IN VITRO ANTIPLORIFERATIVE ACTIVITY, PHYTOCHEMICAL COMPOSITION AND TOXICITY STUDIES OF *FAGAROPSIS ANGOLENSIS* AND *PRUNUS AFRICANA* CRUDE EXTRACTS

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DECLARATION

I declare that, this thesis is my own original work and has not been submitted for any academic award in any academic institution. No part of this study should be used without my knowledge or that of the University of Nairobi.

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DEDICATION

This thesis is dedicated to my beloved wife Seleyian and Lovely sons Leyian and Meikan for their love encouragement, perseverance during the period of my studies.

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LIST OF ABBREVIATIONS AND ACRONYMS

KEMRI	Kenya Medical Research Institute
OECD	Organization for economic co-operation and development
SEM	Standard error of the mean
MTT	3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
UDP	Up-and-Down-Procedure
DMSO	Dimethyl sulfoxide
OD	Optical density
PBS	Phosphate buffered saline
MEM	Eagle's minimum essential media
ATCC	American Type Culture Collection
ELISA	Enzyme-Linked immunosorbent assay
CO_2	Carbon (IV) oxide
CC_{50}	Median cytotoxic concentration dose
IC_{50}	Median inhibitory concentration dose
DNA	Deoxyribonucleic acid
LD_{50}	Median lethal concentration dose
GLOBOCAN	Global burden of cancer website

ABSTRACT

Antiproliferative and Cytotoxicity screening models are the preliminary methods for selection of active plant extracts against cancer. *Fagaropsis angolensis* (FA) and *Prunus africana* (PA) have wide ranging ethno medicinal uses in Kenya. The study aimed at evaluating the *in vitro* antiploriferative activity against selected cancer cell lines, *in vivo* acute toxicity studies and phytochemical constituents of crude of *Fagaropsis angolensis* (FA) and *Prunus africana* (PA).

Water and methanol extracts of different plant parts of FA (leaf, leaf stalk, root bark, root stem and whole root) and PA (Bark) were prepared. Qualitative phytochemical screening was carried out on the extracts to identify compounds of pharmacological value. I*n-vitro* growth inhibition capacity of the plant extracts (water and methanol) were evaluated in vero E6 (normal cell), Hep2 (throat cancer) and CT 26-CL 25 (colon cancer) cell lines using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium (MTT) cell proliferation assayat the following concentrations 0.14, 0.4, 1.24, 3.7, 11.11, 33.33 and 100µg/ml. Cell viability was calculated and dose response curves plotted to determine the effective concentration of the extracts in inhibiting 50% cell proliferation (IC_{50}) against doxorubicin (standard anti-cancer agent). The acute oral toxicity was investigated in 8-12 weeks old healthy female Swiss albino mice using the Organization for Economic Cooperation and Development guidelines number 423, of 2001.

The following phytochemicals were present: alkaloids, flavonoids, glycosides, phenols, tannins, saponins and steroids. It was established that the methanol root stem and whole root extracts of FA had IC_{50} values of 1.10±0.70 µg/ml and 5.10±0.80µg/ml respectively on vero cells which was significantly ($p>0.05$) lower than 6.5±3.25µg/ml of doxorubicin. Further, the methanol root stem and whole root extracts of *FA* had IC_{50} values of 8.33±1.42 µg/ml and 5.25±0.35µg/ml on colon cancer cell lines which was significantly ($p>0.05$) lower than

19.00±9.00 µg/ml of doxorubicin. The IC_{50} values of the aqueous and methanol extracts of *FA* on normal (vero, E6) cell lines was; bark (5.56± 1.5µg/ml, >100µg/ml), respectively compared to (6.5±3.25µg/ml) of doxorubicin (standard anti-cancer agent). On throat cancer (HeP2) cell lines, the IC_{50} values of the aqueous and methanol extracts of *FA* was; bark (18.15±17.85µg/ml, 11.75±2.75µg/ml), respectively compared to 2.5±0.50µg/ml doxorubicin (standard anti-cancer agent). With regard to the CT26 cell lines, the IC_{50} values of the aqueous and methanol extracts of FA was; bark (31.00±21.00µg/ml, 67.50±21.50µg/ml), respectively compared to 19.00±9.00µg/ml of doxorubicin (standard anti-cancer agent). No signs of toxicity or mortality were observed over the test period (LD_{50} >2000 mg/Kg) acute oral toxicity study.

The results of this study suggest that different plant parts of FA and PA have promising anti-cancer activity *in vitro*. Moreover, from the IC_{50} values of methanolic root stem and whole root extracts of *Fagaropsis angolensis* had the best *in vitro* antiploriferative activity. The results of this study justify further investigation of crude methanol extracts of *Fagaropsis angolensis* and crude water extracts of *Prunus africana* as a potential source of anticancer agents. However, further investigations are needed to evaluate the safety and efficacy of the crude extracts of these plants *in vivo* to support their ongoing ethno botanical use in traditional cancer therapy.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Plants use as a source of medications is as old as mankind. Plants either whole or their parts have been used ever since to heal and manage a range of diseases affecting human beings all over the world. Before the invention of synthetic drugs, traditional medicine dominated the world. Studies by WHO have shown that many people are using medicinal plants for cure (WHO, 1988). Globally it is estimated that 80% of the population in the developing countries and 40% of those from developed countries use natural medicines which mainly consists of plants for their primary health care needs (Prabakaran *et al*., 2011). This could be attributed to poverty in these countries which makes many people unable to access modern hospitals and purchase allopathic drugs for their treatment. It is due to this reason that many people turn to plants which are believed to be non-toxic, readily available and affordable to the local population (Ngule *et al*., 2013).

It is believed that medicinal plants and their parts contain active principles of critical significance for human metabolism, disease prevention and healing (Kokwaro, 2009). Internationally, Cancer is a growing concern in developed and developing countries, with the number of cases increasing day by day. More than 7 million people are dying from cancer annually and 11 million new cases expected to rise from 11 million in 2002 to more than 16 million annually by 2020 (WHO, 2002).

Globally, cancer is ranked as the second leading cause of mortality (Joyce *et al.*, 2011). It has consequently been prospected that in the year 2030, around 26 million fresh cancer cases and 17 million cancer deaths per annum will be reported. In the year 1975, low and middle income

countries contributed to about half (51%) of all cancer cases. It is estimated that by 2050, it will reach 61% (IARC, 2008).

There is an immense requirement for innovative and enhanced curative resources, including new chemotherapeutic drugs that are efficacious affordable and with few adverse side effects (Verweij and Jonge, 2001; Karsila *et al*., 2001; Eikesdal *et al*., 2001).

In recent past, the curiosity in doing research on natural products has been greater leading to the invention of new efficient chemotherapeutic medicines meant for cancer management (Calixto, 2000; Rates, 2001; Phillipson, 2001). Imperative milestone in these inventions is exemplified by the taxoid agent composed of docetaxel and paclitaxel componds, discovered from the genera *Taxus*. (Walker and Croteau, 2001) the other is camphtotecin composed of (irinotecan and topotecan), derived from *Camptotheca acuminata* (Mattos *et al.*, 2001). An additional motivating point has been to learn biodiversity tied to species conservation. Furthermore, the absences of current economical drugs for the deprived in the society, majority who are in constant quest for other options, that have to a certain extent distrustful effectiveness, capable of exhibiting miracles with no adverse side effects (Calixto *et al.*, 2000; Taylor and Staden, 2001) there by allowing the fake thoughts of total safety and efficacious natural drugs warrants analysis of these plant materials to ascertain their safety profiles (Chang, 2000).

The unearthing of vinca alkaloids, vinblastine, vincristine and the chemotherapeutic agent called podophyllotoxins in 1950s in plants began the extensive research of anti-cancer drugs from plant sources (Cassady and Duoros, 1980; Noble, 1990). Medicinal plants have been used since 300 BC (Ayensu, 1978) and for thousands of years, plants and other natural products have been used to treat a variety of diseases and as a result, a number of modern drugs have been derived from them (Samuelsson, 1997).

The aim of the research was to evaluate *in vitro* antiproliferative on selected standard and cancer cell lines, phytochemical elucidation as well as acute toxicity effects of *Fagaropsis angolensis* and *Prunus africana* crude extracts. This is envisaged as a benchmark for elucidating safer chemotherapeutic agents, through the isolation of active compounds as well as validation of plants in the treatment and management of cancer.

1.2 Problem statement

Cancer chemotherapy is generally expensive, compounded with severe side effects due to the agents. There is need to develop and invent affordable agents to ensure safe and efficious medicines to the population. Thus, studies of medicinal plants as source of chemotherapeutic agent will go a long way in ameliorating the scarcity and expensive cancer agents.

1.3 Justification of the study

In the present, credible literature and knowledge regarding plant use with medicinal importance in the deterrence, treatment as well as management of various health conditions in Kenya has increased tremendously. In spite of the fact that many herbal medicines have been reported to offer solutions to many diseases, only a handful of them have been investigated and reported to possess potential cancer therapeutic and toxicological effects. Some of them include: *Acanthus pubescens*, *Carissa edulis*, *Spermacoce princeae*, *Toddalia asiatica*, *Clutia abyssinica*, *AsfAragus racemosus* (Jeruto *et al*., 2008 and 2011), *Prunus africana*, *Catunaregam spinosa*, *Flueggia virosa*, *Galium afarinoides*, *Maytenus obscura*, *Ocotea usambarensis* and the Graviola plant (Kokwaro, 2009; Kigen, 2013).

Over the past years, much literature citing cytotoxicity studies of plant extracts has been conducted extensively. Families containing a high proportion of active species with antitumor activity against human cell lines include: Anacardiaceae, Annonaceae, Asteraceae, Leguminosae, Meliaceae, Myrtaceae and Rutaceae (Evans and Trease, 2009). Based on this documentation, this work will address extracts of *Fagaropsis angolensis* and *Prunus africana*

used by herbalists in Kenya to manage cancer related diseases, its safety and cytotoxicity profiles. The obtained data forms basis for further validation of these plants for cancer treatment as well as synthesis of chemotherapeutic agents that have reduced side effects.

1.4 Objectives

1.4.1 General Objective

To evaluate the *in vitro* antiploriferative activity, phytochemical composition and toxicity studies of *Fagaropsis angolensis* and *Prunus africana* crude extracts.

1.4.2 Specific objectives

(a) To determine the qualitative phytochemical composition of *Fagaropsis angolensis* and *Prunus africana* extracts.

(b) To evaluate the *in vitro* antiploriferative activity of *Fagaropsis angolensis* and *Prunus africana* extracts against selected cancer cell lines.

(c) To determine the acute toxicity of *Fagaropsis angolensis* and *Prunus africana* extracts in mice.

1.5 Hypotheses

Null Hypothesis (H_0): The crude extracts of *Fagaropsis angolensis* and *Prunus africanus* do not possess antiploriferative activity and are toxic.

CHAPTER TWO

LITERATURE REVIEW

2.1 Pathophysiology and etiology of Cancer

Cancer is defined as uncontrollable multiplication and proliferation of cells (Cancer society, 2003; American cancer society, 2012). Cancer, a phrase representing a large group of diseases in which cells grow out of control and spread to other parts of the body. It is a disease characterized by cells that keep on dividing without obeying growth signals from other neighboring cells. They stick together and do not differentiate but remain immature and immotile. They metastasize to other parts of the body (World cancer report, 2013). Wherever cancer spreads, it is regarded and managed according to where it initiated. A case in point is the cancer of the prostate glands that has extended to bones is just called cancer of the prostate and not cancer of the bone (Mishra *et al.*, 2000).

Diverse cancers types behave differently, for instance, they proliferate at different rates and respond to therapy differently. Not all tumors or overgrowths are cancerous. Those that don't become cancerous are referred to as benign tumors. If they grow very large, they can press against the normal tissues thus causing problems in functionality. Due to their inability to invade other healthy cells and tissues, they lack the ability to metastasize to other organs (Rang *et al.*, 2012).

There are various causes of cancer but all of them converge to the causation of genetic aberrations leading to uncontrolled cell proliferation. Some of the most important etiologic factors include:

2.1.1. Cancer causing genes

The cell has a definite program and mechanism entailing a period of dormancy, division, differentiation, and senescence respectively. These processes are highly regulated as any hitch can be detrimentally lethal as far as life is concerned. For cell division to occur, 4 major

types of genes are involved; and for tumors to occur there must be faulty copies of more than one of these genes: Oncogenes; under normal circumstances play an important role in cell division (oncogenesis) and growth. When activated, they speed up the rate of cell growth. When one of these oncogenes is damaged, they permanently remain in an 'on' state and continuously cause rapid division of cells.

Tumor suppressor genes; play a critical role in preventing tumor growth and oncogenesis. An important tumor suppressor gene is the p53 gene which is often referred to as the guardian of life.

Suicide genes; are responsible in programming damaged cells to undergo apoptosis hence preventing further damage to neighboring cells. A defect in suicide genes makes cancerous cells to keep multiplying.

DNA repair genes; are proteins located in the cell, and are responsible for the repair of damaged DNA, however if a gene that makes the DNA repair protein is damaged, this ability is reduced, and over a period of time, errors may occur (Devita *et al.,* 2012*).*

2.1.2. Free radicals

A free radical contains an unpaired electron. When electrons are paired, the molecules are kept from flying apart because of the chemical bonds formed. An unpaired electron is very unstable and most often, potent chemical components will drive them to make pairs. Free radicals can also destroy an enzyme, protein, the entire cell, or worse, they can multiply through a chain of reactions to release thousands of the cellular oxidants like Flavin containing monooxygenases (FMOs), damaging the cell to an extent that DNA codes are altered causing cancer and immunity is suppressed to an extent that the tumors formed can evade immunologic surveillance. When cells encounter a free radical, they deteriorate resulting in diseases like cancer (Malcolm, 2001).

2.1.3. Genetic Factors

These are inherited defective tumor suppressor genes and cancer causing genes. This accounts for 5% of cancers. An example is retinoblastoma (George *et al.,* 2010).

2.1.4. Drugs

These are medication, especially those involving immunosuppressant and alkylating agents (George *et al.,* 2010).

2.1.5. Lifestyle

Lifestyle changes including excessive alcohol consumption, tobacco use, lack of exercise and eating unhealthy foods (George *et al.,* 2010).

2.1.6. Environmental factors

Environmental as well as occupational exposure to chemicals like asbestos, benzene, vinyl chloride and ionizing UV radiation can evoke genetic aberrations leading to development of cancer (George *et al.,* 2010).

2.1.7. Viruses

Viruses for instance, the Human Papilloma Virus (HPV), Hepatitis-B-virus (HBV), and Epstein- Burr Virus (George *et al.,* 2010).

2.2. Classification of Cancer

There are more than a hundred fold types of cancers and any part of the body can be affected. The most frequently affected organs include the lung, breast, colon, rectum, stomach and liver. Different types of cancers may be described on the basis of the normal cells from which they arise. The major type of cancer affects the epithelial cells and is termed as carcinoma(s). The other subgroups are sarcomas arising from connective tissues (fat, fibrous tissue, vessels, nerves and muscles) and lymphomas that arise from the lymph nodes. Those arising from body glands are termed as adenomas or adenocarcinomas (Bakharu, 2010).

2.3. Epidemiology of Cancer and effects on patients

Worldwide, the most common five types of cancer that kill men are (in the order of frequency) are: lung, stomach, liver, colorectal and oesophageal cancers. Conversely, in women, breast, lung, stomach, colorectal and cervical cancers respectively are major.

In Kenya, cancer is ranked third in causing deaths after cardiovascular and infectious diseases. It contributes to 7% of all the annual mortalities, with the risk of acquiring the disease before attaining 75years being 14%, while that of dying at 12% (National Cancer Control Strategy Kenya, 2011).

In women, the leading cancers are esophagus, cervical and breast, while in men, Kaposi's sarcoma, esophagus and prostate are the most common. According to the Nairobi Cancer registry, in 2002, 23.3% of all registered cases were of breast cancer, prostate accounted for 9.4% while cervical cancer was 20%. In 2014 and in women, a diagnosis of greater than 2,354 made as having cancer of the cervix resulting in 65% of them dying from the said ailment. Basically, cervical cancer is the second deadliest affecting women in Kenya (International Agency for Research on Cancer - IARC, 2012).

In children, the common cancers in Kenya include: blood cancer (leukemia) and lymphomas (Kenya Health Policy, 2012). Current data in Kenya indicates a mortality of about 50 Kenyans caused by various forms of cancer. In addition, about 82,000 patients are diagnosed out of which about 18,000 die every year. Cancer mortality in Kenya is anticipated to go up to 40,271 in the year 2030 (National Cancer Control Strategy Kenya, 2011).

As per the Regional Cancer Registry at KEMRI, almost 80% of reported cancer cases are at advanced stages, therefore very little gets to be achieved in terms of treatment, and this is attributed to lack of awareness, poor health and diagnostic facilities as well as shortages in both human and financial resources (National Cancer Control Strategy Kenya, 2011).

