ASSESSMENT OF ANTI-PROLIFERATIVE ACTIVITIES OF BIOACTIVE PHYTOCHEMICALS FROM FOUR SELECTED MEDICINAL PLANT EXTRACT'S AGAINST LUNG ADENOCARCINOMA CELL LINE

BY

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DECLARATION

I declare that this thesis is my original work and has not been presented to any other University or Institution for a degree or any other award.

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May God shower you with His blessings and give you good life.

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This work is sincerely dedicated to my parents, dear wife Belieze Atieno and lovely daughter Grace Cayleen in appreciation of their perseverance, encouragement and emotional support.

ABSTRACT

The devastating effect of cancer is a worldwide concern. In Kenya, cancer is ranked third cause of death after infectious and cardiovascular diseases. Unfortunately current therapeutic modalities have been found to possess side effects coupled with the emergence of anticancer drug resistance. This has necessitated the search for novel therapeutic products with better efficacy, safety and affordability through identification of anti-tumor agents from natural products. In this study the laboratory based *in vitro* antineoplastic activity and phytochemical profiles of methanol extracts of four medicinal plants, *Piptadiniastrum africanum, Kigelia africana, Centella asciatica* and *Chaemocrista nigricans* was investigated against NCI-H1155 lung adenocarcinoma cell line. These plants were obtained from ethnopharmacological survey previously done within Lake Victoria Basin. Extraction and concentration of the collected and dried plant samples to obtain crude extracts as well as phytochemical screening of the crude extracts was done following phytochemical standard procedures. The cell line, from American type cell culture (ATCC), was exposed to the extracts at varying concentrations and antiproliferative analysis using 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2-tetrazolium bromide (MTT) colorimetric assay was performed. Statistical differences among fractions of the extract were determined by one way ANOVA. Qualitative Phytochemical analysis of each of the four plants extracts showed presence of terpenoids, alkaloids, saponins, tannins and steroids. Steroids were higher in the bark of *P. africanum* and the fruits of *K. africana*. Flavonoid was absent only in extracts of *C. nigricans* and coumarins present only in the extracts of *K. africana*. The methanolic extracts of the fruits of *K. africana,* leaves of *C. asciatica,* bark of *P. africanum* and *C. nigricans* had inhibitory effects with IC_{50} 8.07ug/ml, 15.69ug/ml, 26.57ug/ml and 28.86ug/ml (p values of 0.055, 0.042, 0.069, and 0.079) respectively. The MTT assay results indicated that all the extracts exerted selected dose dependent inhibition actions on NCI-H1155 cells. The phytochemical compounds were found to trigger morphological changes (blebbing pattern and cell shrinkage) that are associated with apoptosis in the lung adenocarcinoma cell lines. The varying inhibitory activities against tested human lung adenocarcinoma cell line justify the traditional use of these plants in the management of cancers. These data lend support for traditional use of these plants as anti-tumor agents and potential source of natural products for treatment of cancer especially in resource limited countries. However, further phytochemical characterization of the extracts and flow cytometric analysis is necessary to exhaustively describe the mechanisms of cancer cell apoptosis of these four plant extracts.

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ABBREVIATIONS AND ACRONYMS

AIDS - Acquired Immunodeficiency Syndrome

DMEM - Dubelcco's Minimum Essential Media

DNA - Deoxyribonucleic Acid

ELISA - Enzyme Linked Immunosorbent Assay

FBS - Fetal Bovine Serum

HIV - Human Immunodeficiency Virus

IC_{50} - Half Maximum Inhibitory Concentration

MTT - Methyl Thiazolyldiphenyl-Tetrazolium bromide

NNRTIs - Non-Nucleoside Reverse Transcriptase Inhibitors

NRTIs - Nucleoside Reverse Transcriptase Inhibitors

RPMI 1640 - Roswell Park Memorial Institute Media

UNAIDS - United Nations joint Program on HIV and AIDS

WHO - World Health Organization

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CHAPTER ONE: INTRODUCTION

1.1 Background Information

Cancer is an abnormal growth of cells and globally considered as the second cause of death worldwide after Human Immunodeficiency Virus (HIV) and Acquired Immune Deficiency Syndrome (AIDS) (Anand *et al.,* 2008a). In Kenya, it is ranked the third cause of mortality after infectious and cardiovascular diseases and is threatening to move further up the list (Kenya National Bureau of Statistics, 2015). Incidences of cancer are on the increase some of which are of viral etiology such as Kaposi's sarcoma, Burkett lymphoma, and cervical cancer while others are as result of environmental pollution, and changes in lifestyle.

High poverty rate, late detection of cancers, and lack of inexpensive medicines for needy population in the Lake Victoria basin has resulted into malnutrition and increased use of cheap, alternative medicine which is more affordable than the current therapeutics recommended for the management of cancers and catastrophic illnesses (Lamorde *et al.,* 2010). The unfavorable safety profile of existing chemotherapeutics and the issue of drug resistance have fuelled the search for novel plant compounds as potential anti-cancer agents (Dewick, 2005). Therefore, high poverty level in the communities within Lake Victoria Basin has made them unable to afford essential foods which provides adequate antioxidants, micronutrients, proteins, vitamins and carbohydrates to boost their immunity.

In past years, ethnopharmacological studies of extracts or pure compounds from medicinal plants have provided considerable justification for their traditional use to treat

neoplastic diseases (Dewick, 2005; Houghton, 2005 and Patrick, 2005). The application of ethnomedicines to manage cancers has gained public and scientific attention due to low selectivity of current chemotherapeutic agents, different levels of cytotoxicity and rapid emergence of drug resistance to known anti-cancer agents (Joshi *et al.,* 2002). Plants have capacity to produce secondary metabolites which possess many biological activities including antimicrobial activities or growth regulatory molecules (hormone like substances) that stimulate or inhibit cell division and morphogenesis (Cragg and Newman, 2005). The physiological effects of these extracts make some of them sources of therapeutic agents and potentially anti-cancerous agents due to either their direct cytotoxicity on cancer cells or modulation of tumor initiation, promotion, or inhibition.

Plants and other natural products present a large repertoire from which novel anti-proliferative compounds with low cytotoxicity effect can be obtained (Akinmoladun *et al.,* 2007). Most of these promising naturally derived anti-cancer compounds are flavonoids, coumarins, diterpenes, triterpenes, biflavonoids, coumarins, caffeic acid tetramers, hypericin, gallotannins, galloylquinic acids, curcumins, saponins, and alkaloids (Cragg *et al*., 2005). These compounds may inhibit various steps in the cell cycle and induce apoptosis. Examples of natural anti-cancers products currently in the market include the taxoid compounds (docetaxel and paclitaxel) obtained from Pacific Yew, *Taxus brevifolia* (Walker and Croteau, 2001); Camphtotecin derivatives (irinotecan and topotecan) obtained from *Camptotheca acuminate* (Mattos *et al.,* 2001); Vinblastine and Vincristine obtained from *Catharanthus roseus* (Schnkel *et al.,* 1999a). Antitumor area has the greatest impact of plant derived drugs where drugs like vinblastine, vincristine, taxol and camptothecin have improved the chemotherapy of some cancers (Newmann *et al*., 2002). Therefore there is a need to explore more to discover and develop efficient drugs with low toxicity levels from plants. Many of the natural products from plants have medicinal values which affords them further prospective as novel leads for the development of anti-tumors agents.

Cytotoxicity of plant extract should be evaluated before their impact in drug discovery and development are taken into consideration (Lall and Mayer, 2000). Many plant extracts and isolated compounds have been tested *in vitro* for cytotoxicity and antiproliferative activities by using different human cell lines (prostrate, stomach, liver, colon.) as well as animal cells such as monkey kidney cells (Al-Fatimi *et al*., 2005, Jo *et al* 2005; Lamidi *et al*., 2005; Don *et al*., 2006). Cytotoxicity screening models provide important preliminary data to help select plant extracts with potential anti-tumor properties for future studies (Cardellina *et al.*, 1999).

Anti-proliferative assays have been used in oncology research and clinical practice in the assessment of cancer types of individual patients (Edmondson *et al*., 1988; Fotakis and Timbrell., 2006). Methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay is one method which has been described as rapid, simple and reproducible, widely used in the screening anticancer drugs and to measure the tumor cell proliferation (Edmondson *et al*., 1988; Fotakis and Timbrell., 2006). Hence, in the current study, the anti-proliferative properties of the selected plants were evaluated using this assay. The priotized medicinal plants for this study were selected from an ethnobotanical and ethno-pharmacological excursion conducted by Philip Aduma of Maseno University and John Tabuti of Makerere University within the Lake Victoria Basin region under the Phase one VicRes project. The ethnobotanical survey was to identify priority medicinal plants and pure plant-derived active biomolecules from plants used to manage diseases around Lake Victoria basin. However, among the plants identified in the above excursion, there is no information on the antiproliferative properties of the extracts of four medicinal plants that are native to Lake Victoria Basin, including *Piptadiniastrum africanum, Kigelia africana,*

Centella asciatica and *Chaemocrista nigricans.* These plants have been particularly used by Traditional health practitioners within Lake Victoria Basin in the management of human cancers and other related illnesses. This study aimed at screening for the qualitative phytochemical profiles and anti-proliferative effects of methanolic crude extracts of the four-ethnobotanically selected medicinal plants against established lung adenocarcinoma cell lines. Assessing proliferation, cell cycle arrest and apoptotic end-points in established cell line could be first steps for anticancer drug discovery and development.

1.2 Problem Statement

Cancer morbidity and mortality has continued to be a global concern. Over a hundred types of cancer are known, differentiated by etiology, history, pathology and metastasis. In spite of availability of therapies, cancer treatment still poses serious challenge especially in developing countries. In addition to economical and commercial hurdles, cancer patients are faced with multitudinous problems associated with the currently approved therapeutic strategies such as the emergence of drug resistance, bone marrow function inhibition, nausea, vomiting, and alopecia. Moreover, the use of anticancer drugs has been relatively limited by their toxicity, drug resistance development, low bioavailability as well as emergence of cancers associated with viral infections. These issues along with side effects and poor tolerability of these drugs make it apparent that new anti-cancer drugs with high selectivity against only malignant cells, ability to repress tumor metastasis, better efficacy, affordability, acceptable toxicity and resistance profiles and, more importantly, with novel mechanisms of action are required in search of alternative strategies of managing cancers. One of the strategies has been to identify anti-

cancer compounds from natural sources by evaluating the anti-proliferative activities of ethno pharmacologically selected medicinal plants.

1.3 Objectives

1.3.1 General objectives

To assess the *in vitro* anti- proliferative activities of bioactive phytochemicals from four ethnopharmacologically selected medicinal plant extract's against lung adenocarcinoma cell line.

1.3.2 Specific objectives

1. To determine the qualitative phytochemical profiles of the extracts of *Piptadiniastrum africanum, Kigelia africana, Centella asciatica* and *Chaemocrista nigricans*.
2. To determine anti-proliferative activities of the *Piptadiniastrum africanum, Kigelia africana, Centella asciatica* and *Chaemocrista nigricans* on Lung adenocarcinoma cell lines.

