IN VITRO ANTIMICROBIAL, CYTOTOXICITY, AND ANTI-PROLIFERATIVE PROPERTIES OF SELECTED MEDICINAL PLANT EXTRACTS USED IN MANAGEMENT OF AIDS-RELATED OPPORTUNISTIC INFECTIONS IN THE LAKE VICTORIA BASIN OF KENYA

BY

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DECLARATION

I declare that this thesis is my own original work and has not been presented in any university for the award of a degree.

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DEDICATION

This work is sincerely dedicated to my mum Virginia Kinuthia, wife Ann Njoki and my son Andrew Mungai. I appreciated their perseverance, encouragement and emotional support in my academic aspiration.

ABSTRACT

The current management of HIV and its related opportunistic infections involves use of highly active antiretroviral therapy (HAART), however this regimen is characterized by challenges such as toxicity, emergence of drug resistance, mutations, and cost implications because the supply of HAART is donor driven. The quest for alternative medicine has been in progress and crude extracts made from plants obtained from different ecological zones have been explored for their antimicrobial activities, cytotoxicity, and anti-proliferative properties. The chemotherapeutic components of plant preparations in Lake Victoria Basin (LVB) of Kenya has not been fully determined. This study determined the potential antimicrobial properties, cytotoxicity, and anti-proliferative effects of *Piptadeniastrum africanum* (bark), *Chaemacrista nigricans* (leaves), *Kigelia africana* (fruit), and C*entella asiatica* (leaves) extracts used in the management of HIV and AIDS opportunistic infections in LVB. These plants were identified through ethno botanical surveys in Luo-Suba in the LVB. In an *in vitro* experimental laboratory study design, cultures of *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*, were used to investigate the antimicrobial properties of the plant extracts using the susceptibility tests. Mammalian leukemia cell lines (CCRF-CEM) were also cultured and nourished with complete RPMI 1640 media for five days. Aliquots of $1x10^3$ of the cell lines were seeded in 96-well plate and subjected to varying concentrations of the plant extracts to investigate the cytotoxicity and antiproliferative activity on plant extracts on the cells. The cytotoxicity level was determined microscopically for the presence of morphological changes and chemically using 3-(4, 5-Dimethylthiaazol-2-yl)-2, 5-Diphenyltetrazolium Bromide (MTT) assay. Cell viability and MTT assays were used to determine the antiproliferative effects of the plant extracts. Pearson correlation and chi-square test were used to determine the relationship between the plant extract concentrations and the resistance level expressed by the test microorganisms in the antimicrobial susceptibility tests. Nonlinear regression analysis was used to compare the cytotoxicity level of the plant extracts through determination of IC_{50} values. The plant concentrations ≥500mg/mL had antibacterial effect on *S. aureus* (df=3, p=0.019,), *E. coli* (df=3, p=<0.017) and *C. albicans* (df=3, p=<0.017). However, *K. africana* caused toxicity to CCRF-CEM leukemia cells at a concentration of 1096mg/mL. The plant extracts showed antiproliferative effect on CCRF-CEM cells within 72 hours treatment period. The overall findings show that high concentrations of the plant extracts are required in management of HIV and AIDS-related opportunistic infections caused by bacteria and fungi. However, the high concentrations of the plant extracts are not likely to cause toxicity to the cells. Therefore, this study provides crucial information toward clinical application of the traditional uses of these selected plants in the management of HIV and AIDS-related infections.

TABLE OF CONTENTS

ABBREVIATIONS AND ACRONYMS

AIDS	Acquired Immuno-Deficiency Syndrome
CLSI	Clinical and Laboratory Standards Institute
IC_{50}	Half Maximal Inhibitory Concentration
CCRF-CEM	Human CD4+ T-cell line
CD4+	Cluster of Differentiation 4
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic Acid
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
HCMV	Human Cytomegalovrius
HSV	Herpes Simplex Virus
MIC	Minimum Inhibitory Concentration
MBC	Minimum Bactericidal Concentration
SIV	Simian Immunodeficiency Virus
THP	Traditional Health Practitioner
TM	Traditional Medicine
WHO	World Health Organization
MH	Mueller-Hinton
MTT	[3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide]
RPMI	Roswell Park Memorial Institute
FBS	Fetal Bovine Serum
df	degree of freedom

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

1.1. Background Information

Globally, 35.0 million people live with the HIV infection (WHO, 2013). The mortality rate of people dying of AIDS-related illnesses is approximately 1.5 million (UNAIDS, 2016). There are approximately 119 countries in the world that have programs and facilities of preventing the spread of HIV (UNAIDS, 2007). The impoverishment of the sub-Saharan African population, women's economic dependence, deteriorating educational systems, and overloading of health services have contributed dramatically to the spread of HIV (Van de Perre, 1995; Kilmarx, 2009). In Kenya, surveys by Ministry of Health show that high prevalence of HIV infection is along the Lake Victoria Basin with ranges between 14.7% and 25.7% (Ministry of Health, 2013). The Kenyan government in collaboration with Centers for Disease Control and Prevention are in the process of improving the quality of life of HIV-infected individuals along the LVB and also managing the HIV-related diseases. Of the conventional methods used in management of HIV and AIDS and its related illnesses, the use of traditional medicine remains the least understood (Shih *et al.*, 2011; Opio *et al.*, 2013).

HIV is associated with depletion of primary immune cells that include CD4+ T cells, macrophages and Natural Killer (NK) cells, which are critical to the normal functioning of the human immune system that defends the body against all types of illnesses (Eggena *et al.*, 2005). Many approaches are sought by patients to control symptoms of HIV and AIDS, which are escalated by bacteria such as *Staphylococcus aureus* and *Escherichia coli,* and fungi such as *Candida albicans*. The *S. aureus* is frequently found in HIV patients causing endocarditis, pneumonia, cutaneous and subcutaneous infections (Nguyen *et al.*, 1999). *E. coli* which is an

Enterobacteriaceae is a possible cause of severe HIV-related respiratory disease such as Pneumocystis pneumonia. The bacterial pneumonia predominates due to low levels of host immunity, neutropenia, and prolonged use of immune modulators. Candidiasis is a skin infection with *Candida albicans.* The mucocutaneous candidiasis occurs in oropharyngeal, esophageal, and vulvovaginal diseases in HIV and AIDS patients. Presence of oropharyngeal candididasis is an indication of progression of HIV disease (Klein *et al.,* 1984). .

The use of oral and topical Highly Active Anti-Retroviral Therapy (HAART) is the is the primary medication for HIV prevention and has extensively improved the quality of life among the people living with HIV and AIDS (Mayer and Ramjee, 2015). Nonetheless, the HAART treatment faces some challenges to successful clinical outcomes such as lack of adherence to dosage formulation (Al-Dakkak *et al.,* 2013), which result in development of resistance (Bulteel *et al.*, 2014; Shephard, 2015). Other challenges include high cost of HAART in the absence of global support from developed country partners, development of resistance to CD4+ cells due to over-expression of P-glycoprotein, hence the need for alternative medicine. P-glycoprotein is a pump mechanism found on CD4+ cells that pumps out foreign bodies from the cell; HAART is not exempted (Aduma *et al.*, 1995).

It is noted that about 80% of the world uses natural products or their extracts as sources of drugs (Lahlou, 2013). The major plant species that has been in use in the management of HIV and AIDS and its opportunistic infections is *Ancistrocladus korupensis* that is wild in Korup forest in Cameroon but due to its overuse it has almost extict (Manfredi *et al.*, 1991). Many traditional healers (TH) in West Africa and in the LVB are in a continuous process of identifying medicinal plants that are useful in the management of gonorrhea, coughing, syphilis, diarrhea, headache, eczema, and wounds; which are HIV and AIDS-related illness (Hodgson and

Rachanis, 2002). It is a major concern that *S. aures, E. coli* and *C. albicans* have the genetic ability to transmit and acquire resistance to therapeutic drugs, which has led to multidrug-resistance (Kisangau *et al.*, 2007). Studies have shown that the problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the management of HIV and AIDS-related illnesses is still uncertain (Otang *et al.*, 2012; Chinsembu, 2016). Related plant species of *P. africanum, C. nigricans, K. africana* and *C. asiatica* have been investigated in West Africa and Asia, where they have been identified to have a wide spectrum of antimicrobial properties that can be of great significance in therapeutic regimen (Brusotti *et al.*, 2012; Lalitha *et al.*, 2013). However, efficacy of these plants and other potent plants that have active antimicrobial compounds need to be investigated in the LVB given that the chemical potency of plants vary geographically and HIV & AIDS prevalence is relatively higher in the LVB.

The activity-guided approach in traditional medicine research aims at developing a novel compound used in the management of HIV and AIDS and its related opportunistic infections (Copeland, 2005). *In vitro* studies using cancerous cell lines on related plant species used in this study that are found in Asia and Europe have documented presence of pomolic acid, futokadsurin, acridone alkaloid, and flavonoid glycoside that induce cytotoxicity in cells (Rahman *et al.*, 2008; Kuznetsov *et al.*, 2011; Kuete *et al.*, 2015). AIDS patients in LVB are using concoctions of crude plant extracts of *P. africanum, C. nigricans, K. africana*, and *C. asiatica* in the management of HIV and AIDS-related opportunistic infections (Odhiambo *et al.*, 2009; Mutua *et al.*, 2013; Orodho *et al.*, 2014). However, cytotoxicity of *P. africanum, C. nigricans, K. africana*, and *C. asiatica* plant extracts found in LVB in leukemia lymphoid cell lines has not been determined considering that the leukemia lymphoid cells are derived from CD4 cells, which are important in controlling HIV.

Active plant extracts need to be less toxic to the cells and should not alter the normal growth and development of cells. The anti-proliferative screening provides data on the rate and sequence of proliferation which is important in the selection of plant extracts with minimal anti-proliferative effect *in vitro* and *in vivo* (Omosa *et al.*, 2015). Cells exposed to extreme physiochemical or mechanical stimuli die in an uncontrollable manner, as a result of their immediate structural breakdown (Galluzzi *et al.*, 2015). Previous *in vitro* studies have reported superior antiproliferative potential on fractionated extracts of *Mesua ferra, Centella erecta, P. africanum and Solumum torvum* which are closely related species to the plants in this study (Taatjes *et al.*, 2008; Tiwary *et al.*, 2015). Traditional healers in East and West Africa are managing HIV and AIDS-related infections using similar medicinal plants species found in the LVB (Mutua *et al.*, 2013), yet these plant extracts inhibit human cell proliferation (Boulbes *et al.*, 2006). However, antiproliferative properties of the study plant extracts found in LVB has not been determined. Therefore, this study determined the antimicrobial, cytotoxicity, and anti-proliferative properties of *P. africanum*, *C. nigricans*, *K. africana*, and *C. asiatica* plant extracts used in the management of HIV and AIDS-related opportunistic infections along the LVB of Kenya.

1.2. Statement of the Problem

The primary HIV-1 infection and its opportunistic infections is currently being managed by HAART, which is aimed at suppressing replication of HIV and boosting the patient's immune modulators. Recently there have been cases of appearance of viral mutants encoding resistant reverse transcriptase which have reduced susceptibility to available formulations of HAART. The viral mutants have altered to a greater extend the proliferation of opportunistic infections caused by *S. aureus, E. coli* and *Candida albicans*. Moreover, cases on toxicity, mutations, and

carcinogenicity have been reported on people using HAART. Thus, there is a need for alternative treatment approaches and intervention strategies such as the use of extracts of medicinal plants in management of opportunistic infections.

Use of HAART is also characterized by side effects like nausea, anemia and odd distribution of fat in the same persons. In addition, there are other metabolic abnormalities that lead to diabetes-like problems, brittle bones, and heart disease. Withdrawal of donor communities that support the supply of HAART, and lack of awareness campaigns on the potential challenges of HAART are also common scenarios of people living with HIV in the LVB region. The situation is fortified by the aggravated poor economic status of the people in the LVB region. The alternative approach in the study addresses the HAART challenges faced the management of HIV and opportunistic infections in the LVB region.

1.3. General objective

To investigate the antimicrobial, cytotoxicity and anti-proliferative properties of *P. africanum*, *C. nigricans*, *K. africana*, and *C. asiatica* plant extracts used in treatment of HIV and AIDS related opportunistic infection along the Lake Victoria Basin of Kenya.

1.3.1. Specific objectives

1. To determine antimicrobial properties of *P. africanum*, *C. nigricans*, *K. africana*, and *C. asiatica* plant extracts used in treatment of HIV and AIDS related opportunistic infection along Lake Victoria basin, Kenya.
2. To determine cytotoxic properties of *P. africanum*, *C. nigricans*, *K. africana*, and *C. asiatica* plant extracts on mammalian leukemia lymphoid cell lines (CCRF-CEM) in the management of HIV-related opportunistic infection along Lake Victoria Basin, Kenya.

3. To determine anti-proliferative effects on the mammalian leukemia lymphoid cell lines (CCRF-CEM) of *P. africanum, C. nigricans, K. africana,* and *C. asiatica* plant extracts used in treatment of HIV related opportunistic infection along Lake Victoria Basin, Kenya.

1.4. Research questions

1. What are the antimicrobial properties of *P. africanum, C. nigricans, K. africana,* and *C. asiatica* plant extracts used in treatment of HIV and AIDS related opportunistic infection along Lake Victoria Basin, Kenya?
2. What are the cytotoxic properties of *P. africanum, C. nigricans, K. africana,* and *C. asiatica* plant extracts on mammalian leukemia lymphoid cell lines in the management of HIV related opportunistic infection along Lake Victoria Basin, Kenya?
3. What are the anti-proliferative effects on mammalian leukemia cell lines of *P. africanum, C. nigricans, K. africana,* and *C. asiatica* plant extracts used in treatment of HIV-related opportunistic infection along Lake Victoria Basin, Kenya?

1.5. Significance of the study

The study findings on the antibacterial, cytotoxicity properties and anti-proliferative effects of the plant extracts on the mammalian leukemia lymphoid cell lines will provide bases for further analysis in the development of therapeutic drugs from these selected plant extracts. The study findings will also lead to further fractionation of the crude extracts with the aim of identifying the active molecules hence providing the basis of activity-guided approach in developing novel compounds used in the management of HIV and AIDS related opportunistic infections. Moreover, the study findings will provide guidance on determination of ratios to be used in combination therapy with an aim of developing a broad-spectrum dosage formulation. The information from the study will provide bases for dosage formulation for *in vivo* studies, and for the ratios to be used in the drug development.

CHAPTER TWO

LITERATURE REVIEW

2.1. Epidemiology of HIV infection

HIV-1 is a retrovirus having several routes of transmission (WHO, 2013). AIDS is a condition in which the immune function is compromised (UNAIDS, 2007; WHO, 2013). Some of the common routes of infection are sexual transmission, direct injection with HIV contaminated needles, and from HIV-infected mother to foetus (Kilmarx, 2009). Globally, 35 million people live with HIV (WHO, 2013). The mortality rate of people dying of AIDS-related illnesses is approximately 1.5 million (WHO, 2013). There are approximately 119 countries in the world that have programs and facilities of preventing the spread of HIV. The health facilities create awareness of HIV transmission methods, eliminate the mother-to-child transmission of HIV, and steadily provide treatment of HIV-related illnesses. The health programs among the different countries teach the residents the different modes of HIV transmission, prevention, and diagnoses of the HIV infected individuals (WHO, 2005; UNAIDS, 2007).

HIV infection is most prevalent among the sub-Sahara countries of Africa such as Kenya and it is most prevalent in the age group 15-49 years (Kilmarx, 2009; UNAIDS, 2016). HIV is associated with depletion of primary immune cells that include CD4+ T cells, macrophages and Natural Killer (NK) cells (Eggena *et al.*, 2005). Following initial infection by the virus, progression to full blown AIDS varies between individuals and ranges from 3 to 10 years (Kilmarx, 2009). The AIDS defining-illness and their percentage occurrence include oral thrush (60%), tuberculosis (TB) and *Pneumosytis carini*, gastritis and non-hemorrhagic gastroenteritis (65%), Herpes Zooster and Disseminated Herpes Simplex Virus (HSV) (75%), partial loss of sight due to Human Cytomegalovirus (HCMV) (30%), protozoal infection, for example,

Cryptosporodium parvum, Giardia spp, Entamoeba histolytica (10%), generalized wasting due to malnutrition and micronutrient malabsorption (Aduma *et al.*, 1995; Eggena *et al.*, 2005; Chinsembu, 2016).

AIDS-defining illness occurs when CD4+ levels decline to levels below 200 cells/mm^3 and the virus load by PCR increases to levels above 100,000 copies/ml (Giorgi *et al.*, 2002). The patterns of disease progression to AIDS are fast progression, whereby HIV-infected individuals progresses to AIDS within 4 years post infection; typical progression, in which individuals immune system get compromised, consequently giving rise to AIDS-defining illnesses, and no progression to AIDS, which involves individuals living with the HIV-1 for more than 10 years and remains asymptomatic (Morgan *et al.*, 2002). Factors such as viral genetic variability, host susceptibility, immune function, co-infections, and health care may affect the pattern of progression to develop AIDS (Giorgi *et al.*, 2002; Morgan *et al.*, 2002). The AIDS-defining illnesses also include HIV-1 associated cancers such as non-Hodgkin's lymphoma, primary central nervous system lymphoma, lymphoproliferative disorders, Kaposi sarcoma, smooth-muscle tumors-leiomyosarcoma, anal cancer, and cervical cancer (Sitas *et al.*, 2000).

