IN VITRO AND IN VIVO ANTI-PLASMODIAL ACTIVITIES OF SENNA OCCIDENTALIS ROOTS EXTRACTS AGAINST PLASMODIUM FALCIPARUM AND PLASMODIUM BERGHEI

JULIUS W. TEAHTON, JR. (B. Sc.)
I56F/26082/2013

A Thesis Submitted in Partial Fulfillment of the Requirements for the Award of the Degree of Master of Science (Applied Parasitology) in the School of Pure and Applied Sciences of Kenyatta University

September 2016
DECLARATION

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

Signature........................................Date.............................................

Julius W. Teahton, Jr. (156F/26082/2013)
Department of Zoological Sciences
Kenyatta University

SUPERVISORS

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

Signature........................................Date.............................................

Prof. Michael M. Gicheru
Department of Zoological Sciences
Kenyatta University

Signature........................................Date.............................................

Dr. Hastings Ozwara
Department of Tropical and Infectious Diseases
Institute of Primate Research
Nairobi, Kenya
DEDICATION

I dedicate this work to my parents Mr. and Mrs. Julius W. Teahton, Sr. and my entire family for their inspiration during the time of my study.
ACKNOWLEDGEMENTS

I firstly thank the Almighty God for giving me the wisdom and knowledge to carry out this research. I acknowledge the unconditional guidance of my supervisors Prof. Michael M. Gicheru of Kenyatta University and Dr. Hastings Ozwara of the Institute of Primate Research who ensured that this work was well produced. I would also like to thank the IPR fraternity and the Department of Pharmacy, Kenyatta University for providing me with laboratory space, equipments and materials used in this study.

My special appreciation goes to the Ministry of Health of the Republic of Liberia and USAID/Liberia for awarding me scholarship that enabled me to pursue a degree in Master of Science in Applied Parasitology. I also appreciate the Kenyatta University Department of Zoological Sciences, School of Pure and Applied Sciences and Graduate School for facilitating my MSc training.

I acknowledge the Animal Resources Department and Malaria Program (IPR) laboratory staffs for giving me the wonderful opportunity to carry out this work in their laboratory and for being wonderful colleagues. I appreciate the technical assistance of Ms. E. Kagasi and Mr. V. Irungu in carrying out the experimental work, data cleaning and for always ensuring that I had what I needed. I thank my fellow graduate students for their useful discussions and sharing critical scientific input into various aspects of the work which enabled my development as a young scientist.

Finally, I am grateful to my sister, Mrs. Helen T. Kidka and my entire family for their support, encouragements, patience and prayers during the entire period of my studies.
TABLE OF CONTENTS

DECLARATION ....................................................................................... Error! Bookmark not defined.

DEDICATION .................................................................................................................. iii

ACKNOWLEDGEMENTS ................................................................................................. iv

TABLE OF CONTENTS ...................................................................................................... v

LIST OF TABLES .............................................................................................................. ix

LIST OF FIGURES ........................................................................................................... x

ACRONYMS AND ABBREVIATIONS ................................................................................. xii

DEFINITION OF TERMS ................................................................................................. xv

ABSTRACT ....................................................................................................................... xviii

CHAPTER ONE: INTRODUCTION ...................................................................................... 1

1.1 Background information ............................................................................................ 1
1.2 Statement of the problem ............................................................................................. 4
1.3 Justification for the study ........................................................................................... 4
1.4 Research questions ...................................................................................................... 6
1.5 Hypotheses .................................................................................................................. 6
1.6 Objectives of the study ............................................................................................... 6
1.6.1 General objective .................................................................................................... 6
1.6.2 Specific objectives .................................................................................................. 7
1.7 Significance of the study ............................................................................................. 7

CHAPTER TWO: LITERATURE REVIEW ........................................................................ 8

2.1 Classification of malaria parasites ............................................................................... 8
2.2 General Life cycle of \textit{plasmodium} species ........................................................... 8
2.2.1 Vertebrate phase of \textit{Plasmodium} life cycle ....................................................... 9
2.2.2 Invertebrate phase of \textit{Plasmodium} life cycle .................................................... 11
2.3 Plasmodium berghei .................................................................12
2.4 Plasmodium falciparum ..............................................................13
2.5 Malaria epidemiology .................................................................14
2.6 Malaria control measures .........................................................15
  2.6.1 Early and effective diagnosis ..............................................16
  2.6.2 Public health education ......................................................18
  2.6.3 Malaria vector control ......................................................19
2.7 Malaria prevention ..................................................................20
2.8 Malaria management and treatment .............................................21
  2.9.1 Malaria chemotherapy .......................................................22
  2.9.2 Chemoprophylaxis .............................................................34
  2.9.3 Use of traditional medicine in malaria treatment ..................35
  2.9.4 History of herbal medicines ..............................................36
  2.9.5 Herbal plants evaluated for anti-malarial activity .................37
  2.9.6 Current status of herbal medicines ....................................38
  2.9.7 Challenges facing the use of herbal medicines ....................39
  2.9.8 Plant Materials for extraction ............................................41
  2.10 Techniques in herbal extraction ............................................42
    2.10.1 Organic solvent extraction .........................................43
    2.10.2 Maceration .....................................................................43
    2.10.3 Extraction with supercritical gases .................................43
    2.10.4 Steam distillation ..........................................................44
    2.10.5 Cold pressing ...............................................................44
  2.11 Purification and concentration of herbal extracts ....................44
  2.12 Quality assurance in herbal extracts .....................................45
  2.13 Bioassay techniques for anti-malarial drug development ..........45
    2.13.1 In vitro anti-malarial drug testing .................................46
    2.13.2 In vivo anti-malarial drug testing ....................................46

CHAPTER THREE: MATERIALS AND METHODS .........................48

3.1 Study site and plant material ..................................................48

3.2 Preparation of materials and buffers for bioassays ....................48

3.3 Preparation of plant extracts for bioassays with malaria parasites ..49
3.4 Study design ........................................................................................................ 51
3.5 Experimental animals ....................................................................................... 51
3.6 Retrieval and culturing of *P. falciparum* parasites for bioassays ................. 51
3.7 Infection and propagation of *P. berghei* in BALB/c mice............................ 53
3.8 Staining of malaria parasites and determination of parasitaemia .......... 54
3.9 *In vitro* activity of plant extracts on *P. falciparum*................................. 55
3.10 *In vivo* activity of plant extracts on *P. berghei* in BALB/c mice ......... 56
3.11 Statistical data analysis .................................................................................. 60

**CHAPTER FOUR: RESULTS** ........................................................................... 61

4.1 Taxonomy of plant species.............................................................................. 61
4.2 Growth characteristics of *P. falciparum* and *P. berghei* parasites .............. 61
4.3 *In vitro* chemotherapeutic activities of *S. occidentalis* roots extracts on *P. falciparum* ............................................................................................................. 63
   4.3.1 Activity of *S. occidentalis* roots extracts on *P. falciparum* -W2 strain..... 63
   4.3.2 Activity of *S. occidentalis* roots extracts on *P. falciparum* -3D7 strain..... 65
4.5 Summary of IC_{50} values for *S. occidentalis* roots extracts against *P. falciparum* (W2 and 3D7 strains) ............................................................................................................. 66
4.6 *In vivo* activities of the three plant extracts on *P. berghei*...................... 67
   4.6.1 Suppressive activity of the plant extracts on *P. berghei* in mice ............ 68
   4.6.2 Prophylactic activities of plant extract on *P. berghei* in mice ............... 73
   4.6.3 Curative activities of plant extract on *P. berghei* in mice .................... 77
   4.6.4 Survival of mice in suppressive activities.............................................. 82
   4.6.5 Survival of mice in prophylactic activities .......................................... 87
   4.6.6 Survival of mice in curative activities with plant extracts ...................... 92

**CHAPTER FIVE: DISCUSSION** ................................................................. 98

5.1 Plant material and extracts ........................................................................... 98
5.2 *In vitro* anti-plasmodial activities and IC_{50} values.................................. 99
5.3 Suppressive and curative activities of *S. occidentalis* roots extracts .......... 101
5.4 Prophylactic activities of *S. occidentalis* roots extracts .......................... 103
5.5 Survival of mice treated with roots extracts of *S. occidentalis* .................. 104
5.6 Hypothesis Testing ......................................................................................... 106

CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS .......... 107

6.1 Conclusions ..................................................................................................... 107
6.2 Recommendations .......................................................................................... 108
6.3 Recommendations for future research ......................................................... 109

REFERENCES ..................................................................................................... 110

APPENDIX I: Statistical Analysis ................................................................. 123

APPENDIX II: Ethical Approval ....................................................................... 128

APPENDIX III: Roots of *Senna occidentalis* ............................................... 129
LIST OF TABLES

Table 2.1: Summary of Malaria vector control................................................................. 20

Table 4.1: Taxonomy of plant species investigated on Antiplasmoidal Activities.......... 61

Table 4.2: Range of IC$_{50}$ values for plant extracts on $P. falciparum$ (W2 – chloroquine resistance strain and 3D7 –chloroquine sensitive strain)................................. 67

Table 4.3: Effects of crude extract on suppression of $P. berghei$ infected mice for day four and day seven post infection. ................................................................. 70

Table 4.4: Effect of crude extract on prophylactic activities of $P. berghei$ infected mice.... 74

Table 4.5: Effects of crude extract on curative activities of $P. berghei$ infected mice........ 79

Table 4.6: Effects of crude extract on mean survival time of $P. berghei$ infected mice in suppressive activities. ................................................................. 84

Table 4.7: Effects of crude extract on mean survival time of $P. berghei$ infected mice in prophylactic activities. ................................................................. 89

Table 4.8: Effects of crude extract on mean survival time of $P. berghei$ infected mice in curative activities. ................................................................. 94
LIST OF FIGURES
Figure 2.1: A general life cycle of malaria parasite ................................................................. 9
Figure 2.2: Malaria Parasite Erythrocytic Stages ................................................................. 11
Figure 2.3: Schematic representation of the structure of Chloroquine .............................. 25
Figure 2.4: Schematic representation of Primaquine ......................................................... 26
Figure 2.5: Schematic representation of Primaquine .......................................................... 27
Figure 2.6: Structure of artemisinin and its derivatives ....................................................... 31
Figure 2.7: Schematic representation of Proguanil ............................................................. 35
Figure 4.1: Growth characteristics of *P. berghei* and *P. falciparum* in mice and culture..... 62
Figure 4.2: Percentage inhibition of *P. falciparum* (W2 strain) treated with extracts
   from *S. occidentalis* roots, artemeter and chloroquine .............................................. 64
Figure 4.3: Percentage inhibition of *P. falciparum* (3D7 strain) treated with extracts
   from *S. occidentalis* roots, artemeter and chloroquine ........................................... 66
Figure 4.4: Dose-dependent percentage inhibition of parasitaemia in mice placed on 4-
   day/suppressive treatment with aqueous extract and pyrimethamine ................. 71
Figure 4.5: Dose-dependent percentage inhibition of parasitaemia in mice placed on 4-
   day/suppressive treatment with methanol extract and pyrimethamine.......... 72
Figure 4.6: Dose-dependent percentage inhibition of parasitaemia in mice placed on 4-
   day/suppressive treatment with hexane extract and pyrimethamine ............. 73
Figure 4.7: Dose-dependent percentage inhibition of parasitaemia in mice placed on
   prophylactic treatment with aqueous extracts and pyrimethamine ............. 75
Figure 4.8: Dose-dependent percentage of inhibition of parasitaemia in mice placed on
   prophylactic treatment with methanol extracts and pyrimethamine............. 76
Figure 4.9: Dose-dependent percentage inhibition of parasitaemia in mice placed on
   prophylactic treatment with hexane extracts and pyrimethamine .......... 77
Figure 4.10: Dose-dependent percentage inhibition of parasitaemia in mice placed on curative treatment with aqueous extracts and pyrimethamine............................ 80

Figure 4.11: Dose-dependent percentage inhibition of parasitaemia in mice placed on curative treatment with aqueous extracts and pyrimethamine............................ 81

Figure 4.12: Dose-dependent percentage inhibition of parasitaemia in mice placed on curative treatment with hexane extracts and pyrimethamine ....................... 82

Figure 4.13: Survival of P. berghei-infected mice in suppressive regimen treated with aqueous extracts and pyrimethamine......................................................... 85

Figure 4.14: Survival of P. berghei-infected mice in suppressive regimen treated with methanol extracts and pyrimethamine......................................................... 86

Figure 4.15: Survival of P. berghei-infected mice in suppressive regimen treated with hexane extracts and pyrimethamine ......................................................... 87

Figure 4.16: Survival of P. berghei-infected mice in prophylactic regimen treated with aqueous extracts and pyrimethamine......................................................... 90

Figure 4.17: Survival of P. berghei-infected mice in prophylactic regimen treated with methanol extracts and pyrimethamine......................................................... 91

Figure 4.18: Survival of P. berghei-infected mice in prophylactic regimen treated with hexane extracts and pyrimethamine ......................................................... 92

Figure 4.19: Survival of P. berghei-infected mice in curative regimen treated with aqueous extracts and pyrimethamine......................................................... 95

Figure 4.20: Survival of P. berghei-infected mice in curative regimen treated with methanol extracts and pyrimethamine......................................................... 96

Figure 4.21: Survival of P. berghei-infected mice in curative regimen treated with hexane extracts and pyrimethamine ......................................................... 97
### ACRONYMS AND ABBREVIATIONS

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACTs</td>
<td>Artemisinin-Combined Therapies</td>
</tr>
<tr>
<td>ANOVA</td>
<td>Analysis of Variance</td>
</tr>
<tr>
<td>AQ+AS</td>
<td>Amodiaquine and Artesunate</td>
</tr>
<tr>
<td>BALB/c mouse</td>
<td>Mutant albino mice</td>
</tr>
<tr>
<td>BW</td>
<td>Body Weight</td>
</tr>
<tr>
<td>CAM</td>
<td>Complimentary or Alternative Medicine</td>
</tr>
<tr>
<td>CCM</td>
<td>Complete Culture Medium</td>
</tr>
<tr>
<td>Coartem®</td>
<td>Artemether-lumefantrine</td>
</tr>
<tr>
<td>CQ</td>
<td>Chloroquine</td>
</tr>
<tr>
<td>CSP</td>
<td>Circumsporozoite Protein</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethylsulfoxide</td>
</tr>
<tr>
<td>ED$_{50}$</td>
<td>Effective dose that kills 50% of the test organisms in vivo</td>
</tr>
<tr>
<td>HI</td>
<td>Heat Inactivated</td>
</tr>
<tr>
<td>i.p</td>
<td>Intraperitoneal</td>
</tr>
<tr>
<td>i.v</td>
<td>Intravenous</td>
</tr>
<tr>
<td>IC$_{50}$</td>
<td>Drug Concentration killing 50% of test organisms in vitro</td>
</tr>
<tr>
<td>ICM</td>
<td>Incomplete Culture Medium</td>
</tr>
<tr>
<td>IPR</td>
<td>Institute of Primate Research</td>
</tr>
<tr>
<td>IRS</td>
<td>Indoor Residual Spraying</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>IRC</td>
<td>Institutional Review Committee</td>
</tr>
<tr>
<td>KEMRI</td>
<td>Kenya Medical Research Institute</td>
</tr>
<tr>
<td>LLINs</td>
<td>Long Lasting Insecticides Nets</td>
</tr>
<tr>
<td>LD&lt;sub&gt;50&lt;/sub&gt;</td>
<td>Lethal Dose that kills 50% of experimental organisms</td>
</tr>
<tr>
<td>MOH</td>
<td>Ministry of Health</td>
</tr>
<tr>
<td>MST</td>
<td>Mean Survival Time</td>
</tr>
<tr>
<td>MeOH</td>
<td>Methanol</td>
</tr>
<tr>
<td>NMK</td>
<td>National Museums of Kenya</td>
</tr>
<tr>
<td>PATH</td>
<td>Partnership for Appropriate Technology in Health</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate Buffered Saline</td>
</tr>
<tr>
<td>PCV</td>
<td>Packed Volume Cell</td>
</tr>
<tr>
<td>PE</td>
<td>Pre-Erythrocytic</td>
</tr>
<tr>
<td>P&lt;sup&gt;H&lt;/sup&gt;</td>
<td>Power of Hydrogen ion concentration</td>
</tr>
<tr>
<td>RBCs</td>
<td>Red Blood Cells</td>
</tr>
<tr>
<td>RDTs</td>
<td>Rapid Diagnostic Tests</td>
</tr>
<tr>
<td>RPM</td>
<td>Revolutions Per Minute</td>
</tr>
<tr>
<td>RPMI</td>
<td>Rowal Park Memorial Institute</td>
</tr>
<tr>
<td>SEM</td>
<td>Standard Error Mean</td>
</tr>
<tr>
<td>SP</td>
<td>Sulphadoxine-Pyrimethamine</td>
</tr>
<tr>
<td>TI</td>
<td>Therapeutic Index</td>
</tr>
</tbody>
</table>
TM  Traditional Medicine

