IN VITRO AND *IN VIVO* ANTIAMOEBIC ACTIVITY AND PHYTOCHEMICAL SCREENING OF *SENNA DIDYMOBOTRYA* (FRESEN) IRWIN CRUDE ROOT EXTRACTS

BY

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DECLARATION

Declaration by the Candidate

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DEDICATION

I dedicate this thesis to my mum Joyce Gesare, husband Albert Kimutai, our sons Jerry and Ian and all Kenyans.

ABSTRACT

Amoebiasis is a disease of global importance, caused by the eukaryotic parasite *Entamoeba histolytica*. It is the common worldwide cause of mortality from a protozoon after malaria. It is an important health problem, especially in developing countries and highly endemic throughout poor and socio-economically deprived communities in the tropics and subtropics. The most commonly used drug for treatment is metronidazole. However, this drug has been reported to cause mutagenicity in bacteria and is carcinogenic in rodents, with some side effects including metallic taste, nausea, transient neutropenia, interaction with warfarin and peripheral neuropathy. Interactions with alcohol may reduce the level of patient compliance. In addition, amoebae may develop resistance to metronidazole. There is need to investigate substances of natural origin to find new antiamoebic compounds that are effective, safe and affordable. This study, evaluated the *in vitro* and *in vivo* antiamoebic activities and toxicity of crude root extracts of *Senna didymobotrya* and their phytochemical constituents, a plant that is currently used by traditional healers to treat diarrhoea and dysentery in Kenya. The roots were obtained from Bomet County, Kenya where the "Kipsigis" traditional healers have been using the roots to treat diarrhoea. This study also determined the cytotoxicity of the root extracts in mammalian cells and found that the ethyl acetate crude root extract was less toxic while in experimental BALB/c mice the methanol total and dichloromethane crude root extracts were the most toxic with an LD_{50} of 1927mg/kg each. In addition, *in vitro* and *in vivo* bioassays of crude root extracts of *Senna didymobotrya* were performed in order to determine their antiamoebic activities in comparison with metronidazole. The methanol total crude root extract compared favourably well with metronidazole ($\rho>0.05$). There was a significant difference in effectiveness between the different types of crude root extracts with methanol total being the most active crude root extract ($p<0.05$). There was also a significant difference between the different concentrations with the 1000µg/ml being the most active concentration ($\rho <0.05$). The bioactive compounds were determined by phytochemical screening using standard procedures and *Senna didymobotrya* was found to contain steroids, terpenoids, anthraquinones, tannins, saponins, glycosides, alkaloids and phenols. The classes of compounds found to exhibit antiamoebic activity against *Entamoeba histolytica* trophozoites were terpenoids, tannins, saponins, glycosides, alkaloids and phenols. However, some *in vivo* studies with regards to their effects on human also need to be investigated.

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GLOSSARY OF ABBREVIATIONS AND ACRONYMS

AAP -------- American Academy of Pediatrics

Absorp.------- Absorption

AMREF----- Africa Medical Research Foundation

Av. ---------- Average

CBRD ----- Centre for Biotechnology Research and Development

CD ------- Cluster of Differentiation

DMSO ---- Dimethylsulfoxide

ELISA ---- Enzyme Linked Immunosorbent Assay

FBS ---- Foetal Bovine Serum

Gal/NAc ---- N-Acetylgalactosamine

HLA ----- Human Leucocyte Antigen

IC ------ Inhibitory concentration

IC_{50} ---- Concentration required for 50% inhibition

IgA ---- Immunoglobulin A

KEMRI---- Kenya Medical Research Institute

KHPF ----- Kenya Health Policy Framework

LD -------- Lethal Dose

MEM ---- Minimum Essential Medium

MHC ---- Major Histocompatibility Complex

MIC -------Minimum Inhibition Concentration

MTT ---- Thiazolyl Blue Tetrazolium

PAHO ---- Pan American Health Organisation

UNESCO--United Nations Educational, Scientific and Cultural Organization

WHO ---- World Health Organization

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CHAPTER ONE

INTRODUCTION

1.1 Background information

An estimated one billion people are infected with one or more neglected tropical diseases (Kirmizibekmez *et al*., 2011; Martinez & Petersen, 2014; Sosoniuk *et al*., 2014; Hotez, Woc-Colburn & Bottazzi, 2014). Amoebiasis caused by *Entamoeba histolytica*, a protozoan of the family Endomoebidae (Zerpa-Larrauri, Náquira-Velarde & Espinoza, 2007; López-Camarillo, López-Rosas, Ospina-Villa & Marchat, 2014), is associated with high morbidity and mortality and has become a major public health problem worldwide (Bansal, Sehgal, Chawla, Malla & Mahajan, 2006) and is therefore considered as the third parasitosis of medical importance after malaria and schistosomiasis (Pinilla, López & Viasus, 2008). *Entamoeba histolytica* is still endemic in tropical and sub-tropical regions, causing a high incidence of infections in developing countries in Latin America, Asia and Africa (Ximénez, Morán, Ramos & Ramiro, 2007), where poor sanitary conditions, population explosion and inadequate control of reservoirs intensify the development of these infections (Calzada, Yépez-Mulia & Aguilar, 2006).

Amoebiasis is prevalent throughout the developing nations with tropical ecosystems, at times reaching a prevalence of 50% of the general population and is estimated to cause more than 100,000 deaths per year (Ximénez, Morán, Rojas, Valadez & Gómez, 2009; Abhyankar, Shrimal, Gilchrist, Bhattacharya & Petri, 2012). Symptomatic patients typically may develop abdominal pain and tenderness, diarrhea, and bloody stools, but the disease may spread to the liver and other organs resulting in death (Phillipson

& Wright, 1991; Somlata, Bhattacharya & Bhattacharya, 2014).

In Kenya, the prevalence of *E. histolytica* is 21% and although most cases go untreated due to their asymptomatic nature, this high prevalence contributes a lot to amoebiasis (WHO, 2011). A study carried out in rural parts of Thika municipality of Kenya to determine the prevalence and intensity of intestinal parasites in school children showed an overall prevalence of intestinal protozoan parasites of 38.9% of which the most prevalent was *E. histolytica* (Ngonjo, Kihara, Gicheru, Wanzala, Njenga & Mwandawiro, 2012).

Currently, metronidazole is the therapeutic drug of choice for the treatment of amoebiasis (Schlosser, Leitsch & Duchêne, 2013). However, *E. histolytica* has developed resistance to this drug (Samarawickrema, Brown, Upcroft, Thammapalerd & Upcroft, 1997; Wassmann, Hellberg, Tannich & Bruchhaus, 1999). This has led to use of high doses of the drug to effectively treat infections and these have unpleasant side-effects, such as headache, nausea, dry mouth, and a metallic taste, as well as neurotoxicity (Conde-Bonfil & Mora-Zerpa, 1992; Bendesky & Menéndez, 2001; Bautista, Calzada, Ortega & Yépez-Mulia, 2011). Owing to these undesired side effects and taking into account the development of resistant strains of *E. histolytica* against metronidazole, there is an urgent need to develop new, more effective and safer antiprotozoal agents (Singh, Bharti & Mohapatra, 2009; Bautista *et al*., 2011).

Natural products have proved to be an important source of lead compounds in the development of new drugs. For example, artemisinin, quinine and licochalcone A are very effective plant-derived products with antiparasitic activity that have successfully been used in the treatment of malaria (Kayser, Kiderlen & Croft, 2003; Sülsen, Güida, Coussio, Paveto, Muschietti & Martino, 2006). Ethnobotanical knowledge on medicinal plants and their uses by different communities either documented in writing and/or handed down through generations is available. This knowledge provides an initial source from which various plant species can be screened for natural products for treatment of specific ailments, in humans and animals. For example, the "Kipsigis" and "Marakwet" communities in Kenya use a wide range of plants for treating a broad range of diseases (Korir *et al.*, 2012; Kipkore, Wanjohi, Rono & Kigen, 2014). Thus, screening of natural products provides the chance to discover new molecules of unique structure with high activity and selectivity (Newman & Cragg, 2012).

Senna didymobotrya (Fresen) Irwin & Barneby (syn *Cassia didymobotrya*) belonging to the family Fabaceae is a widely used medicinal plant in East Africa (Nagappan, 2012). It is a potential medicinal plant and the medicinal values are explored well in many parts of the world by traditional practitioners. In Congo, Rwanda, Burundi, Uganda and Tanzania, root decoction of this plant has been used for the treatment of malaria, other fevers, jaundice and intestinal worms (Nagappan, 2012). In addition, root or leaf mixed with water or decoction of fresh parts has been used to treat abscess of the skeletal muscle and venereal diseases (Kamatenesi-Mugisha, 2004). The plant is also useful for the treatment of fungal, bacterial infections, hypertension, haemorrhoides, sickle cell

anemia, a range of women's diseases such as inflammation of fallopian tubes, fibroids and backache, to stimulate lactation and to induce uterine contraction and abortion (Tabuti, 2007). In Kenya, the "Kipsigis'' community has traditionally been using this plant to control malaria as well as diarrhoea (Nagappan, 2012). In addition, it has been used to treat skin conditions of humans and livestock infections (Njoroge & Bussmann, 2007).

In the present study, *S. didymobotrya* root extract was selected because this species has routinely been used to cure diarrhoea in the "Kipsigis'' traditional medical practice. It was therefore, of interest to scientifically evaluate its effect on amoebiasis for potential antiamoebic activity *in vitro* and *in vivo*.

1.2 Statement of the problem and justification of the study

Metronidazole; is a highly effective amoebicide and is considered by many clinicians to be the drug of choice for treating acute amoebiasis (Wong-Baeza *et al*., 2010). However, metronidazole has mutagenic effects in bacteria and is carcinogenic to rodents (Bautista *et al*., 2011). The drug is relatively ineffective against asymptomatic infections in the intestinal lumen (Lopez, Palermo, Mudry & Carballo, 2003), and has adverse effects, especially severe nausea, transient neutropenia, interaction with warfarin, metallic taste and peripheral neuropathy (Kurohara, Kwong, Lebherz & Klaustermeyer, 1991). Moreover, amoebae may develop resistance to metronidazole (Singh *et al*., 2009; Bautista *et al*., 2011). In this regard, there is clearly a need for search for alternative antiamoebic agents.

Many plant species are used in traditional medicine to treat dysentery; these plants should be analyzed to determine their efficacy (and toxicity) and thus their potential as sources of new antiamoebic agents. Thus, with the purpose of searching for new antiamoebic agents with high activity, low toxicity, cheaper, and more effective, *S. didymobotrya* that has been used in the "Kipsigis'' traditional practice to control malaria as well as diarrhea was selected and evaluated for its antiamoebic activity *in vitro* and *in vivo*.

1.3 Objectives of the study

1.3.1 General objective

To determine the *in vitro* and *in vivo* antiamoebic activity of crude root extracts of *S. didymobotrya*.

1.3.2 Specific objectives

i) To determine the *in vitro* activity of crude root extracts of *S. didymobotrya* against *E. histolytica.*
ii) To determine the *in vitro* cytotoxicity of *S. didymobotrya* crude root extracts in mammalian cells.
iii) To determine classes of compounds of *S. didymobotrya* crude root extracts that have antiamoebic activity against *E. histolytica*.
iv) To determine the *in vivo* activity of crude root extracts of *S. didymobotrya* against *E. histolytica* infection in BALB/c mice.
v) To determine the *in vivo* toxicity of *S. didymobotrya* crude root extracts in an animal model.

1.4 Research Questions

i) Does the root extracts of *S. didymobotrya* have *in vitro* antiamoebic activity against *E. histolytica?*

ii) Does the root extracts of *S. didymobotrya* have cytotoxic effects on mammalian cells?

iii) Which classes of compounds that are present in root extracts of *S. didymobotrya* have antiamoebic activity against *E. histolytica*?

iv) Does the root extracts of *S. didymobotrya* have *in vivo* antiamoebic activity against *E. histolytica*?

v) Does the root extracts of *S. didymobotrya* have toxic effects in animal models?