The LVB of Kenya has high prevalence of malaria and HIV (Omolo *et al.*, 2010). Additionally, people living in the LVB are prone to many catastrophic diseases and do not have access to clean water (Gitahi-Kamau *et al.*, 2015), have food insecurity that goes for more than six months in a year (Nangami, 2010), and the area has limited access to health care provision (Opio *et al.*, 2013). Recent studies by Nangami (2010) showed that 51% of bed occupancy in the LVB has persons in mid-terminal to terminal stages of HIV and AIDS. The social dimension of the disease in the LVB shows a vicious cycle of disease, malnutrition, poverty, and lack of

economic empowerment (Nangami, 2010; Opio *et al.*, 2013). In addition, the literacy levels in the region is low and generally there is high mortality rate (Nangami, 2010; Nagata *et al.*, 2011).

The trend of HIV and AIDS in Kenya is becoming epidemic. There are 1.6 million Kenyans living with HIV, where 1.4 million are adults, and 191,840 are children aged 0 to 14 years (Ministry of Health, 2013). According to the Ministry of Health (2013) counties around the LVB have highest adult HIV prevalence, which are Homa Bay 25.7%, Siaya 23.7%, Kisumu 19.3%, and Migori 14.7%. The national HIV prevalence is 6%, where male have 5.6% and female 7.6% prevalence (Ministry of Health, 2013). Approximately 88,620 annual new HIV infections occur among the adults and 12,940 among the children. Treatment of HIV in Kenya involves the use of HAART that has been scaling up since 2000, which has averted deaths due to HIV (Day, 2003; Ministry of Health, 2013).

HAART is combination chemotherapy with at least three antiviral classes of drugs such as reverse transcriptase inhibitors, and protease inhibitors (De Clercq, 2002; Chung *et al.*, 2009). Other classes of HIV drugs are Tat inhibitors and Rev inhibitors (Ghosh *et al.*, 2008). Reverse transcriptase inhibitors comprises of nucleoside analogues and non-nucleoside inhibitors, which incorporates di-deoxy nucleoside into the DNA, hence inhibiting the growing polynucleotide chain (el Kouni, 2002). Protease inhibitors cleave to Gag and Gag-Pol precursor protein, consequently inhibiting viral protease (Mao *et al.*, 2000). Although HAART has reduced the rate of deaths due to HIV, the latest evidence in Kenya has shown that people in LVB are using alternative approach of Traditional Medicine (TM) in treating AIDS-related illnesses recording an 80% success rate (Body *et al.*, 1994; Lamorde *et al.*, 2010).

2.2. Bioactive properties of medicinal plant extracts against pathogenic microbial species

Developing countries rely on Traditional Medicine (TM) since it is often accessible and affordable treatment (Mahesh and Satish, 2008). Validation of medicinal plants for HIV and AIDS involve identification of bioactive extracts and compounds against *S. aureus*, *E. coli* and *C. albicans* that cause opportunistic infections such as tuberculosis, oral thrush, and pneumosytis in HIV and AIDS patients. However, the bacteria are highly evolving and are increasingly becoming multidrug resistant, which have also been attributed to poor hygienic condition in the hospitals and at community level (Burkill, 1995; Zou *et al.*, 2012; Agyare *et al.*, 2013). Other multi-drug resistant bacteria are *Pseudomonas aeruginosa*, and *Mycobacterium tuberculosis* (Mahesh and Satish, 2008), which trigger investigations and advanced tests.

Studies of antibacterial activity of *Kigelia africana*, *Strophanthus hispidus,* and *Rhus coriaria* plant extracts indicated antibacterial actions against β-lactamase activity (Adwan *et al.*, 2010; Agyare *et al.*, 2013). The plant extracts were isolated using ethanol extraction method and concentrated using rotary evaporator to remove the excess ethanol on the plant extracts. The Indian traditional medicine is used for a broad spectrum activity such as treating wounds, leprosy, dysentery, cough, skin diseases, and cold. Some of the Indians medicinal ethanolic plants extracts active against Gram positive and Gram negative resistant bacteria are *Holarrhena antidysenterica* (bark), *Plumbago zeylanica* (root), (Burkill, 1995) *Hemidesmus indicus* (stem), *Camellia sinensis* (leaves), and *Delonix regia* (flowers) (Eloff, 1998). Ethanolic extraction method is the most suitable since it selectively isolates active compounds and makes it easy toward concentration process (Eloff, 1998). Herbal plants such as *Acacia nilotica*, and *Ziziphus mauritiana* have shown antifungal activity against *Aspergillus flavus* and *Fusarium verticillioides* (Eloff, 1998). The experiments on medicinal plants in China, India and some parts

of Africa also involve testing for antimicrobial properties. The potential plants further undergo evaluation procedures to determine the active compounds that can be concentrated into a product (Alonso Paz *et al.*, 1995; Nascimento *et al.*, 2000).

Sharing of traditional knowledge is essential toward innovation and practice of biological conservation. It is evident that the use of herbal medicine improves human health and sustains livelihood (Nascimento *et al.*, 2000). Herbal plant studies aim at filling the existing gaps in sustaining optimal utilization of medicinal biodiversity (Nascimento *et al.*, 2000). In this scenario, traditional medicinal plants are rich sources of antimicrobial agents (Nascimento *et al.*, 2000; Mahesh and Satish, 2008). For instance, studies by (Mahesh and Satish, 2008), revealed that *Acacia nilotica* is active against uro-genital diseases, *Sida cordifolia* roots are used in pain management, cardiac diseases and nervous disorders, and *Withania somnifera* is used in cough, rheumatism and gynecological disorders. Adwan *et al.* (2010) found out that the anti-bacterial activity of the methanol and ethanolic plant extracts in the majority of studies undergo testing by the disk diffusion method (Adwan *et al.*, 2010).

The use of traditional medicine in LVB region to manage diseases is receiving attention from mainstream health officials, and training institutions (Tabuti, 2010; Shih *et al.*, 2011). Traditional health practitioners (THPs) use variety of plant materials such as barks, leaves, roots, fruits, and seeds. THPs then make a concoction for the treatment of different infectious and parasitic infections (Tabuti, 2010). Patients visiting the THPs give a positive feedback after consumption of the concoction (Lamorde *et al.*, 2010; Tabuti, 2010). Some of the plants used in the management of bacteria and fungi infections related to HIV and AIDS infections are *Piptadeniastrum africanum* (bark), *Chaemacrista nigricans* (leaves), *Kigelia africana* (fruit) and

Centella asiatica (leaves). The selection of the plants was through the assistants of Traditional Healers from Suba-Luo communities of the LVB of Kenya.

2.2.1. *Piptadeniastrum africanum*

Piptadeniastrum africanum is a tree belonging to the family Leguminosae-mimosoidae. It is an indigenous tree in Senegal, Southern Sudan, Uganda, Angola, and DR Congo (Tafokou *et al.,* 2008). The bark, roots and leaves of *Piptadeniastrum africanum* are used in traditional medicine. The bark concoction is used to treat cough, bronchitis, headache, haemorrhoids, genitor-urinary infections, stomach-ache, fever, pneumonia, skin complaints, rheumatism, and as a purgative. The roots are used for laxatives (Schmelzer and Gurib-Fakim, 2008). The root-bark, and stem-bark are the ingredients of arrow poison in Cameroon and DR Congo. A concoction of the leaves is applied as an enema to treat gonorrhea, and abdominal complaints. Propagation of the tree is through planting of its seeds (Schmelzer and Gurib-Fakim, 2008).

2.2.2. *Chaemacrista nigricans*

Chaemacrista nigricans belongs to family Fabaceae-ceasalpinioideae. *Chaemacrista nigricans* grows wild in tropical Africa, western Asia, and India. Its leaves are used by traditional healers in treatment of measles, inflammation, haemorrhoids, peptic ulcers, headache, meningitis, cough, itching, stomachache, diarrhea, worms, and malaria. The *in vitro* test on the leaves extracts of *C. nigricans* showed significant action against *Herpes simplex* (Schmelzer and Gurib-Fakim, 2008). Ethanolic plant extracts have shown antibacterial activity against *Shigella dysenteriae, Staphylococcus aureus, Staphylococcus faecalis,* and *Vibrio cholera* (Schmelzer and Gurib-Fakim, 2008). *C. nigricans* leaves, and roots show interesting pharmacological actions, hence they warrant further research on the medicinal actions (Schmelzer and Gurib-Fakim, 2008).

2.2.3. *Kigelia africana*

Kigelia africana is a tree belonging to the family *Bignoniaceae*. The tree grows wild in tropical Africa, Eritrea, Chad, Senegal, Namibia, and South Africa. *K. africana* has medicinal properties, and is a potent plant in development of drugs by pharmaceutical industries (Saini *et al.*, 2009; Agyare *et al.*, 2013). The traditional healers use the plant leaves and fruits in treatment of skin ailments such as fungal infections, boils, psoriasis, and eczema. Additionally, the leaves of *K. africana* are used in the treatment of dysentery, ringworm, tapeworm, malaria, diabetes, pneumonia, and toothache. The barks of the tree are used as a remedy for syphilis, and gonorrhea. Venereal diseases are commonly treated with the tree extracts. Laboratory tests of plant extracts of *K. africana* shows large zones of inhibition against *S. aureus,* and *P. aeruginosa*. No inhibitory effects were observed against *C. albicans* (Kwo and Craker, 1996; Saini *et al.*, 2009).

2.2.4. *Centella asiatica*

Centella asiatica is a perennial herb creeper of the family *Umbellifere*. Wild species of *C. asiatica* are present in South Africa, Australia, China, Japan, East Africa, India, and Sri Lanka. The plant is efficient in wound healing. The plant extract also treats skin conditions such as leprosy, eczema, and psoriasis. In addition, *C. asiatica* extracts manages diarrhea, fever, and diseases of the female genitourinary tract (Gohil *et al.*, 2010). *C. asiatica* is widely used as a blood purifier, treats hypertension, and enhances memory. Eastern India traditional healers use the plant extract in treating emotional disorders such as depression. The *in vitro* studies shows that aqueous extracts of *C. asiatica* have both anti-HSV-1 and anti-HSV-2 activities (Body *et al.*, 1994; Yoosook *et al.*, 2000). Crude extract and purified fractions showed cytotoxicity against Ehrlich ascites and Daltons lymphoma ascites tumor cells, in a concentration-dependent manner.

However, no cytotoxic effects were detected against normal human-derived cell lines (Babu *et al.*, 1995; Qureshi *et al.*, 1997).

2.3. Potential antiproliferative and cytotoxicity properties of plant extracts

Medicinal plants excrete compounds that may be harmful to the cultured cell or mammalian metabolic activity. Moreover, the excreted compounds may transform the homeostatic growth and development of cells. The bioactive compounds such as terpene acids, madecassic acid, glycoside, madecassoside among others that possess immunomodulating (Elsyana *et al.*, 2016), antitumor (Khan *et al.*, 2002), antibacterial (Adwan *et al.*, 2010; Agyare *et al.*, 2013), antioxidants (Agyare *et al.*, 2013), antigenotoxic (Angayarkanni *et al.*, 2007), and antiproliferative (Yeh *et al.*, 2012) properties. It is believed that the antioxidation activity and the phenolic activity of the phenolic compound from these plant extracts such as *C. asiatica* can prevent arteriosclerosis, diabetes, cancer, and arthritis. The MTT [3-(4,5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] enzyme-based method is highly used in measuring anti-proliferation effect and cytotoxicity of the medicinal plant extracts. The method is superior to traditionally used methods of measuring cell proliferation based on direct counting of viable cells, since it measures the mitochondrial metabolic activity and cellular DNA content (Khan *et al.*, 2002; Yeh *et al.*, 2012). Traditional cell counting methods involves trypan blue dye exclusion assay using a haemocytometer, which is simple and inexpensive method, but time consuming (Khan *et al.*, 2002).

Testing of plant extracts *in vitro* for antiproliferative and toxicity on continuous cell lines help determine the inhibition concentration (IC_{50}) levels of the extracts (Bussmann *et al.*, 2011). Cells exposed to extreme physicochemical or mechanical stimuli die in an uncontrollable manner, as a result of their immediate structural breakdown (Galluzzi *et al.*, 2015). The harsh

physiochemical conditions do not involve a specific molecular machinery. Cells exposed to extreme physicochemical or mechanical insults die while releasing elevated amounts of damage-associated molecular patterns, that is, endogeneous molecules with immunomodulatory and sometimes cytotoxic activity. Some damage-associated molecular patterns can propagate an unwarranted cytotoxic response that promotes the death of local cells surviving the primary event that caused damage to the cells (Angayarkanni *et al.*, 2007; Galluzzi *et al.*, 2015). For instance, cytotoxicity studies that involved HEP_2 epidermoid cell line treated with *Amorphophallus paeoniifolius* extract showed cells losing cell contacts and changes in the structural morphology, and subsequently high number of cells death at a concentration of 70% after 24 hours of treatment (Angayarkanni *et al.*, 2007).

According to Yeh and colleagues (2012), some plant extracts such as *P. macrocarpus, D. seguine, C. debilis,* and *P. africanum* tend to inhibit cell growth (antiproliferative) *in vitro* on cancerous and continuous cell lines. For instance methanolic extracts of *Gracilaria tenuistipitata* tend to inhibit cell growth on HeLa cells through induced DNA damage (Yeh *et al.*, 2012). In an experiment conducted by Khan and colleagues (2002), it was found out that increasing amounts of *Emblica officinalis* extracts caused intermediate antiproliferative activity on *in vitro* human leukemic K562 cells (Khan *et al.*, 2002; Senthilraja and Kathiresan, 2015). Moreover, bioactivity studies involving *Ononis hirta, Verbascum sinaiticum, Inula viscosa,* and *Salvia pinardi* plant extracts showed that extracts derived from ethanol exhibited higher antiproliferative potential with some significant differences in selectivity ($p<0.05$) against the tested cell lines than aqueous fraction (Talib and Mahasneh, 2010).

Traditionally used plants are currently being used for modern drug development (Omosa *et al.*, 2015). Previous studies have shown that extracts of African plants exhibited interesting

cytotoxicity against sensitive and drug resistant cell lines (Kuete *et al.*, 2011; Omosa *et al.*, 2015). For instance, *Bridelia micrantha* methanolic extract was tested on leukemia most prevalent cell lines due to their high sensitivity towards cytotoxic agents, whereby *B. micrantha* inhibited the proliferation of CCRF-CEM cells by more than 50% following 48 hours incubation. Additionally, ethanol extract of the berries of *Solanum acelestrum* against the drug sensitive human leukemia cells (CCRF-CEM) exhibited cell inhibition with an IC_{50} value of 1.36µg/mL (Omosa *et al.*, 2015; Senthilraja and Kathiresan, 2015).

The study addresses the possibilities of using natural products or derivatives from medicinal plants as potential sources of antiviral drugs and immune modulators. The reports from traditional medicinal men, that some medicinal plants have curative or prophylactic effects in inhibiting the HIV-1 virus and secondary opportunistic infections, are useful in the study. The study shows the developmental stages of progressive increase in the value of traditional herbs to pure compounds that can be converted into products. However, the development of these herbal plants to chemotherapeutic drugs depends on scientific evidence of their efficacy and safety, consequently the need for the current study.

CHAPTER THREE

METHODOLOGY

3.1. Study area

The plants used in the study were collected from Luo-Suba region of Homa Bay and Migori counties between latitude $0^0$53’5”N $1^0$06’67”S and longitude $34^0$45’31”E $34^0$46’67”E. The analysis of the plants was done in Maseno University, which is located on latitude $0^0$00’04”N $0^0$00’67”S and longitude $34^0$58’45”E $34^0$59’85”E (Appendix 7) School of Public Health and Community Development, Biomedical Science Department in Prof. P. Aduma’s Laboratory. The study area, especially the Luo-Suba, has high national prevalence of HIV and AIDS (Ministry of Health, 2013), and has many traditional healers who use medicinal plants in management of HIV and AIDS and its opportunistic infections (Nagata *et al.*, 2011).

3.2. Study design and sample preparation

This was a laboratory-based *in vitro* study that involved collection of plant parts; *P. africanum* (bark), *C. nigricans* (leaves), *K. africana* (fruit), and *C. asiatica* (leaves), drying them and grinding to obtain powdered form of the plant materials. Crude extract of the powdered plant material was obtained using the ethanol extraction method in a waring blender. The crude extract were evaluated for safety and efficacy by testing their antimicrobial properties, cytotoxicity and antiproliferative effects against *in vitro* human leukemia cell lines.

3.3. Sampling procedure

Ethno botanical surveys, which engaged the traditional healers and the consumers of the medicinal concoctions, were conducted on the medicinal plants used in the management of opportunistic bacterial and fungal infections in HIV/AIDS patients in Luo-Suba in LVB of Kenya. This led to collection of the mostly used and abundant plants in moistened plastic bags from the Luo-Suba with the help of known traditional healers and custodians of indigenous knowledge. The study used *P. africanum* (bark), *C. nigricans* (leaves), *K. africana* (fruit), and *C. asiatica* (leaves). The plant materials were authenticated by herbarium staff in the Department of Botany at Maseno University. Air drying of the collected plant parts was done in the Department of Chemistry at Maseno University. The extracted plant materials were stored in bijou bottles to be used for antimicrobial susceptibility assay, cytotoxicity, and antiproliferative assay.

3.4. Extraction and purification of plant extracts

The dried plant samples were ground and crushed homogenously using laboratory pestle and mortar. The ground and crushed plant samples were weighed and the amount recorded. The plants underwent total extraction using ethanol in a warring blender for 2-5 minutes. Blending involved mixing the crushed plant sample (≈1Kg) with absolute ethanol for 5 minutes. The amount of absolute ethanol added (≈2L) completely covered the plant sample. The extract was collected through filtration with an aid of Whatman filter paper (Whatman Inc, Piscataway, NJ) and bruchner funnel. The blending process was repeated until the extract color became colorless. The extracted plants were subjected to Rotary evaporator to remove the solvent used during extraction process, hence concentrating the extracts. The concentrated crude extract collected was used in antimicrobial susceptibility assay, cytotoxicity, and antiproliferative assays.