WHO  World Health Organization
## DEFINITION OF TERMS

<table>
<thead>
<tr>
<th>Term</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alkaloid</td>
<td>Highly active plant constituent containing nitrogen atoms.</td>
</tr>
<tr>
<td>Alternative Therapy</td>
<td>Treatment of disease by means other than conventional medical, pharmacological and surgical techniques.</td>
</tr>
<tr>
<td>Anthraquinone</td>
<td>A glycoside that acts as a laxative.</td>
</tr>
<tr>
<td>Antimalarial</td>
<td>Substance that is therapeutically effective against malaria.</td>
</tr>
<tr>
<td>Antiplasmodial</td>
<td>An agent that kills or inhibits growth of <em>Plasmodium</em> parasite.</td>
</tr>
<tr>
<td>Characterize</td>
<td>To characterize/describe one or more aspects of a phytochemical constituent of a herbal medicine.</td>
</tr>
<tr>
<td>Chemotherapy</td>
<td>The use of drugs to treat disease, especially drug therapy.</td>
</tr>
<tr>
<td>Coartem®</td>
<td>An ACT consisting of artemether-lumefantrine manufacture by Novartis and pre-qualified by WHO for treatment of uncomplicated malaria.</td>
</tr>
<tr>
<td>Complementary and Alternative Medicine (CAM)</td>
<td>Forms of treatment that is used in addition to (complementary) or instead of (alternative), standard treatments.</td>
</tr>
<tr>
<td>Complete Culture</td>
<td>Parasites growth medium consisting of RPMI 1640</td>
</tr>
<tr>
<td>Medium (CCM)</td>
<td>L-glutamine, NaHCO₃ and 20% PAN serum or 20% FCS.</td>
</tr>
<tr>
<td>Cryoprotectant</td>
<td>A freeze medium used in cryopreservation of parasites.</td>
</tr>
<tr>
<td>BALB/c mouse</td>
<td>Mutant strain of albino mouse whose genotype is 114&lt;sup&gt;tmNu/j&lt;/sup&gt;</td>
</tr>
<tr>
<td>Bioactive principle</td>
<td>A phytochemical compound sufficiently efficacious for herbal medicament in which it is</td>
</tr>
<tr>
<td>Term</td>
<td>Definition</td>
</tr>
<tr>
<td>----------------------</td>
<td>----------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Decoction</td>
<td>A water extract of bark or roots prepared by boiling for ten to twenty minutes.</td>
</tr>
<tr>
<td>Effective dose (ED$_{50}$)</td>
<td>Amount of drug kills 50% of the test population.</td>
</tr>
<tr>
<td>Ethnomedicine</td>
<td>Traditional medicines used by different ethnic communities.</td>
</tr>
<tr>
<td>Herbal Medicine</td>
<td>A medicament in which active principle(s) is/are not chemically synthesized and is/are constituent(s) of a plant.</td>
</tr>
<tr>
<td>Incomplete Culture</td>
<td>Parasites growth medium consisting of RMPI 1640 at pH 7.3, L-glutamine and NaHCO$_3$ only.</td>
</tr>
<tr>
<td>Medium (ICM)</td>
<td>L-glutamine and NaHCO$_3$ only.</td>
</tr>
<tr>
<td>Infusion</td>
<td>A preparation made by steeping the plant material in hot water for twenty minutes.</td>
</tr>
<tr>
<td>Lethal dose (LD$_{50}$)</td>
<td>Amount of drug that kills 50% of the test model.</td>
</tr>
<tr>
<td>Lyophilise</td>
<td>Removal of a solvent from a solution to obtain solid extract.</td>
</tr>
<tr>
<td>Maceration</td>
<td>To make soft by soaking or steeping to in a liquid (or) to separate into constituents by soaking.</td>
</tr>
<tr>
<td>Miscella</td>
<td>Crude extracts of a plant material in an extraction solvent.</td>
</tr>
<tr>
<td>Parasiteamia</td>
<td>Level of parasite infection in an organism as detected in blood.</td>
</tr>
<tr>
<td>Pharmacognosy</td>
<td>Screening the plant kingdom for bioactive compounds.</td>
</tr>
<tr>
<td>Phytochemistry</td>
<td>Chemical constituents present in plant fruits and vegetables.</td>
</tr>
<tr>
<td>Placebo</td>
<td>A pharmacologically inactive substance used as a control in scientific experiments.</td>
</tr>
<tr>
<td>Prophylactic</td>
<td>Any agent that prevents an infection or a disease.</td>
</tr>
<tr>
<td>Pulverize</td>
<td>Grind into fine powder.</td>
</tr>
<tr>
<td><strong>Recruscedence</strong></td>
<td>Re-emergence of an infection without new infections.</td>
</tr>
<tr>
<td>---------------------</td>
<td>------------------------------------------------------</td>
</tr>
<tr>
<td><strong>Repository</strong></td>
<td>Ability of a plant to prevent an infection.</td>
</tr>
<tr>
<td><strong>Saponins</strong></td>
<td>Active plant constituents that produce lather in water.</td>
</tr>
<tr>
<td><strong>Schizonticide</strong></td>
<td>A substance that destroys the schizont, a stage in the development of the malaria parasite.</td>
</tr>
<tr>
<td><strong>Therapeutic Index</strong></td>
<td>Correlation in biological activity between effective dose and lethal dose.</td>
</tr>
</tbody>
</table>
**ABSTRACT**

*Plasmodium* is a genus of parasitic protozoa, many of which cause malaria in their host. Five species namely; *P. falciparum, P. malarae, P. ovale, P. vivax* and *P. knowlesi* cause human malaria. Malaria due to *P. falciparum* is one of the most severe public health problems worldwide with an annual estimated 207 million clinical episodes and 627,000 deaths. The use of conventional antimalarial drugs as treatment for malaria is greatly hindered by drug resistance exhibited by the parasite. Consequently, many people in rural areas have opted for the use of medicinal plants despite the fact that there is no sufficient scientific confirmation of their efficacy. However, studies have suggested that plants contain bioactive compounds which have anti-plasmodial activities. One of such plants is *Senna occidentalis* (roots) which is been used by herbalist in different parts of Kenya to treat malaria. This study was therefore carried out to determine the *in vitro* and *in vivo* anti-plasmodial activities of *Senna occidentalis* roots from Oloolua Forest against *P. falciparum* and *P. berghei*. Aqueous, methanol, and hexane crude extracts were prepared from the roots of *S. occidentalis*. The *in vitro* assay using *P. falciparum* were performed each in triplicate in 96 – wells microtiter flat bottomed plates. All the extracts were subsequently assayed for *in vivo* (suppressive, curative and prophylactic) activities against *P. berghei* using a 4-day test in BALB/c mice. A total of 185 mice of both sexes were used in the three regimens in which experimental mice were treated with extracts at dose rates of 1000 mg/kg/day to 200 mg/kg/day for suppressive and 1000 mg/kg/day to 600 mg/kg/day for curative and prophylactic tests respectively being administered orally, while control groups received Phosphate-Buffered Saline and pyrimethamine at 4 mg/kg/day. Mean % parasitaemia and inhibitions were reported as means ± SEM while survivorship values were reported as mean ± SD and compared using one-way analysis of variance (ANOVA) and student t- test. All p-values < 0.05 were considered statistically significant. The results from the *in vitro* assays revealed that there were no significant differences between the both strains of *P. falciparum* (W2 – chloroquine resistant and 3D7- chloroquine sensitive strains) amongst the three extracts (p < 0.05). The crude extracts in all regimens showed a dose-dependent reduction in the levels of parasitaemia in the experimental groups of mice relative to the non-treated mice (p < 0.0001). The three extracts displayed significantly high parasitaemia inhibition at a dose rate of 1000 mg/kg/day (p < 0.05) in suppressive, prophylactic and curative tests respectively. The most effective chemotherapeutic agent was the methanolic extract with an average mean percentage inhibition of parasitaemia of 81.84%, 76.18% and 70.18% for suppressive, prophylactic and curative tests respectively. The extracts prolonged the mean survival time in all the experimental groups relative to the non-treated group (p < 0.0001). The results indicate that *S. occidentalis* roots possess bioactive anti-plasmodial compound. On the basis of this study, it is recommended that further study be undertaken to determine the *in vitro* and *in vivo* cytotoxicity effects of *S. occidentalis* roots extracts on cells and body organs.
CHAPTER ONE: INTRODUCTION

1.1 Background information

*Plasmodium* belongs to the genus of parasitic protozoans of the sporozoan subclass Coccidia that are the causative organisms of malaria. Five species cause human malaria namely: *Plasmodium falciparum, P. malarae, P. ovale, P. vivax* and *P. knowlesi*. Malaria due to *P. falciparum* is one of the most severe public health problems worldwide (WHO, 2005; Muregi, 2007). It is a leading cause of death and disease in many developing countries, where young children and pregnant women are the most affected groups. Half of the world’s population (3.4 billion people) lives in areas at risk of malaria transmission in 106 countries and territories.

In 2012, malaria caused an estimated 207 million clinical episodes and 627,000 deaths. An estimated 91% of deaths in 2010 were in the African Region (WHO, 2013). In sub-Saharan Africa, over 50% of all out patients’ visits and 30% - 50% of all hospital admissions are attributed to malaria (WHO, 2005; Muregi, 2007). Malaria caused by *P. falciparum* is the most dangerous (98.2% cases in Kenya) with the highest rates of complications and mortality (MOH, 2010). *Plasmodium knowlesi* is the fifth species of *Plasmodium* that has been recently identified as a clinically important pathogen in human and found in nature in macaques (monkeys). It accounts for up to 70% of malaria cases in
South East Asia, particularly the forested areas, where it is mostly found (McCutchan et al., 2008).

It is also estimated that economic losses due to malaria in Africa is about USD $12 billion annually (DFID, 2005). *Plasmodium* infects the red blood cells of mammals (including humans), birds, and reptiles. It occurs worldwide especially in tropical and temperate zones. The parasite is transmitted by the bite of the female *Anopheles* mosquito. Although an effective vaccine is the best long term control option for malaria, current work on vaccine development largely remains at preclinical stage. The declining efficacy of classical drugs due to increase of parasite resistant strains, resistance of vectors to insecticides and the difficulty in creating efficient vaccines have led to an urgent need for new anti-malaria drugs (Ridley, 2002). The decline in drug’s efficacy has led to change in treatment policy for malaria in the last 12 years from chloroquine (CQ) to sulphadoxine-pyrimethamine (SP) in 1998 and subsequently to the currently recommended artemisinin-based combination therapies (ACTs) in 2004 (MOH, 2010). While synthetic pharmaceutical agents continue to dominate in Research, attention has been increasingly directed to natural products (Gershell and Atkins, 2003). The success of artemisinin and its derivatives for the treatment of resistance malaria has focused attention to plants as a source of anti-malaria drugs (Tran et al., 1998).

Several studies on anti-malaria activity of traditional medicinal plants have shown that extracts from plants such as *Ajuga remota* (John et al., 2013),
Rubia cordifolia, Harrizonia abyssinica, Leucas Calostachys olive, Sanchus schweinfurthii (Nyambati et al., 2013), Phyllanthus emblica (Asoka et al., 2010), Withania somenifer and Vernonia amygdaline (Benoit, 1996) and Vernonia brachycalys (Oketch-Rabah et al., 2007) among others have anti-plasmodial effects against various species of Plasmodium. Other in vitro studies of Africa Medicinal Plants have also indicated promising anti-plasmodial activities (O’Neil et al., 1985; WHO, 1993). Therefore, plant can be used as a source of anti-malarial drugs development. John et al. (2013) investigated the in vivo anti-malarial activity of Ajuga remota water extracts against Plasmodium berghei in mice. Their findings indicated that the wet leaf extract of Ajuga remota had the active ingredient with the most effective percentage of parasite suppression of 90.2 % as compared to the air-dried and powdered flowers extract which showed less suppression of 17.2% parasitaemia.

Studies also conducted by Nyambati et al. (2013) on the in vitro anti-plasmodial activity of the crude extracts Rubia cordifolia, Harrizonia abyssinica, Leucas calostachys Olive and Sanchus schweinfurthii showed anti-plasmodial activity against P. knowlesi. Their findings also showed that the crude extracts had significant effects on P. knowlesi parasites. This also suggests that there are traditional medicinal plants that can be used synergistically to develop more potent anti-malarials. In addition, the various plants with anti-malarial potency provide a large reservoir for further
development of pharmaceuticals against malaria. *Senna occidentalis* is also one of those medicinal plants used by herbalist to treat malaria in Kenya.

1.2 Statement of the problem

The anti-*plasmodial* drugs have changed over the last 12 years from chloroquine (CQ) to sulphadoxine-pyrimethamine (SP) in 1998 and subsequently to the currently recommended artemisinin-based combination therapies (ACTs) in 2004 (MOH, 2010) due to the continuously increased resistance of different species of *Plasmodium* parasites strains. Resistance of vectors to insecticides and the difficulty in creating vaccines has led to an urgent development of anti-plasmodial drugs (Boland, 2001; Ridley, 2002). The resistance has at the same time increasingly extended to other available anti-*plasmodial* drugs (Peters, 1982). Presently, the commonly used anti-*plasmodium* drugs, *quinolines* and the peroxides anti-plasmodium (artemisinin derivatives) are modelled upon the plant based compounds, quinine and artemisinin respectively. The therapeutic success of these two plants against malaria has inspired researchers to search for new anti-*plasmodial* drugs from plants (Tran *et al.*, 1998).

1.3 Justification for the study

Drugs resistance, exorbitant cost and inaccessibility to antimalarial drugs are some of the major challenges that limit the use of antimalarial drugs. Artemisinin-Combined Therapy (ACTs), which is the most effective available treatments against *P. falciparum* malaria is costly, inaccessible and in short
supply especially in rural areas (WHO, 2005). The therapeutic success of quinine and artemisinin has directed efforts to develop novel antimalarial drugs using plants. Herbal (plant) drugs are available and affordable to majority of the infected and are sometimes perceived as more effective than the conventional anti-plasmodial drugs (Willcox and Bodeker, 2004). Such situation has therefore created a need to use plants as alternative anti-plasmodial therapy.

*Senna occidentalis* is one of the plants used traditionally by herbalist to treat malaria in Msambweni District in Kenya (Nguta *et al*., 2010) and formed the basis of the study. It has been shown to have anti-plasmodial activity and its phytochemical constituents include achrosin, aloemodin, emodin, anthraquinones, anthrones, apigenin, aurantiobtusin, campesterol, cassiollin, chryso-obtusin, chrysophanic acid, chrysarobin, chrysophanol, chrysoeriol (Vijayalakshmi *et al*., 2013). Some of these phytochemicals are polar and non-polar. *In vitro* assay is used to assess the right dosage by directly assessing the drug performance. Though the *in vivo* study serves as the gold standard to assess the performance and dosage of the drug, it also serves as a validation of data from the *in vitro* study thereby providing sufficient data for a drug to be used as malaria chemotherapy (Polli, 2008). Thus, this study was therefore, designed to determine the *in vitro* and *in vivo* effects of the anti-plasmodial activities of *S. occidentalis* roots extracts against *P. falciparum* and *P. berghei*. Mice are used in this study because they are well characterized anatomically and physiologically, possess great genetic diversity, small and prolific breeder.
The parasite (*P. berghei*) is used as a model for human malaria in mice. It is also a laboratory maintained parasite that is useful in screening plant extracts for bioactive compounds.

1.4 Research questions

i. What is the anti-plasmodial activity of *S. occidentalis* roots extracts against *P. falciparum*?

ii. What is the anti-plasmodial activity of *S. occidentalis* roots extracts against *P. berghei*?

iii. What solvent extracts between polar and non-polar show anti-plasmodial activity?

1.5 Hypotheses

i. *Senna occidentalis* plant extracts have no anti-plasmodial effect on *P. falciparum*.

ii. *Senna occidentalis* plant extracts have no anti-plasmodial effect on *P. bergehei*.

iii. There is no difference in the anti-plasmodial activity between polar and non-polar solvents of *S. occidentalis* roots extracts.

1.6 Objectives of the study

1.6.1 General objective

To determine the anti-plasmodial activities of *S. occidentalis* roots extracts against *P. berghei* and *P. falciparum*. 
1.6.2 Specific objectives

i. To determine the \textit{in vitro} anti-plasmodial activity of \textit{S. occidentalis} roots extract against \textit{P. falciparum}.

ii. To determine the \textit{in vivo} anti-plasmodial activity of \textit{S. occidentalis} roots extracts against \textit{P. berghei}.

iii. To compare the anti-plasmodial effects of polar and non-polar extracts.

1.7 Significance of the study

The study provided knowledge on the anti-plasmodial activities of \textit{S. occidentalis} roots extracts to be effective remedies against the fight of malaria thus; it can be used confidently as an alternative treatment of malaria. The results also suggest that there is a potential to make an effective and affordable anti-plasmodial therapy using \textit{S. occidentalis} thereby reducing cost in efficient management and treatment of malaria.
CHAPTER TWO: LITERATURE REVIEW

2.1 Classification of malaria parasites

Malaria causing organisms are grouped into nine subgenera of which three occur in mammals, four in birds, and two in lizards (Martinsen et al., 2008). *Plasmodium knowlesi*, which parasitizes non-human primates, occasionally infects humans. These protozoan haemoparasites fall in the subphylum Apicomplexa and class Aconoidasida (www.sanger.ac.uk). They belong to the order Haemosporidia, family Plasmodiidae and genus *Plasmodium* (Arisue and Hashimoto, 2015).

2.2 General Life cycle of *plasmodium* species

*Plasmodium* species have a complex life cycle, which is shared between a vertebrate host and an insect vector. The parasite enters the bloodstream through the bite of an infected female *Anopheles* mosquito (CDC, 2012). The cyclic development of malaria parasites involves three distinct cycles (Figure 2.1): Sporogonic, which occurs in the mosquito vector, pre-erythrocytic phase that takes place in the liver cells of the vertebrate host, and erythrocytic cycle that takes place in the red blood cells (RBCs).
2.2.1 Vertebrate phase of *Plasmodium* life cycle

There are two stages in which the *Plasmodium* parasites appear in the vertebrate host; the pre-erythrocytic and the erythrocytic stages. The pre-erythrocytic stage, also referred to as exo-erythrocytic (EE) cycle, begins when an infected mosquito injects sporozoites into a vertebrate host as it takes a blood meal. These sporozoites make their way to the liver within an hour where they each enter a liver cell. The surface coat of the sporozoites, the circumsporozoite protein (CSP) bears a ligand that binds specifically to receptors on the basolateral dornmain of the hepatocyte cell membrane (Coppi *et al*., 2011). Within the hepatic cell, these sporozoites transform into feeding trophozoites, maturing within a week to begin schizogony.
During schizogony, the trophozoites transform into tissue schizonts that rupture to release thousands of merozoites into the blood stream, initiating the erythrocytic phase. The merozoites penetrate erythrocytes in the blood and transform into trophozoites by ingesting the hosts’ haemoglobin and cytoplasm. The parasite grows filling more than half of the blood cell and develops asexually into mature schizonts (Figure 2.2), each consisting of a mean of ten erythrocytic merozoites (Ozwara et al., 2003). When development of merozoites is complete, the hosts’ erythrocytes lyse, releasing merozoites, the parasites’ metabolic wastes and haemozoin. Many merozoites are destroyed by the hosts’ reticulo-endothelial system (RES) and leukocytes while others immediately re-invade uninfected red cells (Dimitrov et al., 2015).

Depending on the species, erythrocytic schizogony lasts from one to four days. It takes 24 hours for *P. knowlesi*, 25 hours for *P. berghei* and 48 hours for *P. falciparum* (Breman, 2009). After numerous asexual generations, small proportion of the merozoites transform into macrogametocytes and microgametocytes that appear in the peripheral blood within 8-11 days (Breman, 2009). However, these sexual forms do not multiply like their asexual relations; they rather increase in size, filling almost the blood cell, and circulating within the hosts’ body waiting to be ingested by the next female *Anopheles* mosquito that feeds on the infected vertebrate host.
2.2.2 Invertebrate phase of *Plasmodium* life cycle

The sexual cycle of the *Plasmodium* parasite takes place in the gut of a suitable mosquito. Male and female gametes develop and differentiate when an appropriate mosquito ingests erythrocytes containing gametocytes. Alternatively, they are digested along with the blood or get phagocytised by the RES of the vertebrate host (Breman, 2009). A process involving the shifting of the nucleus towards the periphery occurs thereby resulting to macrogametocyte transforming into macrogamete. On the other hand, microgametocyte undergoes exflagellation. During this process, the microgametocyte divides within 10-12 minutes repeatedly to form six to eight daughter nuclei that
develop into microgametes. The microgamete swims about until it finds a macrogamete that it penetrates and fuses with the ovum to form a diploid zygote. Thereafter, the zygote transforms into a motile ookinete penetrating the peritrophic membrane in the mosquito’s gut and rounding up below the epithelium as an oocyst for two to three weeks (Ozwara et al., 2003).

Meiosis occurs within the oocyst resulting into a number of haploid nucleated masses called the sporoblasts. The sporoblast undergoes sporogeny to yield numerous sporozoites, and upon oocyst rupture, the sporozoites migrate to the salivary glands, ready for inoculation into a new vertebrate host at the next blood meal, beginning the cycle again. Depending on the species and temperature, sporozoites development takes place between ten days to two weeks. Once infected, the mosquito remains infective for life; capable of transmitting malaria to every susceptible vertebrate it bites (Breman, 2009).

