ANTIOXIDANT AND ANTIPROLIFERATIVE ACTIVITIES OF PLANT DERIVED EXTRACTS AGAINST CERVICAL AND PROSTATE CANCER CELL LINES

KEDESANI DOROTHY MOGA

MASTER OF SCIENCE

(Medicinal Chemistry)

JOMO KENYATTA UNIVERSITY OF

AGRICULTURE AND TECHNOLOGY

2018

Antioxidant and Antiproliferative Activities of Plant Derived Extracts against Cervical and Prostate Cancer Cell Lines

Kedesani Dorothy Moga

Thesis Submitted in Partial Fulfilment for the Degree of Master of Science in Medicinal Chemistry in the Jomo Kenyatta University of Agriculture and Technology

2018

DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

Signature…………………………………….. Date………………………

Dorothy Kedesani Moga

This thesis has been submitted for examination with our approval as university supervisors

Signature………………………………… Date…………………………

Dr. Peter G. Kirira, PhD

MKU, Kenya

Signature………………………………… Date ……………………………..

Dr. Esther N. Matu, PhD

KEMRI, Kenya

Signature…………………………….. Date ……………………………..

Dr. Joseph Ng'ang'a, PhD

JKUAT, Kenya

DEDICATION

I dedicate this work to the almighty God for his wisdom, knowledge, understanding, grace and above all his mercies that have made this journey a success.

ACKNOWLEDGEMENT

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF APPENDICES

ABBREVIATIONS AND ACRONYMS

AIDS: Acquired Immune Deficiency Syndrome

ADT: Androgen Deprivation Therapy

ATCC: American Type Culture Collection

ACUC: Animal Care and Use Committee

CAM: Complementary and Alternative Medicine

CC_{50}:   50% Cytotoxic Concentration

CT: Computed Tomography

DMEM: Dulbecco's Modified Eagle's medium

DMSO: Dimethyl Sulfoxide

DNA: Deoxyribonucleic Acid

DPPH: 2, 2-diphenyl-1-picrylhydrazyl

DU145:   Human Prostate Cancer Cell Lines

ELISA: Enzyme Linked Immunosorbent Assay

FBS: Fetal Bovine Serum

FIC: Fractional inhibition concentration

GnRH: Gonadotropin Releasing Hormone

HIV: Human Immunodeficiency Virus

HPV: Human Papilloma Virus

HeLa: Human cervical cancer cell line used in research

IARC: International agency for research on cancer

ICC: Invasive Cervical Cancer

IC$_{50}$:   Half Maximal Inhibitory Concentration

KEMRI: Kenya Medical Research Institute

Kg/bw: Kilograms per body weight

KS: Kaposi"s sarcoma

L-1210:   Leukemia cells

LHRH: Leutenizing Hormone Releasing Hormone

L6:   Normal Rat Skeletal Muscle cell

LD$_{50}$:   Median Lethal Dose

MDE-MB 123: Human Caucasian breast adenocarcinoma

MEM: Eagle"s Minimum Essential Medium

MCF-7:   Michigan Cancer Foundation-**7**

Mcrpc: Metastatic Castration-Resistant Prostate Cancer

MRI: Magnetic Resonance Imaging

MTT: 3-(4, 5- dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide

µg/ml: Microgram per millilitre

NCCAM: National Centre for Complementary and Alternative Medicine

NCI: National cancer institute

NEMA: National Environment Management Authority

OD: Optical Density

OECD: Organization for Economic Co-operation and Development

p388:   Leukemia cell line

PBS: Phosphate Buffered Saline

QOL: Quality of Life

SCAR: Suppressor of cAMP receptor

SD: Standard drug

SEM: Standard error of mean

SERU: Scientific and Ethics Review Committee

SFIC: Sum of Fractional Inhibition Concentration

SI: Selectivity index

SMT: Somatic Mutation Theory

T-Cell: Subtype of a white blood cell

TLC: Thin Layer Chromatography

TOFT: Tissue Organization Field Theory

TRPA1:   Transient Receptor Potential Ankyrin 1

PI: Principle Investigator

WAVE: Wiskott-Aldrich syndrome protein

WHO: World Health Organization

ABSTRACT

Cancer is the third largest cause of mortality in Kenya. Treatment options available for cancer include chemotherapy, radiation and surgical procedures but none presents with minimal side effects and high cure rates. Therefore there is a need to explore new therapies for cancer. For a long time, plants have been used to manage tumor and related ailments in Kenya. The aim of this study was to determine the antioxidant, antiproliferative, acute toxicity and phytochemical composition of organic and aqueous extracts of *Azadirachta indica, Vernonia amygdalina and Galium aparinoides*. The *in vitro* antioxidant activity was determined using DPPH (1, 1-diphenyl-2-picrylhydrazyl) radical scavenging assay. Antiproliferative activity was determined against cervical (Hela), prostate (DU145) and Vero (L6) cancer cells lines using MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5-Diphenyltetrazolium Bromide) assay method and the IC_{50} (Inhibitory concentration) values reported. Cytotoxicity was determined using L6 and CC_{50} (Cytotoxicity concentration) values calculated. Further, the active extracts were combined and assayed *in vitro* using the checkerboard method. Acute oral toxicity was evaluated using (OECD) Organisation of economic cooperation and development guidelines and the lethal dose (LD_{50}) determined. The acute toxicity was carried out on bioactive plant extracts with the highest selectivity index (SI). *A. indica* exhibited the greatest antioxidant activity with the methanol extracts of the stem bark having an IC_{50} value of 69.31 µg/ml, comparable to the positive control of ascorbic acid which had an IC_{50} value of 42.74 µg/ml. *V. amygdalina* highest antioxidant activity was observed in methanol stem parts with an IC_{50} value of 430.8 µg/ml and all *G. aparinoides* extracts had IC_{50} value >500 µg/ml. In the antiproliferative assay, methanol root bark and stem bark extracts of *A. indica* reported IC_{50} values of 1.85 ± 0.01 µg/ml and 2.59 ± 0.29 µg/ml respectively, against Hela cancer cell line and IC_{50} values of 1.53± 0.07µg/ml and 3.26±0.28µg/ml respectively, against DU145 cancer cell line. The results were comparable with the untreated cells (negative control) and 5-Fluorouracil (5-FU), (positive control) with IC_{50} values of 2.04 ± 0.87µg/ml and 5.06± 0.28 µg/ml against Hela and DU145 cell lines, respectively. Methanol extract of *V. amygdalina* aerial parts had IC_{50} values of 430.67 ± 1.17µg/ml and 301.8± 0.93µg/ml 7 µg/ml in Hela and DU145 cell lines, respectively. Notably, the methanol stem bark extract of *A. indica* had a high SI index of 436.52, an indication that the cytotoxic effect of this extract was selective to cancerous cells. In combination assay, all combinations showed synergistic activity except for ethylacetate stem and root bark extracts of *A. indica* against DU 145 cell line. Oral administration of methanol root bark extracts of *A. indica* in mice at the highest dose of 2000 mg/kg body weight demonstrated no mortalities and no adverse effects suggesting that these extracts were non-toxic. Methanol stem bark extracts of *A. indica* on the other hand revealed acute toxicity. All the three plants contained alkaloids, phenolics, flavonoids tannins and terpenoids. The findings of the study demonstrate the potential of *A. indica* in management of cancer and particularly the methanol extracts from the root bark of *A. indica* have great potential as a valuable alternative source for anticancer agent.

CHAPTER ONE

INTRODUCTION

1.1. Background of the study

Cancer is a class of diseases in which a group of cells display uncontrolled growth, with intrusion and destruction of adjacent tissues. Cancer sometimes spreads to other locations in the body via lymph or blood. Cancer cell growth is different from normal cell growth. Instead of dying, cancer cells continue to grow and form new abnormal cells (Jemal *et al.,* 2011).

Cancer is one of the leading causes of death in the world today. In 2012, there were 14.1 million new cancer cases and 8.2 million deaths reported by GLOBOCAN (Erlay *et al.,* 2012). In Africa cancer is the leading cause of death as a result of population growth, aging and adoption of cancer-associated lifestyle including smoking and physical inactivity (Soerjomataram *et al.,* 2012). It is third largest cause of mortality in Kenya, behind infectious and cardiovascular diseases which is ranked first and second respectively (Soerjomataram *et al.,* 2012).

Globally, cervical cancer is the fourth most common cancer in women with an estimated 528,000 new cases and 266,000 deaths (Bray *et al.,* 2012). About 86% of new cases occur in developing countries, where 80-90% of deaths occur within the same year (WHO, 2000). According to the Nairobi Cancer Registry, cervical cancer is the leading cause of death among women of reproductive age with an estimated number of 3,000 cases annually and a death toll of about 1,600. There will be a substantive increase to 4,200 new cases by 2025 (Korir *et al.,* 2015).

Invasive cervical cancer (ICC) is a criterion for a diagnosis of acquired immunodeficiency syndrome (AIDS) as it is mostly associated with human papilloma virus (HPV), though rarely occurs in human immunodeficiency virus (HIV) negative people (Odida *et al.,* 2011). The incidence of cervical cancer precursors is high among women infected by HIV and recurrences are common despite therapy (Massad *et al.,* 2009). About 9.1% of women in the general population are estimated to harbor cervical

HPV-16/18 infection at a given time, and 62.6% of invasive cervical cancers are attributed to HPVs 16 or 18 (Alsbeih, 2014). With HIV/AIDS being a menace in sub-Saharan Africa, invasive cervical cancer has established itself as a major complication in those with HIV/AIDS. Treatment options are pegged on the stage of invasive cancer and comprise of surgery, radiotherapy, and chemotherapy, singly or in combination. The most commonly used medicines include 5 flourouracil (5-FU), Cisplatin, paclitaxel, navelbine, topotecan, carboplatin, docetaxel, gemcitabine, ifosfamide, hydroxyurea, Cyclophosphamide and mitomycin C (O"Dwyer *et al.,* 1999).

In case of limited monetary resources, 5-Floro uracil (5-FU) without or with cisplatin is the recommended treatment. A platinum compound and a taxane can be used in cases where money is not an issue. HIV positive women with T lymphocyte cells (CD4) counts $< 200/\text{mm}^3$ are at risk of complications irrespective of treatment methods (Ntekim & Folasire, 2010).

Prostate cancer is the second most common cancer among elderly male population all over the world, and sixth leading cause of cancer deaths among men globally with over 1.1 million diagnosed cases and 307,000 deaths worldwide. According to GLOBOCAN 2012, new cases of prostate cancer and mortality in Africa were reported to be 23,300 and 17,000 respectively. Kenyatta national hospital has reported approximately 76,500 incidences and 5,800 mortalities of prostate cancer patients per year (Erlay *et al.,* 2012).

Treatment of prostate cancer is also determined to a great extent by the cancer stage. The management options can be low risk, intermediate risk and high risk. Low risk management options include active surveillance, radical prostatectomy and radical radiotherapy. The intermediate risk therapy options can either be radical surgery or radical radiotherapy (Carter, 2011). For high risk management options, an array of therapeutic options may be considered including combined radiation and androgen deprivation therapy, radical prostatectomy, radiotherapy alone, or androgen deprivation therapy alone. This however is dependent upon the patient"s age, general health, and disease-related parameters (Molassiotis *et al.,* 2005).

Prostate cancer is a disease of the aging male, the majority presenting after 65 years (O'Brien *et al.,* 2013). Prostate cancer cells are dependent on the hormone testosterone for their growth. This hormone, majorly produced by the testicles and the adrenal glands in small amounts, is usually a target for therapeutics as its reduction translates to diminished growth of cancer cells. Metastatic castration-resistant prostate cancer (mCRPC) is prostate cancer that is resistant to medical (e.g. hormonal) or surgical treatments that lower testosterone, and has spread to other parts of the body (Chabner & Longo, 2011). Androgen deprivation therapy (ADT) is generally the initial treatment for men with metastatic prostate cancer. Standard approaches include bilateral orchiectomy or medical orchiectomy using a gonadotropin releasing hormone (GnRH) agonist either alone or in combination with an antiandrogen (Nelson & Montgomery, 2003). The common treatment used for treatment in Kenya include goserelin, luprolide, buserelin, bicalutamide, flutamide, docetaxel, estramustine, abiraterone acetate, and diethylstilboestrol. In resource limited settings, diethlystilboestrol is the first line treatment.

Where resources are available, any of the leutenizing hormone (LHRH) agonists like goserelin, with or without an antiandrogen like bicalutamide and flutamide are used (Saidi *et al.,* 2008).These treatment options have certain drawbacks that cannot be overlooked. The most outstanding drawback for chemotherapy and radiotherapy is that they cause undesirable effects to the patients. The post-operative survival rates for surgery on the other hand are low hence patients always tend to have poor prognosis due to late diagnosis when on these treatment options. On top of this, the economic expenditure required so as to get these treatments is so huge that not many that are affected are able to afford (Gomella, 2005)

1.2. Cancer burden

Cancer causes 7% of total national deaths every year (Brown *et al.,* 2006).Although population based data does not exist in the country, it is estimated that the annual incidence of cancer is about 37,000 cases and the annual mortality to be over 22,000 (Brown *et al.,* 2006). Over 60% of those affected are below the age of 70 years. In many developing countries the rapid rise in cancers and other non-communicable

diseases has resulted from increased exposure to risk factors which include tobacco use, harmful use of alcohol and exposure to environmental carcinogens (GOK, 2016). Other risk factors for some cancers include infectious diseases such as HIV/IDS (Kaposi‟s sarcoma and lymphomas), Human Papilloma Virus (HPV), Hepatitis B and C (Liver cancer), bacterial infections such as Helicobacter Pylori (cancer of stomach) and parasitic infestations such as schistosomiasis (cancer of bladder). The leading cancers in women are breast, oesophagus and cervical cancers. In men, oesophagus and prostate cancer and Kaposi sarcoma are the most common cancers.

Based on 2002 data from the Nairobi Cancer Registry, of all the cancers registered breast cancer accounted for 23.3%, cervical cancer for 20% and prostate cancer for 9.4% (GOK, 2016). In 2006, around 2,354 women were diagnosed with cervical cancer and 65% of these died of the disease. Despite the fact that non communicable diseases such as cardiovascular diseases, cancers, diabetes and chronic respiratory diseases are on the increase, the health systems in the country have traditionally concentrated on the prevention and control of communicable diseases. As a result, health and development plans have not adequately invested in the prevention and control of these diseases. The silent epidemic of non-communicable diseases now imposes a „double burden of disease‟ to the country which unless it is addressed will overwhelm us in the near future. This bias in the system has resulted in weakness in programs that should be addressing non communicable diseases and their risk factors in the country (GOK, 2016).

The country has few cancer specialists who are concentrated on a few health facilities in Nairobi. This makes it difficult for a great majority of the population to access cancer treatment services resulting in long waiting times causing some previously curable tumours to progress to incurable stages. The reason for this sad situation is that cancer treatment infrastructure in Kenya is inadequate and some cancer management options are not readily available necessitating some Kenyans to seek cancer treatment abroad. Within the health care systems, cancer is treated through medical, surgical or radiation therapy. Effective treatments require that all these modalities of treatment be available in the same setting to avoid distant referral and delays in treatment administration.

Furthermore, essential drugs list in Kenya does not include chemotherapy for cancer. Some of the very essential drugs for pain management are rare to find in most public hospitals. There is therefore need for clear policies concerning terminal pain management, supportive and palliative care for cancer patients in Kenya. Some of the main impediments to palliative care in Kenya include shortage of financial and human resources, lack of awareness and legal restrictions on the use and availability of opioid analgesics. Cancer research in Kenya is not commensurate with the magnitude of the problem. This is due to inadequate funding and training facilities in cancer research. There is also no comprehensive cancer surveillance system and no population based cancer registry (GOK, 2016).

1.3. Origin of cancer

It has been widely accepted that cancer results from genetic changes that affect cell cycle control. It is driven by somatically acquired point mutations and chromosomal rearrangements thought to accumulate gradually over time. The conventional theory of carcinogenesis is called the Somatic Mutation Theory (SMT). SMT says that cancer starts with a mutation that gives cells a growth advantage, which leads to clonal expansion and successive mutations followed by clonal expansions. The principles of SMT are stated as follows; first, cancer is derived from a single somatic cell that has accumulated multiple Deoxyribonucleic acid (DNA) mutations. Secondly, the default state of cell proliferation is quiescence; and lastly, cancer is a disease of cell proliferation caused by mutations in genes that control proliferation and the cell cycle (Sonnenschein *et al.,* 2014). Under SMT, the adjacent tissue plays a supporting role, affecting the fitness of the clonal expansion or recruiting the surrounding stromal cells to begin an interplay that enhances the neoplastic phenotype (Hanahan & Weinberg, 2011).

1.3.1. Genetic factors of cancer

Somatic Mutation Theory (SMT) began in 1914 with Boveri's theory linking chromosomal changes to cancer (Weinberg, 2008). In the 1950s researchers hypothesized that cancer involved successive mutations interspersed with clonal expansions (Fraga *et al.,* 2004). Based on the age-specific incidence of cancer, some

researchers postulated that six or seven mutations are required for cancer development (Rozhok & DeGregori, 2016). Another theory was that two mutations interspersed by exponential cell growth were needed for cancer development (Dewanji *et al.,* 1991).

Key observational support for SMT came in 1960 with the strong association between the Philadelphia chromosome, a chromosome abnormality, and chronic myeloid leukemia (Baker, 2014). The oncogene theory that viral genes inserted into animal cells cause cancer received a boost in 1976 with the discovery of a close similarity between genes in chickens and genes in the avian sarcoma virus (Morgan & Boyerinas, 2016). Additional support for SMT came in 1982 with experiments showing that introduction of DNA into normal cells could convert the normal cells to cancer cells. A standard classification of mutations thought to cause cancer was either as oncogenes, which cause a gain of function that leads to cancer or tumor suppressor genes, which cause a loss in function that leads to cancer. Perhaps the high point of SMT occurred in 1990 with a genetic multistage model of colorectal tumorgenesis (Cofre & Abdelhay, 2017).

1.3.2. Other causes of cancer

The Somatic Mutation Theory however has loop holes that might suggest cancer could be caused by other factors. The reported number of mutations associated with tumors has increased dramatically. In various studies, investigators have reported cancers with 77 mutations per million base pairs of DNA (Greenman *et al.,* 2007), cancers with over 30 mutations, solid tumors averaging up to 66 mutations (Vogelstein *et al.,* 2013), and tumors with over 10,000 mutations (Lawrence *et al.,* 2013). In another study (Imielinski *et al.,* 2012), only 6% of tumor mutations corresponded to six hallmarks of cancer, and 15% corresponded to no hallmarks of cancer. A further complexity is the recent discovery that some tumors are characterized by sudden catastrophic genetic changes (Stephens *et al.,* 2011), not the slow accumulation of mutations under the "classic" formulation of SMT.