3.5. Antimicrobial susceptibility test assays

3.5.1. Culture procedures

The initial process involved culturing of bacteria and fungi that have high pathogenicity in the HIV infected individuals. The microorganisms used in microbiology assay were Gram positive bacteria - *Staphylococcus aureus* (ATCC 25923), Gram negative bacteria - *Escherichia coli* (EPEC-STG), and *Candida albicans* (ATCC 10231) – fungi. Gram staining technique (Appendix 1) was used to identify pure colonies of Gram positive and Gram negative bacteria. Purification of the contaminated bacteria involved streaking technique on Blood agar for Gram positive bacteria and on MacConkey agar for Gram negative bacteria. The procedure for preparing Blood agar and MacConkey agar was in accordance with the manufacturer's instructions. Briefly, the mixture of the media with sterile distilled water was autoclaved for 15 minutes at 15 lbs and 121^0C. Streaking technique was used followed by incubation at 36^0C for 24 hours. Examination for isolated colonies followed before proceeding with sensitivity testing.

Culturing of *Candida albicans* was done on Potato Dextrose Agar (PDA) for colony isolation in accordance to Liu *et al*., (2002), with slight modifications. The culturing of *C. albicans* involved, measuring 40 grams of the PDA powder and mixing it with one liter of distilled water in a conical flask (Liu *et al.*, 2002). Moderate heating was essential to ensure complete dissolution of the powder. The mixture was sterilized in an autoclave for 15 minutes at 15 lbs and 121^0C. Streaking technique was used followed by incubation at 36^0C for 24 hours. Examination for isolated colonies followed before proceeding with sensitivity testing.

3.5.2. Susceptibility assay

Sensitivity testing of the microbes was conducted on Mueller-Hinton agar by the Kirby-Bauer disk diffusion technique in accordance to Bonev et al., (2008) with slight modifications as

illustrated in Appendix 2, which involved measuring the minimum inhibitory concentration (MIC). The MIC test involved swabbing and growing *S. aureus* and *E. coli* on Mueller Hinton agar and *C. albicans* on PDA. MIC is the lowest concentration of drug that inhibits the growth of the organism. Predominantly, larger zones of inhibition correlate with smaller MIC of antibiotic for that bacteria (Bonev *et al.*, 2008).

The plant extracts were weighed in grams and dissolved in dimethyl sulfoxide (DMSO) at varying volumes in milliliters. The mass of the plant extracts that completely dissolved in the DMSO formed the basis for categorizing the volumes to use in the study. Varying concentrations (500mg/mL, 250mg/mL, 125mg/mL and 62.5mg/mL) of the selected medicinal plant extracts (*P. africanum, C. nigricans, K. africana* and *C. asiatica*) were impregnated on the 6mm diameter filter paper disks and gently placed on the Muller Hinton agar plate that was swabbed with the pathogenic organism. The test was conducted in triplicate and a negative control was included, which was a disk impregnated with DMSO. The plates were incubated at 37^0C for 24 hours then observed for the presence of zone of inhibition. The diameter of the zone of inhibition was measured in millimeters using a ruler.

The study used standard antibiotics and antifungal that are currently being used in the market in management of bacterial infections, and that have recorded least resistance to the infections as the positive controls. These antibiotics used were penicillin and Fungizone (Amphotericin B). The test organisms (*S. aureus, E. coli* and *C. albicans*) were subjected to disks impregnated with the standard antibiotics (positive control) and incubated for 24 hours at 37^0C then observed for the presence of zone of inhibition. The sensitivity of the plant extracts and standard antibiotics were analyzed in reference to the data in Clinical and Laboratory Standards Institute.

MIC of the plant extracts was also determined using the broth dilution method. Pure colonies of *S. aureus, E. coli* and *C. albicans* were emulsified to a turbidity of optical comparison to the 0.5 Mcfarland standard, which corresponds to 1-2 x 10^8 colony-forming units (cfu)/mL. According to Clinical and Laboratory Standards Institute recommendations, standardization of inoculum is essential toward obtaining accurate and reproducible results. Errors in the standardization of the inoculum to correspond to 0.5 Mcfarland may give varying results since it involved use of naked eyes during the standardization process (Lalitha, 2004). The procedure also involved inoculating colonies of *S. aureus, E. coli*, and *C. albicans* into a series of already prepared brain heart infusion (BHI) broth tubes that had twofold dilutions of the plant extracts. For each batch of microorganism, 50mL sterile stock solution of plant extract was prepared using DMSO as the solvent to the highest concentration that was restricted by the available volume at the time of conducting the study, which was 320mg/mL. However, the volume was sufficient considering that dissolved solid mass of the plant extracted did not exceed 500mg/ml. Subsequent dilutions up to 2.5mg/mL were made up using sterile water. Following preparation of the serial plant extract dilution, 2mL of freshly standardized broth culture of the bacterial and fungi strain was inoculated in each tube of the dilution and mixed well for homogeneity. All the MIC tubes and control tubes (negative control-a tube containing BHI media alone, and positive control-a tube containing BHI media + bacterial strain) got incubated at 37^0C for 24 hours. After incubation, the lowest concentration of plant extract that prevented visible growth of the microorganism was the MIC.

Minimum bactericidal concentration (MBC) was conducted as part of antimicrobial susceptibility analysis. During the process of designing a new drug, it is important to determine the concentration of the plant extract that kills the microorganisms rather than inhibiting their

growth (Jorgensen and Ferraro, 2009). MBC was determined by picking a loopful of medium from the tubes used for the MIC test that showed no turbidity and inoculating them onto blood agar plates. The plates were incubated for 24 hours at 37^0C. Any organism that was inhibited but not killed in the MIC test was likely to grow because the plant extract had been diluted significantly. After 24 hours incubation, the lowest concentration that reduced the number of colonies by 99.9% becomes the MBC. The procedures kept in consideration that the bactericidal antibiotics usually have an MBC equal or very similar to the MIC whereas bacteriostatic antibiotics usually have an MBC significantly higher than the MIC (Pankey and Sabath, 2004). Swabbing was done on the following concentration of *P. africanum* on *S. aureus* that showed no turbidity on MIC test: 320, 160, 80, 40 and 20 mg/mL and 320mg/mL of *C. asiatica* on *C. albicans*.

3.6. Cell Culture Assays

3.6.1. Growing Bulk quantities of CCRF-CEM cell line

A vial of CCRF-CEM lymphoid cells was imported from the American Type Culture Collection (ATCC, Manassas, VA). The vial was frozen upon receipt in liquid nitrogen vapor and resuscitated afterwards from the original frozen stock. Cells were maintained in a total volume of 20mL RPMI 1640 medium (ATCC 30-2001) supplemented with 10% FBS (ATCC 30-2020), 100µg/mL gentamicin, and 2.5µg/mL fungizone at 37^0C and 5% CO_2 in 75cm^2 cell culture flasks. The CCRF-CEM cells were observed every 2-3 days to maintain the density of 1.0 x 10^6 viable cells/mL. Daily monitoring was done for growth, contamination or death. The cells were split at a ratio of 1:3 every 2-3 days. A third of the split cells was re-seeded in the flask to maintain the minimum density. Freshly prepared supplemented RPMI 1640 media was added to

the growing cells every 2 to 3 days and the volume maintained to 20% through replacement of medium. Two-thirds of the split cells were frozen in freezing media that contains 5% DMSO and 95% FBS; then dispensed in 1.5mL cryovials. The cryovials were placed in dry ice and transferred to -196^{0}C liquid nitrogen tank.

3.6.2. Determination of cell number and viability

Morphological features of the cells were examined daily using the inverted light microscope (LB-341, Los Angeles, CA) and photomicrographs recorded (Appendix 4). Cell density and viability of cultured cells was calculated at every subculturing procedure using hemocytometer and trypan blue exclusion dye (Appendix 5).

3.6.3. Cytotoxicity and antiproliferative measurements

The assessment of cytotoxicity and proliferation was accomplished through 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) assay while the cell viability and survival capacity of the CCRF-CEM cells was accomplished through cell assays that involved cell counting. The cytotoxicity effects on CCRF-CEM at concentrations of 125µg/mL to 1g/mL of the four plant extracts were quantified using the MTT assay reagents (ATCC® 3010K) according to the instructions of the manufacturing company. Briefly, the cultured CCRF-CEM cells were plated at 1 x 10^3 per mL in 96-well plate using complete RPMI 1640 (Appendix 3). The assay was designed to have a total volume of 200µl per well, and to test forty eight assays of a total of four plant extract concentrations. A 100µl of the plant extract of a defined concentration and CCRF-CEM cells were added into the wells of a microtiter plate. The control wells in the assay contained RPMI 1640 media alone to provide the blanks for absorbance readings. The cells were incubated for 12 hours at 37^{0}C in a humidified atmosphere containing

5% CO_2. To each well, 10µl of 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) reagent was added and the plate returned to cell culture incubator for 2 to 4 hours.

The cells were viewed under inverted light microscope (LB-341, Los Angeles, CA) for the presence of intracellular punctate purple precipitate at regular occurring intervals. When the purple precipitate was clearly visible under the microscope, 100µl of detergent reagent was added to all wells including controls. The plate was swirled gently without shaking. The plate was covered and left in the dark for 2 hours at room temperature. The plate cover was removed and absorbance measured in each well including the blanks at 570nm using an ELISA plate reader (Labomed Inc.® EMR-500 ELISA microplate reader). The average values was determined from the readings and subtracted from the average values for the blank. The IC_{50} value (concentration at which 50% of the cells are killed) was determined from the dose-response curve. The assay was performed in triplicate.

Antiproliferative measurement was also conducted using trypan blue exclusion method. Briefly, CCRF-CEM cells were seeded into 100mm x 15mm petri-dishes at a density of 5.0×10^5 per mL and treated with 500mg/mL and 1g/mL of the plant extracts (*P. africanum, C. nigricans, K. africana* and *C. asiatica*). Controls were also included which contained CCRF-CEM cells at a density of 5.0×10^5 without treatment. The cells were incubated at 37^0C and 5% CO_2. Photomicrographs were recorded after 24 hours, 48 hours and 72 hours, and the cell viability determined using trypan blue exclusion method. Each petri-dish had three repeats of counting and the average concentration was recorded.

3.7. Data management and statistical analysis

The experiments were done in triplicates and results were expressed as mean ±SD. Statistical analyses were done using SPSS version 22.0. (SPSS Inc., Chicago, USA) and Graphpad Prism 5 (GraphPad Software Inc. San Diego CA). Pearson correlation and chi-square test were used to determine the relevance of the plant extract concentrations and the resistance level expressed by the test microorganisms in the antimicrobial susceptibility tests. Nonlinear regression analysis was used to compare the cytotoxicity and antiproliferative properties of the plant extracts through determination of IC_{50} values. All graphs were generated using Graphpad Prism. All P-values of less than or equal to 0.05 ($P \leq 0.05$) were considered statistically significant.

3.8. Study limitations

Kenya faces major challenges in the development and implementation of the regulations of traditional medicines (WHO, 2005). These challenges are regulatory status, assessment of safety and efficacy, quality control, safety monitoring, and lack of knowledge about traditional medicine within the national drug regulatory authority. This being among the initial studies on assessing the antimicrobial, cytotoxicity and antiproliferative properties of medicinal plants found in LVB, it is likely to face challenges of offering conclusive results of the study. This is due to complexity of the requirements and methods for research and evaluation of the antimicrobial, cytotoxicity, and antiproliferation of herbal medicines. Additionally, the study is illegible in determining the quality of the source materials, that is intrinsic factors (genetic) and extrinsic factors (environmental conditions, cultivation, and harvesting) that may challenge the

process of performing quality control of the raw materials in determining the antimicrobial, cytotoxicity, and antiproliferation of the herbal medicines.

CHAPTER FOUR

RESULTS

4.1. Antimicrobial properties of plant extracts using disk diffusion method

Antimicrobial potential of the plant extracts was determined through testing the sensitivity or resistance of the *S. aureus*, *E. coli* and *C. albicans* pathogenic microorganism through measurement of the zones of inhibition. Zones of inhibition varied among the plant species and concentrations used in the study. The plant concentrations range was 65.5 mg/mL - 500 mg/mL, which gave a mean zone of growth of inhibition of 7mm – 19 mm (Table 1).

Table 1: Disk diffusion antimicrobial assay results of plant extracts used in the study

Antibacterial activity (zone of inhibition)				
		Zone of inhibition in mm		
Plant extract	**Plant conc (mg/mL)**	***S. aureus ATCC 25923***	***E. coli EPEC-STG***	***C. albicans ATCC 10231***
P. africanum	65.5	0.0	0.0	0.0
	125	7.0	0.0	0.0
	250	10.0	0.0	0.0
	500	19.0	0.0	0.0
C. nigricans	65.5	0.0	0.0	0.0
	125	0.0	0.0	0.0
	250	0.0	0.0	0.0
	500	10	0.0	0.0
Kigelia africana	65.5	0.0	0.0	0.0
	125	0.0	0.0	0.0
	250	0.0	7.0	0.0
	500	0.0	8.0	0.0
C. asiatica	65.5	0.0	0.0	0.0
	125	0.0	0.0	0.0
	250	0.0	0.0	7.0
	500	7.0	0.0	10.0

Data are mean ± 1.0 SD of three parallel measurements. Mean zones of growth inhibition including the diameter of well are mean in millimeters (mm) of 3 independent experiments. The diameter of well is 6mm. The concentration of each plant extracts were matched with the microorganism inhibition zone.

4.2. Antimicrobial activity of standard drugs

A concurrent test on the antimicrobial activity of standard drugs against *S. aureus, E. coli* and *C. albicans* was also conducted. The standard drugs were selected in accordance to their sensitivity of *S. aureus, E. coli* and *C. albicans* in the management of the opportunistic infections. The standard drugs used were Penicillin (500µg/mL) and Fungizone (500µg/mL). A negative control (DMSO) was also included in the standardization of the study experiments. The zone of inhibition range was 15 mm – 20 mm (Table 2).

4.3. Microorganism susceptibility variations of the plant extracts used in the study

The difference in susceptibility between gram positive, gram negative bacteria and candida were determined for each plant extract. The level of microorganism susceptibility varied among the plant extracts used in the study. The *P. africanum* and *C. nigricans* plant extracts showed a narrow spectrum considering they were most effective only against *S. aureus* (gram positive bacteria). Additionally, *K. africana* exhibited a narrow spectrum since it was most effective only against *E. coli* (gram negative bacteria). However, *C. asiatica* exhibited broad spectrum activity since it inhibited the growth of *S. aureus* and *C. albicans* (Figure 1).

Table 2: Disk diffusion mean zones of reference antimicrobial agents

Antimicrobial activity (zone of activity)			
	Zone of inhibition in mm		
Drug	***S. aureus***	***E. coli***	***C. albicans***
Penicillin (500 µg/mL)	20.0	17.0	nd
Fungizone (Amphotericin B) (500 µg/mL)	nd	nd	15.0
DMSO (negative control)	0.0	0.0	0.0

Data are mean ± 1.0 SD of three parallel measurements of Penicillin, Fungizone and DMSO tested against *S. aureus, E. coli* and *C. albicans.* Mean zones of growth inhibition was measured in millimeters (mm) including the diameter of the well, which was 6mm. nd = not determined

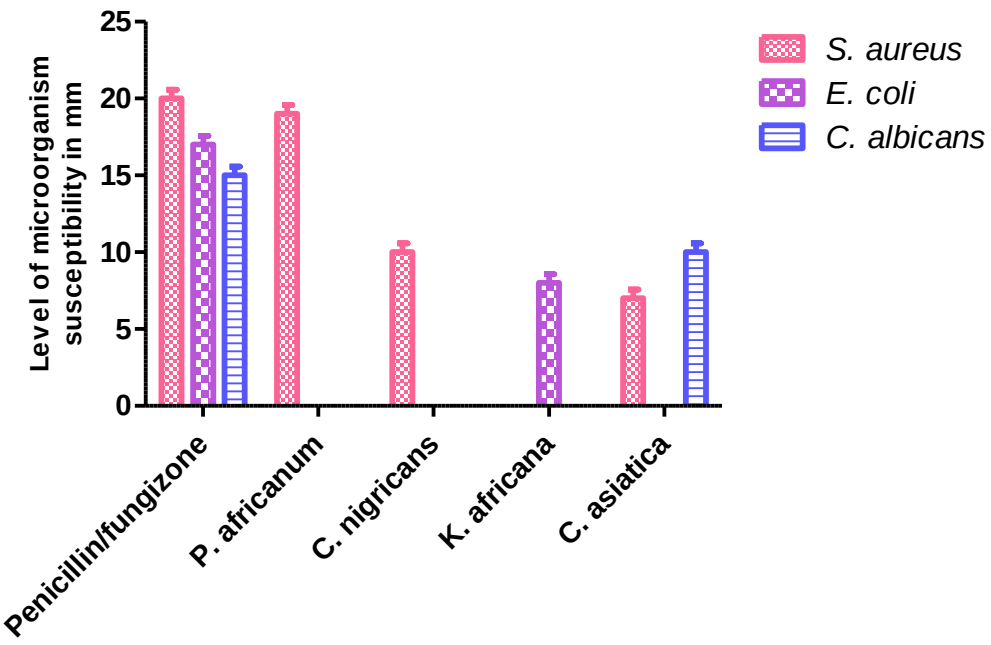


Figure 1: A bar graph of level of antimicrobial susceptibility against the reference antimicrobial standards and the plant extracts; values are expressed in mm. The level of susceptibility was determined through measurement of the diameter of the zone of inhibition in millimeters**.**

The antibacterial and anticandidal activities of the plant extracts increased linearly with increase in concentration of extracts in mg/mL. In comparison with the reference drugs, *P. africanum*, *C. nigricans*, and *C. asiatica* were active against *S. aureus*, while *K. africana* was active against *E. coli*. High concentrations of *C. asiatica* (500mg/mL) was active against *S. aureus* and *C. albicans* (Table 1). The plant extracts induced important inhibitory activities on tested Gram positive, Gram negative and fungi microorganisms with inhibition zone diameters varying from 7 to 19mm. The highest inhibition zone diameter (19mm) was obtained with *S. aureus* (Gram positive). Moreover, *S. aureus* was the most sensitive microorganism (MIC 125 mg/mL) to *P. africanum*. The plant extracts of *C. nigricans* and *C. asiatica* were less active when compared to the *P. africanum* (with inhibition zone diameters = 10mm and 7mm, MIC =

500mg/mL respectively) on *S. aureus.* In general, *P. africanum* and *C. nigricans* extracts were found to be active against gram positive bacteria, *K. africana* extract was effective against gram negative bacteria and *C. asiatica* was effective against gram positive and fungi (Appendix 6).