This disease causes economic derivation as it requires a lot of money for therapy, emotional suppression, hopelessness and other negative effects that accrue it including toxicity as organ dysfunction following therapy (National Cancer Treatment Guidelines Kenya, 2013).

2.4. Cancer management

Cancer diagnosis involves a series of assessments and diagnostic investigations like cytology, histopathology, biochemistry, imaging, endoscopy as well as other laboratory studies (Malcolm, 2001). Upon confirmation of the diagnosis, a series of therapeutic measures are embraced, with multidisciplinary treatments all aimed at improving patient's excellent life. Some of them include radiotherapy, surgery, chemotherapy, hormonal therapy, palliative care or a combination of some or all of these approaches (National Cancer Treatment Guidelines Kenya, 2013).

2.5. Cancer treatment

Cancer therapy entails three major modalities; radiotherapy, chemotherapy, surgery and hormonal therapy. Based on the current methods of treatment, one third of patients are cured effectively with local modalities (surgery or radiation therapy), especially if the cancer has not metastasized to other organs during the course of management (Skipper *et al.*, 1964; National Cancer Treatment Guidelines Kenya, 2013).

Early diagnosis might increase cure rates with such local treatments; however, in the remaining cases, early micrometastasis is a characteristic feature of the neoplasm, indicating that a systemic approach such as chemotherapy is required (often along with surgery or radiation) for effective cancer management (Skipper *et al.*, 1964).

Presently, about 50% of cancer patients can be cured effectively, with chemotherapy contributing to cure in 10–15% of patients. Chemotherapy offers a palliative care to a larger extent more than therapeutic treatment for numerous types of metastasized cancer. Successful palliation leads to a provisional enhancement of the symptoms and signs of cancer as well as

improvement in the overall quality of life. In the past decade, advances in cancer chemotherapy have also begun to provide evidence that chemical control of neoplasia may become a reality for many forms of cancer (Dancey and Arbuck, 2000).

This will probably be achieved through a combined-modality approach in which optimal combinations of surgery, radiotherapy, and chemotherapy are used to eradicate both the primary neoplasm and its occult micrometastases before gross spread can be detected on physical or x-ray examination. Use of hormonal agents to modulate tumor growth is playing an increasing role in hormone-responsive tumors thanks to the development of hormone antagonists and partial agonists. Several recombinant biologic agents have been identified as being active for cancer therapy, including interferon alfa and interleukin-2 (Skipper *et al.*, 1964.; Dancey and Arbuck, 2000; National Cancer Treatment Guidelines Kenya, 2013).

2.5.1. Chemotherapy

Diverse types of chemotherapeutics are used as therapeutic agents for cancer treatment (Dancey ans Arbuck, 2000). These agents can be classified into a variety of categories. The categorization is mainly on their elemental configuration and mode of action on cells that are affected by cancer. The categorization may vary as novel drugs are discovered.

Different types of chemotherapeutic agents that have in recent times entered scientific innovation comprise signal transduction inhibitor, which entails critical signaling pathways vital for cell development and proliferation; inhibitors of microtubule growth aimed against the mitotic spindle apparatus; differentiation drugs, focused to force cancer cells past a maturation block to form end-stage cells with modest or no proliferative effects (Dancey and Arbuck, 2000).

Antimetastatic agents, innovated to disturb surface properties of malignant cells and thus alter their invasive and metastatic effects; antiangiogenic drugs, innovated to hamper the

development of cancer vasculature; hypoxic tumor stem cell-specific agents, designed to exploit the greater capacity for reductive reactions in these often clinically resistant cells; tumor radio sensitizing and normal tissue radio protecting agents, intended at increased clinical efficiency of radiation therapy; cytoprotective agents, focused on protecting certain normal tissues against the toxic effects of chemotherapy; and biologic response modifiers, which alter tumor-host metabolic and immunologic relationships (Dancey and Arbuck, 2000).

2.5.2. Classification of Chemotherapeutic drugs

Chemotherapeutics are usually classified into subtypes. Different kinds of agents in every subclass are based on their mode of action, configuration or origin. A few agents appear to fit into more than one class. Others do not fit to any of the classes (Dancey and Arbuck, 2000).

2.5.3. Phase specific toxicity subtypes

Chemotherapeutic agents can be grouped on to whether they are more likely to target cells in a particular phase of their growth cycle. More crudely, they can also be divided into whether they are more toxic to cells that are actively dividing rather than cells in both the proliferating and resting phases (Skipper e*t al.*, 1964; Dancey and Arbuck, 2000).

2.5.4. Phase-specific chemotherapy

These drugs, such as methotrexate and vinca alkaloids, kill proliferating cells only during a specific part or parts of the cell cycle. Antimetabolites, such as methotrexate, are more active against S-phase cells (inhibiting DNA synthesis) whereas vinca alkaloids are more M-phase specific (inhibiting spindle formation and alignment of chromosomes) (Skipper *et al.*, 1964). Attempts have been made to time drug administration in such a way that the cells are synchronized into a phase of the cell cycle that renders them especially sensitive to the cytotoxic agent. For example, vinblastine can arrest cells in mitosis.

These synchronized cells enter the S-phase together and can be killed by a phase-specific agent, such as cytosine arabinoside. Most current drug schedules, however, have not been

devised on the basis of cell kinetics (Skipper *et al.*,1964; Dancey and Arbuck, 2000; National Cancer Treatment Guidelines Kenya, 2013).

2.5.5. Cell cycle-specific chemotherapy

Most chemotherapy agents are cell cycle-specific, meaning that they act predominantly on cells that are actively dividing (Skipper e*t al.*,1964). They have a dose-related plateau in their cell killing ability because only a subset of proliferating cells remain fully sensitive to drug-induced cytotoxicity at any one time. The way to increase cell kill is therefore to increase the duration of exposure rather than increasing the drug dose (National Cancer Treatment Guidelines Kenya, 2013).

2.5.6. Cell cycle-nonspecific chemotherapy

These drugs, for example alkylating agents and platinum derivatives, have an equal effect on tumor and normal cells whether they are in the proliferating or resting phase. They have a linear dose–response curve; that is, the greater the dose of the drug, the greater the fractional cell kill (Dancey and Arbuck, 2000; National Cancer Treatment Guidelines Kenya, 2013).

2.6 Classification according to mechanism of action

Classifying cytotoxic drugs according to their mechanism of action is the preferred system in use between clinicians.

2.6.1 Alkylating agents

These highly reactive compounds produce their effects by covalently linking an alkyl group ($R\text{-}CH_2$) to a chemical species in nucleic acids or proteins. The site at which the cross-links are formed and the number of cross-links formed is drug specific. Most alkylating agents are bipolar, i.e. they contain two groups capable of reacting with DNA (Dancey and Arbuck, 2000). They can thus form bridges between a single strand and two separate strands of DNA, interfering with the action of the enzymes involved in DNA replication. The cell then either dies or is physically unable to divide or triggers apoptosis. The damage is most serious during

the S-phase, as the cell has less time to remove the damaged fragments. Examples include: Nitrogen mustards (e.g. melphalan and chlorambucil); Oxazaphosphorenes for instance cyclophosphamide, ifosfamide ; Alkyl alkane sulphonates such as busulphan; Nitrosureas for instance carmustine (BCNU), lomustine (CCNU)); Tetrazines such as dacarbazine, mitozolomide and temozolomide; Aziridines (thiopeta, mitomycin C; and procarbazine (National Cancer Treatment Guidelines Kenya, 2013).

2.6.2. Heavy metals

Usually Platinum-based agents used for cancer treatment. These include carboplatin, cisplatin and oxaliplatin (Dancey and Arbuck, 2000). Cisplatin is an organic heavy metal complex. Chloride ions are lost from the molecule after it diffuses into a cell allowing the compound to cross-link with the DNA strands, mostly to guanine groups. This causes intra- and inter-strand DNA cross-links, resulting in inhibition of DNA, RNA and protein synthesis.

Carboplatin has the same platinum moiety as cisplatin, but is bonded to an organic carboxylate group. This leads to increased water solubility and slower hydrolysis that has an influence on its toxicity profile. It is less nephrotoxic and neurotoxic, but causes more marked myelosuppression (Dancey and Arbuck, 2000).

Oxaliplatin belongs to a new class of platinum agents. It contains a platinum atom complexed with oxalate and a bulky diaminocyclohexane (DACH) group. It forms reactive platinum complexes that are believed to inhibit DNA synthesis by forming inter-strand and intra-strand cross-linking of DNA molecules. Oxaliplatin is not generally cross-resistant to cisplatin or carboplatin, possibly due to the DACH group (Dancey and Arbuck, 2000)

2.6.3. Antimetabolites

Antimetabolites are compounds that bear a structural similarity to naturally occurring substances such as vitamins, nucleosides or amino acids. They compete with the natural substrate for the active site on an essential enzyme or receptor. Some are incorporated

directly into DNA or RNA. Most are phase-specific, acting during the S-phase of the cell cycle. Their efficacy is usually greater over a prolonged period of time, so they are usually given continuously. There are three main classes (Dancey and Arbuck, 2000).

2.6.4. Folic acid antagonists

Methotrexate competitively inhibits dihydrofolate reductase, which is responsible for the formation of tetrahydrofolate from dihydrofolate. This is essential for the generation of a variety of coenzymes that are involved in the synthesis of purines, thymidylate, methionine and glycine (Dancey and Arbuck, 2000).

A critical influence on cell division also appears to be inhibition of the production of thymidine monophosphate, which is essential for DNA and RNA synthesis. The block in activity of dihydrofolate reductase can be bypassed by supplying an intermediary metabolite, most commonly folinic acid. This is converted to tetrahydrofolate that is required for thymidylate synthetase function (Dancey and Arbuck, 2000).

2.6.5. Pyrimidine analogues

These drugs resemble pyrimidine molecules and work by either inhibiting the synthesis of nucleic acids which inhibiting enzymes involved in DNA synthesis (e.g. cytarabine, which inhibits DNA polymerase) or by becoming incorporated into DNA (e.g. gemcitabine), interfering with DNA synthesis and resulting in cell death (Dancey and Arbuck, 2000).

2.6.6. Purine analogues

These are analogues of the natural purine bases and nucleotides. 6-Mercaptopurine (6MP) and thioguanine are derivatives of adenine and guanine, respectively. A sulphur group replaces the keto group on carbon-6 in these compounds. In many cases, the drugs require initial activation. They are then able to inhibit nucleotide biosynthesis by direct incorporation into DNA (Dancey and Arbuck, 2000).

2.6.7. Cytotoxic antibiotics

Most antitumor antibiotics have been produced from bacterial and fungal cultures (often Streptomyces species (Dancey and Arbuck, 2000).

They affect the function and synthesis of nucleic acids in different ways. Anthracyclines (e.g. doxorubicin, daunorubicin, epirubicin) intercalate with DNA and affect the topoiosmerase II enzyme. This DNA gyrase splits the DNA helix and reconnects it to overcome the torsional forces that would interfere with replication (Guichard and Danks, 1999).

The anthracyclines stabilize the DNA tomoisomerase II complex and thus prevent reconnection of the strands; Actinomycin D intercalates between guanine and cytosine base pairs. This interferes with the transcription of DNA at high doses. At low doses DNA-directed RNA synthesis is blocked; Bleomycin consists of a mixture of glycopeptides that cause DNA fragmentation; Mitomycin C inhibits DNA synthesis by cross-linking DNA, acting like an alkylating agent (Guichard and Danks, 1999).

2.7 Vinca alkaloids

The two prominent agents in this group are vincristine and vinblastine that are extracted from the periwinkle plant. They are mitotic spindle poisons that act by binding to tubulin, the building block of the microtubules (Guichard and Danks, 1999).

This inhibits further assembly of the spindle during metaphase, thus inhibiting mitosis. Although microtubules are important in other cell functions (hormone secretion, axonal transport and cell motility), it is likely that the influence of this group of drugs on DNA repair contributes most significantly to their toxicity. Other newer examples include vindesine and vinorelbine (Dancey and Arbuck, 2000).

2.8 Taxoids

Paclitaxel (Taxol) is a drug derived from the bark of the pacific yew, *Taxus brevifolia*. It promotes assembly of microtubules and inhibits their disassembly. Direct activation of

apoptotic pathways has also been suggested to be critical to the cytotoxicity of this drug. Docetaxel (Taxotere) is a semisynthetic derivative (Herscher and Cook, 1999).

2.9. Topoisomerase inhibitors

Topoisomerases are responsible for altering the 3D structure of DNA by a cleaving-unwinding-ligation reaction (Guichard and Danks, 1999). They are involved in DNA replication, chromatid segregation and transcription. It has previously been considered that the efficacy of topoisomerase inhibitors in the treatment of cancer was based solely on their ability to inhibit DNA replication. It has now been suggested that drug efficacy may also depend on the simultaneous manipulation of other cellular pathways within tumour cells. The drugs are phase-specific and prevent cells from entering mitosis from G_2 (Guichard and Danks, 1999).

2.9.1. Topoisomerase I inhibitors

Camptothecin, derived from *Camptotheca acuminata* (a Chinese tree), binds to the enzyme–DNA complex, stabilizing it and preventing DNA replication. Irinotecan and topetecan have been derived from this prototype (Guichard and Danks, 1999).

2.9.2. Topoisomerase II inhibitors

Epipodophyllotoxin derivatives for instance etoposide and vespid, are semisynthetic derivatives of *Podophyllum peltatum*, the American mandrake. They stabilize the complex between topoisomerase II and DNA that causes strand breaks and ultimately inhibits DNA replication (Guichard and Danks, 1999).

2.10. Monoclonal antibodies

It is apparent that targeted therapy using monoclonal antibodies (MAb) is vital in the detection and treatment of cancer (Wheeler *et al.*, 1999; Caponigro e*t al*., 2005).

Monoclonal antibodies can be derived from a variety of sources: murine: mouse antibodies; chimeric: part mouse, human antibodies; humanized: engineered to be mostly human; human:

fully human antibodies. Murine monoclonal antibodies may themselves induce an immune response that may limit repeated administration (Green *et al*., 2000; Caponigro *et al*., 2005).

Humanized and, to a lesser extent, chimeric antibodies are less immunogenic and can be given repeatedly. There are several proposed mechanisms of action of monoclonal antibodies.These include: direct effects: – induction of apoptosis; – inhibition of signalling through the receptors needed for cell proliferation/function; – anti-idiotype antibody formation, determinants amplifying an immune response to the tumour cell;: indirect effects: – antibody-dependent cellular cytotoxicity (ADCC, conjugating the 'killer cell' to the tumour cell; – complement-mediated cellular cytotoxicity (fixation of complement leading to cytotoxicity (Caponigro *et al*., 2005).

Antibodies have also been used as vectors for the delivery of drugs and radiopharmaceuticals to a target of tumour cells. The earliest and most successful clinical use of antibodies in oncology has been for the treatment of haematological malignancies (Herbst and Langer, 2002). Interest in the development of antibodies for solid tumours has become increasingly popular, especially with respect to the epidermal and vascular endothelial growth factor receptors. MAb technology has been directed against EGFR (Wheeler *et al.,* 1999; Baselga 2001).

The chimeric IgG antibody cetuximab (C225) has the binding affinity equal to that of the natural ligand and can effectively block the effect of epidermal growth factor and transforming growth factor (Perrotte *et al*., 1999; Herbst and Langer, 2002).

It has been shown to enhance the antitumour effects of chemotherapy and radiotherapy in preclinical models. More recently, cetuximab has been evaluated alone and in combination with radiotherapy and various cytotoxic chemotherapeutic agents in a series of phase II and III studies involving patients with head and neck cancers (Herbst and Langer, 2002).

2.11. Angiogenesis inhibitors

Angiogenesis is the process of new blood vessel formation, triggered by hypoxia and regulated by numerous stimulators and inhibitors. It is vital for cancer development. A tumour cannot extend beyond 2–3mm without inducing a vascular supply. New vessels develop on the edge of the tumour and then migrate into the tumour. This process relies on degradation of the extracellular matrix(ECM) surrounding the tumour by matrix metalloproteinases (MMPs), such as collagenase, that are expressed at high levels in some tumour and stromal cells (Kyzas *et al*., 2005).

Angiogenesis is to a greater extent dependent on the migration and proliferation of endothelial cells. It has been found that antiangiogenic agents tend to be cytostatic rather than cytotoxic, hence stabilizing the tumor and preventing spread. As a consequence, they may be valuable for use in combination with cytotoxic drugs, as maintenance therapy in early-stage cancers or as adjuvant treatment after definitive radiotherapy or surgery (Kyzas *et al*., 2005).

There is evidence to support the fact that suppressing angiogenesis can maintain metastases in a state of dormancy. Interestingly, development of resistance does not appear to be a feature of these drugs (Boehm *et al.,* 1997).

2.12. Traditional medicine in cancer management

Plant materials remain an important resource to combat chronic deseases in the world and pharmacognostic investigations of plants are carried out to find novel drugs development of new therapeutic components for treatment and prevention of diseases including cancer (Jeruto *et al.,* 2010).