Tissue Organization Field Theory (TOFT) (Soto & Sonnenschein, 2011) postulates that cancer arises from the disruption of interactions with adjacent tissue, which can be mediated by intercellular chemical signals, mechanical forces, and bioelectric

changes. Because these adjacent-tissue interactions are thought to play a role in embryonic development, an appropriate summary of TOFT is "development gone awry" (Sonnenschein *et al.,* 2014). The premises of TOFT are that carcinogenesis represents a problem of tissue organization, comparable to organogenesis, and that proliferation is the default state of all cells (Soto & Sonnenschein, 2004). Three corollaries of TOFT are: 1) mutations are not needed for carcinogenesis; 2) cancer can arise in tissue where carcinogen has not been applied; and 3) genetic instability is a byproduct of carcinogenesis.

Plants have been used for medicinal purposes long before recorded history. Ancient Chinese and Egyptian papyrus writings describe medicinal uses for plants as early as 3,000 BC. Indigenous cultures (such as African and Native American) have used herbs in their healing rituals, while others have developed traditional medical systems (such as Ayurveda and Traditional Chinese Medicine) in which herbal therapies are being used. Medicinal herbs and their derivative phytocompounds are being increasingly recognized as useful complementary treatments for cancer (Nelson & Montgomery, 2003). In Kenya, various medicinal plants have been identified and used in treatment and management of cancer (Ochwang'i *et al.,* 2014).

A large volume of clinical studies have reported the beneficial effects of herbal medicines on the survival, immune modulation, and quality of life (QOL) of cancer patients, when these herbal medicines are used in combination with conventional therapeutics. Prostate cancer, specifically, is characterized by a long latency period, a strong dietary influence, and limited treatment strategies for the advanced disease; therefore, many patients turn to complementary and alternative medicine (CAM) with the belief that these represent a viable therapeutic option that may be free of adverse side effects (Nelson & Montgomery, 2003). This folkloric belief, strongly upheld by many, needs to be substantiated with systematic, evidence based research. The study aimed at investigating the anticancer and anti-oxidant properties of three plants species namely, *A. indica, V. amygdalina* and *G. aparinoides* which are traditionally used in the management of cancer in Kenya (Sharma *et al.,* 2014 and Wong *et al.,* 2013). The antioxidant properties were evaluated using the DPPH free radical scavenging assay. *In vitro* anticancer properties were investigated against cervical cancer and prostate

cancer cell lines, singly and then in combinations (blends) using the MTT assay. *In vivo* and acute toxicity was carried out to determine undesirable toxicities of the extracts in mice.

1.4. Statement of the problem

While cancer ranks third in Kenya as the cause of mortality, falling behind infectious diseases and cardiovascular conditions, existing treatment options are not diverse and the high cost of attaining such treatment hinders a majority of the affected population from receiving treatment.

While communicable diseases still remain the leading causes of death in many developing countries, the incidence and mortality from non-communicable diseases is rising rapidly. This has resulted in a "double burden of disease" further straining the existing health systems. In Kenya, it is estimated that the annual incidence of cancer is about 28,000 new cases with an annual mortality of 22,000 cases. Over 60% of those affected are below 70 years while the risk of getting cancer before 75 years of age is 14% and the risk of dying of it is estimated at 12%. The leading cancers in women are breast, cervical and oesophagus. In men, oesophagus, prostate cancer and Kaposi"s sarcoma are the most common. Data from human papillomavirus and related cancers indicate that Kenya has a population of 13.5 million women aged 15 years and older who are at risk of developing cervical cancer. Current estimates indicate that every year 4802 women are diagnosed with cervical cancer and 2451 die from the disease. The Nairobi cancer registry places prostate cancer as the commonest cancer in males at 17.3%. This compares well with 15% reported in developed countries. Despite this significant contribution of cancer to the morbidity and mortality of the Kenyan population, treatment is still not accessible to many, majorly due to the expensive costs.

1.5. Justification of the study

Lack of diversity in the modes of treatment for cancer coupled with the robust economic input required for the treatment of cancer has prompted the shift in focus from conventional medicine to traditional medicine. Surgery, chemotherapy, and

radiotherapy are still the major conventional cancer therapies. Serious drawbacks however can be pegged to these strategies despite the obvious milestones gained in the fight against cancer. Therefore there is an urgent need for the development of novel combination therapies and treatment regimens with higher efficacies.

Medicinal plants have great potential to be used as sources for anti-cancer therapeutics. Recently, there has been a shift in the world of research and drug development to concentrate more on the potential of medicinal plants as sources of anticancer agents. This shift has been driven by the successful potency of plant derived molecules such as the *vinca* alkaloids, vinblastine, vincristine and cytotoxic podophyllotoxins as anticancer agents. The recent success of clinical trials with less toxic plant derived anticancer agents; flavopiridol, isolated from the Indian tree *Dysoxylum binectariferum* and meisoindigo, isolated from the Chinese plant *Indigofera tinctoria,* has propelled research into medicinal plants as sources of anticancer agents.

There is a need to bridge the gaps in such knowledge with regards to the use of *Azadirachta indica, Vernonia amygdalina* and *Galium aparinoides* in cancer management in Kenya. With the investigation of *in vitro* properties of the plant extracts against cell lines for both cervical cancer and prostate cancer, a deeper understanding can be attained of their bioactivity leading to their validation and more informed usage.

1.7. Objectives

1.7.1. Overall objective

To determine the antioxidant properties, antiproliferative activity, acute oral toxicity and phytochemical screening of *A. indica*, *V. amygdalina*, and *G. aparinoides* plant extracts against cervical and prostate cancer cell lines.

1.7.2. Specific objectives

- To determine the antioxidant activity of the extracts of *A. indica, V. amygdalina* and *G. aparinoides.*

9

- To determine the antiproliferative activity of single plant extracts of *A. indica, V. amygdalina* and *G. aparinoides* against cervical cancer and prostate cancer cell lines *in vitro*.

- To evaluate *in vitro* cytotoxicity effects of combined plant extracts against cervical cancer and prostate cancer cell lines *in vitro*.

- To determine the acute oral toxicity of the most potent extracts in mice model *in vivo*.

- To determine the qualitative phytochemical composition of *A. indica, V. amygdalina* and *G. aparinoides*

CHAPTER TWO

LITERATURE REVIEW

2.1. Cancer

The search for cancer therapeutics has come a long way. Since cancer is characterized by the abnormal growth and proliferation of cells, it is important that cancer research concentrates on finding ways to regulate growth of cancer cells and consequently their proliferation. The basic approach towards achieving this has been pegged on either the inhibition of cell division or clearance of cancer cells (Iqbal & Bamezai, 2012). The problem that has been realized with the developed therapeutics so far is that they not only kill cancer cells but they also kill the normal functioning human cells that were not meant to be targets (Iqbal & Bamezai, 2012).

In order to deliver effective therapeutics which cause minimal damage to normal human cells, research has focused on the therapeutic value of medicinal plants of late (Boohaker *et al.,* 2012). Though widely accepted that plants have the potential of synthesizing complex molecules of therapeutic value, the identification, isolation and purification of such molecules requires extensive screening. Phytochemical examination of plants has occurred in the recent past, and many of the plants used in folklore for the treatment of cancer have been found to exhibit antitumor activity (Altemimi *et al.,* 2017).

The plant derived molecules with anticancer activity that have been used clinically include the *Vinca* alkaloids which function by arresting metaphase through interference with microtubule assembly. The *Vinca* alkaloids are derived from *Vinca rosea* and include vinblastine, vincristine and vincamine (Moudi *et al.,* 2013).

They also have synthetic derivatives in the form of vindesine and vinorelbine. Another alkaloid, campothecin, is a topoisomerase inhibitor that has also been found to have anticancer activity (Sisodiya, 2013). The above named are just but a few examples of the existing proven plant derived molecules that exhibit anticancer activity. Due to the potential that is evidently existent in plants when it comes to the sourcing of novel

therapeutic agents, cancer research has seen a major shift towards medicinal plants. In the many cases that the anticancer activity of plants has been established, it has been done so *in vitro*. These compounds, together with those that are yet to be established as having *in vitro* anticancer activity, need to be evaluated *in vivo*. As such, research needs to be carried out using animal models or human models so as to determine the *in vivo* activity of the plant derived molecules.

2.2. Diagnosis and treatment

The key objective to cancer diagnosis and treatment programme is to cure or significantly lengthen the life of patients. Management of cancer is based on the type and its stage, which denotes how far it has grown and weather it has spread from its point of origin (Jiang *et al.,* 2007). Cancer diagnosis begins with a thorough physical examinations and a complete medical history through various laboratory tests. When a tumour is suspected, imaging tests such as X-rays, computed tomography (CT), magnetic resonance imaging (MRI), ultrasound and fiber-optic endoscopy examinations are done to help doctors determine the cancer's location and size (Nabende & Namukhosi, 2015). To confirm the diagnosis of most cancers, a biopsy needs to be performed in which a tissue sample is removed from the suspected tumour and studied under a microscope to check for cancer cells (Karcioglu *et al.,* 1985).

A positive diagnosis implies that cancer is present hence other tests are performed to provide specific information about the cancer in a process called staging. If the biopsy is positive for cancer, the patient is advised to seek a confirming opinion by a doctor who specializes in cancer treatment before any treatment begins. If the initial diagnosis is negative for cancer and symptoms persist, further tests may be needed (WHO, 2014).

2.3. Alternative medicine

Complementary and Alternative Medicine (CAM) is defined by the Cochrane collaboration as" a broad domain of healing resources that encompasses all health system, modalities, and practices and their accompanying theories and beliefs, other than intrinsic to the politically dominant health systems of a particular society or culture in a given historical period" (The Cochrane Library Cochrane Database of

Systematic Reviews). However, the National Centre for Complementary and Alternative Medicine (NCCAM 2006) in America defines CAM as "a group of diverse medical and health care systems, practices and products that are not presently considered to be part of conventional medicine" (Pearson *et al* ., 2006).

There has been a steady increase in the use of complementary and alternative medicine among cancer patients for the past decades (Boon *et al.,* 2000). Among the early studies to ascertain the level of CAM use among cancer patients, it was reported that 16% of cancer patients surveyed in two hospitals in London admitted to using CAM (Downer *et al.,* 1994). This figure is similar to an earlier report in which CAM use was reported at 13% in the United State of America (USA) (Cassileth & Vickers, 2004). However, a recent survey of 127 cancer patients in the United Kingdom (UK) reported that 29% of their sampled population were using some form of CAM (Scott *et al.,* 2005).
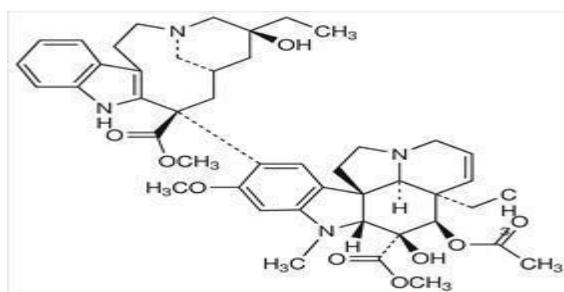
In a systematic review of surveys on the use of CAM among cancer patients in 13 countries, Ernst and Cassileth reported a range of 7% to 64% of CAM use among the adult cancer population and the average of 31.4% across all the studies (Ernst, 1998). Some of the commonly used CAM included visualization, herbs, dietary treatment, meditation, relaxation, homeopathy and hypnotherapy.

Despite the fact that more and more cancer patients are turning to CAM modalities for a number of reasons, few patients disclose this to their health care professionals (Truant & Bottorff, 1999). Studies so far conducted indicated that just about half of the cancer patients who use CAM inform their doctors of such use (Bott, 2007). Patients perceive a lack of interest on the part of health care professionals or their total disapproval of the therapies (Richardson *et al.,* 2007). The lack of communication about CAM between patients and health professionals limits the opportunity to discuss the potential benefits and risk of the therapies.
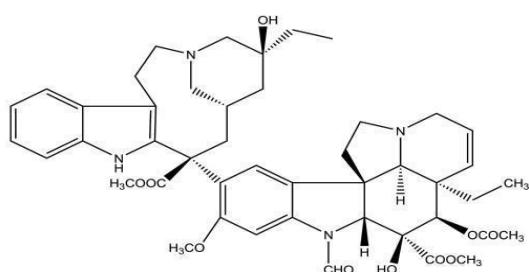
2.4. Plant derived anti-cancer compounds

Despite plants being used as food and spices, they have also been employed as medicines for over 5000 years. Plants have had a vital role in the folklore of ancient

beliefs. Traditional medicine has become a preferred method with an estimate of 70–95% population in developing countries (DeSantis *et al.,* 2014). In the 1950‟s a new trend, that was involved in isolation of plant active compounds begun and led to the discovery of different active compounds that are derived from plants. In the last decades, more and more new materials derived from plants have been authorized and subscribed as medicines, including those with anti-cancer activity (Cragg & Newman, 2005). Anticancer drugs derived from plants that are currently in clinical use can be categorized into four main classes of compounds: *vinca* (or Catharanthus) alkaloids, epipodophyllotoxins, taxanes and camptothecins (Balunas & Kinghorn, 2005). The search for anticancer agents derived from plants started in the 1950s with the discovery and development of the vinca alkaloids and the isolation of the cytotoxic podophyllotoxins. The vinca alkaloids vinblastine (Figure 2.1) and vincristine (Figure 2.2) were isolated from the Madagascar periwinkle (*Catharanthus roseus* or *Vinca rosea*). These alkaloids and their semisynthetic derivatives block mitosis at the metaphase stage causing cell apoptosis and they have been used in the treatment of leukemias, lymphomas, Kaposi‟s sarcoma (KS) and testicular, breast and lung cancers for more than 40 years (Jacobs *et al.,* 2004)

Figure 2. 1: Vinblastine; natural alkaloid isolated from the plant *Vinca rosea*. *Linn.*
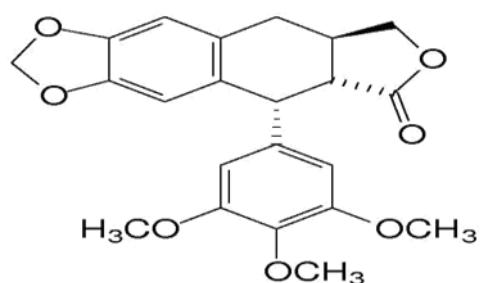
Figure 2. 2: Vincristine; a chemotherapy drug used to treat cancer
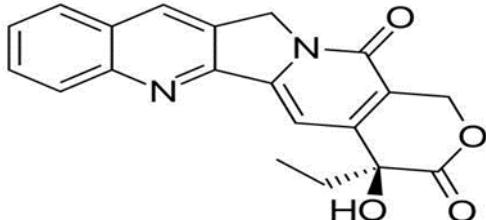
The podophyllotoxins were isolated from the resin of *Podophylum peltatum* and they also block mitosis during the metaphase by inhibiting tubulin polymerization-causing apoptosis. However, their use in clinic failed because of their severe side effects. Further studies lead to the development of semisynthetic derivatives of podophyllotoxins (Figure 2.3) such as etoposide and teniposide that inhibit the catalytic activity of DNA topoisomerase II inducing irreversible breaks in the DNA and apoptosis. Etoposide and teniposide are used in the treatment of lymphomas, as well as acute leukemia and small-cell lung, testicular, ovarian and bladder cancers (Gordaliza *et al.,* 2000).

In 1960, the United States National Cancer Institute (NCI) initiated an extensive plant collection program leading to the discovery of many novel chemotypes including taxanes and camptothecins. The taxanes were isolated from *Taxus brevifolia* and other conifers from the genus *Taxus*. They stabilize the microtubules suppressing their depolymerization to tubulin leading to cell death. Taxanes have been used for the treatment of ovarian cancer, metastatic breast and lung cancers and KS (Oberlies &

Kroll, 2004). The camptothecins (Figure 2.4) are alkaloids isolated from *Campthotheca acuminata*, *Ophiorrhiza pumila* or *Mapia foethida*. Camptothecin inhibits DNA topoisomerase I. The semisynthetic analogs of camptothecin, topotecan and irinotecan, are in clinical use. Topotecan is used for the treatment of ovarian and small-cell lung cancers, whereas irinotecan is used for the treatment of colorectal cancer (Balunas & Kinghorn, 2005).

Figure 2. 3: Podophyllotoxins; antimitotic agent isolated the plant *Podophyllum peltatum*

Figure 2. 4: Camptothecins; used in screening anticancer drugs

Currently, the Natural Products Branch, part of the Developmental Therapeutic Program at the NCI, collaborates with agencies throughout the world to collect thousands of plants and marine organisms for the extraction and screening of antitumor properties of extracts. The NCI is also committed to the conservation of biological diversity. To date, only 1% of the ~500 000 plant species worldwide has been phytochemically investigated (Palombo, 2006). Therefore, it is likely that many new bioactive compounds are waiting to be discovered and developed into treatment for pathologies such as bone metastasis.
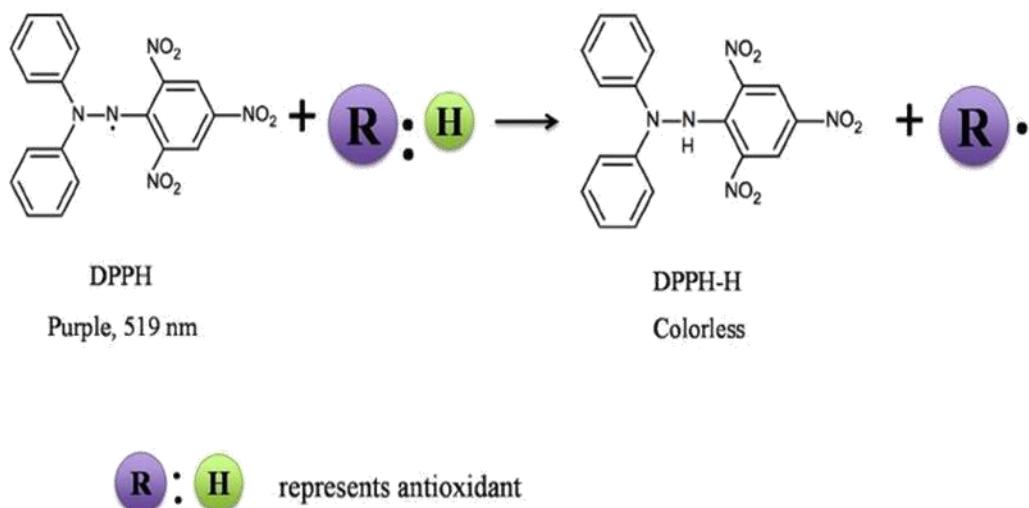
2.5. Antioxidant activity

Antioxidants are chemicals that interact with and neutralize free radicals, thus preventing them from causing damage. Antioxidants are also known as "free radical scavengers. The main characteristic of an antioxidant is its ability to trap free radicals.

