assessment of numerous compounds at the same time (Maji, 2018, Sinha *et al*., 2017). These assays are not affected by confounding host intrinsic factors like immunity, pharmacokinetics and genetics giving them the capacity to analyze various variables at the same time (Nsanzabana *et al*., 2018). In spite of this, *in vitro* assays have been associated with various challenges. These include limited expertise and infrastructure, inability to study drugs that work through active metabolites of the gastrointestinal tract or liver as well as modulation of host responses (Calderón *et al*., 2012, Maji, 2018, Mesia *et al*., 2010, Wells, 2011). They have also been associated with difficulties in standardization and inability to indicate toxicity (Maji, 2018). Homologous malaria parasite strains that have been tested against particular bioactive compounds in different laboratory settings tend to give dissimilar results of IC_{50} probably due to differences in assay protocols (Laufer, 2009, Sinha *et al*., 2017, Wells, 2011, Woodrow *et al*., 2013).

Hypoxanthine method is considered the gold standard for *in vitro* anti-plasmodial assays. Because this method utilizes radioactive material, it has become very unpopular and inapplicable in many laboratory settings (Woodrow *et al*., 2013). There is therefore an urgent need to develop a standardized protocol that would allow uniformity and reproducibility of *in vitro* assays during the bioprospecting of antimalarial drugs as well as in detecting emergence of antimalarial drug resistance. To this effect, the use of developed reference strains of established drug efficacy when conducting such assays has been highly recommended (Laufer, 2009, Woodrow *et al*., 2013). Indeed, the developed *In Vitro* Analysis and Reporting Tool (IVART) for use in antimalarial drug resistance surveillance affirms the feasibility of developing a standardized protocol for *in vitro* drug testing assays. This tool makes use of a Web interface and is automated, rapid and less prone to current protocols that may be subjective (Woodrow *et al*., 2013).

2.20 Animal Models in Malaria Research

Birds, bats, rodents, non-human primates and humanized mice have been used to study malaria. The animal models have been used to unravel various aspects of the parasite biology and malaria processes. Of these models, rodents and non-human primates have been utilized more extensively (Simwela and Waters, 2022).

2.20.1 Avian Malaria

The avian malaria parasites and *P. falciparum* were discovered almost the same time (Simwela and Waters, 2022). As such, avian malaria parasites remained the model of choice for experimental malaria studies until the discovery of rodent malaria parasites when the studies shifted to the use of rodents (Pigeault *et al*., 2015). Avian malaria is caused by mainly *P. relictum, P. gallinaceaum* and *P. lophurae*. *Plasmodium relictum* infection occurs worldwide except in Antarctica whereas *P. gallinaceum* and *P. lophurae* have limited distribution but causes significant pathology in poultry (Simwela and Waters, 2022). More recently, use of avian malaria parasites in experimental malaria has re-emerged especially in the understanding of malaria parasite ecology and evolution because of their profound genetic and phenotypic diversity (Pigeault *et al*., 2015, Simwela and Waters, 2022). Avian erythrocytes are nucleated unlike their mammalian counterparts; provides a constant access to nutrients and transport mechanisms for the metabolites through the host thus contributing to evolutionary divergence (Böhme *et al*., 2018).

2.20.2 Murine Malaria

Murine malaria models have been developed for malaria studies and they give an insight into particular areas of interest in malaria research. They include *Plasmodium chabaudi*, *Plasmodium berghei*, *Plasmodium vinckei* and *Plasmodium yoelii, w*ith *P. berghei* being the most widely used (Arrey Tarkang *et al*., 2014, Jiménez-Díaz *et al*., 2014). The mouse is the most used model in antimalarial drug studies. The model is versatile and readily

accessible. The severity of the disease depends on the parasite strain and genetic background of the mice (Jiménez-Díaz *et al*., 2014).

There is a great genetic difference between human and rodents. In addition, rodent malaria parasites possess a 24 h biological cycle unlike human malaria parasites (48 and 72 h). Furthermore, there are notable differences in specific morphologies between human and rodent malaria parasites (Jiménez-Díaz *et al*., 2014, Simwela and Waters, 2022). Nonetheless, the human and rodent *Plasmodium spp.* share highly conserved genes which provide important insights into the conserved elements of parasite biology. This allows for utilization of these rodent malaria models in studies aimed at understanding the malaria parasite biology, malaria pathogenesis, host-parasite interactions and, antimalarial drugs and vaccine development (Simwela and Waters, 2022). Rodent malaria parasites reproduce a full malaria cycle in mammals, when adapted (Jiménez-Díaz *et al*., 2014). For instance, *P. berghei* causes a fatal disease in BALB/c mice with pathology similar to the one observed in human due to *P. falciparum* (Perlaza *et al*., 2011). Inbred mice are used. Such mice limit chances of variation of results of the variables under investigation (Jambou *et al*., 2011).

2.20.3 Non-Human Primate Malaria

Mouse models are widely used in antimalarial drug discovery studies. However, there exists some vast genetic differences between man and rodents and this hinders the application of the resultant data (Andrews *et al*., 2018, Jiménez-Díaz *et al*., 2014, Lombardini *et al*., 2015, McCarthy *et al*., 2016). On the other hand, non-human primates belonging to genus *Aotus* and *Saimiri* are susceptible to infection by the human malaria parasites; *P. falciparum, P. vivax, P. malariae, P. ovale* and *P. Knowles*i (Jiménez-Díaz *et al*., 2014). Non-human primate models have thus been developed for malarial studies, as reviewed (Yost *et al*., 2023).

In non-human primate malaria models, all stages of the parasites' biological cycle can be reproduced for drug evaluation (Jiménez-Díaz *et al*., 2014). In addition, there exists many

similarities in host-pathogen interactions between the non-human primates and man. For instance, the baboon *(Pabio anubis*) is susceptible to *P. knowlesi;* a parasite that causes pathology similar to *P. falciparum* in human (Onditi *et al*., 2015). The parasite cyto-adheres thus sequestering especially in the placenta just like *P. falciparum* does in human (Onditi *et al*., 2015). However, the life cycle for *P. knowlesi* lasts 24 hours unlike in *P. falciparum* where it lasts 48 hours (Davison *et al*., 1998, Weatherall *et al*., 2002).

2.21 Approaches to *in vivo* Antimalarial Assays

In vivo assays are considered as gold standard tests in antimalarial bioassays (Maji, 2018, Nsanzabana *et al*., 2018). The *in vivo* antimalarial assays include Peters' 4-day Test (The 4-day suppressive test), Prophylactic test and Rane's test which is also known as Curative Test (Maji, 2018).

2.21.1 The 4-Day Suppressive Test

This is done as described (Okokon *et al*., 2017, Tadesse and Wubneh, 2017). It is the most commonly used test for efficacy of compounds. The test is based on comparison of parasitaemia reduction and mice survival of the test groups in relation to the control groups. The aim is to assess inhibition of *Plasmodium* growth during early stages of infection. The mice are infected with *Plasmodium* parasites and the 3 hours later, the test compound and standards administered for 3 consecutive days. Parasitaemia and survivorship is determined starting day 4 onwards. Compounds that achieve 90% parasitaemia suppression between day 4 and 6 are considered effective (Bantie *et al*., 2014, Kalra *et al*., 2006).

2.21.2 Prophylactic Test

This is done as described (Okokon *et al*., 2017). The aim of this experiment is to determine the repository (protective) activities of the compound of interest. The compounds are administered for 3 consecutive days (day 0-2), then the animals infected. Three days post

infection, blood smears are made for determination of parasitemia. In addition, survival time (days) of the experimental animals are recorded.

2.21.3 Rane's Test (Test for Curative activity)

This is done in accordance with protocols described elsewhere (Fenta and Kahaliw, 2019, Okokon *et al*., 2017, Tadesse and Wubneh, 2017). The objective of this test is to determine the potential of the compound in inhibiting *Plasmodium* growth during an established infection. The animals are infected with *Plasmodium* on the first day and confirmation of established infection done on day 3 post-infection. Then 72 h after infection, the animals are administered with compounds of interest and standards for 4-5 consecutive days. Meanwhile mice survival time (days) and parasitaemia suppression are assessed.

2.22 Limitations of *in vivo* Antimalarial Assays

Unlike *in vitro* assays, *in vivo* tests have a standardized protocol and are considered to be a gold standard in antimalarial efficacy assays (Maji, 2018, Nsanzabana *et al*., 2018). In *vivo* tests are generally biased towards the use of rodent malaria parasites like *Plasmodium berghei.* The growth kinetics of rodent malaria parasites are very different from those, *P. falciparum,* which causes the most severe form of human malaria. The existing preclinical standard assay (Peters' test), which is a rodent malarial model is limited in its application as it provides information only on parasitemia and suppression. It does not provide information on parasite killing or clearance as would happen in clinical drug therapy (Andrews *et al*., 2018, Jiménez-Díaz *et al*., 2014, Lombardini *et al*., 2015, McCarthy *et al*., 2016). As such, data obtained from rodent *in vivo* preclinical assays may not be commensurable to data from clinical studies.

There are a lot ethical concerns relating to the use of non-human primates in research. This has limited its application in antimalarial drug studies. In addition, antimalarial drug studies involving the primates are very complex thus requiring highly specialized facilities. On the other hand, *P. falciparum, P. vivax* and *P. malariae* need adaptation, *in*

vivo, before growing reproducibly in the monkeys. In addition, production of infective gametocytes requires splenectomy (Jiménez-Díaz *et al*., 2014), an invasive procedure.

Nonetheless, there have been major technical advancements in preclinical *in vivo* experimental assays for antimalarial drug development (Andrews *et al*., 2018, McCarthy *et al*., 2016). For instance, a humanized *P. falciparum* mouse model (Jiménez-Díaz *et al*., 2014) has been developed to improve the standardization of pre-clinical *in vivo* data and to ensure improved commensurability to clinical data (Langhorne *et al*., 2011, McCarthy *et al*., 2016). This is the only *in vivo* model where parasites grow in human cells. The model is generated by engrafting human erythrocytes intraperitoneally or intravenously into immunodeficient mice (Jiménez-Díaz *et al*., 2014). The application of this humanized rodent malaria model as an IC_{50} substitute in humans has been demonstrated (McCarthy *et al*., 2016). Currently, this is the preferred model for *in vivo* pre-clinical studies. Though expensive and technically challenging (Wells, 2011), it provides data on end point parameters such as time taken for parasite clearance and/or relapse as well as recrudescence. Information on survival rates and parasitemia reduction can also be obtained (Andrews *et al*., 2018). Increased application of such an improved rodent malaria models would be very important in prospective drug development studies.

The use of standard rodent models is instrumental, especially, in preclinical drug development stages (Andrews *et al*., 2018, Langhorne *et al*., 2011, Lombardini *et al*., 2015). However, existing genetic differences between man and rodents hinders their application. Non-human primate (NHP) models are an appropriate alternative because they are genetically closer to humans and are likely to provide data that recapitulate clinical data (Onditi *et al*., 2015). Although NHP models have been developed as excellent tools for malaria research, their application in drug development studies is still very limited. Furthermore, majority of previous studies that have utilized NHPs as models for drug development studies have been limited in accessing experimentally naïve animals. Often, they tend to utilize NHPs that have been previously used in other drug and vaccine development studies. This may affect the outcome of subsequent drug studies and result in generating erroneous data. To overcome this challenge, capacity development for

scientists and young researchers intending to conduct research in drug studies using NHPs is of vital importance (Lombardini *et al*., 2015).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Sites

The plant material was collected from Migori county county (0.9366^{o} S, 34.4198^{o} E), Kenya. Plant material preparation, extraction and fractionation was carried out at the Natural Products Laboratory of the Sino Africa Joint Research Centre (SAJOREC), Jomo Kenyatta University of Agriculture and Technology (JKUAT). Qualitative determination of phytochemical composition of the extract was done at Chemistry Laboratories, JKUAT. The bioassays were done at Animal Science department and, the department of Tropical and Infectious Diseases laboratories in the Kenya Institute of Primate Research (KIPRE).

3.2 Study Design

A laboratory based experimental study design was adopted (Figure 3.1.). Extraction of the plant material was done by maceration in organic solvents of differing polarities, and in water. In addition to maceration, a decoction approach was adopted for aqueous extraction to mimic the traditional way of preparing the antimalarial remedy (Muthaura *et al*., 2011, Muthaura *et al*., 2015, Nguta *et al*., 2010). The resultant crude extracts were screened for phytochemical composition and and assayed for antiplasmodial activity against *P. falciparum, in vitro* and *P. berghei* in mice. The extracts were similarly evaluated for effect on hematological, biochemical and histological parameters in *P. berghei*-infected and uninfected mice. The extract was then successively partitioned into various fractions using column chromatography, and the fractions evaluated for antiplasmodial efficacy against *P. falciparum,* in *vitro.* All experiments were performed in replicates.

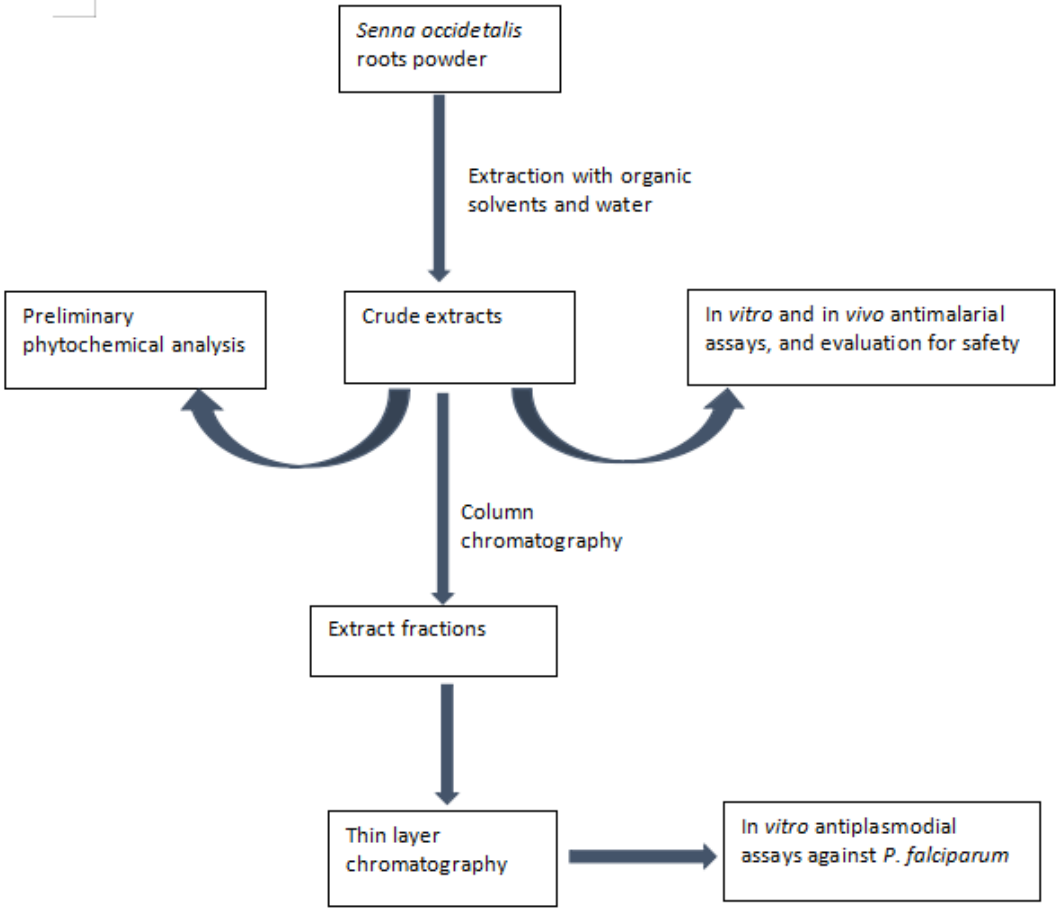


Figure 3.1: Schematic Representation of the Study Design

3.3 Ethical Clearance to Undertake the Study

Approval to conduct this study was granted by the Kenya Institute of Primate Research (KIPRE) Institutional Scientific and Ethics Review Committee (ISERC/02/18). The Kenya Institute of Primate Research facility is registered by the National Commission for Science, Technology and Innovation (NACOSTI), Kenya and accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International.

3.4 Preparation of the Plant Material

Senna occidentalis roots were collected from Migori county (0.9366^{o} S, 34.4198^{o} E), Kenya in the months of August and September, 2019. The plant was identified by Mr. Jonathan Ayayo, a plant taxonomist of the National Museums of Kenya. A voucher specimen (No. 38/81) was stored at the East African Herbarium of the National Museums of Kenya (NMK) for future referencing. Collection of the plant material was done according to the WHO guidelines on Good Agriculture and Collection Practices (GACP) for medicinal plants (WHO, 2003). The roots were cleaned of dust, chopped into small pieces and shade dried. Once dry, they were weighed and ground into powder using an electric plant mill. The resultant powder was stored at 4^{o}C in airtight plastic containers until extraction (Gini and Jeya Jothi, 2018).