The phytochemical components in plants with anti-cancer activity are usually, vinca alkaloids that inhibit cell proliferation by affecting the microtubular dynamics in mitosis.

This characteristic block causes a programmed cell death also known as apoptosis, podophyllotoxins, texanes, camptothecin, berbamine, butulinic acid, bruceatin, beta-

lapachone, colchicines, combretastatin A-4, daphnoretin, cucurbitacin, diazein & genistein, curcumin and elliptine (Joyce *et al.*, 2011).

2.12.1. Rutaceae family

Rutaceae family comprises about 150 genera and 1600 species of trees, shrubs and climbers distributed throughout the tropical and temperate regions of the world. The main genera of this family are Citrus, Ruta, Ptelea, Murrya and Fortunella. In Kenya there are 10 genera and 28 species. The plants rutaceae are characterized by varied secondary metabolites including alkaloids, flavonoids, coumalins, and volatile oil, plants of this family have for a long time been used in the perfumery industry, gastronomy and traditional medicine (Supabphol and Tangjitjareonkun , 2014).

2.12.2. *Fagarospis angolensis* (Engl.) Dale

Fagaropsis angolensis (Engl.) Dale is commonly known as Mukuriampungu (Embu) or Murumu (Meru), Mũkaragatĩ (Kikuyu), Shingulosto (Luhya), Kwiril (Marakwet), Noiywet (Nandi), Myinja.

It is a deciduous tree which grows up to 20 m tall. Its bark is pinkish-grey in colour, slightly rough and sometimes covered with purple corky outgrowths that have white dots. Leaves are compound, glabrous except the midrib, each leaf has 5-11 leaflets measuring 4-9 by 2-5 cm and are smooth with gland dots near the margin. Flowers are cream or yellowish in terminal panicles that are 3-12 cm long, petals are 3.5-6 mm long. The fruit is round, 6-8 mm diameter and is purple when ripe (Beetje, 1994; Gachathi, 2007).

2.12.3. Ethnobotanical and ethnomedical Information of *Fagaropsis angolensis*

In Kenya, *Fagaropsis angolensis* wood is used for construction and making furniture (Gachathi, 2007). Ethno medically the root decoction of *Fagaropsis angolensis* is used for the management of cancer and malaria (Jeruto *et al*., 2010). The leaf decoction is also useful in treatment of malaria and back and joint aches (Jeruto *et al*., 2010; Kareru *et al*., 2007).

2.12.4. Biological Activity of *Fagaropsis angolensis*

Methanol and aqueous extracts of the stem bark has been reported to show considerable in-vitro activity against both chloroquine resistant and chloroquine-sensitive *Plasmodium falciparum* strains. Methanol extracts have significant toxicity in the brine shrimp lethality test. On the other hand, water extracts show only mild toxicity. Important phytochemicals like Canthin-6-one and 5-methoxycanthin-6-one have been reported to exhibit fungicidal activity (Kareru *et al.,* 2006).

2.12.5. Phytochemicals isolated from *Fagaropsis angolensis*

Phenanthrene carboxylic acid derivatives, Hexyl-9, 10-dihydroxydec-5-enoate and Methylheneicosane ester derivatives like Canthin-6-one and 5-methoxycanthin-6-one, alkaloids and limonoids like benzophenanthridine alkaloid nitidine have so far been isolated and characterized (Mudalungu *et al.,* 2013).

Figure 2.1: Parts of *Fagaropsis angolensis* collected from Irangi forest station, Embu county
Source; Photograph taken February, 2016.

2.13. *Prunus africana*

Prunus africana, is an evergreen plant commonly found growing in sub-Saharan Africa. The plant is commonly Africa cherry or red stinkwood. It belongs to the family Rosaceae. The plant has a blackish brown rugged bark which has a red brown inner surface. In Kenya the plant is found mainly on the highland regions of; Tugen hills, Mt. Elgon, Aberdares ranges and Mt. Kenya (Gachie *et al.*, 2012 ; Kadu *et al.*, 2012).

Figure 2.2: Parts of *Prunus africana* collected from Irangi Forest station, Embu County Source; Photograph taken February, 2016.

2.13.1. Ethnomedical uses of *Prunus africana*

Prunus africana is used ethnomedically in the treatment of stomach ache, fever, breast cancer, prostate cancer, colorectal cancer, malaria and bovine babesiosis. The plant is also used as an appetite stimulant (Gachie *et al.*, 2012; Kareru *et al.*, 2007).

2.13.2. Biological activity of Prunus africana

The plant has attracted a lot of attention globally due to its envisaged potential in the treatment of benign prostatic hyperplasia which is common among aged men (Gathumbi *et al.*, 2002).

3.0. CHAPTER THREE

MATERIAL AND METHODS

3.1. Study area

The plant materials (root and leaves) of *Fagaropsis angolensis* and (bark) of *Prunus africana* were collected from Mount Kenya Forest located at Irangi Embu County (1750 m above sea level), Kenya (Figure: 3). The collected specimens were identified and authenticated with the aid of a taxonomist at the University of Nairobi, Chiromo Campus with authentication number ALY2015/01 for *Fagaropsis angolensis* and ALY2015/02 for *Prunus afriana* respectively. The voucher specimens were prepared in duplicates and deposited at the University of Nairobi Herbarium in Chiromo campus.

Figure 3.1: Map of Mount Kenya Forest Area.
Source; Google maps https://www.google.com/maps/place/Irangi+Forest+Station
(Accessed 1/06/2017)

3.2. Extraction

The plants were identified in their natural habitats by a forester from Kenya Forestry Services (KFS), Mt. Kenya forest, Irangi substation in Embu County and authenticated with voucher numbers ALY2015/01 for *Fagaropsis angolensis* and ALY2015/02 for *Prunus afriana* respectively.

The voucher specimens were deposited in the university herbarium at the school of biological sciences, Chiromo campus, University of Nairobi. Plant materials (Leaves, Roots, Stems, and barks) were collected, packaged separately and transported to the Public Health, Pharmacology and Toxicology laboratories, washed thoroughly with running tap water, chopped into small pieces and then dried under shade for a period of 14 days.

The collected plant samples were air-dried and ground. Extraction was done by maceration using methanol and distilled water for aqueous extract. Approximately 50 g of ground materials of *F. angolensis* (Root bark, root stem, whole root, leaves, leaf stalk) and *P. africana* stem bark were soaked separately with enough methanol in a 1 litre beaker and then covered with a foil paper for 48 hours with constant shaking using a magnetic stirrer. Thereafter each extract was filtered and reduced *in vacuo* at 58 ^{0}C and finally dried completely in the oven set at 35 ^{0}C. The aqueous extract was prepared by boiling 30 g of each of the powdered crude in water for five minutes. The extracts were cooled, filtered and then freeze-dried. The dry and lyophilized extracts were weighed and stored in a freezer at - 20^{o}C awaiting biological and chemical assays (Harborne 1976; Bibi *et al*., 2012).

3.3. Investigation of phytochemical composition of crude extracts

Qualitative methods of Savithrama *et al*., 2011; Harborne, 1976 and Trease, 2009, were used with minor modifications to identify the phytochemical composition in the prepared extracts of *Fagaropsis angolensis* and *Prunus africana*.

The criteria for grading the phytochemicals was done on the basis of the intensity of colour produced from reactions observed in the test tubes. Very high concentration was denoted as

(+++), high concentration (++), moderate concentration as (+) and nil (-) represented no observable reaction. Qualitative tests were conducted are as follows:

3.4. Test for Tannins

The crude plant extract (0.5 g) was boiled in 20 ml of distilled water in a test tube and then filtered. Then 0.1 % $FeCl_3$ was added to the filtrate. Bluish- green precipitate indicates presence of tannins (Evans, 2009; Savithrama *et al*., 2012).

3.5. Test for Saponins

Frothing Test was adopted

About 0.5 g of the crude plant extract was dissolved in boiling water in a test tube and allowed to cool and then the tube shaken well. Presence of frothing is indicative of the presence of saponins (Evans, 2009; Savithrama, *et al*., 2012).

3.6. Test for Alkaloids

Crude plant extracts of both plants (each 0.6 g) were mixed with about 8ml of 1 % HCl, warmed, and filtered. 2 ml of filtrate was treated with Mayer's reagent. The appearance of cream colored precipitate is a positive indication for the presence of alkaloids. Similarly, 2ml of the filtered extract was treated with Dragendorff's reagent. (A reddish-brown precipitate indicates a positive test for presence of alkaloids (Evans, 2009; Savithrama *et al*., 2012).

3.7. Test for Glycosides

3.7.1. Cardiac glycosides- Keller-killiani test (test for deoxy sugars)

The crude drugs were extracted with chloroform and evaporated to dryness. 0.4 ml of glacial acetic acid containing trace amour of ferric chloride was added into a small test tube, and carefully, 0.5 ml of concentrated sulphuric acid was added by the side of the test tube. Acetic acid layer showing a blue colour is a positive test for presence of cardiac glycosides (Evans, 2009; Savithrama *et al*., 2012).

3.7.2. Borntrager's test

The test materials were boiled with 1ml of sulphuric acid in a test tube for 5 minutes. Then they were filtered while hot, cooled and then shaken with equal volume of chloroform. Then the lower layer of chloroform was separated and shaken with half of its volume with dilute ammonia. A rose pink to red colour produced in the ammoniacal layer (positive) is an indication of the presence of glycosides (Evans, 2009; Savithrama *et al*., 2012).

3.7.3. Kedde test

Two drops of Kedde reagent were added to one portion of the dry crude plant extract. The presence of purple colour indicates the presence of glycosides whose aglycone moiety has unsaturated lactone ring (Evans, 2009; Savithrama *et al*., 2012).

3.8. Determination of steroids

1 ml solution of ethanol extract was taken and then added Liebermann– Burchard reagent. If reddish purple color is produced, it indicates the presence of steroids (Evans, 2009; Savithrama *et al*., 2012).

3.9. Determination of flavonoids

A few drops of concentrated hydrochloride acid were added to a small amount of an alcoholic extracts of the plant materials. Immediate development of a red color indicates the presence of flavonoids. This was also analyzed by other three means. 10 ml of solution of the extract hydrolyzed with 10% sulfuric acid was divided into three portions: (1) 1 ml dilute ammonia solution was added in one portion. Greenish yellow indicates the presence of flavonoids, (2) 1 ml dilute sodium carbonate solution was added in one portion. Pale yellow indicates the presence of flavonoids, (3) 1 ml dilute sodium hydroxide solution was added in one portion. A yellow indicates the presence of flavonoids (Evans, 2009; Savithrama *et al*., 2012).

3.10. Determination of Phenols

About 1 g of powdered drugs was boiled with 10 ml of 70 % ethanol in a water bath using boiling tubes for 5 minutes. The extracts were filtered while hot and cooled. To 2 ml of each extract 5 % Ferric chloride was added and a green precipitate indicates presence of phenols (Evans, 2009; Savithrama *et al*., 2012).

3.11. Determination of terpenoids

About 2 ml of the test extracts was added to acetic acid anhydride followed by careful addition of concentrated sulphuric acid. The formation of a blue-green ring indicates the presence of terpenoids (Evans, 2009; Savithrama *et al*., 2012).

3.12. *In-vitro* bioassay studies

3.12.1. Preparation of stock extracts

The crude extract and fractions were separately dissolved in DMSO at a concentration of 10 mg/ml. Required serial dilutions were prepared logarithmically under sterile conditions by adding calculated amounts of phosphate buffer solution (PBS) to obtain working concentration of 1000 µg/ml (Bibi *et al*, 2012). All the prepared drugs were stored at 4^{o}C and retrieved only during use.

3.12.2. Determination of *in vitro* antiproliferative activity using MTT Assay.

Vero E6 (normal cell), CT 26-CL 25 (colon cancer), and Hep2 (throat cancer) from ATCC (Manassas, VA, USA) were used. Cells initially stored in liquid nitrogen were obtained and quickly thawed in a water bath at 37 ^{0}C and transferred to growth MEM media with 10 % Fetal Bovine Serum (FBS), 1% L-Glutamine and 1% antibiotic (PS) in a T75 culture bottle and incubated in a high humidity environment at 37 ^{o}C and 5% CO_2. The media was removed after 24hrs and the cells washed three times with phosphate buffered saline (PBS) and new media added.

This step was necessary since during preservation the cells were suspended in media containing 10% dimethyl sulfoxide (DMSO). Dimethyl sulfoxide is toxic to cells at high

concentrations. The cells were then incubated to attain confluence. After attaining confluence, the cells were passaged and counted by tryphan blue exclusion staining with the aid of a hemocytometer. Cells were seeded at a concentration of 50,000 cells per well (in 100 µl of maintenance media) into a flat bottomed micro titer cell culture enabled 96 well plates (Sigma, USA) and incubated at 37 °C and 5 % CO_2 overnight. Samples addition were done by measuring 15µl of the sample solution, followed by addition of 35µl of media to top up the wells' volume at row H to 150µl. This was done to dilute the sample concentration to 100µg/ml starting concentration and also reduce DMSO concentration to 0.01% in order to circumvent its' toxicity to cells. Serial dilution was done by mixing the contents at row H, followed by picking 50µl of the media-drug mixture from row H to row G. This was repeated until row B. Fifty µl of the mixture was also picked from row B and discarded. Row A served as the cell control.

All the plates' columns contained media and cells except column 12 which contained media only. The experiment was done in triplicate. Cells were incubated for 48hours, then 10 µl of MTT dye (5mg/ml) was added and the plates incubated for 3 more hours at 37 °C and 5 % CO_2. Mitochondrial dehydrogenase which is a biomarker of a life cell interacts with MTT dye reducing it to insoluble formazan. The formazan formed is directly proportional to the number of life cells. Formazan formation was confirmed using inverted light microscope and then solubilized with 50 µl of 100 % DMSO and optical density (OD) read at 562 nm in a 96-well microliter plate multiplex reader.

3.13. Determination of *in vivo* acute oral toxicity of study crude extracts

3.13.1. Experimental animals

Twenty four (24) healthy young adult, female Swiss albino mice 8-12 weeks weighing 20-25 g. The animals were nulliparous and non- pregnant were purchased from the Public Health, Pharmacology and Toxicology, University of Nairobi animal house.

3.13.2. Housing and feeding of the animals

Mice cages measuring 35 cm (L) × 25 cm (W) × 18cm (H) were used to house the animals. The cages were lined with wood shavings which served as beddings for the animals.

The mice were housed under standard laboratory conditions (temperature 22^0 C ± 3^oC with natural light and relative humidity between 50-60 %. They were fed on standard pellet diet from a commercial feed supplier (Unga Feeds) and unlimited supply of water (OECD, 2001).

3.13.3. Occupational Health and Personal Protection equipment

Protective clothing, Latex hand gloves and protective masks were used at all times.

Additionally, anti- tetanus and anti- rabies vaccines were made available and stored under refrigerator.

3.13.4. Preparation of animals

Random selection of the animals was done and each animal was marked on the tail for individual identification. They were then kept in the cages for a period of ten days prior to dosing to allow for acclimatization to the laboratory conditions.

3.13.5. Determination of acute oral toxicity studies of the crude extracts in mice

An OECD guideline 423 of 2001 for Acute Oral Toxicity- Acute Toxic Class Method was used. The animals were randomly assigned into two groups of three (3) mice each. They were fasted for four (4) hours by withholding food but not water. Following the period of fasting the animals were individually weighed and marked with picric acid to enable identification. One group which served as the control group were administered 2 ml of physiological buffer saline once daily.

The other group served as the treatment group and was administered with a starting dose of 300mg and 2000mg/kg body weight for *Fagaropsis angolensis* crude extracts as there was no previous information. For *Prunus africanas* the starting dose was 2000mg/kg body weight as available information suggests that mortality is unlikely at the highest starting dose level. This was based on

the judgment with respect to classifying the test substances to a series of toxicity classes defined by LD_{50} range.

Each of the mice in the treatment group was dosed once over 24 hour period to identify the concentration of the extract responsible for mortality of 2-3 (50%) of the animals. Absence or presence of compound related mortality of the animals dosed at one step determined the next step.

Individual observations after dosing at least once during the first 30 minutes, 4 hours, 24 hours and daily thereafter for a total of 14 days was done. Additional physical changes in skin and fur, eyes and mucous membranes, respiration rate, circulatory, autonomic and central nervous systems, somatomotor, activity and behavior pattern. Tremors, convulsions, salivation, diarrhea, lethargy, sleep and coma were observed. Body weight and mortality were recorded at intervals. The data that obtained and presented in tables and the LD_{50} range was determined as per the (OECD, 2001) guidelines.

3.14. Data analysis

The obtained data from antiploriferative studies was expressed as a mean ± standard error of the mean (SEM) of the two independent experiments. Analysis was done by determining IC_{50} (the concentration required to kill 50 % of the cells using linear regression curves.