1.5 Significance of the study

Medicinal plants are popular for people in developing countries because their products are safe and widely available at low cost (Rani, 2011). Some compounds extracted from medicinal plants already play an important role against infectious diseases for instance quinine from *Cinchona* species, and artemisinin from *Artemisia annua*; both are effective against malaria (Hidayathula, Chandra & Chandrashekar, 2011). The Iranian *Thymus vulgaris* has been shown to be effective against the trophozoites of *E. histolytica* (Behnia, Haghighi, Komeilizadeh, Tabaei & Abadi, 2008a). Methanol extract of mature seeds of *Carica papaya* has been shown to exhibit antiamoebic effect on trophozoites of *E. histolytica* (Sarker, Begum, Mondal, Siddique & Rashid, 2010) whereas the methanol extracts from *Piper sarmentosum* root and *Quercus infectoria* nut gall are effective against caecal amoebiasis in mice (Sawangjaroen, Sawangjaroen & Poonpanang, 2004). The root bark of *Adenia cordifolia* extracted in benzene and ethyl acetate has been shown to exhibit antiamoebic activity (Rani, 2011). Many plant species are used in

traditional medicine to treat dysentery. These plants should be analyzed to determine their efficacy and thus their potential as sources of new antiamoebic agents. *Senna didymobotrya* is a medicinal plant and the medicinal values are explored well in many parts of the world by traditional practitioners (Nagappan, 2012). This study sought to establish the toxicity, phytochemical compounds with *in vitro* and *in vivo* antiamoebic activities from *S. didymobotrya* root extracts with an aim of recommending them as potential new antiamoebic agents in man.

CHAPTER TWO

LITERATURE REVIEW

2.1 Incidence of amoebiasis

Amoebiasis is a disease of global importance, caused by the eukaryotic parasite *E. histolytica* (Wilson, Weedall & Hall, 2012). It is the third most common cause of death due to parasitic infection after malaria and schistosomiasis (Obadiah, 2012). It is estimated to cause more than 100,000 deaths annually (Ximénez *et al*., 2009; Abhyankar *et al*., 2012), and 34–50 million cases of severe disease (Araujo, Garc´ıa, Diaz-Su´arez & Urdaneta, 2008). However, fewer than 10% of those infected develop invasive amoebiasis while 90% of infected individuals are asymptomatic (Irusen, Jackson & Simjee, 1992). It is an important health problem, especially in developing countries (Sebastiaan, Stark, Fotedar, Marriott, John & Harkness, 2007). The rate of infection by *E. histolytica* differs among countries, socio-economic and sanitary conditions and populations (Al-Harthi & Jamjoom, 2007). It is highly endemic throughout poor and socio-economically deprived communities in the tropics and subtropics (Duc, Nguyen-Viet, Hattendorf, Zinsstag, Cam & Odermatt, 2011). Environmental, socio-economic, demographic and hygiene-related behaviour is known to influence the transmission and distribution of intestinal parasitic infections (Norhayati, Fatmah, Yusof & Edariah, 2003). Most of the people at risk are those that are living in areas with poor sanitation, as the parasite is transmitted via faecal–oral routes. In such environments, exposure may be very high (Haque, Ali, Sack, Farr, Ramakrishnan & Petri, 2001).

In more affluent countries, where poor living conditions are less common, amoebiasis tends to be seen in certain groups, such as travellers returning from endemic areas (Weinke, Friedrich-J_nicke, Hopp & Janitschke, 1990), men who have sex with men and institutionalized individuals (Nishise *et al*., 2010). Heterosexual and female homosexual activity can also transmit amoebiasis (Salit, Khairnar, Gough & Pillai, 2009). It is hypothesized that, in pathogenic *E. histolytica* infections, resistance to invasion is determined by a relatively small number of host genes (Hamano *et al*., 2008).

2.2 Epidemiology of Amoebiasis

Entamoeba histolytica has diverse distribution and is a significant health risk all over the world where contamination of food and water is high (Raza, Iqbal, Muhammad, Ahmad & Hanif, 2013). The parasite is categorized as third leading cause of death from parasitic disease worldwide and imposes a major threat to public health throughout the world (Stanley, 2003). The prevalence of amoebiasis depends on socioeconomic conditions of the population and varies among countries (Caballero-Salcedo *et al.,* 1994). According to Jackson (2000), this parasitic disease directly affects over 50 million people leading to loss of manpower and economic damage. Poor sanitary and unhygienic conditions are responsible for amoebiasis and people living in such environments are at greater risk in developing amoebiasis (Fuchs, Ruiz-Palacios & Pickering, 1988).

2.3 Management of amoebiasis

Amoebiasis can be controlled by adopting proper sanitation and hygienic measures worldwide (Abd-Alla & Ravdin, 2002). Improvement of sanitation, clean water supplies and health education coupled with treatment reduces amoebiasis transmission in the long

term (Ngonjo, Kihara, Gicheru, Wanzala, Njenga & Mwandawiro, 2012). An analytical review of health progress, and systems performance, 1994 – 2010 by the ministry of public health and sanitation of Kenya reports that improved sanitation which includes provision of safe domestic water, availability of improved latrines and flush toilets, hand washing, contributes greatly in reduction of diarrhoeal diseases including amoebiasis (KHPF, 2010). In a study carried out to determine the prevalence of waterborne protozoan parasites in western Cameroon (Richardson, Callahan, Dondji, Tsekeng & Richardson, 2012), it was observed that due to improved sanitation in Bawa village, infections with *E. histolytica* were lower at 7.1% compared to Nloh village (15.7%) which had unimproved sanitation. In another study carried out to evaluate the risk of pathogenic intestinal infections in Kisii municipality in Kenya, Nyarango, Aloo, Kabiru and Nyanchongi, (2008), reported a prevalence of *E. histolytica* of 11.9% and associated this to poor sanitation amongst food handlers in the municipality. Combined methods of amoebiasis control are the most appropriate approach, which will obtain dramatic reduction in prevalence and intensity of infection. Improved sanitation can result in reduced water and food contamination with human excreta. Such factors as use of toilets and latrines, improved sewage system and personal hygiene if implemented, can greatly reduce infection with *E. histolytica* (AMREF, 2009).

Effective recombinant antigen based vaccines have been developed that prevent liver abscess due to amoebic infections and generate mucosal antiamoebic antibody immune response in animals but the problem is that no single product has yet been tested in human (Stanley, 2000). One of the limitations with development of amoebiasis vaccine

is that natural infection of *E. histolytica* does not elicit long term immunity as reflected by the reinfection of the recovered individuals (Haque *et al*., 2001; Blessmann & Tannich, 2002).

2.4 Diagnosis of Amoebiasis

The World Health Organization has recommended that intestinal infection be diagnosed using an *E. histolytica*–specific test (WHO/PAHO/UNESCO, 1997a). The classic stool ova and parasite examination is therefore obsolete (Petri & Singh, 1999). Laboratory diagnosis of amoebiasis is usually made microscopically (Ali *et al*., 2003; Fotedar, Stark, Beebe, Marriott, Ellis & Harkness, 2007a; Khairnar, Parija & Palaniappan, 2007) and on the basis of serological methods including ELISA, indirect haemagglutination assay and latex agglutination assay (Tanyuksel & Petri, 2003). Accurate diagnosis is not only important for the patients with dysentery but also for the 90% patients of *E. histolytica* that are asymptomatic as they may shed the infective stages of the organism particularly in developing countries with poor hygienic measures (Jackson, 2000). Correct diagnosis will enable correct and prompt treatment.

2.5 Immunology of amoebiasis

Immunity to infection with *E. histolytica* is associated with a mucosal IgA response against the carbohydrate-recognition domain of the Gal/GalNAc lectin (Haque, Huston, Hughes, Houpt & Petri, 2003). Over a one-year period, children with this response had 86% fewer new infections than children without this response (Haque *et al*., 2002). Cell-mediated responses have been described in patients with amebic liver abscess, characterized by lymphocyte proliferation and lymphokine secretion that is amebicidal

in vitro (França-Botelho *et al*., 2011). One study found that in patients with liver abscess, the prevalence of the class II MHC aplotype HLA-DR3 is increased by a factor of more than three, suggesting a role of CD4+ T-cell function in the outcome of the disease (Arellano *et al*., 1996). It is noteworthy, however, that the acquired immunodeficiency syndrome pandemic has not led to increases in invasive amebiasis, although asymptomatic intestinal colonization is undoubtedly common (Fontanet *et al*., 2000). In fact, in the murine model of amebic colitis, the depletion of CD4+ T cells decreases the severity of the disease (Houpt *et al*., 2002).

Development and application of suitable vaccines are the major constraint for the efficient control of infectious diseases. Lack of effective vaccination is one of the major hurdles for the control of amoebiasis that may prevent transmission of the parasite and or at least progression of the infected individuals into active invasive disease (Lotter & Tannich, 2006). Currently, there is no effective vaccine available against *E. histolytica* (Upcroft & Upcroft, 2001). A sum of amoeba proteins has been identified as potential vaccine candidates because of their efficacy to inhibit and prevent liver abscess formation in experimental trials in rodents (Petri & Ravdin, 1991).

2.6 Treatment of amoebiasis

The WHO/PAHO/UNESCO recommendations state that, when possible, *E. histolytica* should be differentiated from morphologically similar species and treated appropriately (Pritt & Clark, 2008). Given the small but substantial risk of invasive disease and the potential to transmit the infection to others, WHO/PAHO/UNESCO recommends treating all cases of proven *E. histolytica*, regardless of symptoms

(WHO/PAHO/UNESCO, 1997b). If *E. dispar* is the only species identified, then no treatment should be given and other causes should be sought as appropriate (WHO/PAHO/UNESCO, 1997b; Huston & Petri, 1999). In resource-poor countries, the standard but less optimal approach is to treat all patients with cysts and trophozoites identified on stool examination without additional testing for speciation (Fotedar, Stark, Beebe, Marriott, Ellis & Harkness, 2007b). This method results in vast overtreatment and may hasten the development of drug resistance in *E. histolytica* (Fotedar *et al*., 2007a).

The cornerstone of treatment for amoebiasis remains the nitroimidazole derivatives (Stanley, 2003; American Academy of Pediatrics, 2006). Amoebic colitis is treated by metronidazole, followed by a luminal agent to eradicate colonization (Wong-Baeza *et al*., 2010). Fulminant amoebic colitis, even with perforation, is managed conservatively, with the addition of antibiotics to deal with bowel flora (Blessmann & Tannich, 2002). Surgically drainage of liver abscess is generally unnecessary and should be avoided as liver abscess in amoebiasis can be treated with single dose of metronidazole without drainage (Akgun, Tacyildiz & Celik, 1999). Asymptomatic individuals with documented *E. histolytica* infection should be treated with a luminal agent to eradicate infection; this recommendation is based both on the known risk for the development of invasive disease in such patients, and the fact that individuals shedding *E. histolytica* cysts are a risk to public health (Haque *et al*., 2001). *Entamoeba dispar* infection does not require treatment but should make the physician vigilant that the individual has been exposed to contaminated food or water (Stanley, 2003).

2.7 Antiamoebic activities of medicinal plants

In recent years, there has been growing interest in alternative therapies and use of natural products, especially those derived from plants (Calixto, 2000; Rates, 2001). For people in developing countries, medicinal plants are popular because their products are safe and widely available at low cost (Rani, 2011). *In vitro* studies have shown antiamoebic activities in a range of plants including Iranian *Thymus vulgaris* (Behnia, Haghighi, Komeilizadeh, Tabaei & Abadi, 2008a), *Carica papaya* (Sarker, Begum, Mondal, Siddique & Rashid, 2010), *Adenia cordifolia* (Rani, 2011) among others. The essential oil and the hexanic extract of Iranian *Allium sativum* are effective on the trophozoites of *E*. *histolytica* species and the essential oil exhibited the greatest antiamoebic activity hence a good antiamoebic candidate for amoebiasis treatment (Behnia, Haghighi, Komeilizadeh, Tabaei & Abadi, 2008b).