2.3 Plasmodium berghei

*Plasmodium berghei* is one of the four species that have been described in murine rodents. Other murine parasites are *P. yoelii*, *P. chabaudi* and *P. vinckei*. *Plasmodium berghei* originated from the forests of Central Africa where its natural cyclic host are the thicket rat *Grammomys surdaster* and the mosquito *An. dureni* (Breman, 2009). The vector is restricted to the Katanga gallery in the Congo Forest at an attitude of between 1000-1700 meters. Due to the restricted freedom of range of its insect vector, *P. berghei* is, therefore, localized in Central Africa (Breman, 2009). In laboratories, the natural hosts
have been replaced by a number of commercially available laboratories mouse strains, and the mosquito *An. stephensi*, which is easily reared and maintained under laboratory conditions (www.sanger.ac.uk).

Rodent parasites are recognised as valuable organisms for the investigation of human malaria because they are similar in most essential aspects of morphology, physiology and life cycle and the manipulation of the complete life cycle of these parasites, including mosquito infections, is simple and safe (WHO, 2010). *Plasmodium berghei* is used in research programs for development and screening of antimalaria drugs and for the development of vaccines against malaria. *Plasmodium berghei* parasites like all other malaria parasites of mammals enter the liver after been injected into the bloodstream by a bite of an infected female *Anopheles* mosquito. These parasites leave the liver and invade the erythrocytes after a short period of development and multiplication (WHO, 2013). Anaemia and damage of essential hosts’ organs are cause by the multiplication of the parasite in the blood. *Plasmodium berghei* has a strong preference for invading reticulocytes with parasites growing in mature RBCs producing 6-12 merozoites per schizont while those growing in reticulocytes produce 12-18 merozoites per schizont. *Plasmodium berghei* (ANKA) in BALB/c mice causes rapidly fulminating infections leading to death within one week (Okokon et al., 2005).

2.4 *Plasmodium falciparum*
*Plasmodium falciparum* is the *Plasmodium* species responsible for 85% of the malaria cases. The four less common and less dangerous *Plasmodium* species are: *P. ovale*, *P. malariae*, *P. vivax* and *P. knowlesi*. Malaria infects over 200 million people annually, mostly in poor tropical and subtropical countries of Africa. It is the deadliest parasitic disease killing over one million people each year. Ninety percent of the deaths occur south of the Sahara desert and most are under five-year-old children. In addition to Africa, malaria occurs in South and Southeast Asia, Central and South America, the Caribbean and the Middle East (Breman, 2009).

### 2.5 Malaria epidemiology

Malaria is a haemoparasitic disease that affects humans, non-human primates, birds and rodents. It is caused by *Plasmodium* (*P*), a haemoparasite protozoan belonging to the subphylum Apicomplexa and transmitted by female mosquitoes of the genus *Anopheles* (*An*).

Human malaria is caused by four parasite species namely *P. falciparum*, *P. vivax*, *P. ovale* and *P. malariae*. *Plasmodium Knowlesi*, which causes malaria in primates, has now been established as a fifth human malaria parasite (Tek *et al.*, 2008).

Malaria is the World’s most important tropical disease. It is prevalent in about 100 countries and around 2, 400 million people are at risk (Kager 2002). Greater than 40% of the World’s population lives in malaria-endemic areas; 60% of malaria deaths worldwide occur in the poorest 20% of the population.
With high levels of transmission, malaria reduces gross domestic product by as much as 1.3% annually and in Africa, malaria is now the fourth-leading cause of years of productive life lost which significantly slows economic growth and development (PATH, 2011). Although malaria incidence has decreased from 244 million in 2005 to 225 million in 2009, this large absolute decrease was not observed in Africa (WHO, 2010). In Kenya, malaria is the leading cause of morbidity and mortality; out of a population of 34 million; 25 million Kenyans are at risk. Malaria also accounts for 30% - 50% of all outpatients’ attendants’ and 20% of all hospital admissions and an estimated 170 million working days are lost to malaria every year (MOH, 2010).

2.6 Malaria control measures

Early diagnosis and treatment of malaria; prevention of deaths; encouraging the use of personal protection materials such as the use of Insecticide Treated Nets (ITNs), forecasting of epidemic areas or countries, early detection and control, monitoring, evaluation and operative research are the most important aspect of many malaria control programs (MOH, 2010). The national malaria control strategy in any malaria-endemic areas or countries must develop a plan with malaria components that reflect the four strategic approaches in controlling malaria, namely; early and effective diagnosis, public health education, malaria vector and chemotherapy (WHO, 2005).
2.6.1 Early and effective diagnosis

In order prevent further spread of the infection, malaria must be recognized at the appropriate time and patient treated urgently. Early and rapid diagnosis of malaria cases are gaining importance in health programmes in endemic areas or countries in response to increasing drugs costs, accurate treatment leading to the reduction in malaria morbidity and mortality (MOH, 2010). There are few techniques employed in the early and effective diagnosis of malaria.

2.6.1.1 Clinical diagnosis

Clinical diagnosis is based on the patient’s symptoms and on physical findings at examination. The first symptoms of malaria include fever, chills, sweat, headaches, muscle pains, nausea and vomiting. For severe malaria; clinical findings such as convulsion, coma, neurologic focal signs, severe anemia and respiratory difficulties are prominent and may increase the suspicion index for malaria (Bartoloni and Zammarchi, 2012). Large proportion of the population can be infected but are not made ill by these parasites thereby making symptomatic diagnosis more complicated in highly endemic areas or countries. Consequently, in most cases the early clinical findings in malaria are not typical and need to be confirmed by a laboratory test (Bartoloni and Zammarchi, 2012).

2.6.1.2 Laboratory diagnosis

The parasites of malaria can be identified through microscopic examination by a drop of suspected patient’s blood, spread out as a blood smear on a
microscope slide. The specimen is stained with Giemsa prior to examination to give the parasites a distinguishing appearance (Bailey et al., 2013). By using this technique, clinicians can easily confirm clinical diagnoses, thus providing important information by identifying parasites species that are in circulation and initiating drug treatment. This technique is the gold standard for laboratory confirmation of malaria cases. Nevertheless, microscopic sensitivity depends on the quality of the reagents, the microscope, and the experience of the technician (Bailey et al., 2013).

2.6.1.3 Molecular diagnosis

The nucleic acids of the parasite are detected by using the polymerase chain reaction (PCR). By means of molecular amplification techniques, multiplex PCR tests have been developed for all four malaria species using circumsporozoite stage deoxyribose nucleic acids (DNA) sequences and target 18S single stranded ribosomal-ribose nucleic acid (rRNA). Despite the fact that it requires a specialized laboratory, this method is more accurate than microscopy (Thakor, 2000).

2.6.1.4 Serology

Antibodies against malaria parasites are detected by serology, using either enzyme-linked immunosorbent assay (ELISA) or indirect immunofluorescence assay (IFA). Both current infections and past experience are measured (WHO, 2015).
2.6.1.5 Rapid diagnostic tests

Rapid diagnostic tests development for malaria has offered a useful alternative to microscopy in situations where reliable microscopy is not accessible. Diagnosis by blood-based assay using lateral-flow immunochromatographic tests commonly called rapid diagnostic tests (RDTs) has offered great promise in extending rapid diagnosis to areas where conventional microscopy diagnosis was impractical. Additionally, *P. falciparum* growing resistance to antimalarial drugs requires the use of expensive combination therapies and accurate diagnosis. These tests use finger-prick or venous blood and take 10-15 minutes (Thakor, 2000).

2.6.2 Public health education

The most important link in the malaria control chain is human. He can be educated in understanding the problem and become useful in helping breaking the chain at multiple points. With great emphasis, people should be educated about the danger of malaria and its control so as to enable common people effectively contributed in controlling the spread of the disease. This, amongst others, includes education of health professionals about the need for early diagnosis and prompt treatment of malaria, personal protection against malaria measure such as use of chemoprophylaxis against malaria and protection against mosquito bites. Encouraging the use of protective measures amongst people living in endemic areas or countries as well as travellers to such territories should be done through public health education. More emphasis must be put on the importance of early diagnosis and treatment of malaria as a
significant aspect in controlling malaria while educating the masses (Bidla et al., 2004). Early detection and treatment of malaria is adequate in itself to control the epidemic in its early stage as it reduces the parasite load within the community, thus reducing the transmission of malaria (WHO, 2015). Early treatment with first line antimalarial drugs plays a major role in reducing parasite load and spread thus preventing deaths. In addition, complete treatment must be encouraged to prevent spread and disease relapse. Due to lack of proper education, adverse effects of the drugs or negligence, many patients fail to complete the treatment (WHO, 2005).

### 2.6.3 Malaria vector control

One of the recommended methods to augment other malaria control interventions to reduce transmission of malaria is the integrated vector management. Vector control should be selective, targeted, site specific and cost effective. Selection of vector control methods must be based on the intensity of the disease transmission, vector, human behaviors, the environment and availability of resources. The inhabitants of the targeted community must be actively involved in the implementation of these vector control measures most especially measures to reduce mosquito breeding sites within their environments (WHO, 2015). Some vector control strategies available include: use of long lasting insecticidal treated nets in homes of malaria endemic areas/countries, indoor residual spraying in endemic and epidemic prone areas/countries, larviciding breeding sites, environmental management for source reduction of vector density (drainage of breeding sites), screening of
house inlets to reduce entry of mosquitoes, feasible use of biological control measure (larvivorous fish, growth regulators, BTI – *Bacillus thuringiensis var israeliensis*), and repellent and fumigants (MOH, 2010; Table 2.1).

### Table 2.1: Summary of Malaria vector control

<table>
<thead>
<tr>
<th>Host</th>
<th>Parasite</th>
<th>Vector</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment using effective Anti-malarials</td>
<td>Chemotherapy</td>
<td>Larviciding and use of ITNs and IRS</td>
</tr>
<tr>
<td>Protection through use of ITNs and health education</td>
<td>Chemoprophylaxis</td>
<td></td>
</tr>
<tr>
<td>Problems: Compliance and Resistance to Limited resources vector</td>
<td>Problems: Drugs resistance and toxicity insecticides</td>
<td>Problems:</td>
</tr>
</tbody>
</table>

#### 2.7 Malaria prevention

Vaccination could have been the suitable preventive measure against the spread of malaria but there is no vaccine for malaria at the moment. However, chemoprophylaxis is recommended to the following high-risk groups: non-immune visitors (tourists), patients with sickle cell disease and patients with tropical splenomegaly syndrome. Travelers and people who live in malaria endemic countries are also encouraged to use other barrier methods (LLINs, insecticide treated materials and repellent) to prevent or reduce bite from mosquito (MOH, 2010). The national malaria control strategy in malaria-
endemic districts have developed business plans from 2003 to 2007 with malaria components that reflect the four strategic approaches, namely: early and effective diagnosis, public health education, malaria vector and chemotherapy.

Malaria control strategies target man, mosquito, and the parasite. Man is a moving host who takes the disease with him everywhere he goes; mosquito, a moving and extremely adaptable insect vector has shown resistance to insecticides; and the parasite, highly adaptable disease agent, hides in humans and mosquitoes and has also developed resistance to drugs (Chauhan and Sanjay, 2001). The use of personal protection measures such as insecticide treated bednets (ITNs), indoor residual spraying (IRS) and chemoprophylaxis through public awareness campaigns are means of targeting man (WHO, 2005). Larviciding in focal areas at risks and epidemic forecasting are aimed at the control of the insect vector while effective drugs and vaccines development are measures that target the parasite in the malaria chain (Chauhan and Sanjay, 2001).

2.8 Malaria management and treatment

The provision of prompt and effective treatment is the cornerstone of malaria case management. The treatment policy for malaria has changed in the last 12 years due to failing therapeutic efficacy from chloroquine (CQ) to sulphadoxine-pyrimethamine (SP) in 1998 and subsequently to the currently
recommended artemisinin-based combination therapies (ACTs) in 2004. ACTs are at present the best treatment for uncomplicated malaria (MOH, 2010). Most of the drugs that are used today are becoming less effective because of the problem of drug resistance (McMorran, 2009). The spate of drugs resistance by the malaria parasite particularly *P. falciparum* has necessitated the scientific evaluation of many traditional medicinal plants for an alternative anti-malarial drug that is effective, safe and affordable, (Oyedeji *et al.*, 2005).

The value of plants in traditional medicine cannot be overemphasized and is still the first point of healthcare for many people in Sub-Saharan Africa (Hostettmann *et al.*, 2000; WHO/TDR, 2007). Plants still provide a source for effective lead compounds against malaria (Midiwo, 2007). Majority of the anti-malarial drugs used today are obtained from plant extracts, for example, the commonly used anti-malarial drugs, quinolines and the peroxide anti-malarial (artemisinin derivatives) are derived from *qing hao* (*artemisinis annua* or sweet wormwood) which is a plant based compounds. The success of the artemesinin and its derivatives for the treatment of resistant malaria has drawn the attention of researchers/scientists to plants as a source of anti-malarial drugs (Nosten and White, 2007).

### 2.9.1 Malaria chemotherapy

Chemotherapy is the use of conventional drugs to kill or inhibit growth of malaria parasites in the vertebrate host. Several anti-malarial drugs have been
used with varying degree of success. Their effectiveness differs with differences in parasites species and with different stages of parasites’ life cycle, due to their different modes of action. Artemisinin and its derivatives, quinolines and antifolates are the three major groups of anti-malarial drugs used today. These anti-malarial drugs differ in a variety of ways which include the stage of the parasite targeted, half-lives, degree of parasite clearance and their metabolic pathways.

2.9.1.1 Aminoquinoline

This is a class of anti-malarial drugs such as chloroquine, amodiaquine and primaquine; with an amine group substituting for a carbon atom in their molecular structures (Chavda et al., 2010). Chloroquine, a 4-aminoquinoline was the drug of choice in most of malaria endemic areas or countries prior to the development of *P. falciparum* resistance. It is a very effective schizonticidal drug against erythrocytic stage of the four *Plasmodium* species. It has no effect on sporozoites, hypnozoites and gametocytes (Singh et al., 2012).

Chloroquine is a lysosomotropic drug having a plasma half-life of 10 days. It carries a positive charge at acidic pH, a weak base and uncharged at neutral pH. Due to this property, CQ selectively accumulates in the lysosomes. The uncharged compound quickly diffuses through the plasma and the lysosomal membranes, where it becomes trapped inside the acidic lysosomal
compartment of the parasite once charged. This may lead to the production of concentration gradient of several orders of magnitude. The intracellular trophozoite feeds on the haemoglobin of the RBC that serves as a source of amino acids (Tilley et al., 2011). Globin protein digestion takes place inside the parasites’ lysosomal resulting to the generation of ferriprotoporphyrin IX (FP), a free haem (Klonis et al., 2010). It is insoluble and as a result, precipitates as a black malaria pigment inside the lysosomes. Haem to harmless haemozoin conversion is done by plasmodial haem polymerase. After the inhibition of chloroquine by this enzyme, a built up of haem kills the parasite by membranolytic action.

Chloroquine (Figure 2.3) is administered orally, unless where not feasible or in severe cases, then it is given by continuous intravenous infusion, frequent intramuscular or through subcutaneous injection. It is totally absorbed and widely distributed throughout the tissues though poorly metabolized in the liver, with about 70% coming out as intact drug. Some of the side effects include nausea, vomiting, diarrhea, rashes, dizziness, psychoses, blurring vision, urticarial symptoms, headache, and prurities (Petersen et al., 2000).
Amodiaquine is a 4-aminoquinoline similar to CQ that has been used extensively in the past to treat and prevent malaria. Its form of action is similar to that of CQ. It is synthesized as amodiaquine hydrochloride. Following oral administration, amodiaquine hydrochloride is quickly absorbed (half-life 5.2 hours), and undergoes rapid and extensive metabolism to desethylamodiaquine that concentrates in the blood cells. Desethylamodiaquine, (not amodiaquine) is expected to be responsible for most of the observed anti-malarial activity, and AQ toxic effects after oral administration may possibly be due to desethylamodiaquine (Tilley et al., 2011).

Primaquine (Figure 2.4) an 8-aminoquinoline is a drug of choice for treating vivax malaria. It is also used to treat relapsing species of Plasmodia. It is a tissue schizonticidal drug which is potent in affecting the mitochondria of the exo-erythrocytic forms and gametocytes of an avian form of P. falciparum.
Taken orally, it is rapidly absorbed and metabolised. Primaquine is given as 15 mg/day for 14 days, and has half-life of 3-6 hours. The main superfluous effect of Primaquine is due to a genetic metabolic condition which is a deficiency of glucose-6-phosphate dehydrogenase (G6PD) in the RBCs and large doses lead to haemolytic anaemia (Chauhan and Sanjay, 2001).

Figure 2.4: Schematic representation of Primaquine (www.drugbank.ca, 2008)

2.9.1.2 Quinoline-methanols

Quinoline-methanols include anti-malarial drugs such as mefloquine and quinine. Mefloquine (Figure 2.5) interferes with the moving of haemoglobin products and other substances from host cell to parasite’s food vacuole (Hoppe et al., 2004). It is a blood schizonticidal of the erythrocytic forms of malaria and as hypnozoites if given as a combination treatment with Primaquine. It is rapidly absorbed when taken orally though it has a slow onset of action. It also
has a very long action with a plasma half-life of 30 days. It is used as a short-term chemoprophylaxis when entering CQ-resistant zones and also as a treatment for uncomplicated CQ-resistant \textit{falciparum} malaria. Some common side effects include convulsion, insomnia, neuropsychiatric reactions and gastrointestinal disturbances (White, 2007).

Figure 2.5: Schematic representation of Primaquine (en.wikipedia.org, 2009)

Quinine is an alkaloid extracted from the bark of \textit{cinchona} tree. It was the first drug of choice in the treatment of malaria till the development of CQ. It is an effective blood schizonticidal drugs against the four species of \textit{Plasmodium}. It is only active against the erythrocytic forms (Titanji \textit{et al.}, 2008; Achan \textit{et al.}, 2011). Its mechanism of action is similar to that of CQ and causes cytotoxicity of the parasite by inhibiting plasmodial haem polymerase with the subsequent built up of toxic haem. It is used in cases of severe \textit{P. falciparum} infection.
where slow intravenous (i.v.) infusion is administered parenterally in patients who are unable to retain oral intake. It clears the blood of parasitaemia and has an antipyrexia activity. About 80% is bound to plasma protein and is well absorbed in the gut. Quinine has half-life of 10 hours and is metabolized in the liver and excreted in urine in 24 hours. Some common side effects include headaches, nausea, vomiting, and depression in heart action, irritation of the gastric mucosa, blindness and hypersensitivity reactions. Other side effects include cardiac dysrhythmias, central nervous system (CNS) disturbances, and hypoglycaemia as well as black water fever, which is a rare but fatal case of acute haemolytic anaemia that is associated with renal failure (Achan et al., 2011).

Halofantrine (Phenanthrene-Methanol) is a blood schizonticidal against the erythrocytic form of *P. falciparum* resistant to CQ. It is active against *P. vivax* erythrocytic form but inactive against the hypnozoites form. It is used to treat the acute form of uncomplicated multi-resistant *falciparum* malaria. It is slowly and intermittently absorbed when orally taken with a peak plasma concentration at 4-6 hours later. Its half-life is about 1-2 days and elimination is through the faeces. Toxicity includes pruritus, headache, abdominal pain, gastrointestinal disturbances, a transient rise in hepatic enzymes and sudden cardiac deaths, haemolytic anaemia and convulsions (Nosten and White, 2007).
2.9.1.3 Artemisinin and its derivatives

The chemical structure of Artemisinin (AR) is relatively different from previously known anti-malarials. The compound is abnormally stable as sesquiterpene lactone bearing a peroxy group. Artemisinin has white needle crystals that are hardly soluble in water or oil and as result formulations other than oral and rectal are not in clinical use (Chaturvedi et al., 2010). Several oil and water-soluble derivatives of AR have been synthesized since the peroxide bridge of the compound is stable under certain chemical reactions (Figure 2.6). These include: dihydroartemisinin, artemether and AS. They were originally developed by Chinese scientists. Artesunate (AR) is a semi-synthetic derivative of AR that is used for treating both severe and uncomplicated malaria. It is formulated for oral, parenteral and rectal administration (Navaratnamt et al., 2000). Artemisinin easily passes through biological membranes and is hydrophobic (Agustijns et al., 1996).