Highly reactive free radicals and oxygen species are present in biological systems from a wide variety of sources. These free radicals may oxidize nucleic acids, proteins, lipids and can initiate degenerative disease. Antioxidant compounds like phenolic acids, polyphenols and flavonoids scavenge free radicals such as peroxide, hydroperoxide or lipidperoxyl and thus inhibit the oxidative mechanisms that lead to degenerative diseases (Mishra *et al.,* 2010).

Free radicals are highly reactive chemicals that have the potential to harm cells. They are created when an atom or a molecule either gains or losses an electron. Free radicals are formed naturally in the body and play an important role in many normal cellular processes (Diplock *et al.,* 1998). At high concentrations, however, free radicals can be hazardous to the body and damage all major components of cells, including DNA, proteins, and cell membranes. The damage to cells caused by free radicals, especially the damage to DNA, may play a role in the development of cancer and other health conditions (Valko *et al.,* 2007).

The principle behind the DPPH (1,1-diphenyl-2-picrylhydarzil) scavenging activity is based on its reduction in presence of a hydrogen donating antioxidant. Due to the influence of hydrogen donating ability of the extracts, DPPH is reduced from its violet color to a colorless color as shown in Figure 2.5 (Molyneux, 2004). DPPH decreases considerably when in contact with a proton radical scavenger due to its proton free radical (Singh & Rajini, 2004).

Figure 2. 5: DPPH Reduction; to diphenyl hydrazine stable solution.

Tumor growth can cause antioxidant disturbances in certain tissues of the tumor host. One of the characteristics of tumor growth and invasion is the increased flux of oxy-radicals and loss of cellular redox homeostasis. Cancer cells can generate large amounts of hydrogen peroxide, which may cause mutation and damage of normal tissues in the body. Antioxidants block carcinogenesis by multiple mechanisms which include prevention of procarcinogenic activation, inhibition of cell proliferation, invasion and angiogenesis and stimulation of apoptosis. Some classes of compounds have been associated with antioxidant and anti-proliferation activity for instance the alkaloids, cardiac glycosides, terpenes, phenolics and flavanoids among others (Aiyelaagbe & Osamudiamen, 2009). This knowledge has resulted in the shift towards medicinal plants due to the fact that they are rich in antioxidant phytochemicals (Hussain *et al.,* 2003).

2.6. *Azadirachta indica Juss.*

2.6.1. Description

Azadirachta indica is a fast-growing tree that can reach a height of 15-20 m, though it occasionally reaches 35-40 m. This plant belongs to the *Meliaceae* family and genus *Azaddirachta*. It is evergreen, but in severe drought it may shed most or nearly all of its leaves. It grows on dry, stony, clayey, and shallow soils having a pH range of 5.0
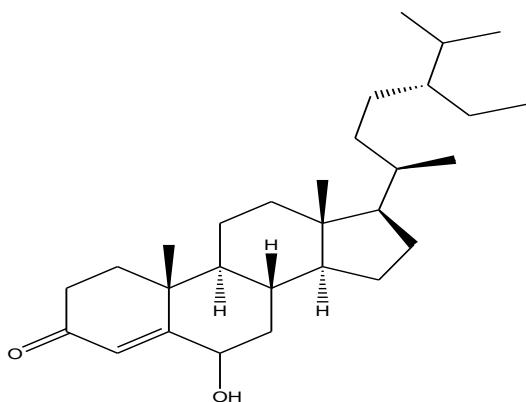
to 8.5. In Kenya it is referred to as *Mwarubaini* loosely translated to a "tree of 40 cures" in reference to the numerous claims of its potential to treat over 40 ailments. *Azadirachta indica* (Neem) is known historically for its miraculous healing properties and has been described an ancient cure for a modern world. Neem is the Chinaberry's miraculous cousin and known as a tree for solving global problems, an epithet which has been recognized by the US National Academy of Sciences (Oaks *et al* ., 1992). It is native to the dry forests of India, Pakistan and Sri Lanka and is cultivated in tropical and subtropical regions worldwide (Othman *et al.,* 2011).

2.6.2. Chemical Compounds
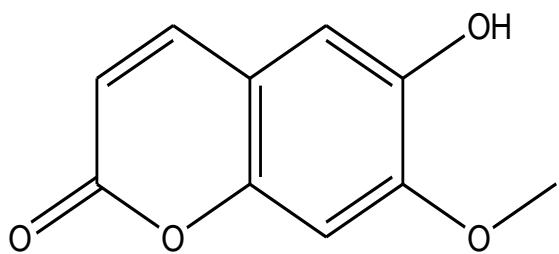
At least 140 different chemical compounds have been found within the different parts of the plant (Atakan, 2012). Studies reveal that *Azadirachta indica* has 16 different groups of organic compounds. Major chemical constituents isolated from the leaves include Tannin, sitosterol and its glucoside, 24-methylene-cycloartenol, 4,14,-dimethyl -5-ergosta- 8, 24(28)-dien-3 -o1 (Figure 2.6), 4 -methyl-5 -ergosta-8, 24(28)-dien-3-o1, Nimatone, Nimbinene, 6-desacetyl Nimbinene, Nimbolins, Vanilic acid, Catechol, Campesterol, Stigmasterol, Sitosterol, -amyrin, Lupeol, Nimbin, Nimbidin, Nimbinin, Sugiol, Kulinone, Kulactone, Kulolactone Methyl Kulonate, 6-hydroxy-4-stigmasten-3-one and 6 -hydroxy-4- campesten-3-one (Paritala *et al.,* 2015).

Gedunin, 7- deacetoxy-7-oxogedunin, fraxinellone, nimbolin cycloeucalenone, and melianin have been found in its stem bark. Besides aesculetin, Campesterol, 6-hydroxy-7-methoxy-coumarin (Figure 2.7), 4 -6 - Dihydroxy-A-homoazadirone,

Isomeldenin, Meldenindiol, 17-AcetoxyMeliacin,6-0-acetylnimbandiol, Desacetylnimbin, Nimocinol, Isonimolicinolide and Nimolinolic have also been found in its root bark (Hossain *et al.,* 2013). Figure 2.6 and Figure 2.7 shows some of the chemical structures found in *A. indica.*

Figure 2. 6: 4, 14,-dimethyl -5-ergosta- 8, 24(28)-dien-3 -o1; meiosis activating sterol.

Figure 2. 7: 6-hydroxy-7-methoxy-coumarin; metabolite generated by BioTransformer

2.6.3. Medicinal uses

Therapeutic properties of *Azadirachta indica* have been recognized since ancient times and have been extensively used in ayurveda, unani, and homoeopathic medicine (Biswas *et al.,* 2002). Many compounds such as limonoids, azadirone, azadirachtin and flavonoids, having therapeutic potential, have been isolated from various parts of neem tree and have been evaluated for their pharmacological actions and plausible medicinal applications along with their safety evaluation. Every part of the tree has been used as traditional medicine for household remedy against various human ailments, from time immemorial (Sofowora *et al.,* 2013).

However, it has previously been reported that *V. amygdalina* aqueous leaf extracts suppressed breast cancer cells growth at 100 μg/ml (Clement *et al.,* 2014). Interestingly, when used in conjunction with chemotherapeutic drugs like cyclophosphamide, isplatin, 5-fluorouracil, or with radiotherapy, it potentiates their

antitumor effects by activating pro-apoptotic signaling and negating survival signaling along with attenuating their side effects (Kpela *et al.,* 2012; Sharma *et al.,* 2014).

Notably, cisplatin, the first member of a class of platinum-containing anticancer drugs, is widely used for treatment of solid malignancies. Cisplatin has a number of side-effects that can limit its use: nephrotoxicity, nausea and vomiting, ototoxicity (hearing loss), electrolyte disturbance, and hemolytic anemia. Also, the majority of cancer patients eventually develop cisplatin-resistant disease necessitating combination therapy approach using multiple chemotherapeutic agents or combining with chemopreventive agents (Sharma *et al.,* 2014). *A. india* can be one such agent.

2.6.4. Biological activity

Recent studies have shown that neem possesses anti-inflammatory, anti-arthritic, antipyretic, hypoglycemic, antigastric ulcer, antifungal, antibacterial, antitumor activities, antidiabetic activity (Dholi *et al.,* 2011). The antineoplastic properties of *A. india* are gaining attention due to its cancer preventive, tumor-suppressive, antiproliferative, apoptosis-inducing, antiangiogenic, and immunomodulatory effects via several molecular mechanisms (Sharma *et al.,* 2014). The alcoholic extract of *A. india* flowers has been reported to possess ideal antifertility agent. (Gbotolorun *et al.,* 2008; Maragathavalli *et al.,* 2012) reported on the antimicrobial activity of the methanolic and ethanolic leaf extracts against human pathogenic bacteria.

2.6.5. Mode of action

Azadirachta indica plants parts shows antimicrobial role through inhibitory effect on microbial growth of cell wall breakdown. Azadirachtin, a complex tetranortriterpenoid limonoid present in seeds, is the key constituent responsible for both antifeedant and toxic effects in insects (Nisbet, 2000).

Results suggest that the ethanol extract of *Azadirachta indica* leaves showed *in vitro* antibacterial activity against *Staphylococcus aureus* with greatest zones of inhibition noted at 100% concentration (Sarmiento *et al.,* 2011). It also plays role as free radical scavenging properties due to rich source of antioxidant. Azadirachtin and nimbolide

showed concentration-dependent antiradical scavenging activity and reductive potential in the following order: nimbolide > azadirachtin > ascorbate (Hossain *et al.,* 2013).

Azadirachta indica ingredient shows effective role in the management of cancer through the regulation of cell signalling pathways. Neem modulates the activity of various tumour suppressor genes, angiogenesis, transcription factors and apoptosis. It also plays role as anti-inflammatory via regulation of proinflammatory enzyme activities including cyclooxygenase and lipoxygenase enzyme.

2.7. *Vernonia amygdalina Del.*

2.7.1. Description
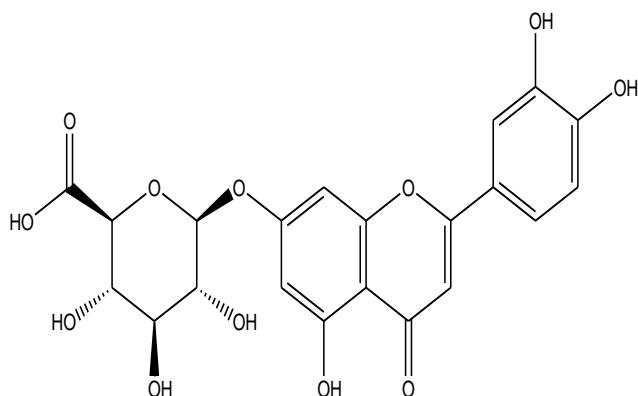
Vernonia amygdalina is a woody shrub that can grow up to 5m tall, belonging to the family of Asteraceae. Native to Nigeria (West Africa) and widely grown in Africa, it is also found in Asia and is especially common in Singapore and Malaysia. The leaves exhibit a characteristic odor and bitter taste, explaining its common English name of „bitter leaf". Different local names have been used in various communities such as Omororia (Kisii), Omulusya/musuritsa (Luhya), Olusia (Luo), Cheburiandet (Nandi), Omuburiri (Nyankore) and Labwori (Acholi).

2.7.2. Chemical compounds
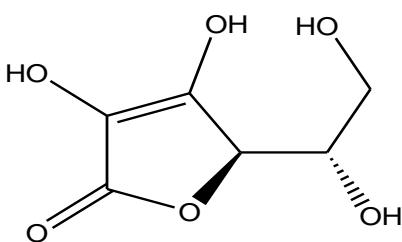
Several investigators have led to the isolation of a number of chemical compounds with potent biological activities from the leaves of *V. amygdalina.* An investigation of *V. amygdalina* showed that the biological-active compounds of *V. amygadlina* include saponins and alkaloids, terpenes, steroids, coumrains, flavonoids like luteolin, luteolin 7-O-glucuronide (Figure 2.8) phenolic acids, lignans, xanthones and anthraquinone, edotides and sesquiterpenes (Erasto *et al.,* 2007).

It has also been reported that *V. amygdalina* contains Thiamine, Pyridoxine, and Ascorbic acid (Figure 2.9), Glycine, Cysteine and Casein. *V. amygdalina* was also reported to possess compounds toxic to common bean aphids (Dunsworth *et al.,* 1982).

Sesquiterpene has been isolated from this herbal plant and shown to possess some active ingredients; vernodalin, vernodalol and 11, 13-dihydrovernodalin. This compounds have been reported have insecticidal properties which act as an insect feeding deterrent (Ileke, 2015). Hydro distillation of the leaves of *V. amygdalina* yielded essential oil containing eucalyptol (1, 8 cineole, 25%), beta pinene (14.5%) and myrtenal (Yeap *et al.,* 2010). Figure 2.8 and figure 2.9 shows some of the chemical structures found in *V. amygdalina.*

Figure 2. 8: Luteolin 7-O-glucuronide; o-glycosidically linked to the flavonoid backbones

Figure 2. 9: Ascorbic acid; vitamin C, functions as a reducing agent

2.7.3. Medicinal uses

The medicinal properties of *V. amygdalina* have been widely recognized and it is a major part of pharmacopeia for Nigeria‟s naturalists (Yeap *et al.*, 2010). The plant has been shown to possess diverse therapeutic effects such as anti-malarial, anti-microbial

(anti-bacterial, anti-fungal and anti-plasmodial), anti-diabetic and anti-cancer effects (Wong *et al.,* 2013). The anti-cancer effect of the plant was first shown in human carcinoma ofnasopharynx and later in leukemia cells P-388 and L-1210 using its chloroform extract (Wong *et al.,* 2013). Different extracts have thus been used in scientific research to reveal the therapeutic properties of this plant. Current research conducted on the anti-cancer effect of *V. amygdalina* has focused exclusively on MCF-7 cells (Ijeh & Ejike, 2011). *V. amygdalina* has been used traditionally to treat cancer (Olatunde & Farombi, 2003) and sexually transmitted diseases such as gonorrhea and malaria (Erasto *et al.,* 2007). The plant has antihelminthic, antitumorigenic, hypoglycaemic and hypolipidaemic activity (Yeap *et al.*, 2010). Both the leaves and the roots are used traditionally in phytomedicine to treat fever, kidney heart disease and stomach discomfort (Farombi & owoeye 2011). This project will further give insight into the bioactivity of the plant and its use in cancer treatment.

2.7.4. Biological activity

Erasto et al. (2007) also reported on the usefulness and prevention of the roots of *V. amygdalina* in treatment of tooth ache and gum decay due to its bactericidal and antimicrobial activities. Many studies have shown that *V. amygdalina* provides anti-oxidant benefit (Khalafalla *et al.,* 2009). *V. amygdalina* aqueous extract has been investigated and identified to possess cell growth inhibitory effects in prostate cancer cell line (Adebayo *et al.,* 2014; Sweeney *et al.,* 2006).

2.7.5. Mode of action

V. amygdalina leaves extracts had been reported to possess protective effect against carbon tetrachloride-induced hepatotoxicity by the antioxidant mechanism of action (Adesanoye & Farombi, 2010). Vernodalin and vernolide had been reported to exhibit potent activity for Leukimia cell lines (IC_{50} P-388 and L-121O cells). Vernolide showed a significant inhibition against gram-positive bacteria and the gram-negative bacterium Salmonella pooni showing its antibacterial activity (Clement *et al.,* 2014). Vernodalin and vernomygdin had been reported to possess antitumor activity against human nasopharynx carcinoma (Luo *et al.,* 2017).

The antifeedant activity was noticed from the isolated 11,13-dihydrovernorodeline, this can resist insects from feeding on the plant but does not result in lethality (Yeap *et al.,* 2010). Vernolepin had also been reported to show antiplatelet property against arachidonic acid and collagen-induced platelet aggregation in rabbits ((Yeap *et al.,* 2010).

2.8. *Galium aparinoides Forssk.*
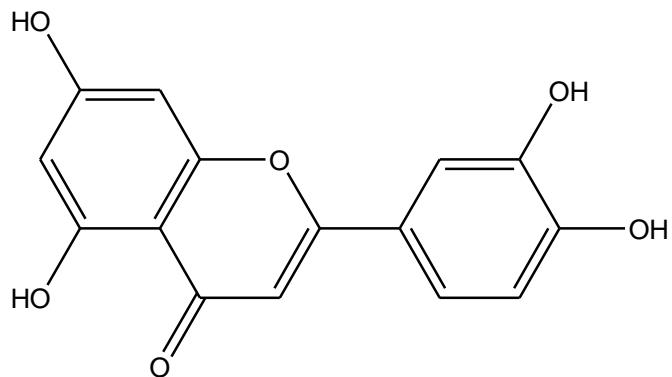
2.8.1. Description

It is a scrambling perennial herb up to 1.8–2.1 m. long; stems usually densely covered with small prickles but otherwise mostly glabrous. Its roots are said to contain a red dye. Leaves and stipules appear in spirals and the blades are narrowly elliptic to 0.4–3 cm long. It has an apical penetration of 0.5–1.5 mm long sparsely hairy on both surfaces. It is known as olengeriantus in Maasai and belongs to family *Rubiaceae*.
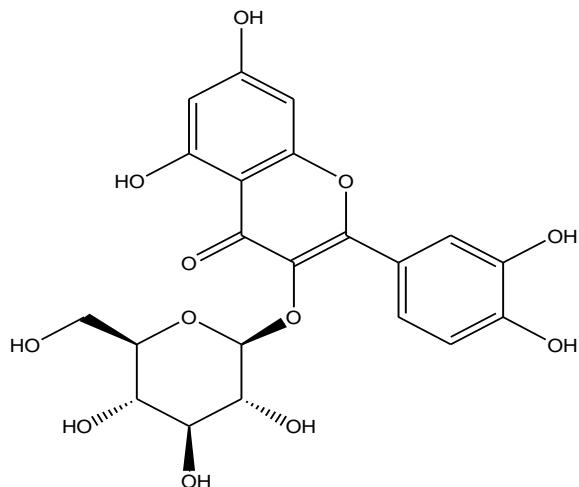
It consists of about 300 herbaceous plant species and have been used in food manufacturing and traditional medicine all over the world (Vlase *et al.,* 2014). *Galium aparine* L. is the most widely spread species of *Galium* and is mostly found growing on moderate climate.