4.4. Determining MIC of the plant extracts using broth dilution in tubes

It was observed that the control tubes of each inoculum had the right number of cfu and inoculum density of the *S. aureus, E. coli* and *C. albicans.* The turbidity of the control tubes of each inoculum had a corresponding optical turbidity to the 0.5 Mcfarland standard. Turbidity was present in all the plant extract concentrations less than and equal to 40mg/ml. Moreover, turbidity was present in all the concentrations used of the *C. nigricans* extracts. However, no turbidity was observed on *P. africanum* extract concentrations greater than and equal to 80mg/ml on *S. aureus.* Turbidity was also not observed on *K. africana* extract concentrations greater than and equal to 160mg/ml on *E. coli*. The *C. asiatica* extract concentrations greater than and equal to 160mg/ml had no turbidity on *C. albicans.* The MIC values of the assayed plant extracts that were useful in evaluation of the activity of the antimicrobial potential of the investigated medicinal plants were presented in the Table 3.

Table 3. MIC of the plant extracts against selected pathogenic isolates. (-) indicates extracts caused no turbidity, (+) indicates extracts caused turbidity.

Minimum inhibitory concentration		Pathogenic isolate		
Plant extract	Concentration (mg/mL)	*S. aureus*	*E. coli*	*C. albicans*
Peptadiniastrum africanum	320	-	+	+
	160	-	+	+
	80	-	+	+
	40	+	+	+
	20	+	+	+
	10	+	+	+
	5	+	+	+
	2.5	+	+	+
Chaemacrista nigricans	320	+	+	+
	160	+	+	+
	80	+	+	+
	40	+	+	+
	20	+	+	+
	10	+	+	+
	5	+	+	+
	2.5	+	+	+
Kigelia africana	320	+	_	+
	160	+	_	+
	80	+	+	+
	40	+	+	+
	20	+	+	+
	10	+	+	+
	5	+	+	+
	2.5	+	+	+
Centella asiatica	320	+	+	-
	160	+	+	_
	80	+	+	+
	40	+	+	+
	20	+	+	+
	10	+	+	+
	5	+	+	+
	2.5	+	+	+

The table shows broth dilution testing by employing serial twofold dilutions between the maximum concentration (320mg/mL) and minimum concentration (2.5mg/mL) of each plant extract. The positive sign (+) indicates the growth of microorganism, whereas the negative sign (-) indicates no growth of the microorganism.

4.5. Bactericidal and bacteriostatic activity of the selected plant extracts

The broth dilution ranges (2.5 – 320mg/ml) gives the target values for determining the susceptibility to the selected plant extracts. The low concentrations (2.5 mg/mL – 40 mg/mL) of the medicinal plant extracts used did not inhibit the growth of *S. aureus*, *E. coli* and *C. albicans* whereas high concentrations (80 – 320mg/ml) inhibited the growth of the *S. aureus*, *E. coli* and *C. albicans.* The tested microorganism shows varying interpretative standards to the tested medicinal plant extracts as evidenced by presence or lack of turbidity at different plant extract concentrations in the broth dilutions as summarized in the graphical presentation in Figure 2.

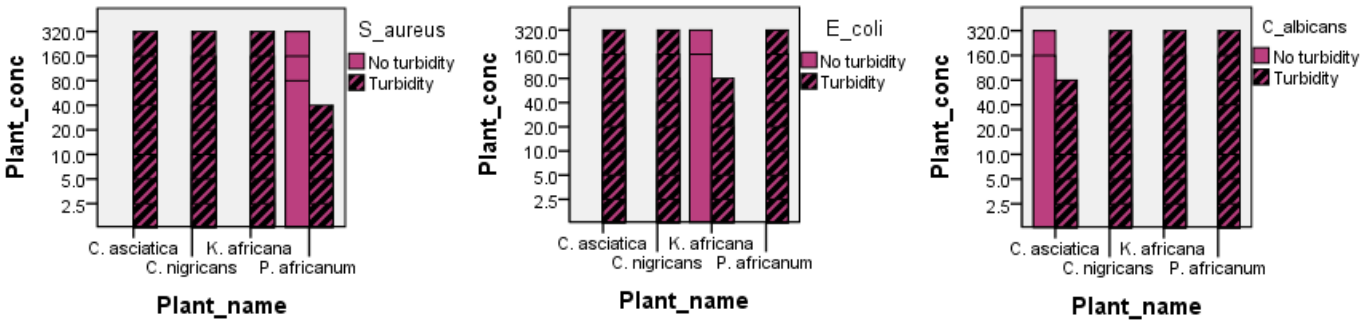


Figure 2: Bar graphs of broth dilution tubes**.** The bar graphs indicates the plant concentrations (mg/mL) and the plant name that caused inhibition (no turbidity) of the tested microorganisms and plant concentrations (mg/mL) and plant name that did not inhibit (Turbidity) of the tested microorganisms in investigative process of determining the MIC of the medicinal plant extracts.

It was observed that the extracts of *P. africanum* had its MIC at 40mg/mL against *S. aureus*, since there was no visible growth of the microorganism after 24 hours incubation at 37^0C. *K. africana* plant extracts had its MIC at 80mg/mL against *E. coli*, whereas *C. asciatica* has its MIC at 80mg/mL against *C. albicans*.

The MBC of *P. africanum* on *S. aureus* was 40mg/mL considering the presence of *S. aureus* colonies observed at the inoculated plate containing the *P. africanum* MIC concentration

of 20mg/mL (Figure 3). MBC of *C. asiatica* on *C. albicans* was 320mg/mL considering no colonies observed after 24 hours incubation of its concentration. After conducting pearson correlation and chi-square test, the correlation of the plant extract concentrations and the antimicrobial effect was significant on *S. aureus* (df=3, p=0.019), *E. coli* (df=3, p=0.017) and *C. albicans* (df=3, p=0.017).

Figure 3: Lowest concentration of *P. africanum* that inhibited growth of *S. aureus* on the plant extract concentration of 20mg/ml inoculum after 24 hours incubation. The presence of colonies at 20 mg/mL resulted to the MBC of *P. africanum* to be the preceding concentration which was 40mg/mL.

4.6. *In vitro* cytotoxicity assessment in CCRF-CEM leukemia cell lines

The *in vitro* cytotoxicity of the plant extracts to the CCRF-CEM mammalian leukemia cells was determined using nonlinear regression analysis. The absorbance values as recorded from the micro plate reader represents the number of the live CCRF-CEM cells after subjecting the cells to varying concentrations of the plant extracts. The *P. africanum, C. nigricans, K. africana,* and *C. asciatica* plant extracts concentrations used were 125, 250, 500, and 1000mg/ml which gave an absorbance range from 3.6 to 1.5. The absorbance values of the MTT assay decreased with increase in the plant extracts concentrations as presented in Figure 4. The

absorbance values and the graph analysis shows that the number of live CCRF-CEM cells decreased with increase in the plant extract concentration.

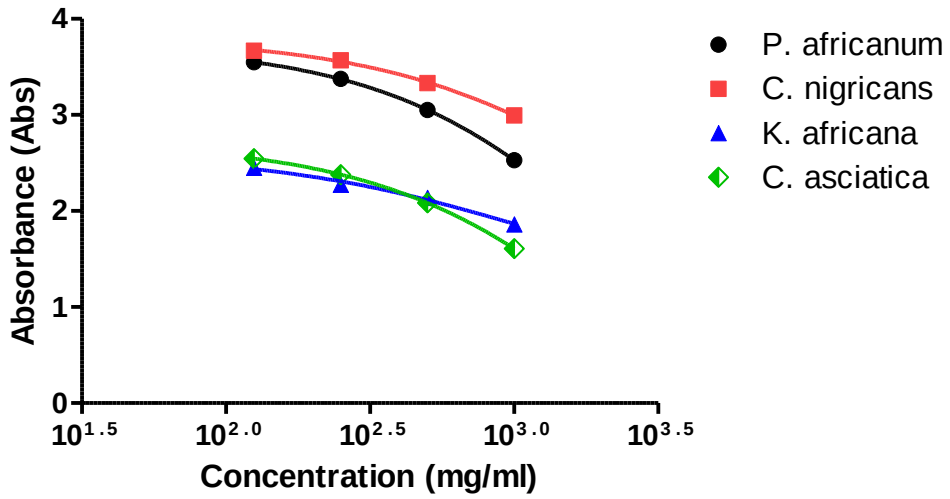


Figure 4: A graph of plant extract concentration against absorbance of CCRF-CEM cells as assessed by MTT assay. Absorbance (Abs) gives an approximate value of the number of live CCRF-CEM cells detected after subjecting the cells to varying concentrations (mg/ml) of the plant extracts, and also became useful in determining the IC_{50} values of the plant extracts as indicated in Table 4.

4.7. The IC_{50} values of the plant extracts used in the study

The MTT assay on the four different concentrations of the plant extracts gave variations in the absorbance of the CCRF-CEM cells. The logarithm of the plant extract concentration and absorbance of the CCRF-CEM cells helps to determine the response of the CCRF-CEM cells half way between the maximal response and maximum concentration of the plant extract as illustrated in Table 4.

Table 4. The half way response (IC_{50}) values of the plant extracts. The IC_{50} values under the Best-Fit value category are of interest in determining the cytotoxicity of the plant extracts to the CCRF-CEM cells.

	P. africanum	*C. nigricans*	*K. africana*	*C. asiatica*
log(inhibitor) vs. response (three parameters)				
Best-fit values				
Bottom	-1.451	0.6930	1.071	-1.650
Top	3.740	3.808	2.591	2.725
$LogIC_{50}$	3.516	3.451	3.040	3.465
IC50	**3281**	**2828**	**1096**	**2920**
Span	5.191	3.115	1.520	4.375
95% Confidence Intervals				
Bottom	-6.569 to 3.667	-16.38 to 17.77	-7.978 to 10.12	-3.136 to -0.1648
Top	3.635 to 3.844	3.346 to 4.269	1.232 to 3.951	2.687 to 2.762
$LogIC_{50}$	2.936 to 4.096	0.09333 to 6.810	-2.284 to 8.363	3.259 to 3.671
IC_{50}	863.5 to 12470	1.240 to 6.450e+006	0.005206 to 2.308e+008	1816 to 4693
Span	0.1597 to 10.22	-13.57 to 19.80	-6.444 to 9.484	2.921 to 5.829
Number of points Analyzed	4	4	4	4

The IC_{50} values (3281, 2828, 1096, and 2920) in the table are in mg/mL, and they indicate the half maximal inhibitory concentration of the plant extracts. Furthermore, the IC_{50} value gives a representation of the minimum plant extract concentration that is likely to cause cell death or morphological alteration of the cultured CCRF-CEM cells.

The algorithm analysis in Table 4 was obtained from the graphical presentation of the plant extract concentrations against absorbance in Figure 4. According to the algorithm analysis in the Table 4, it is evident that *K. africana* has a likelihood of causing toxicity to the cells since its IC_{50} value is 1096mg/mL. Nevertheless, the other three plant extracts (*C. nigricans. P. africanum*, and *C. asiatica*) have no likelihood of causing toxicity to the CCRF-CEM cells since their IC_{50} best fit values are 2828mg/mL, 3281mg/mL, and 2920mg/mL respectively.

4.8. Microscopic determination of cytotoxicity level

Recurrent microscopic observations were made on the plates in the MTT assay setup for up to 72 hours. The plates had equal concentration of CCRF-CEM cells and varying concentrations of the plant extracts (500mg/ml and 1000mg/ml). There was no morphological changes in the *C. nigricans. P. africanum*, and *C. asiatica* plant concentrations for up to 72 hours after observing the plants under inverted microscope. The morphological changes on the CCRF-CEM cells after subjecting 1g/ml of *K. africana*, it was an evident sign that the *K. africana* had slight damaged to the CCRF-CEM cells, which was visible on day 3 of the cell cytotoxicity assay layout as illustrated on Figure 5.

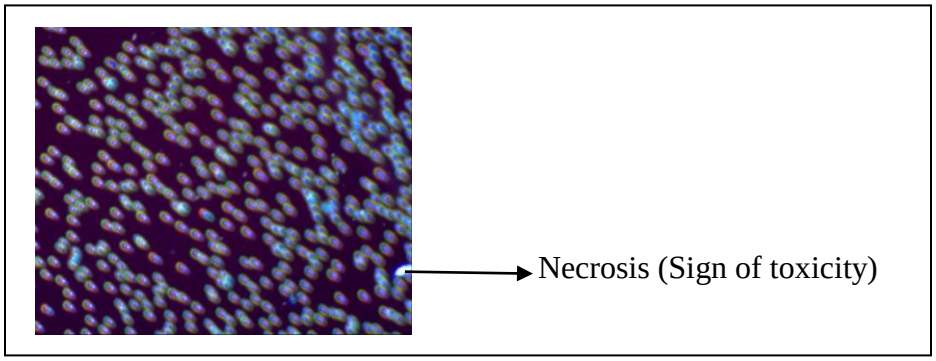
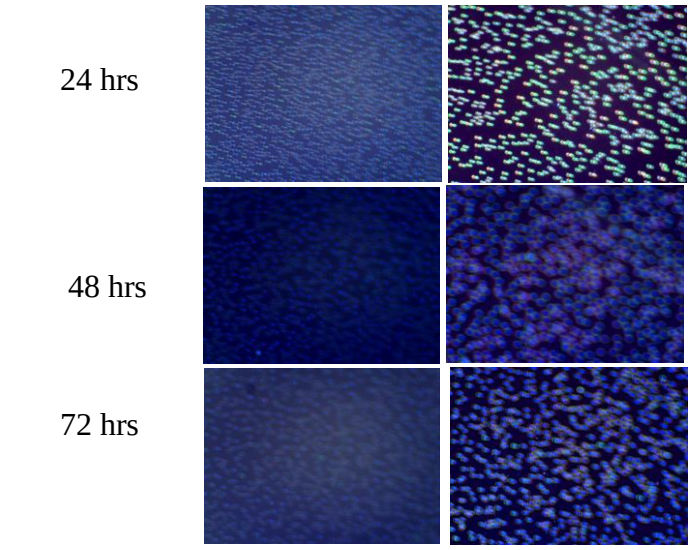


Figure 5: Necrosis of CCRF-CEM cells caused by 1g/mL of *K. africana.* The highlighted cell shows signs of cytoplasmic and plasma membrane swelling.

According to the IC_{50} values obtained from the MTT assay, *K. africana* has a likelihood of causing cytotoxicity to the CCRF-CEM at concentrations above 1096mg/mL. However, the other three plants extracts concentrations used in the study (*C. nigricans. P. africanum,* and *C. asiatica*) had no sign of causing toxicity to the CCRF-CEM leukemia cells after 72 hours incubation even after treating the cells will 1g/ml of the plant concentration. The microscopic assessment of toxicity were in tandem to the MTT assay test, which are showed in Table 4.

4.9. Cell proliferation and viability assessment

Microscopic analysis was used to determine the cell proliferation and trypan blue exclusion test was used to determine the cell viability. Observations under inverted microscope of the co-culture system showed that the CCRF-CEM cells maintained their morphology form from 24 hours to 72 hours. After counting the viable cells using the trypan blue exclusion test at every 24 hours, 48 hours and 72 hours, the treated cells continue to proliferate likewise to the control (untreated) cells, although the plant extracts slightly lowered the rate of cell proliferation (Figures 6 to Figure 9). The cell count for the controls for human leukemia cells were as follows; Day 0: 5.0 X 10^5 cells, Day 1: 9.75 X 10^5 cells and Day 2: 1.5 X 10^6 cells.



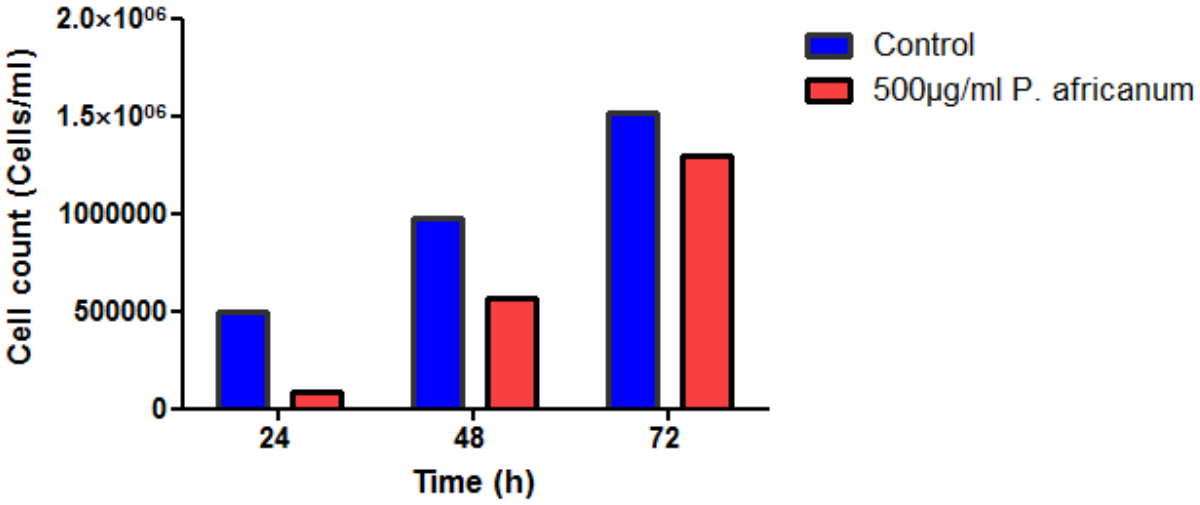


Figure 6(i): Effect of 500mg/mL of *P. africanum* bark extract on the proliferation of CEM-SS cell line under inverted microscope. The cell viability on the treated plates on day 0 was 8.5 X 10^4, day 1 was 5.71 X10^5, and on day 2 was 1.3 X 10^6 cell per mL.