1.4 Research Questions

1. What are the phytochemical components of the extracts from *Piptadiniastrum africanum, Kigelia africana, Centella asciatica* and *Chaemocrista nigricans* plant?

2. What are the anti-proliferative activities of the extracts from *Piptadiniastrum africanum, Kigelia africana, Centella asciatica* and *Chaemocrista nigricans* on Lung adenocarcinoma cells?

1.7 Justification of the Study

Cancer patients are confronted with undesirable side effects resulting from conventional treatments. Morbidity and mortality that result from cancer is still unacceptably high and is currently becoming a growing public health problem. However, most of the drugs in cancer chemotherapy exhibit cell toxicity and can induce genotoxic, carcinogenic and teratogenic effects. Hence there has been need to explore for new alternative drugs which are less toxic and more potent in their mechanism of actions. Moreover, studies on anti-tumor compounds from natural products have yielded promising array of novel chemotherapeutic agents. This is the case with the powerful anti-leukemial drugs; vinblastin and vincristine isolated from the native Madagascar plant, *Catharanthus roseus* and the podophyllins isolated from the roots of the mayapple, *Podophyllum peltatum* among others. An anti-proliferative activity of plant extract against human cell lines is novel and work along this line is highly correlated with traditional plants use against a wide variety of illnesses. However, extracts from; *Piptadiniastrum africanum, Kigelia africana, Centella asciatica* and *Chaemocrista nigricans;* selected from an ethnobotanical and ethno-pharmacological excursion conducted by Philip Aduma of Maseno University and John Tabuti of Makerereke University may have antiproliferative activities against tumor cells. The ethnopharmacological excursion was done within the Lake Victoria Basin region by VicRes project- Phase one, to identify plants used for management of diseases within the Lake Victoria basin (LVB). Similarly, despite the exceptional biodiversity of Africa, few scientific studies have been carried out in the continent regarding the anti-proliferative properties of medicinal plants on established cancer cell lines in comparison to their antimicrobial effects. The present study was

designed to evaluate the *in vitro* anti-proliferative assays of the four ethno botanically and ethnopharmacologically selected medicinal plant extracts on the NCI-H1155 lung adenocarcinoma cell line using MTT assay.

1.5 Significance of the Study

Cancer is one of the leading cause of death worldwide and its incidence is on the rise even within the Lake Victoria basin. Traditional healers are the major source of knowledge on the medicinal plants that are currently being used in the management of enlarged body tissues and cancers in Africa. The findings of this research shows the importance of natural plant products used by traditional healers in management of diseases/illnesses in the Lake Victoria Basin. Moreover, it has addressed the question of whether methanolic extracts of the four selected medicinal plants have phytochemicals that possesses selective mediated suppression of cell viability. Hence knowledge obtained from the current study may lead to further activity guided fractionation and spectroscopic analysis of extracts with the view of developing of an active chemotherapeutic analogs.

1.6 Study Limitations

The study was limited to ethnobotanical and ethnopharmacological survey, conducted by VicRes team, which was majorly based on the traditional knowledge of plants medicinal values. The study also focused on qualitative phytochemical screening, without activity guided purification and characterizing the lead compound. In addition, the antiproliferative cell culture based assays was a single cell system as compared to the multi-cellular and multi-organ system in the *in vivo* assays, hence limited to biotransformation activities and effects in the systemic circulation.

CHAPTER TWO: LITERATURE REVIEW

2.1 Cancer and its Development

Cancer is an abnormal growth of cells caused by changes in gene expression leading to dysregulated balance of cellular proliferation and differentiation (Giri *et al*., 2009). This ultimately evolves into a population of cells that can invade tissues and metastases to distant sites causing significant morbidity and mortality if untreated (Hanahan *et al*., 2000). Cancer is caused by the interaction between genetic and environmental factors (Hugdson, 2008) leading to accumulations of genetic mutations in oncogenes and tumor suppressor genes, which gives cancer cells their malignant characteristics, such as uncontrolled growth (Parera, 1997). Hence is an aberrant net accumulation of typical cells arising from excess proliferation, insufficient apoptosis machinery, or a combination of both (Abdul *et al*., 2009).

Malignant tumor is characterized by abnormal cell growth with potential to invade or spread to other parts of the body (WHO, 2014). Not all tumors are cancerous; the characteristics that delineate a malignant cancer from a benign tumor are the ability to invade locally, spread to regional lymph nodes and to metastasize to distant organs in the body. As the cancerous growth progresses, genetic drift in the cell population produces cell heterogeneity in such characteristics as cell antigenicity, invasiveness, metastatic potential, rate of cell proliferation, differentiation state, and response to chemotherapeutic agents (WHO, 2014).

The major risk factors to cancer acquisition are tobacco use, obesity, poor diet, lack of physical activity, infections, alcohol drinking, exposure to ionizing radiation and environmental pollutants (WHO, 2014). Many cancers can be prevented by not smoking,

eating more vegetables, fruits and whole grains, eating less meat and refined carbohydrates, maintaining a healthy weight, exercising, and being vaccinated against certain infectious diseases (Anand *et al.,* 2008b; Kushi *et al*., 2010). Most chemotherapeutic drugs work by impairing mitosis, effectively targeting fast dividing cells. They prevent mitosis by various mechanisms including damaging DNA, inhibition of cellular machinery involved in cell division (Kehe *et al*., 2009) and inducing apoptosis (Makin *et al*., 2000). Therefore, from the above studies, inhibitors to accumulation of genetic materials or conferment of resistance to the interaction between genetic susceptibility and environmental factors by agents obtained from plant extracts cease cancer growth.

2.2 Characteristics of Neoplastic Cell

Cancer cells show cellular and nuclear pleomorphism, abnormal arrangement of cells, develops changes in the cell membranes and organelles, exhibit abnormal mitoses and chromosomal abnormalities (Pitot, 1968). There is increased motility of malignant cells which may be associated with increased amounts of contractile proteins in their microfilaments, with loss of contact inhibition (probably caused by alteration in calcium ion concentration in the malignant cell membrane) (Fiddler, 1975). Interrupted cellular adhesiveness (for the solid surface) and contact inhibition (among cells) results from changes in the cell surface glycoproteins and the poorly developed tight junctions and desmosomes in malignant cells. Changes in motility, adhesiveness and contact inhibition promotes invasion and subsequent establishment of secondary malignant growth – metastasis (Clement, 1968).

According to Robbins Textbook of Pathology (1963), Cancer cells exhibit differences in metabolism as compared to normal cells. The metabolism of malignant cells is usually more anaerobic than that of normal non-rapidly dividing cells and is greatly accelerated. Malignant cells may be able to withstand hypoxic conditions. They may have increased glucose and amino acid uptake due to high levels of hexokinase. The cancer cells loss capabilities to synthesize specialized proteins typical for differentiated cells. Enzymes and other proteins produced by cancer cells are needed for the tumor growth (Mladosievicova, 2007). Therefore, agents that have potential of preventing metastasis of neoplasm and can inhibit angiogenesis form the basis of future chemotherapeutic agents.

2.3 Cancer Drugs in Clinical Use

2.3.1 Anti-cancer drugs

Chemotherapy is the use of drugs to inhibit or kill proliferating cancer cells. Chemotherapy may also harm cells that divide rapidly under normal circumstances such as cells in the bone marrow, digestive tract, and hair follicles. Resistance to programmed cell death (apoptosis) is an integral part of cancer cell development, and reestablishment of control of apoptosis is a known target mechanism for anticancer drugs (Gibb *et al*., 1997; Joshi *et al*., 2002). Certain products from plants are known to induce apoptosis in neoplastic cells, but not in normal cells, which would be the ideal characteristic of a successful anticancer drug (Hirano *et al*., 1995).

Plant derived compounds comprise a diverse group with different mechanisms of action, but ultimately seem to have the ability to induce apoptosis on rapidly dividing cells (Tharapadar *et al*., 2001). For example, alkylating agents, such as cisplatin, act directly on the DNA by cross-linking the guanine nucleobases and thus hindering the strands of

DNA to uncoil and separate and therefore inhibiting replication. This in turn, triggers apoptosis of the cells (Gibb *et al*., 1997).

2.3.2 Mechanism of antineoplatic drugs

Cancer is a pathologic condition where the normal mechanisms of cell cycle regulation are dysfunctional either due to excessive cell proliferation, insufficient apoptosis or both (Saikumar, *et al*., 1999; Wyllie, *et al*., 1999; Reed, 1999). The recent aim of cancer treatment is to reverse, suppress, or prevent carcinogenic progression through the use of natural dietary agents (Oyagbemi *et al*., 2009; Kundu and Chun, 2014; Thumvijit *et al*., 2014).

Most chemotherapeutic drugs prevent mitosis by various mechanisms including; alkylating agents which bind covalently to DNA via their alkyl group. This follows either cross linking of the two DNA strands preventing replication or DNA damage leading to apoptosis (Siddik, 2005; Damia and D'Incalci, 1998). They are nonspecific and kill rapidly proliferating cells and non-proliferating cells. Examples are mechlorethamine used for the treatment of non-Hodgkin lymphoma and cyclophosphamide (Damia and D'Incalci., 1998). However, because these drugs damage DNA they can cause long term damage to other body tissues or organs.

Anti-metabolites are a group of molecules that inhibit DNA and RNA synthesis. Anti-metabolites resemble either nucleobases or nucleosides, but have altered chemical groups (Parker, 2008). These drugs exert their effect by either blocking the enzymes required for DNA synthesis or becoming incorporated into DNA or RNA (Parker, 2009). By inhibiting the enzymes involved in DNA synthesis, they prevent mitosis because the DNA cannot duplicate itself. This leads to DNA damage inducing apoptosis. Subtypes of

the anti-metabolites are the anti-folates, fluoropyrimidines, deoxynucleoside analogues and thiopurines (Lind, 2008; Parker, 2009). Example is 5-flourouracil which is metabolized to its deoxynucleotide form, 5- Fluorodeoxyuridine, to inhibit the enzyme, thymidilate synthatase which is involved in the methylation of deoxyuridycilic acid to thymidylic acid; arabinosylcytosine blocks the reduction of cytidylic to deoxycytiylic acid inhibiting DNA replication. 6-mercaptopurine and 6-thioguanine prevent purine biosynthesis and interconversion of the purine bases (David and Karnofsky, 1968).

Topoisomerase inhibitors affect the activity of two enzymes: topoisomerase I and topoisomerase II hence interfering with the DNA replication and transcription. Inhibition of these enzymes causes DNA strand breaks, and leads to apoptosis. These agents include etoposide, doxorubicin, mitoxantrone and teniposide (Lodish *et al*., 2000). The second group is catalytic inhibitors which block the activity of topoisomerase II, and therefore prevent DNA synthesis and translation because the DNA cannot unwind properly. This group includes novobiocin, merbarone, and aclarubicin, which also have other significant mechanisms of action. Treatment with topoisomerase inhibitor increases the risk of second cancer, acute myelogenous leukemia (Nitiss, 2009).

