IN VITRO ANTI - PLASMODIAL ACTIVITY OF THE CRUDE EXTRACTS OF *PHYLLANTHUS SEPIALIS* AND *RUMEX STEUDELII*

TESSY FRANCISCA ORANGA,

B. Pharm (UON)

W64/7304/2017

A RESEARCH DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN TROPICAL AND INFECTIOUS DISEASES OF THE UNIVERSITY OF NAIROBI

INSTITUTE OF TROPICAL AND INFECTIOUS DISEASES

UNIVERSITY OF NAIROBI

AUGUST 2020

DECLARATION

This dissertation is my original work and has not been presented in any institution for degree or any other award.

Sign_____ _____ Date:___17th August, 2020_____

TESSY FRANCISCA ORANGA, BPharm

W64/7304/2017

DECLARATION BY SUPERVISORS

We confirm that this dissertation has been submitted for examination with our approval as university supervisors.

PROF. JULIUS O. OYUGI, PhD

INSTITUTE OF TROPICAL AND INFECTIOUS DISEASES, UNIVERSITY OF NAIROBI.

Sign_____ _____ Date ___22nd August, 2020_____

PROF. KENNEDY O. ABUGA, PhD

DEPARTMENT OF PHARMACEUTICAL CHEMISTRY, SCHOOL OF PHARMACY, UNIVERSITY OF NAIROBI.

Sign _____ Date __21st August, 2020_____

DR. LUCY OCHOLA, PhD

TROPICAL INFECTIOUS DISEASES, INSTITUTE OF PRIMATE RESEARCH.

Sign ___ L. Ochola _____ Date ___20th August, 2020_____

DEDICATION

I dedicate this dissertation to my daughter, Mackenzie Njeri Ngugi and my mother, Veronica Eden Oranga, my inspiration.

ACKNOWLEDGEMENTS

I extend my sincere gratitude to my supervisors; Professor Julius O. Oyugi who trusted my capabilities in pursuing this work and keenly guided me throughout the project life and my master's degree; Professor Kennedy O. Abuga who provided the necessary expertise and relevant resources in my plant extraction process and *in vitro* studies; and Dr. Lucy Ochola who worked very closely and tirelessly with me throughout my *in vitro* studies at the Institute of Primate Research. Their constant guidance and mentoring have refined my work and made the whole process worthwhile.

I wish to recognize Hitachi Limited, Kabushiki Kaisha Hitachi Seisakusho (Tokyo, Japan) for providing the industry- academia research grant to the University of Nairobi and Institute of Primate Research that supported discovery science in infectious diseases and hence the finances to undertake my research work. This funding enabled me to access all the resources required in conducting my field and laboratory work.

I am grateful to the School of Pharmacy, University of Nairobi, for the well able team led by Prof. Abuga; Mr. Joseph Nguyo, Mr. Obed King'ondu, Mr. Hannington Mugo and Mr. Raphael Ingwela who shared their expertise and ensured I had a conducive environment and the resources I needed to carry out my plant extraction and phytochemical tests.

I am indebted to the Institute of Primate Research (IPR) team who made me feel like family, led by the well able Dr. Lucy Ochola; Mr. Fred Nyundo, Martin Omondi, Collins Ngudi, Kevin Otieno and Florence Parsimei who worked tirelessly with me, burning the midnight oil to ensure my work was on schedule.

I thank Dr. Jeremiah Gathirwa of KEMRI for ensuring I accessed the required blood and serum for my work.

I am also thankful to the Wellcome Trust, Kilifi for promptly sending the *Plasmodium* parasites when we were in need.

I do appreciate the dedication and great work done by Mr. Patrick Mutiso, the botanist at University of Nairobi; identification of sites of harvest of the plants and spearheading the collection and drying of the same.

I am majorly indebted to my husband, Godfrey Gatacha and my daughter, Mackenzie Njeri, who have been all- round supportive of my work.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

APPENDICES

LIST OF ABBREVIATIONS AND ACRONYMS

℃	Degrees Celsius
µl	Microliter
ACT	Artemisinin-based Combination Therapy
BEI	The Biodefense and Emerging Infections
CDC	Centre for Disease Control and Prevention
COX	Cyclooxygenase
CQ	Chloroquine
DMSO	Dimethyl Sulphoxide
DNA	Deoxyribonucleic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorting
FBS	Fetal Bovine Serum
FCA	Flow Cytometry Assay
FCM	Flow Cytometry Method
FITC	Fluorescein isothiocyanate
FSC-A	Forward scatter- Area
FSC-W	Forward scatter- Width
HEPES	N -2 –hydroxyethylpiperazine –N -2 –ethane sulfonic acid
HTS	High Throughput Screening
IC_{50}	Half Maximal Inhibitory Coefficient
IPR	Institute of Primate Research
IPTp	Intermittent Preventive Treatment in pregnancy
IRBC	Infected erythrocytes
KEMRI	Kenya Medical Research Institute
LM	Light Microscope
mg	Milligram

ml	Milliliter
NIAID, NIH	National Institute of Allergy and Infectious Diseases
nm	Nanometer
NO	Nitric oxide
OD	Optical Density
pf	Plasmodium falciparum
PBS	Phosphate Buffered Saline
RANTES	Regulated on Activation, Normal T cell Expressed and Secreted
RBC	Red Blood Cells
RFU	Relative fluorescence units
RMPI	Roswell Park Memorial Institute
RPM	Rotations per minute
SAR	Structure- Activity Relationship
SSA	Sub -Saharan Africa
SSC- A	Side scatter- Area
SP	Sulphonamide-Pyrimethamine
Spp	Species
V/v	Volume per volume
WHO	World Health Organization

OPERATIONAL DEFINITION OF TERMS

Ethnopharmacology: The systematic study of traditional medicine products and evaluation of their biological activities (Abdolbaset Ghorbani, Farzaneh Naghibi, 2006).

Ethnobotany: Scientific study that relates people's culture, customs and knowledge with medicinal, religious and other uses of plants (Abdolbaset Ghorbani, Farzaneh Naghibi, 2006).

Phytomedicine: A medicinal product derived from plant material or a preparation with identified chemical substances derived from plants that contribute to its biological activity (Pandey, Debnath, Gupta, & Chikara, 2011).

ABSTRACT

Background: Malaria is a disease of high morbidity and mortality, especially in children under five and adults with low immunity. According to the World Health Organization (WHO) Malaria report 2018, there is an average of four million annual malaria incidences in Kenya, with a mortality rate of 5.1% among patients admitted with severe malaria. Drug therapy and vector control are the main modes of malaria control in the absence of an effective vaccine, however, with a growing concern of emerging multidrug- resistant malaria parasites, there is a need to explore for new and effective anti- malarial agents. In this regard, this study involves plant extraction and *in vitro* investigations of anti- plasmodial activity of two medicinal plants; *Phyllanthus sepialis* and *Rumex steudelii,* plants that have been traditionally used in the management of malaria in Kakamega County, Kenya (Mukungu et al., 2016).

Objective: The aim of this study was to establish the phytochemical classes of *Phyllanthus sepialis* secondary metabolites and assess the *in vitro* anti - plasmodial activities of *P. sepialis* and *Rumex steudelii*.

Methods: The two plants were chosen for study through literature review whereby both of them were reported to be used in treatment of malaria in Kakamega County, Kenya. The leaves of *P. sepialis* and the roots of *R. steudelii* were collected from Machakos and Nairobi Counties, respectively. They were then air dried, milled and extracted using distilled water, methanol, hexane, dichloromethane and ethyl acetate, separately. *P. sepialis* plant powder was subjected through various documented phytochemical tests to establish the presence of saponins, tannins, alkaloids, flavonoids, triterpenes, terpenoids, phenols and anthraquinones. The anti- plasmodial activities of all extracts were determined using *P. falciparum in vitro* assessment using light microscopy and flow cytometry. The light microscopy assay involved prepared Giemsa- stained microscope slides that were viewed under a light microscope. The infected erythrocytes were counted and documented against the total number of erythrocytes in the field. The flow cytometry assay involved staining the parasite DNA with fluorescent dye, SYBR Green I, and counting the number of viable parasites using a FACS caliber equipment. Parasitemia was then generated using the protocols of FlowJo software and IC_{50} values calculated using Microsoft Excel (2016).

Results: *Rumex steudelii* is known to contain tannins, phenols, saponins and terpenoids; while *Phyllanthus sepialis* was found to contain phenolic compounds, triterpenes, terpenoids and tannins present. In both tests *R. steudelii,* especially the non-polar secondary metabolites, were found to have activity against the *Plasmodium falciparum* parasite, as indicated by the IC_{50} values of hexane, dichloromethane and ethyl acetate extracts. The water extract also exhibited activity.

P. sepialis also showed activity against *p. falciparum,* with the dichloromethane, methanol, water extracts having moderate anti - plasmodial activity.

Conclusion: The Luhya community of Kakamega County in Kenya have traditionally used *P. sepialis* and *R. steudelii* to treat malaria. This study has confirmed that these plants do inhibit *P.*

falciparum growth *in vitro*, and specifically the active fractions can be found in the leaves and roots of *P. sepialis* and *R. steudelii*, respectively.

CHAPTER 1 : INTRODUCTION

1.1 Background

Malaria is a major health problem worldwide especially in sub-Saharan Africa. 70% of the population in Kenya is at risk of contracting malaria (PMI, 2019). Pregnant women, infants and children under five years of age are mostly affected. In addition, there is the aspect of antimalarial drug resistance which undermines the efforts of effective treatment and management of Malaria, thus increasing the incidence of severe malaria and mortality rates. Mbora and his team (2010) established that malaria was responsible for significant government and out- of- pocket expenditures, additionally, the time spent in hospitals for in- patient and out- patient services, in malaria- endemic countries.

Traditional herbal medicine is mostly used in malaria endemic regions where there is more access to traditional herbs than allopathic medicine. The World Health Organization (WHO) has established that the ratio of a traditional healer to people they serve, in Uganda is 1: 200 – 400 people, this being a sharp contrast to the ratio of a trained medical personnel and the number of patients they serve, i.e. one or less medical personnel serving 20,000 people (Muthaura et al., 2011). Furthermore, the use and misuse of Chloroquine for prevention and treatment of *P. falciparum* malaria in Kenya and other tropical countries led to widespread resistance to this anti-malarial agent (Wellems & Plowe, 2001). Fixed dose Artemisinin Combination Therapy (ACT) was recommended as first-line treatment by WHO for treatment of uncomplicated malaria (WHO, 2015), the currently used antimalarial therapy being effectively used by millions of children annually. It is affordable and accessible in public hospital facilities (Watsierah & Ouma, 2014).

ACT was introduced after the use of artemisinin monotherapy, or its derivatives, was prohibited by WHO to minimize the rate of development of resistance.

In the recent past, emerging resistance to artemisinin has been observed in South east Asia (WHO, 2018), and even as various initiatives work to monitor resistance in Africa, there is an urgent need to seek other more effective, affordable and accessible drugs against the malaria parasite. Medicinal plants have been a source of drugs that have been gold standards in the management of various ailments, inclusive of malaria, such as, quinine that was originally isolated from the bark of Cinchona spp and artemisinin from the plant *Artemisia annua*. The current first line treatment drug for malaria in Kenya is the fixed dose ACT that consists of artemisinin derivative, Artemether (MoH, 2006). The combination therapy has Lumefantrine, which is longer acting, as a partner. Lumefantrine is a synthetic analogue of quinine, which is a natural extract from Cinchona Spp (Wells, 2011). Kenya has a biodiversity of green tropical vegetation and natives have been using plants for traditional medicine for many years.

1.1.1 Ethnopharmacology

The significance of ethnopharmacology cannot be overemphasized: plants have played a pivotal role for centuries in the management of malaria and have provided an alternative source of antimalarial drugs. In developing countries, most health- related requirements are met by traditional medicine men and other traditional healthcare providers as established by WHO in 2013 (Lemma et al., 2017).

Many plants have been analyzed in various studies done in search of potent antimalarial medicines, and advanced studies are ongoing on crude extracts that show significant activity against malaria parasite. By 2015, only about 0.25% of medicinal plants registered botanically had been explored

for anti - plasmodial activity (Justice, 2016). There is insufficient comprehensive ethnobotanical data that would lead to potential plant candidates for *in vitro* and *in vivo* tests (Njoroge & Bussmann, 2006). This is a major indicator that the efforts on ethnopharmacological research need to be scaled up to ensure discovery of new potent agents before resistance to Artemisinin widely spreads.

The studies done so far to unearth the plants used traditionally for treatment of malaria involves information from traditional medicine men, village elders or villagers who utilize these plant extracts and scientific literature that involves published work by researchers. Most researchers use frequency of mention of anti-malarial plant species as the main tool to estimate the scale of use of different medicinal plants in different regions. The data from these studies give pointers for further pharmacological and phytochemical studies (Njoroge & Bussmann, 2006).

1.1.2 Limitations in Ethnopharmacology

After *in vitro* anti- plasmodial assays, most plant materials are found to have little- to- no activity, this is coupled with the fact that the same plant materials have been used traditionally for generations (Lemma et al., 2017). Rejecting such plant candidates may be premature for several reasons that include; host genetic factors that could allow for effective anti- plasmodial activity within the human body with poor activity *in vitro*. In addition, there is the factor of pharmacokinetics of the herb, which includes metabolism of the drug and activation of pro- drugs that are then effective at inhibiting parasite growth. A difference in nutrition, soil quality, weather from geographical region to region, or harvesting season, would also affect concentration of active compounds in plants (Lemma et al., 2017). In addition, two or multiple secondary metabolites may be working in synergy to produce anti- plasmodial activity, a characteristic that cannot be displayed by one component in isolation.

The methods of evaluating drug activity, for instance, the almost exclusive use of IC_{50} values could also be a major contributory factor to getting 'inactive' agents from herbs effectively used traditionally (Lemma et al., 2017). The erythrocytic stages of the *Plasmodium* parasite is the focus of most *in vitro* studies, having the IC_{50} measurements pegged on this stage. This definitely excludes herbs that have mechanism of action linked to extra- erythrocytic stages of the parasite. Therefore, it is mandatory that other techniques are developed to assay all possible targets of action, a more holistic approach (Lemma et al., 2017).