2.8.2. Chemical compounds

Studies have clearly established that *Galium* species have a simple and highly substituted flavone and flavonol compounds including flavonoid, aglycones, glycosides and methylated compounds. Isolated flavone compounds identified were apigenin and its 7-glycoside, luteolin 7-diglycoside, diosmetin and its 7-monoglycoside, as well as its 7-diglycoside. In addition, flavonol compounds were detected and they include kaempferol 3-glycoside (Figure 2.10) , 3-diglycoside and 3,7-diglycoside, quercetin, 3-glycoside (Figure 2.11), 3-rutinoside, 3,7-diglycoside and 3-diglycoside-7-glycoside (Puff, 1975). But none of this compounds have been identified in *Galium aparinoides.* Figure 2.10 and figure 2.11 shows some of the chemical structures found in *G.aparinoides.*

Figure 2. 10: Kaempferol 3-glucoside; astragalin is a chemical compound

Figure 2. 11: Quercetin, 3-glucoside; used in treatment of kidney Cancer

2.8.3. Medicinal uses

It is believed to cure throat cancer in cattle. The whole herb is pounded and soaked in water, or boiled and the decoction given to the sick animal (Kokwaro, 2009). Its species *Galium aparine* (cleavers), has a long history of use as an alternative medicine and is still used widely by modern herbalists. It is an amazing diuretic that is often taken to treat skin problems. It treats many problems such as seborrhoea, eczema, psoriasis. It also acts as a great detoxifying agent in life-threatening ailments like cancer. The fresh plant or juice of this herb is used as a medicinal wonder to treat problems like wounds, skin problems and ulcers. An infusion of the herb has been used

in the treatment of various problems. The infusion is also used to treat liver, bladder and urinary problems (Vlase *et al.,* 2014).

2.8.4. Biological activity

The bioactivity of this specific plant species has not been well documented, though different members of the genus *Galium* have been found to have bioactivity. Studies have shown the presence of hydroxycinnamic acids, coumarins, derivatives of flavones and flavanols in aerial parts of the plant. *Galium verum L.,* yellow "Lady"s bedstraw", with golden yellow flowers, contains polyphenols, flavonoids, phenyl-propanoids compounds and iridoids (Vlase *et al.,* 2014). They are used as diuretic, depurative; light sedative, spasmolytic in kidney stones, and externally for injuries and skin damages as wound healing, psoriasis treatment and rheumatism. Aerial parts of *G. aparine* contain anthraquinones, iridoids, alkanes, flavonoids, tannins, polyphenolic acids, and vitamin C (Vlase *et al.,* 2014). With the potential that *Galium aparinoides Forssk.* has with regards to bioactivity, this project will provide the much needed scientific data to prove their bioactive importance or otherwise.

2.8.5. Mode of action

Although no mode of action has been documented about *Galium aparinoides, l*ittle knowledge is known about its species. . 3,7-diglycoside a flavone, isolated from *Galium verum* has been found to treat kidney stones. Apigenin-7-O-glycoside has also been isolated from gallium species its used as a non-steroidal anti- inflammatory drug (Li *et al.,* 2010). Luteolin 7-diglycoside isolated from *Galium mullugo* and its used to treat diseases such as hypertention cancer and inflammatory disorders (Poppendieck & Kubitzki, 1981). 3′,4′,5,7-tetrahydroxyflavone a Luteolin is able to interfere with almost all of the characteristics of cancer cells thus used as an anticancer agent (Lin *et al.,* 2008).Emetine is a natural product alkaloid from ipecac species. It is one of the main active ingredients in ipecac syrup used as emetic, and has been used extensively in phytomedicine as an antiparasitic drug (Akinboye & Bakare, 2011).

CHAPTER THREE

MATERIALS AND METHODS

3.1. Study site

The study was carried out at Centre for Traditional Medicine and Drug Research (CTMDR) laboratories and the animal house facility at Kenya Medical Research Institute (KEMRI) Nairobi, Kenya.

3.2. Study design

This was a Laboratory based experimental study.

3.3. Ethical considerations

The study was approved by the Kenya Medical Research Institute (KEMRI) Scientific and Ethics Review Unit (SERU) and the animal care use committee an approval certificate number KEMRI/SERU/CTMDR/016/3119 and (KEMRI/ACUC/02.02.15) respectively (Appendix 1 and 2).

3.4. Medicinal plant collection, extraction and phytochemical screening.

3.4.1. Collection and preparation of plant materials

The plant materials of *Azadirachta indica* and *Galium aparinoides* were collected from Ngong' forest and Shimba Hills respectively while *Vernonia amygdalina* was collected from Kakamega forest in its natural habitats. Different parts of the plants, that is, leaves (L), stem barks (SB) and root barks (RB) were collected separately then air-dried in mesh bags and the voucher specimens deposited at the University of Nairobi Herbarium.

A taxonomist was consulted during plant identification and collection. The raw materials were stored separately at the Centre for Traditional Medicine and Drug Research (CTMDR), KEMRI. Plants collected were identified as indicated in Table 3.1.

Table 3. 1: Collection and identification of the plants

Botanical name	Family	Voucher number	Location of collection	GPS Information	Parts Used
Azadirachta indica	*Asteraceae*	PGK/2014/008	Shimba Hills	039ˆ034' 26.57" E and 04ˆ0 19' 08.39"S	Leaves, root bark, stem bark
Vernonia amygdalina	*Asteraceae*	PGK/2014/011	Kakamega forest	020ˆ0 20' 44" N and 34ˆ0 49'57" E	Leave, roots and aerial parts
Galium aparinoides	*Rubiaceae*	PGK/2014/10	Ngong' forest	1ˆ0 18'0" S and 36ˆ0 43'60" E	Aerial parts

Figure 4. 1: *Azadirachta indica* parts.

A; leaves; B; harvesting of the stem bark; C; stem barks

Figure 4. 2: *Vernonia amygdalina* **Parts**

A; leaves. B; harvesting of the leaves. C; plant. D; harvesting of the roots

Figure 4. 3: *Gallium aparinoides* **parts.**

A; Plant shrub. B; harvesting of the plant. C; plant stems, D; harvesting of the stem.

3.4.2. Extraction of the plant materials

3.4.2.1. Aqueous extraction

Accurately weighed 100g of the dried plant extracts were soaked in 1000ml distilled water and put in a water bath at 70°C for 1 hour. It was then covered and left to cool under room temperature. The extracts were decanted in clean 500ml conical flasks. A two layer of sterile gauze filtration was achieved, centrifuged for 5minutes and freeze-dried (Edwards freeze drier Modulyo). Powder-like extracts were collected after 72 hours. The extracts were then weighed, labeled and stored in an airtight container at 4°C till use.

3.4.2.2. Organic extraction

Successive organic extraction was done where one hundred grams of dried plant materials were weighed and soaked in 1000ml of the different solvents of methanol, ethyl acetate and petroleum ether separately in flat bottomed conical flasks and covered tightly by cotton gauze at room temperature for 24 hours. The extracts were filtered through Whatman filter paper no.1, soaked again for another 24 hours. (Sigma Aldrich) and concentrated under reduced pressure using a rotary evaporator, a machine used to remove organic solvents from plant extracts (Buchi Rotavapor R-114). The water bath temperature was set to 60°C, the cooling water temperature was 20°C and the boiling point for the solvent was adjusted to 40°C.The resultant extracts were then weighed, labeled and stored in an airtight container at 4°C till use.

3.5. Preparation of stock solution

One hundred milligrams of the extracts were dissolved in 1ml DMSO (Dimethyl Sulfoxide) to make a stock solution of 100,000 µg/ml in 100% DMSO sterilized by filtration by using a 0.22

um Millex syringe driven filter. The working solution was made by diluting one of the stock solution to 99 parts of Earl's Minimum Essential Medium (MEM) containing 2% Fetal Bovine Serum (FBS) which was 10 µl of the extract in 990 µl of the media

to give a start concentration of the 1000 µg/ml in 1% DMSO which was used in the MTT [3-(4, 5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay.

3.6. Determination of antioxidant activity of the plant extracts

The antioxidant activity of the extracts was measured on the basis of the scavenging activity of the stable 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical with slight modifications (Brand-Williams *et al.,* 1995). Briefly, 1ml of 0.1mM of DPPH solution in methanol was mixed with 1ml of the plant extract solution of varying concentrations (100, 50, 25, 12.5, 6.25, 3.125 and 1.5625 µg/ml). A corresponding blank sample (methanol) was prepared and L-Ascorbic acid was used as reference standard. The decrease in absorbance was measured at 517nm after 30minutes in darkness using a spectrophotometer (UV-VIS Shimadzu). The percentage inhibition effect was calculated using the following equation:

Percentage Inhibition = [1 − OD (DPPH + sample)/OD (DPPH)] x 100

A dose response curve was plotted to determine the IC_{50} values. IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum scavenging capacity. All tests and analyses were done in triplicate.

3.7. Determination of antiproliferative activity of the plant extracts

3.7.1. Cell culturing

Monkey kidney Vero cells (L6), human prostate cancer cell lines (DU145) and Human cervical cancer cell lines (HELA) were sourced from the American Type Culture Collection (ATCC) (Rockville, USA). Vero cell lines are normal cells and they were used to determine the selectivity of cytotoxic plant extracts to cancerous cells. Vero cells and cervical cancer cell lines were maintained in Eagle"s Minimum Essential Medium (MEM) supplemented with penicillin and streptomycin (1%), (Sigma), 10% fetal bovine serum (FBS) and 2 mM L-glutamine at 37°C in a moistened atmosphere of 5% CO_2 to achieve confluence.

3.7.2. 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide assay procedure

The assay was carried out as previously described by (Mosmann, 1983). Vero, prostate and cervical cancer cell lines were cultured and maintained in DMEM supplemented with 10% FBS at 37°C in 5% CO_2. The cells were harvested by trypsinization, pooled in a 50 ml vial and 100μl cell suspension ($2x10^5$ cell/ml) put in the 96-well micro-titer plate. The cells were incubated at 37°C in 5% CO_2 for 24 hours to attach, the medium aspirated and 150μl of the highest concentration of each of the test samples serial dilutions in (MEM)) were added into the same row and a serial dilution was carried out. The experimental plates were incubated further at 37°C with 5% CO_2 for 48 hours. The controls used were untreated cells (no drugs and no cells) and 5-fluorouracil as a positive control. This assay was used to determine cell viability. The MTT assay is a laboratory test which measures changes in color for measuring the activity of the enzyme that reduces MTT to insoluble formazan, giving a purple color. Yellow MTT reduce to purple formazan in living cells (Lau *et al*., 2004). At the end of the incubation time, 10μl of the MTT dye [(5mg of MTT, dissolved in 1ml serum free medium (phosphate buffered saline (PBS) was added into each well and the cells incubated for 2-4 hours until a purple precipitate was clearly visible under a microscope. The medium together with MTT was aspirated off from the wells and 100μl of 100% (DMSO) added and the plates were shaken for 5 minutes to solubilize the formazan crystals. The absorbance was measured for each well at 562 nm and 690 nm as reference using a micro-titer plate reader (Wang *et al.,* 2006).

Cell viability (%) was calculated using a previously described formula; (Njagi *et al., ,* 2016; Patel, G, 2009).

$$\text{Proliferation rate} = \frac{At - Ab}{Ac - Ab}$$

$$\text{Percentage viability} = \frac{At - Ab}{Ac - Ab} \text{ X } 100$$

$$\text{Percentage inhibition} = 100 - \frac{At - Ab}{Ac - Ab} \text{X} 100$$

Where,

At= Absorbance value of test compound

Ab= Absorbance value of blank

Ac=Absorbance value of negative control (cells plus media).

3.8. *In vitro* cytotoxicity assay for combined extracts

To determine the activity of blend extracts, the checkerboard method was applied in this combination experiment and the degree of synergy was evaluated (Berenbaum, 1978). Briefly, the *in vitro* combination studies involved two components a and b. One extract (component a) with a predetermined concentration that needed to inhibit cell growth by 50% (IC_{50}) was kept constant while the other extract also with a predetermined IC_{50} (component b) was varied at different ratios of 50a/50b, 50a/25b and 50a/5b to determine the anticancer effect of the two extracts in combination. The same experiment was repeated with component b as a constant while component a was varied at different ratios. Negative control used was untreated cells (no drugs and no cells).

Fractional inhibition concentration (FIC) calculation: To evaluate the antiproliferative effect of each combination, Σ FIC was calculated:

$$\Sigma \text{ FIC} = Ac/Aa + Bc/Ba$$

Where, Ac and Bc represents equally effective concentrations (IC_{50}) when used in combinations and Aa and Ba was the equally effective concentrations (IC_{50}) when used alone.

The formula used to determine the sum of fractional inhibitory concentrations (FIC).

$$\text{FIC of drug A} = \frac{\text{IC50 (µg/ml) of extract (A) in combination}}{\text{IC50 (µg/ml) of extract (A) alone}}$$

$$\text{FIC of drug B} = \frac{\text{IC50 (µg/ml) of extract (b) in combination}}{\text{IC50 (µg/ml) of extract (b) alone}}$$

Data was interpreted as follows: Σ FIC < 1, synergism; Σ FIC = 1, additive effect and ΣFIC > 1, antagonism (Hall *et al.,* 1983).

3.9. Handling of laboratory animals

The animals were housed at the KEMRI animal house in standard solid bottom cages bedded with wood shavings. Each cage had a maximum of 3 animals and sexes were caged exclusive of each other. The animals were allowed at least a week for acclimatization to housing conditions and handling. They were fed standard pellet diet and aqueous *ad libitum* except where the experiment precluded it. The animals were handled humanely at all times using established methods of restraint and handling. This was performed according to the international accepted guideline conduct of experiment (OECD guidelines, 2005).

3.10. Determination of acute toxicity

In Vivo acute oral toxicity study was performed according to Organization for Economic Co-operation and development (OECD guidelines, 2005) for testing laboratory animals. The proposed protocol was a stepwise method with three groups of animals per step. The test extracts were suspended in Tween® 80. They were administered by an oral gavage needle (Gauge 20, 1.5″ curved shaft, 2.25mm ball diameter). A volume of 1ml/100g body weight was used as a guide to the total volume of administration. Negative control use was phosphate buffered saline (PBS).

The mice were fasted for 3 hours before dosing and 1 hour after dosing, but water was provided *ad libitum*. A starting dose of 300 mg/kg body weight was given to 3 female mice. If 2 or 3 died by the first 48 hours then a lower dose (50 mg/kg) was to be administered to another group of 3 mice; if none or one die, the higher dose in the series but lower than the initial dose of 2000 mg/kg was to be given to another 3-mice group. The animals were closely observed after each dosing for signs of toxicity. Periodically during the first 48 hours and thereafter daily up to the 14th day post-dosing. The initial periodic observation was meant for one to be confident of the survival of one group of animals before proceeding to dose another group.

The 14th day observation period was to provide more information on the onset of toxicity, duration of sub-lethal toxicity and recovery, as well as possible delayed toxicity. Time of onset or disappearance of any signs of toxicity was recorded. Of interest was observation of the cutaneous system, mucus membranes, respiratory function, central nervous system function and behavior. Euthanasia was indicated for any animal showing severe distress or moribund state.

The choice of method for euthanasia was the use of inhalant halothane as per OECD Guidelines 2005.The three plant extracts had good combined antiproliferative activity, *A.indica* methanol and ethylacetate stem bark extracts and *A.indica* methanol root bark extract were screened for acute toxicity. Sixteen groups of three animals were used in the experiment of which four groups of three were used as negative controls.

3.11. Qualitative phytochemical screening

A detailed phytochemical screening was carried out following standard methods to determine presence of different classes of compounds and correlate them to observed bioactivity (Prashanth & Krishnaiah, 2014).

3.11.1. Test of alkaloids

Dilute hydrochloric acid was added to 100g of the plant extract shaken well and filtered and a Mayer's reagent test was performed. Few drops of the reagent was added to 3ml of the filtrate along sides the tube. Formation of a creamy precipitate indicates the presence of alkaloids (Ayoola *et al.,* 2008).

3.11.2. Test for phenolic compounds

To 0.1g of the plant extract distilled water was added. To this solution 2ml of 5% ferric chloride solution was added. Formation of a red, blue or violet colour indicates presence of phenolic compounds (Siddhuraju *et al.,* 2002).

3.11.3. Salkowski's test for terpenoids

Zero point one grams (0.1g) of the extract was treated with 1ml chloroform and filtered. To the filtrate few drops of concentrated sulphuric acid were added shaken and allowed to settle. Presence of a golden yellow layer at the bottom indicates the presence of terpenoids (Edeoga *et al.,* 2005).

3.11.4. Liebermann- Burchad's test for steroids

Zero point one grams (0.1g) of the extract was treated with 1ml chloroform. To this solution few drops of acetic anhydride were added boiled cooled. Concentrated sulphuric acid was added along the walls of the tube. Formation of a brown ring at the junction of two layers, if the upper layer turned green, it indicates the presence of steroids (Edeoga *et al.,* 2005).

3.11.5. Alkaline reagent test for flavonoids

Zero point one grams (0.1g) of the extract was treated with 2-3drops of sodium hydroxide in a test tube. Formation of a deep yellow color, which becomes colorless upon addition the addition of few drops of dilute acid indicates the presence of flavonoids (Sannigrahi *et al.,* 2010).

3.11.6. Test for glycosides

Two milliliters (2ml) of the test solution and 3ml of glacial acetic acid and 1ml drop of 5% ferric chloride were mixed together in a test tube. 0.5ml of concentrated sulphuric acid was carefully added to the solution. Formation of a blue color in the acetic acid layer indicates the presence of cardiac glycosides (Koleva *et al., 2002).*

3.11.7. Test for tannins

Zero point one grams (0.1g) of the extract was mixed with 2ml distilled water and filtered. Few drops of 5% distilled aqueous water was added to the filtrate. Presence of a green colour indicated the presence of tannins (Ruch *et al.,* 1989).