Toxic properties of the crude extracts were assigned and graded in respect to the Loomis and Hayes (1996). The data from acute toxicity studies was expressed as standard error of the mean and compared to the reference drugs and to various cell lines used.

3.15. Ethical Consideration

Approval to use Laboratory Animal was given by Biosafety, Animal use and Ethics committee of the University of Nairobi, Faculty of Veterinary Medicine REF: FVM/BAVEC/2016/109 (Appendix 1). Approval to carry out the study was granted by the Board of Postgraduate studies REF: J56/73786/2014 (Appendix 2)

3.15.1. Disposal of rodent carcasses and used cell line plates

The carcasses were placed in special non- polyvinylchloride, sealable transparent plastic bags. They were then incinerated.

All the used cell lines were disposed in special non- polyvinylchloride, sealable transparent plastic bags. They were then incinerated.

CHAPTER FOUR

RESULTS

4.1. Percentage yields following extraction

The leaf, midrib, root, root bark and root stem of *F. angolensis* and the bark of *P. africana* were extracted with methanol and water according to Haborne (1976). The weights of material extracted, the extract and percentage yields were presented as in tables 4.1 below.

Table 4.1: Percentage yields of methanolic extracts of *F. angolensis* and *P. africana* barks

Sample	Methanolic extracts (%)	Aqueous extracts (%)
PB	**6.68**	**10.37**
FAWR	**8.42**	**14.47**
FARS	**6.48**	**7.81**
FARB	**7.16**	**9.93**
FAL	**6.54**	**13.73**
FALS	**6.58**	**12.60**

KEY: PB-*P. africana* bark; FAWR-*F. angolensis* whole root; FARS- *F. angolensis* root stem; FARB- *F. angolensis* root bark; FAL- *F. angolensis* Leaf; FALS- *F. angolensis* leaf stalk.

4.2. Phytochemical composition of *Prunus africana* and *Fagaropsis angolensis* crude extracts

Five extracts of various parts of *F. angolensis* and the stem bark of *P. africana* were screened for eight phytochemicals. It was observed that these phytochemicals were present variedly across the test extracts with some lacking in some extracts. Table 4.2 shows the composition of various phytochemicals in the test extracts.

Table 1.2: Phytochemical composition of *F. angolensis* and *P. africana* extracts

Sample Extract	Phytochemicals Screened							
	Phenols	**Steroids**	**Glycosides**	**alkaloids**	**flavonoids**	**Terpenoids**	**Saponins**	**Tannins**
FAL MeOH	+++	+++	+	+	+	+	-	++
FALS H_2O	-	++	++	++	++	++	-	++
FAWR H_2O	+	++	+	+	+	+	-	++
FARB MeOH	++	+++	++	+	+++	-	-	+
FAWR MeOH	+	++	+++	+	+	++	-	+
FALS-MeOH	++	++	++	+	+	++	-	+
FARS MeOH	-	+	+++	+++	-	+++	-	++
FAL-H_2O	+	++	+++	+	++	++	-	+++
FARB H_2O	+	++	+	+	++	++	-	++
FARS H_2O	+	++	+	+	+	++		+
PASB-MeOH	+++	-	++	++	+++	+++	+++	+
PASB-H_2O	+	-	+++	+	++	+++	+++	++

Key: FAL- *Fagaropsis angolensis* leaf extracts, FALS- *Fagaropsis angolensis* leaf stem extracts, FAWR- *Fagaropsis angolensis* Whole root extract, FARB- *Fagaropsis angolensis* root bark extracts, FARS- *Fagaropsis angolensis* root stem extracts, PB-*Prunus africana* bark extracts, H2O- water extract, MeOH-Methanol extract, + (present); - (absent).

4.3. *In vitro* antiploriferative activity of *Fagaropsis angolensis* and *Prunus africana* crude extracts on Vero cell lines.

The concentration that inhibits or kills 50% of the cells (IC_{50}) was also calculated for the Vero cell line. The IC_{50} varied with the plant extract and the solvent used for extraction. It was noted that all the aqueous extracts had an IC_{50} value of >100. The methanolic extracts too exhibited an IC50 value of >100 except for the *F. angolensis* root stem and *F. angolensis* whole root that posited IC_{50} values of, 1.10±0.70 and 5.10±0.80 µg/ml respectively in comparison with the reference drug, Doxorubicin which had an IC50 value of >100 µg/ml. Table 4.3 below, shows the IC50 results together with the reference drug, Doxorubicin which had an IC50 value of 6.5±3.25 µg/ml.

Table 4.3: Determination of *in-vitro* antiploriferative of *F. angolensis* and *P. africana* extracts on vero cell lines

Plant part	IC50±SEM µg/ml	
	Methanolic extract	**Water extract**
***F. angolensis* Leaf**	**>100**	**>100**
***F. angolensis* Leaf Stalk**	**>100**	**>100**
***F. angolensis* Whole Root**	**5.10±0.80**	**>100**
***F. angolensis* Root stem**	**1.10±0.70**	**>100**
***F. angolensis* Root bark**	**>100**	**>100**
***P. africana* Bark**	**5.56±1.5**	**>100**
Doxorubicin	**6.5±3.25**	

4.4. *In vitro* antiploriferative activity of *F. angolensis and P. africana* crude extracts on HEP2 cell lines

IC50 values of the extracts of various parts of *F. angolensis* and *P. africana* stem bark were calculated and are presented in table 4.4. Only the Aqueous extracts of the *F. angolensis* leaf and leaf stalk and the methanolic extract of *F. angolensis* leaf had an IC_{50} value greater than 100 µg/ml. Other extracts had varied IC_{50} values with the *F. angolensis* whole root methanolic extract having the least value of 10.05±2.15 µg/ml while that of the *F. angolensis* leaf stalk had the highest value of 89.2. ±3.80 µg/ml as compared to the reference drug Doxorubicin that had an IC_{50} value of 2.5±0.50 µg/ml.

Table 4.4: Determination of *in vitro* antiploriferative activity of *F. angolensis* extracts on HEP2 cell line

Plant part	IC50±SEM µg/ml	
	Methanolic extract	**Water extract**
***F. angolensis* Leaf**	**>100**	**>100**
***F. angolensis* Leaf Stalk**	**89.2. ±3.80**	**>100**
***F. angolensis* Whole Root**	**10.05±2.15**	**21.65±0.05**
***F. angolensis* Root Stem**	**60.50±0.00**	**59.70±3.80**
***F. angolensis* Root Bark**	**60.25±2.75**	**71.80±5.50**
***P. africana* Bark**	**18.15±17.85**	**11.75±2.75**
Doxorubicin	**2.5±0.50**	

4.5. *In vitro* antiploriferative activity of *F. angolensis and P. africana* crude extracts on CT26 cell lines

IC_{50} values of the extracts of various parts of *F. angolensis* and *P. africana* stem bark were calculated and are presented in table 4.5 below. Aqueous extracts of the *F. angolensis* leaf and leaf stalk, root, root stem and the methanolic extract of *F. angolensis* leaf stalk had an IC_{50} value of >100 µg/ml. Other extracts had varied IC_{50} values with the *F. angolensis* whole root aqueous extract having the highest value of 85.20±2.70 µg/ml while that of the *F. angolensis* root stem showing the least value of 8.33±1.42 µg/ml as compared to Doxorubicin that had an IC50 value of 19.00±9.00 µg/ml.

Table 4.5: Determination of *in vitro* cytotoxic activity of *F. angolensis* extracts on CT26 cell line

Plant part	**IC50±SEM µg/ml**	
	Methanolic extract	**Water extract**
***F. angolensis* Leaf**	**80.67 ±2.74**	**>100**
***F. angolensis* Leaf Stalk**	**>100**	**>100**
***F. angolensis* Whole Root**	**5.25±0.35**	**85.20±2.70**
***F. angolensis* Root Stem**	**8.33±1.42**	**>100**
***F. angolensis* Root Bark**	**22.90±1.00**	**>100**
***P. africana* Bark**	**31.00±21.00**	**67.5±21.5**
Doxorubicin	**19.00±9.00**	

The degree of toxicity exhibited by the test extracts was graded according to Hayes and Loomis (1996).

4.6. Dose Response Curves of *Fagaropsis angolensis* extracts on the cell lines

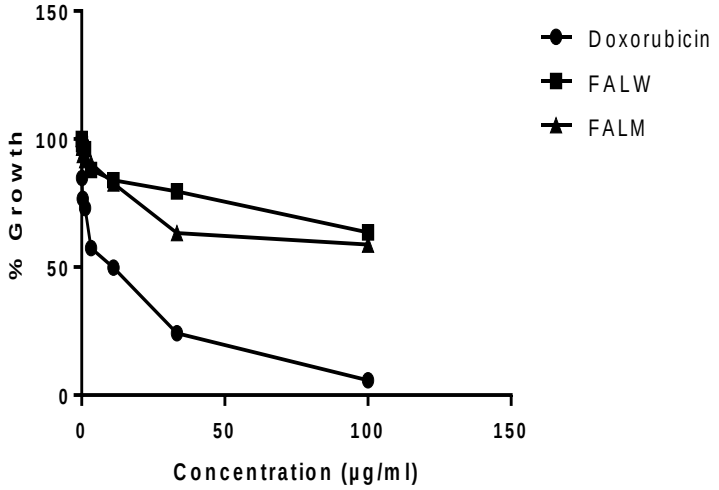
4.6.1 Effect of treatments on vero cell lines

Table 4.6: Effect of treatments on Vero E-199 cell lines cell lines with varying concentrations of *F. Angolensis* extracts

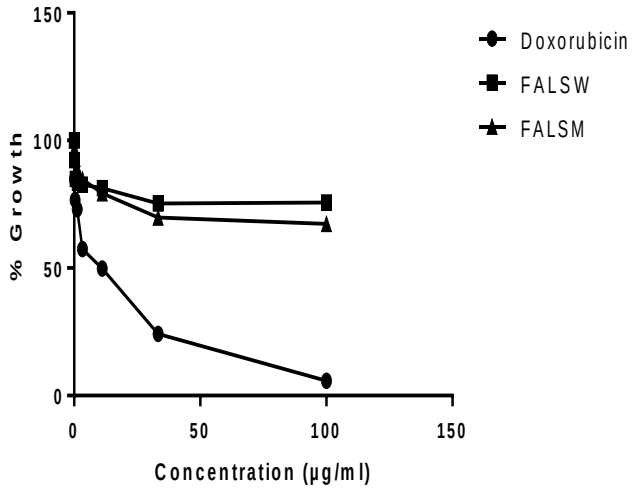
Concentration (µg/ml)	Percentage (%) grow										
	FALW	FALM	FALSW	FALSM	FAWRW	FAWRM	FARBW	FARBM	FARSW	FARSM	Doxorubicin
0.00	100	100	100	100	100	100	100	100	100	100	100
0.14	98.03	96.71	92.28	96.51	99.36	90.83	98.26	91.49	97.08	91.58	84.75
0.40	96.71	93.83	84.93	92.77	96.17	90.42	97.27	90.11	96.11	80.13	76.68
1.24	96.05	91.77	83.46	88.78	91.91	66.67	96.53	85.52	92.94	49.23	73.09
3.33	87.94	90.53	82.72	84.79	89.79	62.92	94.04	67.82	89.54	36.93	57.40
11.11	83.99	82.72	81.25	79.30	87.23	8.33	89.58	62.99	85.89	32.40	49.78
33.33	79.61	63.37	75.37	69.83	83.83	4.79	85.86	42.99	81.51	10.80	24.22
100	63.60	58.85	75.74	67.33	76.17	1.25	79.65	33.79	73.24	4.32	5.83

Key: FALW-*F. angolensis* Leaf water extract; FALM- *F. angolensis* Leaf methanolic extract; FALSW- *F. angolensis* Leaf stalk water extract; FALSM- *F. angolensis* Leaf stalk methanolic extract; FAWRW- *F. angolensis* whole root aqueous extract; FAWRM- *F. angolensis* whole root methanolic extract; FARBW- *F. angolensis* root bark water extract; FARBM- *F. angolensis* root bark methanolic extract; FARSW- *F. angolensis* root stem water extract; FARSM- *F. angolensis* root stem methanolic extract.

The effect of *F. angolensis* leaf extracts on Vero E-199 cell line % growth

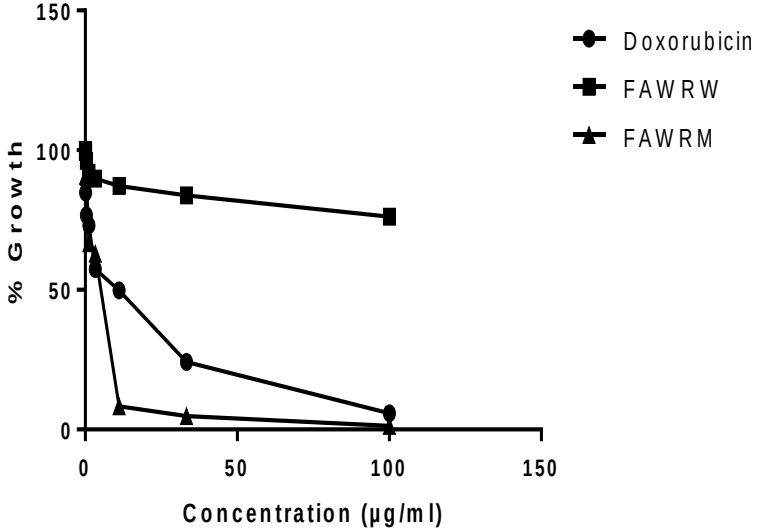


The effect of *F. angolensis* leaf stalk extracts on Vero E-199 cell line % growth



Key: FALW- *F. angolensis* Leaf water extracts, FALM- *F. angolensis* Leaf methanolic extract; FALSW- *F. angolensis* Leaf stalk water extract FALSM- *F. angolensis* Leaf stalk methanolic extract;

The effect of *F. angolensis* whole root extracts on Vero E-199 cell line % growth