1.1.3 Ethnobotany and conservation

Traditional medicine utilizes various plant parts and in Central Kenya majority of the studies show that medicines were obtained from the roots (Njoroge & Bussmann, 2006). This would mean uprooting the whole plant, especially for shrubs and herbs. This is definitely a destructive way of obtaining these medicines. Studies show that most of antimalarial herbal medicines, approximately 77%, are obtained from trees and shrubs (Njoroge & Bussmann, 2006). Njoroge and Bussman, (2006) further noted that some plant species, such as *Warbugia ugandensis* are already over-exploited and is rare in some parts of the country. Hence there's need to conserve the plants to ensure a sustainable supply of this resource.

1.2 Study Problem

Malaria continues to have a high disease burden in the tropical regions, having *P. falciparum* as the major cause of malaria cases in WHO African region, standing at 99.7 %. Similarly, in WHO regions of Western Pacific, Eastern Mediterranean and South-East Asia, where *P. falciparum* accounts for more than 60 % of cases (WHO, World Malaria Report, 2018). The recent world

malaria report indicates that in the year 2017 there were more than two hundred thousand cases of malaria, with an approximate malaria death number of 435,000, with 92 % of these cases and 93 % of the deaths being in the WHO African region (WHO, World Malaria Report, 2018).

Approximately 3.2 billion people worldwide are at risk of malaria infection, with $12 billion per year in economic losses in Africa alone. In 2017, the total funding for malaria control and elimination was approximately US$ 3 billion, with the malaria endemic countries having contributed approximately US$ 900 million (WHO, World Malaria Report, 2018).

Anti- malarial drug resistance is another major concern with evidence of widespread pattern of resistance to older generation anti- malarial drugs such as Chloroquine and Sulphonamide-Pyrimethamine (SP). Artemisinin, being the currently used anti- malarial agent, mainly as Artemisinin based combination therapy (ACTs), has studies showing emergence of resistance patterns around the world. For instance, some areas of the Greater Mekong sub- region as mentioned by WHO (WHO, World Malaria Report, 2018). There are also reports of increasing resistance to the ACT partner drugs. Consequently, novel efforts have to be installed to keep pace with changes of the malaria parasite and disease epidemiology.

1.3 Study Justification

There are higher number scales of traditional healers accessible by the rural communities compared to the number of professional medical personnel that is inadequate to cover the population. As mentioned earlier, studies done in Uganda estimate the ratio of a traditional healer to people they serve to be 1: 200 - 400 people, this being a sharp contrast to the ratio of a trained medical personnel and the number of patients they serve, i.e. one medical personnel serving 20,000 people (Muthaura et al., 2011). This is an indication that traditional medicine plays a vital role in management of diseases in African communities hence ethnomedicine cannot be ignored.

This study employs the ethnopharmacological route to pursue new bioactive plant products because the study plants are traditionally utilized in management of ailments. Selection of medicinal plants follows three different approaches; chemo- taxonomical, random and ethnopharmacological. The ethnopharmacological route has been established to have greater successes in research (Trotter et al., 1983). This study involves *in vitro* anti- plasmodial activity assessment because *in vitro* tests are basic and the most important preliminary step in establishing primary drug activity (Sinha et al., 2017).

1.4 Objectives

1.4.1 General Objectives

This study aimed at assessing the anti - plasmodial activity of crude extracts of *Phyllanthus sepialis* and *Rumex steudelii* using light microscopy and flow cytometry *in vitro* assays.

1.4.2 Specific objectives

1. To investigate the phytochemical classes of secondary metabolites of *P. sepialis.*

2. To determine the *in vitro* anti - plasmodial activities of crude plant extracts of *P. sepialis* and *R. steudelii* using light microscopic assay and flow cytometry.

CHAPTER 2 : LITERATURE REVIEW

2.1 Malaria

Malaria is a life threatening, mosquito-borne disease transmitted by the female *Anopheles* mosquito. Mainly, *A. gambiae*, *A. funestus* and *A. arabiensis* have been known to transmit the *Plasmodium* parasite from a person to another as it feeds on human blood.

The species of *Plasmodium* that transmit Malaria are *P. falciparum, P. malariae, P. ovale, P. knowlesi and P. vivax. P. falciparum* accounts for majority of Malaria infections causing an approximate 99.7% of all Malaria cases in Africa (WHO, World Malaria Report, 2018).

2.2 Symptoms

Malaria symptoms range from mild to moderate and these include fever, chills, headache and flu-like illness. Malaria symptoms occur approximately two weeks after the infective bite of the *Anopheles* mosquito. Without treatment, it may progress to severe illness and finally death (WHO, World Malaria Report, 2018).

Severe/complicated malaria is common in infants and children aged less than 5 years. Symptoms are mainly anemia, respiratory distress and cerebral malaria. There can be organ (s) failure due to hypo- perfusion.

In endemic areas, asymptomatic infection is common due to partial immunity developed by multiple exposures.

2.3 Risk groups

Infants and children under 5 years of age are highly at risk of malaria disease. Similarly, pregnant women, HIV/AIDS patients, as well as non-immune migrants and travelers are prone to the disease (WHO, 2018). Pregnancy decreases immunity hence pregnant women are prone to many infectious diseases. This is inclusive of women who had developed protective immunity before pregnancy. Malaria in pregnancy leads to premature births or low birth weight babies, who consequently struggle to survive in their early life (Guyatt & Snow, 2004).

2.4 Epidemiology of malaria

The worldwide incidence of malaria in 2016 was 216 million with 445,000 deaths, mostly children in the African Region, SSA accounting for more than 90% of malaria cases and deaths by 2017 (WHO, World Malaria Report, 2018).

Globally, approximately half of the world's population, which is more than three billion people, are at risk of contracting Malaria disease (UNICEF/WHO, 2015).

Epidemiology in Kenya

Approximately 14.4 million people in Kenya live in malaria endemic regions (PMI, 2019). Malaria infection has a prevalence of 20% in malaria endemic areas and is responsible for 19% outpatient hospital visits. Furthermore, there are the highland zones prone to seasonal transmission of malaria and epidemics of which may have a higher case- fatality rate compared to endemic zones (PMI, 2019).

Plasmodium falciparum is mostly the causative species but *P. malariae, P. vivax and P. ovale* have also been documented to cause malaria with much lower prevalence (Noor, 2016).

Rainfall, parasite species and altitude are the main influencers of malaria transmission and epidemiology. Malaria endemic regions are the Coast, Western and Nyanza Provinces; they have high transmission rates with an average of one infective bite per person per week throughout the year. Low endemic regions include parts of Eastern Kenya and Rift valley regions (Noor, 2016). Highlands bordering malaria endemic zones experience epidemics, while the areas above an altitude of 1600m, such as Nairobi and Mt Kenya regions do not have malaria, unless when one travels to the endemic zones (Noor, 2016).

2.5 Lifecycle of *P. falciparum*

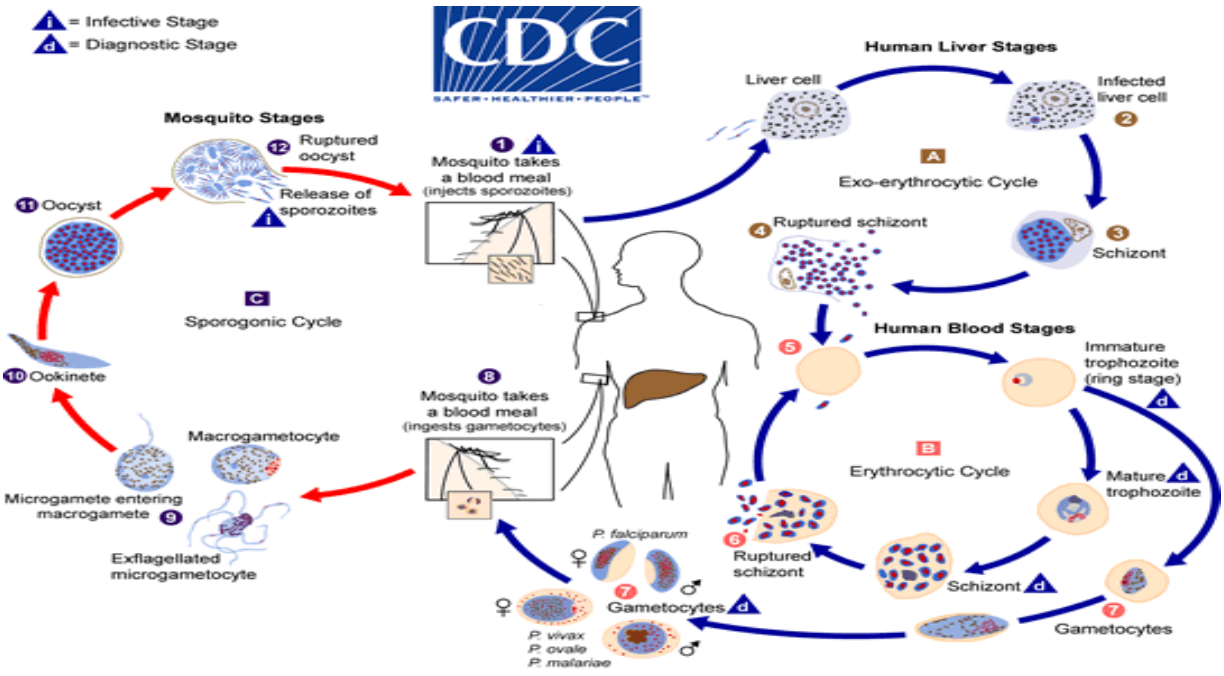

Figure 2.1: Life cycle of *Plasmodium falciparum*. Adapted from: Global Health, Division of Parasitic Diseases and Malaria, CDC (2019).

Male and female gametocytes are ingested by a female *Anopheles* mosquito during a human blood meal. The gametocytes mature within the mosquito; male gametocyte fertilizes the female gametocyte to form a zygote. The zygote transforms to an ookinete which moves to the outer membrane of the gut. It then transforms to an oocyst then divides to form sporozoites which move to the salivary glands of the mosquito. Sporozoites are inoculated into man in the mosquito's next human blood meal.

In man, sporozoites infect hepatocytes within minutes. The parasite divides in the hepatocytes to form a schizont that burst to release merozoites in blood. The parasites then invade erythrocytes, develop from the single ring stages and matures to late stage trophozoites, later develop to multi-nucleated schizonts before the red blood cell lyses to release the merozoites to infect more erythrocytes. Some parasites transform into male and female gametocytes to be ingested by the next bite of the female mosquito.

2.6 The Malaria- Causing Parasites

Different Plasmodium species have different influence on disease occurrence and disease severity. Case in point is *P. vivax* and *P. ovale* that have hypnozoite stage that remains dormant in hepatocytes months to years before reactivation and invasion of blood, causing relapses.

P. falciparum is distributed worldwide but dominates in Africa. It causes severe malaria due to the fact that it multiplies rapidly causing severe anemia. The infected erythrocytes clog small blood vessel and hence can cause cerebral malaria and various organ failure. *P. vivax* is mostly found in Asia, South America, and partly in Africa. *P. ovale* is mostly found in West Africa and the western Pacific islands. *P. malariae* is distributed worldwide, it has a quartan cycle and can cause chronic

malaria infection if untreated, which can last a lifetime. *P. knowlesi* is found in Southeast Asia as a natural pathogen of long-tailed and pig-tailed macaques, causing zoonotic malaria (Autino et al., 2012).

2.7 Study plants

Over the years, many plants have been reported to have ethnobotanical significance. In Kakamega County, *P. sepialis* and *R. steudelii* are being used for malaria management, this was reported for the first time in an ethnobotanical study done in Kakamega County (Mukungu et al., 2016).

2.7.1 *Phyllanthus sepialis*

Phyllanthus sepialis is an open- branched shrub from the family *Phyllanthaceae*. It attains a height of up to 5 meters, but averagely, 1.5 meters. It is largely found in southern Sudan, Ethiopia, Uganda, Kenya, Tanzania and grows well along forest margins, bush-land, extending into drier areas.it grows in well drained soils. It is a dioecious species, meaning both male and female forms are grown to bear fruit and seed and the seeds are used for propagation (Fern, 2019).

It is harvested from the wild and traditionally prepared by boiling the leaves in water (Mukungu et al., 2016). It is used traditionally as a tonic in pregnancy and also in dental hygiene. Its anti-malarial use was first reported in Kakamega East sub- county but so far, no anti- plasmodial activity assay had been done.

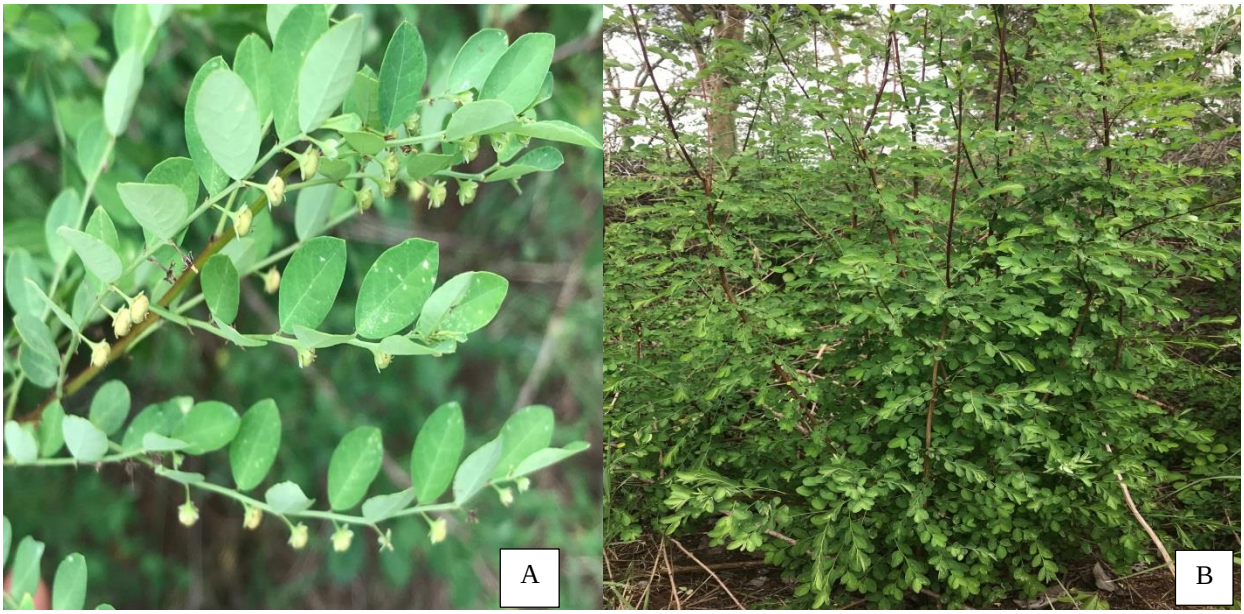

Figure 2.2: *Phyllanthus sepialis*. A; flowering stem; the males have petals. B; The open-branched shrub of *P. sepialis*. (Photo taken at Masii, Machakos County, site of harvest on June 7th, 2019).