3.5 Preparation of the Plant Crude Roots Extracts

The *Senna occidentalis* root powder was extracted with hexane, chloroform, ethyl acetate methanol and distilled water. Extraction was by cold maceration except for distilled water, where decoction was used in addition to maceration. All the organic solvents used were of analytical grade. The resultant extracts were filtered and concentrated by rotary vaporization (BÜCHI R-200 rotary evaporator) to obtain crude extracts. The aqueous extract was concentrated by freeze drying (NANBEI freeze dryer: NBJ-10-1, Zhengzhou, China). Plate 3.1 shows the set ups used for rotary evaporation and lyophilization. The aqueous extracts obtained was in form of dry powder. It was placed in airtight sample bottles and stored at 4^{o}C until required for use. The organic solvent extracts were left to dry in an open place free from dust, until there was no further change in weight. The extracts were then stored in sealed sample bottles at 4^{o}C until use.

Plate 1(a) Plate 1(b)

Plate 3.1: Set-Ups for Rotary Evaporation (a) and Lyophilisation (b)

3.5.1 Extraction by Organic Solvents

Two hundred and fifty (250) grams of the plant root powder was macerated separately, in 1500 ml of each type of menstruum (extraction solvent). The extraction was let to run for 48 hours in an orbital shaker, set at 25^{o}C. After 48 hours, the micelle (solvent of extraction - extract mixtures) were decanted into 1 liter beakers and filtered (Whatman filters No. 1), with the help of a vacuum pump (BÜCHI Vac^{R} V-500). The filtrates were then transferred into a round bottomed conical flasks for rotary vaporization. The rotavapor (BÜCHI R-200 rotary evaporator) was set at 50^{o}C and reduced pressure. The concentrates were then transferred into well-labelled pre-weighed 20 ml beakers and covered with an aluminium foil. Tiny holes were punched at the top of the foil to allow the solvents to evaporate further at room temperature until the extracts attained a constant weight. The marc (resultant powder residue) was re-extracted once again. Once dry, the extracts from both phases of extraction for each solvent were weighed, summed up and yields expressed as percentages of the initial weights of the root powder used for each solvent. The extracts were then transferred into well-labelled airtight sample bottles and stored at 4^{o}C till use.

3.5.2 Extraction by Water

Aqueous extraction of the plant roots powder was done by cold maceration and decoction methods. For cold maceration, 200 grams of the plant root powder was macerated in 1200 ml distilled water. The mixture was shaken and transferred into an orbital shaker set at 25^{o}C. This was left to run for 24 hours. After 24 hours, the mixture was decanted and the solution filtered thrice through a cotton wool plug followed by another filtration (Whatman filters No. 1). The resultant filtrate was dispensed in small volumes into disposable plastic cups and transferred into a fridge set at -27^{o}C for 48 hours to enable the filtrates to solidify. The solidified filtrates, in the plastic cups, were transferred into a lyophilizer (NANBEI freeze dryer: NBJ-10-1, Zhengzhou, China) and left to run for 72 hours. The resultant extract (powder in form) was collected, weighed and yields expressed as percentages of the initial weights of the root powder used. The extract was transferred into sample bottles and stored at 4^{o}C till use.

For decoction, a method (Lima *et al*., 2015) was used with some modifications. One hundred grams (100g) of the root powder was transferred into a 2-liter conical flask containing 600 ml distilled water. The flask was placed in a water bath with a temperature gauge set at 100^{o}C. The powder-water mixture was heated to boiling. It was then left to boil for 30 minutes, with regular mixing at after every 5 minutes. At each mixing point, the temperature of the mixture was monitored. The boiling temperature was recorded as 90^{o}C, on average. The mixture was removed from the heat source and allowed to cool. The micelle was decanted into a beaker and filtered 3 times through a cotton plug followed by a filter paper (Whatman filters No. 1). The boiling and filtration was repeated for the marc (resultant residue). The filtrate was frozen, lyophilized (NANBEI freeze dryer: NBJ-10-1, Zhengzhou, China) and percentage yield calculated as described for the aqueous cold maceration (Section 3.5.1).

3.6 Qualitative Phytochemical Screening for Secondary Metabolites

Plant secondary metabolites are excellent predictors of their bioactivity potential (Okokon *et al*., 2017). In order to predict the bioactivity of *S. occidentalis* roots extract, standard procedures were used to screen the extracts for the presence of saponins, tannins, alkaloids, flavonoids and sterols as described (Gul *et al*., 2017). For saponins, 500 mg of the test sample were added into 1 ml distilled water. Then, 3 drops of olive oil were added, shaken vigorously and observations made for presence of a stable emulsion. For tannins, a sample solution of the extracts were made using the respective solvents, 0.5 ml of 5% ferric chloride added onto them and observed appearance of a dark green colour. In order to check for presence of alkaloids, Meyer's test was performed. Sample solutions of the extracts (1ml) was made using the respective solvents, 1 ml of Meyer's reagent (potassium mercuric iodide) added onto them, and observed for presence of a white precipitate. For flavonoids, drops of ammonia solution were added to 1 ml of the crude extracts. Then, 0.5 ml of concentrated Hydrochloric acid was added and observation made for appearance of a pale brown colouration. In order to check for presence of Sterols, Salkowski test was performed. Sample solutions of the extracts (1 ml each) were made using the respective solvents and 1 ml of chloroform added to each. Then, concentrated sulphuric acid was added carefully by the side of the test tubes to each of the extracts, to form a lower layer and observations made for presence of a red-brown colour (steroidal ring) at the interface.

3.7 Evaluation of Antiplasmodial Activity of Crude Roots Extract of *S. occidentalis* against *P. falciparum, in vitro*

Pyrimethamine sensitive (strain 3D7) malaria parasite strain of *P. falciparum* was used in this experiment. The parasite strain was obtained from Kenya Medical Research Institute (KEMRI) repository. Culturing of the parasites was done as described (Trager and Jensen, 1976) with some modifications (de Andrade-Neto *et al*., 2007).

3.7. 1 Preparation of Culture Media for *P. falciparum* Propagation

The culture medium used in this study is the solution form of the Roswell Park Memorial Institute (RPMI 1640) medium procured from Gibco (Lot:2095766). The 500 ml RPMI 1640 (1X) medium was supplemented to incomplete form as described (Ljungström *et al*., 2008, Radfar *et al*., 2009) with some modifications. Supplementation of the medium was done by adding 18.75ml of 1 molar 4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid (HEPES), (Gibco, Life technologies Ltd.,Paisley, UK), 3 ml of 1 molar Sodium hydroxide (EMD Millipore corporation, Darmstadt, Germany), 5 ml of 20% D-glucose (PANREAC QUIMICA SA Barcelona, Spain), 1.25 ml of 10 mg/ml gentamicin solution (Sigma, lot:070M0764) and 5 ml of L-glutamine (Gibco, Life technologies Ltd.,Paisley, UK lot:2091461A). In essence, each ml of the RPMI 1640 incomplete medium contained 37.5 µl of HEPES (1 molar), 6 µl of Sodium hydroxide (1 molar), 10 µl of D-glucose (20%), 2.5 µl of gentamicin (10mg/ml) and 10 µl of L-glutamine. The medium was kept at 4^{o}C till use. The sodium hydroxide (1 molar) used in reconstituting the medium was prepared by dissolving 4g of the salt in 100 ml double distilled water. The solution was filter-sterilized using 0.22 µm-pore membrane syringe filters (Millipore Co., USA) in a sterile biosafety cabinet. This was then transferred into 50 ml falcon tubes (Corning, USA) and stored at 4^{o}C for use within 3 months. The 20% D-glucose was prepared by weighing 10g of D-glucose and dissolving it in 50 ml double distilled water, filter-sterilized with 0.22 µm-pore membrane syringe filters (Millipore Co., USA), sealed well and stored at 4^{o}C till use.

Complete RPMI 1640 medium for *P. falciparum* culture was prepared by adding one (1) part of heat inactivated human serum (blood group O+) to nine (9) parts of the incomplete RPMI 1640 medium just before use. The actual quantities (ml) were dependent on the amount of complete medium required in the culture at any given time. Thus, 10% of the complete culture medium was serum while 90% was the incomplete medium.

3.7.2 Preparation of Serum for *in vitro* Growth of *P. falciparum*

Serum was prepared from human blood as described (Guerin *et al*., 2010), with minor modifications. Human blood was collected into Red-capped BD Vacutainer® tubes (without anticoagulant). The blood was left at room temperature for 6 hours to coagulate and then kept overnight at 4^{o}C. The blood was then spun (Sigma Laboratory Centrifuge, model 2K15, Germany) at 2000 rpm for 10 minutes at room temperature. Serum (the supernatant) was aseptically transferred (in a sterile biosafety cabinet) into the sterile labelled serum storage tubes (15ml falcon tubes). The serum containing tubes were then placed in a water bath (Precision Scientific, CAT No. 66798) set at 56^{o}C and allowed to heat for 30 minutes. The serum was allowed to cool at room temperature and then stored at -20^{o}C until use.

3.7.3 Preparation of Red Blood Cells for *in vitro* Growth of *P. falciparum*

The aim of this wash step was to remove serum and leukocytes if present in the blood thus providing a pool of fresh red blood cells (RBCs) for the *in vitro* growth of *P. falciparum* (Ljungström *et al*., 2008). The washing was done as described (Radfar *et al*., 2009). Donor blood (blood group O+) was collected in anticoagulant vacutainer and mixed well. Using a serological pipette (Eppendorf), 10 ml of the blood was transferred into 15 ml falcon tubes and centrifuged (Sigma Laboratory centrifuge, model 2K15, Germany) at 1800 rpm for 10 minutes at room temperature. The buffy coat and plasma was aspirated using a serological pipette and discarded into a beaker containing 10% jik (sodium hypochlorite) in water. The packed cells were then supplemented with wash media (the RPMI 1640 incomplete media (described in section 3.7.1) and mixed well. This was centrifuged at 1800 rpm for 10 minutes at room temperature. The supernatant was discarded. The RBCs pellet was re-suspended and centrifuged in fresh wash media two more times. The resultant pellet was re-suspended in an equal volume of incomplete media (ratio of 1:1) giving rise to a 50% PCV (packed cell volume) that was then kept at 4^{o}C till use.

3.7.4 Retrieval of Cryopreserved *P. falciparum* (3D7)

This was done to facilitate continued expansion of the parasite strain (*P.falciparun*, 3D7) to numbers utilizable in the *in vitro* antiplasmodial activity test of the extracts. It was also meant to adapt the parasites to optimum replication rates for use in the assay. Retrieval was done as described (Radfar *et al*., 2009) with some slight modifications. Thawing solutions (12%, 1.6% and 0.9% sodium chloride) were prepared by dissolving 12g, 1.6g and 0.9g separately in 100ml sterile double distilled water and the solutions sterilized by filtering using 0.22 µm syringe filters in a clean and sterile biosafety cabinet. The parasites were obtained from liquid nitrogen (at the KEPRI biobank) and quickly thawed by warming in the hand palm. The outside of the parasite containing vial was wiped with 70% ethanol and transferred to the safety cabinet.

In the safety cabinet, vial contents were transferred into a 50 ml centrifuge tube using a 5 ml sterile serological pipette. Into the thawed blood (approximately 1 ml), 0.2 ml of 12% sodium chloride solution was added drop-wise while mixing constantly and gently. This was let to stand for 3 minutes and 10 ml of the 1.6% sodium chloride solution added drop-wise while constantly mixing gently. Once again, the tube was let to stand for 5 minutes and 10 ml of the 0.9% sodium chloride solution added as above.

The mixture was centrifuged at 1800 rpm for 5 minutes at room temperature and the supernatant removed aseptically by use of a 5ml serological pipette. The pellet (approximately 250 µl) was re-suspended in 8.0 ml of complete RPMI 1640 (described in 3.7.1) culture media (pre-prepared), to achieve a 3% haematocrit. Two hundred microliters (200 µl) of washed fresh (3 days old) O+ Red blood cells were added and mixing done well. The cells were transferred to a T25 culture flask (corning flask, USA) and gassed for 2 minutes using the gas mixture comprising of 90% N_2, 5% O_2 and 5% CO_2. The flask was then sealed and incubated at 37ºC (CO-150, New Brunswick Scientific) for 48 hours.

3.7.5 Continuous Propagation of the *P. falciparum* in Culture

This was aimed at obtaining enough *P. falciparum* parasite load for the *in vitro* drug test and also creating a reserve in the biobank for future use. The parasites were cultured as described (Ljungström *et al*., 2008, Radfar *et al*., 2009), with some slight modifications. After 48 hours, the culture (described in section 3.7.4) was aseptically moved into as sterile biosafety cabinet. The culture was mixed gently by pipetting using a 10 ml serological pipette (sterile) and transferred into a 15 ml falcon tube. It was centrifuged at 1800 rpm for 10 minutes at room temperature and the supernatant discarded. Pre-warmed (at 37^{o}C) complete medium (15 ml) was added to the pellet to achieve a 3% haematocrit. The culture was mixed gently using a sterile serological pipette and approximately 200 µl of the culture transferred into a microfuge tube using a 1 ml serological pipette (sterile), for parasitemia determination. The rest of the culture was transferred into a T25 culture flask, gassed (90% N_2, 5% O_2 and 5% CO_2) for 2 minutes, sealed and incubated at 37^{o}C.

For parasitemia determination, the culture containing microfuge tube was spun at 10,000 rpm for 30 seconds and the supernatant discarded. The pellet was dislodged using a micropipette (p200) and a thin smear made. The smear was air-dried for 5 minutes at room temperature and fixed by dipping it into absolute methanol. It was let to air-dry again and stained with 10% Giemsa solution (Sigma Aldrich) for 15 minutes. The slide was rinsed in running tap water and let to air dry. Once dry, immersion oil was applied onto the slide and observations made under a microscope (100× magnification) for the culture characteristics (presence of parasites, parasitemia level, stage of parasite growth and culture contaminants if any). Parasitemia percentage (%) was computed using the formula; Parasitemia (%) = (y/z)100, where y = number of infected RBCs and z = total number of RBCs counted (at least 1000 cells). This was repeated after every 48 hours. At each point, the culture was replenished with fresh complete medium, gassed, and incubated at 37^{o}C for 48 hours, while maintaining a 3% haematocrit and 2.5% or less parasitemia.

3.7.6 Sorbitol Synchronization of *P. falciparum*

Synchronization of the parasites was aimed at generating parasites of a relatively uniform age (stage) so as to allow for an accurate measurement of the effect of the extracts in inhibiting parasite growth. In this study, sorbitol synchronization (first established in 1979) was used. This method is highly efficient, simple and cost effective (Ranford-Cartwright *et al*., 2010). Trophozoite and schizont infected RBCs develop new permeation pathways (NPPs) that allow sorbitol to enter such infected cells leading to an influx of water followed by lysis of such cells. Such NPPs are inactive in ring infected cell membranes and as such, the ring stage parasites survive sorbitol treatment (Ngernna *et al*., 2019).

A parasite culture at packed cell volume (PCV) of 0.9 ml and 3.97% parasitemia with majority of the infected cells (56%) being at ring stage was considered ideal for synchronization and use in the *in vitro* antiplasmodial testing of the of the extracts. Sorbitol synchronization was performed as described (Radfar *et al*., 2009) with some minor modifications. Five (5) % sorbitol was prepared aseptically by dissolving 2.5g of sorbitol in 50 ml sterile double distilled water followed by filter-sterilization using 0.22 µm syringe filters in a clean and sterile biosafety cabinet and stored at 4^{o}C. The culture was transferred to 15 ml falcon tubes and centrifuged at 1800 rpm for 5 minutes and the supernatant aspirated. The pellet (0.9 ml) was re-suspended in 9ml (10 times volume of pellet) of 5% sorbitol (pre-warmed at 37^{o}C) and incubated at 37^{o}C for 10 minutes. During incubation, the culture was inverted 2 times (at the 4th and 7th minute) to mix. It was then spun at 1800 rpm for 10 minutes to remove sorbitol solution and washed twice by centrifuging at 1800 rpm for 5 minutes in complete RPMI. All the procedures were performed aseptically.

A smear of the synchronized culture was examined microscopically for parasitemia and determination of the proportion of ring-infected cells. Parasitemia was found to have dropped to 2.51% with ring stage parasites being the majority (76.5%). This parasite load

was adjusted to 1% parasitemia and 3% haematocrit, and used for the *in vitro* drug tests. The remainder of the parasite culture was sub-cultured and cryopreserved for future use.