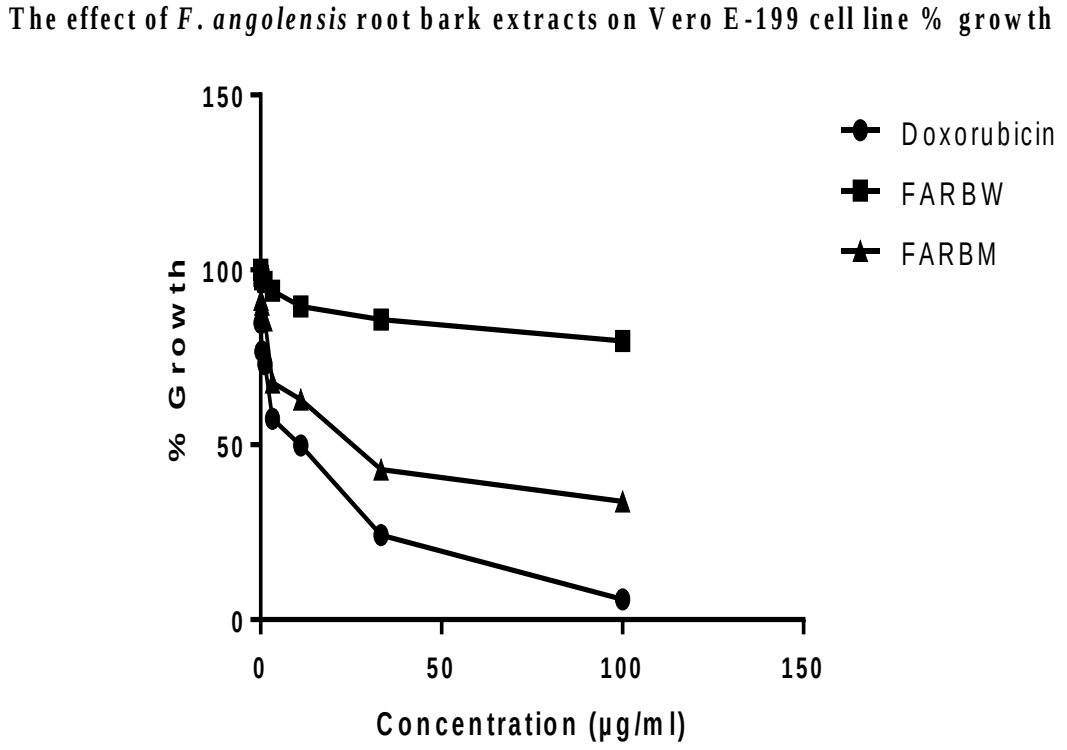


Figure 4.1: Dose response curves of *F. angolensis* extracts on Vero cell lines

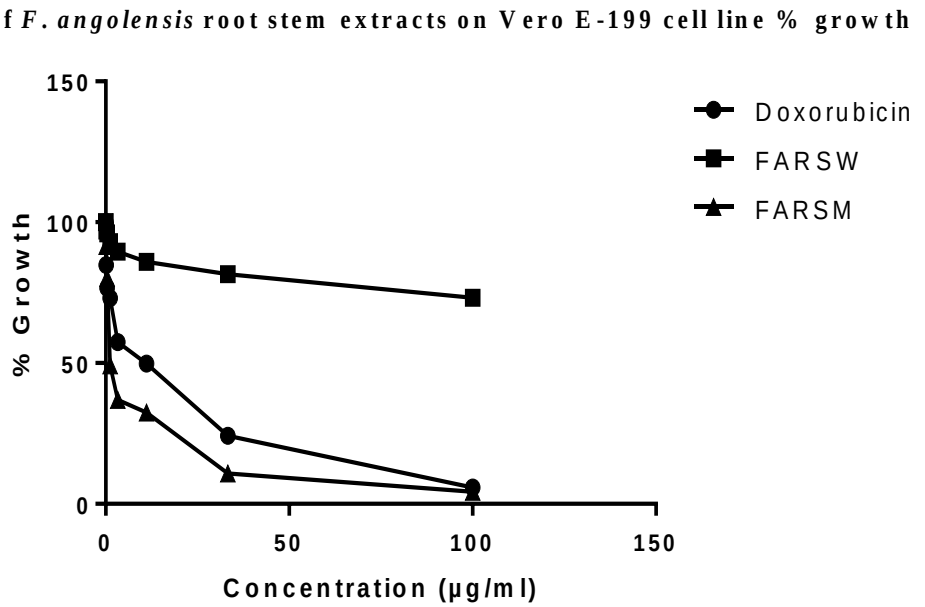


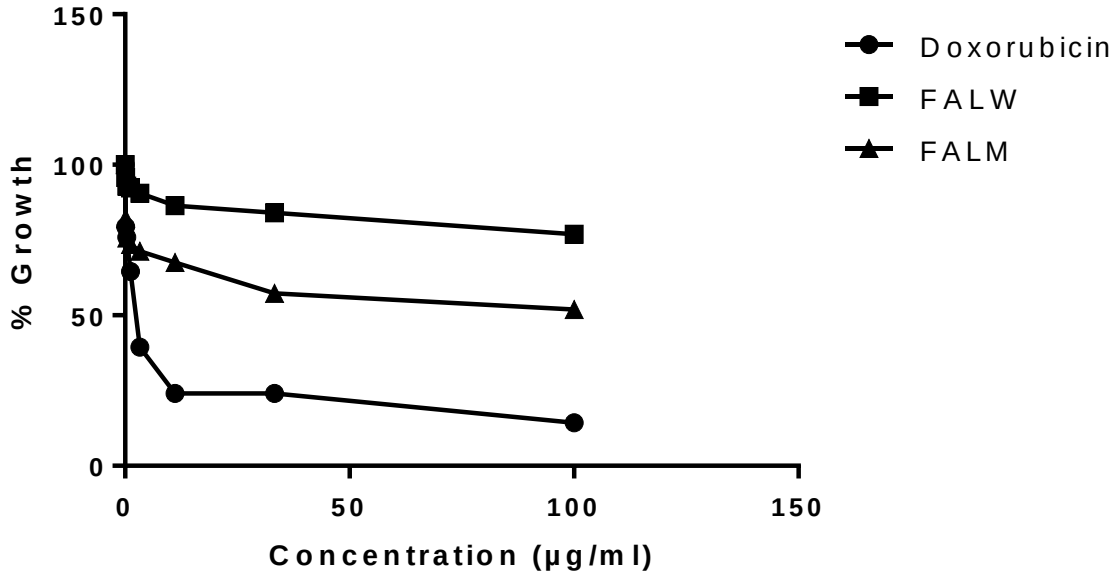
Figure 4.2: Dose response curves of *F. angolensis* root stem extract on Vero cell lines

Table 4.7: Effect of treatments on HEP2 cell lines with varying concentrations of *F. Angolensis* extracts

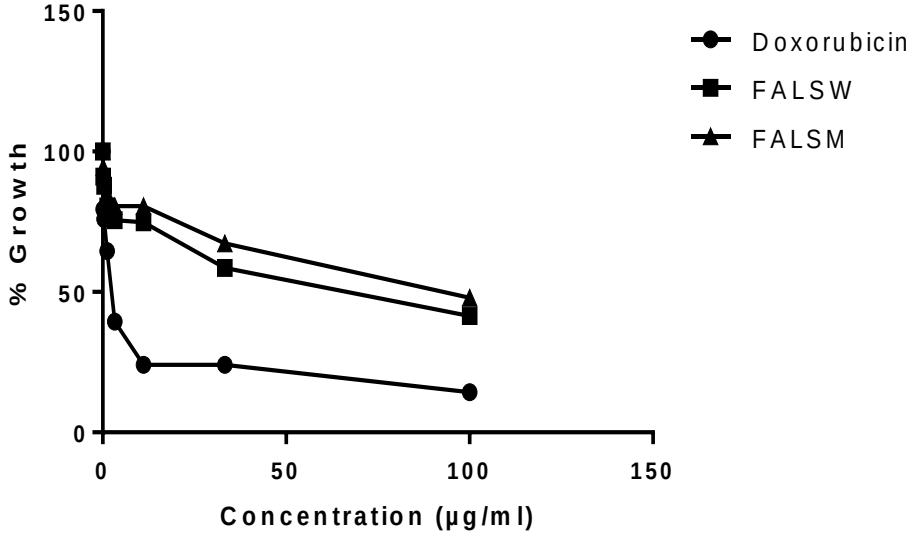
Concentration (µg/ml)	Percentage (%) growth										
	FALW	FALM	FALSW	FALSM	FAWRW	FAWRM	FARBW	FARBM	FARSW	FARSM	Doxorubicin
0.00	100	100	100	100	100	100	100	100	100	100	100
0.14	95.86	82.16	91.06	94.44	93.92	94.64	98.61	96.94	99.53	87.77	79.49
0.40	92.90	75.68	87.81	91.67	90.54	89.29	97.22	96.94	97.65	86.70	75.90
1.24	92.31	73.51	80.49	84.72	85.81	86.31	94.91	90.82	96.24	82.98	64.62
3.33	90.53	71.35	75.61	80.56	81.76	76.79	93.98	89.29	93.90	73.40	39.49
11.11	86.39	67.57	74.80	80.56	77.70	50.60	93.52	87.76	87.32	66.49	24.10
33.33	84.02	57.30	58.54	67.36	22.97	38.10	91.67	73.47	82.63	57.98	24.10
100	76.92	51.89	41.46	47.92	0.68	2.38	7.87	18.37	5.63	28.72	14.36

Key: FAWL-*F. angolensis* Leaf water extract; FALM- *F. angolensis* Leaf methanolic extract; FALSW- *F. angolensis* Leaf stalk water extract; FALSM- *F. angolensis* Leaf stalk methanolic extract; FAWRW- *F. angolensis* whole root aqueous extract; FAWRM- *F. angolensis* whole root methanolic extract; FARBW- *F. angolensis* root bark water extract; FARBM- *F. angolensis* root bark methanolic extract; FARSW- *F. angolensis* root stem water extract; FARSM- *F. angolensis* root stem methanolic extract.

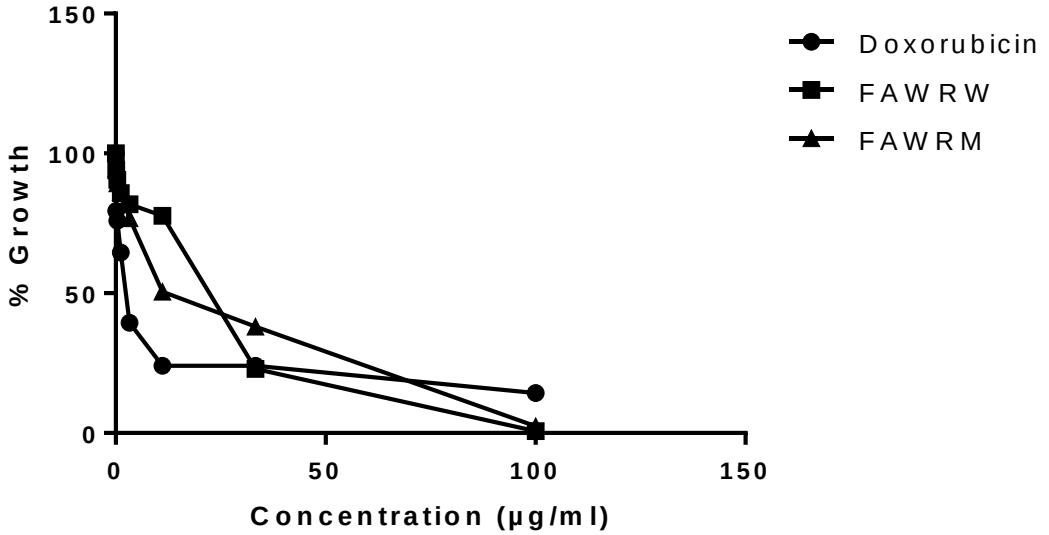
The effect of *F. angolensis* leaf extracts on Hep2 cell line % growth



The effect of *F. angolensis* leaf stalk extracts on Hep2 cell line % growth



The effect of *F. angolensis* whole root extracts on Hep2 cell line % growth



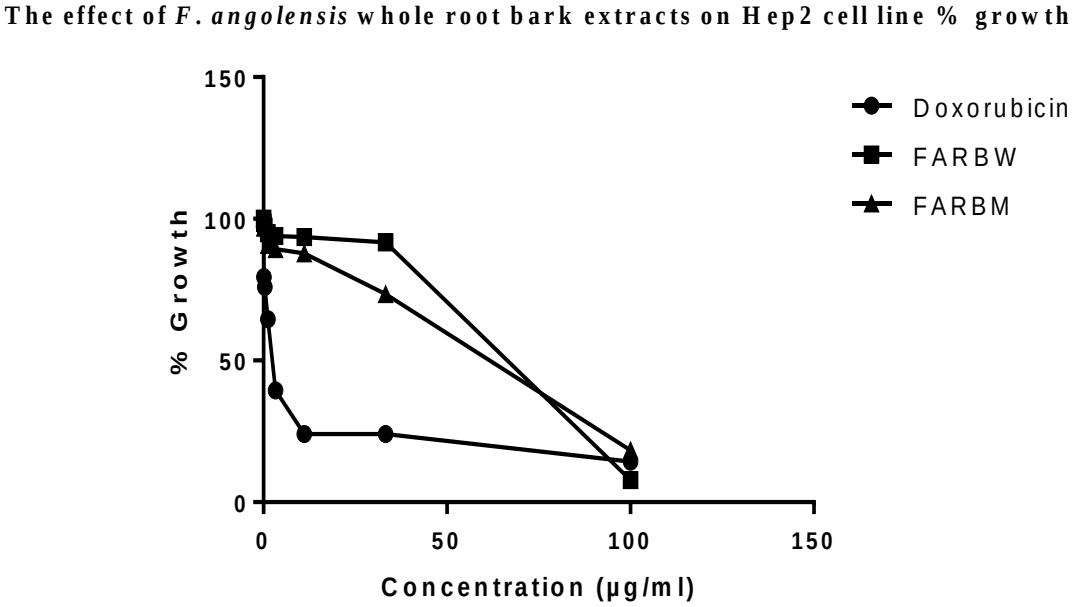


Figure 4.3: Dose response curves of *F. angolensis* extracts on HEP2 cell lines

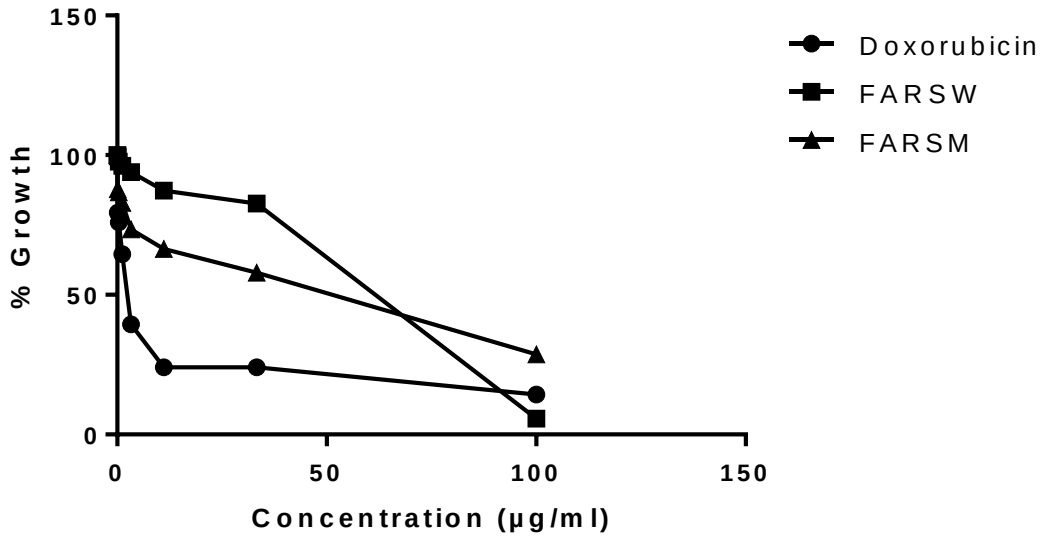


Figure 4.4: Dose response curves of *F. angolensis* root stem extract on HEP2 cell lines

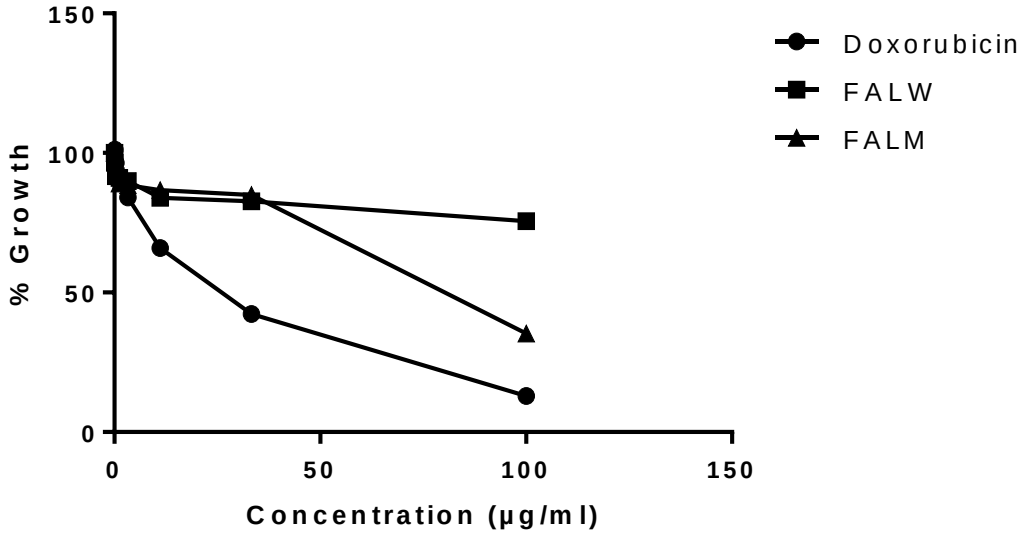
4.6.2. Effect of treatments on CT 26 cell lines

Table 4.8: Effect of treatments on CT26 cell lines with varying concentrations of *F. Angolensis* extracts

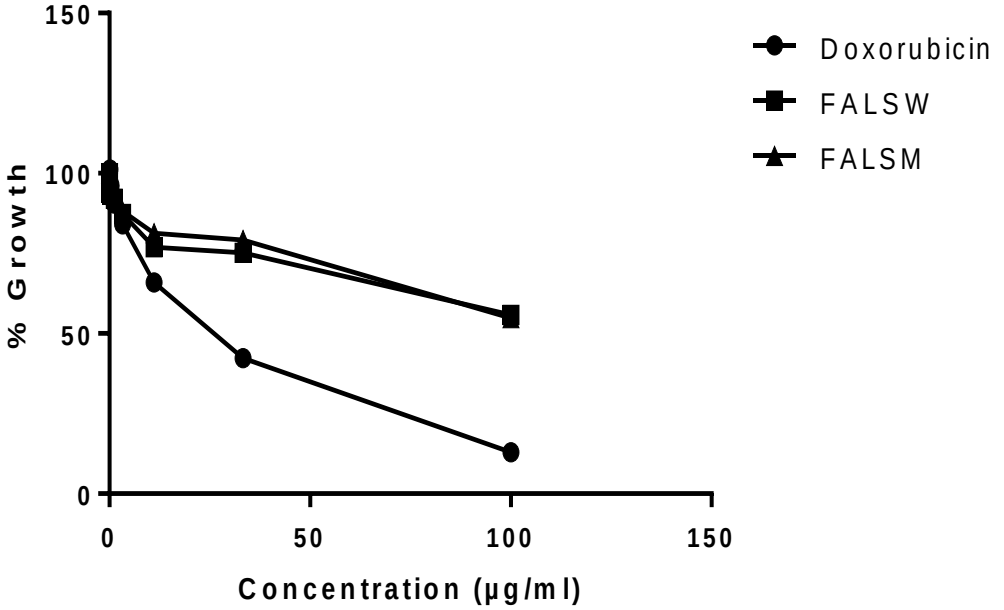
Concentration (µg/ml)	Percentage (%) growth										
	FALW	FALM	FALSW	FALSM	FAWRW	FAWRM	FARBW	FARBM	FARSW	FARSM	Doxorubicin
0.00	100	100	100	100	100	100	100	100	100	100	100
0.14	96.43	97.11	93.94	97.92	95.21	92.68	96.64	95.71	97.5	84.52	101.18
0.40	91.67	91.91	93.33	97.22	92.81	90.85	95.30	94.48	96.25	70.32	96.47
1.24	91.07	89.02	92.12	93.75	90.42	77.44	93.29	86.90	93.13	61.94	90.59
3.33	89.88	88.44	87.27	88.19	89.22	63.41	89.93	80.69	88.75	61.29	84.12
11.11	83.93	86.71	76.97	81.25	84.43	9.15	87.92	71.72	86.25	32.26	65.88
33.33	82.74	84.97	75.15	79.17	73.65	2.44	74.50	31.03	83.75	16.13	42.35
100	75.60	35.26	55.76	54.86	43.71	1.22	49.66	6.90	63.13	8.39	12.94