2.7.2 Rumex steudelii

Rumex steudelii is a herb of the family *Polygonaceae.* Its common name as per area of use i.e. Kakamega County, is *alukhava*. The root is pounded and cold water added before consumption. Frequency of citation according to Mukungu et al. (2016) is 9.5%.

It is used traditionally as an antifertility agent, in managing tonsillitis, wounds, eczema, hemorrhoids and leprosy. Antimalarial activity was first reported in Kakamega East sub- county (Mukungu et al., 2016).

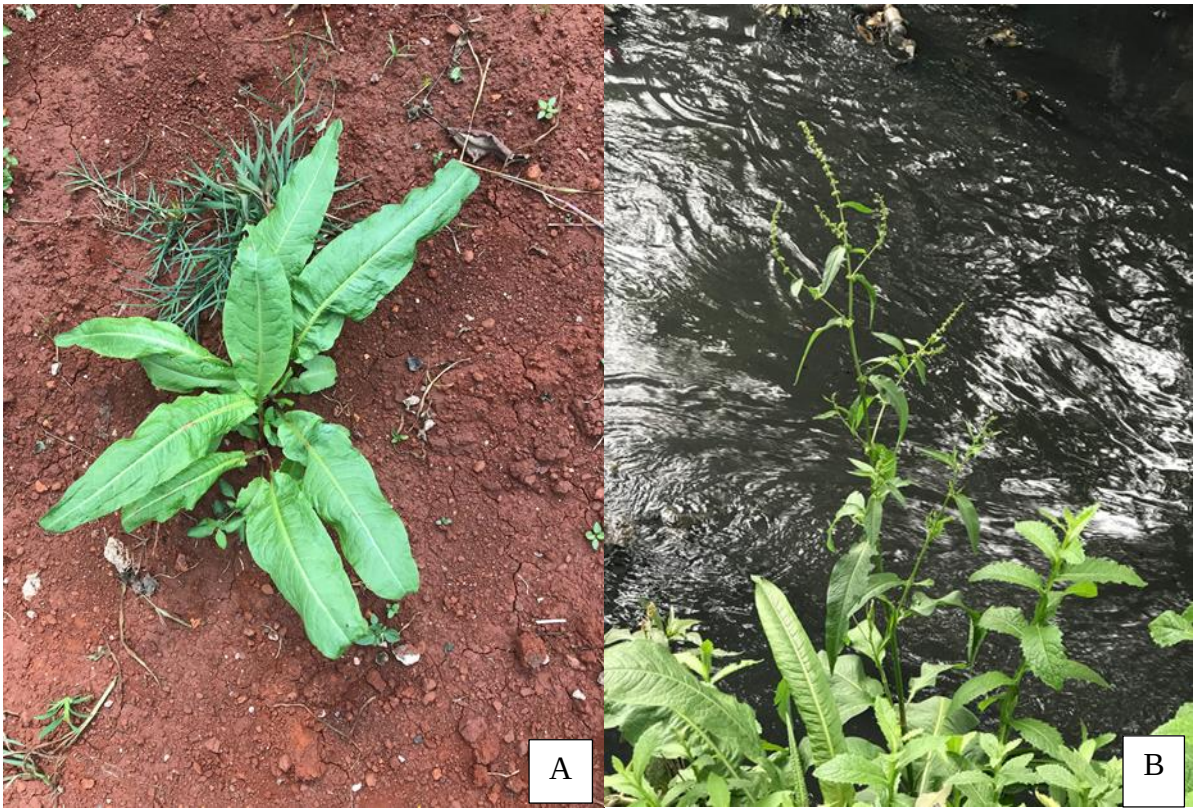

Figure 2.3: *Rumex steudelii*; **A**: *R. steudelii* herb. **B**: The flowering plant growing along the river. (Photos taken along Nairobi River, site of harvest on July 8th, 2019).

2.8 Phytochemistry

Phytochemicals are chemicals produced by plants. Phytochemistry, therefore, is the study of these organic chemicals accumulated by plants; their chemical structure, biosynthesis, distribution, metabolism and function (Harborne, 1998).

The genus rumex is known to accumulate flavonoids, naphthalenes, stilbenoids, triterpenes, carotenoids, anthraquinones, and phenolic acids (Vasas & Orbán-gyapai, 2015). Specifically,

phytochemical tests done on the powdered roots of *R. steudelii* showed presence of saponins, phytosterols, anthraquinones, tannins, and polyphenols (Gebrie et al., 2005).

Some plant species in the *phyllanthaceae* family, such as *Phyllanthus uniraria* have illustrated presence of lignans, tannins, flavonoids, terpenoids, alkaloids, polyphenols, coumarins and saponins (Geethangili & Ding, 2018), phytochemicals that *P. sepialis* might contain considering they both belong to the same genus.

2.9 Solvents for extraction

Polarity of the biomolecules produced by plants determine the choice of solvents to be used for plant extraction, due to the fact that similar polarities between the solute of interest and solvent allows for efficient dissolution of solute into the solvent. A range of solvents of different polarities should be used to extract phytochemicals efficiently (Altemimi et al., 2017).

In this study, five solvents with differing polarities have been used; Hexane, Dichloromethane, Ethyl acetate, Methanol and Water in the order of least polar to most polar solvent.

2.10 *In vitro* drug sensitivity assay methods

There are different developmental stages of *plasmodium* that are targets for anti- malarial drug assays, these include; the liver stage assays which may employ the Infrared fluorescence detection method; the blood stage assays such as Schizont maturation, Microscopic assay, Radioisotopic assay, and Enzymatic assay; gametocyte stage that involves assays such as redox indicator, Alamar

Blue and SMFA; HTS that can use assays such as Fluorescence based assay and *In vitro* beta-hematin formation assay (Sinha et al., 2017).

2.10.1 Microscopic Assay

Light microscopy technique is used to view air dried blood films prepared from the *Plasmodium* culture well plates containing different drug dilutions, methanol is used to fix the cells and staining is done with 10 % Giemsa stain. The number of parasites is counted manually under the microscope and this is used to derive dose- response curves (Karl et al., 2009).

2.10.2 Flow Cytometric Assay (FCA)

This is a high throughput screening method that uses fluorescent SYBR Green dye which intercalates into the parasite DNA, hence staining it (Sinha, Sarma, Sehgal, & Medhi, 2017). The fluorescence is proportional to parasite DNA content, considering human erythrocytes are devoid of DNA. FCA can also determine the developmental stages of the *plasmodium* parasite, a property that can be used to determine the specific stage of the anti- plasmodial activity (Contreras et al., 2004).

CHAPTER 3 : MATERIALS AND METHODS

3.1 Study Design and Site

This was an experimental study that sought to assess the anti- plasmodial activities of crude extracts of *phyllanthus sepialis* and *rumex steudelii*. This study was carried out at the Institute of Primate Research (IPR) in the department of tropical and infectious diseases. The plant extraction and phytochemical tests were done at the School of Pharmacy, University of Nairobi.

3.2 Plant preparation

3.2.1 Collection of study plants

Rumex steudelii and *Phyllanthus sepialis* are used by the Luhya community of Kakamega County in management of malaria. Use of the two plants had been reported for the first time in treatment of malaria with no scientific evidence of anti- plasmodial activity. Although these plants were reported to grow and utilized in Kakamega County, it was established that they also grow naturally in the Nairobi and Machakos areas with *R. steudelii* mostly being riverine or growing wildly along the roads. With this information, the plants were identified by a botanist from the School of Biological Sciences, University of Nairobi and the leaves of *P. sepialis* were harvested from Masii, Machakos County while roots of *R. steudelii* were harvested from along Nairobi River in Nairobi County.

3.2.2 Milling of plant material

The roots of *R. steudelii* were cleaned and air- dried while the leaves of *P. sepialis* were air- dried, after which the two plant parts were separately milled using a hammer mill from the school of

pharmacy, University of Nairobi. This process produced fine powder for experimental work, of which 820 g of each was used in the extraction process.

3.3 Solvent extraction

Cold maceration method was used to extract 200 g of the plant powder of each plant using methanol, dichloromethane, ethyl acetate and hexane separately over a period of 72 hours each with constant magnetic stirring. The ratio of plant material: solvent was 1:6. The extracts were then filtered using a 125 mm Macherey-Nagel (MN 615.125mm REF 431 012) filter papers and each filtrate was reduced by vacuum rotary evaporation (Welch Gardner Denver Heidolph VV 2000 Rotary evaporator) at 50 °C using a 1 L round- bottomed reducing flask. The reduced extracts were then transferred to a 250 ml round- bottomed flask for final extract concentration by the rotary evaporator, to ease the extract- harvesting process.

The reduced extracts were thereafter oven dried at 40 ºC to evaporate the residual extracting solvent. Each dry extract was weighed, yield recorded and finally aliquots were stored in amber colored, glass airtight containers and refrigerated at -20 °C in readiness for anti- plasmodial activity testing.

The water extraction methodology was guided by the mode of preparation of the medicinal plant by the local community whereby the leaves of *P. sepialis* are boiled and the roots of *R. steudelii* are soaked in water for a while, filtered and filtrate consumed by the malaria patients (Mukungu et al., 2016). In this regard, water extraction for *P. sepialis* was done by boiling 20 g of plant powder in 200 ml of distilled water with constant stirring using a magnetic stirrer for 15 minutes

and the water extraction of *R. studelii* was done by cold maceration of 20 g of plant powder in 200 ml distilled water with constant stirring using a magnetic stirrer for 24 hours.

Through lyophilization process, both the water extracts were frozen using dry ice and left standing in the freezer compartment of the fridge for 12 hours and then freeze- dried for 24 hours.

3.4 Phytochemical tests of the plant materials of *R. steudelii* and *P. sepialis*

Portions of the milled leaves of *P. sepialis* were used to investigate the presence of medicinally significant phytochemicals as indicated in literature review using specific prescribed tests. *R. steudelii* had previously been documented to have phytochemicals such as, saponins, phytosterols, anthraquinones, tannins, and polyphenols (Gebrie et al., 2005).

3.4.1 Test for saponins

The froth test was used to determine saponin component of *P. sepialis*. In brief, in a test tube with one gram of sample, 10 ml of distilled water was added and shaken for 1 minute. This was followed by filtration of the mixture and 2.5 ml of filtrate added to 10 ml of distilled water in a test tube that was then stoppered and vigorously shaken for 30 seconds (Dahiru, Onubiyi, & Umaru, 2006). This was let to stand for 30 minutes, results determined visually, interpreted and finally recorded. Presence of saponins was indicated by formation of a persistent honeycomb froth after letting the sample stand for 30 minutes.

3.4.2 Test for Tannins

To test for tannins, one gram of the powdered sample was boiled in 10 ml distilled water for 5 minutes. This was followed by filtration of the mixture and addition of 3 drops of 10 % ferric

chloride solution. A positive result for tannins is indicated when the test sample turns dark green (Dahiru, Onubiyi, & Umaru, 2006). The observations were determined visually, interpreted and finally recorded.

3.4.3 Test for phenols

This test involved adding 2 ml of $FeCl_3$ to 2 ml of extract after boiling one gram of powdered sample in 10 ml distilled water for 5 minutes and the mixture filtered. A positive result was indicated by development of bluish- green color (Dahiru, Onubiyi, & Umaru, 2006).

3.4.4 Test for alkaloids

Mayer's test according to (Prashant Tiwari et al, 2016), with slight modification was used. This involved warming one gram of plant powder in 10 ml 10 % sulfuric acid on a water bath for 5 minutes. This was filtered and a few drops of Meyer's reagent added to the filtrate portion. A positive test was indicated by a white to buffy precipitate.

3.4.5 Test for Flavonoids

To identify flavonoids, one gram of plant powder was boiled in 10ml distilled water for 5 minutes. This was filtered and 5ml dilute ammonia and concentrated sulfuric acid were added to the filtrate. The expected result was the formation of a yellow color that should disappear on standing. This procedure was adopted from (Prashant Tiwari et al., 2016) with minor modifications.

3.4.6 Test for Terpenoids

Salkowki's test as proposed by Edeoga et al. (2005) was used. In brief, one gram of plant powder was boiled in 10 ml distilled water for 5 minutes. This was filtered and 2 ml chloroform added to

the filtrate. 2 ml of concentrated sulfuric acid was also added and the tube shaken gently. Formation of a reddish- brown interface indicates presence of terpenoids.

3.4.7 Test for Anthraquinones

Modified Borntrager's test was used and this involved boiling of 1 g of plant powder in 5 ml 10 % sulfuric acid and 5 ml 5 % ferric chloride for 5 minutes. This was filtered while hot and extracted with 3 ml carbon tetrachloride and shaken gently for 1 minute. 5 ml ammonia was then gently added with the test tube slightly tilted. Positive test was indicated by formation of rose- pink color with ammoniacal layer.

3.4.8 Test for Triterpenes

One gram of plant powder was boiled in 10 ml distilled water for 5 minutes and filtered. Chloroform was added to the filtrate, shaken gently and 2 ml of concentrated sulfuric acid added. This was shaken gently and allowed to stand. The expected outcome was lower layer turning yellow to indicate presence of triterpenes.

3.5 Cultures of *P. falciparum* parasites

3.5.1 Preparation of the O^+ red blood cells from donor blood

Blood type O^+ was obtained from Kenya Medical Research Institute (KEMRI) provided by willing donors whose blood had been previously and routinely screened to ensure the blood fits the research use. The blood donor was bled by a phlebotomist using aseptic technique that involved venipuncture and withdrawal of 30 ml of blood into the syringe that contained 10 USP units of

heparin/ml of blood. This was inverted 10 times after blood collection to ensure complete anticoagulation of the blood. The blood was stored at 4 - 8 ºC in readiness for use.

The blood was centrifuged (DragonLab D3024R Centrifuge) at 1500 rpm for 5 minutes and supernatant and buffy coat was aspirated off. The resulting red blood cell pellet was washed three times with twice the volume of incomplete RPMI media and equal volume of incomplete media (wash media) was added to make 50 % RBC. The wash media was composed of 18.75 ml 1M N - 2 –hydroxyethylpiperazine –N -2 –ethane sulfonic acid (HEPES), 5 ml 20 % glucose, 3 ml 1M NaOH, 250 µl 50 mg/ml Gentamicin and 5 ml 200 mM glutamine.

