IN VIVO ANTIMALARIAL ACTIVITY, TOXICITY AND PHYTOCHEMICAL COMPOSITION OF SECURIDACA LONGEPEDUNCULATA FRESEN. (POLYGALACEAE)

By

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DECLARATION

This thesis is my original work and has not been presented for the award of a degree in any other University

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DEDICATION

This thesis is dedicated to my son, Andy Hawii Ogolla and persons who fund research especially on development of new drugs to combat emerging diseases.
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LIST OF ABBREVIATIONS

ACT: Artemisinin-based Combination Therapy
AL: Artemether-Lumefantrine
ANOVA: Analysis of variance
BSA: Bovine serum albumin
CC\textsubscript{50}: 50\% cytotoxic concentration
CQ: Chloroquine
CTMDR: Center for Traditional Medicine and Drug Research
DMSO: Dimethyl sulphoxide
GHS: Globally harmonized system
GM: Growth media
IC\textsubscript{50}: 50\% inhibitory concentration
IPT: Intermittent preventive treatment in infants
IRS: Indoor residual spraying
LD\textsubscript{50}: 50\% lethal concentration
MEM: Minimum essential media
MM: Maintenance media
MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
NOAEL: No Observed Adverse Effect Level
OD: Optical Density
OECD: Organization for Economic Cooperation and Development
PBS: Phosphate buffered saline
PEG: Polyethylene glycol

PHPT: Department of Public Health, Pharmacology and Toxicology

RBC: Red blood cell

SD: Standard deviation

SEM: Standard error of the mean

SPSS: Statistical package for social sciences

SS: Solvent system

ST: Survival time

TCM: Tissue culture medium

TLC: Thin Layer Chromatography

WHO: World Health Organization
ABSTRACT

The search for new, safe and efficacious agents against malaria is of precedence. This is attributed to resistance of current chemotherapeutic agents to the malaria parasites and increased toxicity of currently available antimalarial drugs. To address this challenge, increased efforts are required aimed at discovering new agents against malaria. Natural products from plant biodiversity have continued to play a pivotal role towards drug discovery against malaria and other diseases. Despite the wide use of medicinal plants to control and treat conditions with malaria-like symptoms, little has been done to evaluate their safety, efficacy and phytochemical composition, to validate the claimed anecdotal efficacy.

The current study was designed to investigate in vivo antimalarial activity and efficacy, in vitro cytotoxicity, in vivo acute toxicity and phytochemical composition of aqueous and organic crude extracts from Securidaca longepedunculata Fresen. (Polygalacea). A four day suppressive standard test was used to investigate in vivo anti-malarial activity of the crude extracts. The in vitro cytotoxicity was evaluated using a tetrazolium salt MTT (3-[4.5-dimethylthiazol-2-yl]-2.5-diphenyltetrazolium bromide) colorimetric method, based on reagent cleavage by mitochondrial dehydrogenase in viable cells. The in vivo acute oral toxicity was carried out on healthy 6-8 weeks old female Swiss albino mice using the Organization for Economic Cooperation and Development (OECD 423) guidelines. Standard methods were used for phytochemical screening.

The standard anti-malarial drug employed for positive control; chloroquine showed percentage parasitaemia suppressions of 96.99% after 4 days of treatment. Chloroquine did not show any significant difference ($p>0.05$) in comparison with that from the percentage parasitaemia
suppression exhibited by the aqueous and organic root extract of *S. longepedunculata* after the 4 days treatment of mice infected with *P. berghei*. The highest level of clearance was observed in chloroquine treated group, with percentage parasitaemia on day 4 as 0.68 ± 0.17 as compared to 22.58 ± 0.84 of the negative control group. *S. longepedunculata* root organic and aqueous crude extracts equally demonstrated a good suppression of parasitaemia on day 4 with a with percentage parasitaemia of 1.93 ± 0.37 and 2.78 ± 0.46 respectively. Both organic and aqueous extracts were non-toxic at 2000 mg/kg of bdw i.e. LD$_{50}$>2000 mg/kg. None of the extract tested was potentially toxic to HEp-2 cell lines, CC$_{50}$ > 100μg/ml. The following phytochemicals were demonstrated to be present in the crude extracts: steroids, alkaloids, anthraquinones, flavonoids, tannins, saponins and cardiac glycosides.

This study has demonstrated that the crude extracts are a potential source of a new class of antimalarial drugs. The extracts have got very good antimalarial activity and are not toxic in vivo mice model. This study recommends further work aimed at hit identification for antimalarial activity through bioactivity guided fractionation, isolation and purification of compounds responsible for the observed antimalarial activity. The purified and identified hits could be used for lead development for antimalarial activity as well as biomarkers for standardization of *S. longepedunculata* herbal preparations for use against malaria. This study recommends in vitro testing of these extracts for their activity against *P. falciparum* in order to consider them as potential sources for antimalarial drug development for human malaria. The study also recommends elucidation of mechanisms of action and investigations of active compounds in terms of structure-activity relationship with modifications of the structure to enhance efficacy.

**Keywords:** *Plasmodium berghei*, antimalarial, *S. longepedunculata*, Swiss albino mice
CHAPTER ONE

INTRODUCTION

1.1 Background information

Malaria results in mortality and morbidity around the world. According to the latest estimates, 198 million cases of malaria occurred globally in 2013 and the disease led to 584,000 deaths. The burden is heaviest in the African Region, where an estimated 90% of all malaria deaths occur and in children aged under 5 years, who account for 78% of all deaths, (WHO, 2014). Despite the strenuous efforts of the international health community, malaria remains a major cause of illness and death especially in tropical countries (WHO, 2012). It is estimated that up to 124 million people in Africa live in areas at risk of seasonal epidemic malaria, and many more in areas outside Africa where transmission is less intense (Hay and Snow, 2006). More than 11.3 million malaria cases are recorded annually in Kenya. Malaria is recognized as a health and socio-economic burden by the Government of Kenya. In 2012, clinically diagnosed malaria accounted for 28% of outpatient hospital visits in Kenya.

Despite efforts to reduce transmission and increase treatment, there has been little change in the areas that are at risk of this disease since 1992 (Hay et al., 2004). Indeed, if the prevalence of malaria stays on its present upwards course, death rate could double in the next twenty years (Desai et al., 2007). Precise statistics of morbidity and mortality are unknown because many cases occur in rural areas where people do not have access to hospitals or the means to afford health care. Consequently, the majority of cases of malaria are undocumented (Desai et al.,
2007). The main cause of the malaria situation recorded in recent years has been the spread of drug resistant parasites, which has led to rising malaria associated mortality (Cheng et al., 2012). Malaria is the most prevalent tropical disease in the world today. It has infected humans for over fifty thousand years, and may have been a human pathogen for the entire history of human species (WHO, 2014). It is a protozoan infectious disease that causes a massive public health burden. It is endemic in 108 countries around the globe and presents a health risk for 50% of the world’s population (WHO, 2014).

Malaria is a disease caused by Plasmodium species. The five Plasmodium species that affect humans are P. falciparum, P. vivax, P. ovale, P. malariae and P. knowlesi, with P. falciparum infection being responsible for the deadliest form of the disease (WHO, 2009). The onset of symptoms tends to occur within 10-15 days of being bitten by the infected mosquito and early symptoms include fevers, chills, headaches and vomiting (Cheng et al., 2012). The high rate of mortality and morbidity associated with malaria is due to rapid growth and multiplication of Plasmodium parasites in the human host blood cells (Cheng et al., 2012).

It is also a cause of poverty and a major hindrance to economic development, (Sachs and Malaney, 2002). The economic impact includes costs of health care, working days lost due to sickness, days lost in education, decreased productivity due to brain damage from cerebral malaria and loss of investment and tourism (WHO, 2009). Moreover, it remains one of the leading causes of death in sub-Saharan regions where Human Immuno deficient Virus (HIV) infection is endemic (Korenromp et al., 2005).
Malaria remains uncontrolled and requires newer drugs and vaccines. The most promising malaria vaccine appears to be the RTS,S/AS01 vaccine that began its development in the early 1980s. The RTS,S/AS01 based vaccine formulation has been demonstrated to be safe, well tolerated, immunogenic and to potentially confer efficacy against malaria. Further research was considered necessary to improve the effectiveness of the vaccine (Casares et al., 2010). The resistance of human malaria parasites to antimalarial compounds has become of considerable concern, particularly in view of the fast speed of emergence of resistant parasites and the shortage of novel classes of antimalarial drugs. Antimalarial drug resistance has emerged as one of the greatest challenges facing malaria control today and has also been implicated in the spread of malaria to new areas and re-emergence of malaria in areas where the disease had been eradicated (Schlagenhauf and Petersen, 2008). Traditional herbal medicine has constituted a good basis for antimalarial lead discovery and drug development (Mwangi et al., 2015).

The need to carry out systematic scientific analyses of herbal medicine aimed at testing plants and to characterize the active principles cannot be over emphasized. Therefore, new approaches for development of new drugs to treat and to prevent malaria will be of interest and medicinal plants constitute incomparable source (Ginsburg and Deharo, 2011). The loss of effectiveness of chemotherapy constitutes the greatest threat to the control of malaria. Therefore, to overcome malaria, new knowledge, products and tools are urgently needed; especially new drugs. Many communities in Kenya are still dependent on traditional medicines to treat malaria which essentially involves the use of plants. Despite the wide use of medicinal plants to control and treat conditions with malaria-like symptoms, little has been done to evaluate their safety, efficacy and phytochemical composition and to validate the claimed anecdotal efficacy.
Therefore, this study was designed to explore this indigenous plant, *S. longepedunculata* for possible anti-malarial activity and safety and authenticate the claims of the traditional healers of this plant which will form the basis for further research. The current study was designed to investigate *in vivo* antimalarial activity, determine *in vitro* cytotoxicity, investigate *in vivo* acute toxicity and finally to analyze phytochemical composition of aqueous and organic crude extracts of *Securidaca longepedunculata*.

1.2 Justification of the study

The world is moving towards reducing incidences of malaria. However, development of drug resistance that is currently being witnessed can reverse this trend. Emerging drug resistant *Plasmodium falciparum* strains are making malaria a resurging infectious disease. Recommended drug therapies, unfortunately have not eluded drug resistant strains of the malaria parasite. There are case reports of resistance against new drug regimens such as Artemesinin Combination Therapies (ACTs) (WHO, 2009).