3.11.8. Froth test for saponins

To 0.1g of the extract, warm aqueous was added shaken well in a graduated cylinder for 15 minutes and allowed to cool. Presence of an obstinate foam formation indicates the presence of saponins (Calabria *et al.,* 2008).

3.12. Data management and statistical analysis

The data collected was both qualitative and quantitative. Data generated from antioxidant activity, *in vitro* antiproliferative and *in vivo* acute toxicity assays of the plant extracts was quantitative data. This data was saved in excel spreadsheets, descriptive statistics done and values recorded as Mean ± SEM. Means among treatment were compared using One-way ANOVA. Tukey"s post-hoc test was carried out for pairwise separation and comparison of means. Values were considered significant where P ≤ 0.05. Dose response curve was plotted and used to determined IC_{50}, CC_{50} and LD_{50}. Analysis of the data was done using Minitab statistical software version 17.0. The data was presented in graphs and tables. Data generated from phytochemical screening of the plant extract was qualitative and was tabulated.

CHAPTER FOUR

RESULTS

4.1. The effect of solvents on the yields of *A. indica*, *V. amygdalina* and *G. aparinoides*

Different parts of the three plant species representing three families were extracted with four different solvents yielding a total of 28 extracts. Organic extracts gave yields in the range of 0-2% while aqueous extracts gave yields in the range of 0.6-3.3% (Table 4.1).

Table 4. 1: Plant species and percentage yields of organic and aqueous species

Plant	Part	Extraction Solvent	Weight after extraction	%yield
Azadirachta indica	Leaves	Methanol	1	1
		Ethyl acetate	1	1
		Petroleum Ether	2	2
		Aqueous	2	2
	Rootbark	Methanol	2	2
		Ethyl acetate	1	1
		Petroleum ether	1	1
		Aqueous	1.3	1.3
	Stem bark	Methanol	1	1
		Ethyl acetate	1	1
		Petroleum ether	0.5	0.5
		Aqueous	0.6	0.6
Vernonia amygdalina	Leaves	Methanol	2	2
		Ethyl acetate	1	1
		Petroleum ether	1.1	1.1
		Aqueous	1.3	1.3
	Roots	Methanol	2	2
		Ethyl acetate	0.5	0.5
		Petroleum ether	0.0	0.0
		Aqueous	3.3	3.3
	Stem	Methanol	1	1
		Ethyl acetate	2	2
		Petroleum ether	0.5	0.5
		Aqueous	1.3	1.3
Galium aparanoides	Aerial parts	Methanol	1	1
		Ethyl acetate	1	1
		Petroleum ether	1	1
		Aqueous	1.3	1.3

The percentage yield was calculated as follows:

$$Percentage\ yield = \frac{total\ weight\ of\ the\ extract\ obtained}{total\ weight\ of\ the\ dried\ plant\ material} \times 100$$

4.2. Determination of the antioxidant activity of three plant extracts

4.2.1. *Azadirachta indica*

At the highest concentration of 500 µg/ml, stem bark (SB), root bark (RB) and leaf extracts (L) had inhibition of 94.75 ± 0.16 % , 86.15 ± 1.59 % and 34.49± 0.37% respectively, while at the lowest concentration of 31.25 µg/ml, the extracts had inhibition of 29.42 ± 0.72 %, 15.84 ± 0.22% and 8.52 ± 0.38% respectively (Table 4.2; Appendix 1).

Table 4. 2: Percentage inhibition of DPPH in *A. indica* methanol extracts

Conc (µg/ml)	Stem Bark	Root Bark	Leaves
500	94.75±0.16	86.15±1.59	34.49±0.37
250	87.66±0.34	56.63±0.85	21.40±0.25
125	73.92±1.26	37.57±1.88	14.40±0.23
62.5	45.51±1.28	23.97±0.59	9.53±0.30
31.25	29.42±0.72	15.84±0.22	8.52±0.38

All values are expressed as Mean±SD, Conc= concentration, Negative control=methanol

Table 4. 3: Percentage inhibition of DPPH in *A. indica* ethyl acetate extracts

Conc (µg/ml)	Stem bark	Root bark	Leaves
500	89.28±2.91	50.26±0.73	7.66±0.39
250	63.34±4.77	34.36±0.60	3.55±0.26
125	36.53±0.97	22.76±1.38	1.33±4.71
62.5	22.70±0.18	18.25±1.05	0.60±0.18
31.25	13.61±0.44	12.20±0.70	0.22±0.01

All values are expressed as Mean±SD, Conc= concentration, Negative control=methanol.

At the highest concentration of 500 µg/ml, SB, RB and L extracts exhibited a radical scavenging activity of 33.74±0.29%, 24.77±0.43% and 33.02±0.66% respectively as compared to the lowest concentration of 31.25 µg/ml that showed a radical scavenging activity where SB, RB and leaves inhibited 1.37±0.08% 1.44±0.01% and 10.33±0.73%, respectively (Table 4.4; Appendix 1).

Table 4. 4: Percentage inhibition of DPPH in *A. indica* petroleum ether extracts

Conc (µg/ml)	Stem Bark	Root Bark	Leaves
500	33.74±0.29	24.77±0.43	33.02±0.66
250	15.37±0.37	12.68±0.18	27.22±2.06
125	7.56±0.33	6.69±0.23	21.33±0.73
62.5	3.42±0.12	3.56±0.26	16.52±1.21
31.25	1.37±0.08	1.44±0.01	10.33±0.73

All values are expressed as Mean±SD, Conc= concentration, Negative control=methanol

At the highest concentration of 500 µg/ml, SB, RB and L extracts exhibited a radical scavenging activity of 59.36±0.57%, 81.61±0.56% and 30.52±0.22% respectively as compared to the lowest concentration of 31.25 µg/ml, that showed a radical scavenging activity where the extracts inhibited 9.70±0.39%, 16.27±0.91% and 10.45±0.76% respectively (Table 4.5; Appendix 1).

Table 4. 5: Percentage inhibition of DPPH in *A. indica* aqueous extracts

Conc (µg/ml)	Stem Bark	Root Bark	Leaves
500	59.36±0.57	81.61±0.56	30.52±0.22
250	35.39±0.99	65.98±1.78	25.43±0.12
125	22.98±1.48	48.92±0.49	20.34±0.43
62.5	11.64±0.12	29.89±1.47	19.46±0.23
31.25	9.70±0.39	16.27±0.91	10.45±0.76

All values are expressed as Mean±SD, Conc= concentration, Negative control=methanol

4.2.2. *Vernonia amygdalina*

At the highest concentration of 500 µg/ml, Stem, Root and Leaf extracts exhibited a radical scavenging activity of 33.74±0.29%, 33.02±0.66% and 24.77±0.43% respectively as compared to the lowest concentration of 31.25 µg/ml, that showed a radical scavenging activity where Stem, Root and Leaf extracts inhibited 10.33±0.73%, 1.44±0.01% and 1.37±0.08% respectively (Table 4.6; Appendix 2).

Table 4. 6: Percentage inhibition of DPPH in *V. amygdalina* methanol extracts

Conc(µg/ml)	Stem	Root	Leaves
500	33.74±0.29	33.02±0.66	24.77±0.43
250	24.54±0.13	26.12±1.64	26.20±1.59
125	13.95±1.59	17.61±0.38	21.53±0.79
62.5	1043±1.87	13.19±1.77	19.14±0.6
31.25	10.33±0.73	1.44±0.01	1.37±0.08

All values are expressed as Mean±SD, Conc= concentration, Negative control=methanol

At the highest concentration of 500 µg/ml, Stem, Root and Leaf extracts exhibited a radical scavenging activity of 63.79±0.52%, 40.29±1.94% and 22.54±1.22% respectively as compared to the lowest concentration of 31.25 µg/ml, that showed a radical scavenging activity where Stem, Root and Leaf extracts inhibited 11.34±0.78%, 8.98±1.43% and 8.65±1.76% respectively (Table 4.7; Appendix 2).

Table 4. 7: Percentage inhibition of DPPH in *V. amygdalina* ethyl acetate extracts

Conc (µg/ml)	Stem	Roots	Leaves
500	63.79±0.52	40.29±1.94	22.54±1.22
250	42.28±1.46	25.22±1.22	19.63±1.44
125	21.07±0.38	6.65±1.44	15.77±1..44
62.5	12.64±0.49	11.67±1.65	10.67±1.43
31.25	11.34±0.78	8.65±1.76	8.98±1.43

All values are expressed as Mean±SD, Conc= concentration, Negative control=methanol

At the highest concentration of 500 µg/ml Stem, Root and Leaf extracts exhibited a radical scavenging activity of 30.99±1.34%, 17.98±1.54% and 20.42±1.87% respectively as compared to the lowest concentration of 31.25 µg/ml, that showed a radical scavenging activity where, Stem, Root and Leaf extracts inhibited 6.77±1.71%, 4.55±1.43% and 7.45±1.54% respectively (Table 4.8; Appendix 2).

Table 4. 8: Percentage inhibition of DPPH in *V. amygdalina* petroleum ether extracts

Conc (µg/ml)	Stem	Roots	Leaves
500	30.99±1.34	17.98±1.54	20.42±1.87
250	23.31±1.37	16.76±1.45	12.22±1.43
125	20.54±1.65	14.85±1.74	10.43±1.29
62.5	12.61±1.43	11.65±1.65	8.23±1.86
31.25	6.77±1.71	4.55±1.43	7.45±1.54

All values are expressed as Mean±SD, Conc= concentration, Negative control=methanol

At the highest concentration of 500 µg/ml, Stem, Root and Leaf extracts exhibited a radical scavenging activity of 61.58±1.84%, 43.83±0.36% and 33.22±0.21% respectively as compared to the lowest concentration of 31.25 µg/ml, that showed a radical scavenging activity where, Stem, Root and Leaf extracts inhibited 3.56±0.26%, 4.33±0.45% and 8.54±1.04% respectively (Table 4.9; Appendix 2).

Table 4. 9: Percentage inhibition of DPPH in *V. amygdalina* aqueous extracts

Conc (µg/ml)	Stem	Roots	Leaves
500	61.58±1.84	43.83±0.36	33.22±0.21
250	34.64±1.59	23.49±0.78	29.54±0.99
125	15.68±0.41	13.94±1.05	21.00±0.85
62.5	7.62±0.19	8.47±0.57	11.99±0.53
31.25	3.56±0.26	4.33±0.45	8.54±1.04

All values are expressed as Mean±SD, Conc= concentration, Negative control=methanol

4.2.3. *Galium aparinoides*

At the highest concentration of 500 µg/ml, Aerial parts (AP) methanol, ethyl acetate, petroleum ether and aqueous extracts exhibited a radical scavenging activity of

11.41±0.67%, 7.73±0.19%, 6.01±0.15% and 9.57±0.05% respectively. While at the lowest concentration of31.25 µg/ml, AP methanol, ethyl acetate, petroleum ether and aqueous extracts exhibited a radical scavenging activity of 0.64±0.04%, 0.23±0.05%, 0.21±0.03% and 0.57±0.17% respectively (Table 4.10).

Table 4. 10: Percentage inhibition of DPPH in *G. aparinoides* extracts

Conc (µg/ml)	Methanol	Ethyl acetate	Petroleum	Aqueous
500	11.41±0.67	7.73±0.19	6.01±0.15	9.57±0.05
250	6.12±0.67	3.66±0.15	2.28±0.26	5.48±0.29
125	3.26±0.13	1.59±0.21	0.98±0.09	2.25±0.09
62.5	1.37±0.26	0.83±0.05	0.71±0.01	1.24±0.10
31.25	0.64±0.04	0.23±0.05	0.21±0.03	0.57±0.17

All values are expressed as Mean±SD, Conc= concentration, Negative control=methanol

4.3. IC$_{50}$ results for antioxidant activity of the plant extracts

In this assay, the following criteria was used to rank the activity, ≤ 10 µg/ml is highly active, 100 µg/ml active, 100-500 µg/ml moderately active and > 500 µg/ml inactive. The ICz value represents the concentration of test extracts that reduced 50% of DPPH solution. Ascorbic acid was used as a reference drug and it exhibited an IC$_{50}$ value of 42.74 µg/ml. Table 4.11 shows IC$_{50}$ values recorded for both organic and aqueous crude extracts of *A. indica* and *V. amygdalina* that were quantitatively assessed using DPPH free radical scavenging. Stem bark (SB) and root bark (RB) methanolic extracts, SB ethyl acetate and aqueous RB extracts of *A. indica,* ranked as the top six most active plant extracts, exhibiting strong antioxidant activity with IC$_{50}$ values of 69.31 1.22 µg/ml, 134.9 0.39 µg/ml, 295.11 1.34 µg/ml, 152.46 0.84 µg/ml, 313.53 1.12 µg/ml and 102.87 0.45 µg/ml respectively. Methanol Stem extract of *V. amygdalina* recorded DPPH scavenging activity that had an IC$_{50}$ value of 430.8 µg/ml while the other remaining plant extracts recorded an activity > 500 µg/ml.

Table 4. 11: IC$_{50}$ results for antioxidant activity of the plant extracts

Plant name	Plant part	Solvent used	IC50
Ascorbic acid	Standard	Methanol	42.74
Azadirachta indica	Leaves	Methanol	>500
		Ethyl acetate	>500
		Petroleum Ether	>500
		Aqueous	>500
	Rootbark	Methanol	152.46
		Ethyl acetate	313.53
		Petroleum ether	>500
		Aqueous	102.87
	Stem bark	Methanol	69.31
		Ethyl acetate	134.9
		Petroleum ether	>500
		Aqueous	295.11
Vernonia amygdalina	Leaves	Methanol	>500
		Ethyl acetate	>500
		Petroleum ether	>500
		Aqueous	>500
	Roots	Methanol	>500
		Ethyl acetate	>500
		Petroleum ether	>500
		Aqueous	>500
	Stem	Methanol	430.8
		Ethyl acetate	>500
		Petroleum ether	>500
		Aqueous	>500
Galium aparanoides	Aerial parts	Methanol	>500
		Ethyl acetate	>500
		Petroleum ether	>500
		Aqueous	>500

4.4. Antiproliferative activity of plant extracts against three cancer cell lines

Eight plant extracts that had good antioxidant activity and were further screened for antiproliferative activity. They included; *A. indica* root bark (aqueous, ethyl acetate and methanol), *A. indica* stem bark (methanol, ethylacetate and aqueous), *V. amygdalina* stem parts methanol and *V. amygdalina* leaves aqueous. Plant extracts of *Galium aparanoides* were not tested in antiproliferaive activity and acute toxicity because their antioxidant activity was inactive.

4.4.1. *Azadirachta indica*

4.4.1.1. Stem bark

At the highest concentration of 1000µg/ml, Stem bark (SB) methanol ethylacetate and aqueous extracts of *A. indica* exhibited proliferation rates of 0.20 ± 0.00, 0.23 ± 0.08, and 0.38 ± 0.01 respectively on prostate cancer cell lines (DU 145). The lowest concentration of 0.00 µg/ml, exhibited proliferation rates of 1.00 ± 0.00, 1.00 ± 0.02 and 1.00 ± 0.00 respectively (Table 4.12; Appendix 3).

Table 4. 12: Antiproliferative activity of *A. indica* SB extracts on DU 145 cell line

Conc. µg/ml	Proliferation Rate 560nm		
	Methanolic Extract	Ethyl acetate Extract	Aqueous Extract
Untreated cells	1.00 ± 0.00^a	1.00 ± 0.02^b	1.00 ± 0.00^a
1.37	0.47 ± 0.02^b	0.96 ± 0.02^b	0.93 ± 0.01^b
4.12	0.36 ± 0.02^c	0.80 ± 0.01^{bc}	0.91 ± 0.00^{ab}
12.35	0.34 ± 0.00^{cd}	0.75 ± 0.02^{bc}	0.86 ± 0.04^{ab}
37.04	0.32 ± 0.00^d	0.37 ± 0.01^d	0.66 ± 0.02^{bc}
111.11	0.31 ± 0.00^d	0.33 ± 0.01^d	0.55 ± 0.03^d
333.33	0.26 ± 0.01^d	0.30 ± 0.00^{cd}	0.43 ± 0.02^c
1000.00	0.20 ± 0.00^e	0.23 ± 0.08^a	0.38 ± 0.01^b

All values are expressed as Mean±SD, All values followed by the same superscript are not significantly different (p>0.05), a, b, c and d are superscripts. Conc= concentration, Untreated cells = negative control

While in HeLa cell line, SB methanol, ethylacetate and aqueous extracts of *A. indica* the extracts of exhibited proliferative rates of 0.12 ± 0.02, 0.08 ± 0.11 and 0.22 ± 0.04 respectively at the highest concentration of 1000µg/ml. The lowest concentration of 0.00 µg/ml SB methanol, ethylacetate and aqueous extracts of *A. indica* exhibited proliferative rates of 1.00 ± 0.43, 1.00 ± 0.01 and 1.00 ± 0.01 respectively (Table 4.13; Appendix 4).

Table 4. 13: Antiproliferative activity of *A. indica* SB extracts on Hela cell line

Conc. µg/ml	Proliferation Rate 560nm		
	Methanolic Extract	Ethyl acetate Extract	Aqueous Extract
Untreated cells	1.00 ± 0.43^a	1.00 ± 0.01^a	1.00 ± 0.01^a
1.37	0.60 ± 0.93^{ab}	0.88 ± 0.01^{ab}	0.87 ± 0.00^a
4.12	0.18 ± 0.04^b	0.72 ± 0.02^{ab}	0.68 ± 0.01^b
12.35	0.17 ± 0.03^b	0.55 ± 0.01^{bc}	0.67 ± 0.01^b
37.04	0.17 ± 0.00^b	0.29 ± 0.02^{cd}	0.61 ± 0.02^b
111.11	0.13 ± 0.01^b	0.10 ± 0.08^{cd}	0.42 ± 0.02^c
333.33	0.15 ± 0.00^b	0.10 ± 0.12^d	$0.30 \pm .0.02^d$
1000.00	0.12 ± 0.02^b	0.08 ± 0.11^d	0.22 ± 0.04^d

All values are expressed as Mean±SD, All values followed by the same superscript are not significantly different ($p > 0.05$), a, b, c and d are superscripts. Conc= concentration, Untreated cells = negative control

Lastly, methanol, Ethylacetate and aqueous SB extracts of *A. indica* had an inhibitory activity exhibiting the proliferation rates of 0.30 ± 0.07, 0.33 ± 0.07 and 0.43 ± 0.09 respectively at the highest concentrations of 1000µg/ml as compared to its lowest concentration of 0.00 µg/ml, that exhibited proliferation rates of 1.00 ± 0.05, 1.00 ± 0.02 and 1.00 ± 0.16 respectively on Vero cell line. (Table 4.14; Appendix 5).