3.7.7 Cryopreservation of *P. falciparum* parasites

This was aimed at storing the remaining parasites in a viable form for future use. The procedure was performed based on the Stockholm Sorbitol method as described (Ljungström *et al*., 2008, Radfar *et al*., 2009) with minor modifications. The freezing solution (100 ml) was prepared by mixing 72 ml of 3% sorbitol in 0.65% NaCl with 28 ml of glycerol followed by filter-sterilization using 0.22 µm syringe filters in a sterile biosafety cabinet, and storage done at 4^{o}C for use within 6 months. Cryopreservation of the parasites was done when the cultures attained a mean parasitemia level of 4.7%, with 35% of the infection being by ring stage parasites. The cultures were transferred into sterile 15 ml falcon tubes and centrifuged at 1800 rpm for 10 minutes. The supernatant was removed and the volume of packed cells (pcv) estimated to be 2.1 ml. An equal volume (2.1 ml) of pre-warmed deep-freeze solution (3% sorbitol in 0.65% NaCl, with 28% glycerol) was added slowly to the cells at room temperature and mixed well to allow glycerol to penetrate cells. The final mixture was then put into cryovials (2.5 ml capacity) in aliquots of 1ml and frozen rapidly by plunging them into liquid nitrogen (-196^{o}C).

3.7.8 Preparation of the Extract and Pyrimethamine Stock Solutions for the *in vitro* Antiplasmodial Assay

Dilution of the plant extracts and the control drug (pyrimethamine) was done as described (Bagavan *et al*., 2011) with some modifications. For the stock solutions (2.5 mg/ml), each extract was dissolved in 0.4% dimethyl sulfoxide (DMSO), vortexed and incomplete RPMI 1640 added to the required volume. Use of DMSO was necessary because the extracts did not readily dissolve in distilled water or even phosphate buffered saline (PBS) as the diluent. The final concentration of DMSO in the extract (0.4%) has been found to be non-toxic to *P. falciparum* culture, *in vitro* (Bagavan *et al*., 2011, Mbouna *et al*., 2018). For extract combinations, the extracts were weighed and blended in a 1:1 ratio

(Tepongning *et al*., 2011). For a positive control, 0.5 mg/ml stock solution of pyrimethamine (Sigma - Aldrich Chemie, Steinheim, Germany) was prepared. The extracts were then filter-sterilized using 0.45 µm followed by 0.22 µm syringe filters in a sterile biosafety cabinet and used in the assay.

3.7.9 The *in vitro* Antiplasmodial Assay of the Extract against *P. falciparum*

The effect of the extracts on *P. falciparum* was determined by measuring the growth inhibition percentage as described (Lima *et al*., 2015). The parasites were propagated as described in section 3.7.5 and used in this assay. The assay was performed aseptically in duplicate. Quantification of the parasites growth was achieved through light microscopy. *In vitro* parasite multiplication inhibition as determined via microscopy is a reliable tool for antimalarial drug susceptibility testing, when done by experienced microscopists, albeit a bit laborious (Kulkeaw, 2021, Lima *et al*., 2015, Sinha *et al*., 2017).

A 96-well cell culture plate was accordingly labelled (extract type and dilution factor) and 100 µl of complete RPMI 1640 introduced into the wells, excluding the first well along which the drugs were to be serially diluted. From the stock solutions of the test drugs, a tenfold dilution was prepared using complete RPMI 1640 culture medium. This resulted to 250 µg/ml and 25 µg/ml as the highest concentrations of the extracts and positive control drug (pyrimethamine) respectively. The concentrations of the extract used here was selected based on previous recommendations (Heinrich *et al*., 2020). The extracts and control drug were introduced into the first wells of the culture plates and serially diluted 7-fold across the plate providing concentrations in the range of 250 µg/ml to 1.95 µg/ml for the test extracts, and 25 µg/ml to 0.20 µg/ml for pyrimethamine (control drug).

Synchronized ring stage parasitized RBCs (1% parasitemia) suspended in complete RPMI (3% haematocrit) were then added to the wells at 100 µl per well. For a negative control, a suspension of non-parasitized red blood cells (npRBCs) and pRBCs in RPMI 1640 culture medium (without the extracts and control drug). Similarly, the cells were cultured in 0.4% DMSO. The plate lids were put back into position and the plates shaken gently to

mix the contents and transferred to a sterile desiccator (with a burning candle placed inside). The desiccator lid was returned to position. After the flame went off (2 minutes' time), the plates (inside the desiccator) were incubated at 37°C for 72 hours.

After 72 hours, thin smears were prepared and parasites quantified by light microscopy. Parasitemia suppression (percentage) was computed and 50% inhibitory concentration (IC_{50}) values determined for each extract. For determination of parasitemia suppression, the number of parasitized red blood cells (pRBCs) was examined per at least 1000 RBCs. Percentage parasitemia and parasite suppression was then computed using the following formulas; Parasitemia (%) = (y/z)100, where, y = number of infected RBCs and, z = total number of RBCs. Parasite suppression (%)was computed as; % suppression = [(a-b)/a]100, where a = percentage parasitemia in untreated control group and, b = % parasitemia in treatment group (Dibessa *et al*., 2020). GaphPad prism software (Version 7.00, California, USA) was used to determine the IC_{50} values for the test extracts and control drug by fitting analysis of a non-linear dose response curve. The obtained IC_{50} values were used to categorize the antiplasmodial activity of the extracts as good (IC_{50} < 10 µg/ml), moderate (IC_{50} > 10 to < 25 µg/ml) and weak (IC_{50} > 25µg/ml), (Bagavan *et al*., 2011).

3.8 Evaluation of the Extract for *in vitro* Cytotoxicity

The aqueous and methanolic extracts of *S. occidentalis* exhibited highest antiplasmodial activity *in vitro* and were as such evaluated for cytotoxicity using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay as described (Kweyamba *et al*., 2019, Waiganjo *et al*., 2020), with some modifications. Vero cell line from the kidney of African green monkey (sourced from KEMRI) were used in evaluating *S. occidentalis* roots extract for *in vitro* cytotoxicity. The cells were retrieved from the KIPRE biobank and adapted to optimum growth, *in vtro*. The cells were grown to confluent monolayer in Minimum Essential Medium Eagle (MEM) containing sodium bicarbonate and L-glutamine. The growth medium was first supplemented with 1% pen-

strep (Sigma), 1% L-glutamine (Gibco), 1% HEPES (Gibco), 10% Fetal bovine serum (FBS).

Ten thousand (10,000) cells contained in 100 µl cell suspension were seeded onto each well of a 96-cell culture plate and incubated for 24 hours at 37^{o}C in a 5% CO_2 incubator. This allowed the cells to achieve a layer of > 90% confluence. The cells were then exposed to 100 µl of the methanolic and aqueous extracts for 48 hours, in concentration ranges of 250-3.91 and 25-0.39 µg/ml for the extracts and pyrimethamine, respectively. The two extracts were selected for cytotoxicity evaluation because they had exhibited highest activity during *in vitro* antiplasmodial assay and were as such candidates for further antiplasmodial activity evaluation in an animal model. Pyrimethamine and DMSO (0.4%) exposed, as well as untreated cells were included as controls. After the 48 hours' incubation, 10 µl of MTT reagent (5 mg/ml) was added aseptically to each well, tapped gently to mix and incubated at 37^{o}C for 3 hours. All the media was then aspirated from the wells and 100 µl DMSO added.

Optical density readings as obtained by a microplate reader at 570 nm (Dynatech, MRX, USA) were then used to compute 50% cytotoxicity concentration (CC_{50}) and selectivity index (SI) of the extract determined using the formula; Cell growth inhibition = [(K-L)/K]100, where K= mean OD of untreated cells and L = mean OD of drug at each concentration. GraphPad prism (Version 7.00, California, USA) was then used to draw dose response curve and a nonlinear regression analysis done to determine the 50% cytotoxicity concentration (CC_{50}). Selectivity index (SI) of the extract was determined using the formula; SI = (A/B)100, where A is the 50% cytotoxicity concentration (CC_{50}) on Vero cells and B is the 50% inhibitory concentrations (IC_{50}) *on P. falciparum.* Substances that exhibit SI < 10 are said to be of low selectivity while those with SI ≥ 10 are of high selectivity (Waiganjo *et al*., 2020).

3.9 Evaluation of the Effect of *S. occidentalis (L.)* Link Roots Extract on Parasitemia and Survival Time of *P. berghei* infected Mice

The effect of *S. occidentalis* root extract in suppressing *P. berghei* parasitemia in mice was determined using a curative test of antimalarial drug efficacy studies. Methanol and aqueous extracts were evaluated because they exhibited high antiplasmodial activity, and high selectivity indices, *in vitro*.

3.9.1 Handling and Care of the Experimental Animals

Inbred BALB/c mice, both males and females aged 8-9 weeks and weighing 27.7g -28.4g were used for evaluation of *in vivo* antimalarial activity of the extract in mice. The animals were obtained from the KIPRE rodent facility. They were maintained in standard Macron type II cages, in a cycle of 12 hours' dark and light, and mean temperature of at 23^{o}C. Food and water was provided *ad libitum*. Handling and care of experimental mice were done in compliance with the international guidelines on the care and use of laboratory animals. In addition, the study was done based on the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) guidelines.

3.9.2 Propagation of the Rodent Malaria Parasites, *P. berghei*

Plasmodium berghei ANKA parasites were used in this experiment. This parasite strain was utilized because it causes severe disease in BALB/c mice with clinical characteristics similar to *P. falciparum* infection (Craig *et al*., 2012). *Plasmodium berghei* ANKA causes death in untreated mice after 7-10 days of infection, with parasitemia reaching 10-20% (Haidara *et al*., 2018). However, factors such as the route of infection and the parasite inoculum may influence the onset of mortality in BALB/c mice with deaths occurring as early as day 6 after infection (Amani *et al*., 1998, Hanum P *et al*., 2003, Rajan and Bagai, 2011). Despite the great phylogenetic distance between rodent and human malaria parasites, *P. berghei* possess conserved genes that permits their use in antimalarial drug efficacy testing (Jambou *et al*., 2011).

The parasites were sourced from Kenya Medical Research Institute (KEMRI) and maintained in mice at KIPRE as described (Janse *et al*., 2006). The parasites were

retrieved from liquid nitrogen, quickly thawed in the hand palm and an equal volume of PBS added to it. The BPS-parasite mixture was inoculated intraperitoneally into 3 donor mice (0.2 ml per mouse). On day 3 post-infection, tail snips were made and tail blood used to make thin blood smears. The smears were fixed with absolute methanol, Giemsa stained (as described in section 3.7.5) and observed under 100× lens of a light microscope. Parasitemia was then determined as described in section 3.7.5. At 5% parasitemia, the mice were euthanized (CO_2 asphyxiation) and blood drawn into heparinized syringes by cardiac puncture. The blood was pooled, parasitemia adjusted by diluting the in PBS and used to infect the experimental mice.

3.9.3 Mice Infection and Treatment

A curative approach, as described (Atsbha *et al*., 2021, Enechi *et al*., 2019) was used with some modification. Mice were inoculated intraperitoneally with 1×10^6 *P. berghei* infected erythrocytes. On day 3 post infection, the mice were checked for patent parasitemia and assigned randomly to five (5) experimental groups (5 mice per group). Treatment (oral) was started on day 4 post-infection (approximately 5% parasitemia) and continued for 4 consecutive days. The oral route was chosen based on documented ethnomedical usage of the plant (Nguta *et al*., 2010). Group A and B were treated with 100 mg/kg and 200 mg/kg of the methanolic extract, respectively. Group C was treated with 200 mg/kg of the aqueous extract. Group D was treated with 1 mg/kg pyrimethamine (Sigma – Aldrich Chemie, Steinheim, Germany), a standard antimalarial drug. Group E was administered with phosphate buffered saline (PBS), the vehicle. Another set of uninfected mice (Groups F and G) were included in the experiment for assessment of the effect of the extract on mice in the absence of the infection. Groups F was administered with the extract (200 mg/kg) while group G was given the vehicle (PBS). The extract dose administered was based on previous recommendations (Ekasari *et al*., 2018, Heinrich *et al*., 2020, Tona *et al*., 2001).

3.9.4 Determination of Parasitemia Suppression

The effect of the extract on *P. berghei* growth suppression in mice was determined as described previously (Atsbha *et al*., 2021). Tail blood was used to make thin smears that were fixed with absolute methanol and observed under 100× lens of a light microscope. Parasitemia suppression was then computed as described in section 3.7.9.

3.9.5 Determination of Mean Survival Time of Mice

The number of days survived by each mouse from the day of parasite inoculation to death was recorded to evaluate the effect of the extract on the survival time of the infected mice. The mean survival time of each group was computed using the formula; Mean survival time (MST) = (A/B)100, where A is the sum of survival time (days) for the mice in a group and B, the total number of mice in the group (Alehegn *et al*., 2020).

3.10 Assessment of the Effect of *S. occidentalis* (L.) Link Roots Extract on Hematological, Biochemical and Histological Parameters of *P. berghei* Infected Mice

Methanolic extract showed highest antiplasmodial activity in *vitro* and in *vivo* and was thus considered for further evaluation of antimalarial potency and safety. *Plasmodium* infection is known to induce reduced or loss of appetite which leads to weight loss (Baah *et al*., 2020). The parasites are known to alter host hematological parameters and induce tissue injury (Silva-Santana *et al*., 2020). On the other hand, the role of hematological indices as predictors of normal biological processes, responses to pharmacological substances during therapy, or adverse effects of foreign compounds is well documented (Kotepui *et al*., 2015, Silva-Santana *et al*., 2020). To assess the effect of *S. occidentalis* root extract on antimalarial potency and safety in *P. berghei* infected mice, the mice were monitored for changes in average body weight, hematological, biochemical and histological parameters.

3.10.1 Infection of Mice with *P. berghei*, Treatment and Collection of Samples for Hematological, Biochemical and Histological Examination

Mice were inoculated intraperitoneally with 1×10^6 *P. berghei* infected erythrocytes. On day 3 post infection, the mice were checked for patent parasitemia and assigned randomly to 4 experimental groups (5 mice per group). Treatment (oral) was started on day 4 post-infection and continued for 4 consecutive days. Group A and B were treated with 100 mg/kg and 200 mg/kg of the methanolic extract, respectively. Group C was treated with 1mg/kg pyrimethamine. Group D was administered with phosphate buffered saline (PBS), the vehicle. Another set of uninfected mice (Groups E and F) were included in the experiment to assess the effect of the extract on mice in the absence of the infection. Groups E was administered with the extract (200 mg/kg) while group F was given the vehicle (PBS). The animals were euthanized by carbon dioxide asphyxiation 24 hours after final dose (day 8 post-infection, after an overnight fasting) and samples collected for hematological, biochemical and histological examination.

3.10.2 Determination of Changes in Body Weight

Plasmodium infection causes decreased appetite which results in weight loss (Baah *et al*., 2020). Studies in experimental animals have demonstrated that drug toxicity is also linked to weight loss (Ugwah-Oguejiofor *et al*., 2019). To assess the effect of the extract on weight changes in *P. berghei* infected and uninfected mice, the average change in body weight of the mice was assessed using a digital weighing balance and comparisons drawn within experimental groups on day 0 (day of infection) and day 8 post-infection (24 hours after the treatment regimen).

3.10.3 Evaluation of Hematological and Biochemical Parameters

The experimental mice were euthanized by carbon dioxide asphyxiation on day 8 post-infection and whole blood collected (cardiac puncture) in ethylenediamine tetraacetic acid (EDTA). The blood was examined using automated hematology analyzer (CC2400Plus

Cell counter) to determine the extract's impact on hematological parameters in *P. berghei* infected and uninfected mice. Red blood cell, white blood cell and platelet indices were recorded and comparisons drawn among the groups. The hematological parameters analyzed include the total white blood cell (WBCs) count, lymphocytes (LYP) and granulocyte (GRAN) populations, hemoglobin (HMG) concentration, hematocrit (HCT) concentration, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), red blood cell distribution width (RDW-SD), total platelet count (PLT), mean platelet volume (MPV) and platelet distribution width (PDW). To assess the effect of the extract on kidney and liver function, blood was collected (in tubes with clot activating factor), processed into serum and analyzed (Humalyzer primus-200) for the levels of urea, creatinine and electrolytes. In addition, measurements and comparison for bilirubin, albumin, total protein, aspartate aminotransferase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) were made.