Validation of traditional medicines as possible antimalarials will encourage their use as therapeutic means to treat malaria. Traditional plants use as herbal remedies, have only been recorded and validated in very few instances. As a result, this knowledge can become lost (Chinsembu and Hedimbi, 2010). The screening of herbal medicines provides a foundation for further exploration of their use as antimalarial medicines (Chinsembu and Hedimbi, 2010). There is a need to record and validate the medicinal uses of these plants to expand their use to include integration into modern medical healthcare systems.
In Kenya, most people use traditional medicine and medicinal plants to treat many diseases including malaria. Traditional herbal medicine has constituted a good basis for antimalarial lead discovery and drug development (Mwangi et al., 2015). Many communities are still dependent on traditional medicines, which essentially involve the use of plants. Despite traditional medicine wide use in malaria therapy, little has been done to evaluate their safety and efficacy and establish their use as conventional therapies of malaria. Toxicological evaluation of phytomedicines to ascertain their safety for consumption and their possible mechanisms of action is thus needed. Few studies have been undertaken to investigate the anti-malarial efficacy and safety of plants claimed to have anti-malarial therapeutic value. Medicinal plants might represent an alternative treatment in non-severe cases of infectious diseases. They can also be a possible source for new potent anti-malarials that can be processed and used conventionally (Du Preez et al., 2011). In the quest to identify new anti-malarial chemotherapeutic agents, many research groups have resorted to plant sources.

Therefore, this study was designed to explore this indigenous plant for possible anti-malarial activity and safety and authenticate the claims of the traditional healers of this plant which will form the basis for further research.
1.3 Objectives of the study

1.3.1 General objective

To assess *in vivo* antimalarial activity, toxicity and phytochemical composition of *Securidaca longepedunculata* organic and aqueous crude extracts.

1.3.2 Specific objectives

1) To determine the *in vivo* antimalarial efficacy of organic and aqueous crude extracts of *Securidaca longepedunculata* in mice.

2) To investigate *in vitro* cytotoxicity of organic and aqueous crude extracts of *Securidaca longepedunculata* using mammalian cell lines.

3) To determine the acute toxicity of organic and aqueous crude extracts of *Securidaca longepedunculata* in mice.

4) To determine qualitatively the phytochemical composition of organic and aqueous crude extracts of *Securidaca longepedunculata*

1.4 Hypotheses

1) Null hypothesis (H₀): Organic and aqueous crude extracts of *Securidaca longepedunculata* do not possess antimalarial activity and are toxic.
CHAPTER TWO
LITERATURE REVIEW

2.1 Resistance to current anti-malarial medicines

The treatment of malaria is confounded by the challenges of widespread resistance of the malaria parasites to cheap and affordable antimalarial drugs. Antimalarial drug resistance has spread and intensified over the years leading to a dramatic decline in the efficacies of the antimalarial drugs. Faced with these reduced efficacies there is urgent need to develop new antimalarial drugs. Several pharmacologically active antimalarial compounds have been isolated from plants and are at different stages of development (Waako et al., 2007; Sebisubi, 2007). Resistance of malaria parasites to existing drugs complicates treatment, but an antimalarial vaccine that could protect against this disease is not yet available. It is therefore necessary to find new effective and affordable medicines. Medicinal plants could be a potential source of antimalarial agents. One way to prevent drug resistance of pathogenic species is by using new compounds that are not based on existing synthetic antimicrobial agents (Kayano et al., 2011). The urgency generated by drug-resistant strains of malaria parasites has accelerated antimalarial drug research over the last two decades. While synthetic pharmaceutical agents continue to dominate research, attention has increasingly been directed to natural products in view to combat drug resistance (Kayano et al., 2011).

There is decreased sensitivity to current first lines drugs such as Artemether-Lumefantrine, an Artemisinin-based combination treatment (ACT), (Kayano et al., 2011). Widely used antimalarial drugs such as quinine and artemisinin were isolated from plants. Therefore,
pharmacognostic investigations of plants for the establishment of complementary medicine for malaria within traditional plants is necessary (Wang et al., 2007). We currently rely on the extraction of artemisinin from the semisynthetic production of artemisinin derivatives. However, the low content of artemisinin that is actually present in each plant, between 0.01 to 0.8% of *A. annua*’s dry weight, makes the production of artemisinin as a drug expensive (Liu et al., 2010).

There is an urgent need to discover new antimalarials, due to the spread of resistance and the limited number of available drugs. To date there are a number of existing drugs to treat malaria, however the spread of resistance to these established antimalarials is of critical concern. There is a widespread resistance to chloroquine and sulfadoxine-pyrimethamine, the two lowest cost antimalarials. In addition to the problem of resistance, some of the established antimalarials have safety issues associated with them or properties that may decrease compliance. Mefloquine continues to be effective in many parts of the world, it is relatively expensive and its side effects, which include seizures, acute psychosis and anxiety neurosis, can make certain individuals think twice before taking it (Ashley et al., 2005). It is important that new antimalarials are safe and inexpensive to produce so that they can be affordable to the populations that need them the most (Gelb, 2007).

Another problem associated with certain antimalarials is the length of treatment; for instance, a recent study focusing on compliance to the 7-day primaquine treatment for *P. vivax* among patients living along the Iquitos-Nauta road in the Peruvian Amazon found that compliance was high during the initial three days of treatment but decreased as symptoms disappeared (Grietens et al., 2010). It is generally thought that the optimal antimalarial drugs would require at most
three days of treatment, either once or twice a day, to be effective (Gelb, 2007). This would be one way to avoid problems associated with compliance and the development of drug resistance.

2.2 Malaria vaccine

A completely effective vaccine is not yet available for malaria, although several vaccines are under development. SPf66 was tested extensively in endemic areas in the 1990s, but clinical trials showed it to be insufficiently effective (Casares et al., 2010). Other vaccine candidates, targeting the blood stage of the malaria parasite's life cycle, have also been insufficient on their own. RTS,S/AS01 is one of several potential vaccines under development that target the pre-erythrocytic stage of the disease. Among them RTSS has shown the most promising results so far (Casares et al., 2010).

RTS,S/AS01 or Mosquirix is an experimental recombinant protein-based vaccine for malaria. The RTS,S/AS01 based vaccine formulation has been demonstrated to be safe, well tolerated, immunogenic and to potentially confer efficacy against malaria. Further research was considered necessary to improve the effectiveness of the vaccine (Casares et al., 2010).

2.3 Recent research on anti-malarial plants

Until recently, there has been a reliance on the cheap antimalarial drugs like Chloroquine and Sulphadoxine-Pyrimethamine. In 2001, the World Health Organization (WHO) recommended Artemisinin combination Therapies (ACTs) as the first line of treatment for uncomplicated malaria. The ACTs which include Artemether-Lumefantrine (AL) and Amodiaquine (AQ) plus Artesunate (AS) have been adopted for treatment of P. falciparum malaria in many African
countries. To protect drugs from resistance, there is now clear evidence that combining them can improve their efficacy without increasing their toxicity (Rahmatullah et al., 2012) and with the development of highly effective Artemisinin derivatives, there is renewed hope for the treatment of malaria in the form of Artemisinin based Combination therapy (ACT).

Most of the antimalarial drugs currently in use were not developed on the basis of rationally selected targets. Some were developed by investigation of traditional medicinal plants (quinine and artemisinin). Some by synthesis of analogues (chloroquine, mefloquine, primaquine and atovaquone). Chemical modification of an active natural product also played a crucial role in drug development (artether, artemether and artesunate). Finally, some antimalarials were developed by assaying drugs that were used against other infectious pathogens (antifolates and antibiotics) (Rahmatullah et al., 2012). The first effective drug treatment against malaria was quinine, which was extracted from the cinchona tree. The structure of quinine was used to synthesize antimalarials like chloroquine and primaquine.

The most significant recent development in naturally occurring antimalarial drugs is arguably the identification of artemisinin as the active component of the plant Artemisia annua, which is used in traditional medicine as an antimalarial agent. The importance of plants as effective antimalarials was further reinforced by the isolation of artemisinin from the Chinese medicinal plant, Artemisia annua. Artemisinins are presently the most effective drug against multi-drug resistant strains of Plasmodium falciparum (Sebisubi, 2007). This unique sesquiterpene contains an endoperoxide group that appears to be an essential requirement for its activity. It is particularly active in vivo against chloroquine resistant P. falciparum and is reported to have
relatively low toxicity (Liu et al., 2010). As artemisinin is a complex molecule, much effort has been put into synthesizing compounds based on the 1, 2, 4-trioxane ring of artemisinin. Many compounds have been produced from artemisinin, some of which have promising in vivo activities in animal models (Wright, 2009).

2.4 Ethnomedicinal anti-malarial plants

Medicinal plants have been used since antiquity to treat various ailments and diseases, (Kinghorn et al., 2004); Even when allopathic medicine is strongly emphasized, 80% of people in developing countries still depend on traditional medicine for primary health care needs, WHO, (2009). People prefer to use medicinal plants over allopathic medicine for various reasons; relatively low cost, effectiveness, perceived safety and minimal side effects (Wells, 2011). Plant materials remain an important source of medicine in the fight against malaria, (Aderounmu, 2007). Traditional medicinal plants have contributed significantly to current malaria therapy. Traditional medicine is extensively practiced in the prevention, diagnosis and treatment of various illnesses (Prashanth et al., 2006). It has attracted increasing public attention over the past 20 years as this type of medicine is easily accessible in some regions (Prashanth et al., 2006). Phytomedicine research is gaining more grounds than ever as majority of people are relying heavily on herbal medicine, which occasionally has been shown to be less toxic and cheaper compared to the synthetic orthodox medicines (Yakubu et al., 2007). The general acceptability of traditional medicines is however limited by lack of dose regimen and adequate toxicity data to evaluate their safety (Palombo, 2006). Herbal drugs have been used since ancient times as medicines for the treatment of a range of diseases. Medicinal plants have played a key
role in world health and in spite of the great advances observed in modern medicine in recent decades; plants still make an important contribution to health care.

Through past experience, herbalists have used herbal preparations either from single plants or as combined proportions (Liu et al., 2004). The use of herbal drugs as combinations has existed for centuries in several cultural systems (Gathirwa et al., 2008). Traditional methods of malaria treatment could be a promising source of new antimalarial compounds. In Africa, more than 80% of people use traditional medicines and most families have recourse to this medicine based on plants extracts for the curative treatment of malaria. In fact, the traditional medicine of this continent constitutes an important source for ethno pharmacological investigations, (Gathirwa et al., 2008).

The use of plants for therapeutic purposes dates back to the human history, (Ogbonna, et al., 2008). Medicinal plants since time immemorial have been used in virtually all cultures as a source of medicine. For a long time, natural products were the only sources of medication, (Bourdy, et al., 2008). Several medicinal plants have been used locally to treat malaria infection. Some of such plants are Enantia chloranta and Nauclea natifolia, (Ogbonna et al., 2008). Acalypha fruticosa, Azadirachta indica, Cissus rotundifolia, Echium rauwolffii, Dendrosicyos socotrana and Boswellia elongate (Merlin et al., 2004; Clarkson et al., 2004 and Alshwash et al., 2007). Alstonia boonei is widely used in Africa for the treatment of various ailments. The stem bark of A. boonei has anti-inflammatory, analgesic and antipyretic activities and is commonly used against malaria (Olajide et al., 2000). Sarcocephalus latifolius (African peach), of the family Rubiaceae, is a multi-stemmed tree or shrub. Reports of its medicinal value include its
effectiveness in the treatment and management of malaria dependent on its phytochemical constituents (Olajide et al., 2000).