In vivo antiamoebic activity of *Piper sarmentosum*t and *Quercus infectoria* nut gall using methanol extracts has been demonstrated (Sawangjaroen *et al*., 2004). According to França-Botelho *et al*., (2011), the reduction in the areas of necrosis observed in experimental amoebic infection of hamsters and rats, together with the increased death of amoebae during leukophagocytosis, suggests that melatonin plays a beneficial role in the control of amoebic lesions and indicates that, as in other diseases, melatonin is useful as an adjuvant in anti-amoebic therapy. The fruits of *Piper longum* used in traditional remedies against intestinal distress have been tested for their efficacy against experimental caecal amoebiasis of rats. The ethanolic extract, hexane fraction, n-butanol soluble fraction exerted *in vitro* amoebicidal action at 1000µg/ml and the chloroform

fraction at 500µg/ml. The ethanolic extract and piperine, a pure compound, from this plant material cured 90% and 40% of rats with caecal amoebiasis, respectively (Ghoshal, Krishna & Laksmi, 1996). Currently, no antiamoebic studies have been done using *Senna didymobotrya.*

2.8 Pharmacology and bioactivity of medicinal plants

People all over the world have used plants as medicines from time immemorial (Korir, Mutai, Kiiyukia & Bii, 2012). It is estimated by WHO that 80% of the population, majority of whom are in developing countries, still rely on plant-based medicine for primary health care (Owoabi, Omogbai & Obasuyi, 2007). Drugs are prepared from various parts of plants including leaves, stem, roots, seeds, tubers or exudates (Mukherjee, 2002; Kokwaro, 2009). Due to their composition plants have been known to possess multiple medicinal properties hence enabling them to have several uses in the pharmaceutical industry (Anthoney *et al*., 2014a). Studies on several plants have been done all over the world and plants have shown great potential in the treatment of diseases affecting both humans and animals (Anthoney, Ngule, Obey, Akumu & Ngule, 2014b). The use of medicinal plants is as old as man (Silva & Fernandes, 2010; Anthoney, Ngule & Obey, 2013a; Anthoney, Ngule, Ngule & Ramesh, 2013b). In the past few decades medicinal plants have been tested extensively and found to have several medicinal uses such as, antibacterial activity, antifungal activity, anti-diabetic activity, anticancer activity, antioxidant activity, hepatoprotective activity, haemolytic activity, anti-inflammatory activity, larvicidal activity, anthelmintic activity, central nervous system activity and pain relief activity (Sukirtha & Growther, 2012; Mir, Sawhney & Jassal, 2013). Many side effects associated with allopathic medicines and

dependencies are common reasons why many people are hospitalized today. In order to counteract the effects, many people are now turning to nature in pure form to prevent and cure diseases using natural medicinal herbs or natural health alternatives (Deshpande, 2010).

2.9 *Senna didymobotrya* and its medicinal uses

Senna didymobotrya is a widely used medicinal plant in East Africa (Nagappan, 2012). It is a native plant to tropical Africa found from Congo east to Ethiopia and extends south to Namibia, Zimbabwe and Mozambique (Tabuti, 2007). In tropical Asia and America this plant was originally introduced as green manure and cover crop (Nagappan, 2012). This East African plant tolerates drought in full sun even with very little water (Bekele-Tesemma, 2007). It is common in deciduous bush land, along lake shores, streams, rivers and other damp localities. The plant is also found in grassland and woodland, from sea-level up to 2500m altitude (Nyaberi, Onyango, Mathoko, Maina, Makobe & Mwaura, 2013). Sometimes it is found in old plantations and in hedges near buildings. In South Africa it has become invasive in grassland, woodland, roadsides, waste-land and on river banks. It tolerates light frost and is hardly attacked by disease or pests (Anthoney, Ngule & Obey, 2014a)**.**

Senna didymobotrya is locally known as "*senetwet*'' by the "Kipsigis'' of Kenya. It is used locally in the flavouring and preservation of "*mursik*'' which is the local name for fermented milk, hence, the name "*mursik*'' plant (Tabuti, 2007; Ngule, Anthoney & Obey, 2013). The plant species is also known as Popcorn *Cassia*, Peanut Butter *Senna* (Singh & Singh, 2010). It is a shrub or small tree producing golden yellow flowers,

opening from buds which are enclosed in greenish black bracts (Plate 2.1). The fruit is a flat, 9-16 seeded pod, linear-oblong 7-12 cm x 1.5-2.5 cm, glabrescent, short beaked, dehiscent or indehiscent when dry, depressed between the seeds, sutures raised, blackish-brown (Schmelzer & Gurib-Fakim, 2008).

Plate 2.1 *Senna didymobotrya* aerial plant parts adapted from www.biodiversityexplorer.org

Senna didymobotrya is a medicinal plant and the medicinal values have been explored well in many parts of the world by traditional practitioners (Nagappan, 2012). In Congo, Rwanda, Burundi, Uganda, Tanzania, root decoction of this plant has been used for the treatment of malaria, other fevers, ringworm, jaundice and intestinal worm (Nagappan, 2012). In Kenya, traditionally the "Kipsigis'' community has been using this plant to control malaria as well as diarrhea (Korir *et al*., 2012). The pastoralists of West "Pokot" peel the bark, dry the stem and burn it into charcoal that they use to preserve milk (Tabuti, 2007). In addition, it has been used to treat skin conditions of humans and

livestock infections (Njoroge & Bussmann, 2007). It is also used in the treatment of animal diseases such as removal of ticks (Njoroge & Bussmann, 2006). The root or leaf mixed with water or decoction of fresh parts has been used to treat abscess of the skeletal muscle and venereal diseases (Kamatenesi-Mugisha, 2004). The plant is also useful for the treatment of fungal, bacterial infections, hypertension, haemorrhoides, sickle cell anemia, a range of women's diseases such as inflammation of fallopian tubes, fibroids and backache, to stimulate lactation and to induce uterine contraction and abortion (Tabuti, 2007). The antibacterial activities of hexane extract against *Microsporum gypsum*, has been reported (Korir *et al*., 2012). According to Reddy, Anjana and Ruveena, (2010), presence of phenolic compounds, flavonoids and carotenoids in the ethyl acetate extract of leaves are responsible for pronounced antibacterial activities.

A decoction or infusion from the leaves, stems and roots of *S. didymobotrya* is drunk as a laxative and purgative for the treatment of abdominal pains, while in large quantities it is taken as an emetic (Singh, Singh & Sandhu, 2003). The leaf sap in water is given as a drink to treat diarrhoea, dysentery, and taken as a diuretic and emetic (Sunarno, 1997). A decoction made from the roots is used as an antidote for poisoning, to expel a retained placenta, and to treat East Coast fever and blackleg (Njoroge & Bussmann, 2007).

2.10 Chemical constituents of medicinal plants

Phytochemicals are non-nutritive plant metabolites that have protective or disease preventive properties (Doss & Anand, 2012). The plants produce these metabolites to protect themselves, however, recent research demonstrate that they can as well protect

humans and animals against diseases (Doss & Anand, 2012). A number of phytochemicals are known, some of which include: alkaloids, saponins, flavonoids, tannins, glycosides, anthraquinones, steroids and terpenoids (Venkatesh, Shanthi, Rajapandian, Elamathi, Thenmozhi & Radha, 2011; Doss & Anand, 2012). They do not only protect the plants but have enormous physiological activities in humans and animals. These include among others cancer prevention, antibacterial, antifungal, antioxidative, hormonal action and enzyme stimulation (Venkataswamy, Doss, Sukumar & Mubarack, 2010; Venkatesh *et al.*, 2011; Doss & Anand, 2012).

2.10.1 Steroids

Steroids are modified triterpenoids which are also derived from squalene by cyclisation, unsaturation and substitution. The nucleus of all steroids is the tetracyclic C_{17} hydrocarbon 1, 2 cyclopentanoperhydrophenanthrene (gonane or sterane) substituted by methyl groups at C_{10} and C_{13}, as well as alkyl side-chain at C_{17} (Ahmad, Aqil & Owais, 2006). Steroids may possess a nucleus derived from the former one by one or more C-C 39 bond scissions or ring expansion or contractions (Donald, Gary, Gorge & Randall, 2005). Plant steroids are known to be important for their cardiotonic activities, posses insecticidal and anti-microbial properties (Ngbede, Yakubu & Nyam, 2008; Anpin, Jeeva, Prakash, Johnson & Irudayaraj, 2011). The diversity of biological activities of steroids includes the development and control of the reproductive tract in humans, the moulting in insect (ecdysis) and the induction of sexual reproduction in aquatic fungi (antheridiol; Anpin *et al*., 2011). In addition steroid contributes to a varied range of therapeutic applications such as cardiotonics (digitoxin), Vitamin D precursors (ergosterol), oral contraceptive agents (some synthetic estrogens and progestins), anti-

inflammatory agents (corticosteroids) and anabolic agent (androgens; Doss & Anand, 2012).

2.10.2 Terpenoids

Terpenes are derived biosynthetically from units of isoprene, which has a molecular formula of C_5H_8 (Donald *et al*., 2005; Ahmad *et al.,* 2006). The basic molecular formulae of terpenes, then, are multiples of it, $(C5H8)_n$, where n is the number of linked isoprene units. The isoprene units may be linked together "head-to-tail" to form linear chains or they may be arranged to form rings. *Terpenes* are classified into – monoterpenes, sesquiterpenes, diterpenes, sesterpenes, triterpenes, tetraterpenes, and rubber – depending upon the total number of carbon atoms or isoprene units in the molecule. *Monoterpenes* consist of two isoprene units and have the molecular formular $C_{10}H_{16}$. They may be linear (acyclic) or may contain rings (Donald *et al*., 2005; Ahmad *et al.,* 2006). Monoterpenes include: linalol, nerol, geraniol, myrcene, ocimene, alloocimene, menthol, limonene.

Sesquiterpenes are fifteen (15) carbon compounds derived by the assembly of three (3) isoprenoid units and are found mainly in higher plants but also in several invertebrates, with a molecular formula of $C_{15}H_{24}$ (Amorim, Gil da Costa, Lopes & Bastos, 2013). Some of the natural sesquiterpenes are: farnesoic acid, methyl farnesoate, juvenile hormone III, farnesol and nerolidol. *Sesterpenes* are also derived from geranyl pyrophosphate and have 25 carbon atoms (Chadwick, Trewin, Gawthrop & Wagstaff, 2013). They were isolated from insect protective waxes and from fungal sources. They include gascardic acid, geranylfarnesol and pimeric acid. *Triterpenes* form a large group

of natural substances which include steroids and consequently sterols. They are made up of six isoprene units having 30 carbon atoms. Squalene is the immediate biological precursor of all triterpenoids (Ngbede *et al*., 2008; Anpin *et al*; 2011). The terpenoid group shows significant pharmacological activities, such as anti-viral, anti-bacterial, anti-malarial, anti-inflammatory, inhibition of cholesterol synthesis and anti-cancer activities (Mahato & Sen, 1997).

2.10.3 Anthraquinones

Anthraquinones occur in various types of plant materials and may occur as free anthraquinones, or as glycosides. Natural products have also been found to contain reduced derivatives of anthraquinones. They are oxanthrones, anthranols and anthrones and compounds formed by union of two conthrone molecules (Donald *et al*., 2005; Ahmad *et al.,* 2006).

Anthraquinones are the main active constituents in herbs often used to relieve constipation. They have an irritant or stimulating laxative effect on the large intestine (Mengs, Schuler & Marshall, 2001). Hence, a decoction or infusion from the leaves, stems and roots of *S. didymobotrya* is drunk as a laxative and purgative for the treatment of abdominal pains, while in large quantities it is taken as an emetic (Singh *et al*., 2003).

2.10.4 Tannins

Chemically, tannins are complex substances, which usually occur as mixtures of polyphenols that are difficult to separate because they do not crystallize. Complex tannins are generally considered to have arisen from simple polyphenols by

polymerization (Donald *et al*., 2005; Ahmad *et al*., 2006). They are oligomeric compounds with multiple structure units with free phenolic groups. They are soluble in water, with the exception of some high molecular weight structures, and are able to bind proteins forming insoluble or soluble tannin-protein complexes. Their astringent property makes them useful in preventing diarrhea and controlling hemorrhage due to their ability to precipitate proteins, mucus and constrict blood vessels (Kokwaro, 2009). This is the reason why traditional healers use *S. didymobotrya* root extracts to rich in tannins to treat diarrhea. In addition tannins have also shown antiparasitic effects (Bajal, 1988).

Extracts from various plant species with high tannin content are used to promote wound healing (Doss & Anand, 2012). This is achieved through encouragement of the formation of new tissues under the leathery layer formed on broken mucosal surface by the action of tannins. Tannins have been reported to exhibit antiviral, antibacterial and anti-tumor activities (Argal & Pathak, 2006; Ngbede *et al*., 2008; Doss & Anand, 2012). It has also been reported that certain tannins are able to inhibit HIV replication selectively and is also used as diuretic (Ngbede *et al*., 2008; Ogbonnia Enwuru, Onyemenem & Oyedele, 2008; Doss & Anand, 2012).