Natural products or Anti-microtubule agents are plant-derived chemicals that block cell division by preventing microtubule function (Rownsky *et al*., 1991). Vinca alkaloids, notably vincristine and vinblastine, derived from *Catharanthus roseus* (formerly known as *Vinca rosea*) bind to specific sites on tubulin, inhibiting the assembly of tubulin into microtubules. The original vinca alkaloids are completely natural chemicals that include vincristine and vinblastine. Following the success of these drugs, semi-synthetic vinca alkaloids were produced: vinorelbine, vindesine, and vinflunine (Yue *et al*., 2010). The

vinca alkaloids prevent the formation of the microtubules, whereas the taxanes prevent the microtubule disassembly. By doing so, they prevent the cancer cells from completing mitosis causeing cell cycle arrest occurs, which induces apoptosis (Lind, 2008; Yue *et al*., 2010). Taxanes are natural and semi-synthetic drugs. This includes paclitaxel, was originally extracted from the Pacific Yew tree, *Taxus brevifolia* and docetaxel, produced semi-synthetically from a chemical found in the bark of another Yew tree; *Taxus baccata*. These drugs promote microtubule stability, preventing their disassembly. Paclitaxel prevents the cell cycle at the boundary of G2-M, whereas docetaxel exerts its effect during S-phase (Yue *et al*., 2010; Bharadwaj and Yu, 2004; NCI, 2006). Further studies are needed to unveil potential inhibitors of proto-oncogene mutations from plant sources to help curb the challenges posed by cancer treatments.

2.3.3. Challenges in the use of anti-cancer drugs

The major problem in cancer chemotherapy is the development of multi drug resistance (MDR) against anti-cancer drugs. It has been discovered that drug resistance in cancer cells results from elevated expression of certain proteins, such as cell membrane ATP-binding cassette transporters (Choi, 2005), which can result in an increased efflux of the cytotoxic drugs from the cancer cells (Ambudkar *et al.,* 1999; Thomas & Coley, 2003). The problems of acquired drug resistance may be circumvented by targeting endothelial cells associated to the tumor instead of the tumor cells themselves (Boehm *et al.,* 1997). The process of angiogenesis is an attractive target of cancer therapy since tumors are dependent on a functioning vascular system, and metastasis is dependent on the formation of new vessels around the cell foci (Griggs *et al.,* 2001). Also already established tumor vasculature is a good target for anticancer therapy since it differs from

normal vasculature in its permeability, the absence of vascular smooth muscle cells and lymphatic drainage (Matsumura and Maeda, 1986). There are a number of promising anti-angiogenic and antivascular agents, some of them originating from higher plants, and are undergoing clinical trials. Among these is the stilbenoid combretastatin-A4 originally isolated from the tree *Combretum caffrum* (Pettit *et al.,* 1987, 1988, 1989 and 1999).

Resistance is a major cause of treatment failure in the use of chemotherapeutic drugs. There are a few possible causes of resistance in cancer, one of which is the presence of small pumps, p-glycoprotein, on the surface of cancer cells that actively move chemotherapy from inside the cell to the outside (Goldman, 2003; Crowley *et al.,* 2009). Cancer cells can also cause defects in the cellular pathways of apoptosis (Luqmani, 2005). This may lead to resistance.

The blood brain barrier poses a difficult obstacle to passage of chemotherapeutic drug to the brain. Only small lipophilic alkylating agents such as lomustine or temozolomide are able to cross this barrier (Deeken *et al.,* 2007; Gerstner *et al.,* 2007). Chemotherapeutic techniques have a range of side-effects that depend on the type of medications used. This includes immune suppression, nausea, alopecia, teratogenicity and infertility (Chen and Clerck, 2009). Therefore, plants which contain alkylating agents as their major phytochemical components can be sourced to overcome blood brain barrier obstructions.

2.4. The Importance of Traditional Medicine in Different African Countries

According to WHO (2000), traditional medicine continues to provide health coverage for over 80% of the world population, especially in the developing world. About 80 – 90 % of the populations in African countries are dependent on traditional medicine for their

primary health care (Hostettman *et al.*, 2000). More than 60% of the population in Tanzania depends on traditional medicines for the management of various diseases including HIV/AIDS (Mhame *et al*., 2004). In Nigeria, traditional medicine is well acknowledged and established as a viable profession (Kafaru, 1994), and almost all plants seem to have some kind of application in traditional medicine (Babayi *et al.*, 2004). In Kenya medicinal plants are now being used for the treatment and alleviation of the symptoms of various diseases including HIV infection (Morris, 2002) and malaria (Njoroge and Bussman, 2006). There is abundant literature which indicates that rural communities across the world depend heavily on plant diversity and have traditionally made judicious selection of these plants for various purposes including control of various ailments affecting human and their domestic animals (Heinrich, 2000; Mahmood *et al*., 2011; Joshi-Kunjani, *et al*., 2010). These traditional methods are sometimes claimed to give fewer side effects than conventional antiretroviral therapy (Morris, 2002). Gradually, traditional medicinal practitioners (TMP) are being officially accepted as part of African health services and their medical knowledge is finding its place in hospitals and clinics (Neuwinger, 2000). The abundance of information on the traditional medicinal uses of plants in Africa is in danger of disappearing since the knowledge of how to use medicinal plants is mostly passed down orally and even to date is poorly documented (Gurib-Fakim, 2006). The oral transfer of knowledge is vulnerable to disruption and interference and may result in the loss and distortion of valuable ethno medical information. Therefore the ethnobotanical and ethnopharmacological survey done by the Lake Victoria Research initiative (VicRes) project documented a number of plants used as herbal remedies in the management of HIV and AIDS and opportunistic

infections within the lake victoria basin. The ethnopharmacological information reported forms the basis for further research to identify bioactive constituents and their anti-cancer effect on established cancer cell lines.

2.5 Traditional Medicines with Anti-Cancer Properties

Natural products have played significant role in drug discovery. Since 1961, nine plant-derived compounds have been approved for use as anticancer drugs in the United States; vinblastine (Velban), vincristine (Oncovin), navelbine (vinorelbine), etoposide (VP-16), teniposide (VM-26), taxol (paclitaxel), taxotere (docetaxel), topotecan (Hycamtin) and irinotecan (Camptosar) (Lee, 1999). Previous studies show that the presence of phytochemical compounds contributes to their antiproliferative activity through antioxidant and free radical scavenging effects (Zakaria *et al.,* 2011). It is believed that phytochemicals may be effective in combating or preventing disease due to their antioxidants effect (Farombi *et al.,* 1988). The antitumor potential of plant extracts and compounds could be attributed to their ability to induce changes in the regulation of target molecules in oncogenic signal transduction pathways implicated in cell growth, replication, apoptosis, as well as in angiogenesis, invasion and metastasis of cancer cells (Amin, *et al*., 2009; Mehta, *et al*., 2010).

The areas of cancer and infectious diseases have a leading position in utilization of medicinal plants as a source of drug discovery. According to report by WHO more than 80% of world's population depend on traditional medicine for their primary healthcare needs (Farnsworth, 1994). Among Food and Drug Administration (FDA) approved anticancer and anti-infectious preparations, drugs of natural origin have a share of 60% and 75% respectively (Cragg *et al*., 2005). It is worth mentioning the current interest in

discovery of natural drugs for cancer treatment and chemoprevention (Balunas and Kinghorn, 2005). Medicinal plants either through systematic screening programs or by serendipity possess an important position in the drug discovery and many modern drugs have their origin in traditional medicine of different cultures. Hence, despite the advantages of the synthetic and combinatorial chemistry as well as molecular modeling, medicinal plants remain an important source of new drugs, new drug leads and new chemical entities (Newman *et al.,* 2000). According to Gilani and Atta-ur-Rahman (2005), the use of plants, plants extracts or plant derived pure chemicals to treat diseases is a therapeutic modality, which has withstood the test of time. The search for biological active agents from plants is part of a wider resurgence of scientific interest to produce new chemotherapeutics. Plants synthesize very complex molecules with specific stereochemistry and can show biological activity with novel mode of action (Houghton, 2005). These complex materials are called secondary metabolites or phytochemicals (Akinmoladun *et al.,* 2007). It is believed that phytochemicals may be effective in combating or preventing disease due to their antioxidant effect (Farombi *et al.,* 1988). Antioxidants protect other molecules (*in vivo*) from oxidation when they are exposed to free radicals and oxygen species which have been implicated in the etiology of many diseases and in food deterioration and spoilage species (Kasaikina, 1997; Koleva *et al.,* 2000).

Herbal medicine have greatly been used and accepted in the treatment of various ailments (Mander, 1998). Communities in the Lake Victoria Basin, like other communities in Kenya practice traditional medicine to a large extent. The diseases dealt with are mainly skin related problems, gastrointestinal diseases, sexually transmitted diseases, malaria,

wounds, eye infections, measles, snake bites, diarrhoea, fever, asthma, and typhoid among others (Kokwaro., 1993; Owuor and Kisangau., 2006; Otieno *et al*., 2007). Natural products can be selected for biological screening based on ethnomedical use, random collection and a chemotaxonomic approach i.e. screening of species of the same botanical family for similar compounds (WHO, 1989).

Despite the rich L. Victoria repertoire from which to select medicinal plants, anti-proliferative activity of traditional herbal medicines are still not well-researched, and African knowledge of herbal remedies used to manage cancer is scanty and not documented. Numerous plants are available in nature which has anticancer properties and majority of them are yet to be examined through screening against established cell lines. Compounds from medicinal plants may not serve as final drug but they comprise of lead compounds for the development of potential anticancer agents. In order to realize the full potential of these medicinal plants it is therefore necessary to verify the claims by the traditional herbalist and make a reliable documentation. This study was designed to qualitatively identify the bioactive constituents and antiproliferative effect of four ethnopharmacologically selected medicinal plants against established NCI-H1155 Lung adenocarcinoma cell line.

2.6 Selected Medicinal Plants

2.6.1 *Piptadeniastrum africanum*

Piptadeniastrum africanum is a large buttress tree called variously as: “Kiryar Kurmi” in Hausa, “Ofie” in Igbo and “agboin or agboyin” in Yoruba (Hutchinson and Daiziel, 1963). The sapwood when fresh is pale reddish – yellow or pinkish – white and

comparatively wide (Tatiana, 2008). *Piptadeniastrum africanum* occurs from Senegal east to southern Sudan and Uganda, and south to DR Congo and northern Angola (Katende *et al*., 1995). In Nigeria, the root and the bark are prepared for use as enema, the bark chipped off for antimalarial use and an infusion of the bark is used to relieve toothache (Hutchinson and Dalziel, 1963). The root of this plant has been reported by (Mengome *et al.,* 2009) to be used against human colonic cancer cell. Phytochemical studies on the root of *Piptadeniastrum africanum* revealed the presence of tannis, flavones, alkaloids, steroids, terpenoids, saponins and glycosides among others (Mengome *et al.,* 2009). Further work is necessary to ascertain the clinical safety of extract from plants (Effraim *et al.,* 2001) and to determine the antiproliferative effect on other established human cell.