A study carried in the Kenyan Lake Victoria basin revealed high usage of herbal drugs in combined proportions to treat ailments such as malaria, sexually transmitted infections, and typhoid. *Toddalia asiatica* (Rutaceae), *Rhamnus staddo* (Rhamnaceae), *Podocarpous falcatus* (Podocarpaceae), *Mormodica foetida* (Cucurbitaceae) and *Aloe sp.* (Aloaceae) are some of the plants used by local communities in the Kenyan Lake Victoria Basin as a combined proportion to treat malaria, (Gathirwa et al., 2008). This is the case of *Azadirachta indica*, *Artemisia annua*, from Asia and *Cinchona officinalis* already known for their antiplasmodial activity, (Tonk et al., 2006).

The anti-malarial potentials of compounds derived from plants is proven by examples such as quinine, obtained from *cinchona* species and *artemisinins* obtained from *artemesia species*. The success of artemisinin isolated from *Artemisia annua* and its derivatives for the treatment of resistant malaria has focused attention on the plants as a source of antimalarial drugs (Merlin et al., 2004). Moreover, plants have been the basic source of sophisticated traditional medicine systems for thousands of years and were instrumental to early pharmaceutical drug discovery and industry (Elujoba, et al., 2005). The world's poorest are the worst affected, and many treat themselves with traditional herbal medicines. These are often more available and affordable, and sometimes are perceived as more effective than conventional antimalarial drugs (Merlin et al., 2004).
Ethnobotanical information about antimalarial plants used in traditional herbal medicine, is essential for further evaluation of the efficacy of plant antimalarial remedies and efforts are now being directed towards discovery and development of new chemically diverse antimalarial agents (Clarkson et al., 2004). Several rural dwellers depend on traditional herbal medicine for treatment of many infectious diseases including malaria. The reputed efficacies of these plants have been recognized and passed on from one generation to the other. About 75% of the population in Africa does not have direct access to chemical treatment, such as chloroquine, but they have access to traditional medicine for treating fevers. Treatment with these remedies has suffered a number of deficiencies; diagnosis is often a problem, identification of plant extracts may be insecure and the chemical content of extracts may vary considerably (Azas, et al., 2002). Natural products isolated from plants used in traditional medicine, which have potent antiplasmodial action in vitro, represent potential sources of new antimalarial drugs. It had been advocated that direct crude drug formulation of the herbs following toxicological absolution may not only produce dosage forms faster but will also lead to cheaper and more affordable drugs for the communities that need them. Also, there is a belief that these medicines are safe because they are natural and have been used traditionally over a period of time (Willcox and Bodeker, 2004). The use of these local herbs for the treatment of malaria has helped to reduce mortality and morbidity rates especially in the rural areas of the developing world where antimalarial drugs are not readily available (Ogbonnaa et al., 2008).

Plant materials remain an important resource to combat serious diseases in the world and pharmacognostic investigations of plants are carried out to find novel drugs or templates for development of new therapeutic agents. In general, natural plant products play a dominant role in
the development of novel drug leads for the treatment and prevention of diseases including malaria.

2.5 Systematics, medicinal uses and phytochemical composition of *Securidaca longepedunculata* Fresen. (Polygalaceae)

2.5.1 Botanical description

The plant *Securidaca longepedunculata* is indigenous to Africa and its leaves, stem and bark have been used in treating a wide spectrum of diseases. It is widely used in African traditional medicine (Dapar et al., 2007; Pallant and Steenkamp, 2008). It is known as violet tree, rhodes’s violet, wild vesteria (English) and mzigi (Swahili). It is a shrub or small (2–10 m high) flowering savannah plant. The flowers are sweet scented, bright purple or violet in racemes, and the fruit is winged. The plant is widespread throughout tropical Africa. The plant belongs to the family Polygalaceae. Its leaves are oblancelate and obtuse at apex. The flowers are purple or blue in coloration and the seeds are winged, (Olofintoye, 2010). The micrograph of the plant is shown in the plate below:
Plate 1: Micrograph of *Securidaca longepedunculata* stem, foliage, roots and plant

2.5.2 Medicinal uses of *Securidaca longepedunculata*

The selection of plants to be screened for anti-malarial activity is done on the basis of traditional reputation of the plants for efficacy in the treatment of malaria (Etuk *et al.*, 2006). It is reported that almost all parts of *S. longepedunculata* (leaves, twigs, stem, bark, root and seeds) are used by man for different purposes (Dapar *et al.*, 2007). Recent studies have also reported antimicrobial activity against protozoa, bacteria and fungi (Dapar *et al.*, 2007). The analgesic,
anti-inflammatory and hypoglyceamic activity of *S. longopedunculata* root bark has also been reported, (Lino and Deogracious, 2006; Maiga *et al*., 2005; Pallant and Steenkamp, 2008).

The root and the bark are taken orally either powdered or as infusion for treating chest complaints, inflammation, abortion, ritual suicide, tuberculosis, infertility, venereal diseases and for constipation (Etuk *et al*., 2006). Tooth ache can also be relieved by chewing the roots. Powdered roots are used to treat head ache by rubbing them on the fore head. Infusions of the roots are used for washing topical ulcers (Meyer *et al*., 2008). Some people take the roots for mental disorders and against children’s illness during breast feeding. They also mix the powdered roots with maize and sorghum beverages for men who are sexually weak. In Zimbabwe, the roots are given to people who are believed to be possessed by evil spirits. The root extracts are also used for menstrual pains and gonorrhea (Dapar *et al*., 2007).

They are also used as fish poison, molluscicide, snake repellent, or as insect repellent, source of fiber, water purifying agent, as an ornamental (Stevenson *et al*., 2009; Olofintoye, 2010). The root extracts are also used for menstrual pains and gonorrhea in Nigeria (Stevenson *et al*., 2009). The root extract is trypanocidal against *Trypanosoma brucei* and *Trypanosoma congolense* (Atawodi *et al*., 2003). In addition, the root bark is used by farmers as a pesticide in stored grain (Stevenson *et al*., 2009). The efficacy of this plant for antimalarial properties has been validated in laboratory studies (Jayasekera *et al*., 2005; Stevenson *et al*., 2009). Molluscidal properties of the root extract has also been recently demonstrated (Olofintoye, 2010).

According to Dapar *et al*., (2007), the aqueous extracts of its roots are used as psyendorsychopharmaceutical agents. The analgesic, anti-inflammatory and hypoglyceamic activity of *S. longopedunculata* root bark has also been reported (Olofintoye, 2010). Its use in bacterial and
malarial chemotherapy has also been documented (Kamba and Hassan, 2010). Natural stands are under considerable pressure from harvesting of roots for its numerous uses (Ouedraogo et al., 2003). This plant is arguably used to treat every conceivable ailment (Avhurengwi and Walter, 2006). The Hausas in Nigeria refer to it as “uwar magunguna” (the mother of all medicines). In general, the claims of the medicinal applications of S. longipedunculata extracts are numerous.

2.6 In vitro cytotoxicity

In vitro cytotoxicity methods are important tools to enhance understanding of hazardous effects caused by chemicals or bioactive components (Prakash et al., 2011). They avoid animal models. In vitro cytotoxicity tests provide useful and necessary information in defining basal cytotoxicity, which is commonly used as a starting point in an integral assessment of potential in vivo toxicity of chemicals or active components in foods (Prakash et al., 2011). The endpoints frequently used in cytotoxicity testing are based on the breakdown of the cellular permeability barrier, reduced mitochondrial function, changes in cell morphology and changes in cell replication (Prakash et al., 2011). Several methods have been developed for measurement of cell proliferation; counting cells that exclude a dye (trypan blue), measuring released Cr-labeled protein after cell lysis, measuring incorporation of radioactive nucleotides thymidine or iododeoxyuridine during cell proliferation, and measuring colorimetric changes of tetrazolium salts in active cells. Among these methods, the colorimetric assay using tetrazolium salts are often employed as it does not involve hazardous radio-active materials and it is suitable for handling a large number of samples (Lee et al., 2000).
MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) is a tetrazolium salt that is reduced to yield a purple-colored water-insoluble formazan product. Since MTT is cleaved only by active mitochondria in living cells and not by dead cells or erythrocytes, MTT reduction is the most widely used method for measuring cell proliferation and viability. The formazan salt is produced in proportion to the active cell number and accumulates within the cell since it is not membrane permeable. However when dimethyl sulfoxide (DMSO), isopropanol or other suitable solvent is added, formazan salt can be quantified calorimetrically. The MTT assay is simple and suitable for a wide variety of cell lines (Barile, 1994). It requires monitoring as duration of MTT treatment, concentration of MTT used, and the number of test cells. These experimental conditions need to be taken into consideration when comparing inter-laboratory results (Lee et al., 2000).

2.7 In vivo acute toxicity

Toxicity is an expression of being poisonous, indicating the state of adverse effects led by the interaction between toxicants and cells. This interaction may vary depending on the chemical properties of the toxicants and the cell membrane (Agbaje et al., 2012).

Evaluation of toxic properties of a substance is crucial because exposure to chemicals can be hazardous and results to adverse effects on human being. It had been advocated that direct crude drug formulation of the herbs following toxicological absolution may not only produce dosage forms faster but will also lead to cheaper and more affordable drugs for the communities that need them (Bellini et al., 2008). Also, there is a belief that these medicines are safe because they are natural and have been used traditionally over a period of time (Agbaje et al., 2012).
Acute toxicity testing is the basic science of poisons. The organization for Economic Cooperation and Development (OECD) defines acute toxicity as the adverse effects that occur within a short time of oral administration of a simple dose of a substance or a multiple doses given usually within 24 hours. It includes observational data gathering and data utilization to predict outcome of exposure in human and animals. The ancient humans categorized some plants as harmful and some as safe, (Agbaje et al., 2012).

Traditional medical practitioners are enjoying widespread use of parts of *Securidaca longipedunculata* for treatment of several ailments, but still little known about their toxicity and safety issue which are always a concern. Investigations on functional plants provide evidence for the presence of substances that are offer potential human health benefits. However, it should be a vital requirement to determine the toxic effects of substances contained in this plant. *S. longipedunculata* root has also been reported to possess some level of toxicity on mice liver and kidney. Intraperitoneal administration of 100 mg/kg body weight resulted in signs of toxicity and death according to Dapar *et al.*, (2007). Also, the cellular enzymes (alanine and aspartate aminotransferases) were elevated in *S. longipedunculata* treated rats. In contrast, there was no observed toxicity following a 28 day oral administration of *S. longipedunculata* root in mice at a dose of 2700 mg/kg body weight (Etuk *et al.*, 2006).