3.5.2 Thawing of the parasites, strain 3D7.

Plasmodium falciparum lines 3D7, the chloroquine- sensitive strain, were used to monitor the anti-malarial activity of plant extracts. The parasites were obtained as part of the BEI resources repository, NIAID, NIH, deposited by SJ Rogerson. The parasites were stored in vials that were then retrieved from liquid nitrogen and immediately thawed at 37 °C in a water bath, followed by transfer to a 50 mL tube. To every 1 mL of parasite suspension the following was added: 200 µl of 12 % NaCl drop wise while shaking, then allowed to stand for 5 minutes, then 10 ml of 1.6 % NaCl and finally 10 ml of 0.9 % NaCl. The mixture was allowed to stand for 5 minutes and centrifuge at 1800 rpm (Sigma laboratory centrifuges. 2K15). The supernatant was aspirated and the pellet washed twice in incomplete RPMI, before transferring pellet into a culture flask.

3.5.3 Parasite viability testing

Before the parasites were subjected to culture, approximately 100 µl of the thawed parasites was withdrawn and a thin smear prepared using the thin smear preparation technique with Giemsa

staining. This was viewed under the light microscope so as to confirm viability of the parasites and parasitemia.

3.5.4 Serum processing from human blood.

Human blood was obtained from KEMRI in blood collection bags free of anticoagulant and left overnight at 4 °C, to allow for separation of serum. In a laminar flow cabinet, the serum was then aliquoted into sterile 50 ml tubes followed by heat inactivation by placing the tubes in a water bath at 56 °C for 1 hour. Once the serum had cooled this was then stored at -20 °C until required for *in vitro* cultures.

3.5.5 Refreshing of 3D7 *P. falciparum* cultures.

The parasite cultures were refreshed every 48 hours during which media was changed and parasitemia assessed by preparing thin blood smears. This process involved transferring culture flasks into a biosafety cabinet, aspirating excess media and adding 10 ml of complete media. Depending on the parasitemia appropriate quantity of erythrocytes (50 % PCV) was added into the media to adjust parasitemia to 2 %. The flasks were then gassed again, sealed and returned to the incubator.

3.6 *In vitro* testing of the anti - plasmodial activity of the extracts

3.6.1 Culturing of the *P. falciparum*, strain 3D7

Into T25 cm^3 culture flask (Corning, UK), 13 ml complete media, which was made up of a mixture of serum: incomplete medium in the ratio of 1: 10, was added followed by 800 µl of 50 % RBC, to make 3 % hematocrit. The 1 ml of thawed parasites were then added and finally gassed with 90 % N_2, 5 % CO_2 and 5 % O_2. The complete media consisted of RPMI 1640 supplemented with 10 % human serum, buffered with 25 mM HEPES, 25 mM $NaHCO_3$, L glutamine and 60 µg/ml gentamicin sulfate. The set up was put in the incubator with the temperature being maintained at 37 °C (Schuster, 2002). Incubation ran for 48 hours after which the media, which was the supernatant, was aspirated off using a pipette. 300 µl of the sediment was put in an eppendorf tube and pulsed from 5000 rpm, supernatant aspirated and discarded. A thin smear was then made from a drop of the sediment in readiness for microscopy. Thin smears were prepared every 48 hours before media was refreshed and checked under the microscope to confirm parasite viability, cell cycle transition and parasitemia.

3.6.2 *In vitro* culturing of the 3D7 *P. falciparum* in presence of extracts

In vitro testing of anti - plasmodial activity involves culturing of the *P. falciparum* parasites in the complete RPMI media together with predetermined concentrations of each of the extracts so as to eventually determine what extracts exhibit anti - plasmodial activity. This method has been used widely in screening for anti - plasmodial activity in plant extracts and allows for parasitemia at various extract concentrations to be determined. In this study the same approach was applied. An aliquot of each plant extract had been earlier thawed and refrigerated at 4-8 °C. The procedure started with the disinfection of the work surface with 70 % alcohol. Phosphate buffered saline

(PBS) was used to dissolve 1 mg of each extract to make a stock solution of 1 mg/ml. The dissolution of the extract was aided by an ultrasonic bath (E/MC^{Corp} RAI RESEARCH) with temperature elevated to 40 ºC for 30 minutes. Ten concentrations of each test sample were obtained by two- fold serial dilutions of the extracts and introduced in the complete medium as arranged in the round- bottomed 96 - microtiter plates, from a range of concentration of 500 µg/ml through to 0.98 µg/ml. 100 µl of the cultured chloroquine - sensitive malaria parasites were added into each well to a final hematocrit of 3 % and parasitemia of 1 % to make up the total volume in each well to 200 µl. The positive control used was chloroquine, prepared from 250 mg tablet of chloroquine-phosphate (Sugaquin® Kampala Pharmaceutical Industries Ltd, Uganda) which had 155 mg of CQ base. One mg of the crushed tablet was used to make the stock solution with a stock concentration of 620 µg/ml. The initial well, therefore, had a concentration of 310 µg/ml, and this was subjected to two - fold serial dilution. Negative control was made by wells with infected erythrocytes (3 % hematocrit and 1 % parasitemia) without extracts. The tests were performed in duplicate.

The plates were then introduced into the candle jar, the candle lit and the jar covered with the lid smeared with petroleum jelly. The set up was then put in the incubator at 37 °C for 48 hours.

3.6.3 Thin smear preparation technique

The supernatant from each well was aspirated, the pellet mixed and a drop of the pellet was used to make a thin smear by putting the drop at one end of a microscope slide, and using another slide at an angle of 60 tilting to 45 degrees for the drop to spread to the edges. The slide was then pushed to the other end to form a thin smear. Methanol was used to fix the smear which was then air dried and stained with freshly prepared 10 % Giemsa stain for fifteen minutes. The slide was then rinsed

with water, air dried and light microscopy technique with oil immersion used to identify and quantify the parasites.

In this experiment, low parasitemia to no viable parasites indicated plant extracts with activity against *p. falciparum*; while high parasitemia indicated low to no activity of the extract. Activity of the extracts were compared to the negative control which was the parasitemia in the cultured parasites without extract interference. This was to help calculate the percentage inhibition of each extract concentration.

3.6.4 Determination of parasitemia in light microscopy assay

Guided by Karl et al. (2009), thin blood films were prepared from every drug dilution on a well plate after an incubation time of 48 hours. To determine the parasitemia, an average of 300 eythrocytes per slide were enumerated at ×100 objective and the number of infected erythrocytes among the 300 erythrocytes was recorded. The smears were prepared in duplicate.

The percentage parasitemia was determined as follows:

$$Percentage\ parasitemia\ = \left(\frac{Number\ of\ infected\ erythrocytes\ counted}{Number\ of\ total\ erythrocytes\ counted}\right) \times 100\%$$

3.6.5 Flow cytometry

Stock solution for SYBR Green I dye was prepared by mixing 200 µl of the dye into 20 ml of PBS, so as to achieve a dilution ratio of 1:100. 100 µl of the diluted SYBR Green I dye was introduced into 1.8 ml of PBS in labelled FACS 4 ml tubes, after which 100 µl from each well of cultured cell suspension was added to its respective tube, to make a final volume of 2 ml. This set up was incubated in the dark for 20 minutes. PBS was used to wash the cultures by centrifuging each tube at 1500 rpm for 5 minutes, 1.5 ml of supernatant aspirated off. 1 ml of PBS was added in each tube

and centrifuged again. The supernatant was removed leaving approximately 400 µl of stained cells. Each sample was then analyzed by flow cytometry (Cytek DxP Athena™ cytometer, Cytek Biosciences®). The initial analysis by flow cytometry involved acquiring fifty thousand events for each tube. Data was first collected with Cell QuestPro® (Chevalley, Coste, Lopez, Pipy, & Valentin, 2010). Followed by analysis using set protocols of the FlowJo v10.6.1 software. Using this software, the data was first gated for singlets so as to exclude multiplets, debris or unintended background noise. This was done by plotting a graph of forward scatter- width (FSC-W) against forward scatter- area (FSC-A). Then gating for the erythrocytes was done, without discriminating between uninfected and infected, by plotting FSC-A against SSC-A. Forward- and side-scatter characteristics are used to distinguish different phenotypes of cells, having erythrocytes on the low side- scatter and low forward- scatter (Perfetto, Chattopadhyay, & Roederer, 2004). Finally, the infected erythrocytes were gated by plotting a graph of side scatter- area (SSC-A) on the y- axis against FITC-A on the x- axis. FITC being the fluorochrome coinciding with the emission wavelength for SYBR Green with an emission peak at 520 nm. Using the negative control, the un-infected erythrocyte culture, the boundary between fluorescing and non- fluorescing erythrocytes was set at 10^3 fluorescing units so that events above this value were considered as fluorescence.

The gating strategy is illustrated in figure 3.1.

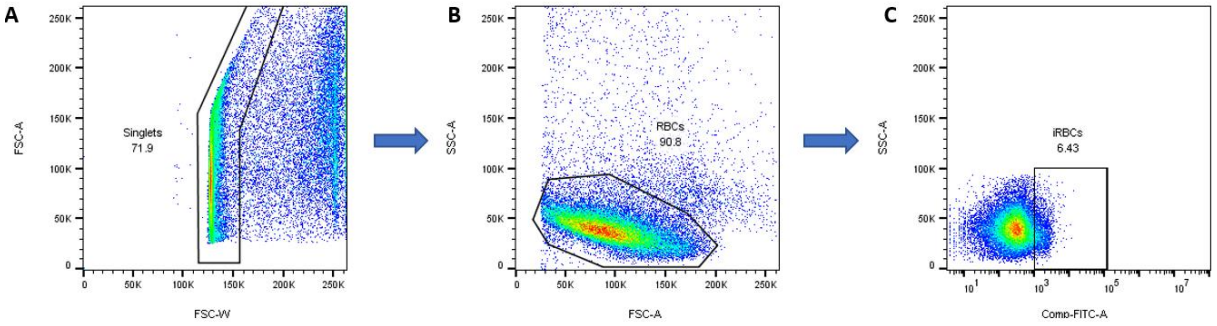

Figure 3. 1: An illustration of the Gating strategy.

Tables were generated containing the fraction of the total number of events detected in each of the gates. The tabular data were further processed using a Microsoft Excel (2016) spreadsheet and IC_{50} values calculated according to standardized procedures.

3.6.6 Freezing of Plasmodium parasites

The parasite culture to be frozen was transferred to a 50 ml tube and centrifuged at 1800 rpm for 5 minutes at room temperature. The supernatant was aspirated and for every 200 µl of pellet, 300 µl of fetal bovine serum (FBS) was added. Lastly 500 µl of freezing solution, (composed of 57 g glycerol, 1.6 g sodium lactate, 30 mg potassium chloride and 35.5 mg sodium phosphate in 100 ml distilled water), was added drop by drop while gently shaking. Approximately 1 mL of the mixture was then aliquoted into labelled cryovials. These were then placed in a freezing chamber and frozen overnight at -80 °C. The vials were transferred to liquid nitrogen the next day for long term storage.

3.6.7 Determination of IC_{50}

The concentration of the extract that would inhibit 50 % of growth of the parasites is known as the IC_{50} value.

Obtaining parasitemia in flow cytometry assay involved gating protocols that streamlined the infected erythrocytes as a fraction of the total erythrocyte population and the parasitemia automatically generated.

The culture of infected erythrocytes without extract (IRBC), in each culturing plate, was used as the negative control in the calculation of percentage inhibition.

Percentage inhibition at each concentration was calculated by the following formula;

$$Percentage\ inhibition\ = \left\{\frac{(IRBC\ Parasitemia\ -Test\ sample\ Parasitemia)}{IRBC\ Parasitemia}\right\} \times 100\%$$

Using Microsoft Excel (2016) spreadsheet, graphs of *Percentage inhibition* on y- axis against *Extract concentration* on x- axis for each extract was plotted and a trendline generated and consequently the equation of $y = mx\ + c$.

The IC_{50} was calculated from each dose- response curve by the equation; $IC_{50} = \frac{(50-c)}{m}$

Where; *c* is the y- intercept and *m* is the trendline gradient.

CHAPTER 4 : RESULTS AND DISCUSSION

P. sepialis and *R. steudelii* are plants used traditionally for management of malaria. In this study, these two plants were put through *in vitro* studies to ascertain their anti - plasmodial activity. Light microscopy and flow cytometry were the methods used for analysis. Phytochemical tests for *P. sepialis* were also done to identify the secondary metabolites present. The phytochemicals for *R. steudelii* had been established through previous studies.

4.1 Plant extract yield

Different solvents were used in the extraction process, namely; water, methanol, ethyl acetate, dichloromethane and hexane. The yields differed depending on the solvent used as shown in Figure 4.1.

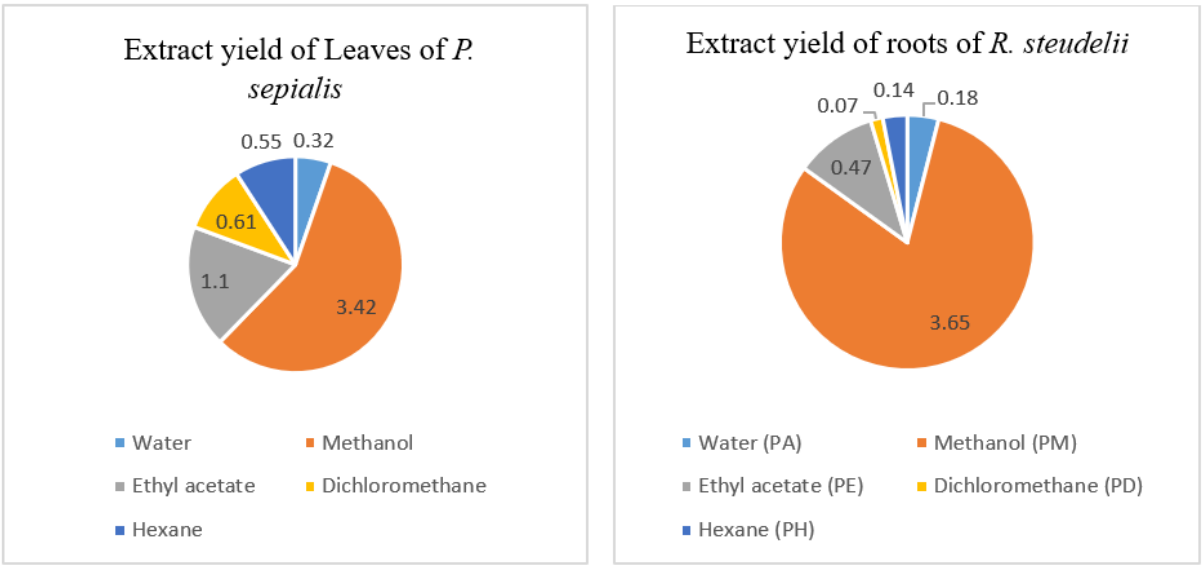

Figure 4. 1: Extract yields (a) leaves of *P. sepialis* as 3.42, 1.1, 0.61, 0.55, 0.32 (% w/w) and (b) root of *R. steudelii* as 3.65, 0.47, 0.07, 0.14 and 0.18 (% w/w) for the methanol, ethyl acetate, dichloromethane, hexane and water solvents respectively.