2.6.2 *Kigelia africana*

Kigelia africana belongs to the family Bignoniaceae. It is called "Yago" in Luo, "Muratina" in Kikuyu, Rotinwo in kalenjin, Kenya. The plant is widely distributed throughout the African tropics. It is also found in China, South East Asia, Australia, Hawahii and South and Central America (Grace and Davies, 2002). *Kigelia africana* is traditionally used in African folk medicine for treatment of various clinical conditions which include malaria, dysentery, constipation, cough and kidney disorders. Analgesic and anti-inflammatory activities of orally administered extracts of the stem bark of *K. Africana* in mice and guinea pigs have been reported (Owalabi and Omogabi, 2007). Powdered fruit is sprinkled on wounds and sores as topical dressings for their antibacterial and antifungal properties (Grace and Davies, 2002). Verminoside and verbascoside are the major constituents of *K. africana* from root-bark used for the

treatment of venereal diseases and naphtoquinones extracted from *K. africana* showed cytotoxic activity against melanoma and renal carcinoma cells (Houghton *et al.,* 1994). There are inadequate reports on the phytochemical studies, phytoanalytical studies and pharmacological screening of the plant. There is enormous scope for the future research of *K. africana* considering the many medicinal purposes it serves. It has a high potential for development into viable drugs as more facts emanates from its uses, especially as a strong anti-cancer agent (Olatunji and Otolani, 2009). It is therefore recommended that more research work should focus on the anti-cancer properties. No data as well shows the distribution and the anticancer property of the methanolic fruit extract this plant within the L. Victoria basin.

2.6.3 *Centella asciatica*

Centella asciatica or "pegaga" belongs to the Umbelliferae family, is widely distributed in Kenya and is well known for its ability in promoting wound healing (Indena, 2011). In India it is also known as Gotu kola or pennywort in English. It is found in Asia (India, Sri Lanka) and East Africa widespread to South America, West Indies and South East Asia like Malaysia, Pakistan, Japan, China, and Australia (Dastur, 1962). Centella contains several active constituents, of which the most important are the triterpenoid saponins, including asiaticoside, centelloside, madecassoside, and asiatic acid. *Centella asciatica* has been reported to possess antiulcer (Asakawa *et al*., 1982; Gohil *et al*., 2010), anti-inflammatory (Guo *et al*., 2004; George *et al*., 2009; Huang *et al*., 2011), immunomodulating (Punturee *et al*., 2005), antitumor (Babu *et al*., 1995), antibacterial (Zaidan *et al*., 2005), antioxidant (Gupta and Flora, 2006), and antigenotoxic (Siddique *et al*., 2007) properties. Besides, *C. asciatica* could act as cardio protective agent that can

enhance myocardial antioxidants and thus prevents the extent of cardiac damage (Gnanapragasam *et al*., 2004).

Wound and ulcer healing are enhanced by the promotion of fibroblast proliferation and collagen synthesis in response to topical treatment with extracts of *C. asciatica* herb (Maquart *et al*., 1990; Maquart *et al*., 1999). *Centella asciatica* increases the production of basic fibroblast growth factor (bFGF), induce angiogenesis and cell proliferation, therefore it promotes wound healing activity (Shukla *et al*., 1999a, 1999b; Cheng *et al*., 2004; Gohil *et al*., 2005). Asiaticoside enhance the burn wound healing at low doses by promoting angiogenesis during skin wound repair (Kimura *et al*., 2008). It is used for the treatment of psoriasis and found to be effective in destroying cultured cancer cells (Maquart *et al.,* 1990). Likewise, Yu *et al* (2006) also found that the extract of the whole plant has a strong anti-cancer activity. In Brazil, *C. asciatica* is used to treat the uterine cancer (Yoshida *et al.,* 2005). The presence of several bioactive components in the extract may possess anticancer activity. Previous studies reported that *C. asciatica* extract possess antipsoriatic effect due to an inhibition of keratinocyte proliferation by its constituent triterpenoid glycosides (Sampson *et al*., 2001). Coldren *et al*. (2003) found the anti-proliferation effect of *C. asciatica* extract on fibroblast cells. Recent study by Babykutty *et al*. (2009) showed that *C. asciatica* extracts induced apoptosis on human breast cancer cells. The *C. asciatica* aqueous extract demonstrated an inhibitory effect towards the proliferation activities of human respiratory epithelial cells (Mutua *et al.*, 2013). No data as well shows the anticancer property of methanolic leaf extract of this plant within the L. Victoria basin.

2.6.4 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 *Chamaecrista nigricans* showed significant action against *Herpes simplex* (Schmelzer and Gurib-Fakim, 2008). *Chamaecrista nigricans* leaves, and roots show interesting pharmacological actions, hence they warrant further research on the medicinal actions (Schmelzer and Gurib-Fakim, 2008).

However, there are no scientific studies on the anticancer properties of the extracts of these plants against lung adenocarcinoma cell lines within Lake Victoria basin. The present study therefore aimed at screening for the preliminary phytochemical and anti-proliferative actions of methanolic crude extracts of four-ethnobotanically selected medicinal plants, including *Piptadiniastrum africanum, Kigelia africana, Centella asciatica* and *Chaemocrista nigricans*, against established lung adenocarcinoma cell lines, NCI-H11551. Assessing proliferation, cell cycle arrest and apoptotic end-points in established cell line could be first steps for anticancer drug discovery and development.

2.7 Anti-Proliferative Activity using MTT assay

Antiproliferative activities was monitored using the 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay. This assay measures the reducing potential of the cell using a colorimetric reaction (Barridge *et al*., 2005). Viable cells will

reduce the MTT reagent to a colored formazan product (Mosmann, 1983). When cells die, they lose the ability to convert MTT into formazan, thus color formation serves as a useful and convenient marker of only the viable cells. The exact cellular mechanism of MTT reduction into formazan is not well understood, but likely involves reaction with NADH or similar reducing molecules that transfer electrons to MTT (Marshall *et al*., 1995). The evidence of cell death may be inferred from morphological changes observed from microscopic evaluation.

In the US National Cancer Institute plant screening program, a crude extract is generally considered to have *in vitro* cytotoxic activity if the IC_{50} value (concentration that causes a 50% cell death) in carcinoma cells, following incubation between 48 and 72 hours, is less than 30ug/ml, while it is less than 4 ug/ml for pure compounds (Boik J, 2001). Screenings of medicinal plants used as anticancer drugs have provided modern medicine with effective cytotoxic pharmaceuticals. Most anti-cancer drugs are designed to eliminate rapidly proliferating cancerous cells, and therefore, they typically show cytotoxicity and induce apoptosis in cancer cells (Kaufmann and Earnshaw, 2000).

It is documented that most cytotoxic anticancer agents induce apoptosis, raising the intriguing possibility that defects in apoptotic programs contribute to treatment failure (Scott and Athena, 2000). The main action of anticancer agents is by triggering the apoptotic pathway. However, intrinsic alterations in the apoptotic pathway are a hallmark of cancer cells and are considered to be a major cause of drug efficacy (Waxman and Schwartz, 2003; Shkreta *et al*., 2008). Apoptosis was initially described by its morphological characteristics, including cell shrinkage, membrane blebbing, chromatin condensation and nuclear fragmentation (Kerr, *et al*., 1994; Thompson, 1995).

Compounds that have cytotoxic effects often compromise cell membrane integrity. Vital dyes, such as Trypan blue or Propidium iodide are normally excluded from the inside of healthy cells; however, if the cell membrane has been compromised, they freely cross the membrane and stain intracellular components (Riss *et al.*, 2004).

CHAPTER THREE: MATERIALS AND METHODS

3.1. Study Area

The priotized medicinal plants for this study were selected from an ethnobotanical and ethno-pharmacological excursion conducted by Aduma and Tabuti within the Lake Victoria Basin region under the VicRes project. Lake Victoria occupies a shallow depression in Africa and has a maximum depth of 84 m (276 ft) and an average depth of 40 m, 130 ft (UN, 1999). Its catchment area covers 184,000 square kilometers (71,040 sq mi). The lake has a shoreline of 4,828 km (3,000 mi), with islands constituting 3.7% of this length and is divided among three countries: Kenya (6% or 4,100 km^2 or 1,600 sq mi), Uganda (45% or 31,000 km^2 or 12,000 sq mi) and Tanzania (49% or 33,700 km^2 or 13,000 sq mi) (Prado *et al*., 1991). This region has high prevalence of HIV and AIDS defining cancers as well as availability of many traditional healers who use medicinal plants in management of HIV, cancers and other infections (Tabuti *et al*., 2003). The Lake Victoria basin covers several sub-counties and counties in the five partner states of the East African Community. These states include Burundi, Kenya Rwanda, Tanzania and Uganda. From the literature, the four plants selected for this study are widely distributed within the Lake Victoria basin (Appendix 1).

3.2. Study Design

A laboratory-based *in-vitro* study was done on the already selected and collected plants from an ethnobotanical and ethnopharmacological excursion conducted by the VicRes under phase one project. During the phase one VicRes excursion, selection of medicinal plants was a cross-sectional study of key informant of the people in the region who use medicinal plants in management of cancer associated with HIV and AIDS infection and

other illnesses. The participants were made to recall and recap the plants used, part of the plant used, and preparation of the plant and the correct quantity of the concoction administered to the patient. Based on this knowledge, each of the plant parts were extracted for evaluation of phytochemical profiles and anti-proliferative activities against established lung adenocarcinoma cell lines (NCI-H1155) in the laboratory.

3.3 Plant Material, Extract Preparation, Fractionation and Formulation

Four plants (*Piptadiniastrum africanum, Kigelia africana, Centella asciatica* and *Chaemocrista nigricans)* materials were collected from different parts in the Lake Victoria basin following ethnobotanical survey and taxonomy. These plants had been ethnopharmacologically and ethnobotanically identified in project under the Lake Victoria Research initiative (VicRes) which focused on value chain analysis and development of plant derived medicinal products for the management of HIV and AIDS in the face of climate change in the Lake Victoria basin. The selected and identified plants were collected for evaluation of phytochemical profiles and anti-proliferative activities of the plant extracts in the laboratory.

3.3.1 Extraction using methanol

Methanol is useful for extraction of phytochemicals due to its polarity index of 5.1 and low boiling point of about 65 degrees. About 2Kg of the bark of *Piptadiniastrum africanum,* 1Kg leaves of *Centella asciatica* and *Chaemocrista nigricans,* and 2kg of the fruit of *Kigelia africana,* was air-dried under room temperature and extracted with 200 mL of 95% methanol using warring blender for eight successions. Each of the extracts was filtered and concentrated by rotary evaporator at room temperature.