*In vitro* studies have suggested an uptake of AR by both healthy and malaria infected red blood cells (Shah et al., 2009). It is identified that AR binds to haem, either in haemoglobin or in haemozoin. Artesunate free radicals are formed through an iron-mediated cleavage of the peroxide bridge. These free radicals destroy different parasite membranes which include mitochondria, rough endoplasmic reticulum, and plasma membranes, thus killing them (Shah et al., 2009). The haem-rich environment of the parasite is believed to be one of the reasons for the selective toxicity of AR towards the malaria parasites as haem has been shown to interact with the compound (Meshnick, 2002). The
account for schizonticidal activity of artemisinin is due to the haem-rich environment of schizonts. Artemisinin has plasma peak concentrations at 1-3 hours post administration and an absorption lag-time of 0.5-2 hours after oral intake. It has a quite short half-life of 1-3 hours (Tituler et al., 2000). It lacks the peroxide bridge and is eliminated by enzymatic metabolism to inactive metabolites (Meshnick, 2002). In healthy volunteers and malaria patients only trace amounts are detectable in urine after oral administrations (Navaratnam et al., 2000). The ether and ester derivatives are metabolized to dihydroartemisinin which accounts for most of the clinical effect of these derivatives after intake (Efferth and Kaina, 2010).
Figure 2.6: Structure of artemisinin and its derivatives (en.wikipedia.org, 2009)

2.9.1.4 Artemisinin resistance in P. falciparum malaria

The anti-plasmodial drugs have changed from chloroquine (CQ) and sulphadoxine-pyrimethamine (SP) to the currently recommended artemisinin-based combination therapies (ACTs) in 2004 (MOH, 2010) due to the continuous increased resistant of Plasmodium parasites strains (Boland, 2001; Ridley, 2002). Artemisinin-based combination therapies are the recommended first-line treatments of P. falciparum malaria in all countries where malaria is endemic. There are recent concerns that the efficacy of such therapies has declined on the Thai-Cambodian border, historically a site of rising anti-malarial-drug resistance (Dondorp et al., 2009). This resistance to the first line
drugs makes it imperative that new drugs are needed for alternative antimalarial therapies.

2.9.1.5 Combination drug therapy

The rapid increasing drug resistant of *P. falciparum* presents an imperative need for alternative ways of managing malaria infections. One alternative is combination therapy. Combination therapy is currently the preferred treatment of malaria, given that it is likely to decrease the development rate of resistance and expand the duration of the drugs (WHO, 2001). Drugs combination is based on various criteria, namely: drugs targeting different stages of the parasites AQ (blood schizonts) and Primaquine [gametocyte]; drugs targeting different metabolic pathways (SP + AQ); and drugs with different half-life; short half life with rapid parasitaemia clearance, such as AS and mefloquine (WHO, 2001). The threat of a patient being infected with a resistant parasite strain to two anti-malarial concurrently is very small (Sinha *et al.*, 2014). Furthermore, the reduction in the number of parasites rapidly by AR or its derivatives reduces the risk of selection for resistance against the second drug of the combination therapy. The developments of these effects have been reported for combinations of AS and mefloquine which halted the progression of mefloquine resistance (Nosten and White, 2007).

Tetracycline and doxycycline are antibiotics that are often combined with pyrimethamine or quinine for 100% cure. Doxycycline is contraindicated in pregnancy and nursing women including children under the age of 12 years.
A combination therapy; an AR compound and a second anti-malarial drug having a longer half-life would as well decrease the danger for recrudescence owing to the long protective action of the second drug. Additionally, such a combination as such results in shorter period of treatment which is beneficial from a compliance point of view (Nosten and White, 2007). Even though different combinations of an artemisinin derivative with mefloquine have been the ones mostly used, however, other compounds have also been used in combination with an artemisinin compound. One of such compound used is lumefantrine which is a new Chinese drug previously called benflumetol. A fixed dose of Coartem® was studied and is currently available for the treatment of uncomplicated *falciparum* malaria (Premji, 2009). Additional clinical studies with other anti-malarial and different combinations of artemisinin compounds are being undertaken.

Recently, a study comparing the monotherapy and the combination therapy was done in Mozambique which showed that, the therapeutic efficacy in monotherapy of AQ (91.6%) was better than that of SP (82.7%) and CQ (47.1%); after 14 days. The study also showed that the combination therapy of AQ+SP, AR+SP and AQ+AR was safe and a 100% efficacy clinically at day 14 of follow up (Abacassamo *et al.*, 2004). Rapidly, the combination therapies decreased fever and reduced the incidence of gametocytaemia during follow up. This study therefore, supports the use of combination therapy for the treatment of malaria. However, unfortunately, ACTs used as the first line drug for treating uncomplicated malaria in Africa is recent and limited; with Coartem® being used as the first line drug for treating uncomplicated malaria

*(Tan *et al.*, 2011)*.
in Kenya currently, and is provided at government supported health facilities and centres at no cost.

2.9.2 Chemoprophylaxis

Several anti-malarial drugs are used to prevent the development of malaria especially among travelers to malaria endemic countries or areas. The kind of chemoprophylaxis used varies depending on the species type and drug resistance prevalent in a country (Chahaun and Sanjay, 2001). Prophylactic drugs are started 2-20 days before departure to an endemic or malarious country or areas and continued for the duration of stay and for 1-4 weeks after return. Some drugs are effective prophylactic that prevent the establishment of infection in the liver by inhibiting PE schizogony while blood schizonticides suppresses the blood forms of the malaria parasite and thus protects against clinical illness. Example of such drugs is proguanil and primaquine (Figure 2.7). However, it is also important to note that none of the chemoprophylaxis is yet 100% effective and some may even have slight side effects that hinder compliance (WHO, 2005).
2.9.3 Use of traditional medicine in malaria treatment

Traditional medicine (TM), referred to as Complementary and Alternative Medicine (CAM) in Western countries, is at the moment in great demand in the developing world for primary health care not because they are inexpensive but for better acceptability, better compatibility with the human body and minimum side effects (WHO, 2013). The use of TM/CAM is continuously gaining popularity in all regions of the developing world, particularly in Africa, Latin America and Asia where it is used to help meet some of the primary health care needs. In Africa, for example, about 80% of the population uses traditional medicine for primary health care. In China, about 30% - 50% account for traditional herbal preparation of the total medicinal consumption at the same time in Mali, Nigeria, Zambia and Ghana, the use of herbal medicines at home as the first line treatment accounts for 60% of children with high fever resulting from malaria (WHO, 2005). The global market for herbal medicines
has further illustrated the ever-increasing importance of TM/CAM. In 2000 according to the USA Commission for CAM, about US $ 17 billion was spent on traditional remedies at the same time in the United Kingdom; about US $ 230 million was used as annual expenditure on alternative medicine. Currently, the global market for herbal medicine stands at over US $ 60 billion annually and is growing steadily (WHO, 2005).

2.9.4 History of herbal medicines

The use of herbal medicines can be traced far back to ancient times. For example, the Egyptians used herbal medicine around the 1500BCs; so far herbal medicine has never been more popular than it is today. About 119 plant-derived drugs accounting for 74% were discovered as a result of chemical studies to isolate the active substances responsible for their traditional use (Haq, 2004). Since 1630, quinine has been used as a natural remedy to malaria. It is extracted from the bark of tropical shrubs of the genus Cinchona while AR, extracted first in 1972 from the Qinghaosu plant, is one of the most effective anti-malarial formulations today. Its use as anti-malarial drug was first recorded in 1596 by Li Shizen. Zhou Hou Bei Ji Feng made reference of this herb (Hand book of prescriptions for emergence treatment) written in 340 AD. The following instructions were found in the book: “In order to reduce fevers, soak one handful of Qinghaou in one liter of water, strain the liquor and drink it all.”
2.9.5 Herbal plants evaluated for anti-malarial activity

Anti-malarial activities of several herbal plants have been determined through a number of screening programs. Some of the studies done on traditional medicinal plants that have been used demonstrating anti-plasmodial activities include the study done on seven EtOH extracts and 20 fractions from the partition of their respective initial EtOH extracts were evaluated. A high anti-plasmodial activity with IC$_{50}$ = 3ug/ml was observed for Cassia occidentalis, Euphorbia hirta and Phyllanthus niruri. Vernonia amygdaline (IC$_{50}$ = 9.7ug/ml), Tetracera poggei (IC$_{50}$ = 36.9ug/ml), and Morinda morindoides (IC$_{50}$ = 94.2ug/ml) were considered less active (Tonia et al., 2004).

Additionally, aqueous extracts of fruits, leaves, stems, and bark from Psidium quajara and Vangueria infausta frequently used as anti-malarial treatment in Kwa Zulu Natal Province of South Africa showed IC$_{50}$ values of 10-20mg/ml against CQ-sensitive P. falciparum (D10). These extracts during phytochemical analysis showed the presence of anthraquinones, secnoides, terpenoids and flavonoids (Nundkumar and Ojewole, 2002). Whereas a hot water extracts of Hydrangea macrophylla reduced parasitaemia in –vivo against P. yoelii stain 17XL, resulting to mice survival (Ishih et al., 2001), and led to disappearance of parasites from the blood within four days (Kamel et al., 2000). In a related study, analysis of aqueous stem extract from Erythrina senegalensis revealed the presence of glycosides and alkaloids that displayed activity against P. berghei when evaluated in-vivo using suppression and curative tests (Saidu et al., 2000).
Many herbalists prescribe a ‘primary herb and others within which it can be mixed (Azas et al., 2001). This may demonstrate the synergistic effects of plant extracts (Us and Us, 2015). Combinations of methanolic fraction from *Feretia apodanthera* and tetrahydroharman from *Guiera senegalensis*, methanolic extract from *F. apodanthera* with total alkaloids from *Mitragyna* and total alkaloids from *M. inermis* with tetrahydroharman showed synergy in their anti-malarial activity (Azas et al., 2001). This also suggests that there are traditional medicinal plants that can be used synergistically to develop more potent anti-malarial. In addition, the various plants with anti-malarial potency provide a large reservoir for further development of pharmaceuticals against malaria.

### 2.9.6 Current status of herbal medicines

At the moment, there is an increasing interest in searching the plant kingdom for new medicines, agrochemicals and other marketable products. Many companies and institutions engaging in this new search are using ethnobotanical information as their clue; to which plants are prime candidates for further screening and chemicals analyses (Njoroge and Bussmann, 2006). Plants used for TM contain a wide range of substances to treat chronic as well as infectious diseases. Developing countries have improved herbal medicines as a result; they have become an alternative solution to health problems and costs of pharmaceutical products. Modern pharmacopoeia still contains at least 25% drugs derived from plants (Njoroge and Bussmann, 2006). Such medicinal plants are more accessible and cheaper than western drugs to many people (Kaur et al., 2009).
2.9.7 Challenges facing the use of herbal medicines

The use of herbal medicines for management of infections and other ailments have had to compete with challenges ranging from lack of information to loss of it.

2.9.7.1 Lack of documented information

Probably the greatest of all is the fact that in many cases the efficacy of medicinal plants has not been fully investigated and established (Azas et al., 2001). For example, the Portuguese have used extracts from *Momordica charanatha* as an anti-malarial for a long time, *in-vivo* testing done for this extract showed no activity (Ueno et al., 1996), thus indicating that not all plants used as medicine are effective. Only 5 to 15% of the 250,000 to 750,000 existing plants species have been estimated through survey contained biologically active compounds which is even over estimated, as those investigated plants have been partially screen for a single or at best, few types of activity (Balandrin et al., 1987). The most intensive areas of natural product research today is the extraction of bioactive agents from plants. Some of the basic compounds associated with plants and reported to have medicinal value are alkaloids, oils, triterpenoids saponins, anthraquinones and glycosides (Njoku et al, 2011).
2.9.7.2 Loss of information

Ethnobiomedical information collected in Kenya has revealed a rich source of agents from which drugs could be developed (Njoroge and Bussmann, 2006). Plants have been and continue to be used as sources of drugs and therefore, the potential disappearance of many species should be of paramount concern. As a result, there is an urgent need for ethnomedicinal studies in Kenya as the possibilities of discovering new drugs from plants are still enormous (Nguta et al., 2010). Additionally, many traditional healers from whom a great deal of information is derived are elderly and lack apprentices. As they die, much of their knowledge of local vegetation also dies.

2.9.7.3 Biodiversity and sustainability

There is a risk that a growing herbal market and its great commercial benefit might pose a threat to biodiversity through over harvesting of the raw material for herbal medicines and other natural health care products. If not controlled, these practices may lead to the extinction of endangered species and destruction of natural habitats and resources (Njoroge and Bussmann, 2006). Uncontrolled harvesting of herbal medicine has resulted to massive exploitation of medicinal plants in Africa. As a result of this, conservationists have raised a major concern. Unfortunately, very few countries in the region have programs of replenishing their popular medicinal plants. Kenya for example, has no legislation governing the production and registration of traditional medicines, some of which have proved to be effective for severe ailments. Consequently, many remedies are obtained in destructive manner and
may need conservation measures to ensure sustainable utilization (Nguta et al., 2010).

2.9.7.4 Pharmaceutical development and pharmacognosy

Drug discovery and development are very expensive and as a result, of all the compounds investigated for use in human, only a small portion is finally approved in most nations by government appointed medical institutions. Every year, only about 25 new chemical entities are approved for marketing and given that a lot of time is needed for discovery, development, and approval of pharmaceuticals, costs can accumulate to nearly half the total expense. Most pharmaceutical companies have re-focused on approved drugs, such as those based on re-formulation of an existing active ingredient that are much less expensive to develop. In addition, most pharmaceutical companies are reluctant to invest in treatments of diseases such as malaria that are less profitable in less economically advanced countries (Nchinda, 1998). A great deal of early-stage drug discovery has traditionally remained the focus of universities and research institutions, which invariably, suffer from poor funding.

2.9. 8 Plant Materials for extraction

2.9.8.1 Collection and identification

It is very important to accurately identify plants used for medicinal purposes collected from the wild before use. During the process of collection, care must be taken to prevent any loss in plant biodiversity. The use of wild plants is
advantageous since they are unlikely to contain any pesticide residues (Bruschi et al., 2003).

2.9.8.2 Cleaning

Cleaning process involves screening, washing, peeling, or stripping leaves from stems. Parts that are unnecessary are removed prior to drying to avoid wasting time and energy.

2.9.8.3 Drying

In some cases, botanicals are used for extraction while fresh, though in most cases they are dried first. The purpose of drying is to reduce the content of water for easy of storage of the plant. Most plants contain 60 to 80 percent moisture when harvested and must be dried within 10 to 14 percent moisture before storage. Immediately after harvest, plant materials must be dried (www.worldagroforestrycentre.org) lest they begin to deteriorate. Plants can be dried naturally or artificially depending on the type of plant or plant part to be used. Natural drying requires no drying equipment and uses solar energy (Bruschi et al., 2003).

2.10 Techniques in herbal extraction

Various techniques used in herbal extraction are organic solvent extraction, supercritical gas extraction and steam distillation.
2.10.1 Organic solvent extraction

Organic solvents are used to separate the desirable substance from the plant material. Plants are grounded and then thoroughly mixed with a solvent such as methanol, hexane, benzene, or toluene inside a tank. The choice of solvent to be used depends on several factors including the characteristics of the constituents being extracted and cost. Once the solvent dissolves the desired substances of the plant, it is called a miscella and can be separated from the plant material in a number of techniques, which include maceration, percolation, and counter current extraction (Bruschi et al., 2003).

2.10.2 Maceration

Maceration technique involves soaking and agitating the solvent and plant materials together before the solvent is drained off. Thereafter, the miscella is removed from the plant material through pressing or centrifuging (Bruschi et al., 2003).

2.10.3 Extraction with supercritical gases

Supercritical gases technique involves the usage of gases for the extraction of active ingredients. The plant material is placed in a vessel that is filled with a gas under controlled temperature and high pressure. The gas dissolves the active ingredients within the plant material, and then passes into a separating chamber where both pressure and temperature are lower. The extract precipitates out and is removed through a control device at the bottom of the chamber. Gases appropriate for supercritical extraction include carbon dioxide,
nitrogen, ethane, ethylene, nitrous oxide, sulphur dioxide, propane and propylene. Supercritical extraction takes place at low temperature, thus preventing the quality of temperature-sensitive components (Philipson, 2001).

2.10.4 Steam distillation

Steam distillation technique involves extracting the active ingredients from medicinal plants (Bruschi et al., 2003). The plant material is loaded onto perforated plates inside a cylindrical tank and steam is injected below. The steam dissolves the desired materials in the plant, and then enters a condenser where it is condensed back into liquid. This condensate then passes into a flask, where the extract either rises to the top or settles to the bottom and is separated from the water (Philipson, 2001).

2.10.5 Cold pressing

Cold pressing technique is used to extract essential oils from plants through pressing. The plant material is spread onto sheets of purified fat to dissolve the essential oils. Experts of herbal medicine prepare aqueous extracts known as decoctions, infusions and maceration through cold pressing. Plant material is mixed, agitated, and soaked in water to dissolve the active ingredients (Bruschi et al., 2003).

2.11 Purification and concentration of herbal extracts

Plant material separated from herbal extracts may contain some unwanted substances such as pigments, tannins, microbial contaminants and residual
solvent. These substances can be removed through decantation, filtration, sedimentation, centrifugation, heating, adsorption, precipitation, and ion exchange. Extracts are then concentrated in order to increase the proportion of the desired substance. This is done through evaporation or vaporization using descending film, thin layer or plate concentrators. Extracts may also be dried completely using vacuum freeze dryers, cabinet vacuum dryers, microwave ovens, or atomizers. The technique for drying depends on the stability of the product and the amount of moisture that must be removed (www.pharmanutrients.ca).

2.12 Quality assurance in herbal extracts

Evaluating the quality and purity of herbal extracts can be done by examining its physical characteristics such as appearance, pH, solubility, total solids content, ash content and particle size. Spectrophotometric and chromatographic techniques are then used to analyze and measure standardized constituents of an extract (Philipson, 2001).

2.13 Bioassay techniques for anti-malarial drug development

Sensitivity tests for the development of anti-malarial drugs are usually performed in both in vitro and in vivo systems. In vitro assays are carried out as described by Desjardins (1979) while in vivo assays are performed using the 4-day suppressive test as described by Peters (1965).
2.13.1 *In vitro* anti-malarial drug testing

The short-term culture technique and drug sensitivity testing is based on the isotopic micro test (Desjardins *et al.*, 1979). Briefly, infected RBCs are suspended in complete culture media (CCM) at a hematocrit of 1.5% and an initial parasitaemia of between 0.2 and 0.6% serially diluted in triplicate in 96-well tissue culture plates precoated with test compounds. The parasites are incubated at 37°C in a gas mixture of 5% O$_2$, 5% CO$_2$ and 90% N$_2$ (Trager and Jansen, 1976) and pulsed with $[^3]$H hypoxanthine after 18 hours of incubation after which the plates are incubated for additional 24 hours before they are frozen to terminate the assays. The plates are then thawed to lyses the infected erythrocytes, and the contents of each well is collected on glass-fibre filters papers, washed, and dried with cell harvester. The filter disks are then transferred into scintillation tubes to which 2 ml of scintillation cocktail are added. The incorporation of $[^3]$H hypoxanthine is quantified using liquid scintillation counter (Ringwald *et al.*, 1999). The 50% inhibitory concentrations (IC$_{50}$), defined as the drug concentration corresponding to 50% of the uptake of $[^3]$H hypoxanthine is measured and compared with drug-free control wells (Desjardins *et al.*, 1979).