The percentage yield of the extracts ranged from 3.65 % to the least yield of 0.07 %, being the dichloromethane extract of *R. steudelii*. The methanol solvent gave the most yield of extract, giving an average of 3.54 %, indicating that either most plant secondary metabolites are polar or the few polar compounds isolated gave the highest quantity. The non - polar solvents relatively gave less yields and so did the water extract.

4.2 Phytochemical tests of the plant materials for *P. sepialis*

As illustrated earlier, *R. steudelii* has been documented to have phytochemicals such as, saponins, phytosterols, anthraquinones, tannins, and polyphenols (Gebrie et al., 2005). However, no previous phytochemical studies had been done on *P. sepialis* and this had to be established in this study.

P. sepialis was subjected through different phytochemical tests, as illustrated in section 3.4 above to identify the specific classes of the phytochemicals present and was found to contain tannins, phenols, terpenoids and terpenes. Table 4.1 shows the phytochemical test results in summary.

Table 4.1: *P. sepialis* phytochemical tests result summary.

Test	**Observations**	***P. sepialis***
Saponins	Persistent honeycomb froth	Negative
Tannins	Dark green color	Positive
Phenols	Bluish - green color	Positive
Alkaloids	White to buffy precipitate	Negative
Flavonoids	Formation of a yellow color that disappears on standing	Negative
Terpenoids	Reddish - brown interface	Positive
Anthraquinones	Pink color with an ammoniacal layer	Negative
Triterpenes	Yellow color	Positive

Terpenoids are modified terpenes that have functional groups with different conjugations; for instance, triterpenes can be converted to saponins, which is a triterpene glycoside obtained by glycosylation of its active sites (Panche, Diwan, & Chandra, 2016). Presence of terpenes and

terpenoids in *P. sepialis* is an indication of potential activity against malaria. Terpenoids have various established medical uses, such as sesquiterpenes which have a medicinal product currently in use i.e. Artemisinin, a sesquiterpene from *Artemisia annua*. Triterpenes also have shown potential in antimalarial activity and work is still ongoing (S & C, 2008).

Terpenoids also have anti- inflammatory activity. Some compounds have been reported to have activity against expression of COX- 1 and 2, production of nitric oxide (NO) and inhibition of release of RANTES (Regulated on Activation, Normal T cell Expressed and Secreted) hence, may be used for treating fever amongst other inflammatory ailments inclusive of its use as an antimicrobial agent.

Both *P. sepialis* and *R. steudelii* have tannins as a constituent. Tannins are poly- phenolic secondary metabolites in plants and are known to be used as astringents, as anti- diarrheal, hemostatic, anti- tumors, antiseptics and as anti- inflammatory agents in Japanese and Chinese communities (Khanbabaee & van Ree, 2001). Tannins have also been found to have activity against bacteria, viruses and parasites (Ashok.P. k & Upadhyaya.Kumud., 2012).

The saponins in *R. steudelii* have been reported to have activity against HIV, bacteria, malaria, helminths, also as anti- inflammatory and anti- oxidants (Guclu-Ustundag & Mazza, 2007).

4.3 *In Vitro* anti - plasmodial tests

4.3.1 Microscopy

Light microscopy allows for quantification of parasitized red blood cells visually (Chevalley et al., 2010). There were two plants; *P. sepialis* and *R. steudelii,* each plant with five extracts; RA, RM, RE, RD, RH, and PA, PM, PE, PD, PH, which are the water, methanol, ethyl acetate, dichloromethane and hexane extracts of *R. steudelii* and *P. sepialis* respectively. Each extract was dissolved in PBS at a stock solution concentration of 1 mg/ml and a two- fold dilution done up to 10 dilutions, hence giving ten different concentrations per extract. After 48- hour culture period, thin smears were prepared in duplicate for each concentration of each extract and the slides were viewed under an optical microscope to identify the parasites as illustrated in Figure 4.2. These were images obtained from low concentration (0.98 µg/ml) of hexane extract of *R. steudelii* (Image A) and high concentration (500 µg/ml) of the same extract (Image B).

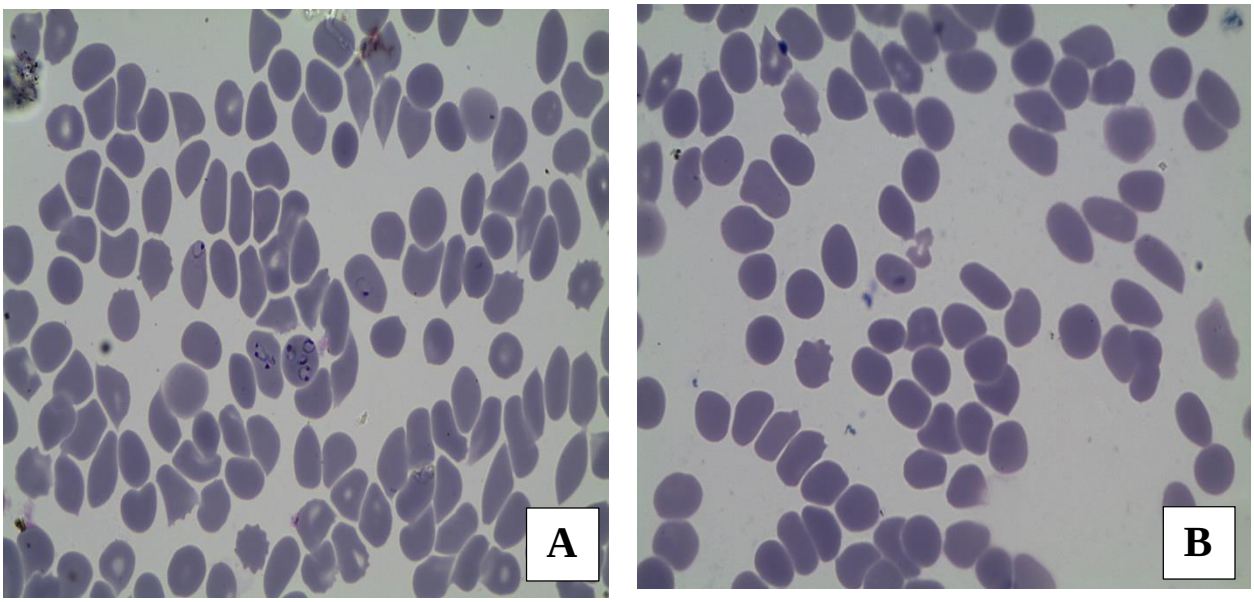

Figure 4.2: Microscopy of infected and normal erythrocytes. A: Erythrocytes infected with ring stages of *P. falciparum*. B: Normal un-infected erythrocytes.

The parasites were counted in a field of around 300 erythrocytes, results obtained were normalized to 100 % (Karl et al., 2009), averaged and tabulated. A plot of concentration of extract was done against the parasitemia, trends developed and each extract activity was compared to the activity of Chloroquine, the positive control in this test. Activity trend of Chloroquine is represented in the red color, while the test sample is in green.

Figure 4.3 illustrates the parasitemia vs extract concentration graphs for *R. steudelii* extracts.

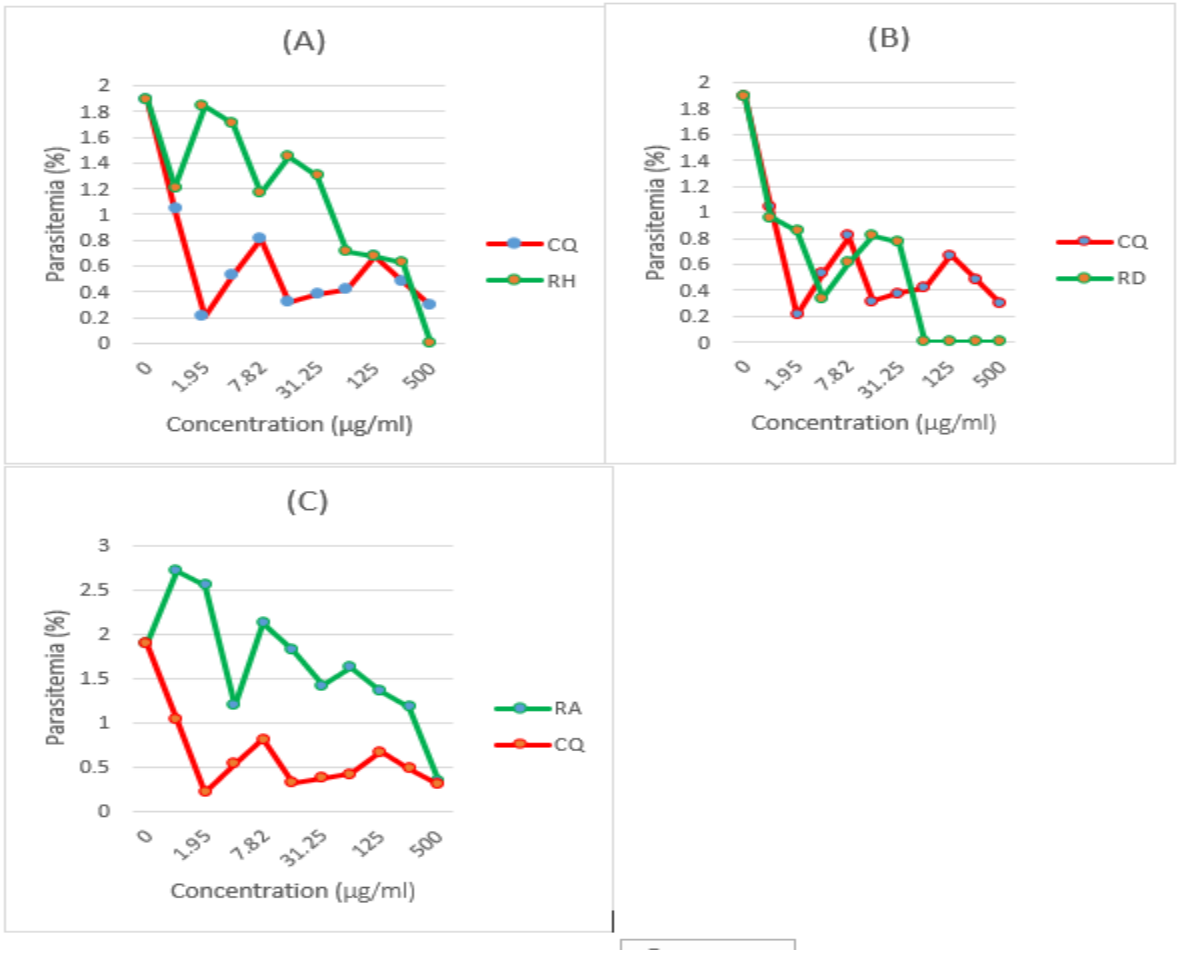

Figure 4.3: An illustration of the trend of parasitemia with increase in concentration for extracts of *R. steudelii*; (A) Hexane extract (RH), (B) Dichloromethane extract (RD), (C) Water extract (RA). The trend of Chloroquine, CQ, as the positive standard.

The trend of parasite growth inhibition was also illustrated across the ten dilutions of each extract, having derived the Percentage inhibition from the equation;

$$Percentage\ inhibition\ = \left\{\frac{(IRBC\ Parasitemia\ - Test\ sample\ Parasitemia)}{IRBC\ Parasitemia}\right\} \times 100\%$$

Figure 4.4 below illustrates the inhibition trend of some of the extracts of *R. steudelii.*

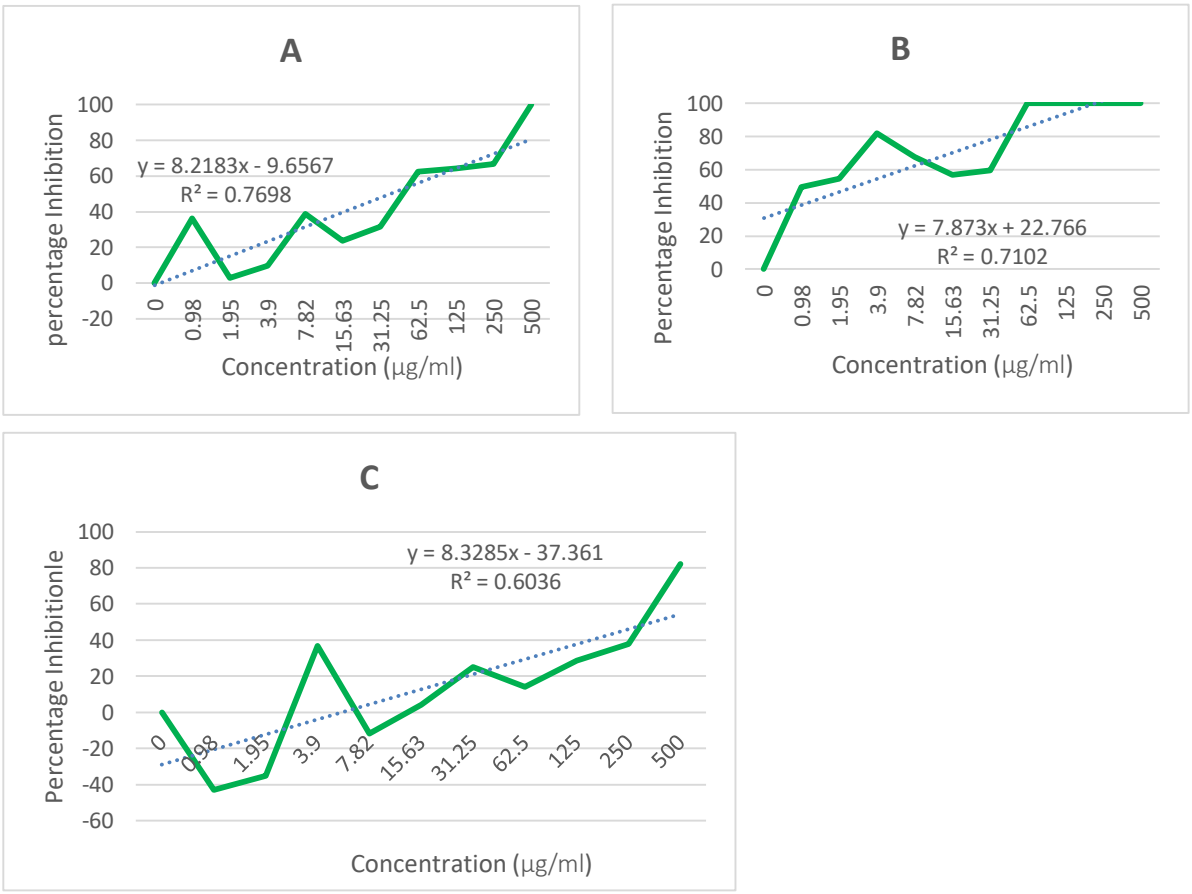

Figure 4.4; Graphs illustrating inhibition trends of (A) hexane extract (B) dichloromethane extract (C) water extract of *R. steudelii*.