2.10.5 Saponins

Saponins are special glycosides with distinctive foaming characteristics; they form froth or foam when shaken with water. They can be said to be natural detergents found in plants because they contain both water-soluble and lipid-soluble components. They consist of a lipid-soluble nucleus, having either a steroid or - 16 - triterpenoid saponins

with one or more side chain of water-soluble carbohydrate (Donald *et al*., 2005; Ahmad *et al*., 2006). Their physiological action depends on the fact that they break up the red blood cells – haemolysis. Saponins have a bitter and acidic taste. Saponins are thought to act as resistant compounds against plant pathogens and are membrane active agents.

The properties of saponins include antioxidant effect, direct and selective cytotoxicity of cancer cell, immune-modulation, acid and neutral sterol metabolism and regulation of cell proliferation (Doss & Anand, 2012; Ngbede *et al*., 2008; Ogbonnia *et al.*, 2008). Their natural tendency to inhibit the growth of microbes may prove to be especially useful for treating those difficult to control fungal and yeast infections. Among the chemical properties of saponins, their polarity, hydrophobicity and nature of the reactive groups seem to be important determinants of their biological properties. They are known to have antibacterial, antitumour, cytotoxic, fungicidal and molluscicidal activities (Ngbede *et al*., 2008; Ogbonnia *et al.*, 2008). Saponins are used as mild detergents and in intracellular histochemistry staining to allow antibody access to intracellular proteins. In medicine, they are used in hypercholestrolaemia, hyperglycaemia, antioxidant, anti-cancer, and anti-inflammatory and weight loss. They are also known to have anti-fungal properties (Ahmad *et al.*, 2006; Ngbede *et al.*, 2008).

Saponins are used to stop bleeding, treating wounds and ulcers as they help red blood cells to precipitate and coagulate (Maobe, Gatebe, Gitu & Rotich, 2013). This can be attributed to ability of saponins to bind with glucose and cholesterol molecules.

Saponins have also been associated with inhibitory effect on inflammation (Maobe *et al*., 2013).

2.10.6 Flavonoids

Flavonoids represent a very wide group of water-soluble derivatives of the basic compound. They are polymeric compounds possessing fifteen carbon atoms, with two benzene rings joined by a linear three-carbon (3-C) chain as its basic structure; the variation is the state of oxidation of the connecting 3-C moiety, which determines the properties and class of each compound. They are generally known to be physiologically active and these include: antioxidant, antimicrobial, anticancer, vasoprotective, anti-inflammatory, anti-viral, antithrombitic and antiallergenic activities (Shirwaikar, Malini & Kumari, 2003; Donald *et al*., 2005; Ahmad *et al*., 2006; Nisar, Ali & Qaisar, 2011; Venkataswamy *et al*., 2010; Venkatesh *et al*., 2011). Flavonoids have been referred to as nature's biological response modifiers because of strong experimental evidence of their inherent ability to modify the body's reaction to allergen, virus and carcinogens (Ogbonnia *et al*., 2008).

Flavonoids are used as antioxidants because of their ability to scavenge free radicals such as peroxide and hydroperoxide of lipid hydroxyl hence inhibiting oxidation that lead to degenerative diseases (Samatha, Shyamsundarachary, Srinivas & Swamy, 2012). According to Cowan, (1999), flavonoids can be used to prevent synthesis of off flavours that are caused by fat oxidation. Flavonoids have been found to have antibacterial activity due to their ability to complex with extracellular and soluble proteins and to

complex with bacterial cell wall (Yadav & Agarwala, 2011). Flavonoids are produced by plants in response to microbial infection and studies have shown that they have antibacterial activity against a wide range of micro-organisms (Ghasemzadeh & Ghasemzadeh, 2011).

2.10.7 Glycosides

Glycosides are secondary metabolites from plant sources in which a sugar is bound to a non-carbohydrate moiety (Anthoney *et al*., 2014b). Glycosides can be considered as sugar-ethers consisting of non-sugar and a component sugar in the same molecule (Donald *et al*., 2005; Ahmad *et al.,* 2006). The sugar and the non-sugar components are known as *aglycone* and *glycone* respectively.

Cardiac glycosides have been used traditionally as arrow poisons or as heart drugs. They are used to strengthen the heart and make it function properly under controlled therapeutic dose. Cardiac glycosides bind to and inhibit Na+/K+-ATPase, which raises the level of sodium ions in cardiac myocytes, leading to an increase in the level of calcium ions and cardiac contraction force (Newman, Yang, Pawlus & Block, 2008).

2.10.8 Alkaloids

Most of the known alkaloids contain a basic nitrogen atom. The term alkaloid is commonly applied to basic nitrogenous compounds that are physiologically active. They nearly contain their nitrogen as part of a heterocyclic system and are often quite complex in structure (Ahmad *et al.,* 2006). According to Karou *et al*., (2006), much study has been done on pharmacological properties of alkaloids and proved to have antiprotozoal,

cytotoxic and anti-inflammatory properties. Alkaloids have been isolated from different plants and their medicinal values tested. The most important use of alkaloids already known with its originality from plants is the use of alkaloid compounds in the treatment of malaria. According to Ameyawn and Duker-Eshun, (2009), many of the antimalarial drugs used today are quinoline derivatives manipulated from cinchona species bark.

In addition, quinine is used in the food industry for bitter flavouring (Ngbede *et al*., 2008; Ogbonnia *et al.*, 2008). Alkaloids have been identified for their functions which include analgesic, antiplasmodic, antioxidant and antibacterial activity (Karou *et al*., 2006). According to Ayitey and Addae (1977), bitter leaves containing alkaloids are capable of reducing headache associated with hypertension.

2.10.9 Phenols

Phenols are associated with diverse functions, including nutrient uptake, protein synthesis, enzyme activity, photosynthesis; structural components and allelopathy (Wu, Haig, Prately, Lemerie & An, 2000). Phenolics show an array of health promoting benefits in human health. The phenolic compounds, have biological and pharmacological properties especially their antimicrobial activity (Anpin *et al*., 2011), antiviral, anti-inflammatory and cytotoxic activity, the antimutagenic and anticarcinogenic activities (Mungole, Awati, Chaturvedi & Zanwar, 2010). Medicinal herbs are enriched with phenolic compounds that have excellent antioxidant properties (Narayana, Reddy, Chaluvadi & Krishna, 2001).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Collection of Plant Material

The roots of *S. didymobotrya* were collected during the month of October-November, 2012 and were authenticated (Plates 3.1a and b). The plant material was taxonomically identified by a taxonomist, Mr Patrick Mutiso of University of Nairobi School of Biological Science Herbarium (Chiromo), with reference number CBRD/Sd/01/2014; the specimen was preserved and deposited at the museum for future reference.

Plate 3.1 a) Collection of *Senna didymobotrya* roots b) *Senna didymobotrya* roots (Photos by Nyamwamu L.B., 2012).

3.2 Preparation of extracts from the medicinal plant

3.2.1 Preparation of the plant material for extraction

The roots were washed, cut into small pieces and air-dried for three weeks under a shed. The dried specimens were shred using an electrical mill in readiness for solvent extractions. The sample preparation and extraction procedures were carried out as described by Harborne, (1994). Sequential extraction was carried out on the plant material with distilled water, ethyl acetate, dichloromethane, hexane and methanol as the solvent systems.

3.2.2 Aqueous Extraction

Three hundred grams (300g) of dried plant root powder were added to 600ml of distilled water in a conical flask and boiled at a temperature of 40^0C for 2h. It was then filtered using No. 1 Whatman filter paper and centrifuged at 5000rpm for 10min. After 6h, the supernatant was collected at an interval of 2h pooled together and concentrated using a rotary evaporator. The residue obtained was collected into a sample bottle and freeze dried into powder; the weight determined and stored at 4°C.

3.2.3 Hexane Extraction

Three hundred grams (300g) of dried plant root powder were taken and mixed with 600ml of hexane in a conical flask, plugged with cotton wool and then kept on a rotary shaker at 190-220rpm for 24h at 30^0C. After 24h the supernatant was collected and the solvent evaporated through a rotary evaporator; the weight determined and stored at

4°C. The process was repeated sequentially for dichloromethane, ethyl acetate and methanol.

3.3 Study design

The *in vitro* studies were carried out using a comparative experimental study design. The efficacy and toxicity of samples were compared with that of metronidazole. *In vivo* studies were carried out using a completely randomized block experimental design. BALB/c mice aged eight weeks old were randomly assigned into six groups of six each and treated using different concentrations of the root extracts, metronidazole and normal saline. The results were compared to determine the efficacy of the test samples against the known drug for treating amoebiasis.

3.4 Ethical consideration

Approval for the study was obtained from the Board of Postgraduate studies of the University of Eldoret and KEMRI ethical review committee. The experiments were done in compliance with KEMRI's Animal Care and Use Committee. Biosafety issues were addressed by autoclaving dead animals in autoclavable disposable bags before transfer to the incinerator. Further precautionary measures involved putting on protective gear and carrying out the experiments in a laminar flow hood.

3.5 Experimental animals

Eight week old (adult stage) BALB/c mice male and female were obtained from Kenya Medical Research Institute's (KEMRI) animal house facility and were infected in

accordance with KEMRI Animal Care and Use Committee approved protocols. The animals were maintained under specific pathogen free conditions.

3.6 *Entamoeba histolytica* trophozoite culture

Entamoeba histolytica HM-1: IMSS strain was used in all experiments. The parasite in culture media was obtained from the University of Boulevard, Manassas, USA. The trophozoites were cultured axenically in screw-capped tubes at 35.5^0C on LYI-S-2 medium, supplemented with 10% (v/v) heat-inactivated bovine serum (Diamond, Harlow & Cunnick, 1978). Subcultures were performed routinely at 48h intervals by replacing the medium without detaching the monolayer. Cells were harvested by replacing the medium with a fresh one, chilled on ice for 20min, and inverting gently to detach the monolayer.

3.7 *In vitro* amoebicidal activity

3.7.1 Preparation of stock solutions

One hundred miligram per milliliter of each extract was dissolved in its solvent and sterilized by passage through a 0.45µm Millipore filter and kept as stock solution. The extracts that were insoluble in water or media were first dissolved in 1% DMSO to avoid solvent carry over (Dorin, Le Roch & Sallicandro, 2001). All the prepared extracts and drugs were stored at 4°C and retrieved only during use.

3.7.2 *In vitro* exposure and evaluation procedures

A concentration range of 1mg-31.25µg (1000µg/ml, 500µg/ml, 250µg/ml, 125µg/ml, 62.5µg/ml, 31.25µg/ml) per milliliter of the plant extract were prepared in test tubes by

serial dilution. To determine the volume of stock solution that was used in the serial dilution the following formula was used:

$C_1V_1 = C_2V_2$ Where: C_1= Concentration of stock solution

V_1= Volume of stock solution

C_2= Concentration of dilute solution

V_2= Volume of dilute solution

A concentration range (250µg/ml, 125µg/ml, 62.5µg/l, 31.25µg/ml) of metronidazole (obtained as stock of 100ml of Trogyl suspension 40mg/ml) was also serially prepared using the above formula. To the test tubes LYI-S-2 medium was added. The contents of the test tubes were then prepared into a suspension by using the ultrasound bath for 30min and kept at rest for 24h at 4°C. To each of the test tubes there was subsequent addition of 1.0×10^3 trophozoites of *E. histolytica* (axenic cultivation of 1ml of media containing 10,000 trophozoites was done in 9ml of media, 33µl of media containing $1x10^3$ trophozoites were inoculated into 300µl of media and drug), followed by incubation at 35.5° C for 24h and 48h as described by Padayachee and Odhav, (2001). The negative and positive experimental controls were assigned in the following manner: I) LYI-S-2 medium + trophozoites II) LYI-S-2 medium.

After the 24h and 48h periods, the tubes were chilled for 20min and the attached trophozoites detached by gentle inversion. The number of viable cells was determined using eosin 0.01%, (Bingham, Jarroll, Meyer & Radulescu, 1979) the microscope and

the hemocytometer. The criterion for viability was assessed by the parasite motility and dye exclusion. The lowest concentration of each plant extract which completely inhibited the growth of trophozoites of *E. histolytica* was considered the minimum inhibition concentration (MIC; Padayachee & Odhav, 2001). Growth rate (GR) was defined as the difference between the number of viable protozoa counted at 0h and after 24h and 48h. The percentage of growth inhibition (%GI) was calculated using the following formula (Muelas-Serrano, Nogal, Martinez-Diaz, Escario, Martinez-Fernandez & Gomez-Barrio, 2000):

$$\% GI = 1 - \frac{GR\ Extract}{GR\ Control} \times 100$$

Where: GI = Growth inhibition;

GR= Growth rate

The experiments were performed in duplicate and repeated three times.