3.3.2 Phytochemical screening for the crude extracts

The qualitative determination methods of phytochemical constituents as described by Trease and Evans (1983); Harbourne (1983) and Sofowora (1993) to test for alkaloids, tannins, flavonoids, steroids, terpenoids, carbohydrates, proteins, anthraquinones, coumarins, glycosides and saponins was used for the phytochemical screening of the four selected medicinal plants in this study.

Test for proteins (Millon's test): 0.5g of crude extract was mixed with 2ml of Millon's reagent, and the appearance of a white precipitate which turns red upon gentle heating confirmed the presence of proteins.

Figure 3.1: Qualitative Phytochemical Assays. A picture of the laboratory experimental assays for qualitative phytochemical analysis of the methanol extracts as was carried out in the Chemistry laboratory, Maseno.

Test for carbohydrates (Molisch's test): Crude extract was mixed with 2ml of Molisch's reagent and the mixture shaken properly. After that, 2ml of concentrated sulfuric acid (H_2SO_4) was then poured carefully along the side of the test tube. Appearance of a violet ring at the interphase showed the presence of carbohydrate.

Test for phenols and tannins: Crude extract was mixed with 2ml of 2% solution of $FeCl_3$. A blue-green or black coloration indicated the presence of phenols and tannins.

Test for flavonoids (Ferric chloride test): 0.5g of the extract was treated with few drops of ferric chloride solution. Formation for blackish red colour indicates the presence of flavonoids

Test for saponins (Foam Test): The extract (0.5 g portions) was shaken with 2 ml of water. Foam produced which persisted for ten minutes indicated the presence of saponins.

Test for glycosides (Salkowski's test for glycosides): 0.5g of the crude extract when mixed with 2ml of chloroform and then 2ml of concentrated H_2SO_4 is added carefully and shaken gently, reddish brown colour indicated the presence of steroidal ring, i.e.,glycone portion of the glycoside.

Test for steroid: Crude extract when mixed with 2ml of chloroform and concentrated H_2SO_4 is added sidewise a red colour produced in the lower chloroform layer indicates the presence of steroids.

Test for terpenoids: 0.8 g of selected plant sample was taken in a test tube, then poured 10 ml of methanol in it, shaken well and filtered to take 5 ml extract of plant sample. Then 2 ml of chloroform was mixed in the extract of selected plant sample and 3 ml of sulphuric acid were added. Formation of reddish brown color indicates the presence of terpenoids in the selected plants.

Test for alkaloids: 0.5g of the crude extract was mixed with 2ml of 1% HCl and heated gently and Mayer's and Wagner's reagents are then added to the mixture, turbidity of the resulting precipitate was an evidence for the presence of alkaloids.

Test for Coumarins: In a test tube, 1 g of each of the extracts was placed and covered with filter paper moistened with dilute sodium hydroxide (NaOH), then heated on water bath for a few minutes. The filter paper was examined under UV light, yellow fluorescence indicated the presence of coumarins.

Test for anthraquinones: 0.5 g of the extract was boiled with 10 ml of sulphuric acid (H_2SO_4) and filtered while hot. The filtrate was shaken with 5 ml of chloroform. The chloroform layer was pipetted into another test tube and 1 ml of dilute ammonia was added. The resulting solution was observed for bright pink or red coloration.

3.4 General Experimental Conditions for Cell Culture Assays

The extracted crude extracts was stored and kept free from any contamination in a restricted lockable cabinet at room temperature in the laboratory. Each of the extracts was dissolved in Dimethyl sulfoxide (DMSO) to form the preparation used for bioassays. After doing Trypan blue viability test, the cell lines were stored in liquid nitrogen, and slowly thawed before being treated with the crude extracts of each of the plants. The bioassays was conducted in level II biohazard biosafety cabinet (Telster BIO II A, en-12469-200) in an ATCC certified and accredited cell culture laboratory contained with a hyper filter Air conditioner and only restricted to the principal investigators.

3.5 Cell Culture

3.5.1 Cell media

The Lung Adenocarcinoma (NCI-H1155; ATCC) culture cells were suspended in RPMI 1640 medium supplemented with 10% fetal calf serum, 1% antibiotic-antimycotic mixture (10.000 U/ml K-penicillin, 10.000 μg/ml streptomycin sulphate 25 μg/ml amphotericin B) and 1% 2uM L-glutamine

3.5.2 Cell culture plate set-up

Approximately 5×10^4 cells were cultured per well in a 96-well plate. Sterility was maintained throughout the culturing period by decontaminating all the working benches using 10% sodium hypochlorite prior to use. Complete culture media, comprised of 88% RPMI 1640 supplemented with 5% AB+ serum, 5% Fetal Bovine Serum (FBS), 50IU/ml Penicillin and 50µg/ml Streptomycin and 1% of 2mM L-glutamine. The complete media was filter sterilized using 0.2µl filter unit and stored at 4^0C. After counting the cells using Trypan blue dye exclusion and hemocytometer, they were diluted up to 5×10^4 cells/ml in a 96-well plate and cultured in complete medium at 37^0C in 5% CO_2, in a water-jacketed incubator and examined after 24hours.

3.6 Antiproliferative Assays

3.6.1 Determination of cell number and viability

Morphological features of the cells were examined every day under an inverted light microscope (Olympus, Shinjuku-ku, Tokyo) and their photomicrograph recorded at every passage. The viability of cells was determined by staining the exponentially growing cells with Trypan blue and counting using a haemocytometer. The cell suspension was prepared and 10 µl was mixed with equal volume of 0.4% Trypan blue and the cells were

checked for viability, where viable cells appeared unstained and non-viable cells were stained blue. Contaminations were minimized by sterilizing the working benches and performing measurements in class II biosafety cabinet.

$$\text{Viable cells (\%)} = \frac{\text{Total number of viable cells (unstained)}}{\text{Total number of cells (stained and unstained)}} \times 100 \text{ / ml of aliquot}$$

3.6.2 Anti-proliferative activity on tumor cell lines

A 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2-tetrazolium bromide (MTT) colorimetric assay (Promega, USA) which is based on a measurement of the biotransformation of the yellow dye 3-(4,5-dimethylthiazol-2-yl)- 2,5-diphenyl tetrazolium bromide to a violet formazan product via the mitochondrial succinate dehydrogenase in viable cells was conducted according to the manufacturer's instructions. Absorbance of the purple formazan developed was measured at 450 nm using ELISA reader spectrophotometer. The activities were compared to 5- fluorouracil as anticancer positive controls and untreated cells as the negative controls.

The tumor cell line, lung adenocarcinoma, acquired from American Type Culture Collection (ATCC), was kept in Roswell Park Memorial Institute media-1640 (RPMI 1640) supplemented with 10% fetal bovine serum (FBS), 1% of 2mM l-glutamine, 100 mg/L streptomycin and 100 IU/ml penicillin at 37^0C in a humidified atmosphere of 5% CO_2. Cell suspensions (100 µL) were dispensed in triplicate in 96-well culture plates at optimized concentrations of ~1.0×10^5 cells/mL per cell line. After 24-hr incubation at 37 ^{0}C, 100 µL culture medium was removed from the wells and 100 µL fresh medium containing the extracts (1000, 500, 250, 125, 50, 25, 0 µg/mL) was added to each well and incubated for another 48 hrs. Wells containing untreated cells were used as the

negative controls. At the end of the treatment period, the medium in each well was aspirated and replaced with 20 µL of 5 mg MTT working solution (MTT stock solution mixed with medium to attain a final concentration of 0.5 mg/mL). Briefly, MTT powder was dissolved in PBS to form an MTT stock solution (5 mg/mL). The stock solution was filter-sterilized through a 0.22 µm filter and stored at −20 ^{0}C until used. The cells were incubated at 37 ^{0}C for 4 hrs, and then the medium was aspirated and replaced with 100 µL DMSO to dissolve the blue formazan crystals formed and stop the reaction. The culture plates were shaken for 1hr and the absorbance (OD) of each well was read using an enzyme-linked immunosorbent assay (ELISA) reader at 450 nm. Each test was done in triplicate and reported as mean.

3.7 Data Analyses

The results were presented as means ± SEM of the triplicate experiments. Statistical differences among fractions of the extract were determined by one way ANOVA using Graph Pad Prism 5 (GraphPad Software Inc., San Diego, USA) to get IC_{50}. All Treatments were compared at 0.05 significant level using turkey's test tool. The data was stored safely in a restricted and password computer excel document.

3.9 Ethical Cconsiderations

Approval to carry out this study was provided by the school of graduate studies of Maseno University. Ethical approval was obtained from Maseno University Ethical Review Committee.

CHAPTER FOUR: RESULTS

4.1. Qualitative Phytochemical Profile

The screening of the methanol extract of the selected different plant species; namely bark of *Piptadiniastrum africanum,* fruit of *Kigelia africana*, leaves of *Centella asciatica* and leaves of *Chaemocrista nigricans;* for phytochemical constituent was performed based on tests of coloration and precipitation as described (Figure 3.1).

The study has revealed the presence of phytochemicals considered as active medicinal chemical constituents. These included tannins, steroids, alkaloids, glycosides, saponins and terpenoids in all the four plant extracts. Flavoinoids was present in *C. asciatica* and *K. africana* whereas coumarin was only present in *K. africana*. Anthraquinone was detected in *P. africanum* and *K. africana*. The results were tabulated as shown in the table 3.1. Methanolic plant extract had most of the phytochemicals required for the antioxidant and anti-cancer activity. These were subjected to further MTT assay antiproliferative activity investigation.

Table 3.1: Phytochemical analysis of the extracts

Plants / Phytochemicals	*P. africanum* *bark*	*C. asciatica* *leaves*	*C. nigricans* *leaves*	*K. africana* *fruit*
Steroids	++	+	+	++
Terpenoids	+	+	+	+
Saponins	+	+	+	+
Alkaloids	+	+	+	+
Carbohydrates	+	-	+	-
Tannins	++	++	++	++
Flavonoids	+	+	-	++
Proteins	+	+	+	-
Glycosides	+	+	+	+
Anthraquinones	+	-	-	+
coumarins	-	-	-	++

Keys: ***(-)*** Negative (Absence of turbidity, flocculation, and precipitation); **(+)** Weak positive (If the reactant has slight opacity); **(++)** test strongly positive (If the test produces a precipitate or heavy color change). Each of the extracts was tested for eleven phytochemical components as tabulated in bold above.

4.2 Antiproliferative activities of the plant extracts

The antiproliferative activity of the extracts against the human Lung adenocarcinoma cell line was tested using the microtitration colorimetric method of MTT reduction. The methanol fraction of *C. nigricans, P. africanum, C. asciatica* and *K. africana* extracts exhibited antiproliferative potential with IC50 values of 28.86µg/ml, 26.57µg/ml, 15.69µg/ml and 8.07µg/ml respectively (Table 2). This falls within the National Cancer

Institute criteria, IC_{50}<30µg/ml *(*Itharat *et al*, 2003; Oskoueian *et al*, 2011), thus are considered as of promising anticancer potential.