The hexane, dichloromethane and water extracts of *R. steudelii* show an increase in inhibition of parasite division with increase in extract concentration. The validity of the data was indicated by use of coefficient of determination, R^2 value, which demonstrates the correlation between the extract concentration and the corresponding parasite percentage inhibition. The R^2 value ranges from 0 to 1, whereby values greater than 0.5 indicate a significant relationship between the variables (Lewis-Beck, Bryman, & Futing Liao, 2012).

4.3.2 Flow cytometry

The use of fluorometric dyes in flow cytometric anti- plasmodial assays benefit from the fact that erythrocytes lack nucleic material and hence the SYBR Green I dye used in this experiment specifically targets the parasite DNA. A higher parasitemia indicates more nucleic material present, hence, the more the dye that binds the DNA and the higher the fluorescence emitted. Active extracts are expected to show a trend of decreased parasitemia with increase in extract concentration.

The *P. falciparum* culture plate without extract was used as the negative control, and it illustrated uninhibited growth. A blank plate was prepared with erythrocytes only. These are illustrated in figure 4.6.

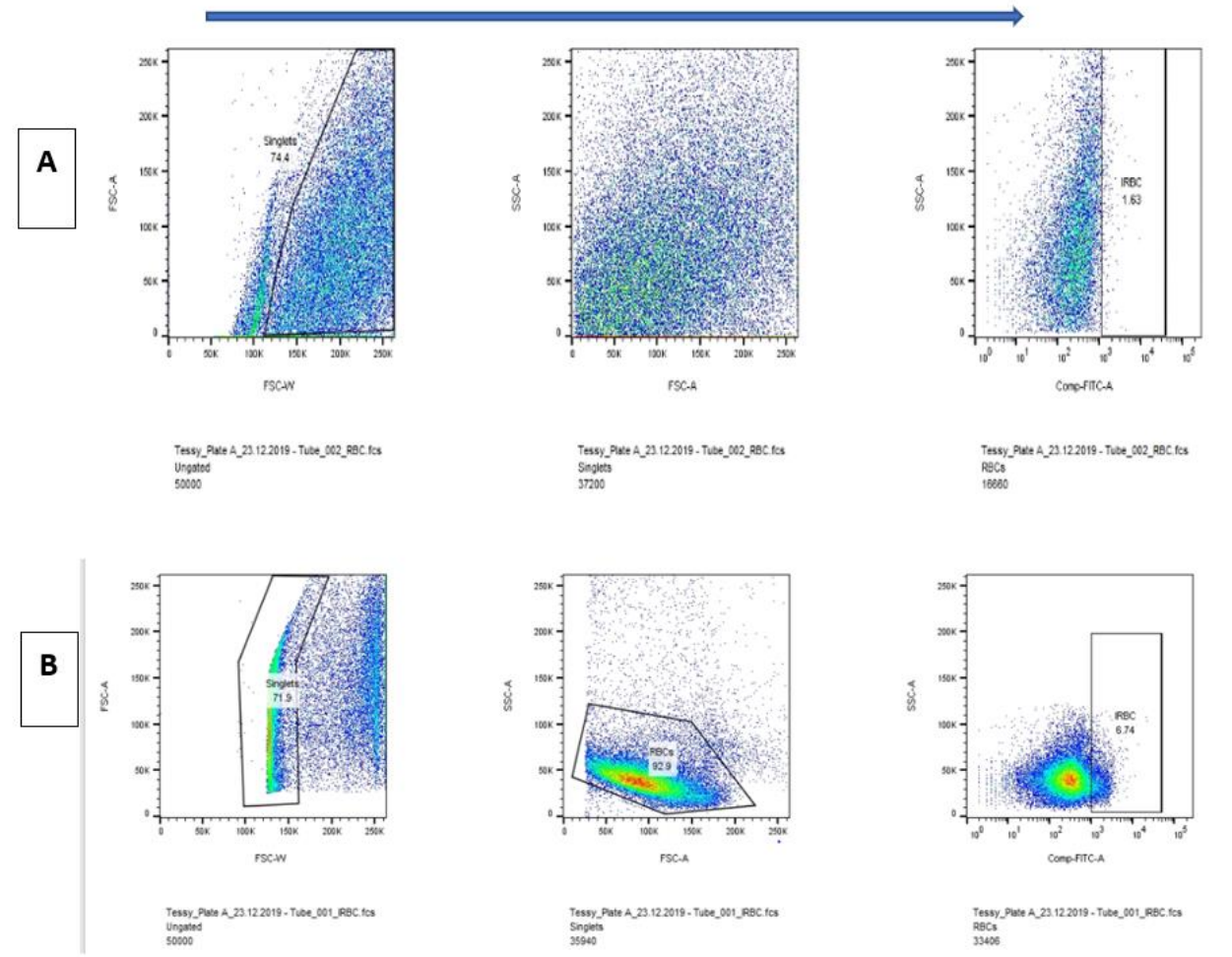

Figure 4.5: **Image A** illustrates the gating sequence of the blank plate. **Image B** illustrates the gating sequence of the negative control (the un- treated but infected erythrocytes), projecting a parasitemia of 6.74 %.

The blank plate showed the boundary between negative and positive fluorescence at 10^3 Fluorescing Units (FU). The value 1.3% was considered as background noise, which could occur due to various factors, such as, use of human serum to culture the parasites, as was done in this experiment. In addition, some cells have native fluorescence molecules. Furthermore, fluorescent reagent measured in a separate channel can have "spill-over" fluorescence (Tung et al., 2007).

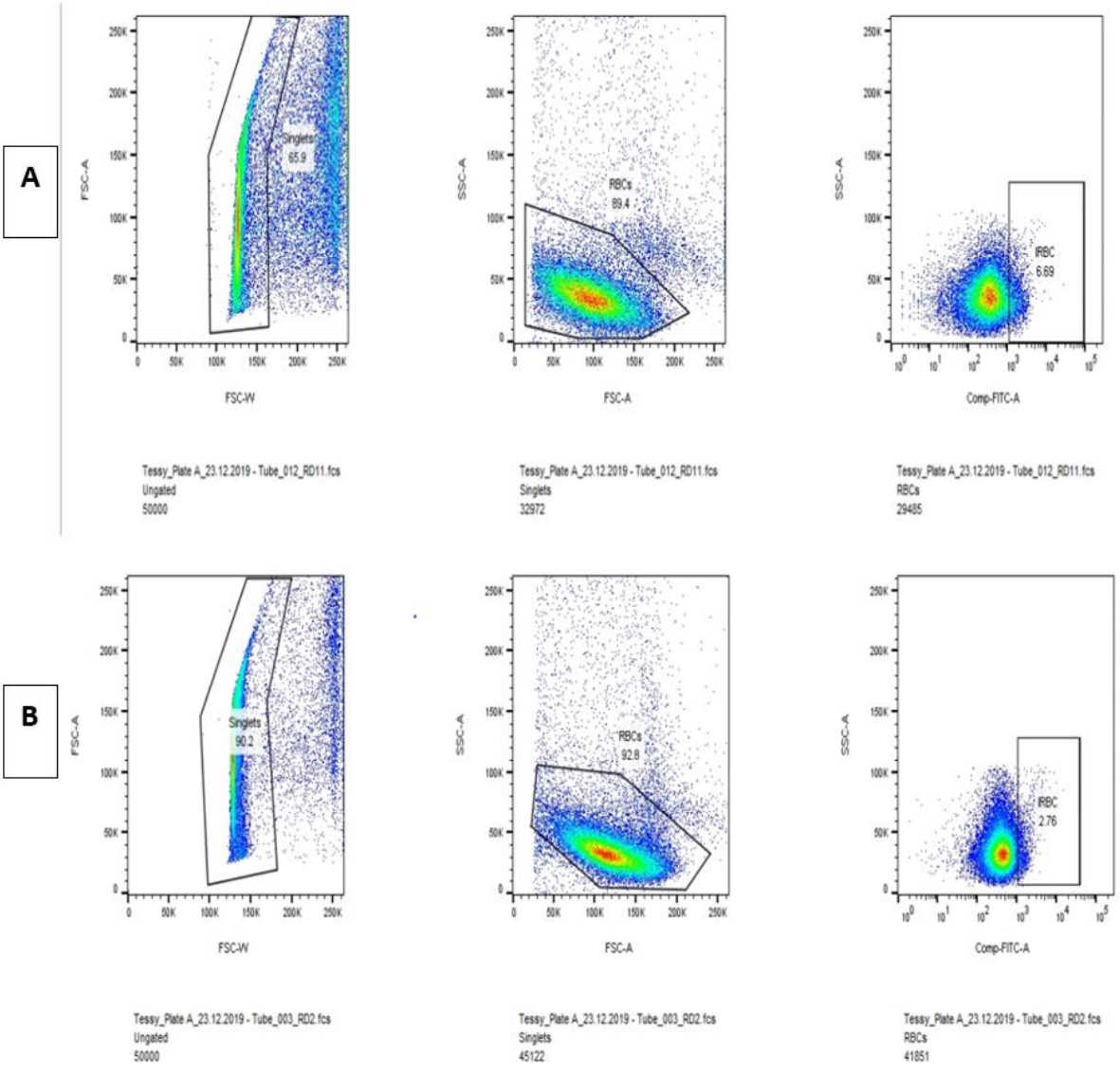

Figure 4.6: A scatterplot illustrating Parasitemia of the dichloromethane extract of *R. steudelii* after 48 hours of cell culture at concentration of 0.96 µg/ml and 500 µg/ml in Image A and B respectively.

The dichloromethane extract of *R. steudelii* showed high parasitemia of 6.69 % coinciding with the lowest extract concentration of 0.98 µg/ml, while at a higher extract concentration of 500 µg/ml the parasitemia was lowered to 2.76 %, an illustration of anti- plasmodial activity.

Trends of parasitemia against the concentration range of the extracts were plotted in comparison to Chloroquine, the positive standard. These are demonstrated in Figure 4.7.

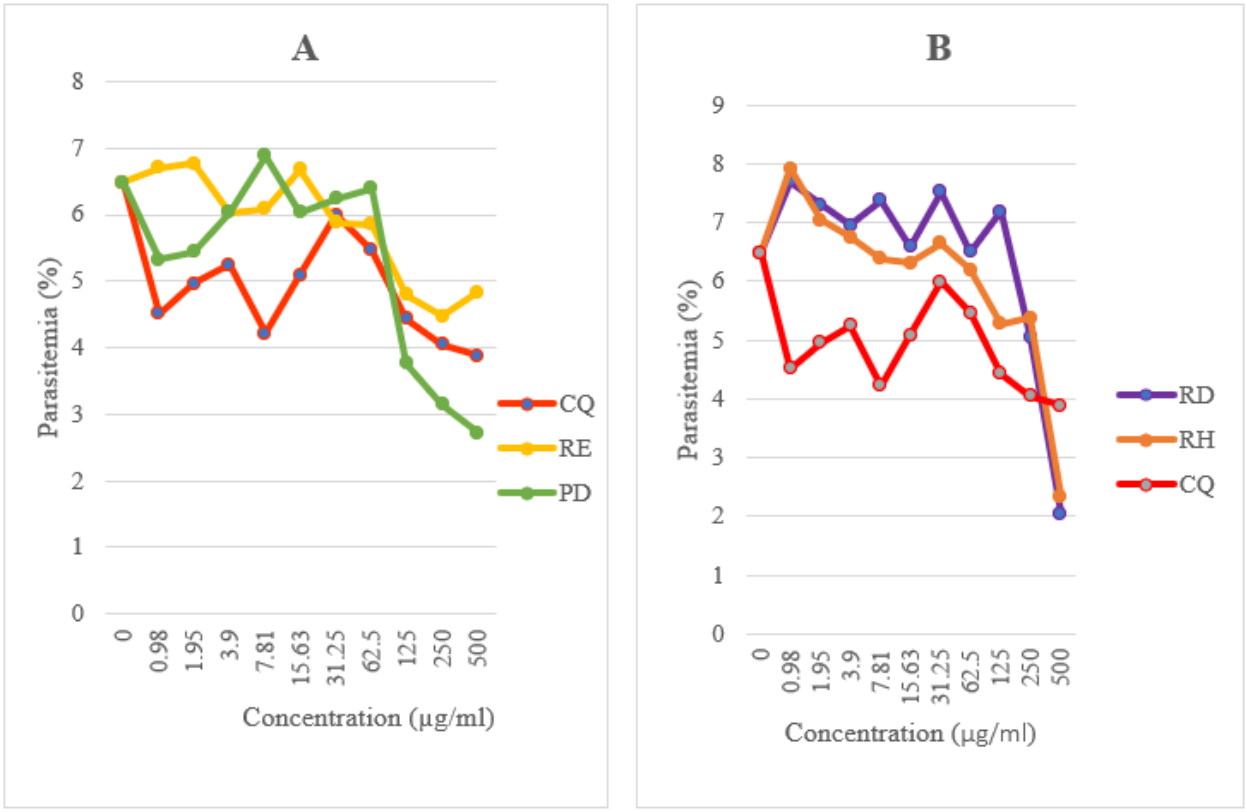

Figure 4.7: The trends of change of parasitemia with increase in extract concentration of the ethyl acetate extract of *R. steudelii* (RE) and dichloromethane extract of *P. sepialis* (PD).