3.7.3 Cell cytotoxicity

The cytotoxic concentration causing 50% cell lysis and death (CC_{50}) was determined for the extracts following the method described by Kurokawa, Hozumi, Tsurita, Kadota, Namba and Shiraki, (2001). The VERO line of African green monkey kidney cells were used for the tests. VERO cells were cultured and maintained in MEM supplemented with 10% FBS. The cells were cultured at 37^0C in 5% CO_2, harvested by trypsinization, pooled in a 50ml vial. One hundred microlitre (100µl) cell suspension (1×10^5 cell/ml) was put into 2 wells of rows A-H in a 96-well micro-titer plate for each sample. The cells were incubated at 37^0C in 5% CO_2 for 24h to attach, the medium aspirated off and 150µl of the highest concentration of each of the test samples serial diluted. The experimental plates were incubated further at 37^0C for 48h. The controls used were cells with no drugs, and medium alone (no drugs and no cells). MTT reagent (10µl) was

added into each well and the cells incubated for 2-4h until a purple precipitate was clearly visible under a microscope. The medium together with MTT was aspirated off from the wells, DMSO (100µl) added and the plates shaken for 5min. The absorbance was measured for each well at 562nm using a micro-titer plate reader (Wang, Ge, Wang, Qian & Zou, 2006). Cell viability (%) was calculated at each concentration via an excel program as described by Mosmann, (1983) using the formula:

$$\% \text{ CV} = \frac{\text{Av. absorp. of duplicate drug wells-Av. absorp. of blank wells}}{\text{Av. absorp. of control wells}}$$

The cell viability percentage values were then fed into the GraFit Software Program to give the 50% inhibitory concentrations (IC_{50}).

3.8 *In vivo* amoebicidal activity assessment

3.8.1 Parasite Inoculation procedure

Eighty four BALB/c mice of both sexes were prepared for *E. histolytica* infection according to the method described by Ray and Chatterjee (1981) with a slight modification. Briefly, 24h before the commencement of the surgery, the mice (20–22g) were starved, and, in the morning and evening, the mice were pretreated orally with 0.5 ml of 25% MgSO4 in distilled water. On the next day, the mice were anesthetized by an intraperitonial injection of pentobarbital sodium 40 mg/kg. Laparotomy was performed to expose the caecum. The suspension of actively motile *E. histolytica* containing 2×10^6 trophozoites was injected directly into the caecum. The caecum was then returned into the peritoneal cavity, the abdominal muscle closed and the skin sutured. Rat pellets and drinking water were provided ad libitum. The mice were randomly selected for the treatment and control groups.

3.8.2 *In vivo* effects of the plant root crude extracts and metronidazole on amoebiasis in mice

The standard drug, metronidazole in syrup form and the extracts of *S. didymobotrya*, were suspended in distilled water. All treatments were administered daily by peroral using a feeding tube, for five consecutive days, beginning 24h after infection with *E. histolytica*. The doses that were used for the plant extracts were 1mg/kg and 500mg/kg body weight per day. The control animals were treated with normal saline 5ml/kg body weight and metronidazole 125mg/kg per day. Six animals were used for each treatment.

On the sixth day, the animals were sacrificed using chloroform and the caecum carefully examined macroscopically for lesions and the content structure (Plate 3.2a and b). The severity of infection was scored according to the method described by Neal, ranging from 0 for normal to 4 for severe structure destruction (Neal, 1951). The presence of *E. histolytica* trophozoites in the caecum was observed under an inverted light microscope. In the absence of *E. histolytica* trophozoites, a small amount of caecum content was transferred into a fresh medium and cultured for 24–48h and this was then examined for trophozoites under an inverted light microscope.

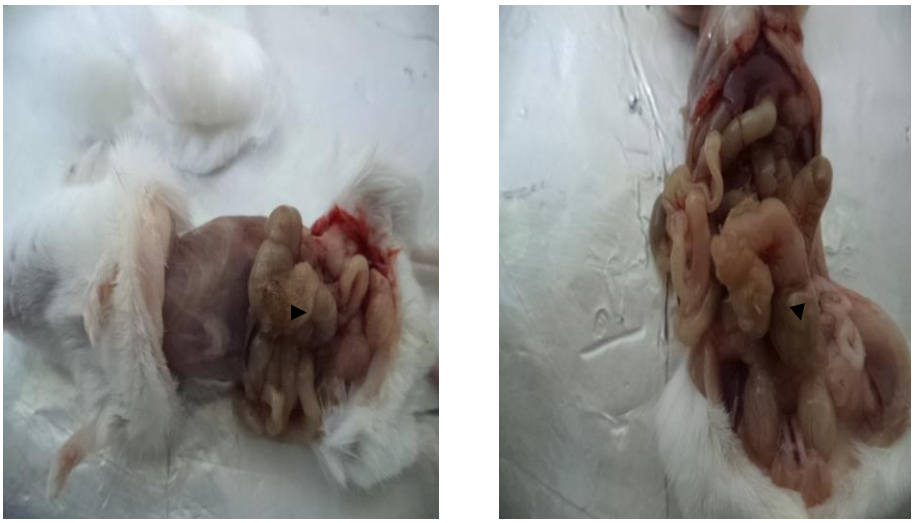

Plate 3.2. a) Caecum exposed with slimy contents b) Caecum with nodules (Photos by Nyamwamu L.B., 2013).

3.8.3 Determination of toxicity

Sixty BALB/c male and female mice were used in the study. Healthy mice weighing 20-22g were divided into two groups (control and treatment) each cage with five mice. The mice were allowed to have access to water and food except for a short fasting period of 12h before oral administration of a single dose of the test extract at a dose of between 1250-5000mg kg^{-1} body weight. In this study, three dose levels of 1250, 2500 and 5000mg kg^{-1} body weight were used. The general behavior of mice was observed continuously for 1h after the treatment and then intermittently for 4h and thereafter over a period of 24h (Twaij, Kery & Al-Khazraji, 1983). The mice were observed further for up to 14 days following treatment for any sign of restlessness and the latency of death (LD_{50}). The LD_{50} value was determined according to a method described by Miller and Tainter, (1944). The percentage deaths were determined using the formula:

$$\% \text{ Death} = \frac{\text{Number of dead mice after treatment with extract}}{\text{Total number mice per treatment in each extract}} \times 100$$

During the experiment all the dead mice were disposed according to KEMRI biosafety guidelines. After the experiment all the mice were sacrificed using chloroform and the carcasses were safely incinerated.

3.9 Phytochemical screening

Phytochemical screening was done for the extracts to identify classes of secondary metabolites that were present. Chemical tests for the screening and identification of classes of secondary metabolites present in the medicinal plant was carried out in the root extracts using the standard procedures as described by Sofowara, (1993) as follows:

3.9.1 Steroids

0.25g of the solvent extract was put in a test tube and 1.25ml of chloroform added and filtered. 1ml of the filtrate was mixed with 1ml of a mixture of acetic acid and concentrated sulphuric acid. Blue green ring indicated the presence of steroids.

3.9.2 Terpenoids

5ml of aqueous extract of each plant sample was mixed with 2ml of chloroform ($CHCl_3$) in a test tube. 3ml of concentrated sulphuric acid (H_2SO_4) was carefully added to the mixture to form a layer. An interface with a reddish brown colouration was formed if terpenoid constituents were present.

3.9.3 Anthraquinones

0.5gm sample of the extract was put in a test tube and 1ml of benzene added. The mixture was shaken and filtered. 0.5ml of ammonia solution was added to the filtrate and the mixture shaken. Presence of violet color in the ammonical phase (lower phase) indicated the presence of anthraquinones.

3.9.4 Tannins

0.125g of powdered sample of each plant was boiled in 5ml of distilled water in a test tube and filtered. 0.1% ferric chloride ($FeCl_3$**)** was added to the filtered samples and observed for brownish green or a blue black colouration which showed the presence of tannins.

3.9.5 Saponins

0.25gm of the crude extract was mixed with 0.625ml of water and vigorously shaken. The formation of stable foam indicated the presence of saponins.

3.9.6 Flavonoids

A few drops of 1% ammonia (NH_3**)** solution were added to the aqueous extract of the plant sample in a test tube. A yellow coloration was observed if flavonoid compounds were present.

3.9.7 Glycosides

Salkowsks' test was used to investigate the presense of glycosides in the root exracts: 0.25gm of extract of the plant material was mixed with 0.5ml of chloroform and 0.5ml

of concentrated sulphuric acid which were carefully added and shaken gently, then the observations were made. A red brown colour indicated the presence of steroidal ring (glycone portion of glycoside).

3.9.8 Alkaloids

50mg of powdered sample was dissolved in 2.5ml of methanol and then filtered. Then 2ml of filtrate was mixed with 5ml of 1% aqueous HCl. One milliliter of mixture was taken separately in two test tubes. Few drops of Dragendorff's reagent were added in one tube and occurrence of orange-red precipitate was taken as positive. To the second tube Mayer's reagent was added and appearance of buff-colored precipitate was taken as positive test for the presence of alkaloids.

3.9.9 Phenolic compounds

The extract (25mg) was dissolved in 0.25 ml of distilled water. To this, few drops of neutral 5% ferric chloride solution were added. A dark green colour indicated the presence of phenolic compounds.

3.10 Data Management and Statistical analysis

All experiments were done in triplicate. The mean and standard deviations were determined. All data were recorded in a laboratory notebook and exported into excel spreadsheet protected by a password. Statistical differences between mean values of experimental groups were determined by analysis of variance (ANOVA) and Student *t* test. *P* values of 0.05 or less were considered significant.

CHAPTER FOUR

RESULTS

4.1 Extraction of compounds of *Senna didymobotrya*

Three hundred grams (300g) of dried powder of *S. didymobotrya* roots yielded 1.77g of dichloromethane, 3.85g of ethyl acetate, 7.35g of methanol total, 6.23g methanol successive, 1.52g of hexane extract and 7.5g of water extract (Figure 4.1). There were some significant differences ($\rho < 0.05$; $\rho = 2.13 \times 10^{-15}$) in the yields obtained by extractions using different solvents. On the basis of yield, the best solvent was found to be water followed by methanol total. The lowest was hexane followed by dichloromethane.

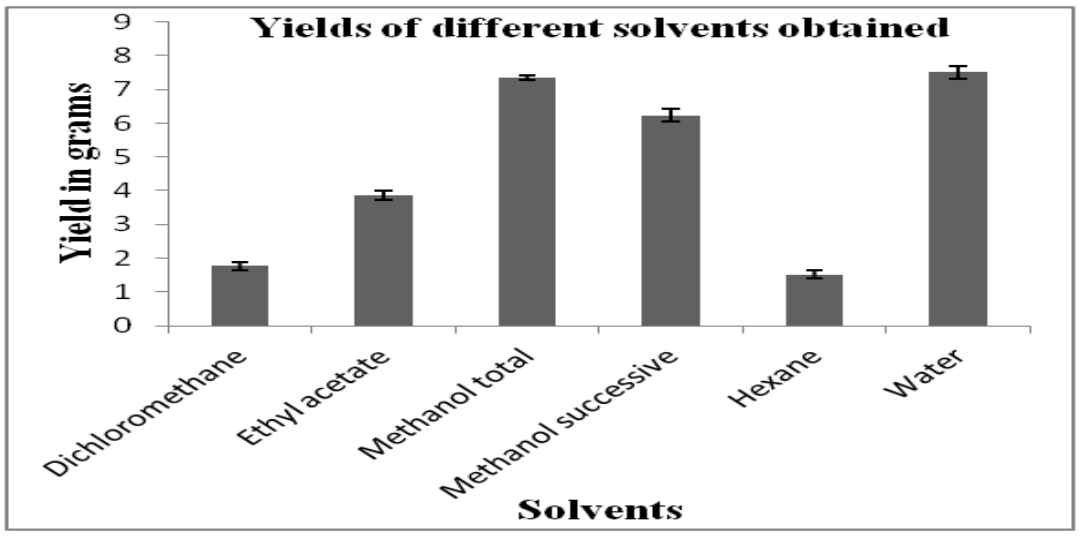

Figure 4.1. Yields of different crude extracts of *Senna didymobotrya* roots obtained using different solvents

4.2 *In vitro* antiamoebic activity of crude extracts of *Senna didymobotrya* on *Entamoeba histolytica* trophozoites

Incubation of *E. histolytica* trophozoites in the concentration range 250-1000mμg/ml of extracts caused growth inhibition. The growth rate of the trophozoites was inversely proportional to extract concentrations. However, population densities of the amoeba in the control tubes were increased on the average. The percentage growth after 24h and 48h are shown in the Figures 4.2 and 4.3. The methanol total was the most active agent against the trophozoites of *E. histolytica* at 24h and 48h period. The MIC results showed that the MIC for methanol total after 48h was 250μg/ml, whereas the MIC for metronidazole, the current drug of choice, was 62.5μg/ml after 48h, respectively (Table 4.1).