2.13.2 *In vivo* anti-malarial drug testing

In order to determine the anti-malarial effect of a test sample, the Peters’ suppressive test of blood schizonticidal action is used (Ryley and Peters, 1970). Infected blood from a donor mouse at 12.4% parasitaemia is diluted in tissue culture medium such that 0.2 ml contain $10^7$ infected cells which is then
administered i.p to experimental animals. The test sample, dissolved by sonication in 2% aqueous solution of tween 80 of 0.5% dimethylsulphoxide (DMSO) is administered orally or subcutaneously (s.c) at a dose rate of 200-1000mg/kg for four successive days. On the fifth day, Giemsa-stained thin smears are prepared from tail blood for examination and used to establish the 50% suppression of parasites (ED$_{50}$ values) in test animals relative to control groups. To establish survival times, the Rane’s test of blood schizonticidal activity is employed (Ryley and Peters, 1970). This test relies on the ability of a standard dose of $P.~berghei$ inoculums to kill the host mice within six days in the presence of the test sample. An extension in survival times by 12 days or longer is considered significant (Okokon et al., 2005).
CHAPTER THREE: MATERIALS AND METHODS

3.1 Study site and plant material

This study was carried out in the Department of Tropical and Infectious Diseases at the Institute of Primate Research (IPR). The Institute of Primate Research is located within Oloolua Forest from where the medicinal plant (*S. occidentalis*) was obtained. The forest is situated at the foot of Ngong Hills; Kajiado District, Kenya. The forest, 22 kilometres west of Nairobi, is a tropical dry forest that offers sanctuary to wide range of flora and fauna. The roots of *S. occidentalis* were selected on the basis of their reputation in the treatment of malaria by herbalists living around the forest. The plant was taxonomically identified by comparing with voucher specimens at the East African Herbarium of the Nation Museums of Kenya (NMK) where voucher specimens (No. 38/81, APPENDIX III) were also stored for future referencing.

3.2 Preparation of materials and buffers for bioassays

This study was carried out in a Biohazard Laboratory (Class II), confining any potential hazardous effects to the laboratory workers with no possibility of spreading to the community. All the relevant safety procedures were performed under sterile conditions in a safety hood. Re-usable glassware was sterilized by auto-claving while 70% ethanol was used for general sterilization of apparatus and equipment.

Erythrocytes, serum, Complete Culture Medium (CCM), cryoprotectants and Phosphate buffers were prepared using standard protocols (Jansen *et al.*., 2006;
Rowe et al., 1968) for use to culture malaria parasites and in bioassays. Erythrocytes for *P. falciparum* were obtained from the Kenya Medical Research Institute (KEMRI). Serum for *P. falciparum* culture was obtained by spinning 50 ml of red blood cells (RBCs) from KEMRI at 1500 RPM for 10 minutes after which the clear serum was aspirated into sterile serum storage and stabilized by inactivating it in a water bath at 56°C for 30 minutes. The heat inactivated (HI) serum and erythrocytes were stored at -20°C and 4°C respectively and used within two weeks after which fresh ones were prepared using the same protocol (Jansen et al., 2006).

### 3.3 Preparation of plant extracts for bioassays with malaria parasites

The procedure for sample extraction was carried out within the Phytochemistry Laboratory, Department of Pharmacy at the Kenyatta University as described by with Aqueous, Methanol and Hexane Solution respectively. A total of 1300 g of fresh dried medicinal plant material (roots of *S. occidentalis*), aqueous, methanol and hexane extracts were prepared, concentrated and weighed before they were reconstituted and assayed against two species of *plasmodium*.

A hundred grams of powdered plant material was soaked in 500 ml of methanol at room temperature for 48 hours. The materials were filtered using Whitman filter paper No.1 and repeated after 48 hours until the filtrate was clear. The filtrate was concentrated under vacuum by rotary evaporation at
40°C. This concentrate was weighed and transferred to an air tight sample bottle and stored at -20°C in the cold room until required for bioassay.

The aqueous extract was obtained by weighing 300 g of the sample and soaking in 1500 ml of distilled water and placed in a water bath at 60°C for 24 hours. The extract was obtained through vacuum filtration using Whitman’s filter papers No. 1 after which the aqueous filtrates were lyophilized for 48 hours. Drying was done through sublimation under vacuum where the extract was subjected to a temperature of -10°C to avoid any qualitative and quantitative changes. The extract was concentrated to crystalline powder form, weighed and stored at 4°C in the cold room until required for bioassays (Tona et al., 2004). Stock solutions of 50 mg/ml were made with de-ionized water and filtered through 0.45 µm and 0.22 µm micro filters in the laminar flow hood. Insoluble extracts were dissolved first in 50 µl of dimethylsulfoxide (DMSO) solvent and vortexed for one minute to dissolve the extract. The extract was dissolved in 50 µl RPMI 1640 culture medium.

The hexane extract was obtained by weighing 900 g of the sample and soaked in 4500 ml of hexane at room temperature for 48 hours. The materials were filtered using Whitman filter paper No. 1. The filtrate was then concentrated under vacuum by rotary evaporation at 45°C. This concentrate was weighed and transferred to an air tight sample bottle and stored at -20°C in the cold room until required for bioassay.
3.4 Study design

This study adopted an experimental study design that determined the anti-plasmodial activities of *S. occidentalis* roots extracts against *P. falciparum* and *P. berghei*. Both *in vitro* and *in vivo* experiments were respectively carried out. The *in vitro* assay to test the anti-plasmodial activities for *P. falciparum* was performed in triplicate in 96-wells microtiter flat-bottomed plates in triplicate. The *in vivo* experiment was carried out in BALB/c mice. This assay involved the treatment of mice with the extracts immediately after mice had been inoculated with *P. berghei*.

3.5 Experimental animals

A total of 185 adult BALB/c mice of both sexes (average weight = 20 ± 25 g) bred in the animal house of the Department of Animal Resources at IPR were used to determine the *in vivo* activities of the three extracts. The mice were kept in plastic cages at room temperature and supplied with adequate drinking water and mice feed. Ethical approval for animal use was granted by the Institutional Scientific and Review Committee (ISERC) of the Institute of Primate Research (IPR).

3.6 Retrieval and culturing of *P. falciparum* parasites for bioassays

*Plasmodium falciparum* parasites were cultured and used to evaluate the *in-vitro* chemotherapeutic activities of the plant extracts. Cryopreserved parasites were retrieved from liquid nitrogen using the Behring-Werk method. Briefly, an ampoule containing parasitized erythrocytes was collected from liquid nitrogen and thawed at 37°C after the parasites were aseptically transferred into
a labelled 50 ml sterile centrifuge tube. Ten milliliter (10 ml) of a sterilized solution containing 1.6% and 0.9% NaCl were added drop wise respectively, to the original culture volume while shaking. In addition, 12% of NaCl was added with a ratio of 1:5 drop wise to the culture volume (cell). The mixture was then spun down at 1500 RPM for 10 minutes and the supernatant was discarded. This step was repeated after which the parasites were transferred to a starting uninfected erythrocytes (RBCs) 100 µl with packed cell volume (PCV) of 50%, 10% human serum and RPMI Medium 1640 (1X) + GlutaMAX (Gibco) in a sterile cell culture flask (Corning® Flask). The total volume of the culture in the cell culture flask was 5 ml.

In order to avoid microbial contamination of the culture, 15 µg/ml of gentamycin was added. The culture was then aerated with a gas mixture of 5% O₂, 5% CO₂ and 90% N₂, for 30 seconds before it was incubated at 37°C (Trager and Jansen, 1976; Butcher, 1979). Finally, the initial parasitaemia and subsequent development of parasites was monitored daily through microscopy with cultures being refreshed every 48 hours for two weeks as Refreshment of the culture, which was preceded with checking of the culture conditions and level of parasitaemia, involved washing off the used culture medium by centrifugation at 1500 RPM for 10 minutes and re-suspending the parasites in fresh culture medium. A normal PCV of 5% was maintained with fresh erythrocytes added within five days of culturing as described by Rowe et al. (1968).
3.7 Infection and propagation of *P. berghei* in BALB/c mice

Experimental mice were infected through mechanical passage using blood stages from donor mouse previously infected with *P. berghei* (ANKA) obtained from the Institute of Primate Research (IPR). A drop of tail blood (5 µl) from door mouse at a parasitaemia of 5 – 17% was collected in 10 ml PBS and 0.1ml of the suspension injected i.p. into naive mouse to begin an infection. Blood stages of *P. berghei* were cultivated in a CCM containing 20% FCS for a single developmental cycle of 18 hours. Collection of blood from the donor mice was done on day seven post-infection between 9:00 a.m. and 12:00 noon when the parasitaemia was about 12.4% and mainly ring forms and young trophozoites (Jansen *et al.*, 2006). The ring forms and young trophozoites were collected from infected mice through cardiac puncture with a sterile syringe and needle earlier flushed with heparin. For a 75 cm² tissue flask, five mice were sacrificed and the infected blood pooled in a 50 ml sterile centrifuge tube to which an equal volume of CCM was added before the mixture was spun down at 1500 RPM for 10 minutes. The supernatant was discarded and the pellet re-suspended in 20 ml of CCM in a tissue culture flask, gassed appropriately before it was transferred to the incubator set at 37°C and fitted with shaker until the following day. By this time, the entire parasite had developed into mature schizonts, which did not rupture in the *in vitro* conditions as described by Jansen *et al.* (2006).

A small sample of the culture was used to prepare Giemsa-stained thin smears that were used to distinguish live from dead parasites through microscopy. The presence of 12-24 free merozoites within each infected erythrocyte and a dark
cluster of haemozoin distinguished healthy and viable schizonts from dead ones, which stained brown and lacked definite free merozoites. For purposes of propagation, schizonts from culture were pelleted (1500 RPM, 10 minutes) and subsequently injected into naive mice where they released merozoites that developed into blood stages as described by Jansen et al. (2006).

3.8 Staining of malaria parasites and determination of parasitaemia

Giemsa-stained thin smear were used to determine percentage inhibition of parasite growth in in vitro cultures by plant extracts and to monitor parasitaemia profiles in mice. From in vitro cultures, 100 µl of the culture were aseptically dispensed into an eppendorf tube and subsequently spun down in a microcentrifuge (Beckman Instrument, USA) at 3500 RPM for 1 minute. Using a micropipette, 2 µl of the erythrocytes pellet was placed near the frosted end of an appropriately labelled slide, which was then evenly spread towards the other end using another slide, held at an angle of 45°. The smears were air-dried and fixed using absolute methanol before staining with 10% Giemsa for 10 minutes as described by Rowe et al. (1968). Excess stain was washed off by running a gentle stream of tap water over the slide tilted at 45°. Air-dried slides were then observed under the microscope. Parasitaemia profiles in P. berghei-infected mice were also monitored by microscopy. A drop of tail blood was collected on a labelled standard slide and used to make Giemsa-stained thin smears for observation as described above. A minimum of 2000 RBCs were counted using at least six fields and the number of infected erythrocytes was recorded and used to calculate the mean percentage parasitaemia as:
% Parasitaemia = \frac{Number\ of\ parasite\ RBC}{Total\ number\ of\ RBCs} \times 100%

3.9 In vitro activity of plant extracts on P. falciparum

The in vitro anti-plasmodial activities were evaluated according to the method described by Tona et al. (1998). The assays were performed in triplicate in 96–well microtiter flat-bottomed plates (Coster Glass Works Cambridge, UK). Aliquots of culture medium (200 µl) were added to all the wells of the 96 plates. Thereafter, 20µl of the test solutions were added in triplicates to the first well and a titertik motor hand diluter was used to make two-folded serial dilution. Serial dilution was made for assays first by dispensing 200µl of complete RPMI into each well followed by 100 µl of 100 µg/ml extract concentration as a start concentration in the first well (A1) of a 96-well microtiter plate.

With a micropipette, 50 µl was drawn from the first well and used to make eight-fold serial dilutions down the plate giving a concentration range of 50, 25, 12.5, 6.25, 3.2, 1.6, 0.8, and 0.4 µg/ml. Similar process was carried for the reference drugs (ATM and CQ) with concentrations range of 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.03125, 0.0156, 0.0078, 0.0039, 0.002 and 0.001 µg/ml. A suspension of 10 µl, 1.5 v/v of parasitized erythrocytes at 1.6% parasitaemia was dispensed to each well bringing the total volume per well to 260 µl. The plates were then transferred into an air tight chamber gas for 3 minutes and
incubated in carbon dioxide condition at 37°C for 24 hours. After 24 hours of incubation, contents of the wells were harvested into eppendorf tubes, centrifuged for 3 seconds and the pellet sucked onto a microscope slide to make a smear. They were Giemsa stained after which the developed schizonts were counted against the total of 2000 erythrocytes. Microscopic examination of Giemsa stained smears were done after every 48 hours to check for the possibility of parasites. Differential counts were done to determine the parasitaemia levels for each extract in each well. Positive controls were carried out using WHO approved herbal drug artemeter and chloroquines which are used to treat malaria while non-treated used the solvent as control.

The anti-malarial activity of *S. occidenatlis* roots extracts were expressed as 50% inhibitory concentration (IC\textsubscript{50}) determined from dose-response curve by non-linear regression analysis (curve-fit) using Graph Pad Prism (version 5.0) software. All experiments were performed in triplicates and the results were expressed as percentage of growth inhibition (Okokon *et al*., 2005).

### 3.10 *In vivo* activity of plant extracts on *P. berghei* in BALB/c mice

The method described by Fidock *et al.* (2004) was used. It involves treatment with extracts immediately after mice are inoculated (early infection). One hundred and eighty-five male and female mice were divided into thirty seven groups of five each. Donor mice infected with *P. berghei* having a parasitaemia of about 20 – 30% was anaesthetized with carbondioxide (CO\textsubscript{2}) and its blood collected by cardiac puncture with a sterile syringe and needle earlier flushed
with heparin. The blood was diluted with normal saline such that 0.2 ml contained about $1 \times 10^7$ infected cells. Each of the 185 mice was inoculated intraperitoneally with 0.2 ml of diluted blood. The extracts at dose levels of 200, 400, 600, 800 and 1000 mg/kg body weight (bw) respectively were administered orally once daily for four days ($D_0$, $D_1$, $D_2$ and $D_3$). A parallel test with pyrimethamine at 4 mg/kg of body weight (bw) in the sixth group was done as a reference. The seventh group was given normal saline which served as control. Thick and thin blood films were made from tail blood from $D_4 – D_7$, fixed with methanol and stained with 10% Giemsa (PH 7.2) for 20 minutes before examining under a microscope. Each slide was examined for the number of infected and uninfected red blood cells (RBCs) counted and means taken. The percent parasitaemia suppression was determined by counting the number of parasitized erythrocytes out of 2,000 red blood cells on random fields under the microscope. The average percentage suppression of parasitaemia was calculated using the formula below as described by Okokon et al. (2005):

$$AS = \left(\frac{[C - T]}{C}\right) \times 100\%$$

Where: $AS =$ average percentage suppression

$C=$ average parasitaemia in the control group

$T=$ average parasitaemia in the test group
The two methods of treating malaria infection, therapeutic and prophylactic described earlier, were applied according to Ryley and Peters (1970) and Peters (1965) respectively.

Mortality was determined daily and the number of days from the time of inoculation of the parasite up to death was recorded for each mouse in the treatment and control groups throughout the follow up period. The mean survival time (MST) for each group was calculated as follows;

\[
MST = \frac{\text{Sum of survival time of all mice in a group(days)}}{\text{Total number of mice in that group}}
\]

To determine the curative effects of the three plant extracts on patent *P. berghei* infection, 50 mice were each injected with 0.2 ml of infected erythrocytes at parasitaemia of 12.9% and treatment withheld for 72 hours to allow parasites to establish. On the fourth day post infection, tail blood from each mouse was used to make Giemsa-stained thin films that were examined under the microscope to confirm infection and percentage parasitaemia. The mice were then randomized into ten groups of five mice per cage. Each mouse in the first nine groups received 0.2 ml of the aqueous, methanol and hexane extracts administered orally at a dose rate of 600, 800 and 1000 mg/kg/day respectively for four consecutive days, with the positive control receiving 0.2 ml of pyrimethamine at 4mg/kg/day for similar period of time.
To determine the suppressive effects of the three plant extracts on patent *P. berghei* infection, 85 mice were each injected with 0.2 ml of infected erythrocytes at parasitaemia of 12.9% and treatment administered 3 hours after infection. The mice were then randomized into seventeen groups of five mice per cage. Each mouse in the first fifteen groups received 0.2 ml of the aqueous, methanol and hexane extracts administered orally at a dose rate of 200, 400, 600, 800 and 1000 mg/kg/day respectively for four consecutive days, with the positive control receiving 0.2 ml of pyrimethamine at 4mg/kg/day and the negative control group also receiving 0.2 ml sterile PBS over a similar period of time. On day four post infection, tail blood from each mouse was used to make Giemsa-stained thin films that were examined under the microscope to confirm infection and percentage parasitaemia.

A third set of 50 mice were used to determine the prophylactic effects of the three plant extracts against *P. berghei*. The mice were divided into ten groups of five each, with each mouse receiving 0.2 ml of the aqueous, methanol and hexane extracts administered orally at a dose rate of 600, 800 and 1000 mg/kg/day respectively for four consecutive days. On the fourth day, each mouse was injected intraperitoneally with an inoculum of *P. berghei* at 12.9% parasitaemia. The prophylactic activities of all three plant extracts were determined in a similar way, with positive control group receiving pyrimethamine at 4mg/kg/day for similar period of time. Starting from the fourth day post-inoculation, Giemsa-stained thin blood films were made from tail blood of each mouse from each group daily for ten consecutive days, and
examined microscopically to assess the levels of parasitaemia. All three treatment regimen had one negative control group receiving 0.2 ml sterile PBS over a similar period of time. The average percentage suppression of parasitaemia was calculated using the formula above as described by Okokon et al. (2005).

3.11 Statistical data analysis
Parasitaemia and survivorship values were recorded as means ± SEM and SD respectively. These were generated in Microsoft excel. One-way analysis of variance (ANOVA) was used to compare suppressive, curative and prophylactic activities of the extracts and controls. Descriptive statistics was used to determine the variation in survivorship between treated groups and the controls. Student t-test was used to compare mean percentage inhibitions of plant extracts on both strains *P. falciparum* (W2 and 3D7) used in the *in vitro* assay. A *P*-value < 0.05 was considered statistically significant.
CHAPTER FOUR: RESULTS

4.1 Taxonomy of plant species

The selected plant was taxonomically identified by comparing with voucher specimen at the herbarium of the National Museums of Kenya (NMK). The identity of the selected plant included botanical, family and local name as well as plant part used by herbalists (Table 4.1).