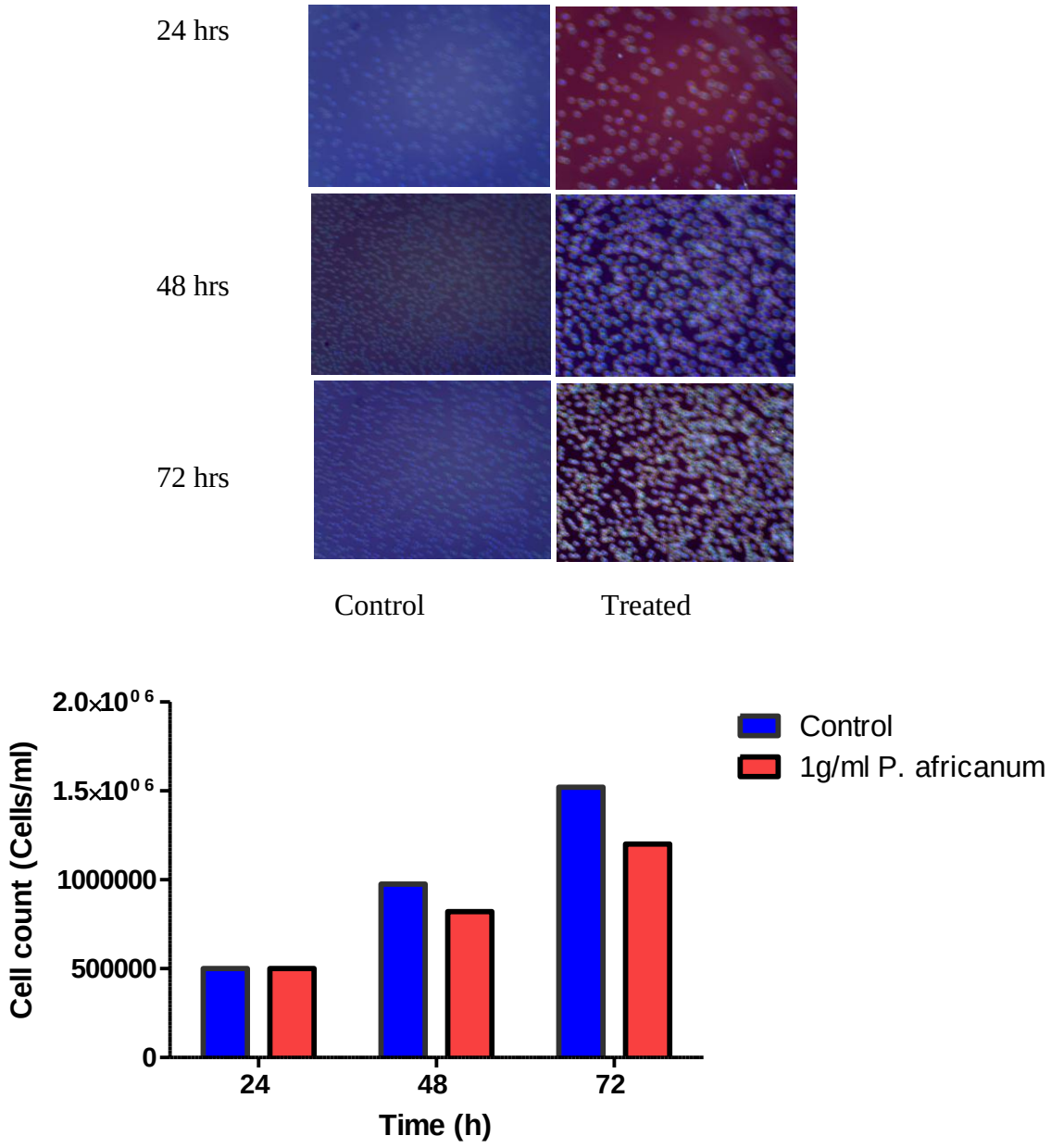
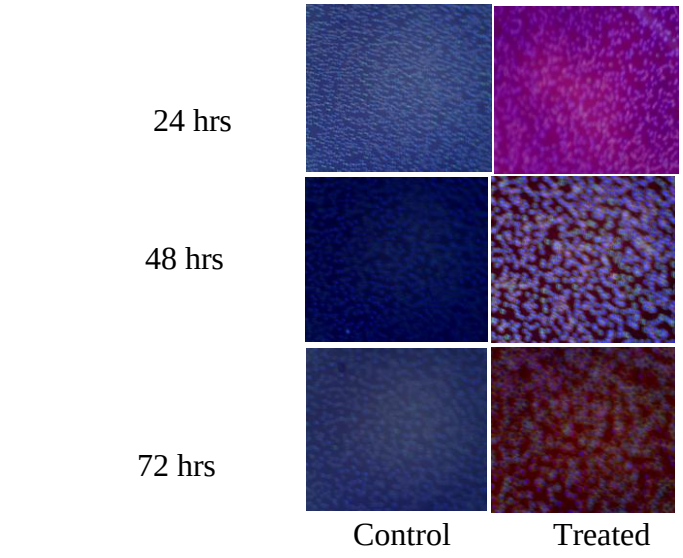


Figure 6(ii): Effect of 1g/mL of *P. africanum* bark extract on the proliferation of CEM-SS cell line under inverted microscope. The cell viability on the treated plates on day 0 was 5.0 X 10^5, day 1 was 8.2 X 10^5, and on day 2 was 1.2 X 10^6 cells per mL.



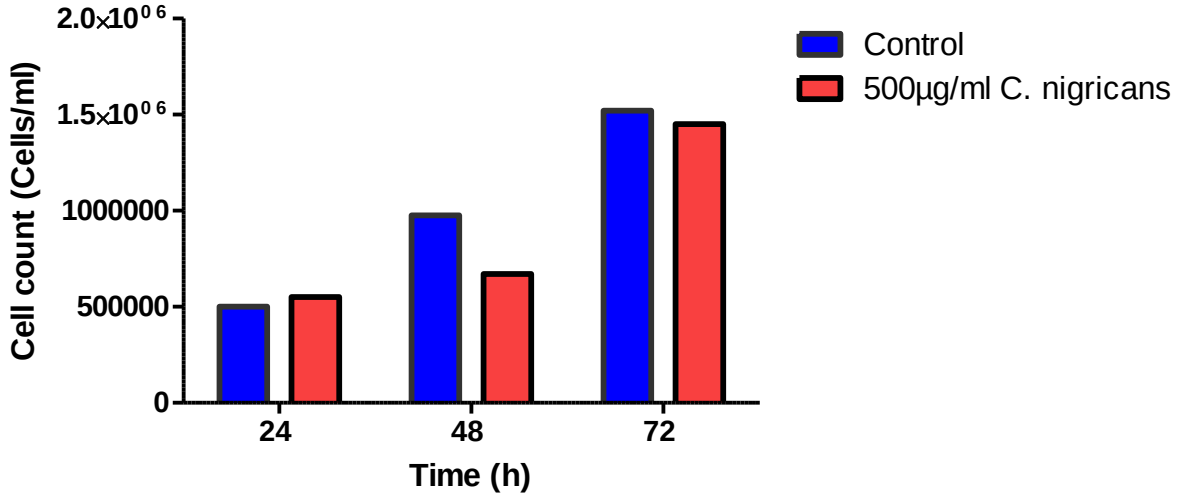


Figure 7(i): Effect of 500mg/mL of *C. nigricans* leaves extract on the proliferation of CEM-SS cell lines under inverted microscope. The cell viability on the treated plates on day 0 was 5.5 X 10^5, day 1 was 6.7 X 10^5 and on day 2 was 1.45 X 10^6 cells per mL.

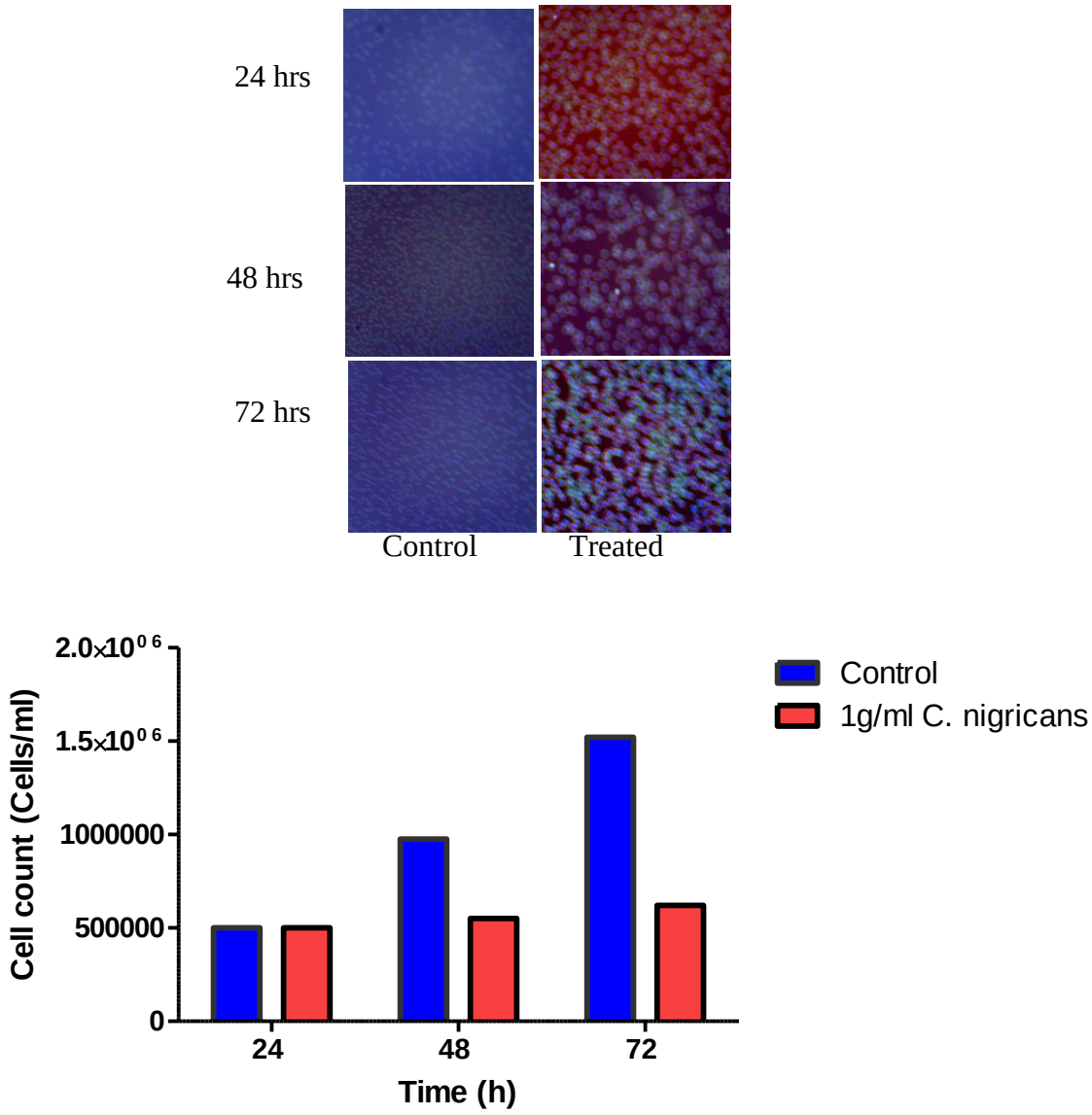
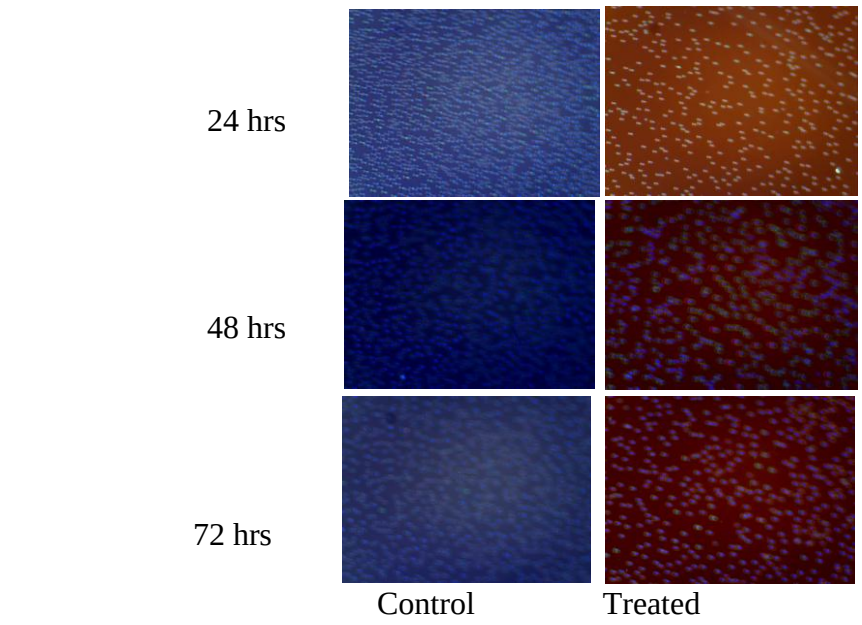


Figure 7(ii): Effect of 1g/mL of *C. nigricans* leaves extract on the proliferation of CEM-SS cell line under inverted microscope. The cell viability on the treated plates on day 0 was 5.0 X 10^5, day 1 was 5.5 X 10^5 and on day 2 was 9.2 X 10^5 cells per mL.



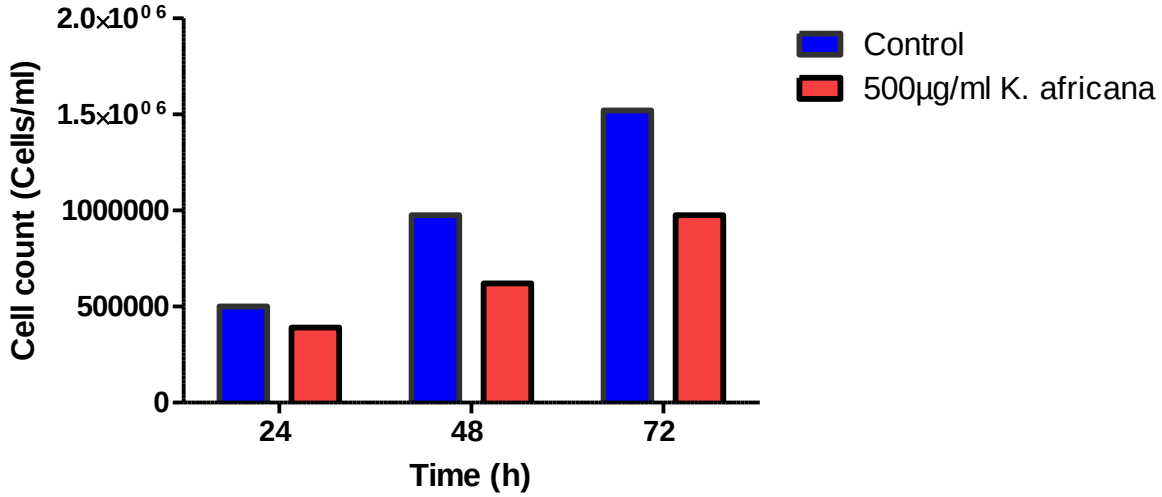


Figure 8(i): Effect of 500mg/mL of *K. africana* fruit extract on the proliferation of CEM-SS cell lines under inverted microscope. The cell viability on the treated plates on day 0 was 3.9 X 10^5, day 1 was 6.2 X 10^5 and on day 2 was 9.75 X 10^5 cells per mL.