There was a significant difference in antiamoebic activity between the different types of extracts with methanol total having the highest activity ($\rho<0.05$; $\rho=5.94x10^{-6}$) and 1000μg/ml being the most active concentration for all the extracts with the exception of ethyl acetate that demonstrated inactivity and with methanol total acting at the lowest concentration. However, there was a significant difference in antiamoebic activity between ethyl acetate and the negative control ($\rho<0.05$; $\rho=0.014$) after 48h. There was also a significant difference between the different concentrations with the 1000μg/ml being the most active concentration (figure 4.3; $\rho <0.05$; $\rho = 0.013$) after 24h and 48h period. However, there was no significant difference in antiamoebic activity between methanol total and the current drug of choice metronidazole ($\rho >0.05$; $\rho=0.211$) after the 48h period.

Dichloromethane, methanol successive, hexane and water extracts were able to destroy the trophozoites of *E. histolytica*, but at higher concentrations after 48h, respectively (500µg/ml; Table 4.1). However, ethyl acetate extract was inactive even at a higher concentration (Table 4.1). The inhibition appeared to be dose-dependent. Moreover, the results revealed that a longer period of exposure to *S. didymobotrya* at the same concentration decreases the number of viable trophozoites (Figures 4.2 and 4.3).

Table 4.1 Cytotoxicity of crude root extracts of *Senna didymobotrya* and metronidazole to VERO cells

Extract	IC_{50} (µg/ml)	MIC (µg/ml)
Dichloromethane	2.15	500
Ethyl acetate	3.63	Not active
Methanol total	0.3	250
Methanol successive	0.46	500
Hexane	0.12	500
Water	0.58	500
Metronidazole	0.03	62.5

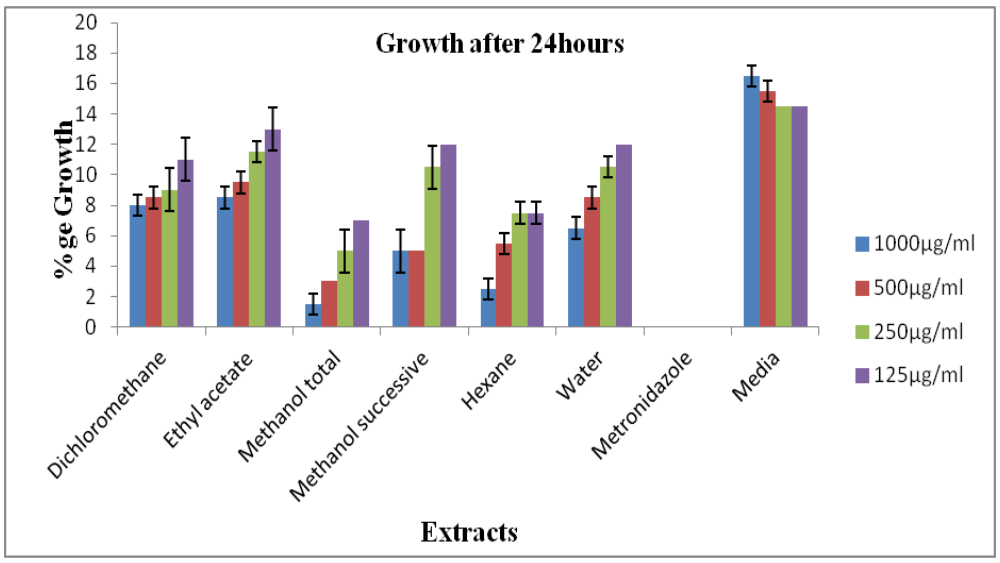

Figure 4.2. Antiamoebic activity of metronidazole and *S. didymobotrya* crude root extracts at different concentrations on *E. histolytica* trophozoites at 24h

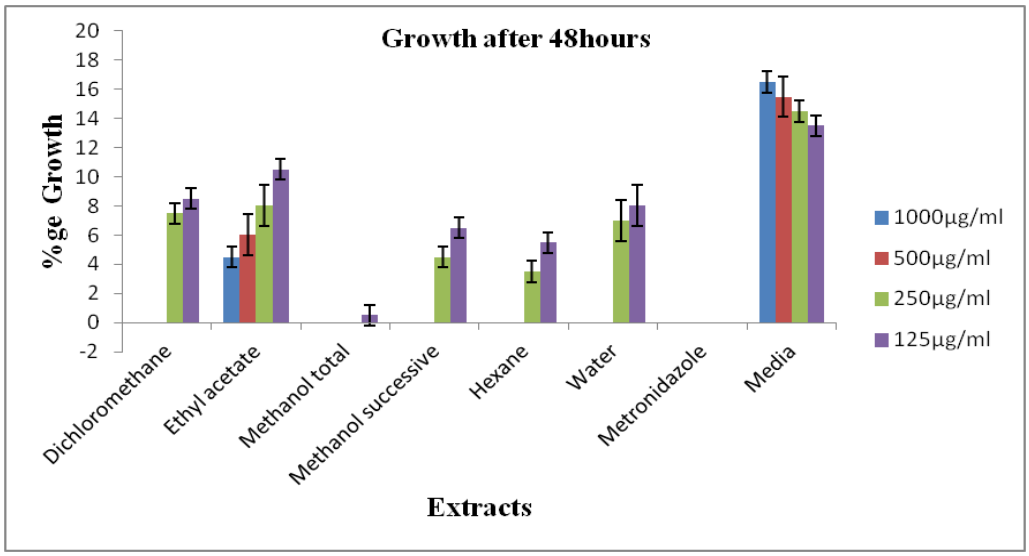

Figure 4.3. Antiamoebic activity of metronidazole and *S. didymobotrya* crude root extracts at different concentrations on *E. histolytica* trophozoites at 48h

4.3 Cytotoxicity of crude root extracts of *Senna didymobotrya*

The IC_{50} values for dichloromethane, ethyl acetate, methanol, hexane and water extracts for *S. didymobotrya* on VERO cells were significantly different ($\rho<0.01$; Table 4.1). The IC_{50} and MIC values for methanol total and hexane samples were significantly lower ($\rho<0.001$). The IC_{50} values of the cytotoxicity of the samples to VERO cells showed that the ethyl acetate extract was less toxic compared to metronidazole (Table 4.1). However, hexane and methanol total were more toxic but less toxic than metronidazole (had lower IC_{50}; Table 4.1).

4.4 Qualitative analysis of phytochemical constituents

The phytochemical characteristics of *S. didymobotrya* crude root extracts using different solvents are summarized in the Table 4.2. The results revealed the presence of medically active compounds in the plant roots. From the table, it can be seen that, tannins were present in the *S. didymobotrya* crude root extracts by all tested solvents though in higher amounts in the methanolic total crude root extract. Steroids were extracted by dichloromethane, methanol total and hexane crude root extracts. However, they were in higher amounts in dichloromethane and low amounts in hexane crude root extracts. Terpenoids were only absent from methanol total crude root extract.

Anthraquinones were moderately present in ethyl acetate and hexane crude root extracts of *S. didymobotrya* while in low amounts in dichloromethane crude root extract. However they were absent in methanol total, methanol successive and water crude root extracts. Saponins were highly present in methanol total and moderately in water crude root

extracts and in low amounts in ethyl acetate extract while they were absent in dichloromethane, methanol successive and hexane crude root extracts. Flavonoids were present in all the crude root extracts except methanol successive crude root extract. However they were highly present in water crude root extracts. Glycosides were present in all the crude root extracts except dichloromethane crude root extract. However they were highly present in methanol total crude root extract. Alkaloids were present in dichloromethane, methanol total and water crude root extracts while phenols were present in all the *S. didymobotrya* crude root extracts except ethyl acetate and water crude root extracts.

Table 4.2 Phytochemical constituents of Senna didymobotrya crude root extracts

Phytochemicals	Dichrolo-Methane	Ethyl acetate	Methanol total	Methanol successive	Hexane	Water
Steroids	+++	-	++	-	+	-
Terpenoids	++	+	-	++	+++	+++
Anthraquinones	+	++	-	-	++	-
Tannins	++	+	+++	+	+++	+
Saponins	_	+	+++	-	-	++
Flavonoids	+	+	++	-	+	+++
Glycosides	-	+	+++	+	++	++
Alkaloids	+	-	++	-	-	++
Phenols	++	-	+++	+	+++	-

Key: +++= highly or greatly present; ++ = moderately or fairly present; + = less present (trace amounts); - = Not present

4.5 *In vivo* effects of crude root extracts of *Senna didymobotrya* and metronidazole on amoebiasis in mice

The effects of crude extracts from *S. didymobotrya* roots against experimental caecal amoebiasis in mice are shown in Table 4.3. The results from the present study demonstrate that methanol total extract was more effective against *E. histolytica* in mice as evaluated by the number of mice cured and the reduction of severity of the mice caecal content and caecal wall lesions in comparison to the untreated mice (Tables 4.3). The antiamoebic effects of all extracts are clearly dose-dependent.

Table 4.3 Effects of crude root extracts of *Senna didymobotrya*, metronidazole and normal saline on caecal amoebiasis in mice

Test materials	Dose (mg/kg/day/bw)	No of mice treated (% cured)	Average caecal score (range)	
			Contents	Walls
Dichloromethane	1	(2/6) 33.3	3 (3-4)	3 (3-4)
	500	(3/6) 50	3 (3-4)	1 (1-2)
Ethyl acetate	1	(2/6) 33.3	4 (4-4)	3 (3-4)
	500	(4/6) 66.7	2 (2-3)	1 (0-1)
Methanol total	1	(5/6) 83.3	0.5(0-1)	1 (0-1)
	500	(6/6) 100	0 (0-0)	0 (0-0)
Methanol successive	1	(0/6) 0	4 (4-4)	4 (4-4)
	500	(4/6) 66.7	3 (2-3)	1 (1-2)
Hexane	1	(4/6) 66.7	3 (2-3)	1 (0-1)
	500	(5/6) 83.3	0.5 (0-1)	0.5 (0-1)
Water	1	(5/6) 83.3	0.5 (0-1)	0.5 (0-1)
	500	(5/6) 83.3	0.5 (0-1)	0.5 (0-1)
Metronidazole	125	(6/6) 100	0 (0-0)	0 (0-0)
Normal saline	5	(0/6) 0	4 (4-4)	4 (4-4)

Caecal scores were graded upon the following criteria (Neal, 1951). Wall: normal, 0; slight thickening, 1; slight contraction, 2; extensive contraction, 3; shapeless caecum, 4. Contents: normal, 0; slightly less solid, 1; slightly mucoid, 2; mucoid, 3; no solid matter, 4.

There was a significant difference between the effects of the different extracts as well as the different concentrations ($\rho=4.86x10^{-6}$; $\rho=0.021$; $p<0.05$; Table 4.4). Methanol total extract had a negative result for *E. histolytica* trophozoites and compared favorably well with the current drug of choice metronidazole at a concentration of 500mg/kg body weight (Table 4.4), whereas dichloromethane, methanol successive, hexane and water extracts had trophozoite levels ranging from moderate to low respectively. The activity

of the *S. didymobotrya* crude root extracts is dose dependent however; ethyl acetate crude root extract was inactive even at a higher concentration by having high to very high trophozoite levels (Table 4.4).

Table 4.4 Trophozoite levels after treatment of mice with metronidazole, *Senna didymobotrya* crude root extracts and normal saline

Extract	Dose (mg/kg)	Trophozoite levels
Dichloromethane		+++
	500	++
Ethyl acetate	1	++++
	500	+++
Methanol total	1	+
	500	-
Methanol successive	1	++++
	500	++
Hexane	1	+++
	500	+
Water	1	+++
	500	++
Metronidazole	125	-
Normal saline	5	++++

Trophozoite levels were graded upon the following criteria:-, Absent; +, Low; ++, Moderate; +++, High; ++++, Very high.