In comparison, was significant difference between the untreated cells and the cells treated with the highest concentration (1000µg/ml) of all the plant extract among all the cell lines ($p < 0.05$). It was observed as the concentration of all the extracts of SB *A.*

indica increased the percentage inhibition increased and percentage cell viability decreased (Appendix 6). Similarly, percentage cell inhibition increased and percentage cell viability decreased as the concentration of the extracts increased (Appendix 6).

Table 4. 14: Antiproliferative activity of *A. indica* SB extracts on Vero cell line

Conc. µg/ml	Proliferation Rate	560nm	
	Methanolic Extract	Ethyl acetate Extract	Aqueous Extract
Untreated cells	1.00 ± 0.05^a	1.00 ± 0.02^a	1.00 ± 0.16^a
1.37	0.73 ± 0.03^b	0.81 ± 0.02^a	0.72 ± 0.13^{ab}
12.35	0.62 ± 0.00^{bc}	0.57 ± 0.01^b	0.60 ± 0.02^{ab}
37.04	0.60 ± 0.01^{bc}	0.53 ± 0.00^b	0.59 ± 0.02^{ab}
111.11	0.52 ± 0.01^{bcd}	0.50 ± 0.00^b	0.58 ± 0.02^{ab}
333.33	$0.39\pm0.0.03^{cd}$	0.45 ± 0.05^{bc}	0.54 ± 0.01^{ab}
1000.00	0.30 ± 0.07^d	0.33 ± 0.07^c	0.43 ± 0.09^b

All values are expressed as Mean±SD, All values followed by the same superscript are not significantly different ($p>0.05$), a, b, c and d are superscripts. Conc= concentration, Untreated cells = negative control

4.4.1.2. Root bark

Ethylacetate, methanol and aqueous RB extracts of *A. indica* had the highest antiproliferative activity against DU145 cell line exhibiting proliferation rates of 0.10 ± 0.03, 0.20 ± 0.14 and0.09 ± 0.01 respectively at the highest concentrations of 1000µg/ml as compared to the lowest concentration of 0.00 µg/ml that exhibited proliferation rates of 1.00 ± 0.07, 1.00 ± 0.14 and 1.00 ± 0.00 respectively. (Table 4.15; Appendix 7).

Table 4. 15: Antiproliferative activity of *A. indica* RB extract on DU145 cell line

Conc. µg/ml	Proliferation Rate 560nm		
	Methanolic Extract	Ethyl acetate Extract	Aqueous Extract
Untreated cells	1.00 ± 0.07	1.00 ± 0.14	1.00 ± 0.00
1.37	0.50 ± 0.13^b	0.81 ± 0.04^b	0.72 ± 0.01^b
4.12	0.41 ± 0.05^{bc}	0.78 ± 0.05^b	0.61 ± 0.00^c
12.35	0.35 ± 0.02^{bc}	0.75 ± 0.15^b	0.56 ± 0.03^d
37.04	0.30 ± 0.03^{bc}	0.63 ± 0.06^b	0.49 ± 0.00^e
111.11	0.28 ± 0.05^{bc}	0.48 ± 0.26^b	0.42 ± 0.00^f
333.33	0.19 ± 0.01^c	0.44 ± 0.26^b	0.26 ± 0.00^g
1000.00	0.10 ± 0.03^d	0.20 ± 0.14^b	0.09 ± 0.01^h

All values are expressed as Mean±SD, All values followed by the same superscript are not significantly different ($p > 0.05$), a, b, c and d are superscripts. Conc= concentration, Untreated cells = negative control

While in HeLa cell lines, RB methanol, ethylacetate and aqueous extracts of *A. indica* the extracts of exhibited proliferative rates of 0.12 ± 0.06, 0.41 ± 0.12 and 0.09 ± 0.09 respectively at the highest concentration of 1000µg/ml, as compared to the lowest concentration of 0.00 µg/ml, that inhibited proliferative rates of 1.00 ± 0.01, 1.00 ± 0.22 and 1.00 ± 0.10 respectively (Table 4.16; Appendix 8).

Table 4. 16: Antiproliferative activity of *A. indica* RB extract on Hela cell line

Conc. µg/ml	Proliferation Rate 560nm		
	Methanolic Extract	**Ethyl acetate Extract**	**Aqueous Extract**
Untreated cells	1.00 ± 0.01^a	1.00 ± 0.22^a	1.00 ± 0.10^a
1.37	0.53 ± 0.02^b	0.68 ± 0.18^{ab}	0.89 ± 0.06^{ab}
4.12	0.46 ± 0.02^{bc}	0.67 ± 0.17^{ab}	0.74 ± 0.08^{abc}
12.35	0.39 ± 0.03^{cd}	0.54 ± 0.03^{ab}	0.61 ± 0.07^{bdd}
37.04	0.36 ± 0.02^d	0.53 ± 0.03^{ab}	0.50 ± 0.07^{cde}
111.11	0.21 ± 0.02^e	0.52 ± 0.02^{ab}	0.27 ± 0.05^{def}
333.33	0.18 ± 0.08^{ef}	0.50 ± 0.02^{ab}	0.22 ± 0.07^{ef}
1000.00	0.12 ± 0.06^f	0.41 ± 0.12^b	0.09 ± 0.09^f

All values are expressed as Mean±SD, All values followed by the same superscript are not significantly different ($p>0.05$), a, b, c and d are superscripts. Conc= concentration, Untreated cells = negative control

Lastly, in Vero cell line, all the extracts inhibited proliferation rate. Methanol, Ethylacetate and aqueous RB extracts of *A. indica* had the highest antiproliferative activity exhibiting proliferation rates of 0.31 ± 0.10, 0.42 ± 0.03 and 0.25 ± 0.16 respectively at the highest concentrations of 1000µg/ml while at the lowest concentration of 0.00 µg/ml, the extracts inhibited proliferation rates of 1.00 ± 0.10, 1.00 ± 0.01 and 1.00 ± 0.04 respectively. (Table 4.17; Appendix 9).

In comparison, there was significant difference between the untreated cells and the cells treated with the highest concentration (1000µg/ml) of all the plant extract among all the cell lines ($p<0.05$). Experimentally, as the concentration of all the extracts of RB *A. indica* increased the percentage inhibition increased and percentage cell viability decreased (Appendix 10). On the contrary, percentage cell inhibition increased and percentage cell viability decreased as the concentration of the extracts increased (Appendix 10).

Table 4. 17: Antiproliferative activity of *A. indica* RB extract on Vero cell line

Conc. µg/ml	Proliferation Rate 560nm		
	Methanolic Extract	**Ethyl acetate Extract**	**Aqueous Extract**
Untreated cells	1.00 ± 0.10^a	1.00 ± 0.01^a	1.00 ± 0.04^a
1.37	0.81 ± 0.03^b	0.96 ± 0.03^a	0.73 ± 0.00^{ab}
4.12	0.78 ± 0.04^b	0.88 ± 0.01^{ab}	0.64 ± 0.00^{ab}
12.35	0.76 ± 0.004^b	0.87 ± 0.01^{ab}	0.62 ± 0.00^{ab}
37.04	0.66 ± 0.10^b	0.78 ± 0.01^{ab}	0.63 ± 0.01^{ab}
111.11	0.53 ± 0.18^b	0.69 ± 0.01^{bc}	0.55 ± 0.01^{ab}
333.33	0.45 ± 0.18^b	0.52 ± 0.01^{cd}	0.43 ± 0.01^{ab}
1000.00	0.31 ± 0.10^b	0.42 ± 0.03^d	0.25 ± 0.16^b

All values are expressed as Mean±SD, All values followed by the same superscript are not significantly different (p>0.05), a, b, c and d are superscripts. Conc= concentration, Untreated cells = negative control

4.4.2. *Vernonia amygdalina*

All the extracts inhibited proliferation rate of all the cell lines. Methanol and aqueous leaf extracts of *V. amygdalina* exhibited antiploriferative activity against DU145 cell line demonstrating proliferative rates of 0.15±0.10 and 0.09±0.05 respectively at the highest concentrations of 1000µg/ml as compared to proliferative rates of 1.00 ± 0.03 and 1.00 ± 0.00 at the lowest concentration of 0.00 µg/ml (Table 4.18; Appendix 11).

Table 4. 18: Antiproliferative activity of *V. amygdalina* Leaf extract on DU 145 cell line

Concentration µg/ml	Proliferation Rate 560nm	
	Methanolic Extract	**Aqueous Extract**
Untreated cells	1.00 ± 0.03^a	1.00 ± 0.00^a
1.37	0.57 ± 0.02^a	0.70 ± 0.01^a
4.12	0.28 ± 0.01^b	0.45 ± 0.01^a
12.35	0.28 ± 0.01^b	0.41 ± 0.01^a
37.04	0.28 ± 0.00^b	0.38 ± 0.02^a
11.11	0.27 ± 0.00^b	0.30 ± 0.02^a
333.33	0.23 ± 0.07^{bc}	0.17 ± 0.00^a
1000.00	0.15 ± 0.10^c	0.09 ± 0.05^b

All values are expressed as Mean±SD, All values followed by the same superscript are not significantly different ($p>0.05$), a, b, c and d are superscripts. Conc= concentration, Untreated cells = negative control

While Hela cell line, all the extracts inhibited proliferation rate. Methanol and aqueous leafextracts of *V. amygdalina* showed antiploriferative activity exhibiting proliferative rates of0.270.04 and 0.17±0.11 respectively at the highest concentrations of 1000µg/ml and at thelowest concentration of 0.00 µg/ml the extracts inhibited proliferation rates of 1.00 ± 0.0and 1.00 ±0.02 respectively (Table 4.19; Appendix 11).

Table 4. 19: Antiproliferative activity of *V. amygdalina* Leaf extracts on Hela cell line

Concentration	Proliferation Rate 560nm	
	Methanolic Extract	**Aqueous Extract**
Untreated cells	1.00 ± 0.09^a	1.00 ± 0.02^a
1.37	0.95 ± 0.10^a	0.70 ± 0.20^a
4.12	0.94 ± 0.09^a	0.57 ± 0.27^a
12.35	0.91 ± 0.08^{ab}	0.39 ± 0.21^{ab}
37.04	0.87 ± 0.09^{ab}	0.27 ± 0.16^a
111.11	0.79 ± 0.09^{ab}	0.28 ± 0.15^{ab}
333.33	0.42 ± 0.12^{ab}	0.23 ± 0.15^{ab}
1000.00	0.27 ± 0.04^b	0.17 ± 0.11^b

All values are expressed as Mean±SD, All values followed by the same superscript are not significantly different ($p>0.05$), a, b, c and d are superscripts. Conc= concentration, Untreated cells = negative control

Lastly, in Vero cell line, all the extracts inhibited proliferation rate. Methanol and aqueous leaf extracts of *V. amygdalina* had proliferative rates of 0.42 ± 0.03 and 0.40 ± 0.03 respectively at the highest concentrations of 1000µg/ml as compared to the lowest concentration of 0.00 µg/ml the inhibited proliferative rates of 1.00 ± 0.01 and 1.00 ± 0.01 respectively (Table 4.20; Appendix 12).

In comparison, significant difference between the untreated cells and the cells treated with the highest concentration (1000µg/ml) of all the plant extract among all the cell lines ($p<0.005$). As the concentration of all the extracts of root bark of *V. amygdalina* increased the percentage inhibition increased and percentage cell viability decreased (Appendix 13). On the contrary, percentage cell inhibition increased and percentage cell viability decreased as the concentration of the extracts increased (Appendix 13).

Table 4. 20: Antiproliferative activity of _V. amygdalina_ Leaf extracts on Vero cell line

Concentration µg/ml	Proliferation Rate 560nm	
	Methanolic Extract	Aqueous Extract
Untreated cells	1.00 ± 0.01^a	1.00 ± 0.01^a
1.37	0.84 ± 0.03^a	0.96 ± 0.03^a
4.12	0.76 ± 0.01^{ab}	0.88 ± 0.01^{ab}
12.35	0.76 ± 0.01^{ab}	0.78 ± 0.01^{ab}
37.04	0.70 ± 0.06^{ab}	0.78 ± 0.06^{ab}
111.11	0.64 ± 0.01^{bc}	0.69 ± 0.01^{bc}
333.33	0.47 ± 0.01^{cd}	0.52 ± 0.01^{cd}
1000.00	0.42 ± 0.03^d	0.40 ± 0.03^d

All values are expressed as Mean±SD, All values followed by the same superscript are not significantly different ($p>0.05$), a, b, c and d are superscripts. Conc= concentration, Untreated cells = negative control

4.5. _In vitro_ cytotoxicity studies of the plant extracts

Eight plant extracts that had a good antioxidant activity were screened for antiproliferative activity. Cytotoxicity of the plant extracts was tested in Vero cell line. Aqueous extract of _A. indica_ RB exhibited the lowest Cytotoxic concentration (CC_{50}) value of 135.34 ± 0.47 µg/ml while aqueous leaves extract of _V. amygdalina_ exhibited the highest CC_{50} value of > 1000 µg/ml as shown in table 4.21. In Hela cell lines _A. indica_ methanol extract of SB had the highest selectivity index of 436.52. This result is very promising in the search of safe anticancer drugs from plants. _A. indica_ ethylacetate RB showed the lowest selectivity index of 1.26 in Hela cell line. This implies that the extract may also cause harm to normal cells. In DU 145 cell line, _A. indica_ ethyl acetate RB extract had the lowest selectivity index of 1.21 while _A. indica_ RB methanol extract had the highest selectivity index of 39.60.

V. amygdalina methanolic stem extract had a selectivity index of 2.29 and *V. amygdalina* aqueous leaves extract reported a selectivity index of 1.72 in Hela cell line. In DU145,*V.amygdalina* methanolic stem extract recorded a SI of 2.25 whereas *V. amygdalina* aqueous leaves extract recorded a SI value of 1.23.Therefore, *A. indica* ethyl acetate RB extract had the lowest SI values in both Hela and DU145 cell lines hence considered non-selective.

V. amygdalina methanolic stem extract and *V. amygdalina* aqueous leaves reported low SI value (< 3) in both Hela and DU145 cell lines hence recognized as non-selective. *A. indica* methanol extract of SB and *A. indica* RB methanol extract had high SI value (>3) in Hela and DU145 cell lines hence defined as selective to cancerous cell lines.

Table 4. 21: Antiproliferative activity against Hela, DU 145 and Vero cells lines

Plant extracts	Hela		DU 145		Vero
	IC_{50}(µg/ml)	SI	IC_{50}(µg/ml)	SI	CC_{50}(µg/ml)
A. indica root bark aqueous	24.3± 0.14	5.6	51.39 ± 0.42	19.46	135.34 ±0.47
A. indica root bark ethylacetate	328.87±1.32	1.26	483.65± 0.58	1.21	480.11±0.28
A. indica root bark methanol	2.59±0.29	135.14	3.26 ± 0.28	39.6	350.02 ±0.13
A. indica stem bark methanol	1.85±0.10	436.52	1.53 ± 0.07	35.94	807.56 ± 0.76
A. indica stem bark ethyl acetate	39.00±0.52	12.72	24.75 ± 0.61	8.31	496.38±0.12
A. indica stem bark aqueous	125.45±1.35	4.8	287.7 ± 0.47	1.49	605.52±0.24
V. amygdalina stem parts methanol	430.67±1.17	2.29	301.87± 0.93	2.25	987.33±0.18
V. amygdalina leaves aqueous	583.09±2.39	1.72	812.72 ±1.15	1.23	> 1000
5-Fluorouracil	2.04 ± 0.87	7.35	5.06± 0.28	3	15 ± 0.12

All values are expressed as Mean ± SD, untreated cells= negative control, 5-Floro uracil=Positive control

4.6. Determination of the synergistic antiproliferative activity of combined extracts

In this study, *A. indica* ethylacetate SB extract and *A. indica* methanol RB extract that exhibited mono-antiproliferative activity were investigated for antiproliferative activity when in combination or a standard drug, 5-fluorouracil. One component was kept constant while the other was varied and vice versa (5-FU). The results of the combination study were presented in Table 4.22.

Combined Inhibitory concentration of *A. indica* plant extracts with a standard drug 5-Fluoro uracil were tested and this was confined to combinations of two extracts. Table 4.22 shows the IC_{50} values (µg/ml) of two extracts in combination (a+b) when extract a is kept constant while extract b is the variable component in prostate cancer cell line (DU 145). For example, in the case of SB+ RB, SB= is the constant component (a) while RB= is the variable component (b).At 50%/ 50%, SB+ SD exhibited a higher inhibitory concentration of 2.22 µg/ml as compared to the combination of RB+SB that displayed an inhibitory activity of 58.03 µg/ml. (A higher concentration of RB+SB combinants was required to achieve the same antiproliferative activity as SB+SD (2.22) at 50%/50%). At 50%/25%, SB+ SD exhibited a higher inhibitory concentration of 2.33 µg/ml as compared to the combination of RB+SB that showed an inhibitory activity of 58.22 µg/ml (A higher concentration of RB+SB combinants was required to achieve the same antiproliferative activity as SB+SD (2.33) at 50%/25%). while at 50%+5%, SB+ SD exhibited a higher inhibitory concentration of 5.54 µg/ml as compared to the combination of RB+SB that displayed an inhibitory activity of 58.83 µg/ml. (A higher concentration of RB+SB combinants was required to achieve the same antiproliferative activity as SB+SD (5.54) at 50%/5%). Generally, SB+ SD had a highest growth inhibition while RB+SB had a lowest growth inhibition at all combinations.

Table 4. 22: IC$_{50}$ values for *A. indica* and 5-Fluorouracil in combination with DU 145

Combination of extract a + extract b	50% a + 50% b	50% a + 25% b	50% a + 5% b
SB + RB	5.23	5.88	7 .02.
SB + SD	2.22	2.33	5.54
RB + SB	58.03	58.22	58.83
RB+ SD	7.34	7.56	7.95
SD+ SB	3.23	3.22	5.81
SD+ RB	6.22	6.44	6.11

Key: SB= ethylacetate stem bark RB= Methanol root bark SD= Standard drug (5-fluorouracil)

Table 4.23 shows the IC$_{50}$ values (µg/ml) of two extracts in combination (a+b) when extract a is kept constant while extract b is varied and vice versa in cervical cancer cell line (Hela). At 50%/ 50%, SB+ SD exhibited a higher inhibitory concentration of 1.22 µg/ml as compared to the combination of RB+SB that exhibited 45.22 µg/ml. At 50%/25%, SB+ SD exhibited a higher inhibitory concentration of 1.31 µg/ml as compared to the combination of RB+SB that displayed 45.75µg/ml and at 50%+5%, SB+ SD exhibited a higher inhibitory concentration of 6.43 µg/ml as compared to the combination of RB+SB that inhibited 45.98 µg/ml. In general, SB+ SD had a highest growth inhibition while RB+SB had a lowest growth inhibition at all combinations.