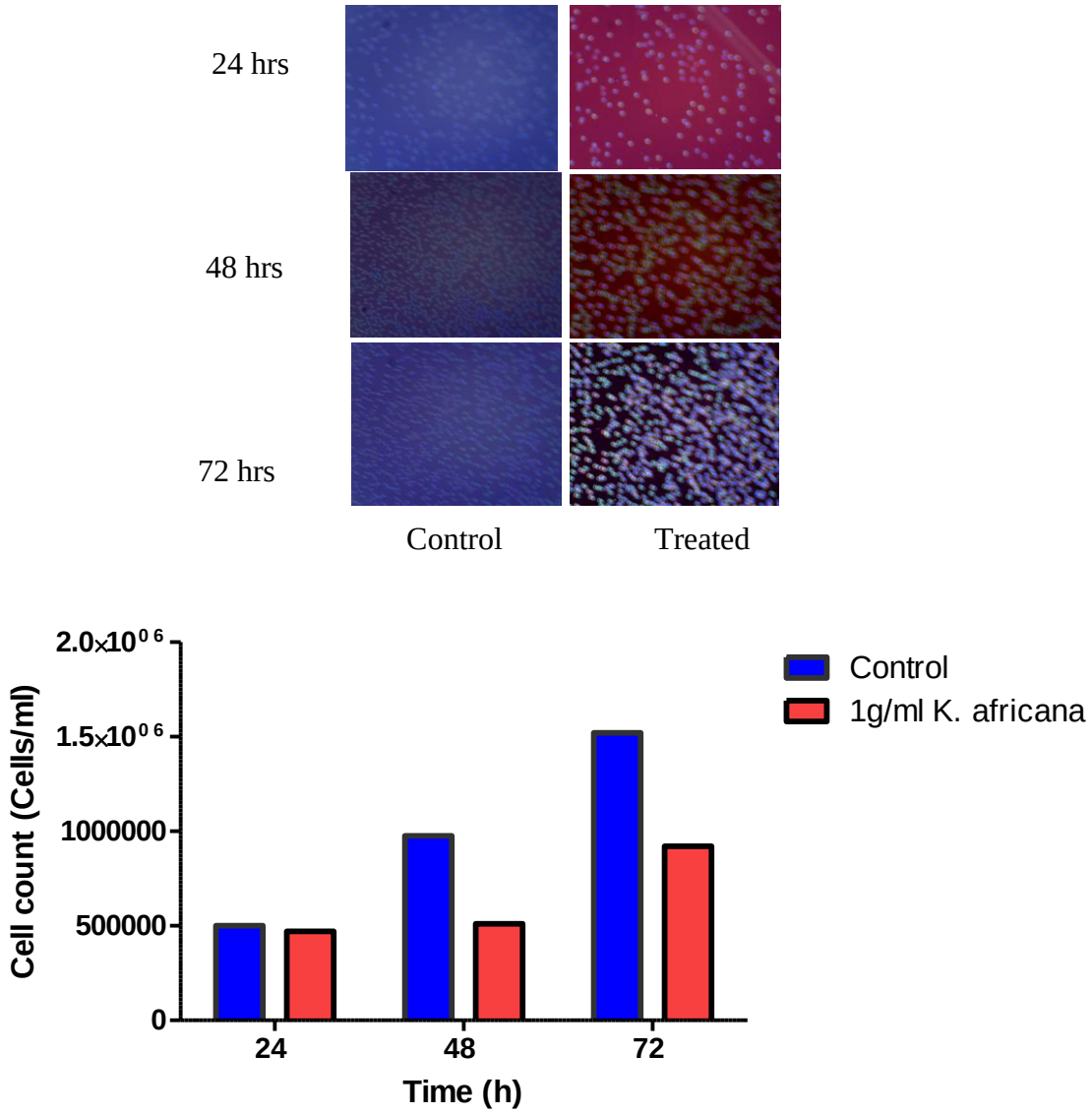
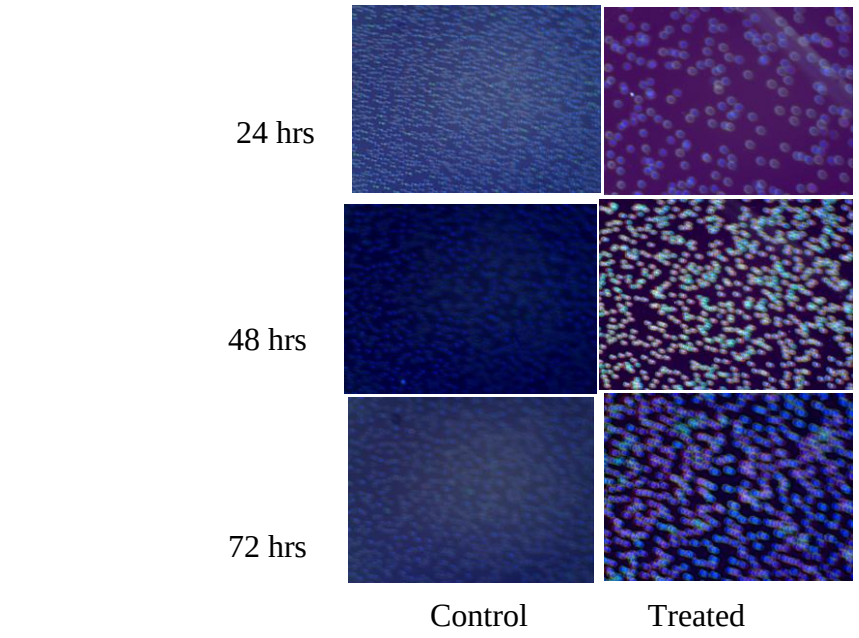


Figure 8(ii): Effect of 1g/mL of *K. africana* fruit extract on the proliferation of CEM-SS cell lines under inverted microscope. The cell viability on the treated plates on day 0 was 4.7 X 10^5, day 1 was 5.1 X 10^5 and on day 2 was 9.21 X 10^5 cells per mL.



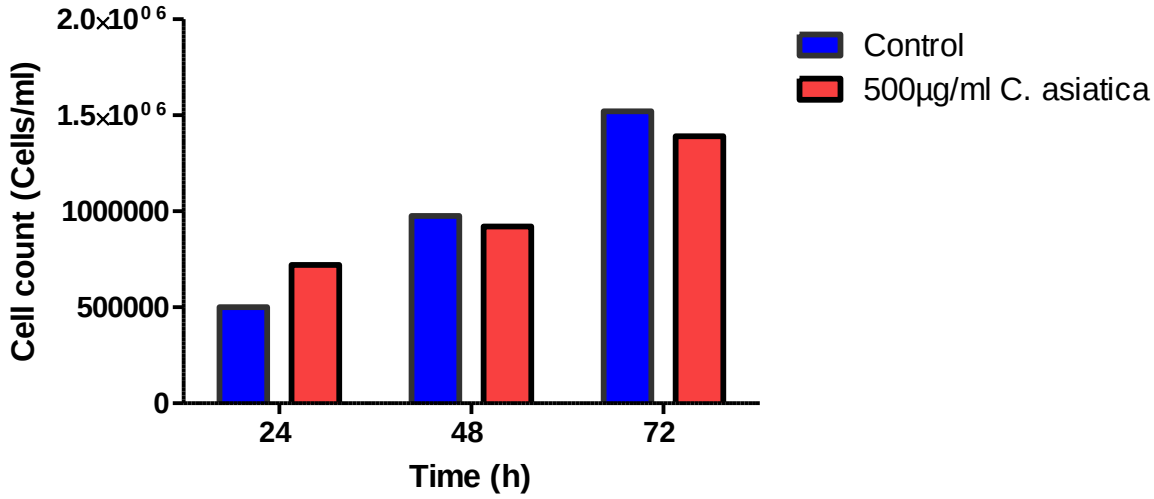


Figure 9(i): Effect of 500mg/mL of *C. asiatica* leaves extract on the proliferation of CEM-SS cell lines under inverted microscope. The cell viability on the treated plates on day 0 was 7.12 X 10^5, day 1 was 9.2 X 10^5 and on day 2 was 1.39 X 10^6 cells per mL.

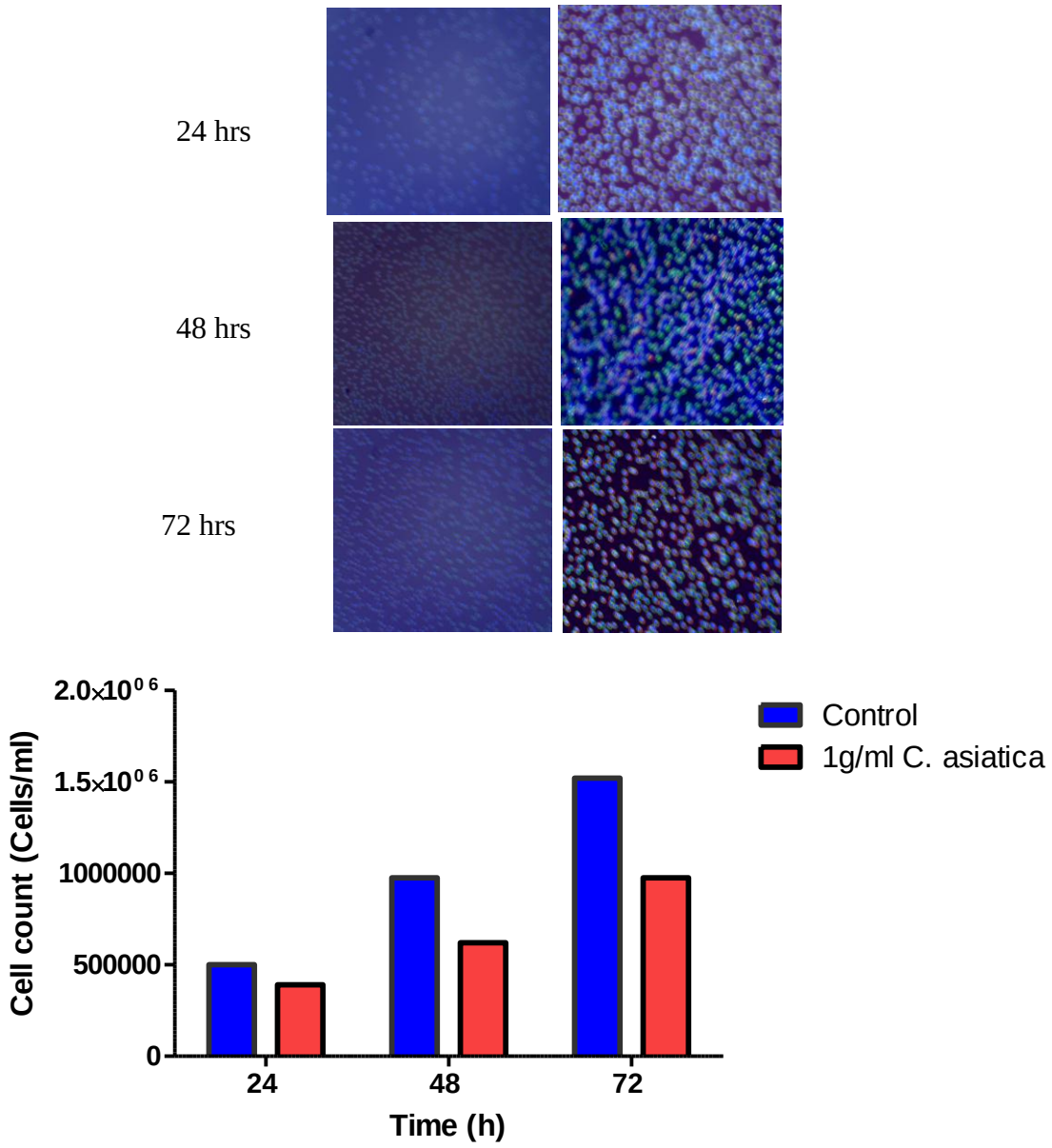


Figure 9(ii): Effect of 1g/mL of *C. asiatica* leaves extract on the proliferation of CEM-SS cell lines under inverted microscope. The cell viability on the treated plates on day 0 was 5.0 X 10^5, day 1 was 8.4 X 10^5 and on day 2 was 1.1 X 10^6 cells per mL.

Cell growth and morphological alterations of CCRF-CEM cells with the tested plant extracts in this study were compared with the control cells (untreated) for 72 hours, which maintained their original morphology form throughout the experiment. After recovery of the cell

growth, it was observed that the antiproliferative effect was dose dependent (Figure 10). The number of CCRF-CEM cells at 72 hours was slightly higher as compared to the number of cells at 24 hours. Additionally, the MTT results (Appendix 3) determined the metabolic activity of the CCRF-CEM cells, which could be summarized by a graph of plant concentration against cell viability as shown in Figure 10. The graph shows that the CCRF-CEM cell viability decreased with increase in plant concentrations of *P. africanum, C. nigricans, K. africana* and *C. asiatica*.

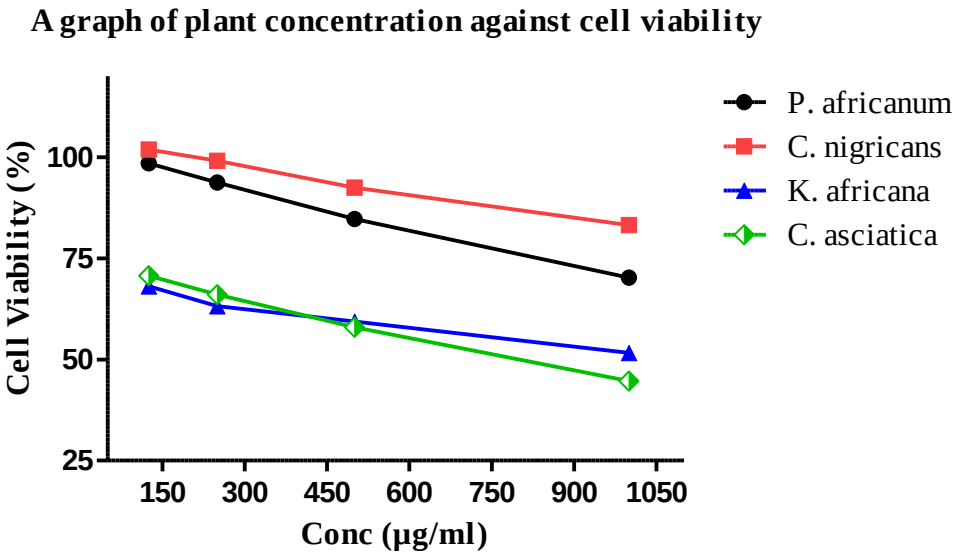


Figure 10: CCRF-CEM cells percentage in the plant extracts treated samples. Cell viability decreased with increase in plant concentration.

CHAPTER FIVE

DISCUSSION

5.1. Antimicrobial properties of the tested plant extracts

The selection of the microbial pathogens used in this study were based on the ease of cultivating them in the level 2 laboratory and infectivity of opportunistic diseases in HIV and AIDS patient. The *S. aureus, E. coli* and *C. albicans* strains are virulent in causing secondary opportunistic infections in HIV and AIDS individuals and have undergone mutations, hence becoming resistant to the available antidotes in the market. All the plant extracts tested showed promising antibacterial and antifungal activity against the test strains. However, the results of disks susceptibility test showed that *S. aureus* and *E. coli* were highly susceptible to penicillin with average diameter zone of inhibition of 20mm and 17mm respectively, whereas, *C. albicans* was highly susceptible to fungizone (Amphotericin B) with an average diameter zone of inhibition of 15mm. According to the Clinical and Laboratory Standards Institute, the acceptable standard diameter zone of inhibition for sensitive organism for antibiotic and antifungal are >16mm and >14mm respectively (CLSI, 2007). The extracts of *P. africanum* plant showed the greatest antimicrobial activity (inhibition zone 19mm at 500mg/ml). It is known that *P. africanum* is rich in saponin and tannins compounds, which have remarkable antimicrobial activity (Brusotti *et al.*, 2012). For the other plant extracts (*C. nigricans, K. africana,* and *C. asiatica*), the average inhibition zones observed against these pathogens ranged from 7mm to 10mm (Table 1). These values fall within the range of resistant and intermediate sensitive when compared with control standards. Several studies have reported bioactivity of crude extracts of medicinal plants within such range of inhibition zones (Eloff, 1998; Oyedeji and Afolayan, 2005; Ayo *et al.*, 2007; Agyare *et al.*, 2013).

The relationship between zone of inhibition and MIC value may or may not be related, since the crude extracts have mixture of phytoconstitutents that may influence the diffusion power of the active constituents (Ahmad and Aqil, 2007). The use of plants to heal infectious diseases has been extensively applied by people considering the widespread resistance to conventional antibiotics. In this study, the MIC values of *P. africanum* and *C. asiatica* were lower than the MBC values, suggesting that the plant extracts were bacteriostatic at lower concentration and bactericidal at higher concentration. However, data from the previous studies shows that plant extracts have great potential for therapeutic treatment of opportunistic infections, although this potential has not undergone complete investigation (Richter *et al.*, 2007; Wiegand *et al.*, 2008; Gohil *et al.*, 2010). The MBC and MIC results exhibit that the extracts inhibit bacteria growth without killing the bacteria. However, the traditional preparations of the plant extracts by the traditional health practitioners involves use of water instead of more lipophilic solvents. This limits the possibility of extracting all the active compounds in the plant contributing to the patient consuming large quantities of the concoction. Dosage is important with regard to the solvent being used during extraction. If water is used, the dosage would be higher, whereas the same dosage using a lipophilic solvent may be toxic. In this study, ethanol was most efficient considering its safety toward conducting antimicrobial assays after extraction as illustrated by Eloff (1998). Determining the antibacterial and antifungal properties of medicinal plants is helpful to the rural communities and informal settlements (Buwa and van Staden, 2006). This study offers scientific rationale on the dosage of using the four ethanol extracts of medicinal plants for treatment of HIV and AIDS related opportunistic infections.