A - dichloromethane extract of *R. steudelii* (RD), **B** - hexane extract of *R. steudelii* (RH). CQ is Chloroquine, the positive standard.

Several extracts, just like in light microscopy, illustrated a trend of decreased parasitemia with increase in extract concentration. These include the hexane, dichloromethane and ethyl acetate extracts of *R. steudelii* and the dichloromethane extract of *P. sepialis*. Chloroquine, however, was more potent than all the extracts.

The trend of anti - plasmodial activity with changing concentration of the extracts is shown in figure 4.8 below.

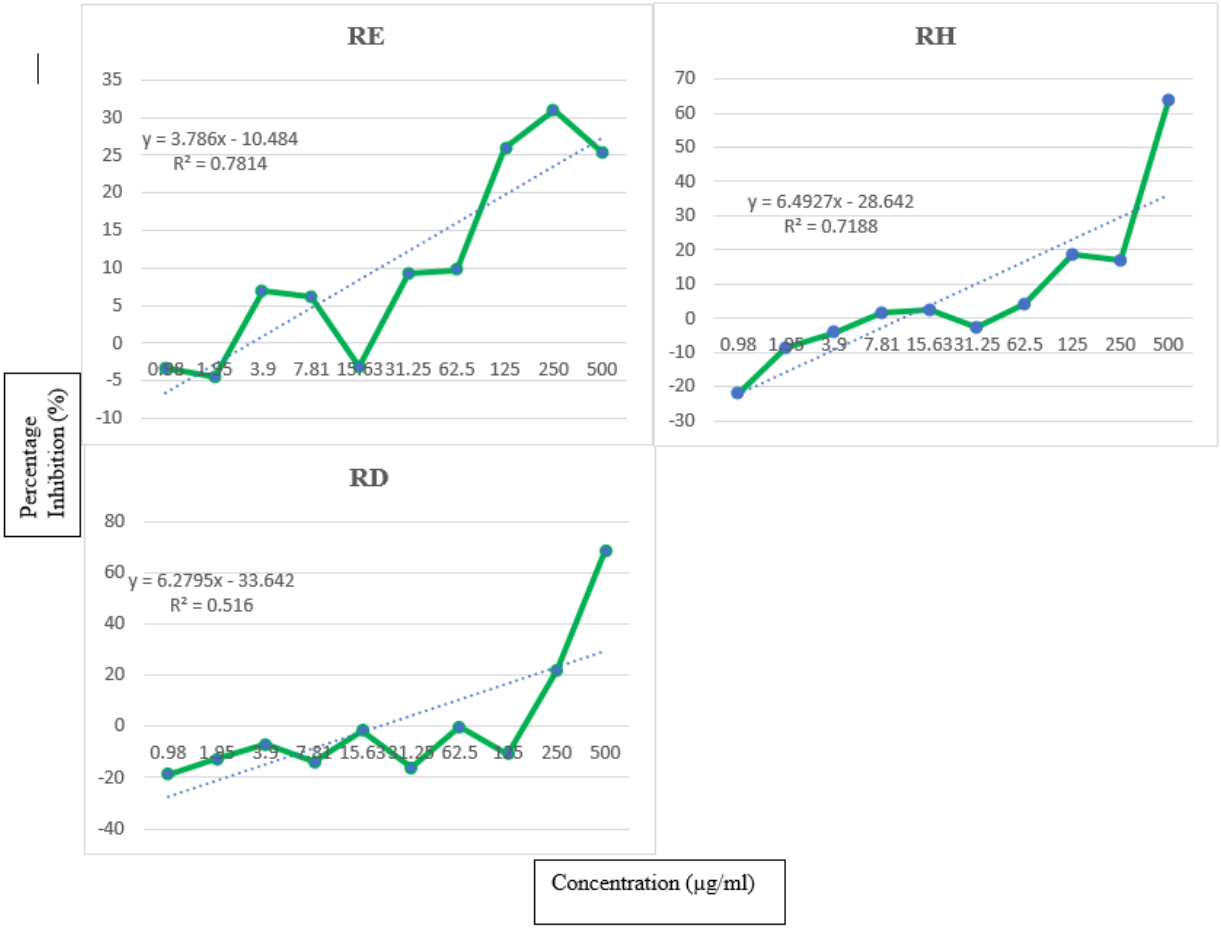

Figure 4.8: Graphs illustrating *pf* inhibition trends of RE, RH and RD, which are the ethyl acetate, hexane and dichloromethane extracts of *R. steudelii*, respectively.

Figure 4.8 above illustrates a trend of increase in *P. falciparum* (pf) inhibition percentage with increase in concentration of the *R. steudelii* extracts of the dichloromethane, hexane and ethyl acetate. The goodness of fit was analyzed using the R^2 value, whereby R^2 value greater than 0.5 was considered an indicator of significant relationship between the variables.

To determine the potency of the plant extracts, IC_{50} values were calculated and potency determined by comparing the values with pre- determined potency categorization. The IC_{50} value is the scientific criterion of determining the anti - plasmodial activity of plant extracts. The categorization of the potency of the test samples was guided by the table 4.2, as adopted by Basco and co- workers and illustrated by (Batista, Jesus, Júnior, & Oliveira, 2009).

Table 4.2: The Categorization of anti - plasmodial activity of plant extracts.

IC_{50} value (µg/ml)	**Category of activity**
<1	Active strong
< 10	Good activity
10– <50	Moderate activity
50-100	Low activity
>100	Inactive

IC_{50} values obtained mostly illustrated moderate activity as illustrated in Table 4.3.

Table 4.2. Table showing the IC_{50} values of the extracts in the study.

Plant	**Solvent**	**Code**	**IC_{50} (µg/ml) (Microscopy)**	**IC_{50} (µg/ml) (FCM)**
P. sepialis	Water	PA	18.45	15.89
	Methanol	PM	34.29	15.93
	Dichloromethane	PD	14.77	12.14
	Ethyl acetate	PE	35.05	-101.98
	Hexane	PH	14.37	-29.84
R. steudelii	Water	RA	10.49	13.83
	Methanol	RM	13.24	46.26
	Ethyl acetate	RE	8.54	15.98
	Dichloromethane	RD	3.46	13.32
	Hexane	RH	7.26	12.11

Flow cytometry demonstrated that several extracts of *R. steudelii* and *P. sepialis* have anti - plasmodial activity and this was also illustrated by optic microscopy, which showed similar drug - response trends of the same panel of extracts. Light microscopy is the gold standard test in anti - plasmodial drug - sensitivity assays (Russell et al., 2013) and in clinical malaria diagnosis (Mukadi et al., 2016). Flow cytometry, however, is a more definitive test of the anti - plasmodial assays as it rids off operator bias that occurs in microscopy (Chevalley et al., 2010); it has more sensitivity and specificity because it automatically identifies the parasites via their nucleic material. Furthermore, use of FACS technology is less labor intensive and quite rapid (Karl et al., 2009).

Flow cytometry had the hexane and the dichloromethane extracts of *R. steudelii* as the most potent showing moderate activity as per the categorization of the anti - plasmodial potency (Batista et al., 2009). These were 12.11 µg/ml and 13.32 µg/ml respectively. Light microscopy illustrated similar drug - response trends for the hexane and dichloromethane extracts of *R. steudelii* as for flow cytometry. The water and ethyl acetate extracts of *R. steudelii* had IC_{50} values of 13.83 µg/ml and 15.98 µg/ml respectively. Dichloromethane, water and methanol extracts of *P. sepialis* had an IC_{50} value of 12.13 µg/ml, 15.89 µg/ml and 15.93 µg/ml respectively. All illustrating moderate anti - plasmodial activity and hence potential sources of anti - malarial agents.

Hexane and ethyl acetate extracts of *P. sepialis* gave negative IC_{50} values of -29.8 µg/ml and -101.9 µg/ml. This may mean that the parasitemia of sample is significantly more than that of control sample. In calculation of the parasite inhibition percentage, the following formula is used;

$Percentage\ inhibition\ = \left\{\frac{(IRBC\ Parasitemia\ -Test\ sample\ Parasitemia)}{IRBC\ Parasitemia}\right\} \times 100\%$. A sample with parasitemia values greater than the control sample (IRBC parasitemia), would generate negative percentage inhibition values, which eventually would give a negative IC_{50} value. This was observed in the ethyl acetate extract of *P. sepialis* whereby at concentrations of 15.63 µg/ml, 31.25 µg/ml, 62.5 µg/ml and 125 µg/ml, the parasitemia was at 6.53 %, 6.87 %, 6.88 % and 7.55 % respectively, above the parasitemia of control sample of 6.2 %, hence giving negative percentage inhibition values. A study was done by (Calabrese & Baldwin, 2003) that indicated that it is possible to obtain enhanced parasitemia at low doses of an anti - plasmodial agent, giving a negative percentage inhibition value. Higher doses of the anti - plasmodial agent may then be inhibitory, a phenomenon known as hormesis. However, as much as this may be a possible explanation for the negative IC_{50} values for the two extracts, this was not proven in this study.

4.4 Comparison of Assays

The anti - plasmodial activity from the two assays, light microscopy and flow cytometric method (FCM), were compared to test for agreement between methods. This analytical tool was adopted from the Bland-Altman method (Bland & Altman, 1999), as previously used by (Karl et al., 2009).

Table 4.4: The data on agreement between methods.

	MICROSCOPY	**FCM**			
SAMPLE	**A** **IC_{50} (µg/ml)**	**B** **IC_{50}** **(µg/ml)**	**Mean** **(A+B)/2**	**A-B**	**[(A-B)/Mean] %**
CQ	2.432878	7.27577	4.854324	-4.84289	-99.7645
RD	3.459164	13.31985	8.389507	-9.86069	-117.536
RH	7.259007	12.11237	9.68569	-4.85337	-50.1086
RE	8.543821	15.9757	12.25976	-7.43188	-60.6201
RA	10.4894	13.83418	12.16179	-3.34477	-27.5023
RM	13.24045	46.25745	29.74895	-33.017	-110.985
PH	14.3668	-29.8444	-7.7388	44.21118	-571.293
PD	14.76839	12.13856	13.45347	2.629832	19.5476
PA	18.45492	15.89253	17.17373	2.562387	14.92039
PM	34.29481	15.92967	25.11224	18.36514	73.13222
PE	35.04654	-101.978	-33.466	137.025	-409.446
Mean				**12.85845**	-121.787

A graph of the difference between the two methods, A-B, against the mean of the two methods, [(A+B)/2] was plotted as shown in the figure 4.9.

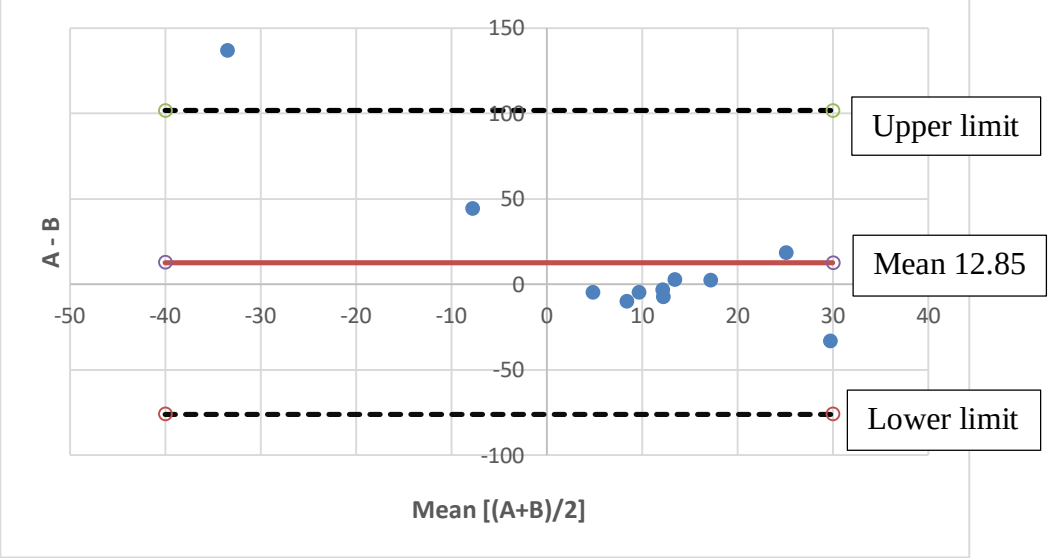

Figure 4.9: A plot of difference between methods, A-B, against the mean of the methods, [(A+B)/2]

From the above calculations guided by Table 4.4 above, the bias between the two methods is 12.85 units, represented by the gap between 0 and 12.85 on the y- axis of figure 4. The bias was obtained by calculating the mean difference, which is the of mean of the sum of differences between the two methods. The standard deviation (SD) was found to be 45.35, hence the upper and lower limits agreement was set at 101.74 and -76.02 respectively (95% CI).

CHAPTER 5 : GENERAL DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 General discussion

Extraction of medicinal plants has occurred for centuries with different plants used in treatment of different ailments. Effective treatment against malaria has always been sought through plant extracts so as to deal with the developing and spreading multi- drug resistant malaria parasites. In this study *P. sepialis* and *R. steudelii* were extracted using five solvents each, varying across the polarity scale to ensure exhaustive extraction of all the secondary metabolites. The solvents ranged from water> methanol> ethyl acetate> dichloromethane> hexane arranged in decreasing polarity.

The phytochemical classes of *P. sepialis* were established by documented tests, as the secondary metabolites for *R. steudelii* had been earlier established through previous studies.

Light microscopy and flow cytometry were used as the anti - plasmodial assay methods. The gold standard for malaria diagnosis and anti - plasmodial assay is light microscopy, while flow cytometry quantitatively establishes the anti - plasmodial activity potency of the extracts. The parasitemia at various extract concentrations was determined and percentage inhibition calculated. The IC_{50} values were derived and categorized according to pre- determined levels of potency.