The roots are a toxicity risk if taken in excess. A saponin found in the roots can cause severe damage to bone marrow and haemolysis when in contact with blood. The solid portion of the root is said to be the most lethal. The root bark also contains 0.42% methyl salicylate. Severe poisoning can result from ingestion of 10-30 ml of methyl salicylate (Etuk *et a.*, 2006).
Toxicity studies are divided into four categories which are acute, sub-acute, sub chronic and chronic. The present work studies the acute toxic effect of Securidaca longipedunculata aqueous and organic extracts.

2.8 Phytochemicals

Plant’s secondary metabolites are also known as phytochemicals. Phytochemicals are chemical compounds that occur naturally in plants but not important for plant’s primary metabolic activities. Studies have indicated that, phytochemicals are mostly produced in response to environmental pressures such as adverse climatic conditions, immune response to infectious agents (Lim and Bowles, 2012). Furthermore, phytochemicals serve many ecological functions such as; defence against microorganisms, insects and herbivores. Some are responsible for odour and flavours while others make the plant pigments (Dharani et al., 2010). There are three main groups of phytochemicals namely; terpenoids and steroids, alkaloids, and phenolics. Major classes of phytochemical with known antiplasmodial properties are; alkaloids, terpenoids, coumarins, flavonoids, chalcones, quinones and xanthones, (Dharani et al., 2010). In this study, emphasis was on selected major classes of antiplasmodial phytochemicals namely; alkaloids, flavonoids, coumarins, terpenoids and anthraquinones.

Studies have shown that phytochemicals isolated from plant sources have been used for the prevention and treatment of many diseases including cancer, heart disease, malaria, diabetes mellitus and high blood pressure (Avwioro, 2010). This has necessitated exploration and screening of medicinal plants with acclaimed therapeutic efficacies. Many plants have been reviewed but satisfactory photochemical analysis are still lacking. Phytochemicals are present in
a variety of plants utilized as important components of both human and animal diets (Awwioro, 2010).

Phytochemical investigations of the root bark of *S. longopedunculata* from literature reviews revealed the presence of flavonoids, alkaloids, saponins, triterpenoids and volatile oils. Other secondary metabolites like sapogenin, presenegenin, indole alkaloid, securinine and some ergot alkaloids have also been reported to be present in the root of this plant (Etuk *et al.*, 2006). Other studies of the root bark revealed the presence of flavonoids, alkaloids, saponins, triterpenoids and volatile oils (Ekpendu *et al.*, 2000). Other secondary metabolites like sapogenins; presenegenin, indole alkaloid; securinine and some ergot alkaloids have also been reported to be present in the root of this plant (Van *et al.*, 1997). The alkaloid securinine confers activity against *Plasmodium falciparum*, the causative agent of malaria (Maiga *et al.*, 2005). The aqueous extract of the root/leaf has high concentrations of glycosides, saponins and tannins (Maiga *et al.*, 2005). The xanthones from its root bark have also shown promise in the treatment of erectile dysfunction, barks and roots have got saponins which have got antiseptic properties, (Meyer *et al.*, 2008).
CHAPTER THREE
MATERIALS AND METHODS

3.1 Collection of the plant materials

The plant materials were collected from Shimoni, Msambweni district. The materials comprised of the roots, stem bark and leaves of S. longepedunculata. The identification and authentication was done at the herbarium section of Botanical Sciences, University of Nairobi by a taxonomist based on international code for botanical nomenclature. The voucher specimen was deposited at the herbarium and given a voucher number (SL040314) for future reference.

3.2 Preparation of plant materials

The S. longepedunculata roots, leaves and stem barks were washed and sliced into pieces and then air dried under shade for 2 weeks to prevent heat destruction of active components. The dried pieces were pulverized with an electric milling machine to powder. The resulting powder was stored in the dark in a big amber glass container in a cool and dry environment. This was done to preserve the presence and the quality of the compounds found within the plant. The moisture content of fresh plant material changes the chemical composition and properties of the plant over time. The crude extracts were obtained by cold maceration technique under constant stirring for 48 hours. Chloroform and methanol (1:1) were used for organic extraction while distilled water was used for aqueous extraction. Filtration was done using Whatman 1 filter paper. The aqueous extracts were lyophilized while the organic crude extracts were concentrated
under reduced pressure in a rotary evaporator at 45°C to give residue. The residue was further
dried in a desiccator to a dry powder. The crude extracts were weighed and stored in sealed
bottles and refrigerated at -21°C.

3.3 In vivo determination of anti-malarial activity- on early infection

In vivo anti-malarial activity was carried out using a four day suppressive standard test described
by David et al., (2004); Frederich et al., (2004) and Jonville et al., (2008) and employing
chloroquine sensitive *P. berghei* ANKA strain. The *Plasmodium berghei* ANKA stain was used
as it infects rodents and it is chloroquine sensitive. A formal study approval and clearance for
performing the experiments on animals was sought from the Center Scientific Committee, of
CTMDR-KEMRI, the Scientific Steering Committee of KEMRI, the Animal Care and Use
Committee and finally Ethical Review Committee both of KEMRI. The experimental protocol
was approved and validated by these committees.

Healthy adult female Swiss mice weighing 18-21g and aged 6-8 weeks were used for this study.
The animals were procured from the Animal House of Kenya Medical Training Institute and
acclimatized at the KEMRI animal house facility for a period of 7 days. The mice were kept in
clean ventilated aluminum cages with relevant environmental conditions (temperature: 23-31°C;
photoperiod: 12 hours natural light and 12 hours darkness; humidity: 50%–55%). The
environmental conditions were measured and determined using relevant apparatus. The mice had
free access to standard mice pellets and water *ad libitum*. Two Swiss mice were infected with *P.
berghei* ANKA strain. Three days later, the percentage parasitaemia was determined. The
parasitaemia was found to be 20.16% and 23.06% respectively. These mice were used as the
donor of parasitized blood. They were sacrificed by putting them in a CO₂ chamber after which they were dissected. The parasitized red blood cells were obtained through cardiac puncture into a heparinised blood tube. The blood was then diluted with sterile saline (0.9% NaCl) to form a working titer. Parasite strain was maintained by serial passage of blood from donor mouse to test mouse. Each mouse was inoculated intra peritoneally with 200 µl of 1 x 10⁷ parasitized erythrocytes containing P. berghei ANKA stain.

Fifteen mice were randomly selected and assigned into 3 treatment groups with each group having 5 mice. The first group represented the negative control. Each mouse in this group orally received placebo which was 0.2 ml/day of distilled water. The second group of five mice represented the positive control and each mouse in the group orally received control drug which was 0.2 ml chloroquine at a dose of 10 mg/kg. The third group of 5 mice represented the test group and each mouse orally received 0.2 ml suspension of root organic extract at a dose of 100 mg/kg. This procedure was done for all the test extracts.

Ten milliliter of distilled water was used to dissolve 0.1 g of the test extracts while DMSO (<1%) was used to dissolve 0.1 g of organic extracts to make working solutions (10,000 mg/ml). The oral administration of the test drugs, control drug and the placebo was done immediately after inoculation of the mice with Plasmodium berghei ANKA stain. This marked the first day of the experiment (D0). The administration of the test drugs, control drug and the placebo were continued from day 0 to day 1, 2 and 3. Each day from day 0 to day 3, a thin blood smear was done from the tail blood of each mouse and stained with Giemsa. This was used to estimate the percentage parasitaemia. Counting was done four times in different fields on the same thin blood
smear. The mice were allowed free access to mice pellets and water *ad libitum* before and after administration of the drugs from the first day to the end of the experiment.

### 3.3.1 Preparation of thin film

Thin smears of blood films were obtained from the tail end of each mouse on day 4 after infection and treatments (David *et al.*, 2004). The working solution was prepared by diluting 1ml of standard Giemsa stain with 19 ml of phosphate buffer (pH 7.2). The solution was prepared prior to use. Thin films (smears) of blood samples were made on clean, greaseless slides and fixed by immersing in absolute ethyl alcohol for 30 seconds. The films were allowed to air dry, immersed in Giemsa working solution for 30 minutes and then further immersed in phosphate buffer for 10 seconds, after which they were air dried in a vertical position. After drying, a drop of oil immersion was put on each slide and examined at x100 objective lens. The percentage parasitaemia was determined by counting the parasitized red blood cells and expressing it as a percentage of the total number of red blood cells per view. The percent of infected RBCs was determined by enumerating the number of infected RBCs in relation to the total number of RBCs. Percent infected RBCs = Number of infected RBCs/Total number of RBCs counted × 100.

### 3.3.2 Estimation of parasitaemia

The percentage suppression of parasitaemia was calculated by comparing the parasitaemia present in infected controls with those of test mice by use of the equation below:

\[
\text{Inhibition} \% = 100\left[\frac{(\text{parasitaemia of control} - \text{parasitaemia of drug})}{\text{parasitaemia of control}}\right]
\]

The extracts were considered active if parasitaemia reduced by 30% or more (Tona *et al.*, 2001).
3.4 *In vitro* cytotoxicity evaluation using MTT assay

Ten milligram of each extract was weighed in 15 ml centrifuge tube. One millimeter of phosphate buffered saline (PBS) solution was added to the aqueous extracts while 8 µl of DMSO (<1%) was added to organic extracts. The extracts were dissolved using a vibrating mixer. PBS was then added to 10 ml mark to obtain a concentration of 1mg/ml (1000µg/ml). The solution was then filtered using 0.22µm membrane syringe filter. Human hepatoma cells ATCC # HB-8065 (HepG2) were obtained from the American Type Culture Collection (ATCC, Manassas, USA). The cells were grown in Eagles Minimum Essential Medium supplemented with 200 mM L-glutamine, 5000 IU/mL penicillin and 5 mg/mL streptomycin, NaHCO₃ (7.5%), HEPES 1M, phenol red (0.4%) and 10% FBS. They were grown within a humidified atmosphere of 5% CO₂ at 37°C over 5 days. Trypnised Hep-2 cells from the source were seeded in a 96 micro titer well plate at 2 x 10⁴ cells/100 µl in columns 1, 2, 4, 5, 7, 8, 10 and 11. 100 µl of maintenance medium containing MEM 5%FBS was added in columns 3, 6, 9 and 12. No cells were added into wells in columns 3, 6, 9 and 12.