3.10.4 Macroscopic and Histopathological Examination of Organs

Plasmodium infection affects mainly the kidney, liver, spleen, lungs and brain (Chin *et al*., 2019). Moreover, the liver and kidney are very vulnerable to toxicity (Nigatu *et al*., 2017). To assess the effect of *S. occidentalis* extract on gross pathological alterations, the organs were collected on day 8 post-infection and examined macroscopically for changes in color and size. For histopathological examination, tissue sections were prepared from the organs and examined for changes in architecture. The preparation of the organs, sectioning and staining were done as described (Chin *et al*., 2019) with some modifications. The organs were collected and preserved in 10% (v/v) solution of buffered formalin (neutral) for 7 days. Samples (5mm thick) were dehydrated in 80, 95 and finally 100% ethanol. This allows for paraffin wax infiltration into tissues in the subsequent step (paraffin wax is hydrophobic in nature). The tissues were then cleared with toluene. The clearing serves to removal ethanol to allow for impregnation with paraffin since ethanol and paraffin are immiscible, and make the tissue translucent for examination by microscopy. The tissue was then impregnated and embedded in paraffin wax. This serves to preserve tissue morphology and give the tissue support during sectioning. From the

embedded tissues, 6µm section ribbons were made (Rotary microtome, Leitz 1512, Germany) and placed on a warm water tissue floatation bath to remove the compression and wrinkles produced by the sectioning. The ribbons were mounted on glass slides, dewaxed to allow for rehydration, cleared with xylene, rehydrated with a descending series of ethanol (100, 95 and 80% ethanol), and stained with Hematoxylin and Eosin solution. Hematoxylin stains blue or dark-purple, the nuclei of cells together with calcified material. Eosin stains pink, the cytoplasm and extracellular matrix. The slides were cleaned in 2 changes of xylene, cover-slipped using Dibutylphthalate polystyrene xylene (DPX) mountant (Sigma) and let to dry. The tissue sections were then examined for changes in architecture and comparisons drawn among the experimental groups.

3.11 Evaluation of Fractions of *S. occidentalis* Methanolic Roots Extract for *in vitro* Antiplasmodial Activity against *P. falciparum*

3.11.1 Preparation of the Methanolic Roots Extract Fractions (Column Chromatography and Thin layer Chromatography)

Senna occidentalis methanolic root extract was fractionated by column chromatography as described (Bajpai *et al*., 2016) with minor modifications and used in this assay. A cylinder shaped glass column was assembled (Plate 3.2) and some chloroform passed through to clean it. The column was packed with silica gel (60-120 mesh size). For column packing, the gel was mixed with chloroform to form a sludge that was poured gently into the column. The gel was then let to settle. The extract was prepared for fractionation by mixing it with silica gel. The sample mixture was then loaded on top of the column using a funnel taking care to ensure that the column is not disturbed. The sample was then layered using a piece of cotton wool. Solvents of different gradient systems (Table 3.1) were passed through the column at a uniform flow rate to facilitate fractionation by gravity. Fractions (approximately 200 ml, each) were collected separately into conical flasks.

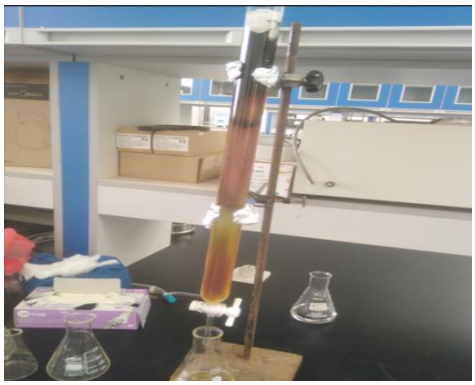


Plate 3.2: The Chromatography Column Displaying the Separation

Table 3.1: Gradient Solvent System Used in the Fractionation of *S. occidentalis* Methanolic Root Extract

Solvent system	Organic solvents and ratio (v/v)
S1	100% chloroform
S2	90% : 10%; chloroform : methanol
S3	70%: 30%; chloroform : methanol
S4	50% : 50% ; chloroform : methanol
S5	30% : 70% ; chloroform : methanol
S6	100% methanol

3.11.2 Analysis of the Fractions by Thin layer Chromatography (TLC)

The collected fractions were analysed by Thin layer chromatography (TLC) as described (Gwatidzo *et al*., 2018) with a few modifications. Pencil lines were drawn 1.5cm from the edge of the TLC plate (TLC Silica gel 60 F_{254}). The eluted fraction samples were spotted along the pencil line (0.5cm apart) using glass capillary tubes and the plates let to air dry. To select an appropriate solvent system for the separation of the spots, some spotted plates (trials) were placed into a development chamber containing the different solvent systems. Solvent front was allowed to move up to about 1 cm from the top edge and the plates visualized for appearance of spots.

Chloroform: methanol (3:7, v/v) exhibited the best separation of the spots. This solvent system was used to develop the rest of the plates. The plates were removed from the chamber and the solvent front marked using a pencil. They were air dried and visualized under uv at 254 nm for normal spots) and 366 nm for fluorescence (DESAGA-SARSTEDT-GRUPPE-MinUVIS). The chromatograms were marked and retention factors (R_f) computed as: R_f = Distance (cm) of the sport from the origin ÷ Distance (cm) of the solvent front from the origin. Fractions with similar chromatograms and retention factors were pooled (Plate 3.3), concentrated by rotary vaporization, dried and stored at 4^{o}C. until use.

Plate 3.3: Some of the Pooled Extract Fractions Following TLC Analysis

3.11.3 The *in vitro* Antiplasmodial Assay of the *S. occidentalis* Fractions

3.11.3.1 Propagation of the Malaria Parasites, *P. falciparum*

The extract fractions were evaluated for growth inhibitory activity against *P. falciparum*, strain 3D7, using the PfLDH assay. The parasites used here were propagated and synchronized as described in sections 3.7.5 and 3.7.6 respectively.

3.11.3.2 Extract Dilutions and the Antiplasmodial Assay

Parasite growth *in vitro* in presence of the extract and control drugs was measured using the *Plasmodium* lactate dehydrogenase (PLDH) assay as described (Noor Rain *et al*., 2007, Verma *et al*., 2011). The assay was performed in quadruplets. To prepare a stock solution of the extract (100 mg/ml), 50 mg of the extract was dissolved in 500 µl DMSO. The extract stock solution was then diluted further in DMSO providing 10, 3, 1, 0.3, 0.1., 0.03, 0.01, 0.003, 0.001, 0.0003 mg/ml as the working concentrations. The positive control was composed of artemisinin (250 µM, as stock solution). The control drug was diluted in double distilled water to provide 100, 30, 10, 3,1, 0.30.1., 0.03, 0.01 and 0.003 µM as the working concentrations. Four microliter (0.4 µl) of the extract working solutions was transferred into a 96-well cell culture plate. The standard drug and DMSO (0.4 µl, each) were included as controls. Then 100 µl of sorbitol synchronized ring stage parasite culture suspension (0.3% parasitemia and 2% hematocrit) was transferred to each well containing

the extract and standard drug. This provided 40 - 0.0012 µg/ml and, 0.4 – 0.000012 nanomolar (nM) as the final extract and standard drug concentration ranges, respectively. The culture plate was covered and gassed (90% N_2, 5% O_2 and 5% CO_2) for 10 seconds in a gas chamber. The plate was then incubated at 37^{o}C for 72 hours.

After incubation, cold phosphate buffered saline (200 µl) was added onto each well. The plate was then centrifuged at 800g for 5 minutes. Two hundred and sixty microliter (260 µl) of the supernatant was carefully aliquoted from each well without disturbing the pellet. The plate was then kept at 30^{o}C for 24 hours to facilitate erythrocytes lysis. After 24 hours, the plate was obtained from the freezer and let to thaw for 1 hour. The contents of the plate were mixed (650 rpm) for 1 minute in an orbital shaker. Ninety microliter (90 µl) of PfLDH assay mixture constituting PfLDH buffer, nitro-blue tetrazolium (NBT), 3-acetylpyridine dinucleotide (APAD) and diaphorase was added per well and the plate covered with aluminium foil to prevent degradation of the assay mixture. The contents of the plate were mixed (650 rpm) for 20 minutes in an orbital shaker. The plate was then read for optical densities using a micro-plate reader set at 650 nanometres and dose response curves (Version 7.00, California, USA) used to demonstrate the effect of the extract on the parasites growth.

3.12 Statistical Analysis

The data were recorded as means ± standard error of the means (M±SEM). For determination of half-maximal inhibitory concentration (IC_{50}) of the extracts, the dose values were log transformed and their respective parasite % suppression values normalized to % scale. GaphPad prism software (Version 7.00, California, USA) was then used to determine the IC_{50} values for the extracts and control drug by fitting analysis of a non-linear dose response curve. The obtained IC_{50} values were used to categorize the antiplasmodial activity of the extracts as good (IC_{50} < 10 µg/ml), moderate (IC_{50} > 10 to < 25 µg/ml) and weak (IC_{50} > 25µg/ml) as described by (Bagavan *et al*., 2011).

Data on parasite suppression *in vivo*, hematology and clinical chemistry were analyzed through Ordinary One-Way ANOVA followed by Tukey's multiple comparisons test, and the student's t-test. Comparisons of survival time of mice in the different treatment groups was achieved through log rank analysis. Data on changes in weight between day 0 and day 8 post-infection were analyzed using student's t-test. GraphPad prism (Version 7.00, California, USA) was used for the analysis. The differences in means were considered significant when, $p < 0.05$.

CHAPTER FOUR

RESULTS

4.1 Characteristics of *S. occidentalis* Roots Extract and Yields Based on the Nature of the Extraction Solvent

To obtain polar and nonpolar root extracts of *S. occidentalis* for evaluation of antimalarial potential and safety, the plant roots powder was extracted with solvents of differing polarity. Table 4.1 shows the characteristics and yields of the extracts obtained. All organic extracts were dark brown, and in form of paste. The aqueous extracts were brown and powdery. Aqueous and hexane extraction provided maximum and minimum yields respectively. The percentage yield for aqueous extraction was 3-fold that of hexane. The results suggest that *S. occidentalis* roots contain mainly polar compounds.

Table 4.1: Characteristics of *S. occidentalis* Roots Extract and the Percentage Yields

Extraction method	**Weight of plant material(g)**	**Colour of extract**	**Texture of extract**	**Weight of extract(g)**	Percentage yield
Aqueous (decoction)	250	Brown	Powder	17.7	7.08
Aqueous (maceration)	250	Brown	Powder	15.98	6.39
Methanol (maceration)	250	Dark brown	Paste	14.24	5.7
Ethyl acetate (maceration)	250	Dark brown	Paste	8.73	3.49
Chloroform (maceration)	250	Dark brown	Paste	6.85	2.74
Hexane (maceration)	250	Dark brown	Paste	5.33	2.13

4.2 Qualitative Pytochemical Composition of *S. occidentalis* Roots Extract

To predict antimalarial properties of *S. occidentalis* roots, the methanol, ethyl acetate, chloroform, hexane and aqueous extracts of the plant were screened for presence of secondary metabolites. All extracts contained secondary metabolites (Table 4.2). The results suggest that the extracts were potentially bioactive and were therefore assayed for biological activity against malaria parasites.

Table 4.2: Phytochemical Content of *S. occidentalis* Roots Extracts

Type of extract	Saponins	Tannins	Alkaloids	Flavonoids	Sterols
Methanolic	+	+	+	+	+
Aqueous	+	+	+	+	+
Aqueous decoction	+	+	+	+	+
Ethyl acetate	+	+	+	+	+
Chloroform	+	+	+	+	+
Hexane	+	+	+	+	+

(+) Presence of secondary metabolite

4.3 *In vitro* Antiplasmodial Activity of *S. occidentalis* Roots Extract against *P. falciparum*

Senna occidentalis roots were screened *in vitro* for growth inhibitory activity against *P. falciparum,* strain 3D7. To determine the antiplasmodial activity of both polar and nonpolar metabolites of the plant extract, polar and non-polar extracts were investigated for *P. falciparum* parasitemia suppression, *in vitro*. Table 4.3 shows the growth inhibitory activity of *S. occidentalis* root extracts against the human malaria parasites, *in vitro*. The activity of the extracts ranged from good to moderate. Methanol, aqueous, ethyl acetate extracts, and their combinations exhibited good activity against *P. falciparum*. Chloroform, hexane extracts and their combinations showed moderate activity against the parasites. Methanoic extract exhibited minimum IC_{50} value whereas hexane extract exhibited maximum IC_{50} value implying that methanolic extract was more active against the parasites compared to hexane extracts. When the antiplasmodial activity of the other extracts was compared to that of hexane, the efficacy was 10, 8, 4, 2.7 and 1.5-fold higher for methanol, aqueous macerate, ethyl acetate, aqueous decoction and chloroform respectively. This suggests that polar extracts of *S. occidentalis* roots are more active against *P. falciparum, in vitro,* compared to non-polar ones. Furthermore, these findings

demonstrate that both polar and non-polar metabolites of *S. occidentalis* roots are potent against *P. falciparum in vitro*.

Table 4.3: Growth Inhibitory Activity of *S. occidentalis* Root Extracts against *P. falciparum, In vitro*

Type of extract and controls	Range in percentage parasitemia (Mean±SEM)	Range in growth suppression (%)	IC_{50} (µg/ml)
Methanol	0.46±0.03 to 1.23±0.02	82.44 to 53.05	1.756
Aqueous macerate	0.69±0.02 to 1.38±0.11	73.66 to 47.33	2.283
Methanol + Ethyl acetate	0.73±0.00 to 1.38±0.04	72.14 to 47.33	4.209
Ethyl acetate	0.75±0.07 to 1.39±0.06	71.37 to 46.95	4.728
Aqueous macerate+ ethyl acetate)	0.70±0.09 to 1.42±0.06	73.28 to 45.80	5.831
Aqueous decoction	0.74±0.04 to 1.53±0.01	71.75 to 41.60	6.831
Chloroform	0.74±0.06 to 1.49±0.00	71.76 to 43.13	12.130
Methanol+ Hexane	0.76±0.02 to 1.81±0.11	70.99 to 30.91	14.560
Aqueous macerate + hexane	0.78±0.00 to 2.05±0.00	70.22 to 21.75	17.650
Hexane	0.89±0.05 to 1.73±0.04	66.03 to 33.97	18.470
Pyrimethamine	0.18±0.00 to 0.82±0.06	93.03 to 68.70	0.003
Infected RBCs only	2.62±0.02	-	-
Infected RBCs and DMSO	2.53±0.01	-	-

RBCs: Red blood cells; DMSO: Dimethyl sulfoxide; (%): percentage

4.4 *In vitro* Cytotoxic Activity of *S. occidentalis* Roots Extract against Vero cells

Based on their high antiplasmodial activity, *in vitro*, methanolic and aqueous extracts were selected for further evaluation of antimalarial activity in a rodent malaria model. Before evaluation for antimalarial activity, *in vivo*, the two extracts were assessed *in vitro* for possible cytotoxicity using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Substances that exhibit a selectivity index ≥ 10 are said to be highly selective. Both the methanolic and aqueous extracts exhibited selectivity indices greater than 10 (Table 4.4), an indication of high selectivity for the malaria parasites. This

observation implies that *S. occidentalis* root extract is potentially non-toxic to animal cells at the tested concentrations.

Table 4.4: Selectivity Indices of the Methanolic and Aqueous Extracts of *S. occidentalis* Roots Extract

Type of extract	IC_{50} (µg/ml) on *P. falciparum*	CC_{50} (µg/ml) on Vero cells	SI
Methanolic	1.756	247	140
Aqueous (Macerate)	2.283	1786	781

IC_{50} (50% inhibitory concentrations); CC_{50} (50% cytotoxicity concentration); SI (selectivity index)

4.5 Antimalarial Activity of *S. occidentalis* Roots Extract Against *P. berghei* in Mice

The *Plasmodium berghei*-BALB/c mouse model was utilized to assess the antiplasmodial activity of *S. occidentalis* polar extracts *in situ* in an animal system. Only the methanolic and aqueous extracts were evaluated following their high antiplasmodial activity, *in vitro*.

4.5.1 Effect of the Extracts on *P. berghei* Parasitemia in Mice

The effect of *S. occidentalis* extract on *P. berghei* parasitemia was determined by monitoring parasite multiplication in infected mice upon treatment with the extract. There was a significant difference in parasite suppression on day 8 ($F_{4, 20} = 83.64$, $p < 0.0001$), day 10 ($F_{4, 20} = 58.41$, $p < 0.0001$) and day 12 ($F_{4, 20} = 30.60$, $p < 0.0001$). At a dose of 200mg/kg body weight, the extracts (both methanolic and aqueous) significantly suppressed *P. berghei* growth ($p < 0.05$) on days 8, 10 and 12, relative to the phosphate buffered saline (vehicle) group. The inhibitory activity of methanol extract (200mg/kg) and aqueous extract (200mg/kg) was similar ($p > 0.05$). However, on day 6 post-infection, the methanolic extract depicted higher activity ($p = 0.03$). The suppressive activity of 100mg/kg methanol extract was comparable to that of 200mg/kg aqueous extract ($P > 0.05$). Similarly, 100mg/kg methanolic extract suppressed parasitaemia in mice at equal

levels to 200mg/kg extract of the same solvent ($p > 0.05$). This suggests that 100mg/kg body weight methanolic extract as optimum for use in mice. Parasite multiplication increased steadily in the placebo group until day 8 post-infection when it began to lag. The pyrimethamine control group achieved 100% parasite clearance by day 9 post infection. Overall, these findings show that aqueous and methanolic extracts of *S. occidentalis* roots suppress *P. berghei* propagation, *in vivo* and that the aqueous extract is as effective as the methanolic extract at the tested concentrations. The parasite suppression levels for the different treatment groups are as shown in Table 4.5. Figure 4.1 shows parasitemic profiles of *P. berghei* in mice treated with the extract and the controls.