4.6 Toxicity studies

During the 24h period dichloromethane crude root extract of *S. didymobotrya* had a LD_{50} of 4433mg/kg and killed 60% of mice at a dose of 5000mg/kg and 20% at a dose of 2500mg/kg, ethyl acetate crude root extract of *S. didymobotrya* had a LD_{50} of 4092mg/kg and killed 60% of mice at a dose of 5000mg/kg and 40% at a dose of

2500mg/kg, methanol total crude root extract of *S. didymobotrya* had a LD_{50} of 2342mg/kg and killed 80% of mice at a dose of 5000mg/kg, 60% at a dose of 2500mg/kg and 20% at a dose of 1250mg/kg (Table 4.5). Methanol successive crude root extract of *S. didymobotrya* had a LD_{50} of 3892mg/kg and killed 60% of mice at a dose of 5000mg/kg, 40% at a dose of 2500mg/kg and 20% at a dose of 1250mg/kg, hexane and water crude root extracts of *S. didymobotrya* had a LD_{50} of 5329mg/kg and killed 40% of mice at a dose of 5000mg/kg and 20% at a dose of 2500mg/kg.

During the 14 days period dichloromethane and methanol total crude root extracts of *S. didymobotrya* had a LD_{50} of 1927mg/kg and killed 80% of mice at a dose of 5000mg/kg body weight, 60% at a dose of 2500mg/kg and 40% at a dose 1250mg/kg (Table 4.6). Ethyl acetate crude root extract had a LD_{50} of 3892mg/kg and killed 60% of mice at a dose of 5000mg/kg body weight, 40% at a dose of 2500mg/kg and 20% at a dose of 1250mg/kg. Methanol successive and hexane crude root extracts of *S. didymobotrya* had a LD_{50} of 4092mg/kg and killed 60% of mice at a dose of 5000mg/kg body weight and 40% at a dose of 2500mg/kg, while water crude root extract of *S. didymobotrya* had a LD_{50} of 4433mg/kg and killed 60% of mice at a dose of 5000mg/kg and 40% at a dose of 2500mg/kg.

Table 4.5 Toxicity of *Senna didymobotrya* crude root extracts in BALB/c mice after 24h

	Mortality after 24h						
	5000 mg/kg		2500 mg/kg		1250 mg/kg		
Test extracts	Deaths	% Deaths	Deaths	% Deaths	Deaths	% Deaths	LD50
Dichloro-Methane	3/5	60	1/5	20	0/5	0	4433
Ethyl Acetate	3/5	60	2/5	40	0/5	0	4092
Methanol Total	4/5	80	3/5	60	1/5	20	2342
Methanol successive	3/5	60	2/5	40	1/5	20	3892
Hexane	2/5	40	1/5	20	0/5	0	5329
Water	2/5	40	1/5	20	0/5	0	5329

Table 4.6 Toxicity of *Senna didymobotrya* crude root extracts in BALB/c mice after 14 days

	Mortality after 14 days						
	5000 mg/kg		2500 mg/kg		1250 mg/kg		
Test extracts	Deaths	% Deaths	Deaths	% Deaths	Deaths	% Deaths	LD50
Dichloro-Methane	4/5	80	3/5	60	2/5	40	1927
Ethyl Acetate	3/5	60	2/5	40	1/5	20	3892
Methanol Total	4/5	80	3/5	60	2/5	40	1927
Methanol successive	3/5	60	2/5	40	0/5	0	4092
Hexane	3/5	60	2/5	40	0/5	0	4092
Water	3/5	60	1/5	20	0/5	0	4433

CHAPTER FIVE

DISCUSSION

5.1 Extraction of compounds of *Senna didymobotrya*

The aqueous extraction produced the highest yields compared to the other solvents. This is because water is more polar compared to the organic solvents hence it is able to extract more compounds from a plant material (Behnia *et al*., 2008b). This is in agreement with the study by Kigondu, (2007), who obtained similar results. Water extract produced the highest yields as compared to the organic solvents. However, there was no significant difference between water and methanol total crude root extract (ρ=0.067; ρ>0.05). When the organic solvents were compared, methanol total produced the highest yields compared to the other organic solvents. This concurs with the study carried out by Korir *et al*., (2012). This activity could be explained by the ability of methanol to extract both polar and non-polar compounds (Korir *et al*., 2012).

5.2 *In vitro* antiamoebic activity of crude root extracts of *Senna didymobotrya* on *Entamoeba histolytica* trophozoites

In this study, methanol total extract demonstrated the highest antiamoebic activity with the lowest MIC (250µg/ml). The extract showed 100% lysis of trophozoites of *E. histolytica* at a concentration of 250µg/ml. On the other hand, ethyl acetate extract did not exhibit any antiamoebic activity. Analysis of the crude root extract demonstrated that ethanol total crude root extract was rich in tannins, saponins, glycosides, phenols and moderate in flavonoids and alkaloids. These metabolites have been shown to inhibit the growth of *E. histolytica* trophozoites. Previous studies have shown that tannins

interfere with energy generation by uncoupling oxidative phosphorylation (Mute, 2009; Sharma, Sharma, Singh, Sutar & Singh, 2010; Sutar, Garai, Sharma & Sharma, 2010) while saponins cause changes in membrane permeability and pore formation (Tiwari, Kumar, Kumar, Kaur, Debnath & Sharma, 2011).

According to Roongruangchai, Kummalue, Sookkua and Roongruangchai, (2010), phenolic compounds cause damage to the plasma membrane, which results in leakage of intracellular constituents from the cell. In addition phenolic compounds act as oxidizing agents causing cell membrane damage by reacting with cellular proteins, lipids, nucleic acids and carbohydrates. They also interfere with the glycoprotein of the cell surface of parasites and cause death (John, Mehta, Shukla & Mehta, 2009). Flavonoids have been shown to inhibit the growth of *E. histolytica* trophozoites *in vitro* (Ardalan, Mushref & Ahmed, 2011) while alkaloids have shown to possess antiprotozoal properties.

The dichloromethane crude root extract which was rich in steroids showed less antiamoebic activity as steroids have been shown to stimulate proliferation of *E. histolytica* trophozoites and therefore provoke amebic activity (Escobedo, Homedes, Aldana, Alt, Serrano & Garcia, 2003). The antiamoebic activity of *S. didymobotrya* crude root extracts of dichloromethane, methanol successive, hexane and water was lower than that of methanol total although higher than that of the ethyl acetate extract which showed no activity against *E. histolytica* trophozoites. This corroborates with the study carried out by Rani, (2011), whereby the highest activity was found in methanol extracts. It is therefore possible that the active components in *S. didymobotrya* have high

polarity since the highly polar solvent (methanol) seems to extract compounds with activity against *E. histolytica* parasites as suggested in an earlier study (Kigondu, 2007).

5.3 Cytotoxicity of crude root extracts of *Senna didymobotrya*

The IC_{50} values of the cytotoxicity of *S. didymobotrya* extracts to VERO cells showed that ethyl acetate was significantly less toxic compared to metronidazole. However, hexane and methanol total were significantly more toxic but less toxic than metronidazole the current drug of choice for amoebiasis. In similar results the positive control that contained metronidazole had an IC_{50} of 0.0846µg/ml for *E. histolytica* while the methanol extract had an IC_{50} of 203.9µg/ml for VERO cells (Leos-Rivas *et al*., 2010). However, it is worth noting that methanol total crude root extract was rich in tannins, phenols and saponins. Tannins, phenols and saponins have been shown to possess antiproliferative activity in VERO cells (Njagi *et al*., 2016).

5.4 Qualitative analysis of phytochemical constituents

From the study results the roots of *S. didymobotrya* were found to contain steroids, terpenoids, anthraquinones, tannins, saponins, glycosides, flavonoids, alkaloids and phenols. This is in agreement with the study carried out by Kitonde, Fidahusein, Lukhoba and Jumba, (2014) on the roots of *S. didymobotrya* that demonstrated the presence of sapogenins, terpenoids, quinones and flavonoids. This is also in conformity with a study carried out on the bark of S. *didymobotrya* by Korir *et al*., (2012), which was found to contain terpenoids, anthraquinones, flavonoids and alkaloids.

5.5 *In vivo* effects of crude root extracts of *Senna didymobotrya* and metronidazole on amoebiasis in mice

This study shows that the selected medicinal plant extracts reduced the severity of caecal infection by *E. histolytica*. The pooled controls of 6 mice were all positive for amoebae at the time of sacrifice (Table 4.4). This amoebic infection generally produced high caecal content and caecal wall score of 4 (Table 4.4). This is an indication of the high virulence of the strain of *E. histolytica* used in this study.

In the present study, mice treated with metronidazole at a concentration of 125mg/kg body weight per day for 5 days were successfully cured from amoebiasis, confirming that this strain of *E. histolytica* was still sensitive to this drug (Table 4.4). The results on efficacy of metronidazole were similar to the studies of several investigators whose studies on caecal amoebiasis were performed, both in rats and mice models (Bhopale, Pradhan, Masani & Kaul, 1995; Sohni, Kaimal & Bhatt, 1995; Ghoshal, Krishna & Laksmi, 1996).

The *S. didymobotrya* methanol total crude root extract that was rich in tannins, saponins, glycosides, phenols and moderate in flavonoids and alkaloids was the most effective at a dose concentration of 500mg/kg body weight per day after 5 days, as this dose cleared all *E. histolytica* from the intestine of mice on the day of examination. This is comparable to metronidazole at a dose 125mg/kg body weight per day.

Tannins have an astringent property that makes them useful in preventing diarrhea and controlling hemorrhage due to their ability to precipitate proteins, mucus and constrict

blood vessels (Kokwaro, 2009). This is the reason why traditional healers use *S. didymobotrya* root extracts rich in tannins to treat diarrhea. In addition tannins have also shown antiparasitic effects (Bajal, 1988). Many extracts with high tannin content are used to promote wound healing (Doss & Anand, 2012). This is achieved through encouragement of the formation of new tissues under the leathery layer formed on broken mucosal surface by the action of tannins. Tannins have been shown to exhibit high inhibitory growth of trophozoites of *E. histolytica* (Segura, Morales-Ramos, Verde-Star & Guerra, 1990). This conforms with this study whereby the methanol total extract that was highly rich in tannins was able to suppress the growth of *E. histolytica* trophozoites and thus having normal caecal wall and contents. Tannins have also been shown to interfere with energy generation by uncoupling oxidative phosphorylation (Mute, 2009; Sharma, *et al*., 2010; Sutar *et al*., 2010).

The main actions of saponins are changes in membrane permeability and pore formation (Tiwari, *et al*., 2011). Saponins have been known to affect permeability of cell membranes of parasites and cause vacuolation and disintegration of teguments (Melzig, Bader & Loose, 2001; Wang, Han, Zhao, Jiang, Liu & Liu, 2010). In the present study, saponins were highly present in the methanol total and hexane crude root extracts leading to normal caecal wall and content due to their ability to cause red blood cell coagulation and help in blood clotting, treating wounds and enteric ulcers (Foster & Duke, 1990).

Methanol total extract with a lot of glycosides caused marked and local thickening and contraction thus preventing invasion of *E. histolytica* trophozoites in 1mg/kg body weight per day. This could be as a result of glycosides which have been shown to bind to and inhibit Na+/K+-ATPase, which raises the level of sodium ions, leading to an increase in the level of calcium ions and contraction force (Newman *et al*., 2008).

In this study, methanol total and hexane crude root extracts that were rich in phenols were able to cure 100% and 83.3% of mice at a concentration of 500mg/kg body weight per day with caecal wall and content ranging between normal to slight thickening respectively. Phenols have been shown to exhibit antiamoebic activity against trophozoites of *E. histolytica in vitro* (Degerli & Tepe, 2015). According to Roongruangchai *et al*., (2010), phenolic compounds cause damage to the plasma membrane, which results in a leakage of intracellular constituents from the cell. Plant phenolics are also known to interfere with energy generation mechanism by uncoupling oxidative phosphorylation and also interfere with the glycoprotein of the cell surface of parasites and cause death (John *et al*., 2009).

Although treatment with the extract from methanol total at a concentration of less than 500mg/kg body weight per day did not cure all animals, the caecal content and caecal wall of these mice appeared normal indicating the effectiveness of the extract against the parasites. The use of this extract to treat amoebiasis may at least help in reducing severity that occurred in the intestine.