Then the cells were incubated for 12 hrs to allow cells to attach and form a confluent monolayer. After 12 hrs of incubation, the media was removed from the plates. The drug extracts were added each at a volume 150 µl and a concentration of 1000 mg/ml in rows H1 to H8. Root organic, stem organic, leaves organic and root aqueous crude extracts were added in plate1. Stem aqueous and leaves aqueous were added in plate 2. Serial dilution was carried out using a multichannel pipette by removing 50µl from wells of row H and adding to wells of row G. After mixing, another 50µl was transferred from row G to wells of row F and mixed well. This procedure was continued up to row B. The last 50µl of row B was discarded. A threefold dilution was achieved.
After this serial dilution, the micro titer plates were incubated at 37°C, 5% CO₂ for 48 hours to allow drug to take effect. The evaluation was done after 48 hours of incubation. The growth of the cells in the plate was examined under an inverted microscope. The maintenance media was emptied and 10μl of 5mg/ml MTT (3-[4,5-dimethylthiazol-2-yl]-2.5-diphenyltetrazolium bromide) reagent added to each well including controls. The micro titer plates were further incubated for 4 hrs.

After 4 hrs all the media in all wells was removed taking care not to dislodge the cells. DMSO less than 1% at a volume of 100 μl was added into each well to dissolve the purple formazan precipitate. The absorbance in each well including the blanks was measured at 562 nm in a micro titer plate reader and at a reference wavelength at 690 nm (Han, 2010). The cytotoxicity of the extracts was assessed using a tetrazolium salt MTT colorimetric method, based on reagent cleavage by mitochondrial dehydrogenase in viable cells as described by Han, (2010). Cytotoxicity was scored as the percentage reduction in absorbance at 570 nm versus that of the untreated control culture. The cytotoxic activity of S. longepedunculata extracts was expressed by the cytotoxic concentrations 50 (CC₅₀), representing the concentration of the test substance (μg/mL) required for the reduction of cell viability by 50%. The percentage cell viability following the addition of varying concentrations of the extracts in relation to untreated controls was calculated. The CC₅₀ (cytotoxicity) values were calculated as the concentration of compounds resulting in 50% reduction of absorbance compared to untreated cells. Tests were carried out in triplicate.
% inhibition = 100 (1-At/Ac); Where: At is the absorbance of treated wells (means); Ac is the absorbance of control wells (means). Percentage inhibitions were calculated and plotted on a curve with concentrations on the x-axis.

3.5 Acute toxicity in mice

The acute oral toxicity testing of *S. longepedunculata* organic and aqueous extracts was carried out on non-infected healthy 6-8 weeks old female Swiss mice weighing between 18-21g using the Organization for Economic Cooperation and Development (OECD 423) guidelines. The mice were housed in cages. Randomly selected ones were marked on the tail for individual identification. All mice were maintained on relevant environmental conditions (temperature: 23°C -31°C; photoperiod: 12 hrs natural light and 12 hrs darkness; humidity: 50%–55%). The environmental conditions were measured and determined using relevant apparatus. The mice were allowed to acclimatize to laboratory conditions for a week before starting the experiment. Drinking water and food were provided ad libitum throughout the experiment, except for the short fasting period where the drinking water was still in free access but no food supply was provided 12 hrs prior to treatment.

Each extract was orally administered sequentially at a concentration of 50mg/kg, 300mg/kg and 2000 mg/kg. Each concentration was given to a treatment group of three mice. If mortality was observed in more than half of the test animals, then the following higher dose was not administered. The crude extract was suspended in a vehicle (distilled water). Following the fasting period, body weight of the mice was determined and the dose was calculated in reference
to the body weight. The volume of the extracts solution given to the mice was 0.2 mls. Another cage of three female mice was given distilled water as a placebo and regarded as the control group. Food was provided to the mice approximately an hour after treatment. The mice were observed in detail for any indications of toxicity effect within the first six hours after the treatment period and daily further for a period of 14 days. Surviving animals were weighed and visual observations for mortality, behavioural pattern, changes in physical appearance, injury, pain and signs of illness were conducted daily during the period.

Observations started immediately after administration and then observed continuously for, 30 minutes, one hour after the treatment; intermittently for four hours, and thereafter over a period of 24 hours. The mice were observed for changes such as feeding habits, pilo erection, lacrimation, weight loss, mortality, loss of righting reflex, urination, increased or reduced activity. Other observations included changes in health status of the mucous membranes, respiratory system, circulatory system, autonomic system, central nervous system and gross behavioural changes. The LD$_{50}$ was determined.

### 3.6 Phytochemical screening

*S. longepedunculata* organic and aqueous extracts were tested for the presence or absence of seven classes of secondary metabolites using standard methods. The seven classes of compounds tested were alkaloids, steroids, anthraquinones, glycosides, flavonoids, saponins and tannins. Qualitative thin layer chromatography (TLC) was carried out. Aliquots of between 50–75μl of the organic and aqueous extracts were applied 1 cm from the base of the TLC plates pre-coated
with silica gel. Serial gradients of acetone and pet ether in the ratio 2:8 was used as eluent for organic extracts while for aqueous extracts butanol, acetic acid and water in the ratio 4:1:1 was used as eluent. Developments of the chromatograms were done in a closed tank in which the atmosphere had been saturated with the eluent vapor by lining the tank with filter paper wetted with the eluent. The phytochemical screening was done according to procedures described by Florey, (2005).

3.6.1 Test for tannins

The TLC plate was sprayed with 3 drops of 5% ferric chloride solution in a safety fume chamber or cabinet. A green black or blue-black coloration indicated the presence of tannins.

3.6.2 Test for flavonoids

The TLC plate was sprayed with 25% aqueous solution basic lead acetate. The positive spot fluoresced in long-wave UV light. A confirmation was done by dissolving 0.5g of the plant material in petroleum ether to remove the fatty materials (lipid layer). The defatted residue was dissolved in 20mls of 80% ethanol and filtered. Three milliliters of the filtrate was mixed with 4mls of 1% potassium hydroxide in a test tube and the color was observed. A dark yellow color indicated the presence of flavonoids.

3.6.3 Test for alkaloids

The TLC plate was sprayed with Dragendorff reagent composed of two solutions. The first solution consisted of 0.85g basic bismuth nitrate dissolved in a mixture of 10ml acetic acid and
40ml water. The second solution consisted of 8 g potassium iodide in 20ml of water. Equal volumes of solutions 1 and solution 2 were mixed to make Dragendorff reagent. 1ml of Dragendorff reagent was mixed with 2ml acetic acid and 10ml water and then sprayed to the TLC plate that has undergone separation. The positive spot appeared as an orange red turbidity.

3.6.4 Test for saponins

This was evaluated by adding 5mls of distilled water to about 0.5g of the powder in a test tube and shaking vigorously. A persistent froth lasting for at least 15 minutes indicated the presence of saponins.

3.6.5 Test for quinones

The TLC plate was exposed to ammonia fumes. Red, orange, yellow or brown coloration indicated the presence of quinones.

3.6.6 Test for cardiac glycosides

This was evaluated by adding 5mls of the aqueous extract to 2mls of glacial acetic acid containing one drop of Ferric chloride (FeCl₃) solution, followed by the addition of 1ml of concentrated sulphuric acid. Brown ring was formed at the interface which indicated the presence of deoxysugar of cardenoloides. A violet ring appeared beneath the brown ring, while in the acetic acid layer, a greenish ring appeared just gradually throughout the layer.
3.7 Data analysis

The data was entered into excel, then exported to SPSSS 15.0 and explored for normal distribution. In vivo antimalarial data was analyzed using independent Student’s t-test. The observed means were compared with that of the control. The results of the study were expressed as mean ± standard error of mean (M ± SEM). All data was analyzed at a 95% confidence interval ($p = 0.05$). Percentage parasitaemia and percentage suppression were calculated. The CC$_{50}$ (cytotoxicity) values were calculated as the concentration of compounds resulting in 50% reduction of absorbance compared to untreated cells. Data was expressed as means and percentage inhibitions calculated. This was plotted on a dose-response curve with concentrations on the x-axis against percentage inhibitions. In vivo acute oral toxicity response data was tabulated. Parameters such as mortality, body weight changes and signs of toxicity were compared with untreated controls and analysed. Significant differences and trends were noted. LD$_{50}$ was determined. Phytochemical investigation data was tabulated and analyzed.
CHAPTER FOUR

RESULTS

4.1 Extraction yields

The yields of extracts after extraction and drying are given in table 4.1 below. The percentage yields were obtained as percentages of mass yield. The weight of the dry extract was expressed as a percentage of the total mass of dry plant powder.

Table 4.1: The percentage yields of crude organic and aqueous extracts of *S. longepedunculata* leaves, stem and roots

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<th>Organic crude extracts</th>
<th>Aqueous crude extracts</th>
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<td></td>
<td>Leaves</td>
<td>Root</td>
</tr>
<tr>
<td>Weight of screw cup bottle + sample extract</td>
<td>104.82g</td>
<td>102.44g</td>
</tr>
<tr>
<td>Weight of screw cup bottle</td>
<td>79.97g</td>
<td>81.39g</td>
</tr>
<tr>
<td>Weight of sample extract</td>
<td>24.85g</td>
<td>21.05g</td>
</tr>
<tr>
<td>Weight of material before extraction</td>
<td>200g</td>
<td>200g</td>
</tr>
<tr>
<td>Yield (% w/w)</td>
<td>12.42%</td>
<td>10.52%</td>
</tr>
</tbody>
</table>

High yields were obtained with organic extraction: leaves (12.42%), roots (10.52%) and stem (9.99%). Low yields were obtained by aqueous extraction: leaves (6.73%) roots (3.4%) and stem (2.61%).
The organic extracts resulted in the highest quantity of crude dry extract, while the aqueous extracts gave the least quantity. The order was as follows: (leaves organic > root organic > stem organic > leaves aqueous > root aqueous > stem aqueous).

4.2 Determination of *in vivo* anti-malarial activity

The 4 day standard suppressive test is commonly done for antimalarial screening and the investigation of percentage of suppression of parasitaemia is used to determine the efficacy and potency of the test drug towards malaria. The percentage parasitaemia was determined by counting the parasitized red blood cells and expressing as a percentage of the total number of red blood cells per view. The percentage suppression of parasitaemia was calculated by comparing the parasitaemia present in infected controls with those of test mice by use of the equation below.

\[
\text{Inhibition} \% = 100 \left[ \frac{(\text{parasitaemia of control} - \text{parasitaemia of drug})}{\text{parasitaemia of control}} \right]
\]

The extracts were considered active if parasitaemia reduced by 30% or more (Tona *et al.*, 2001). The results are summarised in the table 4.2 below.
Table 4.2: Suppression of *P. berghei* by organic and aqueous extracts of *S. longepedunculata* in mice after day 4 post inoculation.