Table 4. 23: IC$_{50}$ values for *A. indica* and 5-Fluorouracil in combination with Hela

Combination of extract a + extract b	50% a + 50% b	50% a + 25% b	50% a + 5% b
SB+ RB	2.34	2.65	5.25
SB+ SD	1.22	1.31	6.43
RB+SB	45.22	45.75	45.98
RB+ SD	5.44	5.65	5.88
SD+ SB	1.43	1.56	8.71
SD + RB	4.19	4.22	4.37

Key: SB= ethylacetate stem bark RB= Methanol root bark SD= Standard drug (5-fluorouracil)

On HeLa cell lines, SB+SD had a highest inhibition value of 1.09±0.24 µg/ml in comparison to SB+RB that had the lowest inhibition value of 2.50±0.13 µg/ml while on DU145 cell lines, SD+SB showed the highest inhibition value of 2.04±0.07 µg/ml as compared to SB+RB that exhibited the lowest inhibition value of 4.55±0.41 µg/ml (Table 4.24).

Table 4. 24: *In vitro* cytotoxic concentration on different cell lines

Extract a+b Each at 50µg/ml	Hela IC$_{50}$ (µg/ml)	DU145 IC$_{50}$ (µg/ml)	L6 CC$_{50}$ (µg/ml)	SI (CC$_{50}$/IC$_{50}$)	
SB+RB	2.50±0.13	4.55±0.41	245.09	160.13	94.63
SB+SD	1.81±0.33	2.83±0.44	212.15	138.66	103.99
SD+SB	1.09±0.24	2.04±0.07	190.24	37.59	102.83

Where SB= ethylacetate stem bark RB= Methanol root bark SD= Standard drug (5 fluorouracil)

Further the fractional inhibitory concentration (FIC) was calculated to ascertain their relationships. On HeLa cell lines, SB+RB exhibited the highest with a FIC value of 0.89 as compared to SB+SD that had a lowest FIC value of 0.40, while on DU145,

SB+RB exhibited the highest with a FIC value of 1.23 as compared to SD+SB that showed the lowest FIC value of 0.53 (Table 4.25).

Table 4. 25: Fractional inhibitory combination of the selected plant extracts

Combination a+b	IC_{50} of (a) in combination	FIC (Ac/Aa)	IC_{50} of (b) in combination	FIC (Bc/Ba)
SB+RB	4.55	0.89	2.50	1.23
SB+SD	2 .83	0.55	1.81	0.88
SD+SB	2.24	0.403	1.29	0.534

Where SB= ethylacetate stem bark RB= Methanol root bark SD= Standard drug (5-fluorouracil).

4.7. Investigation of the acute toxicity of the bioactive extracts in a mouse model

Table 4.26 shows the body weights of mice before and after drug administration at 300 mg/kg/bw and 2000mg/kg/bw concentrations. It was noted that there was an increase in the body weight of mice treated with the ethylacetate extract of *A. indica* SB at both doses and a decrease in the body weight of mice treated with *A. indica* methanol extracts of RB and SB at both doses.

Table 4. 26: Acute toxicity of *A. indica* extracts in the swiss albino mice model

Treatment	BODY WEGHT (gms)		
300mg/kg/bw	Before drug administration	After drug administration	Mortality
Negative controls	18.93± 0.47	23.49± 0.79	0/3
A. indica (stem bark) EoAt	20.00± 0.34	24.77±0.43	0/3
A. indica (Root bark) MeoH	18.25±0.47	16.00±1.05	0/3
A. indica (stem bark) MeoH	19.00±0.44	17.35± 1.36	0/3
2000mg/kg/bw			
Negative controls	18.33±0.47	22.00±1.63	0/3
A. indica (stem bark) EoAt	20.00±0.82	25.3±1.25	0/3
A. indica (Root bark) MeoH	19.33±0.47	17.00±0.812	0/3
A. indica (stem bark) MeoH	18.66±0.47	17.33±0.94	3/3

All values are expressed as Mean± SD, n=3 kg=Kilogram, gms=grams, bw=body weight, MeoH = methanol, EoAt= ethylacetate, negative control=phosphate bovine serum (PBS).

4.8. Qualitative phytochemical screening

In the current study all the plants revealed the presence of phyto-components with all the plants showing flavonoids as the most prominent as shown in table 4.27. *A. indica* and *V. amygdalina* exhibited almost the same compounds but glycosides and saponins were absent in *G. aparinoides.*

Table 4. 27: Qualitative phytochemistry

Classes of compounds	Alkaloids	Phenolics	Terpenoids	Steroids	Flavanoids	Gycosides	Tannins	Saponins
A. indica	+	+	+	_	+	+	+	+
V. amygdalina	+	+	+	_	+	+	+	+
G. aparinoides	+	+	+	_	+	_	+	_

+ is Present   - is absent

CHAPTER FIVE

DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1.1. IC$_{50}$ Values for antioxidant activity of the plant extracts

In this assay, the following criteria was used to rank the activity; IC$_{50}$ ≤ 10 µg/ml is highly active, IC$_{50}$ value of 10-150 µg/ml active, IC$_{50}$ value of 150-500 µg/ml moderately active and IC$_{50}$ > 500 µg/ml inactive. The IC$_{50}$ value represents the concentration of test extracts that reduced 50% of DPPH solution.

In the current study, *A.indica* extracts exhibited the best antioxidant activity as compared to *V. amygdalina* plant extracts that had moderate activity and *G. aparinoides* plant extracts that showed inactive results. The standard drug, ascorbic acid had an antioxidant activity that was higher than that of the test extracts. It was dissolved in methanol as documented in other studies (Kaneria *et al.,* 2012). Compared to the other solvents used, the solubility of methanol was higher than that of ethyl acetate and petroleum ether.

Previous studies have demonstrated antioxidant activity of various medicinal plants. (Pandey *et al.,* 2014) demonstrated antioxidant activity of ethanolic leaf extract of *A. indica* reporting an IC$_{50}$ value of 110.36μg/ml. While the antioxidant activity of *A. indica* flowers and seed oil ethanolic extracts has been carried out and observations reported that they had a free radical scavenging activity with 64.17 ± 0.02% and 66.34 ± 0.06% respectively (Nahak & Sahu, 2011). Studies have also revealed that the aqueous leaf and ethanolic flower extracts from Siamese neem tree were screened for antioxidant activity and they exhibited IC$_{50}$ values of 26.5 µg/ml and 27.9 µg/ml respectively (Sithisarn *et al.,* 2005).

Ocimum basillcum, *Cassia acutifolia* and *Beta vulgaris* ethanolic extracts have been reported to exhibit antioxidant activity with IC$_{50}$ values of 0.19 µg/ml, 0.59 µg/ml and 0.48 µg/ml respectively (Koksal *et al.,* 2011; Nassr-Allah *et al.,* 2009) reported that Aqueous and ethanolic leaf extracts of *Melissa officinalis* were evaluated for their

radical scavenging activities and they gave IC$_{50}$ values of 31.4 μg/ml and 202.7 μg/ml respectively.

Ethylacetate, acetone and aqueous leaf extract of *Manikara zapota* showed strong activity on scavenging DPPH radical, exhibiting IC$_{50}$ values 22μg/ml, 7.6μg/ml and 42.2μg/ml respectively which involves a vital resistance against the free radicals (Kaneria *et al.,* 2012). (Ayoola *et al.,* 2008) reported that ethanolic leave extracts of *Carica papaya, Psidium guajava*, *Vernonia amygdalina* and stem bark extract of *Mangifera indica* were screened for free radical

scavenging activity and their IC$_{50}$ values recorded as 0.58mg/ml, 0.040mg/ml, 0.313mg/ml, 2.30mg/ml and 0.58mg/ml respectively where *P. guajava* was found to be the most potent.Even though very little antioxidant activity was found in *G. aparinoides,* antioxidant activity has been reported on other *Galium* species namely; *G. aparine, G. verum*, *G.mullugo and G. odoratum* (Vlase *et al.,* 2014). Generally the results conclude that medicinal plants have an impending source of antioxidants of natural origin.

.1.2. Determination of antiproliferative activity

The extracts that displayed a high antioxidant activity were further tested for antiproliferative activity against Hela, DU145 and Vero cell lines (*A. indica* root bark (aqueous, ethyl acetate and methanol), *A. indica* stem bark (methanol, ethylacetate and aqueous), *V. amygdalina* stem parts methanol and *V. amygdalina* leaves aqueous). An extract is defined to be highly active if it has IC$_{50}$ < 10 μg/ml, active when IC$_{50}$ is between 10 μg/ml and 100 μg/ml, moderately active if the IC$_{50}$ is between 100μg/ml and 500μg/ml and low activity if the IC$_{50}$ is > 500 μg/ml (Webb *et al.,* 2007).

Generally, *A. indica* SB and RB methanolic extracts showed antiproliferative activity in all the cell lines. It has been reported before that the anticarcinogenic activity of the extracts from the leaves of *A. indica* is due to its major production of an antioxidant, glutathione that is a carcinogen detoxifying enzyme and other valuable active compounds such as azadirachtin (Rajkumar *et al.,* 2011). In a recent study, methanol and aqueous neem leaf extracts were determined to inhibit growth of Hela cell lines

with IC_{50} values of 7 µg/ml and 22 µg/ml respectively (Shukla *et al.,* 2016). The methanolic extracts from the SB and RB of the Kenyan species have demonstrated more than 2-6 fold improved activity against prostate and cervical cancer cell lines (Prashanth & Krishnaiah, 2014). This demonstrates that *A. indica* SB and RB can be used for the development of locally affordable and effective alternative to synthetic drugs. Generally, there was significant difference between the untreated cells (negative controls) and the cells treated with the highest concentration (1000µg/ml) of all the plant extract among all the cell lines ($p<0.005$). As the concentration of all the extracts increased, the percentage inhibition increased and percentage cell viability decreased. On the contrary, percentage cell inhibition increased and percentage cell viability decreased as the concentration of the extracts increased.

Methanolic stem part of *V. amygdalina* and the aqueous leaves extracts had minimal antiproliferative activity against all cell lines. However, it has previously been reported that *V. amygdalina* aqueous leaf extracts suppressed breast cancer cells growth at 100 µg/ml (Kupchan *et al.,* 1969). Most available reports of anticancer potential of *V. amygdalina* are against breast cancer cell lines but little has been reported on cervical and prostate cancer cell lines. *V. amygdalina* has also been reported to have two active lactones, vernodalin and vernomygdin that have cytotoxic activities (Nabende & Namukhosi, 2015).

5.1.3. *In vitro* cytotoxicity effects of the plant extracts

In vitro cytotoxicity of the plant extracts was tested in Vero cell line. Generally aqueous extract of *A. indica* RB exhibited the lowest CC_{50} value, while aqueous leaves extract of *V. amygdalina* exhibited the highest CC_{50} value. The CC_{50} were useful in determination of selectivity index ($SI = CC_{50} / IC_{50}$) a value that indicates how selective the extract is to the test cell lines. An extract is defined as highly selective if the $SI > 3$ and less harmful to normal healthy cell lines while an extract with low selectivity index of $SI < 3$ is defined as less selective between cancer and normal healthy cell lines hence more toxic (Nabende & Namukhosi, 2015).

In Hela cell lines *A. indica* methanol extract of SB demonstrated selectivity with an index value that is very promising in the search of a safe anticancer drugs from plants.

A. indica ethyl acetate RB was not selective and this implies that the extract may also cause harm to normal cells. Similarly, in DU 145 cell line, *A. indica* RB methanol extract was selective while ethyl acetate RB extract was not selective.

In Hela cell lines *V. amygdalina* methanolic stem and aqueous leaves extracts were not selective. Likewise, in DU145 cell lines *V. amygdalina* methanolic stem and aqueous leaves extracts did not show any selectivity.

5.1.4. Combined antiproliferative activity of extracts and 5-fluorouracil

Chemotherapy has undergone a steady transition from a mono-remedy to multidrug treatment. Interactions of chemical components within the drug combinants may improve efficacy and combinations that have been well formulated tend to enrich synergy (Vlase *et al.,* 2014). Generally, synergy is defined as the interaction of two or more agents to produce a combined effect greater than the sum of their individual effects (Van Vuuren & Viljoen, 2011).

In medicinal research field , however pharmacodynamics synergy is where more agents working on the same receptors or biological targets results in enhanced therapeutic outcomes through there positive interactions (Spinella, 2002). Studies have shown that a combination of botanical and chemical agents can reveal enriched efficacies with fewer side effects (Mbatchi *et al.,* 2006).

In this study, ethyl acetate SB extract and methanol RB extract that exhibited mono-antiproliferative activity were investigated for antiproliferative activity when in combination or with a standard drug, 5-FU a fluorinated analog of uracil, mostly used in treatment of various tumors including gastrointestinal and head and neck carcinomas.

Some combinations such as those in RB + SB, RB + SD and SD + RB did not show any significant difference in their activity. However an enhanced activity was observed between SB + RB, SB + SD and SD + SB. It has been shown that some of these components may have acted synergistically (Meletiadis *et al.,* 2010).

In most cases, increased activity was noted whenever ethylacetate SB extract was one of the component used in combination. The strongest activity was recorded in combinations of the SD and SB ethylacetate extracts.

The three combinations of SB+RB, SB+ SD and SD+SB showed promising anticancer activity were selected and tested against L6 cell line. There was no significant difference and the combinations were not cytotoxic to L6 cell lines at the concentrations tested.

In order to ascertain the relationships between the selected combinations, their FIC were assessed as described by (Hall *et al.,* 1983). When the FIC value is less than 1 this indicates synergism, when its equals to 1 it indicates additivity and when it's greater than 1 it indicates antagonism (Wagner, 2011). The combinations between the SD and SB ethylacetate extracts, whose activity was enhanced in the experiment, showed an FIC value less than 1 against both Hela and DU-145 cell lines. In this study, all the combinations showed a synergistic effect except combination between SB ethylacetate extract and RB ethylacetate extract on DU145 cell line that showed an antagonist effect. Generally, the antiproliferative activity of the combinations recorded against HeLa cell lines was higher than those of DU145 cell line in all the combinations.

Previously, a study conducted on the combinational use of *Taxus cuspidata* extract with 5-FU displayed a strong synergistic activity with combination index values ranging from 0.93 µg/ml to 0.13 µg/ml at different effect levels from IC_{40} to IC_{90} in PC-3M-1E8 prostate cancer cells with a combination index values ranging from 0.26 µg/ml to 0.90 µg/ml for IC_{50} to IC_{90} in MCF-7 breast cancer cells (Shang *et al.,* 2011).

Sharma *et al.* (2014) reported that a combination of ethanolic leaf extracts of *A. indica* with cisplatin demonstrated a synergistic interaction with a combination index <1 for both MCF-7 and Hela cell line. Muddukrishnaiah & Singh, (2015) reported that *A. indica* leaf extract and

W. somnifera Rhizome exhibited a very good synergistic activity against multidrug resistant strain. Therefore, combinations of chemo preventive drugs and

chemotherapeutic drugs may have immense prospects for the development of therapeutic approaches to overcome resistance and side effects brought about by chemotherapy in human cancers.

5.1.5. Determination of acute oral toxicity for the plant extracts in Swiss albino mice

Acute toxicity is a safety parameter used in toxicological analysis of herbal medicine. Toxicological evaluation is carried out in experimental animals to foretell its toxicity and to provide guidelines for selecting a safe dose in humans (Raghavendra & Reddy, 2011). Even though medicinal plants are known and used in management of several diseases in humans, very little is known about their toxicity. Safety should be an overriding criterion in the selection of medicinal plants for use in the healthcare systems (Latha *et al.,* 2010).

In this study, the methanolic SB, methanolic RB and ethyl acetate SB extracts revealed high antiproliferative activity and were therefore investigated for acute toxicity studies. Body weights of mice before and after drug administration at 300 mg/kg/bw and 2000mg/kg/bw concentrations were determined. It was noted that there was an increase in the body weight of mice treated with SB ethylacetate extract at both doses and a decrease in the body weight of mice treated with methanol extracts of RB and SB at both doses.

No mortality was reported in experimental mice when the three extracts were administered orally at a dose of 300mg/kg/bw but at higher doses of 2000 mg/kg/bw, the methanol SB extract led to mortality. The study showed that at 2000mg/kg/bw of methanol RB and ethylacetate SB extract were safe. Methanol RB and ethyl acetate SB extracts had no significant changes observed in behavior neither did they show any general appearance of distress during the 14-day study period. Body weight gain was recorded, food intake and water consumption was found to be normal during that period. Even though groups of mice treated with methanol RB and ethylacetate SB extract showed decreased in body weight after oral administration of the drug, these changes were found to be insignificant, indicating that the administration of the drug

to the mice did not possess any toxic effects on the growth of the animal. This results were found to have LD_{50} values ≥ 300 mg/kg/bw, but ≤ 2000 mg/kg/bw.

The design and observations from the acute oral toxicity assay were based on OECD protocol, (2005). LD_{50} value is the dose at which 50% of the test population is dead. The LD_{50} value of the methanol extract of SB was more than 300 mg/kg but less than 2000 mg/kg/bw. The routine and insignificant variations in wellness parameters in body weight shows the safety of methanol extract of RB at a dose of 300 mg/kg body weight. Methanol RB and ethyl acetate SB extract recorded no mortality at the highest concentration of 2000 mg/kg/bw. Therefore, there LD_{50} was more than 2000 mg/kg/bw.

Other studies shows that the LD_{50} value ethanol extract of neem leaf in mice was 4.57g/kg/bw. (Kupradinun *et al.,* 2010; Subapriya & Nagini, 2003) revealed that the aqueous extracts of neem leaves was not toxic to mice upto the dose of 1g/kg/bw. Okpanyi & Ezeukwu (1981) also reported that the LD_{50} value of ethanol extract of neem bark and leaf in mice was about 13g/kg/bw.

Mokkhasmit *et al.,* (1971) also reported, by subcutaneous injection at a dose of 1000 mg/kg the neem leaf caused no toxicities to the mice. (Hosseinzadeh, *et al.,* 2000) reported no mortality of albino mice for the ethanolic extracts of *A. indica* at a dose of 2000 mg/kg.