5.2 Conclusions

R. steudelii, especially the non- polar secondary metabolites, have shown activity against the *Plasmodium falciparum* parasite, as indicated by the IC_{50} values of hexane, dichloromethane and ethyl acetate extracts. The water extract also exhibited moderate activity, and this confirms its use ethnobotanically. The activities of these extracts established by flow cytometry ranged from an IC_{50} value of 12.11 μg/ml to 46.26 μg/ml.

P. sepialis also exhibited activity against *p. falciparum,* with the dichloromethane, methanol, water extracts having moderate anti - plasmodial activity, with an IC_{50} value ranging from 12.14- 15.93 μg/ml.

These IC_{50} values are backed up with good data from phytochemical test results which indicated that these two plants have secondary metabolites in classes that are documented to have good therapeutic value in malaria management, such as triterpenes, phenols, tannins and saponins.

5.3 Recommendations

This being a study based on activity of crude extracts, potency may not be clearly demonstrated, unless pure compounds are isolated and tested. Hence further processing is required for these extracts that were found to be moderately active to isolate the pure compounds and test for their separate activity and synergy, because two or multiple secondary metabolites may be working in synergy to produce anti - plasmodial activity, a characteristic that cannot be displayed by one component in isolation.

The use of IC_{50} values could also contribute to getting 'inactive' outcomes from plant extracts that are effectively used traditionally, due to the common practice of basing the *P. falciparum* cultures on the erythrocytic stage, as also observed by Lemma et al. (2017), and consequently the IC_{50} values generated are based on the same parasite cycle stage. This definitely excludes secondary metabolites that have mechanism of action linked to extra-erythrocytic stages of the parasite. Therefore, it is mandatory that the extra- erythrocytic techniques are also used to assay all possible targets of action and give a more holistic approach.

BIBLIOGRAPHY

Abdolbaset Ghorbani, Farzaneh Naghibi, M. M. (2006). Ethnobotany, Ethnopharmacology and Drug Discovery. *Iranian Journal of Pharmaceutical Sciences*, *2*(2), 109–118. Retrieved from https://www.researchgate.net/publication/228617295_Ethnobotany_Ethnopharmacology_and_Drug_Discovery

Autino, B., Noris, A., Russo, R., & Castelli, F. (2012). Epidemiology of malaria in endemic areas. *Mediterranean Journal of Hematology and Infectious Diseases*, *4*(1). https://doi.org/10.4084/MJHID.2012.060

Batista, R., Jesus, A. De, Júnior, S., & Oliveira, A. B. De. (2009). Plant-Derived Antimalarial Agents: New Leads and Efficient Phytomedicines. Part II. Non-Alkaloidal Natural Products. *Molecules*, *14*, 3037–3072. https://doi.org/10.3390/molecules14083037

Calabrese, E. J., & Baldwin, L. A. (2003). Chemotherapeutics and hormesis. *Critical Reviews in Toxicology*, *33*(3–4), 305–353. https://doi.org/10.1080/713611041

Chevalley, S., Coste, A., Lopez, A., Pipy, B., & Valentin, A. (2010). Flow cytometry for the evaluation of anti-plasmodial activity of drugs on Plasmodium falciparum gametocytes. *Malaria Journal*, *9*(1), 1–7. https://doi.org/10.1186/1475-2875-9-49

Contreras, C. E., Rivas, M. A., Domínguez, J., Charris, J., Palacios, M., Bianco, N. E., & Blanca, I. (2004). Stage-specific activity of potential antimalarial compounds measured in vitro by flow cytometry in comparison to optical microscopy and hypoxanthine uptake. *Memorias Do Instituto Oswaldo Cruz*, *99*(2), 179–184. https://doi.org/10.1590/S0074-02762004000200011

Dahiru, D., Onubiyi, J. A., & Umaru, H. A. (2006). Phytochemical screening and antiulcerogenic effect of Moringa oleifera aqueous leaf extract. *African Journal of Traditional, Complementary and Alternative Medicines*, *3*(3), 70–75.

Fern, K. (2019). Phyllanthus sepialis. Retrieved from Tropical Plants Database website: tropical.theferns.info/viewtropical.php?id=Phyllanthus+sepialis

Gebrie, E., Makonnen, E., Debella, A., & Zerihun, L. (2005). Phytochemical screening and pharmacological evaluations for the antifertility effect of the methanolic root extract of Rumex steudelii. *Journal of Ethnopharmacology*, *96*(1–2), 139–143. https://doi.org/10.1016/j.jep.2004.08.026

Geethangili, M., & Ding, S. T. (2018). A review of the phytochemistry and pharmacology of Phyllanthus urinaria L. *Frontiers in Pharmacology*, *9*(OCT), 1–20. https://doi.org/10.3389/fphar.2018.01109

Guyatt, H. L., & Snow, R. W. (2004). Impact of Malaria during Pregnancy on Low Birth Weight in Sub-Saharan Africa Helen. *American Society for Microbiology*, *17*(4), 760–769. https://doi.org/10.1128/CMR.17.4.760

Harborne, J. B. (1998). Phytochemical Methods A Guide to Modern Techniques of Plant Analysis.

In *Chapman and Hall* (3rd ed.). Retrieved from https://books.google.co.ke/books?id=vCWHUU6iobwC&printsec=frontcover&dq=phytochemistry&hl=en&sa=X&ved=0ahUKEwid4rGH-PDjAhWGy4UKHRuVCX4Q6AEITDAI#v=onepage&q=phytochemistry&f=false

Justice, D. (2016). The role of botanical gardens in plant conservation©. *Acta Horticulturae*, *1140*(February), 127–129. https://doi.org/10.17660/ActaHortic.2016.1140.27

Karl, S., Wong, R. P., St Pierre, T. G., & Davis, T. M. (2009). A comparative study of a flow-cytometry-based assessment of in vitro plasmodium falciparum drug sensitivity. *Malaria Journal*, *8*(1), 1–11. https://doi.org/10.1186/1475-2875-8-294

Lemma, M. T., Ahmed, A. M., Elhady, M. T., Ngo, H. T., Vu, T. L. H., Sang, T. K., … Karbwang, J. (2017). Medicinal plants for in vitro antiplasmodial activities: A systematic review of literature. *Parasitology International*, *66*(6), 713–720. https://doi.org/10.1016/j.parint.2017.09.002

Lewis-Beck, M., Bryman, A., & Futing Liao, T. (2012). R-Squared. *The SAGE Encyclopedia of Social Science Research Methods*, 1187–1190. https://doi.org/10.4135/9781412950589.n877

MoH. National guidelines for the diagnosis, treatment and prevention of malaria in kenya. , EAC IRC Repository § (2006).

Mukadi, P., Lejon, V., Barbé, B., Gillet, P., Nyembo, C., Lukuka, A., … Jacobs, J. (2016). Performance of microscopy for the diagnosis of malaria and human African trypanosomiasis by diagnostic laboratories in the democratic Republic of the Congo: Results of a nation-wide external quality assessment. *PLoS ONE*, *11*(1), 1–15. https://doi.org/10.1371/journal.pone.0146450

Mukungu, N., Abuga, K., Okalebo, F., Ingwela, R., & Mwangi, J. (2016). Medicinal plants used for management of malaria among the Luhya community of Kakamega East sub-County , Kenya. *Journal of Ethnopharmacology*, *194*, 98–107. https://doi.org/10.1016/j.jep.2016.08.050

Muthaura, C. N., Keriko, J. M., Derese, S., Yenesew, A., & Rukunga, G. M. (2011). Investigation of some medicinal plants traditionally used for treatment of malaria in Kenya as potential sources of antimalarial drugs. *Experimental Parasitology*, *127*(3), 609–626. https://doi.org/10.1016/j.exppara.2010.11.004

Noor, A. (2016). The epidemiology and control profile of malaria in Kenya. *Ministry of Health*, *june*, *2016*(June), 1–78.

Pandey, M., Debnath, M., Gupta, S., & Chikara, S. K. (2011). Phytomedicine: An ancient approach turning into future potential source of therapeutics. *Journal of Pharmacognosy and Phytotherapy*, *3*(2), 27–37. Retrieved from https://www.researchgate.net/publication/215511510_Phytomedicine_An_ancient_approach_turning_into_future_potential_source_of_therapeutics

Perfetto, S. P., Chattopadhyay, P. K., & Roederer, M. (2004). *Seventeen-colour flow cytometry:*

unravelling the immune system. *4*(August), 1160–1163.

PMI. (2019). *President ' s Malaria Initiative: Malaria Operational Plan for Kenya 2019* (2019th ed.). Retrieved from https://www.pmi.gov/docs/default-source/default-document-library/malaria-operational-plans/fy19/fy-2019-kenya-malaria-operational-plan.pdf?sfvrsn=3

Russell, B., Malleret, B., Suwanarusk, R., Anthony, C., Kanlaya, S., Lau, Y. L., … Renia, L. (2013). Field-based flow cytometry for ex vivo characterization of plasmodium vivax and P. falciparum antimalarial sensitivity. *Antimicrobial Agents and Chemotherapy*, *57*(10), 5170–5174. https://doi.org/10.1128/AAC.00682-13

Sinha, S., Sarma, P., Sehgal, R., & Medhi, B. (2017). Development in Assay Methods for in Vitro Antimalarial Drug Efficacy Testing : A Systematic Review. *Frontiers in Pharmacology*, *8*(October), 1–14. https://doi.org/10.3389/fphar.2017.00754

Trotter, R. T., Logan, M. H., Rocha, J. M., & Boneta, J. Lou. (1983). Ethnography and bioassay: combined methods for a preliminary screen of home remedies for potential pharmacological activity. *Journal of Ethnopharmacology*, *8*(1), 113–119. https://doi.org/10.1016/0378-8741(83)90092-2

Tung, J. W., Heydari, K., Tirouvanziam, R., Sahaf, B., Parks, D. R., Herzenberg, L. A., & Herzenberg, L. A. (2007). Modern Flow Cytometry: A Practical Approach. *Clinics in Laboratory Medicine*, *27*(3), 453–468. https://doi.org/10.1016/j.cll.2007.05.001

UNICEF/WHO. (2015). Reversing the Incidence of Malaria 2000–2015. *WHO Global Malaria Programme*, 1–40. https://doi.org/10.1007/s00267-009-9280-1

Vasas, A., & Orbán-gyapai, O. (2015). The Genus Rumex: Review of traditional uses, phytochemistry and pharmacology. In *Journal of Ethnopharmacology*. https://doi.org/10.1016/j.jep.2015.09.001

Watsierah, C. A., & Ouma, C. (2014). Access to artemisinin-based combination therapy (ACT) and quinine in malaria holoendemic regions of western Kenya. *Malaria Journal*, *13*(1), 1–7. https://doi.org/10.1186/1475-2875-13-290

Wellems, T. E., & Plowe, C. V. (2001). Chloroquine-Resistant Malaria. *Journal of Infectious Diseases*, *184*, 770–776.

Wells, T. N. C. (2011). Natural products as starting points for future anti-malarial therapies : going back to our roots ? *Malaria Journal*, *10*(Suppl 1), S3. https://doi.org/10.1186/1475-2875-10-S1-S3

WHO. (2015). Guidelines for the treatment of Malaria. *WHO*, *35*(3), 1–317. https://doi.org/10.1046/j.1472-765X.2002.01179.x

WHO. (2018). World Malaria report 2018. *WHO Global Malaria Programme*, *1*(1), 1–210. Retrieved from https://www.who.int/malaria/publications/world-malaria-report-2018/en/

APPENDICES

Appendix A. 3: Parasitemia values (%) obtained in Optic Microscopy for the plant extracts at different concentrations.

CONCENTRATION (µg/ml)	**RD**	**RH**	**RA**	**RM**	**RE**	**PD**	**PA**	**PH**	**PE**	**PM**	**CQ**
0	1.89	1.89	1.89	1.89	1.89	1.89	1.89	1.89	1.89	1.89	1.89
0.98	0.96	1.21	2.71	1.7	1.55	1.98	1.52	1.51	1.2	0.84	1.67
1.95	0.86	1.84	2.56	1.9	2.1	2.1	1.19	1.45	1.91	1.61	0.34
3.9	0.34	1.71	1.2	2.1	2.22	1.6	1.42	1.26	0.78	1.2	0.85
7.82	0.61	1.16	2.12	1.63	0.83	1.49	1.96	1.14	1.98	2.16	1.31
15.63	0.82	1.45	1.82	2.28	2.43	2.58	1.96	1.29	1.52	2.51	0.51
31.25	0.77	1.3	1.42	2.88	2.17	1.91	2.18	1.81	1.98	1.58	0.61
62.5	0	0.71	1.63	1.81	0.68	1.68	1.19	2.03	2.44	1.66	0.68
125	0	0.68	1.35	2.1	0.67	1.57	1.36	1.57	0.76	2.46	1.08
250	0	0.63	1.18	0.77	0.67	1.82	1.65	1.74	1.57	1.84	0.77
500	0	0	0.34	0	0	0.29	0.68	0.4	1.16	0	0.48

Appendix A. 4: Parasitemia values (%) obtained in Flow Cytometry for the plant extracts at different concentrations.

SAMPLE CONCENTRATION (µg/ml)	**RD**	**RH**	**RM**	**RE**	**RA**	**PD**	**PH**	**PM**	**PE**	**PA**	**CQ**
0	6.48	6.48	6.48	6.48	6.48	6.48	6.48	6.48	6.48	6.48	6.48
0.98	7.7	7.91	7.13	6.7	6.63	5.32	4.01	7.01	6.24	6.3	4.51
1.95	7.3	7.04	7.27	6.77	7.95	5.44	5.18	5.82	4.3	6.81	4.96
3.9	6.95	6.75	7.88	6.03	6.76	6.04	5.59	5.21	5.34	6.58	5.25
7.81	7.37	6.38	8	6.08	5.09	6.89	5.26	5.93	4.2	6.41	4.22
15.63	6.59	6.31	5.37	6.68	7.04	6.04	6.53	5.42	5.67	8.82	5.09
31.25	7.53	6.65	7.34	5.88	6.88	6.24	6.87	6.94	5.05	5.84	5.99
62.5	6.5	6.2	6.5	5.85	7.13	6.4	6.88	6.75	5.22	6.8	5.47
125	7.18	5.27	7.07	4.8	5.68	3.76	7.55	5.5	5.75	5.03	4.43
250	5.06	5.37	7.6	4.47	1.71	3.14	5.36	4.6	6.59	4.96	4.05
500	2.04	2.34	5.91	4.84	5.31	2.72	3.54	2.94	4.33	3.5	3.88