<table>
<thead>
<tr>
<th>Drug</th>
<th>% Parasitaemia*</th>
<th>% Suppression</th>
</tr>
</thead>
<tbody>
<tr>
<td>Organic root extract</td>
<td>1.93 ± 0.37</td>
<td>91.46</td>
</tr>
<tr>
<td>Aqueous root extract</td>
<td>2.78 ± 0.46</td>
<td>87.64</td>
</tr>
<tr>
<td>Organic stem extract</td>
<td>18.27 ± 0.74</td>
<td>19.09</td>
</tr>
<tr>
<td>Aqueous stem extract</td>
<td>19.07 ± 0.86</td>
<td>18.42</td>
</tr>
<tr>
<td>Organic leaves extract</td>
<td>10.34 ± 0.91</td>
<td>54.04</td>
</tr>
<tr>
<td>Aqueous leaves extract</td>
<td>13.98 ± 0.68</td>
<td>38.05</td>
</tr>
<tr>
<td>Positive control</td>
<td>0.68 ± 0.17</td>
<td>96.99</td>
</tr>
<tr>
<td>Negative control</td>
<td>22.58 ± 0.84</td>
<td></td>
</tr>
</tbody>
</table>

*S.l.* = *Securidaca longepedunculata*; N = 5 per group; *Data are means ± SEM

The results of this study demonstrated suppression of parasitaemia in treated groups. The percentage parasitaemia ranged from 1.93 % to 13.98 % in mice treated with the plant extracts. In positive control group and negative control group, the percentage parasitaemia was 0.68 % and 22.58 % respectively. The percentage suppression of parasitaemia ranged between 18.42 % and 91.46% in mice treated with the plant extracts. In positive control group, the percentage suppression of parasitaemia was 96.99%.
4.2.1 The mean survival time post infection

The percentage mean survival time (ST) of each treated group, consisting of 5 mice was recorded for a period of 14 days and expressed in the Table 4.3 and figure 4.1. The survival time of *S. longepedunculata* treated groups was compared with that of chloroquine treated group. The effect of *S. longepedunculata* on percentage increase in life span of mice infected with *P. berghei* was calculated on the basis of mortality of the experimental mice and total number of animals in the group.
Table 4.3: Effects of aqueous and organic extracts of *S. longepedunculata* at a dose of 100mg/kg on survival time of mice infected with *P. berghei* compared with chloroquine from day 0 to day 14.5

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Survival time in days</th>
<th>% Survival (day 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D0</td>
<td>D1</td>
</tr>
<tr>
<td>Root organic</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>Stem organic</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>Leaves organic</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>Root aqueous</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>Stem aqueous</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>Leaves aqueous</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>+ve control</td>
<td>5/5</td>
<td>5/5</td>
</tr>
<tr>
<td>-ve control</td>
<td>5/5</td>
<td>5/5</td>
</tr>
</tbody>
</table>
Four-day suppressive test revealed that both organic and aqueous extracts of *S. longepedunculata* administered at a dose of 100mg/kg caused increased survival time compared with non-treated control infected mice. All mice in the root organic and root aqueous treated groups survived to the end of the experiment. A similar observation was made in positive control chloroquine treated group. None of the mice in both stem organic and aqueous and leaves aqueous treated groups survived till the end of the experiment. Only one mouse in leaves organic treated group survived till the end of the experiment.

Parasitaemia suppression of the root organic extract was higher than that of root aqueous extract (87.64 ± 0.46%). However, infected mice treated with the root organic extract had the same survival time compared of root aqueous extract treated mice.
4.3 *In vitro* cytotoxicity evaluation using MTT Assay

The cytotoxic activity of *S. longepedunculata* extracts was expressed by the cytotoxic concentrations 50 (CC$_{50}$), representing the concentration of drug eliciting a 50% inhibition of cell proliferation. The CC$_{50}$ values were determined graphically on dose-response curves shown in Figures 4.2 to 4.7 (concentration on the x-axis versus percentage inhibition). The CC$_{50}$ values for organic extracts and aqueous extracts are shown in Tables 4.4 and 4.5 respectively. NOAEL values were extrapolated from the dose response curves to generate highest dose levels that does not produce adverse response and ascertain minimum risk levels.

![Root Organic Extracts](attachment:image.png)

**Figure 4.2:** Percentage inhibitions of HEp-2 cells against tested concentrations of *S. longepedunculata* crude organic roots extracts
Figure 4.3: Percentage inhibitions of HEp-2 cells against tested concentrations of *S. longepedunculata* crude organic stem extracts

Figure 4.4: Percentage inhibitions of HEp-2 cells against tested concentrations of *S. longepedunculata* crude organic leaf extracts
Table 4.4: CC$_{50}$ values of parts of *S. longepedunculata* organic extracts

<table>
<thead>
<tr>
<th>Code</th>
<th>Plant part</th>
<th>CC$_{50}$ (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JRO</td>
<td>Root organic</td>
<td>140</td>
</tr>
<tr>
<td>JSO</td>
<td>Stem organic</td>
<td>128</td>
</tr>
<tr>
<td>JLO</td>
<td>Leaf organic</td>
<td>132</td>
</tr>
</tbody>
</table>

Figure 4.5: Percentage inhibitions of HEp-2 cells against tested concentrations of *S. longepedunculata* crude aqueous roots extracts
Figure 4.6: Percentage inhibitions of HEp-2 cells against tested concentrations of *S. longepedunculata* crude aqueous stem extracts

![Stem Aqueous Extract](image)

Figure 4.7: Percentage inhibitions of HEp-2 cells against tested concentrations of *S. longepedunculata* crude aqueous leaf extracts

![Leaf Aqueous Extracts](image)
### Table 4.5: CC<sub>50</sub> values of parts of *S. longipedunculata* aqueous extracts

<table>
<thead>
<tr>
<th>Code</th>
<th>Plant part</th>
<th>CC&lt;sub&gt;50&lt;/sub&gt; (μg /ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>JRW</td>
<td>Root aqueous</td>
<td>137</td>
</tr>
<tr>
<td>JSW</td>
<td>Stem aqueous</td>
<td>123</td>
</tr>
<tr>
<td>JLW</td>
<td>Leaf aqueous</td>
<td>115</td>
</tr>
</tbody>
</table>

#### 4.4 *In vivo* acute toxicity

#### 4.4.1 Cage side observations

The examination of the behavior of animals after administration of the drug was reported. General behavior of each animal was recorded on a 30 minute interval for 6 hours and then after 24 hours. Any changes or abnormalities recorded were treated as an indication of toxicity. Details of the behavioural changes are summarised in the Table 4.6.
Table 4.6: Behavioral changes observed during acute toxicity studies of the extracts of *S. longepedunculata* crude extracts in Swiss albino mice

<table>
<thead>
<tr>
<th>Observations</th>
<th>30 mins</th>
<th>1 hr</th>
<th>2 hrs</th>
<th>3 hrs</th>
<th>6 hrs</th>
<th>24 hrs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inactivity</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Pilo erection</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Diarrhoea</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Vomiting</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Rolling movements</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Urination</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Breathing abnormalities</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Tremors</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Sedation</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Bluing of the skin</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Coma</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

**Key:** + present, - absent

### 4.4.2 Mortality

Mortality is the main criteria in assessing the acute toxicity (*LD*$_{50}$) of any drug. A sequential testing procedure was used using only three animals per a given dose. The extracts were administered with objective of reaching a standard dose known to cause marked distress and toxicity. Extracts were orally administered sequentially at a concentration of 50 mg/kg, 300
mg/kg and 2000 mg/kg as shown in Table 4.7 below. Each concentration was given to a treatment group of three mice. If mortality was observed in more than half of the test animals, then the following higher dose was not administered.

Table 4.7: Mortalities observed at different concentrations of organic and aqueous extracts of *S. longepedunculata*

<table>
<thead>
<tr>
<th>Dose (mg/kg)</th>
<th>Log Dose</th>
<th>No of Mice Dead</th>
<th>Root organic</th>
<th>Stem organic</th>
<th>Leaves organic</th>
<th>Root aqueous</th>
<th>Stem aqueous</th>
<th>Leaves aqueous</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>1.69</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>300</td>
<td>2.48</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
<tr>
<td>2000</td>
<td>3.3</td>
<td>1/3</td>
<td>0/3</td>
<td>0/3</td>
<td>1/3</td>
<td>0/3</td>
<td>0/3</td>
<td>0/3</td>
</tr>
</tbody>
</table>

No mortality was observed at 50 mg/kg and 300 mg/kg. At 2000 mg/kg, one mortality was observed in root organic and root aqueous treated groups. Higher doses above 2000mg/kg were not tested because less than half of the test animals were killed at standard dose of 2000 mg/kg. An exposure range where mortality lies was estimated to be above 2000mg/kg.
4.4 Phytochemicals screening

Composition of phytochemicals present in the plant is directly proportional to its pharmacological activity. The extracts were tested for seven known anti-malarial compounds. These compounds include alkaloids, steroids, anthraquinones, glycosides, flavonoids, saponins and tannins. The results are summarized in Tables 4.8 and 4.9 below.

Table 4.8: Phytochemical constituents of the aqueous root, stem and leaves extracts of *Securidaca longipedunculata*

<table>
<thead>
<tr>
<th>Samples</th>
<th>Steroids</th>
<th>Alkaloids</th>
<th>Tannins</th>
<th>Anthraquinones</th>
<th>Flavonoids</th>
<th>Saponins</th>
<th>Cardiac glycosides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root</td>
<td>+</td>
<td>-</td>
<td>+++</td>
<td>+</td>
<td>-</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Stem</td>
<td>+</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>-</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Leaves</td>
<td>++</td>
<td>-</td>
<td>+++</td>
<td>-</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
</tbody>
</table>

Key: +++ present, ++ moderately present, + weakly present, - absent
Table 4.9: Phytochemical constituents of the organic root, stem and leaves extracts of *Securidaca longipedunculata*

<table>
<thead>
<tr>
<th>Samples</th>
<th>Steroids</th>
<th>Alkaloids</th>
<th>Tannins</th>
<th>Anthraquinones</th>
<th>Flavonoids</th>
<th>Saponins</th>
<th>Cardiac glycosides</th>
</tr>
</thead>
<tbody>
<tr>
<td>Root</td>
<td>+++</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>++</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Stem</td>
<td>+++</td>
<td>+</td>
<td>+++</td>
<td>++</td>
<td>+</td>
<td>+++</td>
<td>+</td>
</tr>
<tr>
<td>Leaves</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>+</td>
</tr>
</tbody>
</table>

Key: +++ present, ++ moderately present, + weakly present, - absent
CHAPTER FIVE
DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

5.1.1 In vivo anti-malarial activity

The plant kingdom continues to provide new and important leads against various pharmacological targets, (Balunas and Kinghorn, 2005). Several antimalarial drugs have been derived from medicinal plants. These include quinine from cinchona tree, artemether and artemisinin isolated from Artemisia annua (Chin et al., 2006). Plants have enormous chemical diversity and further exploration is necessary to discover new drugs. The results of in vivo study demonstrated inhibition of development and multiplication of Plasmodium berghei in mice in the treated groups. The standards anti-malarial drug employed for the positive control; chloroquine at a dose of 10 mg/kg showed percentage parasitaemia suppressions of 96.99% after 4 days of treatment. Chloroquine did not show any significant difference ($p>0.05$) in comparison with percentage parasitaemia suppression exhibited by the aqueous and organic root extract of S. longepedunculata after the 4 days treatment of mice infected with P. berghei. There was a significant difference ($p<0.05$) between chemo suppressive percentages on day 4 post infection of the treated groups and the negative control (22.58 ± 0.84).