Table 4.4: Selectivity Indices of the Methanolic and Aqueous Extracts of *S. occidentalis* Roots Extract

Treatment	Parasitemia (Mean±SEM) and suppression (%)				Survival time in days
	Day 6	Day 8	Day 10	Day 12	(Mean±SEM)
A	9.41±2.82 (13.11*)	12.18±1.72 (73.63*)	28.03±2.13 (47.11*)	34.13±5.14 (41.74*)	19±1.14
B	9.12±1.68 (15.79*)	14.62±2.36 (68.35*)	26.87±3.46 (49.30*)	36.13±6.29 (38.32*)	18.8±0.73
C	9.89±0.65 (8.68*)	18.77 ±1.64 (59.36*)	29.22 ±1.52 (44.87*)	43.15 ±1.04 (26.34*)	17.4±1.75
D	6.35±1.45 (41.37*)	1.26±0.17 (97.27*)	0.00 ±0.00 (100*)	0.00 ±0.00 (100*)	30.0±0.00
E	10.83±0.93 (0*)	46.19±2.34 (0*)	53.0±3.73 (0*)	58.58±2.9 (0*)	13±1.67

A (Methanol extract: 200mg/kg body weight); B (Methanol extract: 100mg/kg body weight); C (Aqueous extract: 200mg/kg body weight); D (Pyrimethamine:1mg/kg body weight); E (Phosphate buffered saline); Suppression percentage (*)

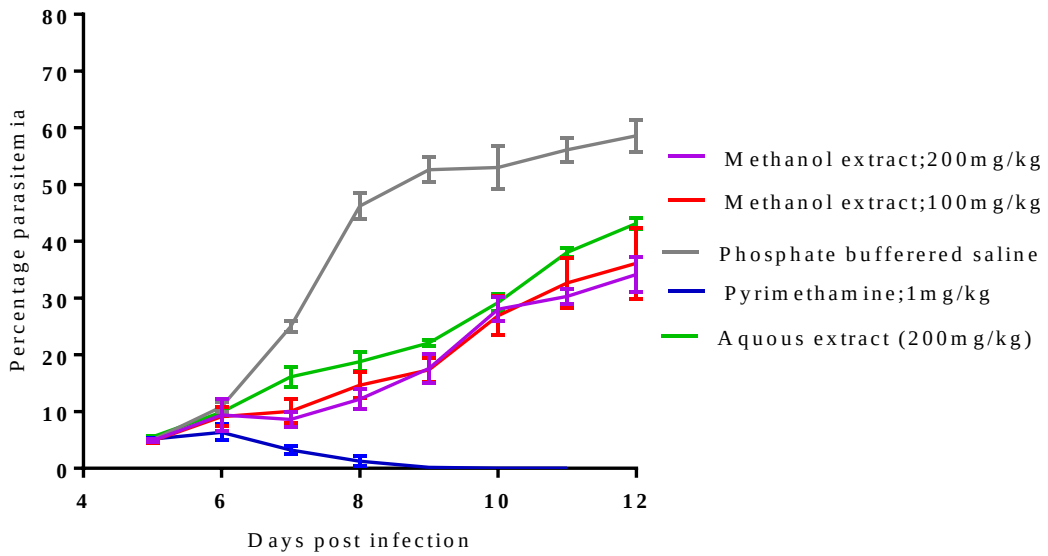


Figure 4.1: Parasitemic Profile of Pyrimethamine-Sensitive *P. berghei* 7 Days upon Extract Treatment, in Comparison with Controls

4.5.2 Effect of the Extract on Survival Time of *P. berghei* Infected Mice

The effect of *S. occidentalis* extract on the survival of *P. berghei* infected mice was assessed by monitoring survival time of the extract-treated animals for 30 days, post-infection. The extract prolonged the survival time of the infected mice in the range of 4 to 6 days, which is approximately 0.3- to 0.5-fold extension of survival time relative to the vehicle only-treated animals. Treatment with the standard drug, pyrimethamine, was associated with 100% survival of the infected mice. When compared to the vehicle only-treated group, the increase in survival time of the extract treated mice was significant ($F_{4, 20} = 25.63$, $P < 0.0001$). The number of days survived by the infected animals post infection is as shown in Table 4.5 (above). Figure 4.2 represents survivorship curves for the animals in the different treatment groups.

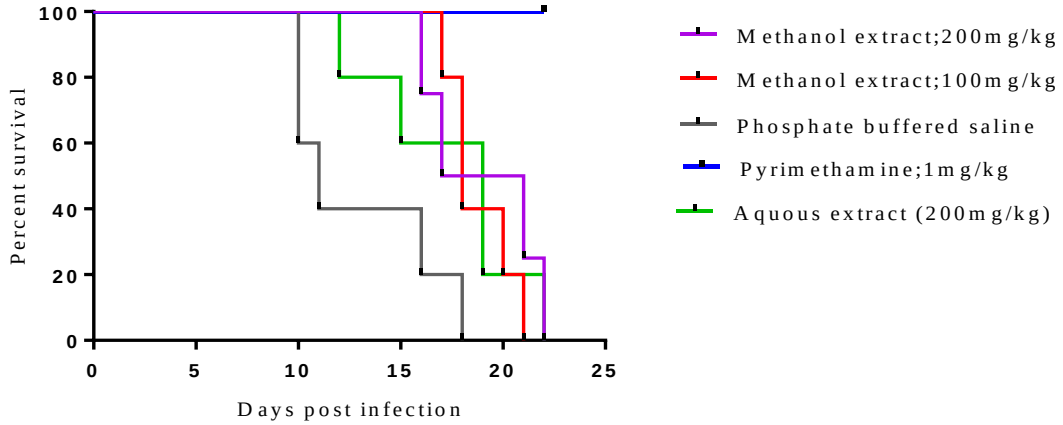


Figure 4.2: Survivorship Curves of *P. berghei* Infected upon Treatment with the Extracts

4.5.3 Effect of *S. occidentalis* Roots Methanolic Extract on Weight, Hematological, Biochemical and Histopathological Parameters of *P. berghei* Infected Mice

Given that methanolic extract depicted highest antiplasmodial activity *in vitro* and *in vivo*, it was evaluated further for safety and potential to alleviate the effects of *P. berghei* infection in mice.

4.5.3.1 Effect of *S. occidentalis* Methanolic Roots Extract on Weight Changes in Mice

To determine the effect of methanolic extract of *S. occidentalis* roots on weight changes in *P. berghei* infected and uninfected mice, animal weights were recorded and comparisons drawn between the day of infection (day 0) and day 8 post-infection (24 hours after administering the final dose of treatment), as illustrated in Table 4.6. Mice in group D (infected but not treated) and the extract treated mice (groups A and B) lost weight significantly: ($F_{4, 4} = 2.507$, $p = 0.007$), ($F_{4, 4} = 1.891$, $p = 0.007$) and ($F_{4, 4} = 2.890$, $p = 0.006$) respectively during the experimental period. On the other hand, pyrimethamine-treated animals gained weight during the experimental period, although this change was not significant when compared to the untreated animals ($F_{4, 4} = 2.605$, p

= 0.2259). The difference in weight loss between 100 and 200 mg/kg doses of the extract was not significant (p = 0.084). Likewise, there was no significant change in weight between extract and vehicle administered uninfected mice (p > 0.05). Although the extract treated mice lost weight significantly during the infection period, a comparison of weight loss between extract treated and untreated mice shows that the extracts minimized weight loss resulting from *P. berghei* infection by at least 3.67%. Further, results from the uninfected mice show that the animals tolerated the extract dose.

Table 4.6: Weight Changes in *P. berghei* Infected and Uninfected Balb/c Mice Administered with *S. occidentalis* Methanolic Root Extract

Experimental groups	Weight (g) day 0 (Infection day)	Weight (g) on day 4 post-infection	Weight (g) on day 8 post-infection)	Change in body weight(g)	Percentage change in weight
A	27.21±0.32	27.16±0.65	25.23±0.44(0.01^{p})	-1.98	7.28
B	28.4±0.51	28.21±0.34	26.20±0.30(0.01^{p})	-2.20	7.75
C	27.73±0.57	27.69±0.86	28.65±0.92(0.42^{p})	+0.92	3.32
D	27.75±0.48	27.63±0.37	24.50±0.76(0.01^{p})	-3.25	11.71
E	28.12±0.82	28.85±0.66	29.45±0.61(0.23^{p})	+1.33	4.72
F	27.84±0.72	28.53±0.71	29.13±0.83(0.27^{p})	+1.29	4.63

A: Infected and given methanolic extract (100mg/kg); B: Infected and given methanolic extract (200mg/kg); C: Infected and given pyrimethamine; D: Infected and given PBS; E: Not infected but given extract (200mg/kg); F: Not infected but given PBS; (^{p}): p-value; (-): Decrease; (+): Increase

4.5.3.2 Effect of *S. occidentalis* Methanolic roots Extract on Hematological Parameters in Mice

To evaluate the effect of *S. occidentalis* methanolic root extract on hematological indices in *P. berghei* infected and uninfected mice, the animals were administered with the extract and blood samples collected and analyzed for leucocyte, erythrocyte, and platelet indices.

4.5.3.2.1 Effect of the Extract on White Blood Cell Indices

There was a significance difference in the levels of total white blood cell count among the experimental groups ($F_{4, 24} = 50.42$, $p < 0.0001$). The composition of lymphocytes and granulocytes, however, was not significantly different among the groups ($F_{4, 24} = 2.24$, p

= 0.1172 and $F_{4, 24}$ = 1.89, p = 0.1691 respectively). The total white blood cells increased significantly in Balb/c mice infected with *P. berghei* ($p < 0.05$) compared with the uninfected animals. Infected mice treated with the extract and the standard drug pyrimethamine experienced a significant drop in their total white blood cell count ($p < 0.01$) compared with the infected animals that were administered with vehicle only. Neither the extract nor pyrimethamine treatment significantly altered the lymphocyte ($p = 0.514$) and granulocyte ($p = 0.627$) composition. Mice administered with 200 mg/kg extract had a significantly lower total leucocyte count compared to the 100 mg/kg extract administered group ($p = 0.006$). However, there was no significant difference in lymphocyte and granulocyte population between mice administered the two extract doses ($p > 0.05$). The administration of the extract to uninfected mice did not induce any remarkable changes in total leucocyte ($t_4 = 2.052$, $p = 0.110$), granulocytes ($t_4 = 0.4393$, $p = 0.683$) and lymphocytes ($t_4 = 0.777$, $p = 0.483$) counts. These findings affirm that treatment with the extract reduced the negative effect of *P. berghei* infection on the total leucocyte count. Absence of leukocytosis or leukocytopenia in the uninfected animals given the extract demonstrate that differences in parasitemia burden may be responsible for the observed changes in leucocyte indices in infected mice. Table 4.7 shows the effect of *Senna occidentalis* root extract on white blood cell indices of *P. berghei* infected and uninfected Balb/c mice compared with controls.

Table 4.7: White Blood Cell Indices in *P. berghei* Infected and Uninfected Balb/c Mice Administered with *S. occidentalis* Methanolic Root Extract

White blood cell parameters	Experimental groups					
	A	B	C	D	E	F
WBC (×10^9/l)	15.85±2.25(^{p*})	6.55±1.15(^{p*})	3.45±0.65(^{p*})	28.85±1.65	8.2±0.8	5.95±0.75
LYM (%)	88.9±0.1	87.05±1.55	92.9±0.5	81.1±5.6	84.1±0.6	86.5±3.05
GRAN (%)	4.75±0.15	7.5±1.8	3.05±0.85	8.9±2.1	7.35±0.65	6.3±2.3

(^{p*}): Statistically significant change; A: Infected and given methanolic extract (100mg/kg); B: Infected and given methanolic extract (200mg/kg); C: Infected and given pyrimethamine; D: Infected and given PBS; E: Not infected but given extract (200mg/kg); F: Not infected but given PBS

4.5.3.2.2 Effect of the Extract on Red Blood Cell Indices

There was a significance difference in the levels of hematocrit ($F_{4, 24}$ = 3.134, p = 0.487), hemoglobin ($F_{4, 24}$ = 10.200, p = 0.001) and RDW-SD ($F_{4, 24}$ = 3.982, p = 0.023) among the experimental groups. On the other hand, there was no significance difference in total red blood cell counts ($F_{4, 24}$ = 2.575, p = 0.083), MCH ($F_{4, 24}$ = 0.826, p = 0.555), MCHC ($F_{4, 24}$ = 0.560, = 0.729) and MCV ($F_{4, 24}$ = 1.460, p = 0.273) among the groups. The *Plasmodium berghei*-infected mice exhibited decreased levels of total red blood cell count, hemoglobin, percentage hematocrit, and mean corpuscular hemoglobin concentration, which was not significant (p > 0.05) compared with uninfected animals. Red blood cell distribution width (RDW-SD) was insignificantly increased (p > 0.05) in the infected animals. Infected mice treated with the extract had insignificantly (p > 0.05) elevated total RBC count and hematocrit percentage compared with the animals given the vehicle only. Mice treated with the extract (200mg/kg) and pyrimethamine exhibited a significantly higher hemoglobin concentration compared to the untreated infected mice (p < 0.001). Whilst the total red blood cell count and hematocrit level were both greater in the pyrimethamine treated group compared with the vehicle only administered animals, the observed difference was not significant (p = 0.441 and p = 0.250 respectively). In comparison to the infected group that received the vehicle only, the extract (200mg/kg) and pyrimethamine treated mice displayed a significantly low RBC distribution width (p < 0.01). With the exception of hemoglobin concentration (p = 0.045) and RDW-SD (p < 0.01), there was no significant difference between the effects of 100 and 200 mg/kg extract on red blood cell indices (p > 0.05). Uninfected mice that received the extracts did not display any noticeable change in the total RBCs count (t_4 = 0.1406, p = 0.895), hematocrit (t_4 =0.5522, p = 0.610), hemoglobin (t_4 = 0.333, p = 0.756), MCH (t_4 = 0.833, p = 0.452), MCHC (t_4 = 0.904, p = 0.417), MCV (t_4 = 0.992, p = 0.377) and RDW-SD (t_4 = 0.389, p = 0.717). These findings imply that using the extract as an antimalarial agent reduces the negative effect of *P. berghei* infection in Balb/c mice on some red blood cell indices. The findings further show that the animals tolerated the extract given that there was no stimulation or suppression of erythropoiesis in the uninfected animals. Table 4.8 shows

red blood cell indices in *P. berghei* infected and uninfected Balb/c mice administered with *S. occidentalis* methanolic root extract.