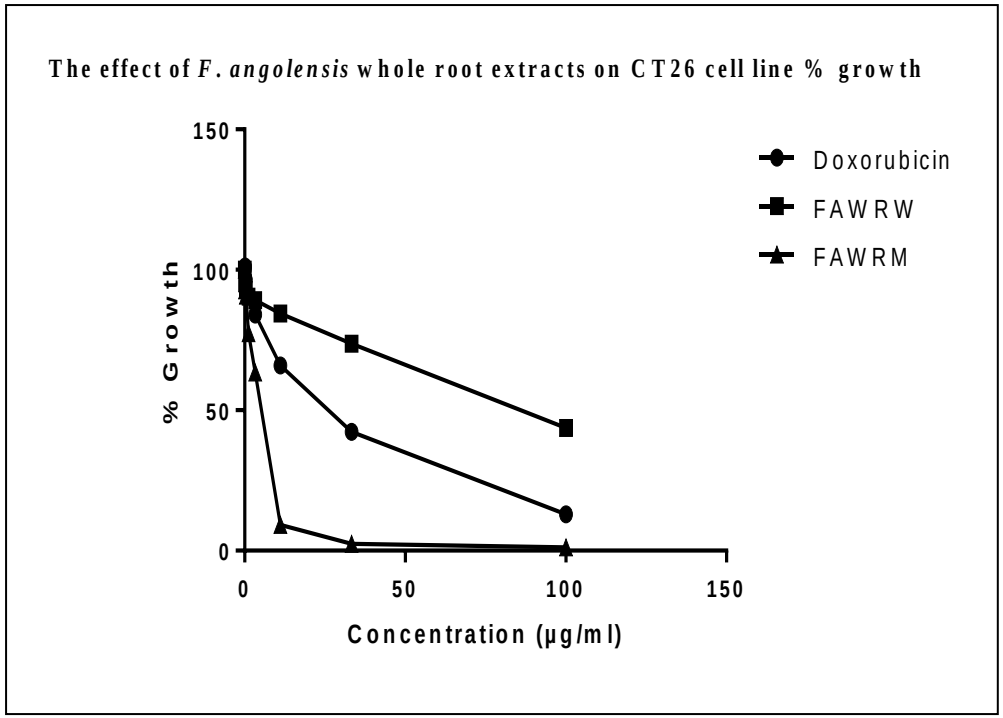
Key: FAWL-*F. angolensis* Leaf water extract; FALM- *F. angolensis* Leaf methanolic extract; FALSW- *F. angolensis* Leaf stalk water extract; FALSM- *F. angolensis* Leaf stalk methanolic extract; FAWRW- *F. angolensis* whole root aqueous extract; FAWRM- *F. angolensis* whole root methanolic extract; FARBW- *F. angolensis* root bark water extract; FARBM- *F. angolensis* root bark methanolic extract; FARSW- *F. angolensis* root stem water extract; FARSM- *F. angolensis* root stem methanolic extract.

The effect of *F. angolensis* leaf extracts on CT26 cell line % growth



The effect of *F. angolensis* leaf stalk extracts on CT26 cell line % growth





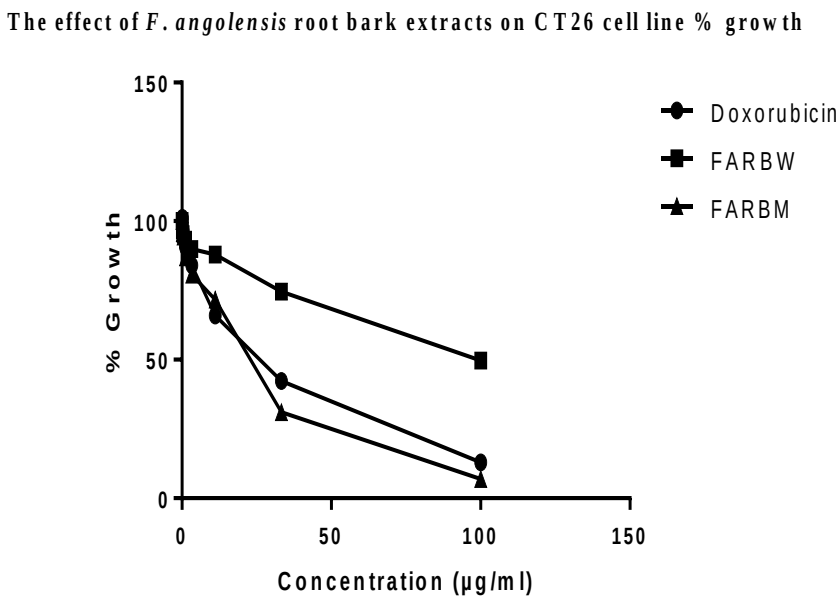


Figure 4.5: Dose response curves of *F. angolensis* extracts on CT26 cell lines

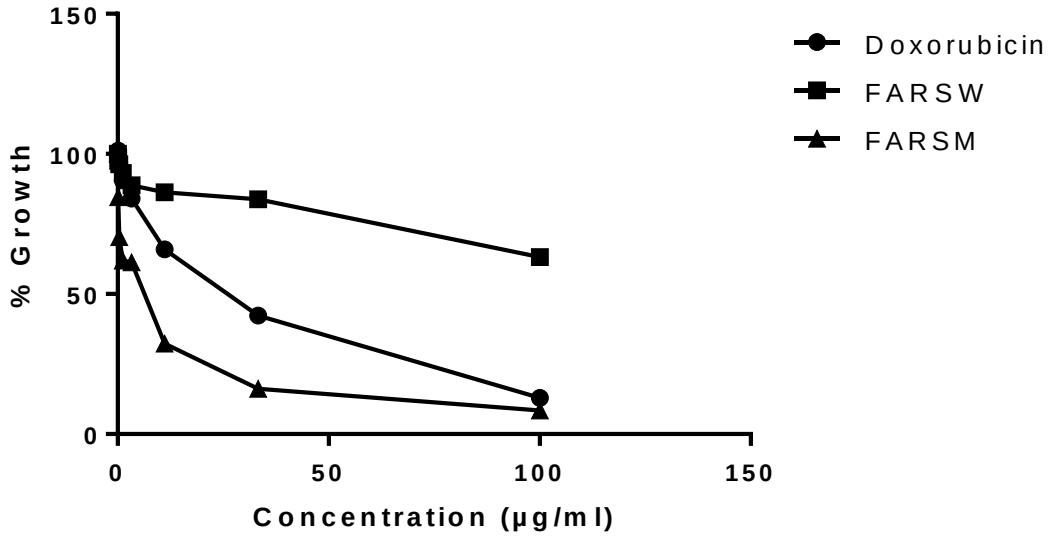


Figure 4.6: Dose response curves of *F. angolensis* root stem extract on CT26 cell lines

4.7. Dose response curves of *Prunus africana* extracts on the cell lines

4.7.1. Effect of treatments on Vero cell lines

Table 4.9: Effect of treatments on Vero E-199 cell lines with varying concentrations of *P. africana* extracts

Concentration (µg/ml)	Percentage (%) growth		
	PABW	PABM	Doxorubicin
0.00	100	100	100
0.14	95.14	98.21	84.75
0.40	91.05	90.82	76.68
1.24	87.21	85.99	73.09
3.33	85.68	59.18	57.40
11.11	81.59	36.73	49.78
33.33	78.26	27.30	24.22
100	74.68	16.58	5.83

Key: PABW-*P. africana* bark water extract; PABM- *P. africana* bark methanolic extract

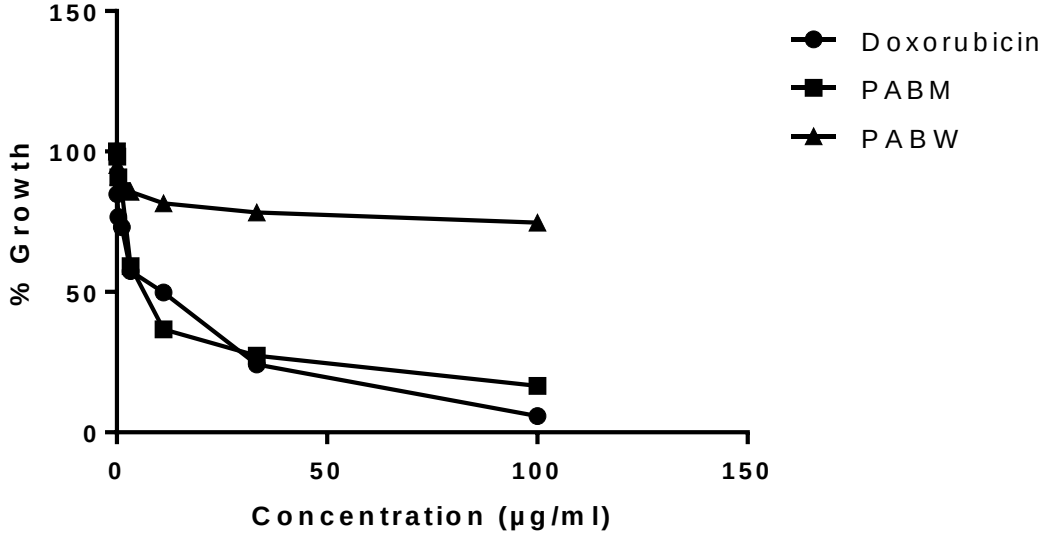


Figure 4.7: Dose response curves of P. africana extracts on Vero cell lines

4.7.2. Effect of treatments on HEP2 cell lines

Table 4.10: Effect of treatments on HEP2 cell lines with varying concentrations of *P. africana* extracts

Concentration (µg/ml)	Percentage (%) growth		
	PABW	PABM	Doxorubicin
0.00	100	100	100
0.14	94.79	98.44	79.49
0.40	89.06	70.83	75.90
1.24	83.33	62.5	64.62
3.33	78.13	52.60	39.49
11.11	51.04	42.71	24.10
33.33	10.94	32.81	24.10
100	4.17	11.46	14.36

Key: PABW-*P. africana* bark water extract; PABM- *P. africana* bark methanolic extract

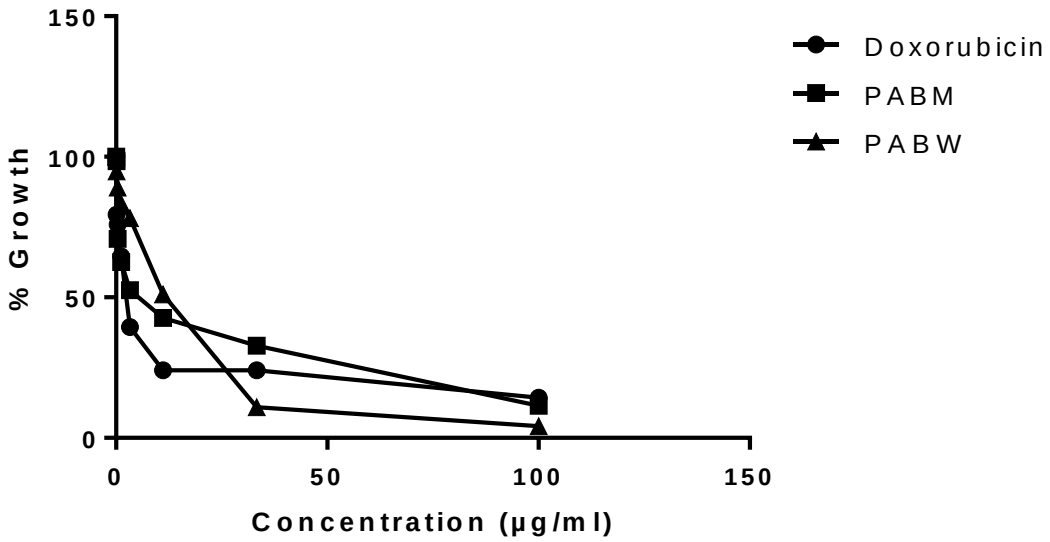


Figure 4.8: Dose response curves of *P. africana* extracts on HEP2 cell lines

4.7.3. Effect of treatments on CT 26 cell lines

Table 4.11: Effect of treatments on CT26 cell lines with varying concentrations of *P. africana* extracts

Concentration (µg/ml)	Percentage (%) growth		
	PABW	PABM	Doxorubicin
0.00	100	100	100
0.14	86.62	99.33	101.18
0.40	82.80	93.96	96.47
1.24	81.53	90.60	90.59
3.33	80.89	82.55	84.12
11.11	73.25	64.43	65.88
33.33	61.78	55.70	42.35
100	42.04	15.44	12.94

Key: PABW-*P. africana* bark water extract; PABM- *P. africana* bark methanolic extract

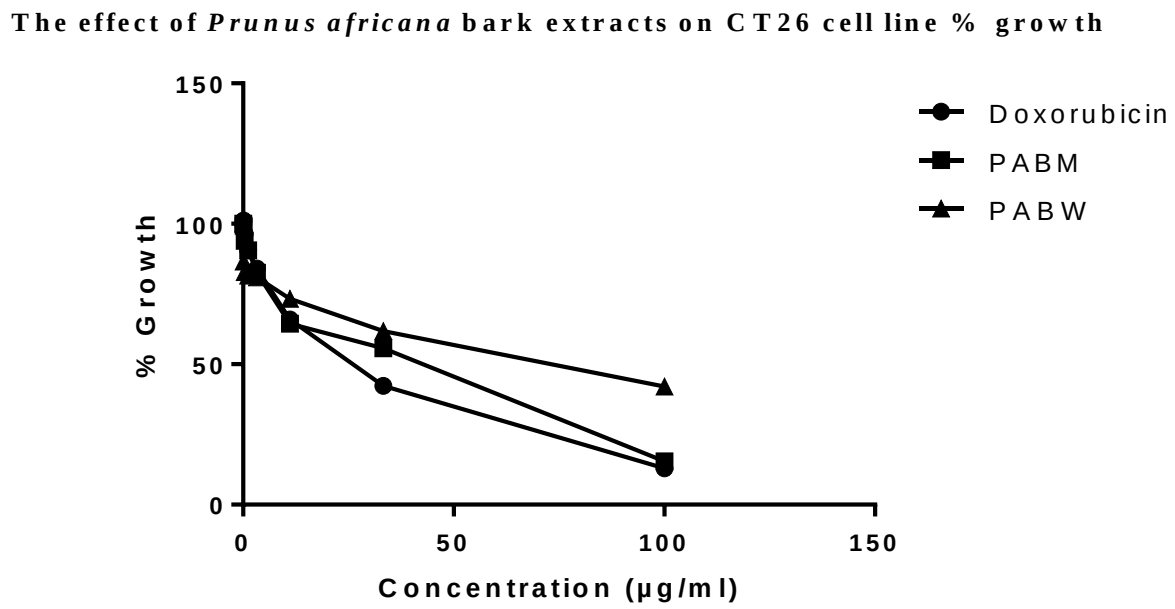


Figure 4.9: Dose response curve of *P. africana* extracts on CT26 cell lines

Table 4.12: Classification of toxicities of test extracts' IC_{50} according to Loomis & Hayes (1990)

Dosage (mg/Kg)	Deduction/toxicity level	Test Plant extract
$IC_{50} \leq 1$	Extremely toxic	None
$1 > IC_{50} < 50$	Highly toxic	*F. angolesnsis* root stem methanolic extract (Vero, CT26)
		F. angolesnsis whole root methanolic extract (Vero, HEP2, CT26)
		F. angolensis whole root aqueous extract (HEP2)
		F. angolensis root bark methanolic extract (CT 26)
		P. africana bark methanolic extract (Vero, HEP2, CT26)
		P. africana bark aqueous extract (HEP2)
$50 > IC_{50} < 500$	Moderately toxic	*F. angolesnsis* leaf, leaf stalk, whole root, root bark, root stem, aqueous extracts (Vero)
		F. angolesnsis leaf and leaf stalk, methanol and aqueous extract (HEP2, CT26)
		P. africana bark aqueous extract (Vero)
$500 > IC_{50} < 5000$	Slightly toxic	*F. angolesnsis* root stem methanolic and water extracts (HEP2)
$500 > IC_{50} < 15000$	Practically non-toxic	*F. angolesnsis* root stem aqueous extract (CT 26)
$IC_{50} > 15000$	Relatively harmless	*F. angolensis* whole root aqueous extract (CT26)
		F. angolensis root bark methanolic and water extracts (HEP2)
		F. angolensis root bark Aqueous extract(CT 26)
		P. africana bark aqueous extract (CT26)

4.8. *In vivo* acute oral toxicity of crude extracts on adult Swiss albino mice

4.8.1. Mortality

Mortality is the main criteria in assessing the acute toxicity (LD_{50}) of the extracts. The extracts were sequentially using three animals per given dose. The extracts were administered with the objective of achieving a standard dose known to cause marked distress and toxicity.