The highest level of clearance was observed in chloroquine treated group, with percentage parasitaemia on day 4 as 0.68 ± 0.17 as compared to 22.58 ± 0.84 of the negative control group S. longepedunculata root organic and aqueous crude extracts equally demonstrated a good suppression of parasitaemia on day 4 with percentage parasitaemia of 1.93 ± 0.37 and 2.78 ± 0.46 respectively. There was no significant difference ($p>0.05$) between observed suppression of
chloroquine and root organic and aqueous extracts. This study demonstrated that organic and aqueous root extracts of *S. longepedunculata* had very good activities against *P. berghei* malaria parasite in Swiss albino mice.

The antimalarial potency was defined according to Willcox and Bodeker, (2004). According to this classification, *in vivo* antiplasmodial activity of an extract or a fraction can be classified as having very good activity if the parasite growth inhibition is between 100%-90%, good to moderate activity if the parasite growth inhibition is 90% to 50%, moderate to weak activity, if the inhibition is 50%-10% and is considered inactive if the inhibition is 0%. Root organic extracts had very good antiplasmodial activity. Organic leaves and root aqueous extracts had good to moderate activity while stem organic and aqueous extracts had moderate to weak activity. The findings of this study have corroborated the findings of a previous study done by Oketch-Rabah, (2003) which indicated significant suppression of parasitaemia by the methanol extract of *S. longepedunculata*.

Parasitaemia suppression above 90% was attained with the root organic extract at a dose of 100 mg/kg/day. According to WHO, (2012) extracts displaying very high level of chemo-suppression (>90%) at 250 mg/kg/day may be recommended for potential therapeutic development. This study demonstrated that the phytochemicals in these extracts suppressed the growth of *Plasmodium berghei* in Swiss albino mice inhibiting their development and multiplication. Antimalarial activity observed in this study could be due to a single phytochemical or synergistic effect of the phytochemicals already screened. Phytochemical screening demonstrated presence of alkaloids, tannins, steroids, saponins, flavonoids and cardiac glycosides. However, the
pharmacological active principle that causes antimalarial activity should be identified and separated using bioactivity methods.

Aqueous extracts of *S. longepedunculata* demonstrated slightly lower antimalarial activities than organic extract (root organic > root aqueous and leaves organic > leaves aqueous). This contradicts reports by Willcox and Bodeker, (2004) who demonstrated that aqueous extracts usually have got the highest antimalarial activity. The activity of stem and leaves extracts could be improved on further fractionation to yield extracts with strong antimalarial activity.

Traditional medical practitioners do not have standardized extraction methods. To maneuver this, traditional herbal preparation techniques often employ mixtures of plant parts or mixtures of plants. Patients take whole plant extracts which contain mixtures of active ingredients which act synergistically to treat the disease. This maybe more effective than taking an extract from one part of the plant or taking a single isolated ingredient. This study has demonstrated that root extracts are more effective in parasitaemia suppression than other parts of the plant. In traditional setting, the herbalist would give a mixture of plant parts.

The safety and efficacy of any drug is dependent on the ratio between the toxic or lethal concentration (LD$_{50}$) and the minimum effective concentration (EC$_{50}$). The drug should suppress the parasite but possess no toxicity or adverse effect to the animal or human models. This study has demonstrated a higher therapeutic index of *S. longepedunculata* extracts with LD$_{50}$ more than 2000 mg/kg body weight and EC$_{50}$ less than 100 mg/kg. The result of this study is therefore in concurrence with the traditional use of this plant for antimalarial therapy. Even though the rodent malaria model, *P. berghei*, is not exactly similar to that of the human *Plasmodium*
parasites, it is the first step to screen most of the \textit{in vivo} antimalarial activities of new molecules and new therapeutics.

\textbf{5.1.2 \textit{In vitro} cytotoxicity}

The reduction of MTT ([3-(4, 5-dimethylthiazol-2-yl) 2, 5-diphenyl tetrazolium bromide]) in the cell assesses the functional intactness of mitochondria based on the enzymatic reduction of the tetrazolium salt by the mitochondrial dehydrogenase in viable cells. The cytotoxicity was defined according to Smee \textit{et al.}, (2002). CC$_{50}$ < 10μg/ml, high toxicity; CC$_{50}$ 11-50μg/ml, moderate toxicity; CC$_{50}$ 51-100μg/ml, mild toxicity; and CC$_{50}$ > 100μg/ml not toxic. None of the extract tested was potentially toxic to HEp-2 cell lines. The achieved results indicate that none of the extracts inhibited metabolic activity of HEp-2 cells and showed no signs of cytotoxic activity. The dose-response dependencies had a typical sigmoid form. The CC$_{50}$ values extrapolated from the graphs were 132μg/ml, 140μg/ml and 128μg/ml for leaves, root and stem organic extracts respectively. For aqueous extracts CC$_{50}$ values were 115μg/ml, 123μg/ml and 137μg/ml for leaves, stem and root extracts respectively. MTT and neutral red are probably the most commonly used colorimetric indicators of cell viability. In this study, MTT was used to evaluate cytotoxicity in a quantitative way in contrast with cell morphology evaluation by inverted light microscopy which is qualitative and more subjective (Smee \textit{et al.}, 2002).

According to a study done by Lawal \textit{et al.}, (2012), \textit{S. longopedunculata} aqueous extract was cytotoxic to Ehrlich ascites cancer cells with IC$_{50}$ of 67μg/ml. \textit{In vitro} studies reported mild to no cytotoxicity at the molecular level. However this may not be reflected \textit{in vivo}. According to Waako \textit{et al.}, (2005), some compounds that do not show \textit{in vitro} cytotoxicity may possess \textit{in vivo} toxicity due to pharmacokinetic and immunological factors. This calls for further studies to
investigate safety of this plant in an *in vivo* system. Sometimes MTT underestimates the toxicity of certain substances. As this dye undergoes enzymatic conversion in viable cells, it is possible that certain compounds might inhibit this process making them to appear less cytotoxic than they really are (Smee *et al.*, 2002). Moreover, compounds that are naturally colored may interfere with a test based upon colorimetry. Therefore these two factors may interfere with the cytotoxic results obtained in this study. Another method to investigate cytotoxicity should be employed before declaring this extracts complete safe in *in vitro* models.

**5.1.3 *In vivo* acute toxicity**

Assessment of toxicity is clearly an important part of the evaluation of a potential anti-malarial agent since a useful compound should show neither acute nor long-term toxicity against the host. Such a compound should be completely selective for plasmodium specific processes with no or few effects on cellular metabolism. Acute toxicity studies are those carried out within 24 hours with single dose administration of the drug. It is aimed at establishing the therapeutic index (LD$_{50}$/ED$_{50}$). The higher the index the safer the compound. A chemical is considered to be extremely toxic if it has LD$_{50}$ of 1mg/kg and practically non-toxic if it has an LD$_{50}$ of 1500mg/kg and above (Gosh, 1984).

The extracts at the highest dose of 2000 mg/kg did not show any significant changes in behavior after administration of the drug as compared to the control. The observed behavioral changes were prominent in the first one hour after which they gradually declined. The extracts from *S. longipedunculata* at a dose of 2000 mg/kg did not adversely affect behavior of Swiss albino mice. Observations such as inactivity, diarrhea, rolling movements and vomiting are consistent with normal behavioral patterns. This study also corroborates another study done by Agbaje
et. al., (2012) which documented sedation and weakness within 30 min post *S. longepedunculata* root extracts administration and the same persisted for two to three hours. Agbaje et. al., (2012) reported similar results with this study with oral LD$_{50}$ values of 3162 mg/kg. Urination observed may be an indication of diuretic effects however more research is needed to verify this. Observed sedation and decreased motor activity may demonstrate depression of the CNS. These extracts can therefore interact with CNS depressants and other sedatives and therefore concomitant use should be avoided.

5.1.3.1 Mortality

No mortality was observed in any of the experimental groups at 50 mg/kg and 300 mg/kg. At 2000 mg/kg, one mortality was observed in root organic and root aqueous treated groups. Higher doses above 2000 mg/kg were not tested because less than half of the test animals were killed at standard dose of 2000 mg/kg. An exposure range where mortality lies was estimated to be above 2000mg/kg. Both organic and aqueous extracts were non-toxic at 2000 mg/kg of bdw i.e. LD$_{50}$>2000 mg/kg. The results of oral studies are important for drugs, food and accidental domestic poisonings. In general, the smaller the LD$_{50}$ value, the more toxic the chemical is. Also, the larger the LD$_{50}$ value, the lower the toxicity (Senin, 2006). The toxicity was ranked and classified according to the Globally Harmonized System for the classification of chemicals which cause acute toxicity. It was ranked under category 5 with toxicity above 2000mg/kg. This is regarded as safe according to toxicity classification/scale of toxic substances (Hodge and Sterner, 2005).

This study also agrees with another study done by Agbaje et. al., (2012) which demonstrated LD$_{50}$ at a dose of 3162.27 mg/kg after oral administration of *S. longepedunculata* root extracts.
This study therefore demonstrates that *S. longepedunculata* organic and aqueous extracts are safe and has no any significant toxic effect in normal doses in Swiss albino mice. This study corroborates another study by, Etuk *et al.* (2006), which indicated that there was no observed toxicity following a 28 day oral administration of *S. longepedunculata* root in mice with the LD$_{50}$ above 2000 mg/kg body weight. Etuk *et al.*, (2006), observed toxicity with LD$_{50}$ of 2700 mg/kg. These two studies contrast another study done by Dapar *et al.*, (2007) which reported that *S. longepedunculata* root extracts possess some level of toxicity on mice liver and kidney. Intraperitoneal administration of 100 mg/kg body weight resulted in signs of toxicity and death, Dapar *et al.*, (2007). Disparity in findings could be due to different routes of drug administration. According to Lorke (1983), LD$_{50}$ values cannot be measured exactly but the knowledge of its specific numerical value is not very important since it cannot be directly extrapolated from the experimental animals to humans. However, it still serves a great purpose as a first pointer to the safety or toxic potential of a substance whose toxicity profile is not yet known (Kagbo and Ejebe, 2010).

Literature review revealed large scale use of this plant parts to treat various illnesses. Therefore evaluation of toxicities caused by this plant is of great significance. According to Dapar *et al.*, (2007), LD$_{50}$ is not a very reliable procedure in the determination of toxicity. There is a wide variation in results between different species and even in the same species under different experimental conditions. One species of rodents was used in acute toxicity studies however the results may not be true for other species. Additionally, acute toxicity measures lethality within 24 hours and there is no information on the long term effects of the plants. The LD$_{50}$ provides no information on what system failure led to the death of the animals. Some deaths may have been
due to the quantity of the test substance causing gastric rupture or other morbidity unrelated to the toxicity of the extract.