Table 4.8: Red blood Cell Indices in *P. berghei* Infected and Uninfected Balb/c Mice Administered with *S. occidentalis* Methanolic Root Extract

Red blood cell indices	Experimental groups					
	A	B	C	D	E	F
RBCs(10^12/l)	5.56±1.21	6.92±.11	7.15±0.34	5.48±0.67	7.59±0.28	7.55±0.05
HGB (g/dl)	10.45±0.65	12.65±0.25(^{p}*)	12.9±0.3(^{p}*)	9.3±0.9	13.3±0.3	13.4±0
HCT (%)	38.1±3	38.7±0.65	41.5±1.8	33.65±3.45	44.9±2.7	43.4±0.3
MCV (fl)	70.7±10	58.1±0.35	58.1±0.2	61.55±1.25	59.1±1.4	57.5±0.8
MCH (pg)	19.4±3	14.2±3.5	18.1±0.4	17.05±0.45	17.5±0.3	17.75±0.15
MCHC (g/dl)	27.45±0.35	24.45±8.13	31.1±0.5	27.65±0.15	29.7±1.2	30.8±0.2
RDW-SD (fl)	27.35±4.65	21±0(^{p}*)	20.75±0.65(^{p}*)	30.75±2.05	21.5±0.9	21.1±0.5

(^{p}*): Statistically significant change; A: Infected and given methanolic extract (100mg/kg); B: Infected and given methanolic extract (200mg/kg); C: Infected and given pyrimethamine; D: Infected and given PBS; E: Not infected but given extract (200mg/kg); F: Not infected but given PBS

4.5.3.2.3 Effect of the Extract on Platelet Indices

There was a significance difference in the total platelet count among the experimental groups ($F_{5, 24}$ = 4.354, p = 0.017). However, the MPV and PDW-SD was not significantly different ($F_{5, 24}$ = 1.493, p = 0.2632, and $F_{5, 24}$ = 1.450, p = 0.2761) among the groups. Infection with *P. berghei* caused a significant reduction in platelet count in Balb/c mice (p = 0.0499) but had no discernible effect on mean platelet volume (p = 0.726) or platelet distribution width (p = 0.932) compared with uninfected animals. The total platelet count, mean platelet volume and platelet distribution width did not change significantly after the extract and pyrimethamine treatment of the infected mice compared to the vehicle-only administered animals (p > 0.05). Similarly, the extract had no notable impact on total platelet count (t_4 = 0.712, p = 0.516) of uninfected mice. However, the extract administered mice exhibited a significantly elevated MPV (t_4 = 4.919, p = 0.008) and PDW-SD (t_4 = 5.000, p = 0.008). Table 4.9 shows the effect of the *S. occidentalis* extract on platelet indices. The results demonstrate that the extract did not have any remarkable impact on platelet indices of both infected and uninfected mice.

Table 4.9: Platelet Indices in *P. berghei* Infected and Uninfected Balb/c Mice Administered with *S. occidentalis* Methanolic Root Extract

Platelet indices	Experimental groups					
	A	**B**	**C**	**D**	**E**	**F**
PLT (×10^9/l)	338.5±9.5	335±17.5	375.5±17.5	341±22	419±33	451.5±31.5
MPV (ft)	6.55±0.05	7.7±0.5	7.25±1.15	7.8±0.3	6.75±0.05	6.2±0.1
PDW (%)	8.6±0.2	9.05±1.05	9.5±0.3	8.25±0.15	9.55±0.25	8.3±0

A: Infected and given methanolic extract (100mg/kg); B: Infected and given methanolic extract (200mg/kg); C: Infected and given pyrimethamine; D: Infected and given PBS; E: Not infected but given extract (200mg/kg); F: Not infected but given PBS

4.5.3.3 Effect of *S. occidentalis* Methanolic Roots Extract on Biochemical Indicators in *P. berghei* Infected and Uninfected Mice

Methanolic root extract of *S. occidentalis* was administered orally to both *P. berghei* - infected and uninfected mice to assess its impact on kidney and liver function parameters.

4.5.3.3.1 Effect of Roots Extract on Kidney Function Parameters

There was no significance difference in the levels of chloride ($F_{5, 24} = 0.995$, $p = 0.4415$), sodium ($F_{5, 24} = 3.068$, $p = 0.052$) and potassium ($F_{5, 24} = 0.644$, $p = 0.6713$) among the experimental groups. On the other hand, there was a significance difference in the levels of serum urea and creatinine ($F_{5, 24} = 12.490$, $p < 0.001$, and $F_{5, 24} = 33.51$, $p < 0.0001$) among the experimental groups. *Plasmodium berghei* infection in mice did not significantly alter the serum electrolytes ($p > 0.05$) in the animals compared with uninfected animals. However, the infection significantly increased serum urea and creatinine levels in the mice ($p < 0.001$). Treatment with extract and control drug, pyrimethamine, had no significant impact on electrolyte levels of the infected mice ($p > 0.05$) compared with the vehicle only administered animals. However, the infected mice treated with the extract, control drug exhibited significantly reduced urea, and creatinine levels ($p < 0.01$) compared with the vehicle only administered animals. There was no significant difference between the effect of 100 and 200 mg/kg extract on kidney function

parameters ($p > 0.05$) in the infected animals. Administration of extract to uninfected mice had no marked impact on chloride ($t_4 = 3.130$, $p = 0.035$), sodium ($t_4 = 0.894$, $p = 0.422$), potassium ($t_4 = 0.121$, $p = 0.910$), urea ($t_4 = 0.781$, $p = 0.479$) and creatinine ($t_4 = 2.002$, $p = 0.116$) compared with vehicle only administered animals. The effect of the extract on renal function parameters is illustrated in Table 4.10. These observations affirm that *S. occidentalis* methanolic root extract lessened the negative effect of *P. berghei* infection on urea and creatinine levels in Bab/c mice. In addition, the results show and that the extract is well tolerated in the mouse model at the doses investigated.

Table 4.10: Kidney function indices in *P. berghei*-infected and uninfected Balb/c mice administered *S. occidentalis* methanolic root extract

Kidney function parameters	Experimental groups					
	A	B	C	D	E	F
Sodium(mmol/L)	156.5±2.5	160.3±1.5	159±2	153.5±0.5	155±1	153±2
Potassium (mmol/L)	9.95±0.75	12.9±1.45	10.35±0.15	12.35±2.42	10.9±0.3	10.65±2.05
Chloride(mmol/L)	106±3	110±2	110±5	109.5±0.5	112±1	105±2
Urea(mmol/L)	2.9±0.2(^{p*})	3.45±0.15	2.9±0.1(^{p*})	3.9±0.2	2.35±0.25	2.1±0.2
Creatinine (Umol/L)	55.8±2.5(^{p*})	53.75±2.45(^{p*})	46.4±2(^{p*})	74.05±2.45	44.65±1.15	41.25±1.25

(^{p*}): Statistically significant change; A: Infected and given methanolic extract (100mg/kg); B: Infected and given methanolic extract (200mg/kg); C: Infected and given pyrimethamine; D: Infected and given PBS; E: Not infected but given extract (200mg/kg); F: Not infected but given PBS

4.5.3.3.2 Effect of the Extract on Liver Function Parameters

The levels of ALP ($F_{5, 24} = 57.78$, $p < 0.0001$), ALT ($F_{5, 24} = 17.84$, $p < 0.0001$), AST ($F_{5, 24} = 134.3$, $p < 0.0001$), total bilirubin ($F_{5, 24} = 216.2$, $p < 0.0001$), direct bilirubin ($F_{5, 24} = 55.75$, $p < 0.0001$) and total protein ($F_{5, 24} = 7.857$, $p = 0.002$) was significantly different among the experimental groups. The level of albumin was not significantly different among the groups ($F_{5, 24} = 0.531$, $p = 0.751$). The levels of total bilirubin, direct bilirubin, ALT and ALP were significantly elevated in the *P. berghei*-infected mice ($p < 0.05$) compared with the uninfected animals. The level of total protein was also raised significantly ($p < 0.05$) in the infected animals, compared to uninfected ones. Albumin and AST levels were similarly elevated in the infected mice but not significantly ($p = 0.099$, and $p = 0.064$ respectively). An increase in AST/ALT ratio correlates with

diminishing liver function (Scaccabarozzi *et al*., 2018). In this study, the AST/ALT ratios were 2.08, 2.05, 1.76, 2.16, 1.47 and 1.34 for animals in experimental groups A, B, C, D, E and F, respectively. Mice that were infected with the *P. berghei* parasites and treated with the extract or standard drug, pyrimethamine, had significantly lower levels of bilirubin and ALP compared to the infected mice that were given the vehicle only ($p < 0.05$). The extract had no marked impact on total protein and albumin levels in the infected animals ($p > 0.05$). The standard drug, pyrimethamine, significantly lowered the total protein levels ($p = 0.032$) in the infected animals. The infected mice that were given the extract and control drug exhibited insignificantly reduced levels of AST and ALT ($p > 0.05$). Similarly, the impact of 100 and 200 mg/kg extract on liver function parameters did not differ significantly ($p > 0.05$), except for the bilirubin level, which was significantly higher ($p < 0.05$) in infected animals that were administered the latter dose. The administration of the extract to uninfected mice had no distinguishable impact on albumin ($t_4 = 1.647$, $p = 0.179$), ALP ($t_4 = 0.515$, $p = 0.634$), AST ($t_4 = 0.000$, $p = 0.100$), ALT ($t_4 = 1.567$, $p = 0.192$), total bilirubin ($t_4 = 1.118$, $p = 0.326$), direct bilirubin ($t_4 = 0.469$, $p = 0.664$) and total protein ($t_4 = 0.751$, $p = 0.495$). The effect of the extract on hepatic function is displayed in Table 4.11. Low levels of ALP, bilirubin and AST/ALT ratio in the infected mice that were treated with the extract is indicative of reduced liver injury resulting from *Plasmodium* infection. Further, the results demonstrate that the animals tolerated the extract doses administered.

Table 4.11: Liver Function Indices in *P. berghei*-Infected and Uninfected Balb/c Mice Administered with *S. occidentalis* Methanolic Root Extract

Liver function parameters	Experimental groups					
	A	B	C	D	E	F
Total bilirubin(Umol/L)	5.7±0.1(P*)	6.55±0.25(P*)	5.4±0.3(P*)	10.75±0.25	2.4±0.1	2.15±0.15
Direct bilirubin(Umol/L)	2.4±0.2(P*)	3.1±0.1(P*)	2.15±0.15(P*)	5.15±0.25	1.25±0.25	1.1±0.2
Total proteins(G/L)	70.2±1.1	71.3±0.4	66.75±1.75	71.65±1.65	64.85±1.65	63.25±1.35
Albumin (G/L)	19.8±1.4	18.65±4.13	19.1±0.3	17.85±1.85	22.3±1.7	19.05±1.05
AST (U/L)	227±4	224.25±2.25	190.65±7.35	251.4±4.4	138.25±3.25	130.5±2.5
ALT (U/L)	109.85±1.15	109.4±1	108.3±2.7	116.75±2.95	94.35±0.85	97.9±2.1
ALP (U/L)	256.65±6.95(P*)	282.4±11.2(P*)	272.1±2.9(P*)	342±6.8	203.95±5.85	207.55±3.83

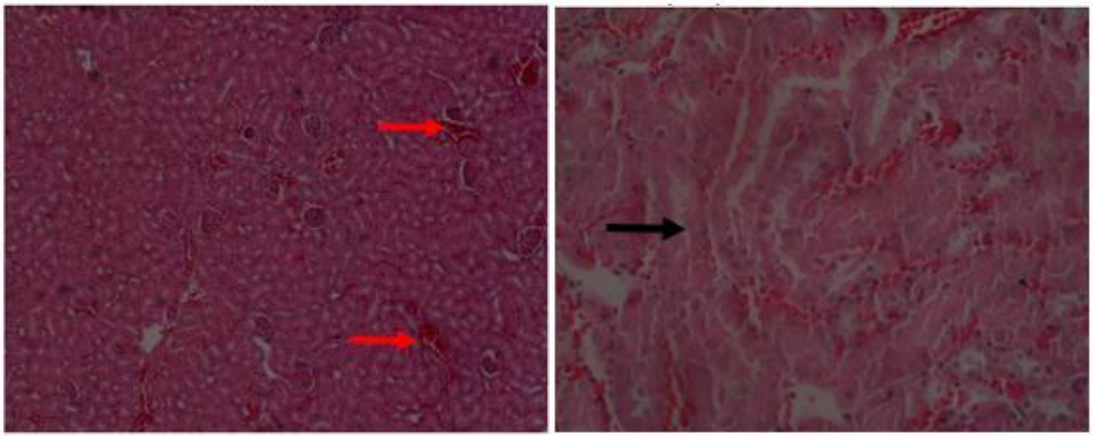
(P*): Statistically significant change; A: Infected and given methanolic extract (100mg/kg); B: Infected and given methanolic extract (200mg/kg); C: Infected and given pyrimethamine; D: Infected and given PBS; E: Not infected but given extract (200mg/kg); F: Not infected but given PBS

4.5.3.4 Effects of *S. occidentalis* Methanolic Roots Extract on Organ Pathological changes in Mice

Organs (kidney, liver, spleen, lungs and brain) harvested from *P. berghei* infected and uninfected mice were examined macroscopically for changes in color and size to determine the effect of the administered *S. occidentalis* methanolic root extract. The kidney, liver, spleen, lungs and brain of the mice treated with extract or pyrimethamine appeared normal. The organs were similarly normal for uninfected mice that received the extract. Infected animals that received the vehicle only had normal kidneys, dark and enlarged livers and spleens, dark lungs, and pale brains.

In the histological examination of the organs, animals in groups A, B, C, E, and F did not exhibit histopathological changes suggestive of organ damage. However, animals in group D, which were infected and received the vehicle only, displayed severe tissue pathology changes in the organs that were examined. Tubular and glomerular hemorrhages with tubular degeneration were observed in the kidneys of the animals (Plate 4.1a and, b). Hemorrhages, hepatocyte degeneration, loss of hepatic cord pattern, and general loss of

lobular architecture were evident in the liver (Plate 4.2a and, b). There was interstitial cellular infiltration, collapsed alveoli, emphysema, interstitial hemorrhages, and congested blood vessels in the lungs of these animals (Plate 4.3a and, b). Other observations included the proliferation of lymphocytes with hyperplasia of germinal centers in the spleen and congestion of the cerebral vasculature. These results suggest that the extract and the control drug, pyrimethamine, minimized pathology resulting from *P. berghei* infection in Balb/c mice. Additionally, these findings imply that there was no detectable organ damage caused by the extract, implying that the short-term use of the extract doses used here was safe and well tolerated by the animals.



Pate 4.1a (40x)

Plate 4.1b (100x)

Plate 4.1: Histological Tissues Showing (a) Hemorrhages in the Kidney (Red Arrows), and (b) Tubular Degeneration (Black Arrow) and Hemorrhages

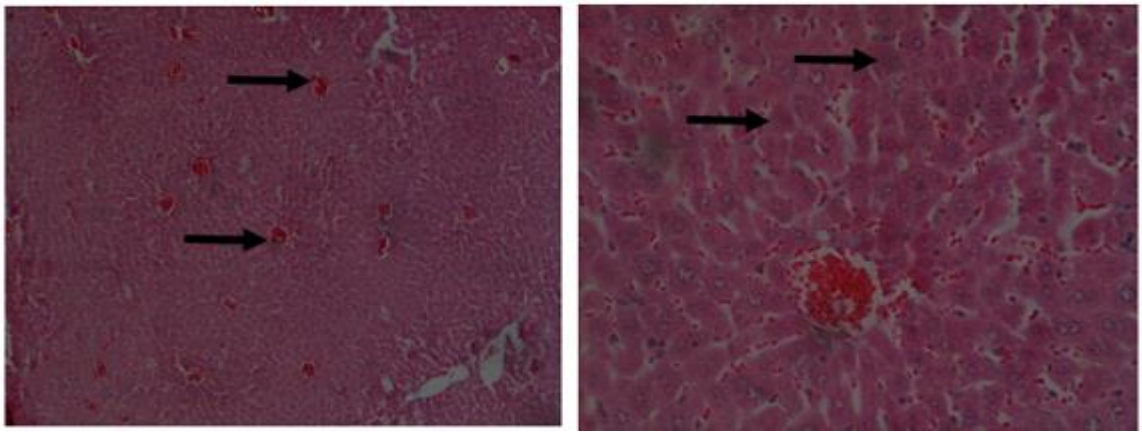


Plate 4.2 a (40x)

Plate 4.2b (100x)

Plate 4.2: Histological Tissues Showing (a) Congestion of Hepatic Blood Vessels, and (b) Hepatocyte Degeneration without a Visible Nucleus (Black Arrows) and Hemorrhages

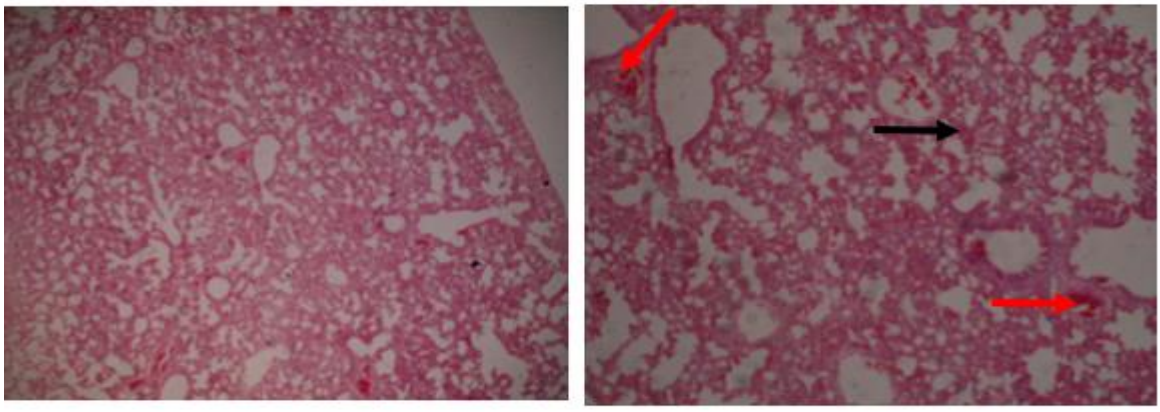


Plate 4.3a (40x)

Plate 4.3b (100x)

Plate 4.3: Histological Tissues Showing (a) Interstitial Cellular Infiltration, Collapsed alveoli, and emphysema, and (b) Interstitial Hemorrhages (Black Arrow) and Congested Blood Vessels (Red Arrows)

4.6 *In vitro* Antiplasmodial Activity of *S. occidentalis* Methanolic Roots Extract Fractions against *P. falciparum*

In modern drug development practice, pure isolated compounds are preferred relative to unrefined products. In this study, *S. occidentalis* methanolic root extract was fractionated through column chromatography and the fractions evaluated for antiplasmodial activity against *P. falciparum* using the PfLDH assay. A total of 12 fractions (fraction A - L) were obtained in the chromatographic separation (Table 4.12). TLC analysis of the fractions culminated to pooling of the 12 fractions into 4 main fractions (fractions 1, 2, 3 and 4) where fraction A and B, were pooled and renamed fraction 1 and fractions C - F, pooled and renamed fraction 2. Similarly, fractions G - J, were pooled and renamed fraction 3, and fractions K and L, pooled and renamed fraction 4. All the 4 main fractions exhibited antiplasmodial activity against *P. falciparum*, 3D7 (Figure 4.3). Fraction 3, a chloroform-methanolic fraction (ratio of 1:1 and 3:7) exhibited higher activity (1.5-fold) compared to the parent extract. These results suggest that a bioactive compound of the extract may be more potent than the parent crude extract.