Table 4.1: Taxonomy of plant species investigated on Anti-plasmodial Activities

<table>
<thead>
<tr>
<th>Botanical Name</th>
<th>Plant Family</th>
<th>Local/Common Names</th>
<th>Community</th>
<th>Part of Plant Used</th>
</tr>
</thead>
<tbody>
<tr>
<td>Senna occidentalis</td>
<td>Fabaceae (Leguminosae)</td>
<td>Negro coffee</td>
<td>Kikuyu* (Mu-Nyua) &amp; Maasai* (Eseneto)</td>
<td>Roots</td>
</tr>
</tbody>
</table>

*The two Kenyan communities living around Oloolua Forest, Ngong Division

4.2 Growth characteristics of P. falciparum and P. berghei parasites

Plasmodium falciparum and P. berghei were cultivated and used to determine the chemotherapeutic and chemoprophylactic effects of plant extracts. Two stains of *P. falciparum* (W2 strain – sensitive to CQ and 3D7 strain –resistant to CQ) were used in this study. Both strains of *P. falciparum* (W2 & 3D7) grew in culture over a period of two weeks (Figure 4.1), attaining a maximum parasitaemia of 100 parasites per $10^4$ erythrocytes between the fifth and seventh day post-incubation. On the other hand, *P. berghei* (ANKA) was
propagated in mice since it does not expand in culture. Figure 1 shows that *P. berghei* increased exponentially to a parasitaemia of 100% within one week of inoculation. Tests with both W2 and 3D7 strains of *P. falciparum* were, therefore, performed between the sixth and eighth day of culturing while assays with *P. berghei* were carried out when the mean parasitaemia was between 10 - 14%, before disease characteristics became obvious.

![Figure 4.1: Growth characteristics of *P. berghei* and *P. falciparum* in mice and culture. Each value is in a mean of two observations.](image-url)
4.3 *In vitro* chemotherapeutic activities of *S. occidentalis* roots extracts on *P. falciparum*

4.3.1 Activity of *S. occidentalis* roots extracts on *P. falciparum* - W2 strain

Three extracts from *S. occidentalis* were assayed for *in vitro* activity against *P. falciparum* (W2 strain – chloroquine resistant) at eight–fold serial dilutions. The mean parasitaemia inhibition levels were not significantly different between the crude aqueous and methanolic extracts (p > 0.05; Figure 4.2). On the other hand, crude aqueous extracts had significantly higher antiplasmodial activities than those of hexane (p < 0.05; Figure 4.2). Similarly, methanol extracts showed significantly higher antiplasmodial activities when evaluated against hexane extracts (p<0.001). The results of the *in vitro* antiplasmodial activity of the extracts tested indicate that polar solvents were responsible for the activity since the greatest activity was noted in organic extraction (Figure 4.2).
Figure 4.2: Percentage inhibition of *P. falciparum* (W2 strain) treated with test extracts from *S. occidentalis* roots and positive controls – artemeter (ATM) and chloroquine (CQ).

The W2 strain was tested against artemeter (ATM) and chloroquine (CQ). The mean inhibition of parasitaemia for artemeter (ATM) was 61.8±2.95% which was significantly higher than that of hexane (p<0.001; Figure 4.2) while being no significant difference from the inhibition levels recorded for aqueous extracts (p > 0.05; Figure 4.2) and methanol extracts (p > 0.05; Figure 4.2). Chloroquine also had a higher level of anti-*plasmodial* activity compared to hexane (p<0.001; Figure 4.2) but not when compared to aqueous (p> 0.05; Figure 4.2) and methanol extracts (p> 0.05; Figure 4.2). Generally, there was an increase in the inhibitory activities of ATM, CQ and medicinal plant extracts with an increase in the concentrations. The mean parasitaemia inhibition by CQ was 58.9±1.16% (Figure 4.2).
4.3.2 Activity of *S. occidentalis* roots extracts on *P. falciparum* -3D7 strain

Similar assay for *in vitro* activity against *P. falciparum* (3D7 strain – chloroquine sensitive) by the three extracts from *S. occidentalis* roots was carried out at eight-fold serial dilutions. The methanolic extract again showed the highest level of inhibition of development of parasitaemia followed by the aqueous and hexane extracts. The IC$_{50}$ values for the three extracts were 2.288, 6.283 and 15.650 µg/mL respectively. Variations in parasitaemia inhibition levels amongst the crude herbal extracts (methanol, aqueous and hexane) revealed that there was a statistically significant difference ($p < 0.05$). Methanolic extract had the highest mean inhibition (54.0±2.12%) followed by aqueous (48.8±1.32%) and hexane extract (45.0±1.62%). No statistically significant differences were observed between the inhibitory activities of aqueous extract when assessed against the crude extract of hexane ($p > 0.05$) and methanol ($p > 0.05$). Significantly higher levels of anti-plasmodial activity were reported in the methanolic extract when compared to hexane extract ($p < 0.05$). Additionally, there was no significant differences observed in the *in vitro* tests of extracts between the both strains of *P. falciparum* (W2 and 3D7) ($p > 0.05$; $p > 0.05$ and $p > 0.05$) for aqueous, hexane and methanol respectively ($p < 0.05$; Figure 4.3).
Figure 4.3: Percentage inhibition of *P. falciparum* (3D7 strain) treated test with extracts from *S. occidentalis* roots and positive controls - artemeter (ATM) and chloroquine (CQ).

The IC$_{50}$ value for ATM was 0.002467 µg/mL while that of CQ was 0.0007121 µg/mL. Overall, the proportions of inhibition of parasitaemia at various concentrations were higher in ATM compared to CQ though not statistically significant (p > 0.05). However, when comparing CQ and ATM, the three extracts showed significantly lower levels of anti-plasmodial activity (Figure 4.3; p < 0.05 and p < 0.05 respectively).

### 4.5 Summary of IC$_{50}$ values for *S. occidentalis* roots extracts against *P. falciparum* (W2 and 3D7 strains)

Table 4.2 gives IC$_{50}$ values for the plant extracts and positive controls assayed for *in vitro* chemotherapeutic activity against *P. falciparum* (W2 – chloroquine
resistance strain) and *P. falciparum* (3D7 – chloroquine sensitive strain). Of the three selected herbal extracts assayed against the two strains of *P. falciparum*, the lowest IC$_{50}$ value was recorded in methanol extract followed by the aqueous extract while the highest was obtained from hexane extract. Additionally, the lowest IC$_{50}$ values obtained from the positive control drugs assayed against W2 –chloroquine resistance strain was in ATM while CQ being the lowest obtained from 3D7 – chloroquine sensitive strain respectively from the positive controls. These results also confirmed that the both strains of parasites (*P. falciparum*) used were indeed the resistance and sensitive strains.

**Table 4.2: Range of IC$_{50}$ values for plant extracts on *P. falciparum* (W2 – chloroquine resistance strain and 3D7 –chloroquine sensitive strain)**

<table>
<thead>
<tr>
<th>Plant Extract/ Drugs</th>
<th>IC$_{50}$ values (µg/ml)/W2</th>
<th>IC$_{50}$ values (µg/ml)/3D7</th>
</tr>
</thead>
<tbody>
<tr>
<td>MeOH</td>
<td>0.6529</td>
<td>2.288</td>
</tr>
<tr>
<td>Aqueous</td>
<td>1.832</td>
<td>6.283</td>
</tr>
<tr>
<td>Hexane</td>
<td>8.785</td>
<td>15.650</td>
</tr>
<tr>
<td>CQ</td>
<td>0.129</td>
<td>0.0007121</td>
</tr>
<tr>
<td>ATM</td>
<td>0.065</td>
<td>0.002467</td>
</tr>
</tbody>
</table>

MeOH: methanol; CQ: chloroquine; ATM: Artermeter.

4.6 *In vivo* activities of the three plant extracts on *P. berghei*

On account of their *in vitro* anti-plasmodial activities on both strains of *P. falciparum* (W2 strain – chloroquine resistant and 3D7 strain – chloroquine sensitive), all three extracts were further assayed for *in vivo* suppressive,
curative and prophylactic activities against *P. berghei* in BALB/c mice. Parasitaemia profiles and mean survival time (MST) in treated as well as non-treated mice were recorded and used as indices of activity.

### 4.6.1 Suppressive activity of the plant extracts on *P. berghei* in mice

A 4-day test or suppressive treatment involving co-treatment starting on the day of infection with parasite and continued for four days was carried out. All the extracts assessed showed dose-dependent suppressive activities on parasitaemia. On day four, the mean percentage suppression for aqueous extracts were 75.43% (1000 mg/kg), 73.80% (800 mg/kg), 67.87% (600 mg/kg), 63.27% (400 mg/kg) and 57.20% (200 mg/kg); methanol extracts were 69.49% (1000 mg/kg), 65.49% (800 mg/kg), 65.04% (600 mg/kg), and 64.24% (200 mg/kg); and the hexane extracts were 67.53% (100 mg/kg), 66.52% (800 mg/kg), 64.88% (600 mg/kg), 63.27% (400 mg/kg) and 59.12% (200 mg/kg) respectively. By day seven, there was continual increase in the mean percentage suppression for all the treated groups’ mice throughout the study period but with slight decrease in aqueous 1000 mg/kg/bw (Table 4.3).

For mice treated with aqueous extracts, there was no significant difference observed in parasitaemia values between day four and day seven (p > 0.05), however, there was a significant difference in dose responses between the two days (p < 0.05). On the other hand, for mice treated with methanol extracts, there was a significant difference observed in parasitaemia values between day four and day seven (p < 0.05) and no significant difference observed between
dose responses (P > 0.05). For mice treated with hexane extracts, there was no significant difference observed in parasitaemia values between day four and seven (P > 0.05) and dose responses the two days (P > 0.05; Table 4.3; Figure 4.4, 4.5 and 4.6).
Table 4.3: Effects of crude extract on suppression of *P. berghei* infected mice for day four and day seven post infection.

<table>
<thead>
<tr>
<th>Treatment/Extract</th>
<th>Dose(mg/kg)</th>
<th>Mean (%)</th>
<th></th>
<th>Mean (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Suppression Day 4</td>
<td></td>
<td>Suppression Day 7</td>
</tr>
<tr>
<td>Aqueous</td>
<td>1000</td>
<td>75.43 ± 2.859</td>
<td>74.76 ± 0.93</td>
<td></td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>73.80 ± 2.522</td>
<td>74.15 ± 2.10</td>
<td></td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>67.87 ± 7.187</td>
<td>73.05 ± 1.73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>63.27 ± 2.490</td>
<td>71.73 ± 1.80</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>57.20 ± 3.679</td>
<td>70.40 ± 1.76</td>
<td></td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>4</td>
<td>99.08 ± 0.191</td>
<td>99.69 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td>1000</td>
<td>69.49 ± 1.030</td>
<td>81.84 ± 1.74</td>
<td></td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>65.95 ± 1.370</td>
<td>80.64 ± 5.73</td>
<td></td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>65.04 ± 1.145</td>
<td>75.05 ± 1.92</td>
<td></td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>64.24 ± 13.370</td>
<td>74.34 ± 5.19</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>63.89 ± 0.0000</td>
<td>72.68 ± 0.40</td>
<td></td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>4</td>
<td>99.08 ± 0.191</td>
<td>99.69 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Hexane</td>
<td>1000</td>
<td>67.53 ± 2.316</td>
<td>76.92 ± 3.86</td>
<td></td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>66.62 ± 1.940</td>
<td>74.55 ± 3.43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>64.88 ± 1.872</td>
<td>73.89 ± 11.6</td>
<td></td>
</tr>
<tr>
<td></td>
<td>400</td>
<td>63.27 ± 2.490</td>
<td>70.84 ± 2.93</td>
<td></td>
</tr>
<tr>
<td></td>
<td>200</td>
<td>59.12 ± 10.27</td>
<td>64.05 ± 5.14</td>
<td></td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>4</td>
<td>99.08 ± 0.191</td>
<td>99.69 ± 0.03</td>
<td></td>
</tr>
</tbody>
</table>

Data represented as mean ± SEM; P value < 0.05 significant
Figure 4.4: Dose-dependent percentage inhibition of parasitaemia in mice placed on 4-day suppressive treatment with aqueous extract and pyrimethamine (p < 0.05). Each point is an average count from five infected mice (±SEM).
Figure 4.5: Dose-dependent percentage inhibition of parasitaemia in mice placed on 4-day-suppressive treatment with methanol extract and pyrimethamine (p < 0.05). Each point is an average count from five infected mice (±SEM).
Figure 4.6: Dose-dependent percentage inhibition of parasitaemia in mice placed on 4-day/suppressive treatment with hexane extract and pyrimethamine (p < 0.05). Each point is an average count from five infected mice (±SEM).

4.6.2 Prophylactic activities of plant extract on *P. berghei* in mice

The crude extracts showed a dose-dependent reduction in levels of parasitaemia in the experimental groups of mice. The highest mean inhibition of parasitaemia was in the methanol extract (76.18±1.734%) at 1000mg/kg body weight followed by aqueous extract at similar dose concentration (74.69±1.862%). Hexane extract at similar dose concentration of 1000 mg/kg body weight showed the lowest amongst the three extracts but higher than the pyrimethamine 4mg/kg with an inhibitory activity of 71.53±1.517% and
71.48±3.720% respectively (Table 4.4). The prophylactic effects showed statistical significance (p < 0.05).

**Table 4.4: Effect of crude extract on prophylactic activities of P. berghei infected mice.**

<table>
<thead>
<tr>
<th>Treatment/Extract</th>
<th>Dose (mg/kg)</th>
<th>Prophylaxis (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>74.69 ± 1.862</td>
<td></td>
</tr>
<tr>
<td>800</td>
<td>72.74 ± 2.035</td>
<td></td>
</tr>
<tr>
<td>600</td>
<td>69.21 ± 2.236</td>
<td></td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>76.18 ± 1.734</td>
<td></td>
</tr>
<tr>
<td>800</td>
<td>72.39 ± 1.878</td>
<td></td>
</tr>
<tr>
<td>600</td>
<td>69.34 ± 2.583</td>
<td></td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>4</td>
<td>71.48 ± 3.720</td>
</tr>
<tr>
<td>One way</td>
<td>F</td>
<td>0.798</td>
</tr>
<tr>
<td>ANOVA</td>
<td>P</td>
<td>0.51</td>
</tr>
<tr>
<td>Hexane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>71.53 ± 1.517</td>
<td></td>
</tr>
<tr>
<td>800</td>
<td>66.97 ± 2.900</td>
<td></td>
</tr>
<tr>
<td>600</td>
<td>61.21 ± 4.209</td>
<td></td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>4</td>
<td>71.48 ± 3.720</td>
</tr>
<tr>
<td>One way</td>
<td>F</td>
<td>2.257</td>
</tr>
<tr>
<td>ANOVA</td>
<td>P</td>
<td>0.113</td>
</tr>
</tbody>
</table>

Data represented as mean ± SEM; P value < 0.05 significant.
Generally, the trend observed was that the prophylactic activities of the extracts increased with increased concentration of the herbal extracts (Figure 4:7, 4:8 and 4:9 respectively). Higher proportions of prophylactic activities were observed in the hexane extracts followed by methanol extract while aqueous extract showed the lowest levels of inhibitory effects (p > 0.05).

Figure 4.7: Dose-dependent percentage inhibition of parasitaemia in mice placed on prophylactic treatment with aqueous extracts and pyrimethamine (p > 0.05). Each point is an average count from five infected mice (±SEM).
Figure 4. 8: Dose-dependent percentage of inhibition of parasitaemia in mice placed on prophylactic treatment with methanol extracts and pyrimethamine (p > 0.05). Each point is an average count from five infected mice (±SEM).
Figure 4. 9: Dose-dependent percentage inhibition of parasitaemia in mice placed on prophylactic treatment with hexane extracts and pyrimethamine (p > 0.05). Each point is an average count from five infected mice (±SEM).

4.6.3 Curative activities of plant extract on *P. berghei* in mice

Evaluation of the curative activities (Rane’s test) of the herbal extracts revealed a dose-dependent reduction of parasitaemia in all the experimental groups relative to the controls. The lowest levels of parasitaemia were observed at the highest concentrations of the extracts (1000mg/kg/bw). The mean percentage parasitaemia at the highest concentrations of the extracts (1000mg/kg/bw) were 15.31 ± 0.879%, 18.3 ± 1.237% and 18.33 ± 0.529% for methanol, aqueous
and hexane extracts respectively (Table 4.5). The extracts treated mice had a significantly lower parasitaemia when compared to the non-treated group (p<0.05). Statistically, using one-way ANOVA, the dose-dependent curative effects of aqueous extracts exhibited at a dose of 800 mg/kg/bw was comparable to that of 1000 mg/kg/bw. However, there was a significant difference in the curative effects exhibited at a dose of 1000 mg/kg/bw vs 600 mg/kg/bw and 800 mg/kg/bw vs 600 mg/kg/bw (p < 0.05; Figure 4.10). In addition, there was also a significant difference in the curative effects at various doses of aqueous extracts as compared to pyrimethamine at 4mg/kg/bw (p < 0.0001; Figure 4.10).

The hexane extract at various doses demonstrated no significant difference in their curative effects between different doses (p > 0.05; Figure 4.12). Nevertheless, there was also a significant difference when comparing their curative effects to that of the pyrimethamine at 4mg/kg/bw (p < 0.0001; Figure 4.12). Unlike aqueous and hexane extracts, there was no significant difference at a dose of 1000 mg/kg/bw vs 800 mg/kg/bw; and 800 mg/kg/bw vs 600 mg/kg/bw but a significant difference was observed between a dose of 1000 mg/kg/bw vs 600 mg/kg/bw in the methanolic extraction (p < 0.05; Figure 4.11). However, similar to aqueous and hexane extracts, methanol extract had a significant difference when comparing their curative effects to that of the pyrimethamine at 4mg/kg/bw (p < 0.0001; Figure 4.11).
### Table 4.5: Effects of crude extract on curative activities of *P. berghei* infected mice

<table>
<thead>
<tr>
<th>Treatment/Extract</th>
<th>Dose (mg/kg)</th>
<th>Curative (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aqueous</strong></td>
<td>1000</td>
<td>64.05 ± 4.052</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>61.45 ± 4.724</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>48.80 ± 4.482</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>4</td>
<td>99.79 ± 0.121</td>
</tr>
<tr>
<td><strong>Methanol</strong></td>
<td>1000</td>
<td>70.18 ± 2.842</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>62.98 ± 3.758</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>58.31 ± 4.808</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>4</td>
<td>99.79 ± 0.121</td>
</tr>
<tr>
<td><strong>Hexane</strong></td>
<td>1000</td>
<td>62.04 ± 2.688</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>63.39 ± 2.227</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>62.11 ± 2.407</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>4</td>
<td>99.79 ± 0.121</td>
</tr>
</tbody>
</table>

One Way F 32.56
ANOVA \( P \) < 0.0001

One way F 30.64
ANOVA \( P \) < 0.0001

One Way F 77.31
ANOVA \( P \) < 0.0001

Data represented as mean ± SEM; \( P \) value < 0.05 significant
Figure 4.10: Dose-dependent percentage inhibition of parasitaemia in mice placed on curative treatment with aqueous extracts and pyrimethamine (p < 0.05). Each point is an average count from five infected mice (±SEM).
Figure 4.11: Dose-dependent percentage inhibition of parasitaemia in mice placed on curative treatment with methanol extracts and pyrimethamine (p < 0.05). Each point is an average count from five infected mice (±SEM).
Figure 4.12: Dose-dependent percentage inhibition of parasitaemia in mice placed on curative treatment with hexane extracts and pyrimethamine (p < 0.05). Each point is an average count from five infected mice (±SEM).