5.1.4 Phytochemical screening

The organic extracts revealed the presence of steroids, alkaloids, tannins, anthraquinones, flavonoids, cardiac glycosides and saponins. Saponins, steroids and tannins were detected in higher quantities followed by cardiac glycosides, anthraquinones, flavonoids and alkaloids which were detected the least. This study corroborates a previous study done by Haruna et al., (2013) which demonstrated the presence of steroids, alkaloids, tannins, anthraquinones, flavonoids, cardiac glycosides and saponins. Alkaloids, flavonoids and anthraquinones demonstrated by this study to be present in this plant have got documented antimalarial properties. Auwal et al., (2012), detected the presence of alkaloids, cardiac glycosides, flavonoids, saponins and tannins. The same study detected saponins and tannins to be present in larger quantities than alkaloids and cardiac glycosides which is in agreement with the findings of this study.

The results demonstrate that the phytochemical constituents are more concentrated in the organic extracts than aqueous extracts. Organic solvents are able to extract broad spectrum phytochemicals due to their non-polarity and lipophilicity. However, water is usually the main solvent used by traditional healers to prepare plant extracts. Phytochemistry of S. longepedunculata appears to be dominated by saponins. Tailang and Sharma, (2009), reported that there is greater solubility of saponins and tannins in aqueous solutions. This has been demonstrated by this study. Saponins and tannins were detected in high quantities in aqueous extraction. This study confirms a previous study by Kamba and Hassan, (2010) which demonstrated that the root bark contains steroids, flavonoids, tannins, and saponins. Agbaje
et.al., (2012) also demonstrated presence of tannins, alkaloids, flavonoids, saponins and cardiac glycosides. This study corroborates with another study which illustrated that plant phytochemicals are more soluble in organic solvents (Willcox and Bodeker, 2004).

This study agrees with another study done by Agbaje et.al., (2012) which documented presence of tannins, phlobatannins, alkaloids flavonoids, saponins and cardiac glycosides in methanolic root extracts of S. longepedunculata. A study done by Evans, (2009) demonstrated that plants of families Polygalaceae, Moraceae and Cannabinaceae have got secondary metabolites namely tannins, cardiac glycosides, triterpenes and flavonoids. This is in agreement with the results of this study which has demonstrated the presence of the same phytochemical constituents in S. longepedunculata (Polygalaceae). Cardiac glycosides are a major source of toxicities and poisoning (Raffi and Mark, 2009). When they are taken at therapeutic doses, they improve circulation in the heart. Antimalarial activity observed in this study could be due to a single phytochemical or synergistic effect of the phytochemicals already screened. However, the pharmacological active principle that causes antimalarial activity should be identified and separated by bioactivity methods for maximum therapeutic effect.

The flavonoids have played a major role in successful medical treatments of ancient times and their use has persisted up to now. Flavonoids have gained recent attention because of their broad biological and pharmacological activities including antimicrobial, cytotoxicity, anti-inflammatory as well as antitumor activities (Eisenbrand et al., 2002). Flavonoids have capacity to act as powerful antioxidants which can protect the human body from free radicals and reactive oxygen species. Flavonoids from Artemisia annua have been shown to have anti-plasmodial

Alkaloids have some pharmacological effects and are used as medications, recreational drugs, or in entheogenic rituals e.g. the local anaesthetic and stimulant cocaine, the stimulant caffeine, the analgesic morphine or the antimalarial drug quinine (Tailang and Sharma, 2009). They are natural products that contain heterocyclic nitrogen atoms and are basic in character. Almost all the alkaloids have bitter taste. The alkaloid quinine for example is one of the bitterest tasting substances known (Eisenbrand et al., 2002). Alkaloids have many pharmacological activities including antimalarial activity e.g. quinine alkaloid which occurs in the bark of cinchona tree. It has been used for centuries for treatment of malaria. It was first isolated from *Cinchona ledgeriana* (Rubiaceae) in 1820. It is among the most pharmacologically potent plant derived natural products in clinical use (Eisenbrand et al., 2002). It has also been used as a template for synthetic amino-quinoline based anti-malarial analogues such as chloroquine, amodiaquine, primaquine and mefloquine.

Tannins are a heterogeneous group of high molecular weight polyphenolic compounds with the capacity to form reversible and irreversible complexes with proteins. Several health benefits have been recognized for the intake of tannins however antimalarial properties of tannins have not been well documented (Evans, 2009). The tannin containing plant extracts are used as astringents, against diarrhea, as diuretics, against stomach and duodenal tumors and as haemostatic pharmaceuticals (Eisenbrand et al., 2002). There are two types of quinones namely; naphthaquinones and anthraquinones. Anthraquinones have three aromatic with a ketone group
in the middle. Naphthaquinones have two aromatic rings and two ketone groups. The antiplasmodial drug atovaquone is a naphthaquinones derivative.

This study agrees with the study done by Ekpendu et al. (2000), which investigated the phytochemicals constituents in the root bark of *S. longepedunculata* and revealed the presence of flavonoids, alkaloids, saponins, triterpenoids and volatile oils. Flavonoids are present widely in the plant kingdom and possess many functions including antiinflammatory, antimicrobial, enzyme inhibition, antioxidant and antimalarial (Cushnie *et al.*, 2005). Flavonoids are characterized by low toxicity since they are widely distributed in edible plants (Cushnie *et al.*, 2005). Flavonoids are potent water-soluble antioxidants and free radical scavengers. They prevent oxidative cell damage and have strong anticancer activity as well as antimalarial properties (Meyer *et al.*, 2008).

Other secondary metabolites like sapogenins, presenegenin, indole alkaloid, securinine and some ergot alkaloids have also been reported to be present in the root of this plant. The alkaloid securinine confers activity against *Plasmodium falciparum*, the causative agent of malaria. Alkaloids possess analgesic, antispasmodic, antiplasmodial and bactericidal effects. The saponins have got antiseptic properties (Meyer *et al.*, 2008). Saponins are capable of neutralizing some enzymes in the intestine that can become harmful, building the immune system and promoting wound healing. This study explains the pharmacological significance of this plant. It has got numerous active chemical constituents that are used to treat numerous diseases. This plant is arguably used to treat every conceivable ailment (Avhurengwi and Walter, 2006). Natural stands are under considerable pressure from harvesting of roots for its numerous uses.
(Ouedraogo et al., 2003). The Hausas refer to it as “uwar magunguna” (the mother of all medicines). The claims of the medicinal applications of *S. longepedunculata* extracts are numerous. This is evidenced by numerous phytochemical constituents demonstrated in this study. This is further supported by the fact that *S. longepedunculata* belongs to the family Polygalaceae. Plants in this family are well known for their ethno medical value and pharmacological significance. Alkaloids, flavonoids and anthraquinones demonstrated by this study to be present in this plant have got documented antimalarial properties. Most of these phytochemical constituents are potent bioactive compounds found in medicinal plant parts which are precursors for the synthesis of useful drugs. The study has demonstrated the presence of bioactive principles that can be used to explain the activities of this plant. To consider pharmacological potential of these phytochemicals, different studies should be carried out including activity against *Plasmodium falciparum* and elucidation of mechanism of action.
5.2 CONCLUSIONS

Currently, the cornerstone of malaria research across the globe and especially in Kenya is the exploitation of effective and inexpensive drugs particularly those from plant sources. The evidence demonstrated in this study, has indicated the potential of this plant as a source to develop new compounds against malaria. This therefore validates the use of *S. longipedunculata* in the treatment of malaria by traditional practitioners. The root organic and root aqueous extracts are very efficacious and have potential use as an effective anti-malarial in consonance with its use in folkloric medicine. The high activity of *S. longipedunculata* on malaria parasites which has been well elucidated in this study and also cited in scientific literature could be alluded to its phytochemical chemical constituents. Cytotoxicity investigation revealed that the extracts were not toxic to HEp-2 cell lines. The study has established that the extracts have antiplasmodial activity at concentrations that can be achieved without inducing toxic effects to cells.

The plant extracts were found to be relatively safe for use as far as lethality is concerned in mice. *S. longopedunculata* organic and aqueous extracts were classified in category 5 of Globally Harmonized System for the classification of chemicals which cause acute toxicity. The LD$_{50}$ is above 2000mg/kg. This is regarded as safe according to toxicity classification/scale of toxic substances. Phytochemical analysis revealed presence of known antimalarial compounds: alkaloids, flavonoids and anthraquinones. Even though the study on the specific class of phytochemical constituents implicated and their activity guided isolation was not carried out, all the identified classes have been reputed to exert good activity on malaria parasites. The results obtained from the phytochemical analysis provide preliminary data on some known anti-malarial
compounds that are found in this plant that have potential antiplasmodial activity. Further studies would be required to isolate the specific active principles of the plant responsible for the antimalarial activity to standardize the plant for commercial exploration. The active constituents of the plants should be isolated, identified and characterized.

The evidence demonstrated in this study, has indicated the potential of natural plant sources to develop new compounds against malaria. This therefore validates the use of *S. longepedunculata* in the treatment of malaria by traditional practitioners.
5.3 RECOMMENDATIONS

The study also recommends elucidation of mechanisms of action of this plant as malaria therapeutic. Such clarification is necessary to optimize therapeutic activities. Active compounds need to be investigated in terms of structure-activity relationship with modifications of the structure to enhance efficacy.

The results demonstrated that this plant is potent antimalarial with limited systemic toxic effects. The study recommends more investigations to be done using other mammalian cell lines to verify selectivity. The in vitro antiplasmodial activity and cytotoxic activity values would be used to generate the selectivity index. It is important that the extracts have maximum antiplasmodial activity with minimal cell toxicity. This study recommends in vitro testing of these extracts for their activity against \textit{P. falciparum} in order to consider them as potential sources for antimalarial drug development for human malaria.

This study investigated acute in vivo toxicity. The study recommends further investigations on sub-acute and chronic toxicities before declaring the crude extracts completely safe for use in humans.

This study recommends further work aimed at hit identification for antimalarial activity through bioactivity guided fractionation, isolation, purification and characterization of compounds responsible for the observed antimalarial activity. The purified and identified hits could be used for lead development for antimalarial activity as well as biomarkers for standardization of \textit{S}. 
*longepedunculata* herbal preparations for use against malaria. The plant phytochemical constituents need to be standardized for commercial exploration.

Finally due to medical importance of this plant exhibited by the above investigations, this study recommends conservation of this plant. *S. longepedunculata* should be conserved and propagated by communities in the ecosystems where they naturally grow. Rapid population growth and charcoal burning in Msambweni district threatens the natural existence of this plant. The government should enact policies to cultivate *S. longepedunculata* due to medicinal importance demonstrated by this study.
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