Table 4.12: Thin Layer Chromatography Pattern of Fractions Obtained from Column Chromatography of *S. occidentalis* Methanolic Root Extract

Solvent systems	Fractions	Number of spots	Retention factor (R_f) values	Pooled fractions
S1	A and B	2	0.11, 0.08	Fraction 1
S2 and S3	C – F	4	0.78, 0.69, 0.45, 0.18	Fraction 2
S4 and S5	G-J	4	0.86, 0.60, 0.29, 0.24	Fraction 3
S6	K and L	3	0.85, 0.67, 0.11	Fraction 4

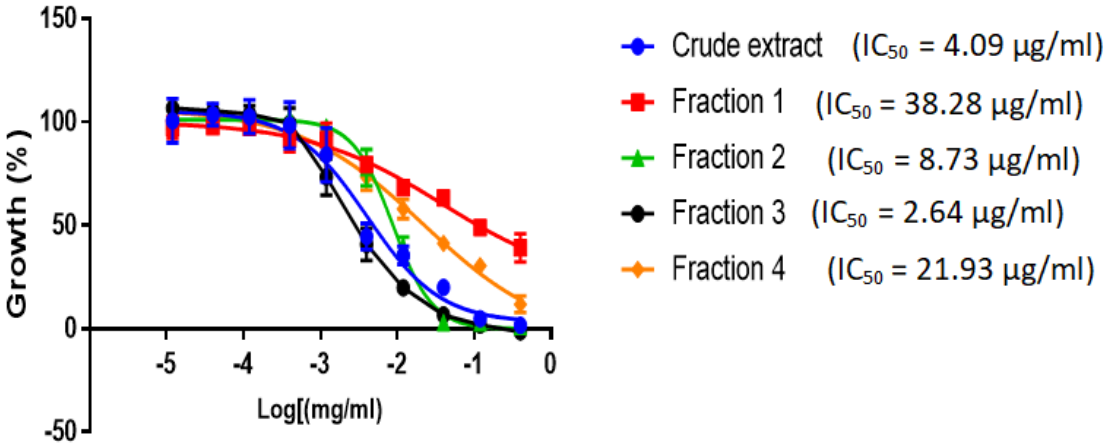


Figure 4.3: Growth Inhibitory Activity of Fractions of *S. occidentalis* Methanolic Root Extract against *P. falciparum*, 3D7

CHAPTER FIVE

DISCUSSION

This study shows that *S. occidentalis* roots extract inhibits the growth of *P. falciparum in vitro* and *P. berghei* in mice. It also shows that the plant root extract is well tolerated in mice and may therefore be a safe treatment against malaria. The choice of this plant was informed by documented ethnomedical use of the plant roots decoction as a cure for malaria in Kenya (Muthaura *et al*., 2015, Nguta *et al*., 2010). This ethnopharmacological approach is advantageous because it has been associated with high success rates of discovering active compounds from natural sources (Valdés *et al*., 2010). The approach is also cost effective and time saving compared to mass screening of plants for specific biological activity (Kigen *et al*., 2013, Pan *et al*., 2013).

5.1 *In vitro* Antiplasmodial Activity of *S. occidentalis* Roots Extract against *P. falciparum*

The various solvent extracts used in the current study suppressed *P. falciparum* parasitemia *in vitro*. The good antiplasmodial activity observed in the current study concur with previous findings regarding *in vitro* activity of leaf extracts of the same plant species (Tona *et al*., 2004). However, a different study showed a weak antiplasmodial activity for the plant leaf (IC_{50} = 49µg/ml), (Murugan *et al*., 2015), an observation that is contrary to the current results. The plant part used, place of origin, season of collection and method of preparation may account for the observed differences in activity (Garg *et al*., 2012, Ginsburg and Deharo, 2011, Oliveira *et al*., 2009, Sadiq *et al*., 2012). In a study conducted in Africa on the phytochemical composition of the extracts of *S. occidentalis*, the stems, roots, bark and leaves collected from Ivory Coast had no alkaloids while the same samples collected from Ethiopia had a lot of alkaloids (Manikandaselvi *et al*., 2016). When investigating medicinal plants as antimalarials, extracts that exhibit IC_{50} values below 15µg/ml are regarded as potential sources of new antimalarial agents (Kweyamba *et al*.,

2019). Therefore, the present results of the *in vitro* antiplasmodial assay suggest that *S. occidentalis* roots are a potential source of new antimalarial principles.

Solvents ranging from polar to non-polar were used for extraction in the current study with the observed *in vitro* antiplasmodial activity appearing to be dependent on the polarity of solvents used. Extracts of polar solvents depicted higher antiplasmodial activity than non-polar ones. These results suggest that polar solvents may be desirable for extraction of bioactive antimalarial principles from *S. occidentalis* roots. Other researchers have similarly reported on the high potency of polar solvents in extraction of antiplasmodial principles from leaves of the same plant species (Murugan *et al*., 2015, Tona *et al*., 2004), and other plant species (Berthi *et al*., 2018, Dhawan and Gupta, 2017, Kweyamba *et al*., 2019, Nyandwaro *et al*., 2020). It is reported elsewhere that extraction efficiency is affected by among other factors, the properties of solvents used (Zhang *et al*., 2018). This may account for the variations in antiplasmodial activity based on the solvent used for extraction as observed in the *in vitro* assay of the current study. Differences in extraction temperature may account for the variation in bioactivity between aqueous macerate and decoction extracts. High temperatures lead to loss of thermolabile compounds or even transformation of the phytochemicals (Zhang *et al*., 2018).

5.2 *In vitro* Cytotoxicity against Vero cells

In this study, *S. occidentalis* roots extract was found to be non-toxic to Vero cells at the concentrations tested suggesting that the extract is potentially safe in animals. These results corroborate an earlier report on the toxicity of the plant root bark (Tona *et al*., 2001). Leaf extracts of the plant have been reported to be similarly non-toxic to rodents when administered orally (Aragão *et al*., 2009, Silva *et al*., 2011, Tanimu and Wudil, 2012). On the other hand, there are reports that *S. occidentalis* seeds are toxic to animals (Barbosa-Ferreira *et al*., 2011, Gebrezgi *et al*., 2020, Gotardo *et al*., 2017, Teles *et al*., 2015). The toxicity of this plant species is influenced by among other factors, the plant part used, the dose administered, the method of preparation, the animal species involved and the length of exposure (Barbosa-Ferreira *et al*., 2005, Gotardo *et al*., 2017, Vashishtha

et al., 2009). This may explain the variation in toxicity of this plant species as reported in the different studies. However, studies on safe use of this important medicinal plant when used to treat different diseases are largely lacking.

5.3 Effect of *S. occidentalis* Roots Extract on *P. berghei* Parasitemia and Mice Survivorship

The *in vivo* results show that root extracts of *S. occidentalis* suppresses *P. berghei* multiplication in mice. This is in agreement with an earlier observation regarding the antimalarial activity of the same plant species (Tona *et al*., 2001) in an early stage malaria infection model (4-day suppressive test). In the vehicle treated group, parasite multiplication increased steadily but then lagged off starting day 9 post infection. This was indicative of reticulocyte depletion resulting in fewer cells available for *P. berghei* infection (Cromer *et al*., 2006, Thakre *et al*., 2018).

In assessment of survival rates, compounds that lead to survival rates greater than those of the negative control are classified as active (Anosa *et al*., 2014, Du Plessis *et al*., 2015). In the current study, the *S. occidentalis* root extract was active, and remarkably prolonged the survival time of mice infected with *P. berghei*. The extended survival time of mice treated with the extract in the current study corresponds with enhanced parasite suppression in the animals. The prolonged survival of the extract treated mice may be attributed to the effect of the extract in suppressing parasite multiplication and subsequent alleviation of pathologic effects, as observed in the current study (Kotepui *et al*., 2015). Indeed, there exists a positive correlation between parasite density, disease severity and mortality due to malaria (Kotepui *et al*., 2015, Phillips *et al*., 2009). The *in vivo* results corroborated the *in vitro* findings thus confirming the antiplasmodial activity of this plant extract against malaria parasites. This gives further credence to the traditional use of *S. occidentalis* root in management of malaria.

The suppressive activities of the extract against malarial parasites as observed in the present study may be explained by the presence of secondary metabolites as evident in the

extracts investigated herein. Roots extract of *S. occidentalis* evaluated in the present study contained tannins, sterols, saponins, alkaloids and flavonoids. Other researchers have similarly reported the presence of these secondary metabolites in *S. occidentalis* (Issa *et al*., 2020, Nde *et al*., 2022, Ogunkunle and Ladejobi, 2006). Some of these phytochemicals have been implicated in antimalarial activities of plant extracts (Enechi *et al*., 2019, Okokon *et al*., 2017, Uzor, 2020, Zahari *et al*., 2016). For instance, flavonoids have been shown to have antioxidant potential and kill *Plasmodium* parasites by chelating nucleic acids of malaria parasites (Al-Adhroey *et al*., 2010, Bonkian *et al*., 2018). It is also reported elsewhere that antioxidants can inhibit haem polymerization. Unpolymerized haem is very toxic to *Plasmodium* parasites and as such leads to malaria parasites killing (Monti *et al*., 1999). Antiplasmodial activity of *S. occidentalis* leaves extract has similarly been associated with the presence of some of these phytochemicals (Tona *et al*., 2004).

5.4 Effect the Extract on Changes in Body Weight of *P. berghei* Infected Mice

Reduced water and feed intake, coupled with loss of body weight are indicators of poor health status in animals (Ugwah-Oguejiofor *et al*., 2019). One of the hallmarks of malaria infection is loss of body weight (Reece and Prior, 2018). An ideal antimalarial drug should be able to prevent severe weight loss during infection (Chaniad *et al*., 2019). In this study, mice infected with *P. berghei* experienced a significant weight loss, as expected. Documented evidence suggest that weight loss during malaria infection may be attributed to a reduction in feed intake and metabolism accompanied with hypoglycemia (Baah *et al*., 2020, Reece and Prior, 2018). Treatment of *P. berghei*-infected mice with *S. occidentalis* methanolic root extract minimized weight loss in the current study, though insignificantly. Other studies have reported restricted weight loss in murine malaria infected mice following treatment with plant extracts with antimalarial properties (Bihonegn *et al*., 2019, Chaniad *et al*., 2019, Girma *et al*., 2015, Mohammed *et al*., 2014). In uninfected mice, the current study demonstrated that administration with *S. occidentalis* root extract does not affect weight gain. This observation concurs with the findings of a previous study where leaf and stem extracts of *Cassia occidentalis* (Synonym: *S. occidentalis*) were found not to induce weight changes in Wistar rats (Silva *et al*., 2011).

5.5 Effect of *S. occidentalis* Roots Extract on Hematological Parameters of *P. berghei* Infected Mice

Hematological indices play a well-established role in predicting normal biological processes and responses to pharmacological substances during therapy or adverse effects of foreign compounds (Kotepui *et al*., 2015, Silva-Santana *et al*., 2020). Infection with malaria parasites alters the host's hematological parameters (Kotepui *et al*., 2015). On the other hand, parameters such as white blood cells count, platelets count, red blood cells count and hemoglobin concentration are important indicators of antimalarial drug efficacy (Enechi *et al*., 2019). In the current study, *P. berghei* infection in mice deviated various hematological indices from normal status. Using *S. occidentalis* methanolic root extract to treat the infected mice ameliorated some of these parameters, demonstrating its effectiveness in reducing malaria pathogenesis.

Plasmodium berghei infection was associated with an elevation of host white blood cell count in the current study. This observation is consistent with findings of previous studies of *P. falciparum* in humans (Maina *et al*., 2010, Rojanasthien *et al*., 1992). However, a low to normal leucocyte count is more common during *Plasmodium* infection (Kotepui *et al*., 2015, McKenzie *et al*., 2005). Reduction in leucocyte count in the infected animals result from the sequestration in the spleen, which in turn limits their peripheral circulation (Erhart *et al*., 2004, Kotepui *et al*., 2015). In the current study, *P. berghei* infected animals that were treated with the extract and standard drug, pyrimethamine, demonstrated a reduction in their white blood cell count. This reduction in leucocyte number was in tandem with the decreased parasite load. Occurrence of leucocytosis or leucopenia during *Plasmodium* infection depends on the parasite burden with leucocytosis positively correlating with a high parasite density (Kotepui *et al*., 2015). Leucocytosis or leucocytopenia may also result from the malfunctioning of myeloid and lymphoid stem cells. Factors that alter the production of colony stimulating factors and interleukins would affect production of leucocytes (Silva-Santana *et al*., 2020). In this study, *S. occidentalis* extract had no impact on the production of leukocytes in uninfected mice. On the contrary, it was previously reported that a stem bark extract from *S. occidentalis* promoted white

blood cell production in rabbits (Adedapo *et al*., 2009). The observed variation in leucocyte synthesis activity between the two studies may be explained by differences in plant part and dosage used (Garg *et al*., 2012, Vijayalakshmi *et al*., 2013).

Anemia resulting from reduced erythrocyte count is the most common complication of malaria (Enechi *et al*., 2019). As expected in this study, *P. berghei*-infected mice exhibited a reduced erythrocyte count. The reduction in red blood cell count could be attributed to the phagocytosis of erythrocytes as well as the inhibition of erythropoiesis by the accumulated malaria pigment, hemozoin (Baah *et al*., 2020, Berthi *et al*., 2018, White, 2018). The sequestration and rosetting of the infected cells in various organs may also contribute to this reduction (Kotepui *et al*., 2015). These events may lead to deviations in some erythrocyte indices of the infected host. During the erythrocytic stage, malaria parasites degrade host hemoglobin in order to obtain amino acids for their own use (Gavigan *et al*., 2001). This may account for the observed decrease in hemoglobin concentration during *Plasmodium* infection in the current study. The potential of an antimalarial drug candidate to restore normal hematological parameters following infection is highly sought after (Baah *et al*., 2020). The significantly high hemoglobin concentration and the reduced red blood cell distribution width in the extract treated group compared to the untreated group is desirable even though the plant extract used here did not cause a notable change in many of the red blood cell indices of infected mice. Animal tissues require oxygen for cellular respiration, which is transported to the tissues by hemoglobin. It also carries carbon dioxide away from the tissues (Enechi *et al*., 2019).

Plasmodium infection led to a significant reduction in platelet count in the current study. A reduction in platelet count during malaria infection has similarly been observed in other studies (Maina *et al*., 2010). Thrombocytopenia, as observed in the current study, may have resulted from the engulfment of *Plasmodium*-infected erythrocytes by platelets, thus marking them for destruction by phagocytes (Ezenyi and Salawu, 2016, Ifeanyichukwu and Esan, 2014, Kotepui *et al*., 2015). It may also have occurred due to disseminated intravascular coagulation (DIC) following the infection (Maina *et al*., 2010). In the present study, treatment with *S. occidentalis* methanolic root extract did not accrue any

remarkable beneficial or detrimental effects on platelet indices of both infected and uninfected animals. Similar to the results of the current study, *C. occidentalis* leaf and stem extracts failed to alter the hematological profiles of uninfected wistar rats, in a separate study (Silva *et al*., 2011).