Table 3.2: *In vitro* antiproliferative activities (IC_{50} µg/ml) of the crude extract from the four selected medicinal plant against NCI-H1155 Lung adenocarcinoma cell line determined by MTT assay

Plants extract	**IC_{50} ± SEM**	**P- value**
C. nigricans	28. 86 ± 0.143	0.079
P. africanum	26.57 ± 0.127	0.069
C. asciatica	15.69 ± 0.135	0.042
K. africana	8.07 ± 0.125	0.055

Table 3.2: MTT antiproliferative assays of the four selected medicinal plant extract on Lung adenocarcinoma cell line. The absorbance was read after 48 hours of incubation using an ELISA microplate reader at 450nm. The data indicates that *P. africanum* had the lowest half inhibitory (IC_{50}) attributed to its rich phytochemical constituents while *C. nigricans* had the highest IC_{50}. The treatments were compared at 0.05 significant level using Turkey's test tool. The differences obtained in comparison to the positive control are not statistically significant except for *C.asciatica*.

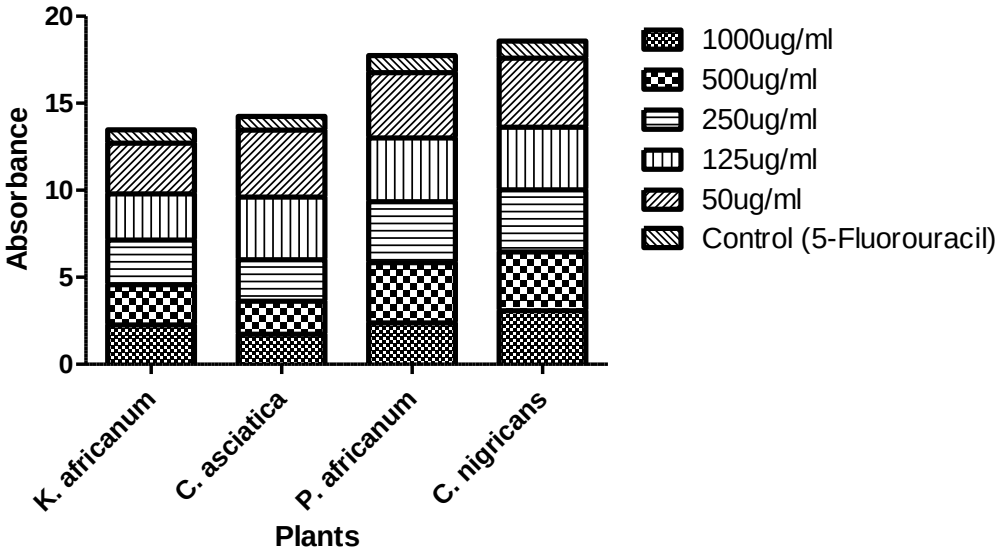

Figure 3.2a: Graphical representation of the antiproliferative effects of the plants extracts against Lung adenocarcinoma cell line. The absorbance of each plants extract was carried out in serial dilution of the crude extract concentration (0, 50,125, 250, 500, 1000g/mg) and a positive control, 5-Fuorouracil. Highest absorbance was with *C. nigricans* doses of the crude extract indicating an increase in cell proliferation. This show its low activities compared to other extracts.

A.

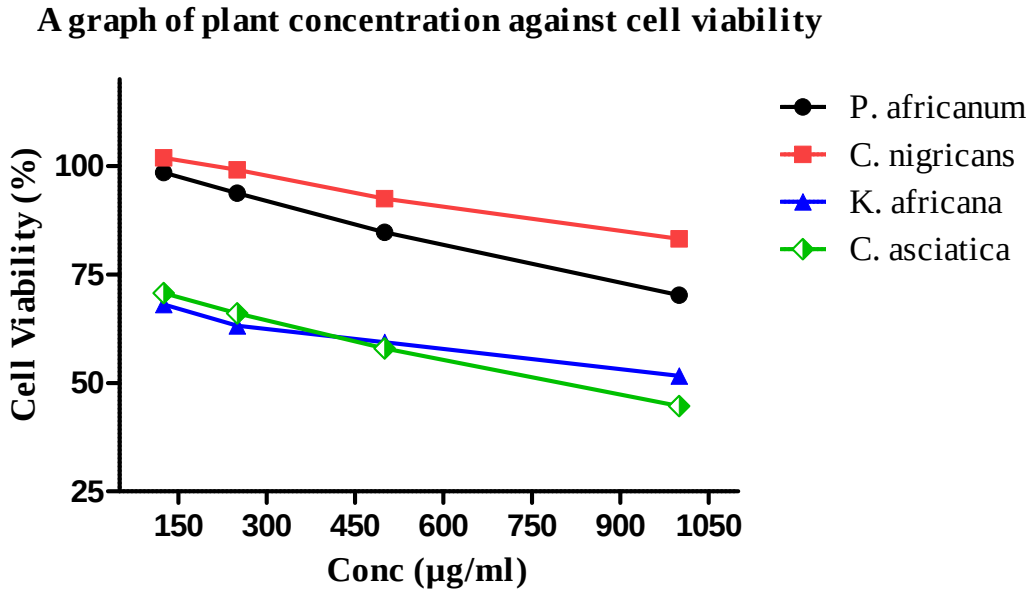

Figure 3.2b: Survival effect of NCI-H1155 grown for 48h in the presence of increasing concentration of *Piptadiniastrum africanum, Kigelia africana, Centella asciatica* and *Chaemocrista nigricans* extracts. There is a dose dependent survival of the cells induced by the extracts.

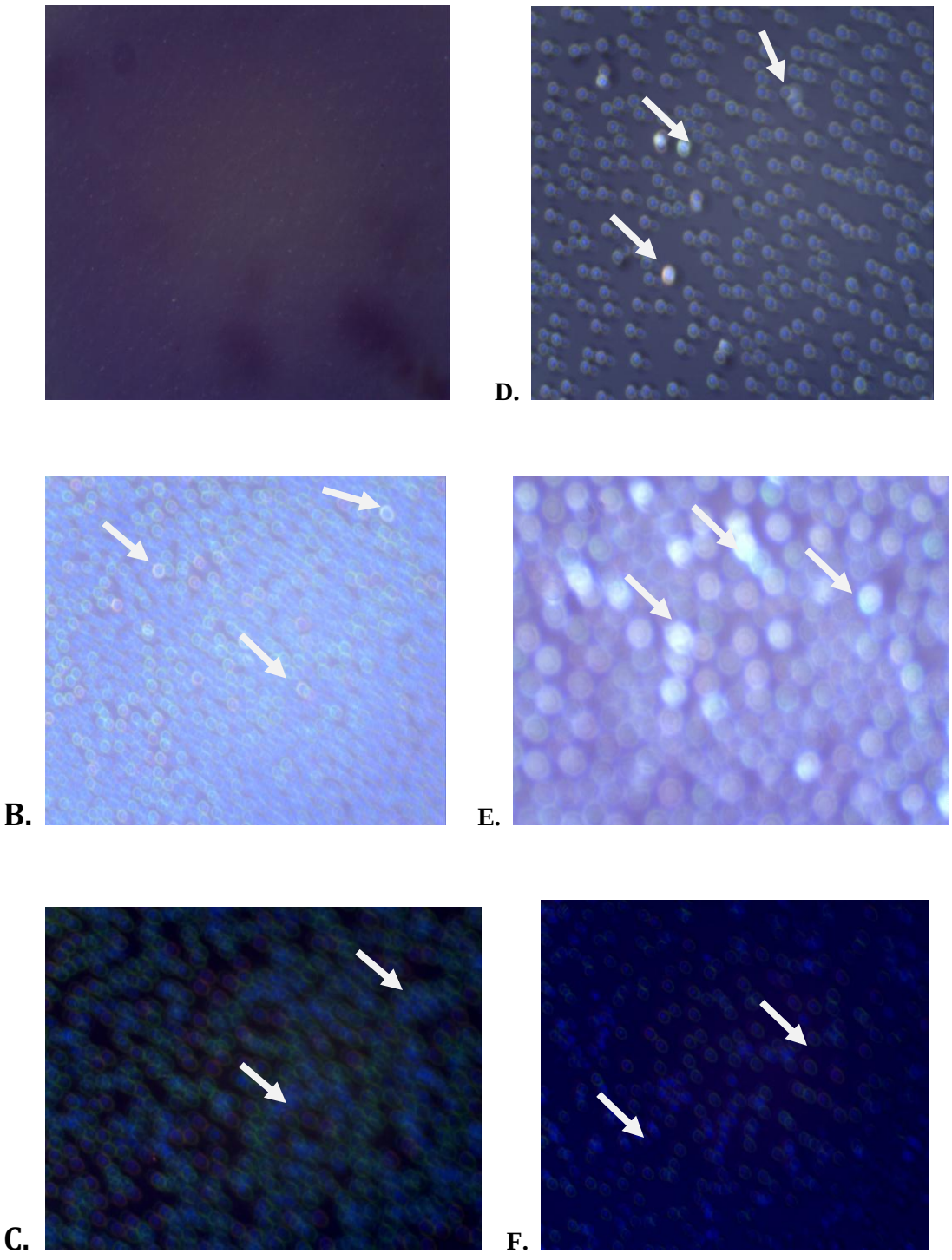

Figure 3.3: Morphological changes in Lung adenocarcinoma cells assayed to assess cell apoptosis. (A) Negative control; (B) Cells treated with 125µg/ml of *C. nigricans* methanol extract; (C) Cells treated with 125µg/ml of *C. asciatica* (Leaves*)* methanol extract. (D) Cells treated with125µg/ml *K. africana* (fruits) methanol extract; (E) Cells treated with 125µg/ml of *P. africanum* (F) Positive control as observed under the inverted light microscope. Dark stained nuclei which indicate nuclear fragmentation, cytoplasmic blebbing and nuclear condensation (Arrows)

CHAPTER FIVE: DISCUSSION

5.1 Qualitative Phytochemical Profiles

Phytochemicals often play an important role in plant defense against prey, microorganism, stress as well as interspecies protections and have been used as drugs for millennia. Hence, screening of phytochemicals serves as the initial step in predicting the types of potential active compounds from plants (Chew *et al*., 2011). The Secondary metabolites include phenolic compounds, alkaloids, terpenoids, steroids, quinones, saponins, flavonoids, with complex structures that are known to support bioactive activities in medicinal plants extracts (Krishnaiah *et al*., 2009). The phytochemical analysis was done to detect qualitatively the presence of eleven secondary metabolites in the four plants, namely *Kigelia africana, Piptadiniastrum africanum, Centella asciatica, and Chaemocrista nigricans* as tabulated in Table 3.1. Overally, tannins, steroids, alkaloids, glycosides, saponins and terpenoids were detected in all the four plant extracts. Flavonoids were present in *C. asciatica* and *K. africana* whereas coumarins were only present in *K. africana*. Anthraquinones were detected in *P. africanum* and *K. africana*. The phytochemical analysis revealed the presence of many chemical groups in the extracts that could be responsible of their antioxidant, anti-cancer activity and other bioactivities.