5.2. Cytotoxicity of the tested medicinal plants

Assessment of the tested plant extracts for their IC_{50} (dose that inhibits cell growth by 50%) values was at the concentration range of 500mg/mL and 1000 mg/mL. The selection of the concentration range depended on the microbial concentration that would inhibit growth of microbial pathogens (Kuete *et al.*, 2011). Concentration measurements have demonstrated the toxicity of different medicinal plants that are substitutes toward curing diseases like malaria, cancer, AIDS among others (Kuete *et al.*, 2015). The potential of the plant extracts to be active against resistant pathogens has minimized the toxicity effects caused by high dosage of conventional drugs (Abdelwahab *et al.*, 2009; Elsyana *et al.*, 2016). A recent study by Tiwary and colleagues, showed that organic extracts of the *p. africanum* and *K. africana* plant species had low cytotoxicity levels at a single dose of 250µg/ml against MCF7 and Vero cell lines (Tiwary *et al.*, 2015). Earlier studies conducted by Tiwary and colleagues had showed that organic extracts of *K. africana* had low cytotoxicity levels with a potent cytotoxic activity of <50% at a single dose of 300µg/ml against cultured KB cells (Tiwary *et al.*, 2015). Teixeira *et al.* (1984) have reported that infusions prepared from the medicinal plants *Solanum torvum, Oscbekia nepalensis, Kigelia africana, Selaginella monospora,* and *Mesua ferrea,* showed cytotoxic levels of <50% at a single dose of 450 µg/ml and no activity for *Piptadeniastrum africanum, Centella erecta, Chamaecrista fasciculata* which are comparatively similar to this study. In this study, *P. africanum* (R^2=0.9962, *P*=0.0019), *C. nigricans* (R^2=0.9935, *P*=0.0032) and *C. asiatica* (R^2=0.9954, *P*=0.0023) had no remarkable observable cytotoxic effect against CCRF-CEM cells from the microscopic results and IC_{50} (mg/mL) values from MTT assay, although it is evident that, 1096mg/mL of *K. africana* (R^2=0.9706, *P*=0.0148) damages <25% of the total CCRF-CEM cells used in the study layout. The morphological changes of CCRF-CEM

cells as a result of cytotoxicity of *K. africana* was confirmed through microscopic observations. The CCRF-CEM cells indicated signs of cell apoptosis after administering 1g/mL of *K. africana* plant extract and incubation for 72 hours (Figure 5).

Cytotoxicity assays such as MTT measure DNA damage and apoptosis (Taatjes *et al.*, 2008; Smith-Hall *et al.*, 2012). In this study the novel sign of toxicity was necrosis as illustrated in Taatjes *et al.,* (2008). However, high concentration of *K. africana* resulted in slight morphological changes to CCRF-CEM, which is a novel sign of toxicity (Figure 5). The necrosis in this case was evidenced by cytoplasmic and plasma membrane swelling (Copeland, 2005). These observations means that the selected plant extracts has toxic effects at high dosage of ≥1096mg/mL on CCRF-CEM cells.

5.3. Antiproliferative effects of the tested plant extracts

The CCRF-CEM cells used in the study maintained their cell growth and morphology throughout the assay due to the presence of intrinsic growth factors that were not affected by the active compound of the plant extracts. The active molecules and compounds in the plant extracts would have caused the variations on the standardized growth of the cultured cells. The increase in concentration of the tested compound had slight increase on antiproliferative effect. The plant extract concentrations slightly decreases the growth and proliferation of the CCRF-CEM but did not alter the morphology of the CCRF-CEM cells. The results are in tandem with previous studies (Teixeira *et al.*, 1984; Syed Abdul Rahman *et al.,* 2013; Tiwary *et al.,* 2015), whereby after the cell growth recovered from 24 hours treatment, the MCF-7, and HCT-116 cells had no observable morphological changes (Syed Abdul Rahman *et al.,* 2013). The findings by Tiwary *et al*., (2015) agrees with the findings of this study, hence confirming that the tested plant extracts used in this study were not capable of causing cytotoxicity in CCRF-CEM cells (Tiwary *et al.,*

2015). These observations could mean that the plants extracts slightly reduced the multiplication of CCRF-CEM cells without causing cell death.

The MTT assay applied the principle of formazan crystal formation through dehydrogenesae mitochondria activities in living cells. The rate of formation of MTT crystal formazan was directly proportional to the number of living cells. According to Mutua *et al.,* (2013), *C. asiatica* and *K. africana* aqueous extracts suppressed the proliferation of keritinocytes and human dermal fibroblasts cell lines. The ethanolic extracts of the roots of *C. nigricans* had the lowest antiproliferative activities, however, the leaves of the same plant showed similar results as illustrated in this study (Mutua *et al.,* 2013; Elsyana *et al.,* 2016). The plant extracts in this study did not alter the actively proliferating CCRF-CEM cells even after inducing the cells with the highest plant extract concentration of 1g/ml (Figure 6 to Figure 9). The lack of alteration in the cell proliferation was due to the presence of secondary metabolites such as saponins and polyphenols which increased the metabolic activity of the CCRF-CEM cells (Naik and Kabnoorkar, 2013; Tiwary *et al.*, 2015). It is also evident that the plant extracts did not release toxic molecules that may result to diminishing activity of the CCRF-CEM cells (Copeland, 2005).

High concentration of *K. africana* have a high likelihood of releasing toxic molecules and diminishing the cellular metabolic activities of mammalian cells. The other plant extracts used in the study (*C. nigricans. K. africana,* and *C. asiatica*) showed no signs of releasing toxic molecules and diminishing the cellular activities of the cultured cells. Although all the plant extracts concentrations used in the study did not inhibit cell proliferation, there was decrease in cell multiplication with increase in the plant concentration. It is evident that the tested plant extracts have a high efficacy and have less noxious side effects, in dealing with infections (Babu

et al., 1995; Smith-Hall *et al.*, 2012). According to the study observations, the cell proliferation decreased with increase in plant concentration. This could be taken to suggest that the plant extracts had the potency of causing cell death at high concentration.

In vitro assay is an indicator of mammalian cell survival and growth (Smith-Hall *et al.*, 2012). Cell viability assay was conducted through counting the remaining viable cells after every 24 hours up to 72 hours and viability graphs obtained (Figure 6-9). It is evident that the treated cells continue to proliferate also to the control (untreated) cells, although the plant extracts lowered the rate of cell proliferation. Inclusion of control wells in the MTT viability assays (Appendix 3) was essential to compare possibilities of cell impairment, membrane integrity and changes in cell morphology which elicit strong likelihood of cell growth inhibition (Copeland, 2005; Smith-Hall *et al.*, 2012). According to this study results, there were no signs of cell growth inhibition during the 72 hours of incubation. The results of this study suggest that the tested plant extracts had the sheer potency of treating HIV and AIDS opportunistic infections caused by *S. aureus, E. coli* and *C. albicans*, also the plant extracts were relatively nontoxic and were safe for traditional or complementary medicine.

CHAPTER SIX

SUMMARY OF FINDINGS, CONCLUSION AND RECOMMENDATION

6.1. Summary of Findings

In this study, the ethanolic extracts of *P. africanum, C. nigricans, K. africana,* and *C. asiatica* were found to have antibacterial, antiproliferative properties, and low toxicity on *in vitro* lymphoid CCRF-CEM cell lines. *P. africanum* showed antimicrobial inhibitory activity on *S. aureus, C. nigricans* on *S. aureus, K. africana* on *E. coli,* and *C. asiatica* on *S. aureus* and *C. albicans*. Cytotoxicity of the plant extracts was only depicted by *K. africana* at an IC_{50} value of 1096mg/ml. It was found out that there was no signs of cell growth inhibition although the cell proliferation of the CCRF-CEM decreased with increase in plant extract concentration.

6.2. Conclusions

These results demonstrate that:

i. The four plant extracts (*P. africanum, C. nigricans, K. africana,* and *C. asiatica*) can be an important source of antibacterial and antifungal properties which are essential in management of HIV and AIDS related opportunistic infections. The antibacterial and antifungal activity vary with the species of the plant extracts. The extracts of *P. africanum,* and *C. nigricans* have high affinity against *S. aureus,* extracts of *K. africana* have high affinity against *E. coli*, and extracts of *C. asiatica* have high affinity against *S. aureus* and *Candida albicans*. The effect of the antimicrobial activity increases with increase in the plant extract concentration.

ii. The plant extracts are not toxic to the leukemia cell lines (CCRF-CEM) at concentration less than 1g/ml. The cytotoxicity effect vary with the species of the plants. The extracts of *K. africana* have a high likelihood of causing toxicity to the leukemia cells at a

concentration of 1g/mL unlike the other plant extracts (*P. africanum, C. nigricans,* and *C. asiatica*) which have no likelihood of causing toxicity at the concentration of 1g/mL.

iii. The ethanolic extracts of *P. africanum, C. nigricans, K. aficana,* and *C. asiatica* possesses anti-proliferative activities and slightly inhibit the growth (<25%) of CCRF-CEM cells *in vitro.* The effect of cell proliferation vary with the species of the plants. However, the ethanolic plant extracts used in the study decreased the cell proliferation rate of the cultured CCRF-CEM cells.

6.3. Recommendation from the study

i. Studies need to be conducted to isolate the active compounds with antibacterial and antifungal activity in *P. africanum, C. nigricans, K. africana* and *C. asiatica,* which will be helpful to lower the required doses and quantities from the crude extracts.

ii. Conduct morphological assessment of apoptosis using the isolated active compounds from the plant species that showed high inhibitory activity during screening, and before being used in new therapeutic treatment.

iii. Conduct phytochemical analysis of these extracts to determine the synergism of the possible compounds that cause antiproliferation activity and cytotoxicity to the cultured cells.

6.3. Recommendations for future studies

i. Fractionation of bioactive compounds from the plant extracts need to be conducted. This will help to determine the minimal inhibitory concentration and minimal bactericidal concentration of each fraction with an aim of reducing the quantity required for consumption in the management of HIV and AIDS-related opportunistic infections.

ii. The antiproliferation rate patterns and assessment need to be conducted on the activity profile of the plant extracts to extensively determine the plant potency based on dose response using both lymphoid and epithelial cells.

iii. The duration of effect and potential forms of toxicity need to be determined through incorporation of IC_{90} and IC_{50} in the study framework. This will help to depict the morphological alterations on the cells in a dose-dependent response.

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APPENDICES

Appendix 1: Gram Staining

Gram stain is a technique used in microbiology to classify and differentiate bacteria into two categories, gram positive and gram negative bacteria. For each microorganism; the following procedure was conducted to identity pure gram positive and gram negative bacteria:

i. Cleaning of the slide using 70% alcohol and then labeling.
ii. Placing a drop of normal saline on the slide.
iii. Sterilizing wire loop, picking of a single-colony from the plate, and making a thin film on the slide.
iv. Air-drying followed by heat-fixing by passing the slide over the flame.
v. Flooding of the slide with crystal violet for one minute, then washing with running water.
vi. Flooding the slide with Gram's iodine for one minute, then washing with running water.
vii. Decolourising the slide with drops of acetone for 20 seconds over running water.
viii. Flooding the slide with neutral red for one minute, then wash with running water.
ix. Drying the slide between blotting paper.
x. Observing the slide over a light microscope using oil immersion at X100.

Appendix 2: Kirby-Bauer disk diffusion protocol

Kirby-Bauer disk diffusion antibiotic sensitivity testing is a susceptibility test used in microbiology to test the effectiveness of an antibiotic on a specific microorganism. The following procedure is a brief description of the preparation as illustrated by Bonev *et al*., (2008).

i. Allowing MH agar plate for each organism to be tested to come to room temperature
ii. Labeling appropriately each MH agar plate for each organism to be tested
iii. Touching 4-5 isolated colonies of the organism to be tested using a sterile inoculating loop
iv. Suspension of the organism in 2mL of sterile saline
v. Vortexing the saline tube to create a smooth suspension
vi. Dipping s sterile swab into the inoculum tube
vii. Rotating the swab against the side of the tube to remove excess fluid

viii. Inoculating the dried surface of MH agar plate by streaking the swab three times over the entire agar surface

ix. Rimming the plate with the swab to pick up any excess liquid

x. Appropriate placing of plant extract-impregnated disks on the surface of the agar using sterile forceps

xi. Replacing the lid, inverting the plates and placing them in a 37^0C incubator for 18-20 hours

xii. Following incubation is measuring the zone sizes to the nearest millimeter using a ruler

xiii. Recording the zone size on the recording sheet

xiv. Interpreting and reporting of the results

Appendix 3: Cytotoxicity assay

The cytotoxicity assay involved using the MTT assay kit that measured the cell viability and proliferation. Cells were resuspended in a 96 well plate at 1 x 10^3 per mL. The test cells concentrations were arranged as shown in the table below in triplicates. The columns of the plate are represented by numbers 1 to 12 whereas the rows are represented by alphabets A to H.

	1	2	3	4	5	6	7	8	9	10	11	12
A	RPMI Media Alone	RPMI Media Alone										RPMI Media Alone
B		RPMI+10^3 Human leukemia	P. afr (125mg/ml)	C. nigr (125mg/ml)	K. afr (125mg/ml)	C. asc (125mg/ml)	P. afr (500mg/ml)	C. nigr (500mg/ml)	K. afr (500mg/ml)	C. asc (500mg/ml)	RPMI+10^3 Human leukemia	
C												
D												
E			P. afr (250mg/ml)	C. nigr (250mg/ml)	K. afr (250mg/ml)	C. asc (250mg/ml)	P. afr (1g/ml)	C. nigr (1g/ml)	K. afr (1g/ml)	C. asc (1g/ml)		
F												
G												
H		RPMI Media Alone										

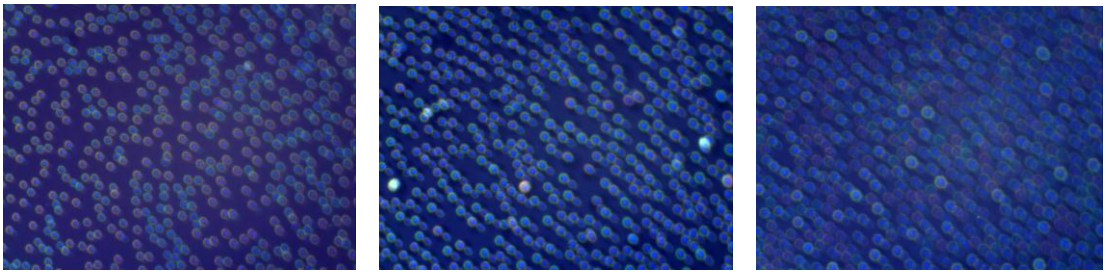
96-well plate cytotoxicity assay template

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.806	1.064	0.805	0.706	0.553	1.182	0.752	0.640	0.681	0.753	0.947	0.878
B	0.555	1.07	3.545	3.619	3.255	2.623	2.989	3.269	2.073	2.267	0.751	0.901
C	0.707	1.01	3.54	3.698	1.972	2.515	3.014	3.336	2.172	1.837	0.787	0.642
D	0.669	0.751	3.557	3.687	2.127	2.499	3.151	3.388	2.171	2.153	0.776	1.086
E	1.065	1.09	3.298	3.515	2.363	2.549	2.487	3.021	1.543	1.71	0.753	0.683
F	1.182	1.03	3.357	3.691	2.233	2.462	2.575	3.054	2.015	1.581	0.789	0.773
G	1.067	0.753	3.472	3.503	2.23	2.124	2.521	2.912	2.023	1.534	0.778	0.949
H	0.929	0.937	0.807	0.709	0.558	1.066	0.682	0.948	0.641	0.724	0.948	0.751

96-well plate cytotoxicity assay results. The table shows the results obtained after running the MTT cell proliferation assay.

Appendix 4: Samples of photomicrographs of cultured CCRF-CEM cells

The photomicrographs were obtained from an inverted microscope connected to a digital camera. The CCRF-CEM cells were induced with plant extracts and monitored for 3 days. The photomicrographs were taken after the end of each day to examine the cell morphology, cell proliferation, and cell viability. The photomicrographs showed a continuous process of cell proliferation without any alteration of the cell morphology.



Day 1 Day 2 Day 3

Appendix 5: Cell number and viability determination using a hemocytometer

Cells count was as follows:

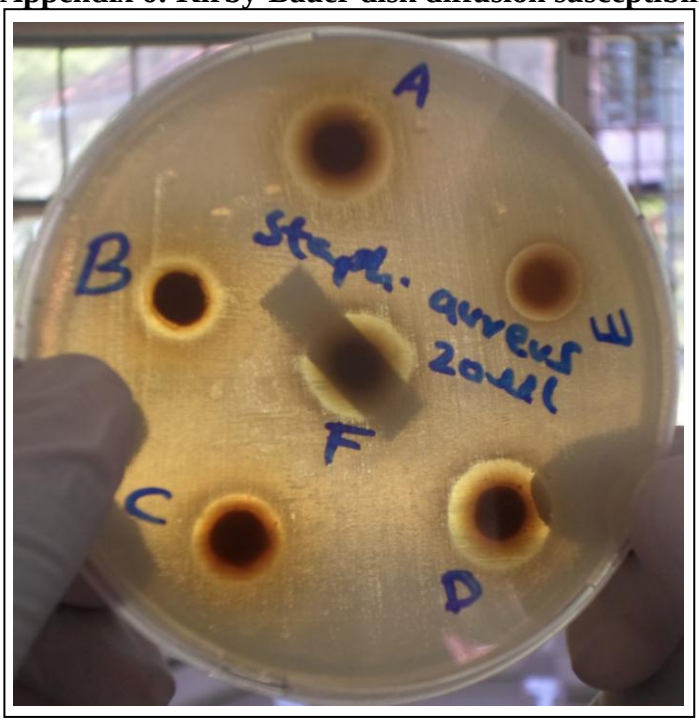
1. The hemocytometer with the cover slip were cleaned, dried and assembled.
2. A small amount of cell suspension was transferred to the edge of each of the two counting chambers.
3. The hemocytometer was placed under an inverted microscope and the cells viewed at 100X magnification.

4. Focus was on the four quandrants and the number of cells in each quadrant recorded.
5. The number of cells were averaged and multiplied by 10^4 cells/ml.