5.6 Effect *S. occidentalis* Roots Extract on Kidney and Liver Function Parameters of *P. berghei* Infected Mice

An increase in protein in urine and elevated levels of urea, creatinine, and electrolyte imbalances are all indicators of a dysfunctional kidney (Ekasari *et al*., 2022, Silva-Santana *et al*., 2020). In this study, *P. berghei*-infected mice exhibited an increase in urea and creatinine levels. Other studies have similarly reported elevated renal function parameters in malaria infected individuals (Sharma *et al*., 2012). Similar to an observation made previously regarding infection of humans with *P. falciparum* (Das *et al*., 2019), *P. berghei* infection did not alter the level of serum electrolytes in the present study. Treatment of infected mice with methanolic root extract of *S. occidentalis* and pyrimethamine ameliorated some renal function parameters in the animals. The improvement in renal function parameters was in tandem with reduced parasite burden. Similar to the observations herein, high parasite density has been associated with diminished renal function during *Plasmodium* infection (Sharma *et al*., 2012).

Liver injury is characterized by an increase in the blood levels of aspartate aminotransferase (AST), alanine transaminase (ALT) and Alkaline phosphatase (ALP) (Couto *et al*., 2008, Mei *et al*., 2020, Nigatu *et al*., 2017, Silva-Santana *et al*., 2020). In the current study, total protein, bilirubin, ALT, and ALP were significantly elevated in *P. berghei*-infected mice, an indication of decreased liver function. This observation concurs with previous reports regarding *P. falciparum* infection in human (Das *et al*., 2019, Kotresh and Suresh, 2016). The AST/ALT ratio was at least 1.4-fold higher in the untreated *P. berghei*-infected mice compared with the uninfected animals. Elevation of the AST/ALT ratio in infected mice could be explained by hemolysis of RBCs due to the infection, since erythrocytes contain AST (Scaccabarozzi *et al*., 2018). Erythrocytes

hemolysis lead to the generation of hemozoin, which plays an important role in oxidative damage of the liver (Scaccabarozzi *et al*., 2018). In the present study, *S. occidentalis* methanolic root extract improved the outcome of *P. berghei* infection on bilirubin and ALP levels in mice, an observation that affirms its antimalarial potency. The extract did not significantly alter liver function parameters in uninfected mice. This observation concurs with previous finding involving leaf and stem extracts of the plant in which the extracts were found not to alter biochemical profiles in Wistar rats (Silva *et al*., 2011). Contrary to these results, a leaf extract from the plant species significantly altered the liver function parameters in uninfected Wister rats in a different study (Nuhu and Aliyu, 2008), an indication of toxicity.

There are emerging concerns regarding the safety of phytomedicicines (Koduru *et al*., 2006, Ugwah-Oguejiofor *et al*., 2019). In the present study, uninfected mice that received the methanolic extract from *S. occidentalis* roots had no appreciable unfavourable changes in their weight, hematological, biochemical, and histological parameters. Our findings concur with previous observations on the toxicity of *S. occidentalis* stem and leaf extracts (Aragão *et al*., 2009, Silva *et al*., 2011, Tanimu and Wudil, 2012). Contrary to the current findings, *S. occidentalis* extract was shown to significantly alter some the investigated biochemical and hematological parameters in previous studies (Barbosa-Ferreira *et al*., 2005, Essa'a and Medoua, 2013, Gotardo *et al*., 2017, Nuhu and Aliyu, 2008, Saidu *et al*., 2011, Vashishtha *et al*., 2009). The observed variation in these studies may be explained by differences in the plant's place of origin, the season of collection, the part used, the method of preparation, the dose administered and the length of exposure (Barbosa-Ferreira *et al*., 2005, Gotardo *et al*., 2017, Vashishtha *et al*., 2009). The current study shows that the plant root extract may be safe at the tested concentrations because there were no noticeable harmful effects in the uninfected mice that were given the extract. However, it will be crucial to investigate the safety of the extract in the context of long-term use as an antimalarial remedy.

5.7 Effect of *S. occidentalis* roots extract on organs of *P. berghei* infected mice

The liver, kidney, brain, lungs and spleen constitute the major organs affected during severe malaria infection (Chin *et al*., 2019, Rungruang *et al*., 2010). In the current study, hyperplasia was observed in the spleen. The spleen filters out parasitized erythrocytes and is usually, the first organ to be altered morphologically during *Plasmodium* infection (Buffet *et al*., 2011). Parasitized RBCs sequester/accumulate in the spleen microvasculature and hemozoin deposition and congestion occurs in pulp element, which distorts the structural and functional integrity of the organ (Chin *et al*., 2019).

There was congestion of the cerebral vasculature in the brain of the *P. berghei*-infected mice in the current study. It has been observed previously that parasitized RBCs sequester within the brain cerebral microvascular endothelium accompanied by accumulation of hemozoin leading to vascular obstruction and cerebral hypoxia (Chin *et al*., 2019). Lungs of the infected animals in the current study exhibited pulmonary edema and hemorrhage. Though not observed herein, this observation is usually characterized by hyaline membrane formation and sequestration of parasitized RBCs in the microvasculatures of the organs (Chin *et al*., 2019). This may block blood circulation affecting gaseous exchange at the alveolar membrane.

In the liver of *Plasmodium* infected animals, there occurs hydropic degeneration, fibrosis, congestion of central veins and liver sinusoids (Nigatu *et al*., 2017). There also occurs hyperplasia (Chin *et al*., 2019). Hyperplasia may explain the loss of hepatic cord pattern and general loss of lobular architecture as seen in the current study. Liver macrophages (Kupffer cells) engulf parasite infected RBCs lysing the blood cells. This leads to formation of hemozoin which is deposited in the liver parenchymal (Chin *et al*., 2019). There also occurs microvascular congestion and hepatocyte necrosis (Chin *et al*., 2019, Ekasari *et al*., 2022). Sequestration of parasitized RBCs is rare in the liver (Brugat *et al*., 2014). In the kidney cortex and medulla, there occurs hyaline casts formation, vacuolation, congestion and haemorrhage as evident in the current study, often leading to glomerulonephritis and acute tubular necrosis (Chin *et al*., 2019, Ekasari *et al*., 2022).

Malaria nephropathy is believed to be due to sequestration and cytoadherence of the parasitized RBCs as well as oxidative stress resulting from toxic heme, a product of the ruptured erythrocytes (Chin *et al*., 2019, Elias *et al*., 2012).

There was no observable change in macroscopic and microscopic appearance of organs from uninfected mice that were administered the extract. Similarly, stem and leaf extracts from the plant did not alter the appearance of the kidney, liver, spleen, lungs, brain, small intestines, adrenal gland, stomach and oesophagus among others, in a previous study (Silva *et al*., 2011). However, seeds from the plant have been shown to cause tissues alterations in some organs (Chileski *et al*., 2021, Mariano-Souza *et al*., 2010). The results on organ pathology affirm further the antimalarial potency and safety of a methanolic extract of *S. occidentalis* roots as observed in the antiplasmodial, hematological and biochemical investigations herein.

5.8 Antiplasmodial Activity of *S. occidentalis* Roots Extract Fractions against *P. falciparum, in vitro*

In modern drug development practice from natural products, isolated compounds are preferred relative to unrefined products (Ginsburg and Deharo, 2011). Isolation of pure compounds allows for structural analysis and modifications that may enhance the efficacy and safety of the identified bioactive compound (Fabricant and Farnsworth, 2001). Pure compounds are believed to be safer compared to their respective parent extracts given that the inactive compounds, which are assumed to be toxic, are removed during the isolation (Ginsburg and Deharo, 2011, Kunle *et al*., 2012). Moreover, the isolated compounds are considered to be of relatively higher efficacy than the parent extracts.

In the current study, partially purified fractions of *S. occidentalis* methanolic extract exhibited antiplasmodial activity against *P. falciprarum*, strain 3D7 with one of them (chloroform-methanol fraction) exhibiting a 1.5-fold increase in activity compared to the parent extract. However, in some studies, fractions and isolated compounds have been shown to be less efficacious when compared to their parent extracts (Ferreira *et al*., 2010,

Rasoanaivo *et al*., 2011, Wagner and Ulrich-Merzenich, 2009). Some multicomponent therapeutic products achieve their bioactivity through the effect of different biomolecules acting on different targets on the pathogen or even via synergistic or additive effects between or among its phytochemical constitutes (Ezenyi and Salawu, 2016, Kunle *et al*., 2012). Separation of the constituent phytochemicals may thus lead to loss of bioactivity (Ezenyi and Salawu, 2016, Kunle *et al*., 2012). Reduced efficacy of the fractions/compounds could be due to (i) loss of inert compounds which may serve to enhance stability of the bioactive compounds (ii) loss of pharmacodynamic synergy/additivity (iii) loss of multidrug resistant inhibitory compounds (iv) loss complementary killing mechanisms such as immunostimulation and (v) loss of compounds that induce modulation of adverse effects (Ginsburg and Deharo, 2011, Kunle *et al*., 2012, Rasoanaivo *et al*., 2011). The results of the current study suggest that a bioactive compound of the extract may be more potent than the crude extract. *Senna occidentalis* roots can thus be developed further into antimalarial therapy through bioactivity guided fractionation.

5.9 Conclusions and Recommendations

5.9.1 Conclusions

This study has demonstrated that;

i. Polar and non-polar extracts of *S. occidentalis* inhibit the propagation of *P. falciparum,* strain 3D7, *in vitro*, with polar extracts being more potent than non-polar ones
ii. *Senna occidentalis* (L.) Link root extract suppresses *P. berghei* parasitemia in mice thereby alleviating some of the pathological changes and extending the survival time of the infected animals
iii. *Senna occidentalis* (L.) Link root extract does not induce adverse effects on the hematological, biochemical and histological parameters of uninfected Balb/c mice, suggesting that the extract is well tolerated in mice when used in short term

iv. A partially purified fraction of *S. occidentalis* (L.) Link methanolic root extract is more potent against malaria parasites, *in vitro*, than the crude extract of the plant

5.9.2 Recommendations

Given the good antimalarial efficacy and safety profile exhibited by *S. occidentalis* (L.) Link roots extract in the current study, there is need for further development of the plant root into an antimalarial therapy. The plant root should be subjected to;

i. Antimalarial studies in a prophylactic model, and also, in a non-human primate malarial model for a more complete pre-clinical testing
ii. Chronic toxicity studies so as to establish the safety of the extract when used as an antimalarial therapy over an extended period of time (long-term use)
iii. Bioactivity guided isolation and identification of the antimalarial principles from the plant root so as to facilitate their further development into lead antimalarial drug candidates

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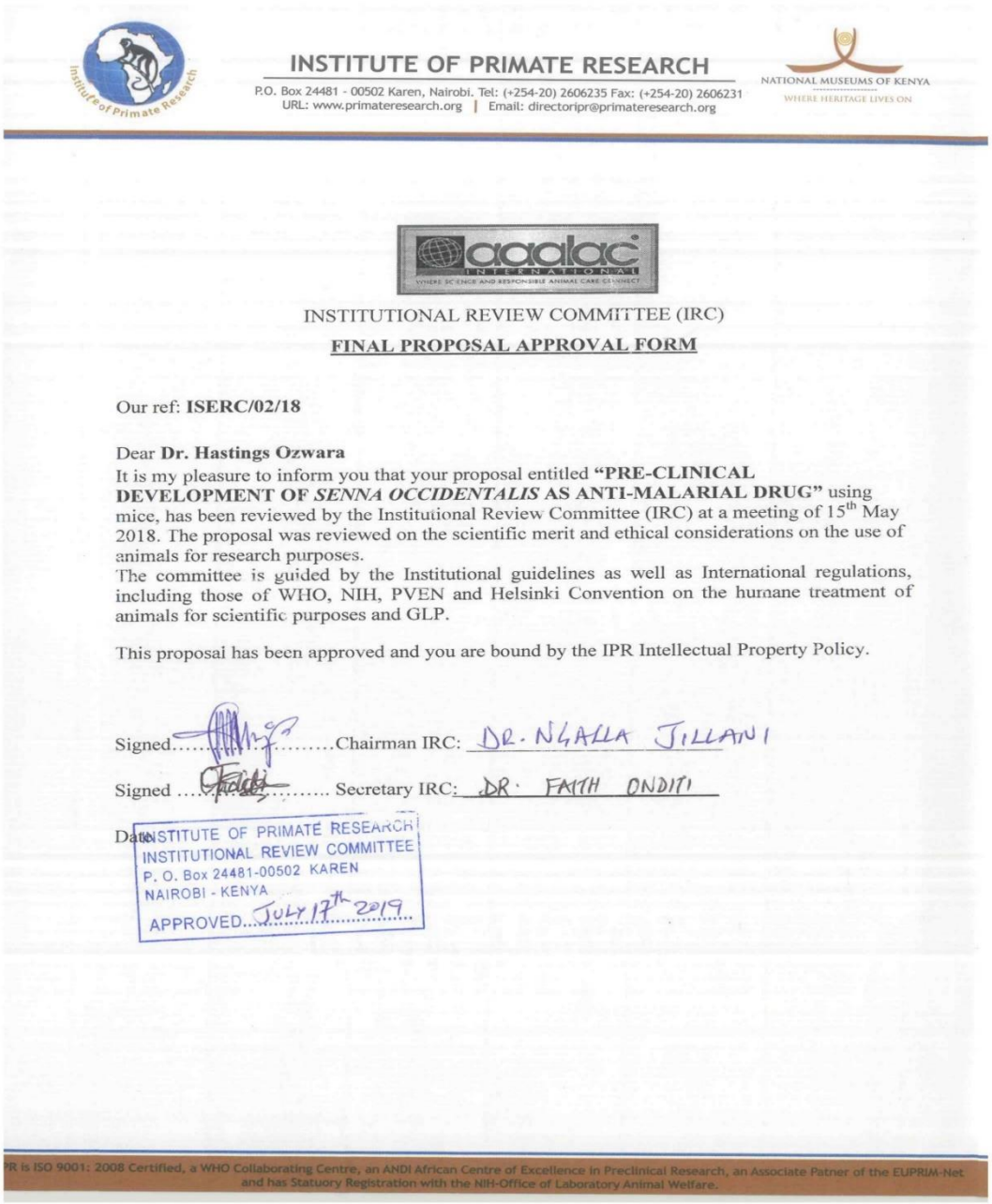
APPENDICES

Appendix I: Research Proposal Approval by the Institutional Scientific and Ethics Review Commettee



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NATIONAL MUSEUMS OF KENYA
WHERE HERITAGE LIVES ON

INSTITUTIONAL REVIEW COMMITTEE (IRC)

FINAL PROPOSAL APPROVAL FORM

Our ref: **ISERC/02/18**

Dear **Dr. Hastings Ozwara**

It is my pleasure to inform you that your proposal entitled **"PRE-CLINICAL DEVELOPMENT OF *SENNA OCCIDENTALIS* AS ANTI-MALARIAL DRUG"** using mice, has been reviewed by the Institutional Review Committee (IRC) at a meeting of 15th May 2018. The proposal was reviewed on the scientific merit and ethical considerations on the use of animals for research purposes.

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

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

Signed.....Chairman IRC: DR. NGALLA JILLANI

Signed .....Secretary IRC: DR. FAITH ONDITI

Date

INSTITUTE OF PRIMATE RESEARCH
INSTITUTIONAL REVIEW COMMITTEE
P. O. Box 24481-00502 KAREN
NAIROBI - KENYA
APPROVED... JULY 17th 2019

IPR is ISO 9001: 2008 Certified, a WHO Collaborating Centre, an ANDI African Centre of Excellence in Preclinical Research, an Associate Partner of the EUPRIM-Net and has Statuory Registration with the NIH-Office of Laboratory Animal Welfare.

Appendix II: Certificate for a Training on Animal Ethics in Research

Appendix III: Publications Emanating from the Thesis

1

Mogaka *et al.*
BMC Complementary Medicine and Therapies (2023) 23:71
https://doi.org/10.1186/s12906-023-03854-8

BMC Complementary Medicine and Therapies

RESEARCH Open Access

Senna occidentalis (L.) Link root extract inhibits *Plasmodium* growth in vitro and in mice

Simeon Mogaka[1,2*], Halkano Molu[1], Esther Kagasi[1], Kenneth Ogila[2], Rebeccah Waihenya[2], Faith Onditi[1,3] and Hastings Ozwara[1]

2

Hindawi
BioMed Research International
Volume 2023, Article ID 8296195, 12 pages
https://doi.org/10.1155/2023/8296195

Research Article

Antimalarial Efficacy and Safety of *Senna occidentalis* (L.) Link Root Extract in *Plasmodium berghei*-Infected BALB/c Mice

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