The phytochemical results obtained from these plants corroborate the results from other studies (Rehab *et al*., 2015; Akinlamini *et al.,* 2012; Thangavel *et al.,* 2011; Mengome *et al.*, 2009). As suggested by Rui (2004) it is possible that the anticancer activities of these plant extracts could be due to a synergy between their various chemical constituents exerting total antioxidant activity.

5.2 Antiproliferative Activity

Traditional medical practices in Lake Victoria basin use various plants for the treatment of various diseases. Though these plants and their extracts have been used, information from organized published literature or ethno-pharmacology survey by VicRes does not provide the evidence for antitumor activities of the four selected medicinal plants used in this study. Also the increase in the use of these medicinal plants and their phytoconstituents in the recent past as well as the scarcity of scientific studies on their safety and efficacy have raised concerns in the scientific community such as VicRes and there is need to assess the potential effects of these plants.

With the above perspective in mind, the present study was designed to assess the anticancer potential of methanolic extracts of four medicinal plants, *K. africana, P. africanum, C. asciatica* and *C. nigricans*, from the Lake Victoria basin. These plants are used by traditional health practitioners for treating a variety of ailments. The antiproliferative activity of the extracts against the human lung adenocarcinoma, NCI-H1155, cell line was tested using the microtitration colorimetric method of MTT reduction. The highest absorbance exhibited by *C. nigricans* indicated an increase in cell proliferation and its low activities compared to other extracts (Figure 2a and b). Antiproliferative activities of plant extract against human cancer cell lines could be due to their phytochemicals which induces cell death or inhibit the progression of the growth of cancer cells.

Apoptosis is the common mode of cell death by therapeutic drugs used in cancer treatment and based on this fact, the cellular and nuclear morphological changes induced

by the extracts treatment in lung adenocarcinoma cells were observed in this study. The presence of active medicinal chemical constituents is thought to trigger morphological changes associated with apoptosis in the lung adenocarcinoma cell lines (Figure 3). Extracts of *Piptadiniastrum africanum, Kigelia africana, Centella asciatica* and *Chaemocrista nigricans* induced morphological changes in the cell line (Figure 3.3). Apoptotic cells show morphological changes such as cell shrinkage, pyknosis and extensive plasma membrane blebbing leading to the formation of apoptotic bodies, which are subsequently phagocytosed by macrophages parenchymal or neoplastic cells. Suppression/inhibition of apoptosis during carcinogenesis is known to play a role in the development and progression of cancers (Kurosaka, *et al*., 2003). Many chemo-preventive agents have been shown to induce apoptosis in transformed cells both *in vitro* and *in vivo*. Induction of apoptosis appears to be associated with their effectiveness in modulating carcinogenic processes (Sun *et al*., 2004). In fact, activation of apoptosis in pre-cancerous cells is one of the most important mechanisms of cancer chemoprevention by dietary factors (Martin, *et al*., 2006).

Morphological examinations of NCI-H1155 lung adenocarcinoma cells treated with four different plant extracts were compared to untreated cells and visualized using an inverted light microscope. In comparison to untreated cells, the treated cells showed typical features of cell death at the morphological level such as rounding off of cells, cell shrinkage and blebbing pattern thus supporting indication that these extracts induces cell death by apoptosis (Figure 3.3). Untreated cells showed large and prominent nuclei indicating no significant characteristics of apoptosis. This suggested that cytotoxicity of the extracts might involve the apoptosis pathway. This is in agreement with how

commercially available chemotherapeutic agents exert their anticancer activity by inducing cell apoptosis (Yu *et al*., 2007; Yan-Wei *et al*., 2009).

Subsequent MTT cell viability tests on the cell lines revealed that the plant extracts were able to exhibit antiproliferative effect at different concentrations. Results obtained indicated that an inhibitory trend was detected in response to the *Piptadiniastrum africanum, Kigelia africana, Centella asciatica* and *Chaemocrista nigricans* extracts (Figure 3.2). The higher the concentration of the extract the more inhibitory effect was seen. This indicated that the inhibitory effect of the methanolic extracts on NCI-H1155 Lung adenocarcinoma cells is concentration-dependent.

According to the standard National Cancer institute criteria, crude extracts possessing an IC50 < 30 µg/ml *(*Itharat *et al,* 2003 and Oskoueian *et al*, 2011) are considered active against tested cancer cell lines. The methanolic extracts of the fruits of *K. africana,* leaves of *C. asciatica,* bark of *P. africanum* and leaves *C. nigricans* had inhibitory effects with IC_{50} 8.07ug/ml, 15.69ug/ml, 26.57ug/ml and 28.86ug/ml and p values of 0.055, 0.042, 0.069 and 0.079 respectively. *C. nigricans* extract containing less phytochemicals possessed weak antiproliferative activity with IC50 of 28.86µg/ml and reduced morphological changes on NCI-H1155 cells (Table 3.2 and Figure 3.3B).The higher activity of methanolic extract of *K. africana, P. africanum,* and *C. asciatica* are thought to be as a result of the various important phytochemicals constituents which induces cell death by apoptosis.

From previous studies these bioactive phytochemicals have the ability to inhibit cell proliferation and induce morphological changes associated with apoptosis in cancer cell lines. Tannins and polyphenolic compounds are useful compounds that have remarkable

cancer prevention and anti-cancer activities (Aiyegoro *et al*., 2010). Laboratory rodent studies have shown that polyphenols have cancer-preventing properties and considered to be potential chemo preventive agents (Ramos, 2008; Lambert, *et al*., 2005; Fresco, *et al*., 2006). They can influence important cellular and molecular mechanisms associated with multiple carcinogenic steps, such as expression of key proteins in signal transduction pathways (e.g., mitogen activated protein kinases or activator protein (AP)-1), the transcription factor nuclear factor-kappa B (NF-κB) and its downstream gene products, modulation of cell-cycle regulation and induction of apoptosis (Fresco, *et al*., 2006), which affect cell differentiation, proliferation and apoptosis, immune responses and metabolism of carcinogens (Nichenametla, *et al*., 2006). Many chemopreventive agents are able to block or delay the promotion and/or progression of premalignant or malignant cells by modulating cell proliferation and/or differentiation (Sun, *et al*., 2004; Hail, 2005). Hence, the results of this study suggest the presence of tannin in the four extracts contributing to their antiproliferative effects on the cell line.

Flavonoids are ingredients of many vegetables and fruits and the association of vegetable and fruit consumption with reduced cancer risk has been reported (Ferguson *et al*., 2004; Kinadaswami *et al*., 2005). Antiproliferative activity recorded in the present study is in accordance with this finding, since the phytochemical evaluation indicated the presence of flavonoids in three plant species with promising activity except in *C. nigricans*. The ability of flavonoids to scavenge hydroxyl radicals, superoxide anion radicals, and lipid peroxyl radicals, which is important for preventing diseases associated with oxidative damage of membranes, proteins, and DNA (Aiyegoro *et al*., 2009). The results of this

study are in accordance with this finding since the phytochemical screening showed the presence of flavonoids in all active extracts (Table 3.1).

The presence of alkaloids with flavonoids in *P. africanum, C. asciatica* and *K. africana* extracts may explain higher activity compared with *C. nigricans* extract in the present study. Park *et al*. (2008) reported that flavonoids would induce apoptosis in cancer cells. Reed and Pellecchia, (2005) work in this direction stressed on inducing apoptosis as a desired strategy of controlling cancers. Antiproliferative activities of these plants may also be ascribed to the presence of terpenoids in all the four plant extracts. It is Probable that these antioxidant compounds trigger intracellular signaling pathways which induce cell death through apoptosis.

The extract of *C. asciatica* consists of bioactive terpene acids such as Asiatic acids, madecassic acid and their respective glycoside, asiaticoside and madecassoside which are considered as antitumor (Babu *et al*., 1995, Inamdar *et al*., 1996). Previous studies reported that *C. asciatica* extract possess antipsoriatic effect due to an inhibition of keratinocyte proliferation by its constituent triterpenoid glycosides (Sampson *et al*., 2001). It is believed that the antioxidation activity of the phenolic compound from *C. asciatica* can prevent certain diseases like arteriosclerosis, cancer, diabetic and arthritis (Zainol *et al*., 2003).

More effective anticancer drugs with high selectivity against only malignant cells and with ability to repress tumor metastasis are desired. As candidates for such drugs, cytotoxic, antitumor or anticancer natural products have been often sought, and plant components such as Vinca alkaloids, taxoids, etoposide and irinotecan are now used in clinical treatments (Montvale, 1998).

Furthermore, the extract contained saponins, anthraquinones and coumarins which have an inhibitory effect on inflammation, hypocholesterolemia, and diabetes (Kusuma *et al.,* 2011). Coumarins compounds might inhibit cell proliferation by interfering with mitotic spindle microtubule function (Irene, 2005. However, the leaf of *C. nigricans* extract did not contain anthraquinones and coumarins hence could be the reason for its low activity.

The pharmacological activity of the *C. nigricans* leaves has been investigated only in rats, rabbits and mice (Chidume *et al.,* 2001) but not in cancer cell lines. Absence of flavonoids and other phytochemicals in *C. nigricans* could be attributed to its low inhibitory activities.

Due to the high mortality rates of cancer and the absence of effective chemotherapy, there is a continued need to explore for new alternatives for treatment and prevention of cancer, and natural products play a dominant role in the discovery of such new drugs (Cragg *et al.,* 1997). However, no studies have reported the antiproliferative activity of these extracts in lung adenocarcinoma cells in Kenya and Lake Victoria Basin. The present study provides evidence that methanolic extracts of the four medicinal plants contain various phytoconstituents such as flavonoids, tannins, steroids, terpenoids, saponins and alkaloids which have the ability to inhibit cell proliferation and induce apoptosis in lung cancer at different dose levels. It could also be possible to speculate that the anticancer activities of the four plant extracts could be due to a synergy between their various chemical constituents. This represents the first instance in which the antiproliferative potential of the four plants has been demonstrated on NCI-H1155 Lung adenocarcinoma cells.