Cell viability was as follows:

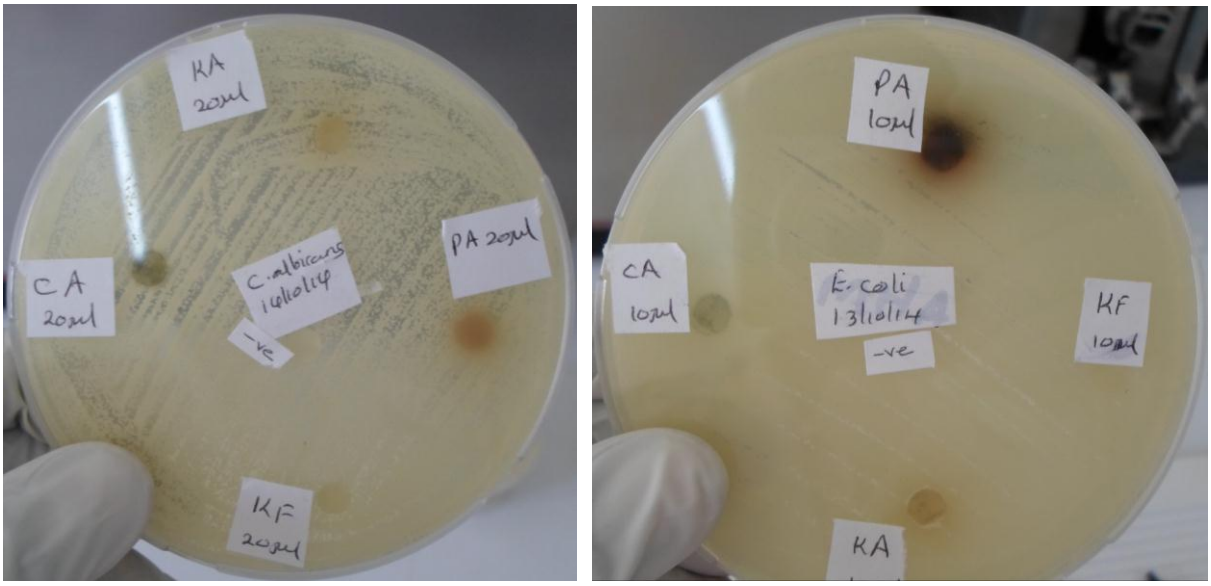
1. 10µl of cell suspension was mixed with 10µl of 0.4% trypan blue solution.
2. The cells were loaded into a clean, dry hemocytometer and observed under light microscope at 100X magnification.
3. The number non-viable cells will be stained dark blue whereas the viable cells do not take the dye. Cell viability was calculated as the number of viable cells divided by the total number of cells and expressed as a percentage.

Appendix 6: Kirby-Bauer disk diffusion susceptibility test results



A=*P. africanum*, B=*B. aegyptica*, C=*C. nigricans* D=*B. pilosa*, E=*C. asiatica* F=*K. foetidissima*

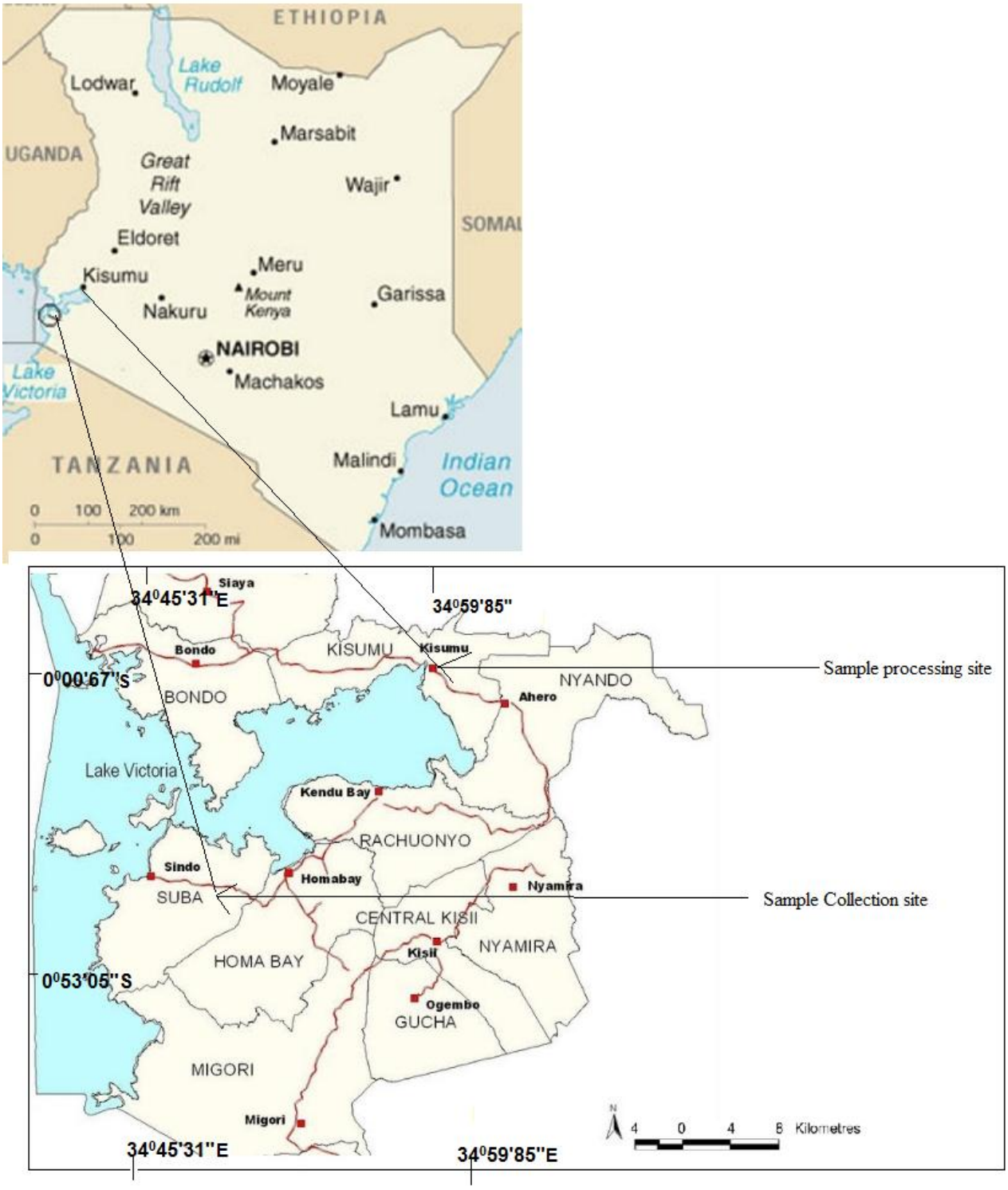
The Kirby-bauer disk diffusion susceptibility test results shows a plate swabbed with 24 hour old culture of *S. aureus.* Filter paper disks were impregnated with the 1g/ml of the plant extracts labelled A-F and carefully placed on the plate swabbed with *S. aureus.* The zones of inhibition were observed after 24 hours and their measurements recorders in millimeters.



KA=*K. africana*, PA=*P. africanum*, KF=*K. foetidissima*, CA=*C. asiatica*

Similary, the test results showed are obtained from plates swabbed with 24 hour old cultures of *C. albicans* and *E. coli*. Filter paper disks were impregnated with the 1g/ml of the plant extracts labelled KA, PA, KF, and CA and carefully placed on the swabbed plates. The zones of inhibition were observed after 24 hours and their measurements recorders in millimeters

Appendix 7: Study cite map



Appendix 8: Plant pictures and local names

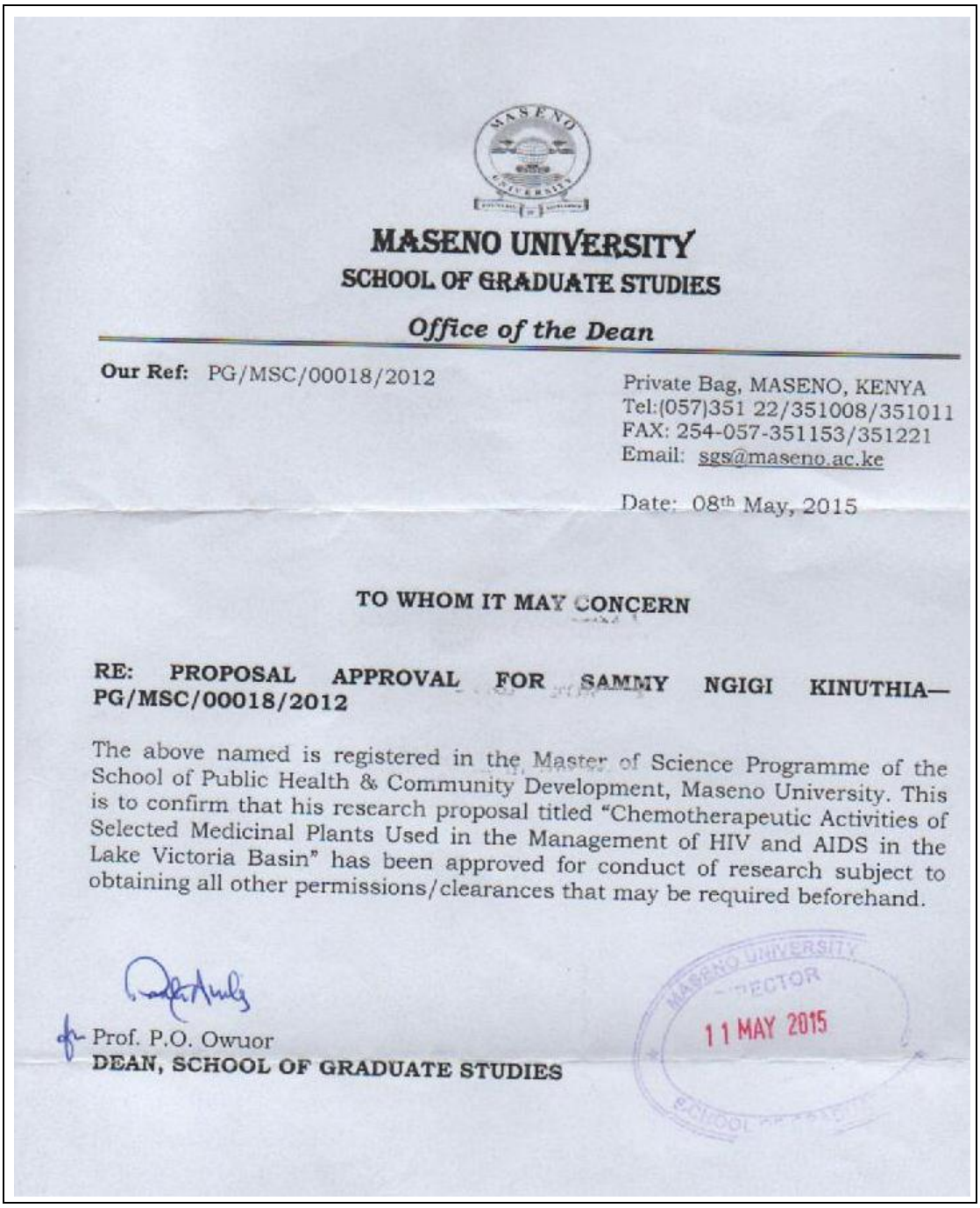
Scientific name: *Piptadeniastrum africanum*
Local name: Mpewere (Bantu)

Scientific name: *Kigelia africana*
Local name: Kumufunga (Luhya)

Scientific name: *Chaemacrista nigricans*
Local name: Achak (Dholuo)

Scientific name: *Centella asiatica*
Local name: Chikombe za chui (Digo)

Appendix 9: SGS research proposal approval letter



MASENO UNIVERSITY
SCHOOL OF GRADUATE STUDIES

Office of the Dean

Our Ref: PG/MSC/00018/2012

Private Bag, MASENO, KENYA
Tel:(057)351 22/351008/351011
FAX: 254-057-351153/351221
Email: sgs@maseno.ac.ke

Date: 08th May, 2015

TO WHOM IT MAY CONCERN

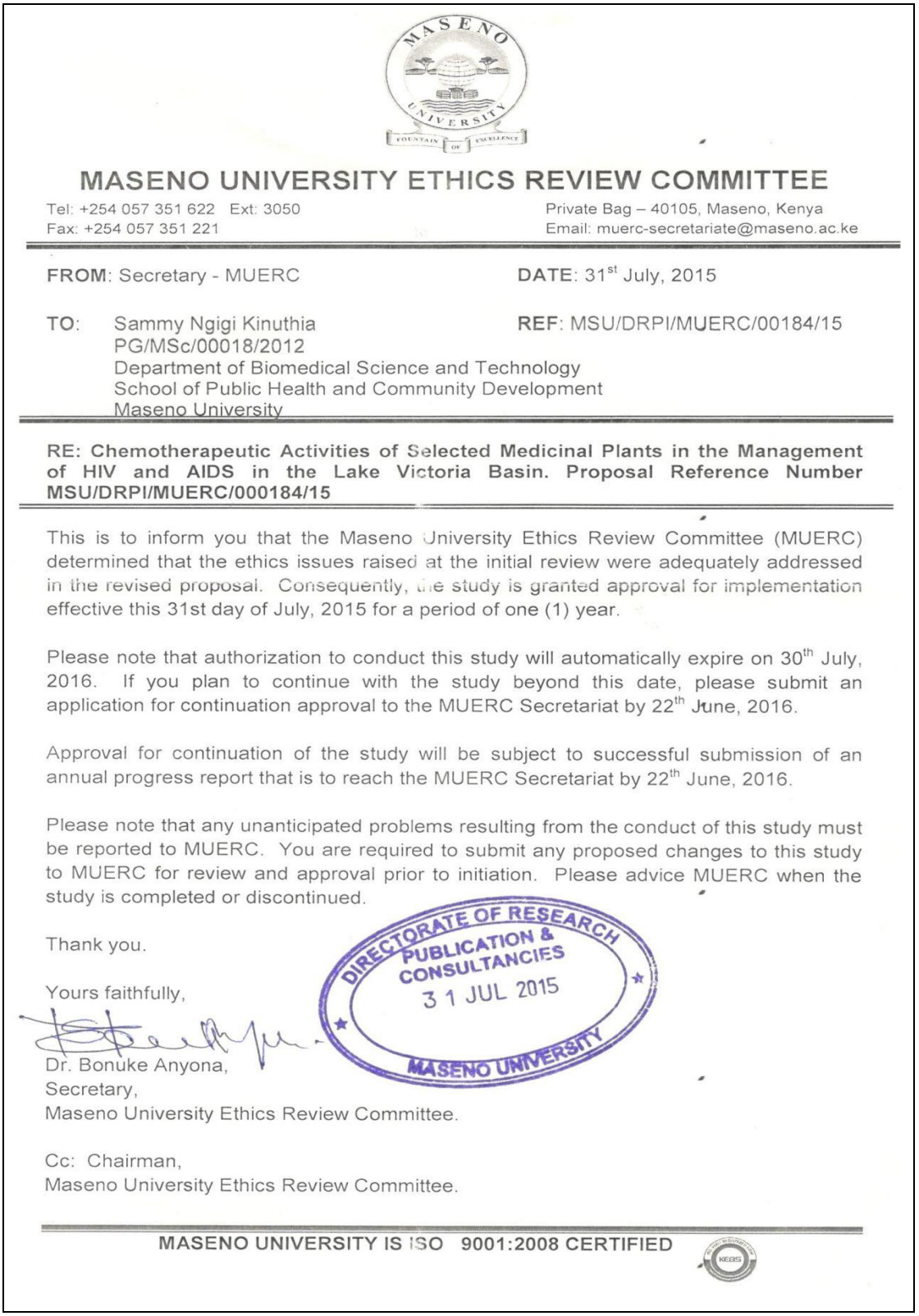
RE: PROPOSAL APPROVAL FOR SAMMY NGIGI KINUTHIA— PG/MSC/00018/2012

The above named is registered in the Master of Science Programme of the School of Public Health & Community Development, Maseno University. This is to confirm that his research proposal titled "Chemotherapeutic Activities of Selected Medicinal Plants Used in the Management of HIV and AIDS in the Lake Victoria Basin" has been approved for conduct of research subject to obtaining all other permissions/clearances that may be required beforehand.

Prof. P.O. Owuor
DEAN, SCHOOL OF GRADUATE STUDIES

MASENO UNIVERSITY
DIRECTOR
11 MAY 2015
SCHOOL OF GRADUATE STUDIES

Appendix 10: MUERC research approval letter



MASENO UNIVERSITY ETHICS REVIEW COMMITTEE

Tel: +254 057 351 622 Ext: 3050
Fax: +254 057 351 221

Private Bag – 40105, Maseno, Kenya
Email: muerc-secretariate@maseno.ac.ke

FROM: Secretary - MUERC

DATE: 31st July, 2015

TO: Sammy Ngigi Kinuthia
PG/MSc/00018/2012
Department of Biomedical Science and Technology
School of Public Health and Community Development
Maseno University

REF: MSU/DRPI/MUERC/00184/15

RE: Chemotherapeutic Activities of Selected Medicinal Plants in the Management of HIV and AIDS in the Lake Victoria Basin. Proposal Reference Number MSU/DRPI/MUERC/000184/15

This is to inform you that the Maseno University Ethics Review Committee (MUERC) determined that the ethics issues raised at the initial review were adequately addressed in the revised proposal. Consequently, the study is granted approval for implementation effective this 31st day of July, 2015 for a period of one (1) year.

Please note that authorization to conduct this study will automatically expire on 30th July, 2016. If you plan to continue with the study beyond this date, please submit an application for continuation approval to the MUERC Secretariat by 22th June, 2016.

Approval for continuation of the study will be subject to successful submission of an annual progress report that is to reach the MUERC Secretariat by 22th June, 2016.

Please note that any unanticipated problems resulting from the conduct of this study must be reported to MUERC. You are required to submit any proposed changes to this study to MUERC for review and approval prior to initiation. Please advice MUERC when the study is completed or discontinued.

Thank you.

Yours faithfully,

DIRECTORATE OF RESEARCH PUBLICATION & CONSULTANCIES 31 JUL 2015 MASENO UNIVERSITY

Dr. Bonuke Anyona,
Secretary,
Maseno University Ethics Review Committee.

Cc: Chairman,
Maseno University Ethics Review Committee.

MASENO UNIVERSITY IS ISO 9001:2008 CERTIFIED