There was no mortality recorded within 24 hours and during the 14 day period of observation in all mice groups that received the water and methanol extracts of various *F. angoensis* and the stem bark of *P. africana.* It was deduced that their respective LD_{50} values were therefore >2000 mg/kg body weight. There was an increase in body weight in all mice groups as shown in tables 4.12 and 4.13. Mice that received 300 mg/kg (group 1) and 2000 mg/kg (group 2) had no significant difference compared to the control group

Table 4.13: Toxicity of different crude extracts at 2000mg/kg concentration from *Prunus africana* and *Fagaropsis angolensis* at different time intervals to swiss albino mice

Sample	Initial weight	7th day	14th day	Mortality	Toxicity symptoms
PB MeOH	**23.85±2.45**	**29.28±2.35**	**27.99±2.31**	**0/3**	**Nil**
FAWR MeOH	**22.39±3.46**	**23.99±3.25**	**25.51±3.25**	**0/3**	**Nil**
FARS MeOH	**22.91±1.45**	**24.70±1.57**	**25.98±1.99**	**0/3**	**Nil**
FARB MeOH	**22.97±2.13**	**24.08±2.12**	**25.62±2.57**	**0/3**	**Nil**
PB H2O	**23.46±2.67**	**N/A**	**N/A**	**0/3**	**Nil**
FAWR H2O	**22.50±1.57**	**23.75±1.51**	**24.74±2.19**	**0/3**	**Nil**
FARS H2O	**24.11±2.15**	**25.78±2.05**	**27.05±2.76**	**0/3**	**Nil**
FARB H2O	**21.03±1.79**	**23.07±2.00**	**23.07±2.18**	**0/3**	**Nil**
FAL MeOH	**17.88±2.15**	**18.75±2.11**	**20.18±2.54**	**0/3**	**Nil**
FALS MeOH	**18.43±3.17**	**20.74±2.97**	**21.59±3.19**	**0/3**	**Nil**
FAL H2O	**18.84±2.01**	**19.13±2.67**	**20.27±2.79**	**0/3**	**Nil**
FALS H2O	**17.84±1.58**	**19.87±1.34**	**21.21±1.79**	**0/3**	**Nil**

Table 4.14: Toxicity of different crude extracts at 300 mg/kg concentration from *Prunus africana* and *Fagaropsis angolensis* at different time intervals to swiss albino mice

Sample	Initial weights	7th day	14th day	mortality	Symptoms
PB MeOH	**23.37±2.45**	**24.05±2.25**	**25.79±2.54**	**0/3**	**Nil**
FAWR MeOH	**21.02±3.36**	**22.59±3.15**	**23.78±2.25**	**0/3**	**Nil**
FARS MeOH	**23.27±1.45**	**25.19±1.79**	**26.16±1.96**	**0/3**	**Nil**
FARB MeOH	**24.98±2.15**	**26.44±2.15**	**28.10±2.59**	**0/3**	**Nil**
PB H2O	**20.78±2.57**	**25.27±2.14**	**25.80±2.53**	**0/3**	**Nil**
FAWR H2O	**21.50±1.57**	**22.75±1.51**	**23.74±2.19**	**0/3**	**Nil**
FARS H2O	**22.11±2.15**	**23.78±2.05**	**25.05±2.76**	**0/3**	**Nil**
FARB H2O	**20.03±1.79**	**22.07±2.00**	**23.07±2.18**	**0/3**	**Nil**
FAL MeOH	**19.58±2.35**	**20.92±2.11**	**23.70±2.54**	**0/3**	**Nil**
FALS MeOH	**19.44±3.17**	**21.08±2.97**	**22.21±3.19**	**0/3**	**Nil**
FAL H2O	**18.59±2.45**	**19.23±2.59**	**19.23±2.57**	**0/3**	**Nil**
FALS H2O	**17.97±2.00**	**20.04±2.11**	**20.44±1.79**	**0/3**	**Nil**

4.8.2. Behavioral changes observed during oral acute toxicity studies of the extracts.

The examination of the behavior of the animals after administration of the crude extracts was reported. General behavior of each animal was recorded during the first 30 minutes, 4 hours, periodically during the first 24 hours and daily thereafter for total of 14 days. It was also observed that behavioral changes in all the test mice were normal and thus no signs of toxicity.

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1. DISCUSSION

Following extraction, it was observed that, generally, the aqueous extracts produced a higher yield as compared to the methanolic extracts. The whole root of *F. angolesis* aqueous extract had the highest yield of 14.47 % as compared to its methanolic counterpart that had a yield of 8.42 %. The aqueous extract of *P. africana* bark presented a yield of 10.37 % as compared to its methanolic extract that gave a yield of 6.68 %.

This was in agreement with the studies done by Kokwaro, (2009). Which showed that water had a higher extractivity value as compared to methanol. It was deduced that, the plants perhaps habour a vast or high concentration of polar compounds that easily dissolves in water and thus extracted.

From his studies, he deduced that water was the commonly used solvent of choice in the preparation of concoctions and decoctions of these two plants used in the management of various diseases. Thus the results, support the use of water in the extraction of active principles from these plants as it was evident that water had a higher extractivity than methanol which is commonly used for isolation and analysis in modern science.

Moreover, the use of water in preparation of herbals may in part be associated to the reduced toxicity of the preparations. This indicates that water plays a role in the neutralisation, and reduction of toxicity in these plants since most toxins that are able to dissolve in water so that they are easily transported and excreted leaving no chances of accumulation within the body (Kokwaro, 20009).

5.1.1. Phytochemical screening

Photochemistry is a distinct discipline juxtaposed in between organic chemistry, plant biochemistry and closely related to natural products. It deals with a variety of organic substances accumulated in plants and to a greater extent provide health benefits for humans further than those attributed to macronutrients and micronutrients (Hasler and Blumberg, 1999).

These biologically active compounds protect plants from stress like diseases and damage. They also contribute to the aroma, flavor and colour of plants. Apart from their roles in plants, research has indicated that plant phytochemicals play critical roles in the protection of human health especialy when their dietary consumption is significant. Over 4000 phytochemical compounds have been documented classified according to their functions as protective, based on physical and chemical properties. Of these, only approximately 150 phytochemicals have been researched into detail. (American cancer society, 2000; Meagher, 1999). Phytochemicals are deposited in different parts of plants, including stems, leaves, roots, flowers and seeds (Costa *et al.,* 1999).

In the current study, eight phytochemical compounds were screened in various parts of *F. angolensis* and *P. africana* methanolic and aqueous extracts.

Qualitative tests were performed to determine the presence or absence of phytochemicals in the various extracts under study and the intensity of the colour formed or frothing was treated as degee of concentration of the phytochemicals in that particular extract. The leaf, leaf stalk, whole root, root bark and root stem of *F. angolensis* and the bark of *P. africana* plants for various phytochemicls.

It was observed that the methanolic extract of *F. angolesis* leaf had the highest concentration of phenols based on the intensity of the green precipitate formed and steroids based on the redish-purple color developed.

Glycosides (moderate red color in the ammonical layer), tannins (bluish green precipitate), alkaloids (redish-brown precipitate) favonoids geenish yellow colour and terpenoids (blue green ring) respectively were detected. The saponins were vividly absent in all the *F. angolensis* extracts. The

aqueous extract of *F. angolensis* leaf on the other hand appeared to possess higher content of glycosides and tannins in contrast to the methanolic extract.

This could be partly attributted to the difference in polarities of the two solvents. Methanol has a polarity index of 5.2 while water has 10.2. Because glycosides are polar, they were better extracted with water as compared to steroids that are slightly nonpolar thus extracted with methanol better. It was also noted that in contrast with the methanolic extract, phenols were least in regard to the precipitate formed. This could be due to their phenolic structure that is nonpolar. It was deduced that the phytochemicals that were present in these extracts (methanolic and aqueous) had differential extractivity thus varied color intensities following their determination.

The aqueaus extract of *F. angolensis* leaf stalk did not show presence of phenols and saponins but all other phytochemicals were moderate. Strikingly, the methanolic extracts of the *F. angolensis* showed that all the tested for phytochemicals were present except the saponins. This means that water did not extract phenols in the leaf stalk but methanol did. The difference in solubilities of the two solvents used could be responsible. In comparison with the whole leaf, phenols were predominant in the leaf as opposed to the leaf stalk. It was notable that all the *F. angolesis* parts screened, phenols and tannins were more sound.

This conform with an earlier study by Kimutai *et al.,* 2015, that also implicated the absence of saponins in *F. angolesis* extracts. The presence of terpenoids in these plant supports its use as an antimicrobial drug. Terpenoids are bioactive and are thought to be bacteriocidal by disrupting the bacterial membranes by lipophilic compounds. The presence of flavanoids and their derivatives in plants has for long been associated with antimicrobial protection against plant pathogenic microbes and insect attack. (Kimutai *et al.,* 2015; Cowen, 2008).

Phenols and phenolic compounds are known to habor germicidal activity and are utilized in the formulation of disinfectants. Alkaloids are regarded as one of the largest groups of plant secondary metabolites that have been linked to antimicrobial activities. They also have been found to imbert

some effects on humans which has led to the development of potent pain killers, anesthetic and stimulants for instance, cocaine, caffeine, nicotine, and antimalarial drug quinine (Omulokoli *et al.*, 1997).

The presence of alkaloids in *F. angolensis* supports its ethnomedical use in malaria management and antiprotozoal activity as earlier reported by (Kareru *et al.,* 2006). The presence of these phytochemicals could have contributed to the medicinal activities claimed by the traditional medical practitioners as observed by (Jeruto *et al*., 2008). Owing that the *F. angolensis* contains more of these metabolites, it is not surprising that this plant is being utilized for its various medicinal uses. The observations made is in this study conforms with the findings by (Geyid *et al,* 2005).

Just like in *F. angolesis* extracts the bark of *P. africana* methanolic and aqueous extracts were also tested for their phytochemical constituents. It was determined that this plant contained an array of all the investigated phytochemicals except steroids that were absent. The presence of tannins in this plant may confer the astringent property which makes it useful in preventing diarrhea and controlling hemorrhage due to their ability to precipitate proteins, mucus and constrict blood vessels (Kokwaro 2009).

This is could be the reason why traditional practioners use herbal medicines rich in tannins to treat wounds and burns since they are able to induce blood clotting. This shows how traditionally used medicinal plants rich in tannins can be used to control life threatening and debilitating diseases. Additionally, tannins have also been demonstarted to exhibit antiparasitic effects (Akiyama *et al.*, 2001).

The anticarcinogenic and antimutagenic potentials of tannins may be related to their antioxidative property which is important in protecting cellular oxidative damage including lipid peroxidation. The growths of many fungi, yeast, bacteria and viruses has been proven to be inhibited by tannins (Chung *et al.*, 1998).

Terpenoids have shown great potency in the treatment against microorganisms. According to Andrew ,(1980) terpenoids have been studied in the in vivo environment and found to inhibit the growth of various bacteria. They have also shown potency in the treatment against *Plasmodium falciparum* which is the causative agent of malaria. Terpenoids have been found to inhibit the growth of fungi *Candida albicans* (Murata *et al.*, 2008).

Flavonoids are known to contain specific compounds called antioxidants which protect human, animal and plant cells against the damaging effects of free radicals. Imbalance between free radicals and antioxidants leads to oxidative stress which has been associated with inflammation, autoimmune diseases, cataract, cancer, parkinson's disease, aging and arteriosclerosis (Sharma, 2006).

Alkaloids on the other hand have been found to have analgesic, antispasmodic activity, antihypertensive effects, anti-malarial activity, anticancer and antiinflammatory activities (Banzouzi *et al.,* 2004;Kareru *et al.,* 2006).

The current study is also in conformity with previous studies in which the plant was found to contain flavonoids (Dixon *et al.,*1983). However apart from flavonoids the plant was also found to have diverse spectrum of phytochemicals which previous studies have labelled them to have great medicinal value (Ghasemzadeh, 2011). The presence of these phytochemicals has been associated with its use in the management of Benign prostate hyperplasia (BPH). BPH is a progressive non-cancerous urologic condition that leads to enlargement of the prostate gland (Parsons and Kashefi, 2008). The condition is more prevalent in most men over 50 years of age and manifests itself as increased frequency in urination, pain in passing urine, inability to empty the bladder and post urinary dribbling (Garnick, 1994).

Allopathic medical therapy for BPH includes drugs, surgical and non-surgical treatments. Drugs used include the antiandrogens; terazosin hydrochloride and finasteride which are synthetic inhibitors of the 5-α-reductase enzyme (Bartsch *et al.,* 2000). Non-surgical therapy includes thermotherapy, balloon dilation and stents. Treatment by surgery involves excision of excess tissue.

All these methods have a number of side effects and thus phytotherapy is the primary treatment in European countries.

The bark extracts from *P. africana* also inhibit bladder hyperactivity. The use of the bark in traditional medicine includes the treatment of chest pain, urinary and bladder infections, stomach aches, kidney disease and malaria. The bark is either chewed or crushed into powder and drunk as tea (Stewart, 2003).

The results reported in this study show that bought plants have active phytochemicals that confer either solely or in synergy their diverse use in the management of cancer and other diseases in traditional medicine.The traditional medicinal practitioners or medicinemen/medicinewomen use water as an extractant to preparee concoctions and decoctions that are normally medicinal active probably because water is very polar and it extracted some compounds that are not extracted by methanol (Nostro *et al.,* 2000). This was also in agreement with studies done by Rainer *et al.,* 2009 where they stated that some compounds used in medicinal plants may not always be extracted by methanol.

Flavonoid and their derivatives are known for their anti-allergic properties as well as a wide variety of activity against bacteria, fungi and viruses as described by Afolayan and Meyer, (1997). The biochemical mechanisms thought to be responsible for phenolic toxicity to microbes include enzyme inhibition by the oxidized compounds, possibly through reaction with sulfhydryl groups or through more nonspecific interactions with the proteins of the involved microorganisms. The activity of flavonoids is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls. The lipophilic flavonoids disrupt microbial membranes.

This is in agreement with the studies done by Duke, (1992) who considered Eugenol to be fungistatic and bacteriostatic against both fungi and bacteria respectively.

Terpenes or terpenoids are active against bacteria and fungi. The triterpenoid betulinic acid is just one of several terpenoids which have been shown to inhibit HIV. The mechanism of action of

terpenes is not fully understood but is speculated to involve membrane disruption by the lipophilic compounds (Mendoza *et al.,* 1997).

5.1.2. *In vitro* antiproliferative activity.

Cell culture technique has for long been used to screen for toxicity of drugs both by estimation of the basal functions of the cell , for instance those processes common to all types of cells or by tests on specialized cell functions (Ekwall, 1983). Chemical inventories, available in several countries have demonstrated that many chemicals are being produced from either chemistry or natural sources and marketed in many countries. For instance, about 55 000 commercial substances are listed in the US EPA TSCA inventory. Although about 9.5 per cent of the total number of substances reported (i.e. about 5000 chemicals) account for 99.9 per cent of the total production (Blair and Bowman, 1983) it is evident that exposure to relative low volume chemicals could be a major health crisis for specific population groups under certain circumstances. Toxicological data are currently either inadequate or non-existent for most existing natural products and chemicals (Grossblatt *et al.*,1984; Silano *et al.*,1986).

In the absence of data regarding the health-related parameters, *in vitro* cytotoxicity data, which can be produced in a relatively short time and at a relative low cost, is very useful for ranking chemicals with a similar exposure potential according to their potential toxicity and, possibly, to confirm other predictions based on consideration of their chemical structures.

In vitro antiploriferative data could also indicate the need for specific kinds of additional toxicity tests that would be required. Since industrial chemicals are not primarily of interest because of their specific biological activities, *in vitro* antiploriferative data may provide a more useful criterion for priority ranking these chemicals than for other groups of chemicals such as drugs or pesticides.

An established cell line can be used to provide tests with little variation, normal basic functions and metabolic capacity, respectively. General toxicity tests, aimed mainly at detection of the biological activity of test substances, can be carried out on many cell types.