CHAPTER SIX: SUMMARY OF THE FINDINGS, CONCLUSIONS AND RECOMMENDATIONS

6.1. Summary of the Findings

The phytochemical analysis and antiproliferative activities of methanol extracts of four medicinal plants from the Lake Victoria Basin, including *Piptadiniastrum africanum, Kigelia africana, Centella asciatica* and *Chaemocrista nigricans* which had been identified through ethno botanical surveys and taxonomy was investigated. Overally; tannins, steroids, alkaloids, glycosides, saponins and terpenoids were detected as the major phytochemical components in all the four plant extracts. Flavonoids were present only in *C. asciatica* and *K. africana* whereas coumarins were only present in *K. africana*. Anthraquinones were detected in *P. africanum* and *K. africana only*. The phytochemical analysis revealed the presence of many chemical groups in the plant extracts that are responsible for their antioxidant, anti-cancer activity and other bioactivities like antimicrobial effects. The antiproliferative data indicates that all the plants extracts showed inhibitory activities. Among the Methanolic extracts, *K. africana, C. asciatica* and *P. africanum* had the highest inhibitory effects with IC_{50} 8.07±0.125µg/ml, 15.69±0.135µg/ml and 26.57±0.127µg/ml respectively. The highest IC_{50} value was obtained with the leaf extract of *C. nigricans* against lung adenocarcinoma cells of 28.86±0.143µg/ml. *Kigelia africana* had the lowest half inhibitory attributed to its rich phytochemical constituents whereas *C. nigricans* had the highest half inhibition probably due to its low phytochemical contents. Observation under inverted microscope showed extracts of *Piptadiniastrum africanum, Kigelia africana, Centella asciatica* and *Chaemocrista nigricans* induces morphological changes such as cell shrinkage, pyknosis

and extensive plasma membrane blebbing which are typical features of cell death at the morphological level thus supporting indication that these extracts induces cell death by apoptosis.

6.2 Conclusions

In conclusion, these findings demonstrate that:

1. Phytochemical analysis of the four methanol plant extracts showed that tannins, steroids, alkaloids, glycosides, saponins and terpenoids were present in all the four plant extracts. Flavoinoids was present in *C. asciatica* and *K. africana* whereas coumarins were only present in *K. africana*. Anthraquinone was detected only in *P. africanum* and *K. africana*. These findings show the potential therapeutic capability of the traditional preparations obtained from these plants.
2. The extracts of *Piptadiniastrum africanum, Kigelia africana, Centella asciatica* and *Chaemocrista nigricans* demonstrated a dose-dependent antiproliferative effect and typical features of cell death at morphological level of the NCI-H1155 lung adenocarcinoma cultured cell line.

6.3 Recommendations from the Study

1. It is recommended that the information reported in this study forms the basis for further research to identify and elucidate the actual bioactive compounds of these plants that can be developed to anticancer drugs for cancer management.
2. To exhaustively describe the mechanisms of antiproliferative activities of the these four plant extract used in the current study, further flow cytometric studies is

necessary for more extensive biological evaluation and describe the mechanisms of cancer cell apoptosis of the most active ingredients from the four plants.

6.4 Recommendation for future Study

1. There is need in the future to evaluate the antiproliferative activities of these four plant extract on other human cancer cell lines and on animal models.

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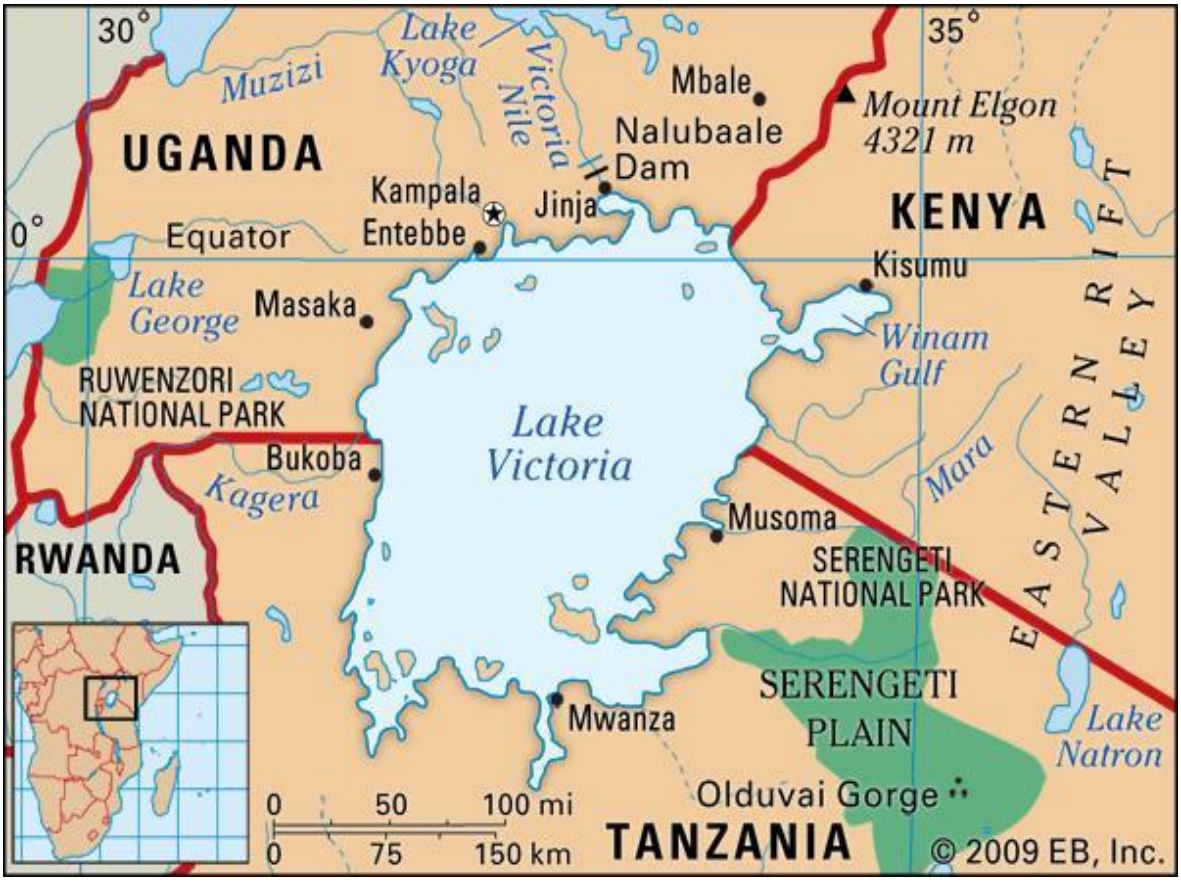
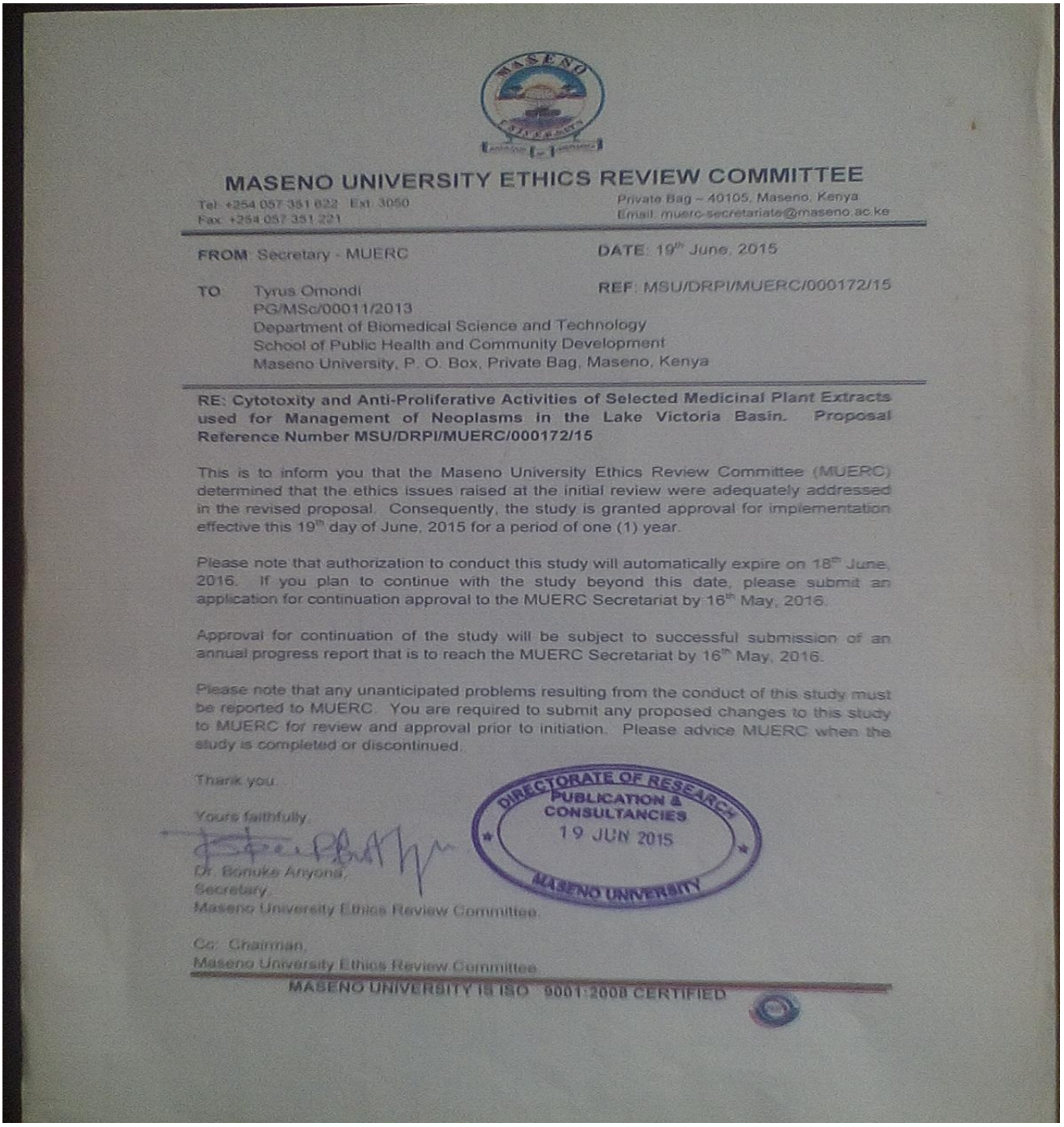
APPENDICES

Appendix 1: Lake Victoria basin



Adapted from map https:/lakevictoriabasinmaps.google.com/

Appendix 2: Research approval letter



MASENO UNIVERSITY ETHICS REVIEW COMMITTEE

Tel: +254 057 351 622 Ext. 3050
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Private Bag – 40105, Maseno, Kenya
Email: muerc-secretariate@maseno.ac.ke

FROM: Secretary - MUERC

DATE: 19th June, 2015

TO: Tyrus Omondi
PG/MSc/00011/2013
Department of Biomedical Science and Technology
School of Public Health and Community Development
Maseno University, P. O. Box, Private Bag, Maseno, Kenya

REF: MSU/DRPI/MUERC/000172/15

RE: Cytotoxity and Anti-Proliferative Activities of Selected Medicinal Plant Extracts used for Management of Neoplasms in the Lake Victoria Basin. Proposal Reference Number MSU/DRPI/MUERC/000172/15

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 19th day of June, 2015 for a period of one (1) year.

Please note that authorization to conduct this study will automatically expire on 18th June, 2016. If you plan to continue with the study beyond this date, please submit an application for continuation approval to the MUERC Secretariat by 16th May, 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 16th May, 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 19 JUN 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