This study shows that the effect of methanol total on *E. histolytica* are consistent with those previously reported that the methanol extracts from *Piper sarmentosum* root and *Quercus infectoria* nut gall were effective against caecal amoebiasis in mice at a concentration of 1000mg/ml per day (Sawangjaroen *et al*., 2004*),* ethanol extract of *Piper longum* fruit at a concentration of 1000mg/ml per day can cure 90% of rats infected with *E. histolytica* (Ghoshal *et al*., 1996).

Terpenoids were present in relatively high quantities in hexane and water crude root extracts of *S. didymobotrya* that cured 83.3% of mice with caecal wall and content ranging from normal to slightly thickening. This finding is consistent with the study carried out by Lakshmi and Ghoshal, (2015), whereby terpenoids isolated from *Psedoplenaura wagenaari* have been shown to exhibit antiamoebic activity *in vitro.*

Water crude root extract that was rich in flavonoids inhibited the growth of *E. histolytica* trophozoites and this could be attributed to the inhibitory effects of flavonoids *in vitro* (Ardalan, *et al*., 2011). Flavonoids have been known to inhibit enzymes of glycolysis and glycogenolysis and disturb the calcium ions homeostasis and nitric oxide activity in parasites (Stepek, Buttle, Duce & Behnke, 2006). Alkaloids that were moderately present in methanol total and water crude root extracts have been shown to possess antiprotozoal properties.

Anthraquinones, however, absent from methanol total crude root extract but moderately present in ethyl acetate and hexane crude root extracts have been shown to possess

antiprotozoal activity (Moo-Puc, Mena-Rejon, Quijano & Cedillo-Rivera, 2007; Loonjang, Duangjinda, Phongpaichit, Sawangjaroen, Rattanaburi & Mahabusarakam, 2015). However, the findings from this study were contrary to those reported as ethyl acetate crude root extract exhibited no antiamoebic activity.

Steroids have been shown to stimulate proliferation of *E. histolytica* trophozoites and therefore provoke amebic activity and cause colitis (Escobedo *et al*., 2003). The study results concur with the findings as the dichloromethane crude root extract which was rich in steroids had mice with shapeless caecum, extensive ulceration and abscess formation and caecal wall and contents ranging from 3 to 4 respectively.

5.6 Toxicity studies

Crude root extracts of *S. didymobotrya* were found to be toxic to animal cells. They were considered safe when the death of mice was less than 50% which was observed at a lower dose of less than 5000mg/kg body weight except methanol total and dichloromethane whose safety were below 2500mg/kg. It was found out that the water crude root extract of *S. didymobotrya* (LD_{50} of 4433mg/kg) was less toxic and this is in agreement with a study carried out by Muthaura *et al*. (2007), who found out that the water extract was less toxic compared to dichloromethane extract. *Senna didymobotrya* is widely used among the "Kipsigis'' community in treating and managing skin and diarrhea infections. However, the method of preparation such as burning into ashes and then mixing with margarine for skin conditions and mixing with hot water and milk for stomach problems may be a way of reducing toxicity (Korir *et al*., 2012).

CHAPTER SIX

CONLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

i) The methanolic total extract proved to be a good antiamoebic candidate for amoebiasis treatment *in vitro* and *in vivo* and inhibition of growth of *E. histolytica* is dose-dependent.

ii) The IC_{50} values of the cytotoxicity of the samples to VERO cells showed that the ethyl acetate extract was less toxic compared to metronidazole. However, hexane and methanol total were more toxic (had lower IC_{50}) but less toxic than metronidazole.

iii) From the study the roots of *S. didymobotrya* were found to contain terpenoids, saponins, tannins, glycosides, alkaloids and phenols which have antiamoebic activity against *E. histolytica.*

iv) The root extracts of *S. didymobotrya* have toxic effects in animal models at a dose of 5000mg/kg body weight except methanol total and dichloromethane that had a toxicity of less than 2500mg/kg.

6.2 Recommendations

a) The methanolic total crude root extract proved to be a good antiamoebic candidate for amoebiasis treatment both *in vitro* and *in vivo*, however, some *in vivo* studies with regards to its effects on human need to be investigated;

b) Further investigations are necessary to isolate and identify the bioactive compounds in this extract on *E. histolytica* and to determine whether the alteration of enzyme activity is its mode of action.

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APPENDICES

Appendix I Approval Letter

Kituyi Christine

P.O. BOX 6200-00200,

Nairobi, Kenya.

7/4/2015.

TO: WHOM IT MAY CONERN

RE: *IN VITRO* AND *IN VIVO* ANTIAMOEBIC ACTIVITY OF *Senna didymobotrya Irwin* (Fresen) CRUDE ROOT EXTRACTS AGAINST *Entamoeba histolytica*

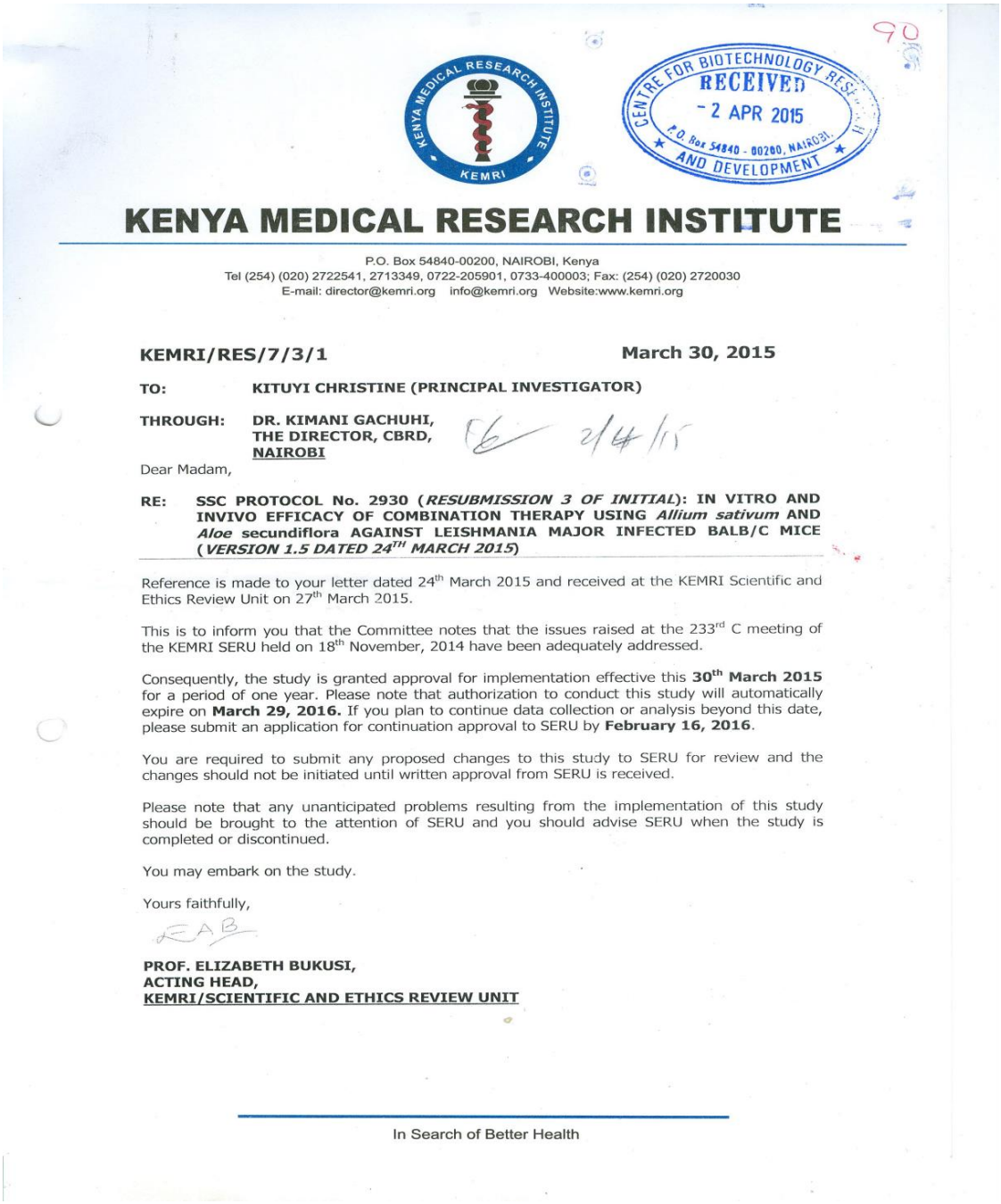
The above named project is a nested proposal from the main proposal titled *In vitro* and *in vivo* efficacy of combination therapy using *Allium sativum* and *Aloe secundiflora* against *Leishmania major* infected Balb/c mice which had been approved with reference number KEMRI/RES/7/3/1 dated 30th March, 2015.

Yours faithfully,

Christine Kituyi

Principal Investigator

Appendix II KEMRI Ethical Review Committee Research Approval



KENYA MEDICAL RESEARCH INSTITUTE

P.O. Box 54840-00200, NAIROBI, Kenya
Tel (254) (020) 2722541, 2713349, 0722-205901, 0733-400003; Fax: (254) (020) 2720030
E-mail: director@kemri.org info@kemri.org Website:www.kemri.org

KEMRI/RES/7/3/1 **March 30, 2015**

TO: **KITUYI CHRISTINE (PRINCIPAL INVESTIGATOR)**

THROUGH: **DR. KIMANI GACHUHI, THE DIRECTOR, CBRD, NAIROBI**

Dear Madam,

RE: **SSC PROTOCOL No. 2930 (*RESUBMISSION 3 OF INITIAL*): IN VITRO AND INVIVO EFFICACY OF COMBINATION THERAPY USING *Allium sativum* AND *Aloe* secundiflora AGAINST LEISHMANIA MAJOR INFECTED BALB/C MICE (*VERSION 1.5 DATED 24TH MARCH 2015*)**

Reference is made to your letter dated 24th March 2015 and received at the KEMRI Scientific and Ethics Review Unit on 27th March 2015.

This is to inform you that the Committee notes that the issues raised at the 233rd C meeting of the KEMRI SERU held on 18th November, 2014 have been adequately addressed.

Consequently, the study is granted approval for implementation effective this **30th March 2015** for a period of one year. Please note that authorization to conduct this study will automatically expire on **March 29, 2016.** If you plan to continue data collection or analysis beyond this date, please submit an application for continuation approval to SERU by **February 16, 2016**.

You are required to submit any proposed changes to this study to SERU for review and the changes should not be initiated until written approval from SERU is received.

Please note that any unanticipated problems resulting from the implementation of this study should be brought to the attention of SERU and you should advise SERU when the study is completed or discontinued.

You may embark on the study.

Yours faithfully,

PROF. ELIZABETH BUKUSI,
ACTING HEAD,
KEMRI/SCIENTIFIC AND ETHICS REVIEW UNIT

Appendix III KEMRI Animal Care and Use Committee Approval

KENYA MEDICAL RESEARCH INSTITUTE

Centre for Virus Research, P.O.Box 54628 - 00200 NAIROBI - Kenya
Tel: (254) (020) 2722541, 254 02 2713349, 0722-205901, 0733-400003 Fax (254) (020) 2726115
Email: cvr@kemri.org

KEMRI/ACUC/ 03.03.15 **24th March, 2015**

Kituyi Christine
P. O. Box 62000-00200
Nairobi, Kenya.

Kituyi,

RE: Animal use approval for SSC 2930 (Revised) – "In vitro and in vivo efficacy of combination therapy using *allium sativum* and *aloe secundiflora* against leishmania major infected balb/c mice" protocol

The KEMRI ACUC committee acknowledges the resubmission of the above mentioned protocol. It has been confirmed that all the issues raised earlier have been addressed appropriately.

The committee grants you the approval to use laboratory mice in your study but recommends that you proceed after obtaining all the other necessary approvals that may be required.

Approval is granted for a period of one year starting from when the final ethical approval will be obtained. The committee expects you to adhere to all the animal handling procedures as described in the protocol.

The committee wishes you all the best in your work.

Yours sincerely,

Dr. Konongoi Limbaso
Chairperson KEMRI ACUC

KENYA MEDICAL RESEARCH INSTITUTE
★ 24 MAR 2015 ★
ANIMAL CARE AND USE COMMITTEE
Signature: .....