The current study sought to determine the antiploriferative profiles of different parts of *Fagaropsis angolensis* methanolic and aqueous extracts (Leaf, leaf stalk, whole root, root stem and root bark) as well as the stem bark of *Prunus africana* methanolic and aqueous extracts as these two plants have been used ethnomedically for management of various diseases including cancer and malaria.

The Internation Organization for Standard (ISO) describes the remarkable recommendation of vero E-6 cell lines in *in vitro* antiploriferative assays and in the study of cell-substrate associations in biological research. (ISO, 1992).

Based on the results, both the *F. angolensis* and *P. africana* aqueaous extracts showed an IC_{50} value that was greater than 100 µg/ml. Similarly, methanolic extracts of the *F. angolensis* leaf, leaf stalk and root bark exhibited an IC_{50} of >100 µg/ml. Of most important, the methanolic extracts of the *F. angolensis* root stem posted the lowest IC_{50} value of 1.10±0.70 µg/ml, followed by the whole root with IC_{50} value of 5.10±0.80 µg/ml.

In comparison with the methanolic extract of *P. africana* bark that showed an IC_{50} value of 5.56±1.50 µg/ml and reference drug, Doxorubicin that had an IC_{50} value of 6.5±3.25 µg/ml against Vero E-6 cell lines.

The *in vitro* screening methodology recommended by the National Cancer Institute (USA), employed in this work, allow the evaluation of drugs in various types of neoplastic cells and the discovery of new specific agents. Other advantages are the rapidness, efficiency and reproducibility of the method (Monks *et al.,* 1991).

The test extracts were tested against two cancer lines namely the throat cancer (HEP2) and colon cancer (CT 26). On the Throat cancer (HEP2) cell line, the methanolic and water extracts of *F. angolensis* whole root showed the lowest IC_{50} value of 10.05±2.15 µg/ml and 21.65±0.05 µg/ml respectively. The other extracts had varied values with the Leaf of *F. angolensis* having IC_{50} values of >100 µg/ml in comparison to the standard drug that had a value of 2.5±0.50 µg/ml. This is in reference to table 4.4 above.

On the colon cancer cell line (CT 26), the methanolic extract of the *F. angolensis* whole root had the lowest IC_{50} value of 5.25±0.35 µg/ml but its aqueaous extract showed a higher value of 85.20±2.70 µg/ml; the methanolic extract of the root stem followed closely with a value of 8.33±1.42 µg/ml and root bark with IC_{50} value of 22.90±1.00 µg/ml . This is contrast with the water extracts that had an IC_{50} value of >100 µg/ml as compared with Doxorubicin that had an IC50 value of 19.00±9.00 µg/ml.

Based on the (Loomis and Hayes, 1996) classification of toxicity, no extract exhibited extreme toxicity to the three cell lines. Of keen Interest, the methanolic extract of *F. angolensis* was highly toxic to vero cell lines as compared to the other cell lines and to the standard drug, Doxorubicin.

It was also noted that, the methanolic extract of the root stem of *F. angolensis* had a low IC_{50} value as compared to that of the whole root that was 5.10±0.80 and the root bark that was > 100 with Doxorubicin that had 6.5±3.25 µg/ml. This could partly mean that the active principles responsible for causung toxicity to normal cells, are concentrated in the root stem.

It could also mean that the other phytochemicals present in the root bark could be having antagonistic activity to the ones responsible for toxicity culminating in the increase in the IC_{50} values of the whole root. This calls for careful and urgent regulation of the plant especially when root barks are peeled off and only the root stem used in traditional medicine as there exists a higher propensity of lethality. Following antiproliferative assay, the root stem of *F. angolensis* methanolic extract was highly toxic against colon cancer cell line CT26. This means that this part of the plant can be a potential source of antineoplastic agents especially in comparting colon cancer.

The whole root of *F. angolensis* methanolic extrct was equally highly toxic against CT26. Generally, the methanolic extract s of the *F. angolensis* root were highly cytotoxic against CT26 and moderately to HEP 2 cell lines. The search for anticancer drugs is inevitable. Medicinal plants have been used since time memorial in the treatment of diseases traditionally thought to be tumors.

Additionally, over 60% of the current antineoplastic drugs have either direct origin from plants or templates of plant compounds.

The current study sort to provide scientific justification on the use of *Fagaropsis angolensis* and *Prunus africana* which are used traditionally used in the treatment of conditions believed to be tumors. Colorectal cancer causes over 694, 000 deaths globally in developing countries. High cost and increased attrition rates derail its treatment. Previous studies have demonstrated anticancer activity of *Prunus africana* on cancer cell lines. (National Cancer Control Strategy Kenya, 2011). The antiproliferative activity observed from the current study could be attributed to the pharmacologically important compounds which were found to be present in the plants.

The aqueous extracts of the same part were relatively safe based on the IC50 values. This partly means that water neutralizes the toxic principles in this plant and methanol concentrates them. In traditional medicne, water is the solevent mainly used to prepare concotions and decoctions of *F. angolensis* roots in the management of diseases like malaria. (Kireru *et al.*,2006). The toxicity profile reported in this work partly supports an earlier study demonstarting antiplasmodial (Kireru *et al.*,2006)., and antimicrobial activities (Marta *et al.*, 2011).

The results of *P. africana* bark were comparable to that of the root of *F. angolesis* methanolic extract ranging from highly toxic to harmless. This supports its use for the management of other neoplasms apart from the BPH as there was toxicity against the colon and throat cancer cell lines used in this study. The two plants were selected based on their ethnobotanical use therefore the report provides a partial scientific justification on the use of the two plants in treatment and management of neoplastic conditions. Medicinal plants are attracting a lot of attention today based on the believe that they are safe than their synthetic counterparts.

Naturally derived compounds have continued to influence the organic chemistry world with the various structures derived from them not only contributing towards finding new synthetic drugs posing a big challenge but might be the basis for new biologically active leads (Sukari *et al.*, 1992;

Singh, 2012). Though commonly believed to be non-toxic plant extracts may pose perilous systemic side effects to consumers including mutagenic potentials (Singh, 2012).

The other extracts were moderately toxic based on their IC_{50} values. The difference in toxicities is attributable to the diffence is the distribution of various phytochemicals that are synthesised and deposited in these regions. In addition, this study provides an important framework for further investigation into the isolation, characterization and elucidation of the mechanism of cytotoxic compounds from the screened *F. angolensis* and *P. angolensis* extracts, thus these plants could be used as a source of new lead structures in drug design to combat cancer.

5.1.3. *In vivo* acute oral toxicity

The utilization of plant concoctions and decoctions to manage diseases in traditional medicine has offered valuable leads to new areas of research and biodiversity conservation. Oral administration of methanolic and aqueous extracts of *F. angolensis* and *P. africana* at single doses of 300 mg/Kg body weight and 2000 mg/Kg body weight according to the OECD showed no mortality even at the highest concentration.

The mice had normal increase in weight an indication that the extracts supported the growth of the animals (Ogwal- Okeng *et al.,2003*) .This is in agreement with study done by Ogwal-Okeng *et al.*, 2003, that showed that the *F. angolesnsis* plants might also be safe for oral consumption.

The increase in weight observed in the test groups was comparable to that observed in the control groups. Throughout the 14 days no change in behavioral pattern was observed in both the test groups and the control.

Prunus africana extracts at 300mg/kg concentration were safe to the animals as no signs of toxicity were observed. The mice in these concentration groups gained weight normally.

There was also no mortality observed in mice that received the aqueous and methanolic extracts of *P. africana* bark. This was in conformity with studies done by Gathumbi *et al., 2002* which demonstrated that *P. africana* extracts are non-toxic. The study continued to state that, chloroform

extracts of *P. africana* did not cause clinical signs or pathology in rats at daily dosages of up to 1000mg/kg for 8 weeks.

A similar study done by (Bombardelli and Morazzoni, 1997) that involved acute and chronic toxicity of *P. africana* in mice and rats showed no adverse reactions observed after intragastric administration of a single dose of a lipophilic extract of the trunk bark (1–6g/kg body weight in mice and 1–8g/kgbody weight in rats).In the study there was also no adverse reactions observed in mice and rats after chronic intragastric administration of the extract (60 and 600mg/kg bodyweight, respectively, daily for 11 months) (Bombardelli and Morazzoni, 1997).

Toxicology studies in human trials demonstrated a low incidence of toxicity; this explains why the bark extracts from *P. africana* have been used in the management of prostate disorders in men both traditionally and in modern medicine (APA citation, 2012).

Since neither signs of toxicity nor mortality were reported, based on the scale of (Loomis and Hayes, 1996) classification of toxicity, the methanolic and water extracts of both *F. angolensis* and the bark of *P. africana* were relatively harmless with LD_{50} of >2000mg/kg body weight.

From the results, it was determined that most extracts of both plants exhibited various degrees of toxicity towards the selected cell lines *in vitro* as opposed to *in vivo* assays. Deviations from a linear relationship between cytotoxic concentrations of a substance *in vitro* and toxic dose *in vivo* can result from the fact that effective concentrations *in vitro* are irrelevant for the concentrations that cause toxicity in target organs *in vivo*.

An important reason is a compound's bio kinetic behavior, which determines the concentrations reached in target organs. This can be augmented further by the fact that body systems harbor various ways of detoxifying a toxic agent before it can cause any notable harm.

The higher extractivity of aqueous extracts based on the percentage yields may as well mean that water plays a great role in the dilution of toxic components in these plants rendering them safe. In addition, water being an important transporter of many molecules and compounds including toxins

in the body helps in their excretion. This evades toxin accumulation in the body that may lead to notable symptoms of toxicity.

5.2. CONCLUSION

Based on the findings of these studies, the following conclusions were made;

1. The methanol root stem and whole root extracts of *Fagaropsis angolensis* had the best *in vitro* antiploriferative activity on colon cancer cells.
2. The safety profile of methanol and water extracts of *Fagaropsis angolensis* and *Prunus africana* was observed.
3. Isolation of phytochemical bioactive compounds, elucidation of their efficacy is important.

5.3. RECOMMENDATIONS

Based on the findings of the present study, the following recommendations may be drawn:

1. The methanol root stem and whole root extracts of *Fagaropsis angolensis* which had best invitro activity should be confirmed in animal cancer models.
2. Safety studies such as sub acute studies on the extract with confirmed antiploriferative potential should be done.
3. Further research to quantitatively determine the phytochemical constituents of the active plant extract

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APPENDICES

APPENDIX 1: ETHICAL APPROVAL

UNIVERSITY OF NAIROBI
FACULTY OF VETERINARY MEDICINE
DEPARTMENT OF VETERINARY ANATOMY AND PHYSIOLOGY

P.O. Box 30197,
00100 Nairobi,

Tel: 4449004/4442014/ 6
Ext. 2300
Direct Line. 4448648

Dr Antony L. Yiaile
c/o Department of PHPT

REF:FVM BAUEC/2016/109

06/08/2016

Dear Dr Yiaile,

RE: Approval of Proposal by Biosafety, Animal use and Ethics committee

A study of *in vitro* anticancer efficacy and toxic effects of *Fagaropsis angolensis* (Engl.) Dale extract
By Antony L. Yiaile (Reg.No.: J56/73786/2014)

We refer to your revised proposal that you submitted to our committee for review. We have noted that you have reduced the proposed numbers of animals to be used for your experiments. Furthermore, we note that that the animals will be humanely treated as you carry out your experiments. We require that all animal experiments will have to be carried out under the supervision of your supervisors who are veterinarians.
We hereby approve your work as per your revised proposal.

Rodi O. Ojoo BVM M.Sc Ph.D
Chairman,
Biosafety, Animal Use and Ethics Committee,
Faculty of Veterinary Medicine

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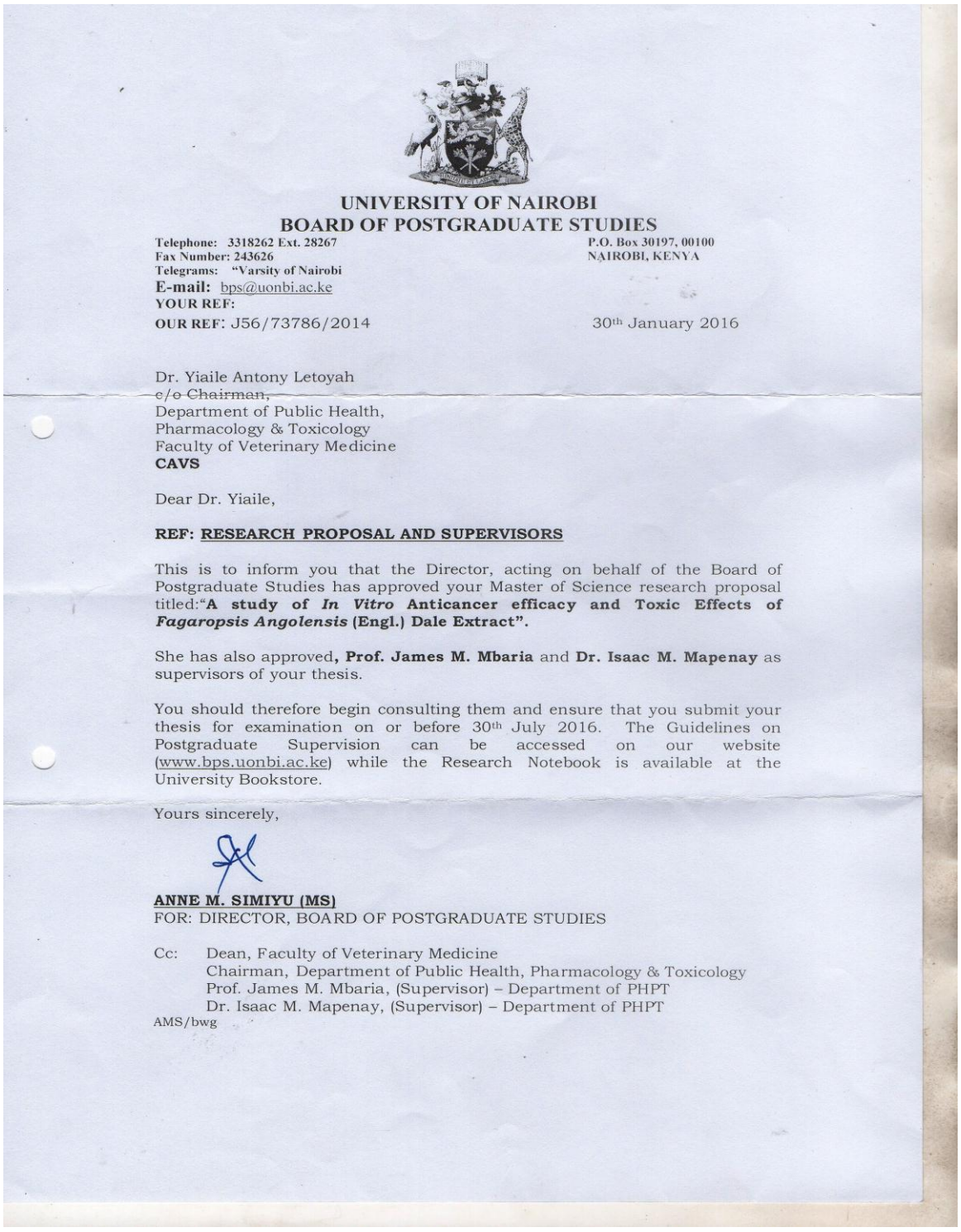
APPENDIX 2: PROPOSAL APPROVAL



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OUR REF: J56/73786/2014

P.O. Box 30197, 00100
NAIROBI, KENYA

30th January 2016

Dr. Yiaile Antony Letoyah
c/o Chairman,
Department of Public Health,
Pharmacology & Toxicology
Faculty of Veterinary Medicine
CAVS

Dear Dr. Yiaile,

REF: RESEARCH PROPOSAL AND SUPERVISORS

This is to inform you that the Director, acting on behalf of the Board of Postgraduate Studies has approved your Master of Science research proposal titled:**"A study of *In Vitro* Anticancer efficacy and Toxic Effects of *Fagaropsis Angolensis* (Engl.) Dale Extract".**

She has also approved**, Prof. James M. Mbaria** and **Dr. Isaac M. Mapenay** as supervisors of your thesis.

You should therefore begin consulting them and ensure that you submit your thesis for examination on or before 30th July 2016. The Guidelines on Postgraduate Supervision can be accessed on our website (www.bps.uonbi.ac.ke) while the Research Notebook is available at the University Bookstore.

Yours sincerely,

ANNE M. SIMIYU (MS)
FOR: DIRECTOR, BOARD OF POSTGRADUATE STUDIES

Cc: Dean, Faculty of Veterinary Medicine
Chairman, Department of Public Health, Pharmacology & Toxicology
Prof. James M. Mbaria, (Supervisor) – Department of PHPT
Dr. Isaac M. Mapenay, (Supervisor) – Department of PHPT

AMS/bwg