4.6.4 Survival of mice in suppressive activities

The suppressive test or 4-day test involved co-treatment with extracts (for 4 days) starting on the day of infection (3 hours post infection) with parasite. The survivorship of *P. berghei* infected mice in the suppressive regimen was recorded over a 16-day period. The total numbers of days survived by each mouse were cumulatively average to yield the mean survival time (MST) and standard deviation (SD). The mean survival time for mice treated with aqueous extracts survived for lower days than mice treated with pyrimethamine at 4 mg/kg/bw with the exception of mice treated at a dose concentration of 600 mg/kg (14.40±2.302 days), which survived for a favorably comparable
numbers of days (14.60±2.191 days) similar to pyrimethamine at 4 mg/kg/bw-treated group.

Unlike mice treated with aqueous extracts, methanol extracts at a dose concentration of 1000 mg/kg/bw and 200 mg/kg/bw prolonged the mean survival time of mice (15.60±0.894 and 16.00±0.00 days) respectively as compared to mice treated with pyrimethamine at 4 mg/kg/bw (14.60±2.191 days). In addition, hexane at 800 mg/kg/bw prolonged the mean survival time of mice (16.00±0.000 days) as compared to mice in the pyrimethamine at 4 mg/kg/bw treated group (14.60±2.191 days). Generally, all the extracts and pyrimethamine 4mg/kgbw – treated mice prolonged the mean survival time of mice as compared to mice in the infected non-treated group ( P < 0.05; Table 4.7). It was observed that pyrimethamine and methanol extracts treated mice had the highest percentage of mean survival time of mice up to day 16 as compared to aqueous and hexane extracts (Table 4.7 and Figure 4.13, 4.14 and 4.15).
Table 4.6: Effects of crude extract on mean survival time of *P. berghei* infected mice in suppressive activities

<table>
<thead>
<tr>
<th>Treatment/Extract</th>
<th>Dose (mg/kg)</th>
<th>Survival Time (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>10.20 ± 5.119</td>
<td></td>
</tr>
<tr>
<td>800</td>
<td>10.80 ± 5.450</td>
<td></td>
</tr>
<tr>
<td>600</td>
<td>14.40 ± 2.302</td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>11.20 ± 5.357</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>13.20 ± 3.701</td>
<td></td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>4</td>
<td>14.60 ± 2.191</td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td>6.20 ± 2.864</td>
</tr>
<tr>
<td>One way</td>
<td>F</td>
<td>6.755</td>
</tr>
<tr>
<td>ANOVA</td>
<td>P</td>
<td>&lt; 0.0108</td>
</tr>
<tr>
<td>Methanol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>15.60 ± 0.894</td>
<td></td>
</tr>
<tr>
<td>800</td>
<td>14.00 ± 4.472</td>
<td></td>
</tr>
<tr>
<td>600</td>
<td>14.60 ± 3.130</td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>14.40 ± 2.608</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>16.00 ± 0.000</td>
<td></td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>4</td>
<td>14.60 ± 2.191</td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td>6.20 ± 2.864</td>
</tr>
<tr>
<td>One way</td>
<td>F</td>
<td>28.97</td>
</tr>
<tr>
<td>ANOVA</td>
<td>P</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Hexane</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000</td>
<td>12.60 ± 4.775</td>
<td></td>
</tr>
<tr>
<td>800</td>
<td>16.00 ± 0.000</td>
<td></td>
</tr>
<tr>
<td>600</td>
<td>11.80 ± 4.550</td>
<td></td>
</tr>
<tr>
<td>400</td>
<td>12.40 ± 5.128</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td>13.80 ± 3.493</td>
<td></td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>4</td>
<td>14.60 ± 2.191</td>
</tr>
<tr>
<td>PBS</td>
<td></td>
<td>6.20 ± 2.864</td>
</tr>
<tr>
<td>One way</td>
<td>F</td>
<td>8.067</td>
</tr>
<tr>
<td>ANOVA</td>
<td>P</td>
<td>&lt; 0.0060</td>
</tr>
</tbody>
</table>

Data represented as mean ± SD; P value < 0.05 significant
Figure 4.13: Survival of *P. berghei*-infected mice in suppressive regimen treated with aqueous extracts and pyrimethamine (p < 0.05). Data are expressed as mean ± SD for five mice per group.
Figure 4.14: Survival of *P. berghei*-infected mice in suppressive regimen treated with methanol extracts and pyrimethamine (p < 0.05). Data are expressed as mean ± SD for five mice per group.
Figure 4.15: Survival of *P. berghei*-infected mice in suppressive regimen treated with hexane extracts and pyrimethamine (p < 0.05). Data are expressed as mean ± SD for five mice per group.

4.6.5 Survival of mice in prophylactic activities

Unlike the suppressive regimen which involved co-treatment with extracts 3 hours post infection, in the prophylactic activities, extracts were first administered for four days after which parasite inocula were intraperitoneally administered. It was observed that the aqueous extracts at dose concentrations of 1000, 800 and 600 mg/kg/bw in the suppressive regimen gave slightly higher mean survival time as compared to similar dose concentrations in the prophylactic regimen (p > 0.05; Table 4.8). It was noted that methanolic extracts at dose concentrations of 1000, 800 and 600 mg/kg/bw gave the
highest mean survival time in the prophylactic regimen (11.00 ± 6.856, 9.40 ± 6.189 and 10.60 ± 4.827 days respectively) but lower than similar dose concentrations in suppressive regimen (p < 0.05; Table 4.7 and 4.8). However, for both suppressive and prophylactic regimens, mice treated with methanol extracts gave the highest mean survival time when compared to mice treated with aqueous, hexane extracts and pyrimethamine 4mg/kg/bw-positive control (p < 0.05; Table 4.7 and 4.8). Similar to mice treated with aqueous and methanol extracts, mice treated with hexane extracts in prophylactic regimen also gave a lower mean survival time (Table 4.8) when compared to those at similar dose concentrations in suppressive regimen (p < 0.05; Table 4.7). Overly, all extracts treated mice prolonged the mean survival time of mice thereby obtaining the highest percentage of surviving mice as compared to mice treated with pyrimethamine at 4mg/kg/bw and non-treated groups (p > 0.05; Table 4.8; Figure 4.16, 4.17 and 4.18).
Table 4.7: Effects of crude extract on mean survival time of *P. berghei* infected mice in prophylactic activities

<table>
<thead>
<tr>
<th>Treatment/Extract</th>
<th>Dose (mg/kg)</th>
<th>Survival Time (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Aqueous</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>8.60 ± 6.768</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>9.40 ± 6.189</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>7.00 ± 5.431</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>4</td>
<td>6.00 ± 3.162</td>
</tr>
<tr>
<td>PBS</td>
<td>-</td>
<td>6.20 ± 2.864</td>
</tr>
<tr>
<td>One way ANOVA</td>
<td>F</td>
<td>0.4906</td>
</tr>
<tr>
<td><strong>Methanol</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>11.00 ± 6.856</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>9.40 ± 6.189</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>10.60 ± 4.827</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>4</td>
<td>6.00 ± 3.162</td>
</tr>
<tr>
<td>PBS</td>
<td>-</td>
<td>6.20 ± 2.864</td>
</tr>
<tr>
<td>One way ANOVA</td>
<td>F</td>
<td>1.844</td>
</tr>
<tr>
<td><strong>Hexane</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1000</td>
<td>10.60 ± 3.847</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>7.60 ± 5.030</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>6.40 ± 5.128</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>4</td>
<td>6.00 ± 3.162</td>
</tr>
<tr>
<td>PBS</td>
<td>-</td>
<td>6.20 ± 2.864</td>
</tr>
<tr>
<td>One way ANOVA</td>
<td>F</td>
<td>3.073</td>
</tr>
<tr>
<td><strong>Data represented as mean ± SD; P value &lt; 0.05 significant</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
Figure 4.16: Survival of *P. berghei*-infected mice in prophylactic regimen treated with aqueous extracts and pyrimethamine (p > 0.05). Data are expressed as mean ± SD for five mice per group.
Figure 4.17: Survival of *P. berghei*-infected mice in prophylactic regimen treated with methanol extracts and pyrimethamine (p > 0.05). Data are expressed as mean ± SD for five mice per group.
Figure 4.18: Survival of *P. berghei*-infected mice in prophylactic regimen treated with hexane extracts and pyrimethamine (*p* > 0.05). Data are expressed as mean ± SD for five mice per group.

4.6.6 Survival of mice in curative activities with plant extracts

Unlike the suppressive regimen, the curative regimen (Rane’s test or test of established infection) involved post treatment after 72 hours of infection with parasite. It was observed that mice treated with aqueous extracts at dose concentration of 1000, 800 and 600 mg/kg/bw prolonged the mean survival time (11.00±6.462, 12.80±4.147 and 10.40±5.505 days respectively) of mice as compared to mice treated with methanol and hexane extracts at similar dose concentrations (*p* > 0.05; Table 4.9). Additionally, mice treated with aqueous extracts gave the highest mean survival time in the curative regimen (Table
4.9) when compared to mice at similar dose concentrations of the prophylactic and suppressive regimens (p > 0.05; Table 4.8 and 4.7 respectively). However, mice treated with methanol and hexane extracts in the prophylactic regimen (Table 4.8) prolonged the mean survival time of mice when compared to mice in the curative regimen (p > 0.05; Table 4.9). Generally, mice treated with extracts and pyrimethamine 4mg/kg prolonged the mean survival time of mice and higher percentage of mice surviving during the study period relative to the non-treated (p > 0.05; Table 4.9; Figure 4.19, 4.20 and 4.21).
Table 4.8: Effects of crude extract on mean survival time of *P. berghei* infected mice in curative activities

<table>
<thead>
<tr>
<th>Treatment/Extract</th>
<th>Dose (mg/kg)</th>
<th>Survival Time (Day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous</td>
<td>1000</td>
<td>11.00 ± 6.462</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>12.80 ± 4.147</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>10.40 ± 5.505</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>4</td>
<td>14.40 ± 2.302</td>
</tr>
<tr>
<td>PBS</td>
<td>-</td>
<td>6.20 ± 2.864</td>
</tr>
<tr>
<td>One way ANOVA</td>
<td>F</td>
<td>4.629</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.0324</td>
</tr>
<tr>
<td>Methanol</td>
<td>1000</td>
<td>10.00 ± 7.778</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>12.00 ± 6.519</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>6.80 ± 5.586</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>4</td>
<td>14.40 ± 2.302</td>
</tr>
<tr>
<td>PBS</td>
<td>-</td>
<td>6.20 ± 2.864</td>
</tr>
<tr>
<td>One way ANOVA</td>
<td>F</td>
<td>3.414</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.0670</td>
</tr>
<tr>
<td>Hexane</td>
<td>1000</td>
<td>8.00 ± 5.431</td>
</tr>
<tr>
<td></td>
<td>800</td>
<td>7.00 ± 5.523</td>
</tr>
<tr>
<td></td>
<td>600</td>
<td>7.20 ± 5.357</td>
</tr>
<tr>
<td>Pyrimethamine</td>
<td>4</td>
<td>14.40 ± 2.302</td>
</tr>
<tr>
<td>PBS</td>
<td>-</td>
<td>6.20 ± 2.864</td>
</tr>
<tr>
<td>One way ANOVA</td>
<td>F</td>
<td>6.479</td>
</tr>
<tr>
<td></td>
<td>P</td>
<td>0.0124</td>
</tr>
</tbody>
</table>

Data represented as mean ± SD; *P* value < 0.05 significant
Figure 4.19: Survival of *P. berghei*-infected mice in curative regimen treated with aqueous extracts and pyrimethamine *(p < 0.05).* Data are expressed as mean ± SD for five mice per group.
Figure 4.20: Survival of *P. berghei*-infected mice in curative regimen treated with methanol extracts and pyrimethamine (p > 0.05). Data are expressed as mean ± SD for five mice per group.
Figure 4.21: Survival of *P. berghei*-infected mice in curative regimen treated with hexane extracts and pyrimethamine (p < 0.05). Data are expressed as mean ± SD for five mice per group.
CHAPTER FIVE: DISCUSSION

5.1 Plant material and extracts

The botanical identity of anti-malarial plant investigated was authenticated by a taxonomist as an essential prerequisite. The selected medicinal plant for investigation and plant part collected were those commonly used in traditional preparations as guided by a herbalist. To prevent enzymatic degradation and minimize post-harvest chemical changes plant material was air-dried at room temperature before extraction as recommended by (Sofowara, 1996).

Successive extractions with solvents in increasing polarity are a common procedure in many screening programmes (Gessler et al., 1995). It involves removing fatty oils from plant materials using petroleum ether, cyclohexane or heptanes. The residue is then extracted with ethyl acetate or chloroform, then methanol and finally water. In the successive extractions, non-polar compounds and polar compounds are extracted with their respective solvents. An alternative approach would be to use all-purpose aqueous-alcohol mixture.

Extraction in this study was based on the scientific principle in which non-polar solvents (hexane) was used to dissolve and extract non-polar compounds while polar solvents; methanol and water were used to extract polar compounds (Willcox and Bodeker, 2000).
5.2 In vitro anti-plasmodial activities and IC$_{50}$ values

Numeral experimental models for detecting antiplasmodial activity of plant extracts in erythrocytic stage of malaria parasites are available. The standard method, the radioactive microdilution technique (Desjardins et al., 1979) is based on the inhibition of hypoxanthine uptake by *plasmodia* cultured on human blood. The results are expressed as percentage inhibition with respect to controls or as median inhibitory concentration of 50% of the culture (IC$_{50}$).

The grade of efficacy of extracts according to IC$_{50}$ values are ranked as: very good (IC$_{50}$<0.1 µg/ml); good (IC$_{50}$ values of 0.1 -1.0 µg/ml); good to moderate (IC$_{50}$ values of 1.1 – 10 µg/ml); weak (IC$_{50}$ values of 11 – 25 µg/ml); very weak (IC$_{50}$ value range of 26 – 50 µg/ml); and inactive (amid IC$_{50}$ < 100 µg/ml) (Rasoanaivo et al., 1994). The three extracts in this study were within the IC$_{50}$ range of 1 – 10 µg/ml, indicating a good to moderate anti-*plasmodial* activity. This makes the extracts good candidates for fractionation.

Activity indices are determined by comparing test IC$_{50}$ values with a reference to give a comparison in anti-*plasmodial* activities during *in vitro* test involving the erythrocytic stages of *plasmodial*. When the IC$_{50}$ value of the most active extract-MeOH (IC$_{50}$ = 0.653 µg/mL) for *P. falciparum* W2 is divided by the standard reference (ATM), activities indices of 10.04 is found respectively. It is however higher when compared with an ethanolic extract from *Cassia occidentalis*, with observed activity index of 3.5 (Tona et al., 2004). It is
however notable that the IC$_{50}$ values of the hexane and methanol extracts used in the present study against *P. falciparum* -3D7 strain are comparably lower than *Cassia occidentalis* - MeoH (IC$_{50} = 88.2 \pm 2.2 \mu$g/mL), *Senna didymobotrya* – MeOH (IC$_{50} = 56.0 \pm 9.9 \mu$g/mL); *Cassia occidentalis* – EtoAC (IC$_{50} = 31.9 \pm 4.5 \mu$g/mL); *Cassia occidentalis* – n-hexane (IC$_{50} = 19.3 \pm 2.0 \mu$g/mL), *Senna didymobotrya* –n-hexane (IC$_{50} = 57.6 \pm 22.3 \mu$g/mL), that were in fact considered (IC$_{50} \geq 10 \mu$g/mL and $\leq 50 \mu$g/mL moderately active and $< 50 \mu$g/mL inactive respectively) (Ramalhete *et al.*, 2008).

In this study, the lowest IC$_{50}$ values were recorded in methanolic extractions for both strains of *P. falciparum*. Additionally, test on the antiparasmodial activities of the three extracts against both strains of *P. falciparum* (W2 and 3D7) gave hexane extract the highest IC$_{50}$ (8.785 and 15.650µg/mL) followed by aqueous extract (1.832 and 6.283µg/mL) and methanol extract obtaining the lowest (0.6529 and 2.288 µg/mL) respectively, implying that methanol and aqueous extracts are more active than hexane extract against *P. falciparum*. These results disagree with finding from another study of a plant in similar family (*Cassia occidentalis*) in which the IC$_{50}$ value for hexane extract (IC$_{50} = 19.9\mu$g/mL) was lower than methanol (IC$_{50} = 88.9\mu$g/mL) and ethyl acetate (IC$_{50} = 31.9\mu$g/mL) extracts respectively (Ramalhete *et al.*, 2008). In a related study, an ethanolic extract of *C. occidentalis* (plant in the same family) gave an IC$_{50}$ value of 4.5µg/mL (Choudhary *et al.*, 2014 and Tona *et al.*, 2004).
The observations by Chouldhary et al. (2014) and Tona et al. (2004) are corroborated by this study whereby alcoholic extracts (methanolic) exhibited the highest anti-plasmodial activities ($IC_{50}=0.653\ \mu g/ml$ for W2 and $IC_{50}=2.29\ \mu g/ml$ for 3D7) as compared to aqueous and hexane extracts. This difference in the bioactivity over aqueous and hexane extracts points to the minimal destruction of other active compounds and/or a refining effect of synergistic properties of compounds in the methanolic fraction. The high anti-plasmodial activity by the methanol extract could be attributed to the presence of quinones and anthraquinone as previously reported by Tona et al. (2004) on ethanolic extract C. occidentalis.

5.3 Suppressive and curative activities of S. occidentalis roots extracts

The potent anti-malarial activity of the plant extracts in in vitro assay, were replicated in vivo using the 4-Day suppressive test. The mean percentage inhibition of parasitaemia in the mouse model was highest for the polar solvent extracts than the non-polar solvent extract. In the 4-day suppressive activity, the three extracts significantly reduced parasitaemia in mice in a dose-dependent manner with methanol extract exhibiting the highest anti-plasmodial activity. The high activity by methanolic extracts can be attributed to the minimal destruction of other active compounds as previously reported by Tona et al. (2004). The highest mean percentage inhibition of parasitaemia was observed at a dose of 1000 mg/kg while 200 mg/kg exhibited the lowest activity across the three extracts. The overall 60% suppression by the three extracts was comparable to a study reported by Tona et al. (2004).
It was observed in this study that aqueous extracts was more potent than the methanolic extracts at day 4. This is in contrast to the findings by Tona et al. (2004) where ethanolic extracts of *C. occidentalis* were more active than the aqueous extracts. There is therefore, an implication that although polar extracts are potent; there may be a variation in their potency or anti-malarial activities base on the solvent used (Chouldhary *et al.*, 2014). The plant metabolites that are suspected to be responsible for the bioactivity exhibited by the three extracts tested include alkaloid, terpenes, anthaquinones, emodin, flavonoid and quinones as reported by Kolewale *et al.* (2010) and Berhan *et al.* (2012).

From the curative test, it was observed that the three extracts exhibited over 60% mean parasite inhibition at doses above 800 mg/kg body weight. The significant decrease in parasitaemia over time in the curative test implies that bioactive compounds in the tested extracts had a significant bioavailability. These plant metabolites like the quinones as earlier noted may be inferred to have some residual effect in the mice. This may explain the significant decrease in parasitaemia as observed 6 days post treatment. The three extracts exhibited significant antiplasmodial activities in the suppressive and curative tests when compared to the infected non treated mice however, the extracts was not as effective as the reference drugs – pyrimethamine at dose of 4 mg/kg body weight in parasite clearance.
5.4 Prophylactic activities of *S. occidentalis* roots extracts

The three extracts exhibited a significant prophylactic activity against *P. berghei* infected mice as demonstrated by the reduction in the levels of parasitaemia dose dependently. It is evident based on these findings that *S. occidentalis* roots extracts possess anti-plasmodial effect justifying its folkloric usage in the management of malaria. This effect may be attributed to the presence of alkaloids, terpenes and flavonoids that have been implicated in anti-plasmodial activity as reported by Alexandru *et al.* (2007), Asres *et al.* (2001b), Saxena *et al.* (2003) and Hilou *et al.* (2006).

The mean percentage inhibition of parasitaemia in the prophylactic activity was again high in the polar solvent extracts (methanol extract) than the non-polar solvent extract. The high activity by methanolic extracts can be attributed to the minimal destruction of other active compounds as previously reported by Tona *et al.* (2004). This difference in the bioactivity over aqueous and hexane extracts points to the minimal destruction of other active compounds and/or a refining effect of synergistic properties of compounds in the methanolic fraction. The high anti-plasmodial activity by the methanol extract could be attributed to the presence of quinones and anthraquinone as previously reported by Alexandru *et al.* (2007) on ethanolic extract *C. occidentalis*.

*Senna occidentalis* has been reported to contained terpenoids (Chouldhary *et al.*, 2014). Phytochemical compounds such as terpenoids are commonly implicated in the anti-protozoa and anti-plasmodial activity of many plants.
(Abduleleh and Zainal-Abidin, 2007). An example of common terpenoids is Artemisinin. These phytochemical compounds are can be attributed to the prophylactic effect exhibited by *S. occidentalis*. Flavonoids are the other form of bioactive compounds present in the *S. occidentalis* plant that are further implicated in the antiparasitic activity as observed by Berhan *et al.* (2012). These chemical compounds may be acting singly or in synergy with one another to exert the observed anti-malarial activity of *S. occidentalis*.

### 5.5 Survival of mice treated with roots extracts of *S. occidentalis*

The survivorship of *P. berghei* infected mice was recorded over a 16-day period. There was significant parasitaemia suppression in mice treated with the three extracts. This resulted to lowering and delaying parasitaemia onset and progression that led to increased survival time of treated mice compared to the infected non-treated mice. It was observed that chemosuppression was dose-dependent, thus, survivorship of the extracts treated mice was also dose-dependent. Overall, mice groups treated with extracts had 80%, 70% and 60% survivorship in the suppressive, curative, and prophylactic regimen respectively. This indicated a better survival rates after treatment with the three extracts. The high percentage of survivorship observed in the suppressive and curative treatment regimen could be attributed to that simultaneous presence of the bioactive compounds in the extracts as well as the parasites. This in turn may have actively affected the growth and reproduction of the parasites (Kolawole *et al.*, 2010). On the other hand, prophylactic treatment with the extracts resulted in a fairly low survivorship possibly due to the metabolism
and excretion of the bioactive plant compounds prior to the establishment of *P. berghei* infection (Saxena *et al.*, 2003). However, the 60% survivorship is indicative of potential residual action of the plant extracts.

The improved survivorship could be attributed to the observed high chemosuppressive activity of the extracts on parasites that, in turn, reduced the overall parasite burden on the mice. This probably gave the immune systems of the mice time to recover and help control the parasite infection. Chemosuppression is inversely related to parasitaemia. In this study, the three extracts have shown to reduce parasitaemia to low levels demonstrating corresponding high chemosuppression. The methanolic extracts had the highest chemosuppression in the three treatment regimen recorded in the experiment from the three extracts tested. Subsequently, the infected mice treated with methanol extracts had the highest mean survival time compared to those treated with aqueous and hexane extracts. This high mean survival time of mice treated with the methanol extract could be attributed to its high anti-*plasmodial* activity on parasitaemia as earlier observed.

The reduced parasitaemia and corresponding high survival rates could be through effects of bioactive compounds on the parasites such as reduction of parasite nutrient intake, interference of parasite metabolic pathways like heme metabolic pathway which is involved in the metabolism of iron (Abdulelah and Zainal-Abidin, 2007). The extracts could be further attributed to negative effects on parasite reproduction and growth as explained by Asres *et al.* (2001b) on drugs. Overall, the extracts led to decreased parasitaemia and
subsequent relief from symptomatic malaria thus the high survivorship by day 16.

The study therefore establishes that the plant evaluated were suppressive (the 4-day test or suppressive treated involving co-treatment starting on the day of infection with parasite and continue for 4-days), prophylactic which involved pre-treatment stage (4 days before infection with parasite) and curative (Rane’s test or established infection which involved post treatment after 72 hours of infection with parasite; lowering and delaying parasitaemia onset and progression that resulted in increased survival of treated mice. These observations confirm the traditional use as medicinal plant in malaria therapy and demonstrate the potential as a source of anti-malarial drugs.

5.6 Hypothesis Testing

This study reports that *S. occidentalis* plant extracts have anti-plasmodial effects on *P. falciparum* and *P. berghei*. The finding of this study therefore rejects the first null hypothesis. The finding of this study also reports that there is difference in the anti-plasmodial activities between polar and non polar solvents of *S. occidentalis*. This finding therefore rejects the second null hypothesis.
CHAPTER SIX: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

From the *in vitro* chemotherapeutic test;

i. The three extracts; aqueous, methanol and hexane from *S. occidentalis* roots possess anti-plasmodial activities in the *in vitro* assay against *P. falciparum*.

ii. The three extracts from *S. occidentalis* roots tested were found to be more potent against the resistance strain of *P. falciparum* (W2-chloroquine resistance) when compared to the sensitive strain (3D7-chloroquine sensitive) of *P. falciparum*.

iii. The polar extracts (methanol and aqueous) were found to be potent than the non-polar extract (hexane) in *in vitro*.

From the *in vivo* bioassay;

i. The three extracts; aqueous, methanol and hexane from *S. occidentalis* roots possess anti-plasmodial activities in the *in vivo* assay against *P. berghei* in BALB/c mice.

ii. The three extracts increased mice survivorship by maintaining low parasitaemia profiles and delaying parasitaemia onset and progression.
iii. The anti-plasmodial activity of the three extracts is not affected by treatment regimen, as the extracts were equally active in the suppressive, curative and prophylactic tests.

iv. The polar extracts (methanol and aqueous) were found to be more potent than the non-polar extract (hexane) in \textit{in vivo}.

6.2 Recommendations

Based on this study it is recommended that:

i. Traditional ethnomedicinal knowledge and information are still relevant and should be harnessed and standardized in the continued search for active lead plant molecules in the development of anti-malarial drugs.

ii. Isolation and characterization of bioactive compounds from \textit{S. occidentalis} against \textit{P. berghei} and \textit{P. falciparum}.

iii. Laboratory maintained strains of \textit{P. berghei} and \textit{P. falciparum} remain useful in screening plant extracts for bioactive components given their sensitivity to the extracts.

iv. Combinational regimen of polar and non-polar solvent extracts of \textit{S. occidentalis} roots at different doses should be carried out \textit{in vitro} and \textit{in vivo}.
6.3 Recommendations for future research

i. To determine the *in vitro* and *in vivo* cytotoxicity levels/effects of *S. occidentalis* roots extracts on cells and body organs.

ii. Combination studies of *S. occidentalis* roots extracts with other anti-malarial plant extracts or standard anti-malarial on drug-resistant malaria parasites.

iii. The formulation of *S. occidentalis* tablet to be evaluated against *P. knowlesi* in baboons.

i. Comparative studies need to be carried out on various parts of *S. occidentalis* to determine which plant parts have the most potent anti-malarial activities.

ii. This study recommends that further study be undertaken on *S. occidentalis* obtained from different regions/countries of Africa.
REFERENCES


APPENDIX I: Statistical Analysis

i. Analysis of Variance (Two-way ANOVA) of the effect of aqueous crude extract for suppressive activity of *P. berghei* infected mice for day four and seven post infection.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>% of total variation</th>
<th>df-value</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>P-value</th>
<th>F-value</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>0.33</td>
<td>1</td>
<td>52.93</td>
<td>13.23</td>
<td>0.6723</td>
<td>1.214</td>
<td>No</td>
</tr>
<tr>
<td>Dose (mg/kg)</td>
<td>27.64</td>
<td>4</td>
<td>166.7</td>
<td>41.69</td>
<td>0.0111</td>
<td>3.823</td>
<td>Yes</td>
</tr>
<tr>
<td>Total</td>
<td>27.97</td>
<td>5</td>
<td><strong>219.63</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

ii. Analysis of Variance (Two-way ANOVA) of the effect of methanol crude extract for suppressive activity of *P. berghei* infected mice for day four and seven post infection.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>% of total variation</th>
<th>df-value</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>P-value</th>
<th>F-value</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>14.92</td>
<td>1</td>
<td>46.29</td>
<td>46.29</td>
<td>0.0514</td>
<td>4.322</td>
<td>No</td>
</tr>
<tr>
<td>Dose (mg/kg)</td>
<td>15.37</td>
<td>4</td>
<td>47.68</td>
<td>11.92</td>
<td>0.3795</td>
<td>1.113</td>
<td>No</td>
</tr>
<tr>
<td>Total</td>
<td>30.29</td>
<td>5</td>
<td><strong>93.97</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

iii. Analysis of Variance (Two-way ANOVA) of the effect of hexane crude extract for suppressive activity of *P. berghei* infected mice for day four and seven post infection.

<table>
<thead>
<tr>
<th>Source of variation</th>
<th>% of total variation</th>
<th>df-value</th>
<th>Sum of squares</th>
<th>Mean square</th>
<th>P-value</th>
<th>F-value</th>
<th>Sig.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>0.30</td>
<td>1</td>
<td>3.762</td>
<td>3.762</td>
<td>0.7662</td>
<td>0.06452</td>
<td>No</td>
</tr>
<tr>
<td>Dose (mg/kg)</td>
<td>9.21</td>
<td>4</td>
<td>115.8</td>
<td>28.94</td>
<td>0.6028</td>
<td>0.09019</td>
<td>No</td>
</tr>
<tr>
<td>Total</td>
<td>9.51</td>
<td>5</td>
<td><strong>119.562</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
iv. **Analysis of variance (One-way ANOVA) of the effect aqueous crude extract for prophylactic activity of *P. berghei* infected mice.**

<table>
<thead>
<tr>
<th>ANOVA Table</th>
<th>df - value</th>
<th>Sum of Squares</th>
<th>Mean of Square</th>
<th>P-value</th>
<th>F-value</th>
<th>Sig.</th>
<th>95% CI of diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment (between columns)</td>
<td>3</td>
<td>27.89</td>
<td>9.296</td>
<td>0.6989</td>
<td>0.4815</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Residual (within columns)</td>
<td>20</td>
<td>386.2</td>
<td>19.31</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>414.1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bonferroni’s Multiple Comparison Test</td>
<td></td>
<td>Significant? P&lt;0.05?</td>
<td>95% CI of diff.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000mg/kg vs 800mg/kg</td>
<td>No</td>
<td>-8.489 to 6.363</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000mg/kg vs 600mg/kg</td>
<td>No</td>
<td>-10.37 to 4.482</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000mg/kg vs Pyrimethamine (4mg/kg)</td>
<td>No</td>
<td>-9.283 to 5.569</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>800mg/kg vs 600mg/kg</td>
<td>No</td>
<td>-9.306 to 5.546</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>800mg/kg vs Pyrimethamine (4mg/kg)</td>
<td>No</td>
<td>-8.220 to 6.632</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>600mg/kg vs Primethamine (4mg/kg)</td>
<td>No</td>
<td>-6.340 to 8.512</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

v. **Analysis of variance (One-way ANOVA) of the effect methanol crude extract for prophylactic activity of *P. berghei* infected mice.**

<table>
<thead>
<tr>
<th>ANOVA Table</th>
<th>df - value</th>
<th>Sum of Squares</th>
<th>Mean of Square</th>
<th>P-value</th>
<th>F-value</th>
<th>Sig.</th>
<th>95% CI of diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment (between columns)</td>
<td>3</td>
<td>39.85</td>
<td>13.28</td>
<td>0.5667</td>
<td>0.6775</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Residual (within columns)</td>
<td>20</td>
<td>392.1</td>
<td>19.31</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>432.0</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bonferroni’s Multiple Comparison Test</td>
<td></td>
<td>Significant? P&lt;0.05?</td>
<td>95% CI of diff.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1000mg/kg vs 800mg/kg</td>
<td>No</td>
<td>-9.388 to 5.578</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
vi. Analysis of variance (One-way ANOVA) of the effect hexane crude extract for prophylactic activity of *P. berghei* infected mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Mean of Square</th>
<th>P-value</th>
<th>F-value</th>
<th>Sig.</th>
<th>95% CI of diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment (between columns)</td>
<td>3</td>
<td>119.7</td>
<td>39.91</td>
<td>0.392</td>
<td>1.277</td>
<td>No</td>
<td></td>
</tr>
<tr>
<td>Residual (within columns)</td>
<td>20</td>
<td>624.9</td>
<td>31.24</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>744.6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Bonferroni’s Multiple Comparison Test

Significant? P<0.05? 95% CI of diff.

1000mg/kg vs 800mg/kg  No  -11.94 to 6.953
1000mg/kg vs 600mg/kg  No  -15.03 to 3.863
1000mg/kg vs Pyrimethamine (4mg/kg) No  -9.741 to 9.151
800mg/kg vs 600mg/kg  No  -12.54 to 6.356
800mg/kg vs Pyrimethamine (4mg/kg) No  -7.248 to 11.64
600mg/kg vs Primethamine (4mg/kg) No  -4.158 to 14.73

vii. Analysis of variance (One-way ANOVA) of the effect aqueous crude extract for curative activity of *P. berghei* infected mice.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>df</th>
<th>Sum of Squares</th>
<th>Mean of Square</th>
<th>P-value</th>
<th>F-value</th>
<th>Sig.</th>
<th>95% CI of diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment (between columns)</td>
<td>2</td>
<td>179.0</td>
<td>89.49</td>
<td>0.0073</td>
<td>6.551</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Residual (within columns)</td>
<td>18</td>
<td>245.9</td>
<td>13.66</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>df-value</td>
<td>Sum of Squares</td>
<td>Mean of Square</td>
<td>P-value</td>
<td>F-value</td>
<td>Sig.</td>
<td>95% CI of diff.</td>
</tr>
<tr>
<td>------------------</td>
<td>----------</td>
<td>----------------</td>
<td>----------------</td>
<td>---------</td>
<td>---------</td>
<td>------</td>
<td>----------------</td>
</tr>
<tr>
<td>Treatment</td>
<td>2</td>
<td>127.8</td>
<td>89.49</td>
<td>0.0061</td>
<td>6.858</td>
<td>Yes</td>
<td></td>
</tr>
<tr>
<td>Residual</td>
<td>18</td>
<td>167.7</td>
<td>13.66</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>295.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Descriptive ANOVA Table

Bonferroni’s Multiple Comparison Test

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Significant? P&lt;0.05?</th>
<th>95% CI of diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000mg/kg vs 800mg/kg</td>
<td>No</td>
<td>-6.548 to 3.881</td>
</tr>
<tr>
<td>1000mg/kg vs 600mg/kg</td>
<td>Yes</td>
<td>-11.97 to -1.537</td>
</tr>
<tr>
<td>800mg/kg vs 600mg/kg</td>
<td>Yes</td>
<td>-10.63 to -0.2035</td>
</tr>
</tbody>
</table>

viii. Analysis of variance (One-way ANOVA) of the effect methanol crude extract for curative activity of *P. berghei* infected mice.

ix. Analysis of variance (One-way ANOVA) of the effect hexane crude extract for curative activity of *P. berghei* infected mice.
<table>
<thead>
<tr>
<th>Treatment (between columns)</th>
<th>2</th>
<th>5.742</th>
<th>2.871</th>
<th>0.1476</th>
<th>2.132</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Residual (within columns)</td>
<td>18</td>
<td>24.24</td>
<td>1.346</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>20</td>
<td>29.98</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Bonferroni’s Multiple Comparison Test

<table>
<thead>
<tr>
<th>Bonferroni’s Multiple Comparison Test</th>
<th>Significant?</th>
<th>P&lt;0.05?</th>
<th>95% CI of diff.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1000mg/kg vs 800mg/kg</td>
<td>No</td>
<td></td>
<td>-2.264 to 1.009</td>
</tr>
<tr>
<td>1000mg/kg vs 600mg/kg</td>
<td>No</td>
<td></td>
<td>-2.918 to -0.3562</td>
</tr>
<tr>
<td>800mg/kg vs 600mg/kg</td>
<td>No</td>
<td></td>
<td>-2.290 to -0.9835</td>
</tr>
</tbody>
</table>

x. Analysis of the effects of crude extracts on the in vitro antiplasmodial activity of both strains of *P. falciparum* (W2 and 3D7) using Student t-test.

<table>
<thead>
<tr>
<th>Table Analyzed</th>
<th>P-value</th>
<th>t-value</th>
<th>df</th>
<th>Sig.</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aqueous 3D7 vs Aqueous W2</td>
<td>0.2464</td>
<td>1.284</td>
<td>6</td>
<td>No</td>
<td>-17.80 to 5.547</td>
</tr>
<tr>
<td>Hexane 3D7 vs Hexane W2</td>
<td>0.3732</td>
<td>0.9620</td>
<td>6</td>
<td>No</td>
<td>-11.58 to 5.044</td>
</tr>
<tr>
<td>Methanol 3D7 vs Methanol W2</td>
<td>0.2473</td>
<td>1.282</td>
<td>6</td>
<td>No</td>
<td>-13.46 to 4.206</td>
</tr>
</tbody>
</table>
APPENDIX II: Ethical Approval

Institute of Primate Research

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

INSTITUTIONAL SCIENTIFIC AND ETHICS REVIEW COMMITTEE
(ISERC)

FINAL PROPOSAL APPROVAL FORM

Our ref: ISERC/10/16

Dear Julius W. Teahion, Jr.

It is my pleasure to inform you that your proposal entitled “In vitro and In vivo Antiplasmodial Activity of S. occidentalis Extracts against Plasmodium falciparum and Plasmodium berghel” in collaboration with Dr. Hastings Ozwara of IPR and Prof. Michael M. Gicheru of Kenyatta University, has been reviewed by the Institutional Review Committee (IRC) at a meeting of 28th June 2016. The proposal was reviewed on the scientific merit and ethical considerations on the use of animals for research purposes. The committee is guided by the Institutional guidelines as well as International regulations, including those of WHO, NIH, PVEN and Helsinki Convention on the humane treatment of animals for scientific purposes and GLP.

You are bound by the IPR Intellectual Property Policy.

Signed: [Signature] Chairman IRC: DR. I. NIANI

Signed: [Signature] Secretary IRC: DR. NDIAMA JILLANI

Date: 31/07/2016

INSTITUTE OF PRIMATE RESEARCH
INSTITUTIONAL REVIEW COMMITTEE
P. O. Box 24481-00502 KAREN
NAIROBI - KENYA
APPROVED
APPENDIX III: Roots of *Senna occidentalis*

Fresh leaves of *Senna occidentalis*

Roots of *Senna occidentalis* with voucher specimen No. 38/81