Prasanna Krishna (2011) reported, ethanol extract of *Ficus glomerata L.* assessed by OECD guidelines No.423, was found to be non-toxic in rats when administered orally in doses up to 2000 mg/kg/bw. Mir *et al.,* (2013) reported that methanolic extracts from *Tridex procumbens* evaluated by OECD guidelines 2005, was found to have LD_{50} values ≥ 300 mg/kg/bw, but < 2000 mg/kg bw.

5.1.6. Phytochemicals

In the current study, phytochemical screening was performed to confirm the antioxidant and anticancer properties of *A. indica,* the phyto-components revealed. The results are almost in agreement with the results published in other research studies (Koleva *et al.,* 2002). Flavonoids which were components of *A. indica* extracts have

been shown to possess antioxidant, anti-proliferative, anti-inflammatory, anti-allergic and antimicrobial activities (Pietta, 2000 and Edeoga *et al.,* 2005). Therefore the antiproliferative and antioxidant activities of the plant extracts could be attributed to the phytochemicals present in the extracts. Other studies shows that the presence of flavonoid by phytochemical evaluation of *A. indica* may be accounted for its antioxidant activity (Siddhuraju *et al.,* 2002). Flavanoids isolated from medicinal plants, such as *Enhydra fluctuans* exhibited anticancer activitity against Ehlrich"s ascites carcinoma (EAC) (Sannigrahi *et al.,* 2010).

Generally, phenolic and flavonoid content have been shown to contribute significantly to antioxidant activity (Mahmood *et al.,* 2011). Tannins have also been reported to be useful in the treatment of inflamed tissues and they have a remarkable activity in cancer prevention (Ruch *et al.,* 1989).

Nine triterpene, a saponin isolated from the leaves and stem of *Silphuim radula* was found to be cytotoxic against human breast cancer cell line MDA-MB-231 (Calabria *et al.,* 2008). Alkaloid being one of the present phytocomponent in the current study, has also been one of the most active component in natural herbs and some of its compounds have been developed into chemotherapeutic drugs such as camptothecin (Huang *et al.,* 2007). Generrally, high free radical scavenging activity of *A. indica* barks is due to their possession of high phenolic content (Siddhuraju *et al.,* 2002).

5.2. Conclusion

- The present study demonstrated antioxidant activity of *A. indica, V. amygdalina* and *G.aparinoides*
- The study showed significant antiploriferative activity on *A. indica* and *V. amygdalina.*
- Antiproliferative activity of combined extracts of *A. indica* was higher in cervical cancer cell lines as compared to prostate cancer cell lines.
- In acute toxicity studies *A. indica* plant extracts were safe at 300mg/kg/bw.
- The classes phytochemicals present in *A. indica, V. amygdalina* and *G.aparinoides* have been known to possess antioxidant and antiproliferative activity.

5.3. Recommendations

Following the identification, extraction and testing of methanol, ethylacetate, petroleum ether and aqueous extracts of *A. indica, V. amygdalina* and *G.aparinoides*, it has demonstrated that methanol extracts of *A. indica* exhibited numerable antioxidant, antiploriferative and safety in mice. It is therefore recommended that:

- This experiment should be carried out using the murine models of prostate and cervical cancer.
- A detailed bioassay guided fractionation and identification of bioactive compounds should be carried out.

REFERENCES

Adebayo, O. L., James, A., Kasim, S. B. and Jagri, O. P. (2014). Leaf Extracts of *Vernonia amygdalina Del.* from Northern Ghana Contain Bioactive Agents that Inhibit the Growth of Some Beta-lactamase Producing Bacteria *in vitro. British Journal of Pharmaceutical Research, 4*, 192-202.

Adesanoye, O. A. and Farombi, E. O. (2010). Hepatoprotective effects of *Vernonia amygdalina* (astereaceae) in rats treated with carbon tetrachloride. *Experimental and Toxicologic Pathology. 62*(2), 197-206.

Aiyelaagbe, O. and Osamudiamen, P. M. (2009). Phytochemical screening for active compounds in *Mangifera indica* leaves from Ibadan, Oyo State. *Plant Science Research. 2*(1), 11-13.

Altemimi, A., Lakhssassi, N., Baharlouei, A., Watson, D. G. and Lightfoot, D. A. (2017). Phytochemicals: Extraction, Isolation, and Identification of Bioactive Compounds from Plant Extracts. *Plants. 6*(4), 42

Alsbeih, G. (2014). HPV Infection in Cervical and Other Cancers in Saudi Arabia:Implication for Prevention and Vaccination. *Front Oncology. 4,* 65.

Atakan, Z. (2012). Cannabis, a complex plant: different compounds and different effects on individuals. *Therapeutic Advances in Psychopharmacology. 2*(6), 241-254.

Ayoola, G. *et al.,*(2008). Phytochemical screening and antioxidant activities of some selected medicinal plants used for malaria therapy in Southwestern Nigeria. *Tropical Journal of Pharmaceutical Research. 7*(3), 1019-1024.

Baker, S. G. (2014). A cancer theory kerfuffle can lead to new lines of research. *Journal of the National Cancer Institute. 107*(2), 405

Balunas, M. J. and Kinghorn, A. D. (2005). Drug discovery from medicinal plants. *Life Sciences. 78*(5), 431-441

Berenbaum, M. (1978). A method for testing for synergy with any number of agents.

Journal of Infectious Diseases. 137(2), 122-130.

Biswas, K., Chattopadhyay, I., Banerjee, R. K. and Bandyopadhyay, U. (2002). Biological activities and medicinal properties of neem (*Azadirachta indica*). *Current Science-Bangalore. 82*(11), 1336-1345.

Boohaker, J. R., Lee, W. M., Vishnubhotla, P., Perez, L. M. J. and Khaled, A. (2012).The use of therapeutic peptides to target and to kill cancer cells. *Current Medicinal Chemistry. 19*(22), 3794-3804.

Boon, H., Stewart, M., Kennard, M. A., Gray, R., Sawka, C., Brown, J. B. and Aaron, D.(2000). Use of complementary/alternative medicine by breast cancer survivors in Ontario: prevalence and perceptions. *Journal of Clinical Oncology. 18*(13), 2515-2521.

Bott, J. (2007). An analysis of paper-based sources of information on complementary therapies. *Complementary Therapies in Clinical Practice. 13*(1), 53-62.

Brand-Williams, W., Cuvelier, M. E. and Berset, C. (1995). Use of a free radical method to evaluate antioxidant activity. *LWT-Food Science and Technology. 28*(1), 25-30.

Bray, F., Jemal, A., Grey, N., Ferlay, J. and Forman, D. (2012). Global cancer transitions according to the Human Development Index (2008–2030): a population-based study.*The Lancet Oncology. 13*(8), 790-801.

Brown, M. L., Goldie, S. J., Draisma, G., Harford, J. and Lipscomb, J. (2006). Health service interventions for cancer control in developing countries. *Disease Control Priorities in Developing Countries*. 2, 569-589.

Calabria, L. M., Piacente, S., Kapusta, I., Dharmawardhane, S. F., Segarra, F. M., Pessiki, P. J. and Mabry, T. J. (2008). Triterpene saponins from *Silphium radula.Phytochemistry. 69*(4), 961-972.

Carter, H. B. (2011). Management of low (favourable)-risk prostate cancer. *British Journal of Urology International. 108*(11), 1684-1695.

Cassileth, B. R. and Vickers, A. J. (2004). Massage therapy for symptom control: outcome study at a major cancer center. *Journal of Pain and Symptom Management. 28*(3), 244-249.

Chabner, B. A. and Longo, D. L. (2011). Cancer Chemotherapy and Biotherapy*: Principles and Practice*: Lippincott Williams and Wilkins.

Chemicals, D. (2005). OECD Guideline for testing of chemicals.

Clement, E., Erharuyi, O., Vincent, I., Joy, A., Christopher, A., Anthony, A. and Abiodun, F. (2014). Significance of Bitter Leaf (*Vernonia Amagdalina*) In Tropical Diseases and Beyond: A Review. *Malaria Chemotherapy, Control and Elimination Journal. 3*(120), 2

Cragg, G. M. and Newman, D. J. (2005). Plants as a source of anti-cancer agents. *Journal of Ethnopharmacology. 100*(1), 72-79.

Cofre, J. and Abdelhay, E. (2017). Cancer Is to Embryology as Mutation Is to Genetics: Hypothesis of the Cancer as Embryological Phenomenon. *The Scientific World* Journal. 2017.

DeSantis, C. E., Lin, C. C., Mariotto, A. B., Siegel, R. L., Stein, K. D., Kramer, J. L. and Jemal, A. (2014). Cancer treatment and survivorship statistics, *CA: A Cancer Journalfor Clinicians. 64*(4), 252-271.

Dewanji, A., Moolgavkar, S. H. and Luebeck, E. G. (1991). Two-mutation model for carcinogenesis: joint analysis of premalignant and malignant lesions. *Mathematical Biosciences. 104*(1), 97-109.

Dholi, S. K., Raparla, R., Mankala, S. K. and Nagappan, K. (2011). *In vivo* Antidiabetic evaluation of Neem leaf extract in alloxan induced rats. *Journal of Applied Pharmaceutical Science. 1*(4), 1.

Diplock, A., Charuleux, J.-L., Crozier-Willi, G., Kok, F., Rice-Evans, C., Roberfroid, M. and Vina-Ribes, J. (1998). Functional food science and defence against reactiveoxidative species. *British Journal of Nutrition. 80*(1), S77-S112.

Downer, S., Cody, M., McCluskey, P., Wilson, P., Arnott, S., Lister, T. and Slevin, M.(1994). Pursuit and practice of complementary therapies by cancer patients receiving conventional treatment. *British Medical Journal. 309*(6947), 86-89.

Dunsworth, T., Rich, S., Swanson, J. and Barbosa, J. (1982). No evidence for linkage between diabetes and the Kidd marker. *Diabetes. 31*(11), 991-993.

Edeoga, H., Okwu, D. and Mbaebie, B. (2005). Phytochemical constituents of some Nigerian medicinal plants. *African Journal of Biotechnology. 4*(7), 685-688.

Erasto, P., Grierson, D. S. and Afolayan, A. J. (2007). Evaluation of antioxidant activity and the fatty acid profile of the leaves of *Vernonia amygdalina* growing in South Africa. *Food Chemistry. 104*(2), 636-642.

Erlay, J., Ervik, M., Dikshit, R., Eser, S. and Mathers, C. (2012). Cancer incidence and mortality worldwide: IARC *GLOBOCAN. 1*(11).

Ernst, E. (1998). The prevalence of complementary/alternative medicine in cancer. *Cancer. 83*(4), 777-782.

Farombi, E. O. and Owoeye, O. (2011). Antioxidative and chemopreventive properties of *Vernonia amygdalina* and *Garcinia* biflavonoid. *International Journal of Environmental Research and Public Health. 8*(6), 2533-2555.

Fraga, M. F., Herranz, M., Espada, J., Ballestar, E., Paz, M. F., Ropero, S. and Niveleau, A. (2004). A mouse skin multistage carcinogenesis model reflects

the aberrant DNA methylation patterns of human tumors. *Cancer Research. 64*(16), 5527-5534.

Gbotolorun, S., Osinubi, A., Noronha, C. and Okanlawon, A. (2008). Antifertility potential of Neem flower extract on adult female Sprague-Dawley rats. *African Health Sciences. 8*(3), 168-173.

GOK. (2016). *Kenya Nationa Cancer Control strategy* 2011-2016.

Gomella, L. G. (2005). Addressing the needs of the high-risk prostate cancer patient.

Reviews in Urology. 7(5), S18.

Gordaliza, M., Castro, M. d., Miguel el Corral, J. and Feliciano, A. S. (2000).Antitumor properties of podophyllotoxin and related compounds. *CurrentPharmaceutical Design. 6*(18), 1811-1839.

Greenman, C., Stephens, P., Smith, R., Dalgliesh, G. L., Hunter, C., Bignell, G. and Stevens, C. (2007). Patterns of somatic mutation in human cancer genomes. *Nature 446*(7132), 153-158.

Hall, M., Middleton, R. and Westmacott, D. (1983). The fractional inhibitory concentration (FIC) index as a measure of synergy. *Journal of Antimicrobial Chemotherapy. 11*(5), 427-433.

Hanahan, D. and Weinberg, R. A. (2011). Hallmarks of cancer: the next generation. *Cell. 144*(5), 646-674.

Hossain, M. A., Al-Toubi, W. A., Weli, A. M., Al-Riyami, Q. A. and Al-Sabahi, J. N.(2013). Identification and characterization of chemical compounds in different crude extracts from leaves of *Omani* neem. *Journal of Taibah University for Science. 7*(4), 181-188.

Hosseinzadeh, H., Ramezani, M. and Salmani, G. (2000). Antinociceptive, anti-inflammatory and acute toxicity effects of *Zataria multiflora Boiss.* extracts in mice and rats. *Journal of Ethnopharmacology. 73*(3), 379-385.

Huang, J., Li, Q., Sun, D., Lu, Y., Su, Y., Yang, X. and He, N. (2007). Biosynthesis of silver and gold nanoparticles by novel sundried *Cinnamomum camphora* leaf. *Nanotechnology. 18*(10), 105104.

Hussain, S. P., Hofseth, L. J. and Harris, C. C. (2003). Radical causes of cancer. *Nature Reviews Cancer. 3*(4), 276-285

Ijeh, I. I. and Ejike, C. E. (2011). Current perspectives on the medicinal potentials of *Vernonia amygdalina Del. Journal of Medicinal Plants Research. 5*(7), 1051-1061.

Ileke, K. D. (2015). Entomotoxicant Potential of Bitter Leaf, *Vernonia Amygdalina* Powder in the Control of Cowpea Bruchid, *Callosobruchus Maculatus* (*Coleoptera: Chrysomelidae*) Infesting Stored Cowpea Seeds. *Octa Journal of Environmental Research. 3*(3).

Imielinski, M., Berger, A. H., Hammerman, P. S., Hernandez, B., Pugh, T. J., Hodis, E. and Sivachenko, A. (2012). Mapping the hallmarks of lung adenocarcinoma with massively parallel sequencing. *Cell. 150*(6), 1107-1120.

Jacobs, D. I., Snoeijer, W., Hallard, D. and Verpoorte, R. (2004). The Catharanthus alkaloids: pharmacognosy and biotechnology. *Current Medicinal Chemistry. 11*(5), 607-628.

Jemal, A., Bray, F., Center, M. M., Ferlay, J., Ward, E. and Forman, D. (2011). Global cancer statistics. *CA Cancer Journal for Clinicians. 61*(2), 69-90.

Jiang, Y., Liu, C., Li, J. Y., Huang, M. J., Yao, W. X., Zhang, R. and Xie, K. (2007). Different attitudes of Chinese patients and their families toward truth telling of different stages of cancer. *Psycho‐Oncology. 16*(10), 928-936.

Kaneria, M., Kanani, B. and Chanda, S. (2012). Assessment of effect of hydroalcoholic and decoction methods on extraction of antioxidants from

selected Indian medicinal plants. *Asian Pacific Journal of Tropical Biomedicine. 2*(3), 195-202.

Karcioglu, Z. A., Gordon, R. A. and Karcioglu, G. L. (1985). Tumor seeding in ocular fine needle aspiration biopsy. *Ophthalmology. 92*(12), 1763-1767.

Khalafalla, M. M., Abdellatef, E., Daffalla, H. M., Nassrallah, A. A., Aboul-Enein, K. M.,Lightfoot, D. A. and El-Shemy, H. A. (2009). Antileukemia activity from root culturesof *Vernonia amygdalina. Journal of Medicinal Plants Research. 3*(8), 556-562.

Khan, S. A., Khan, L., Hussain, I., Marwat, K. B. and Akhtar, N. (2008). Profile of heavy metals in selected medicinal plants. *Pakistan Journal of Weed Science Research. 14*(1-2), 101-110.

Koksal, E., Bursal, E., Dikici, E., Tozoglu, F. and Gulcin, I. (2011). Antioxidant activity of *Melissa officinalis* leaves. *Journal of Medicinal Plants Research. 5*(2), 217-222.

Kokwaro, J. O. (2009). *Medicinal Plants of East Africa*: University of Nairobi press.

Koleva, I. I., van Beek, T. A., Linssen, J. P., Groot, A. d. and Evstatieva, L. N. (2002). Screening of plant extracts for antioxidant activity: a comparative study on three testing methods. *Phytochemical Analysis. 13*(1), 8-17.

Korir, A., Okerosi, N., Ronoh, V., Mutuma, G. and Parkin, M. (2015). Incidence of cancer in Nairobi, Kenya (2004–2008). *International Journal of Cancer. 137*(9), 2053-2059.

Kpela, T., Ekanem, T. B. and Akpantah, A. O. (2012). Protective effect of ethanol extract of neem leaves on cisplatin-induced kidney damage in wistar rats. *Journal of Medical* Sciences. *1*(9), 249-254.

Kupchan, S. M., Hemingway, R. J., Karim, A. and Werner, D. (1969). Tumor inhibitors. XLVII. Vernodalin and vernomygdin, two new cytotoxic

sesquiterpene lactones from *Vernonia amygdalina Del. The Journal of Organic Chemistry. 34*(12), 3908-3911.

Kupradinun, P., Tepsuwan, A., Tanthasri, N., Meesiripan, N., Tunsakul, S., Tompat, W. and Kusamran, W. R. (2010). Toxicity testing of flowers of neem tree (*Azadirachta indica A. Juss*). *The Thai Journal of Veterinary Medicine. 40*(1), 47-55.

Latha, L. Y., Darah, I., Jain, K. and Sasidharan, S. (2010). Toxicity study of *Vernonia cinerea. Pharmaceutical Biology. 48*(1), 101-104.

Lau, C., Ho, C., Kim, C., Leung, K., Fung, K., Tse, T. and Chow, M. (2004). Cytotoxicactivities of *Coriolus versicolor* (Yunzhi) extract on human leukemia and lymphomacells by induction of apoptosis. *Life Sciences. 75*(7), 797-808.

Lawrence, M. S., Stojanov, P., Polak, P., Kryukov, G. V., Cibulskis, K., Sivachenko, A., and Roberts, S. A. (2013). Mutational heterogeneity in cancer and the search for new cancer-associated genes. *Nature. 499*(7457), 214-218.

Li, J., Chen, L., Li, B., Liu, S. and Dong, J. (2010). Study on the chemical constituents of *Galium aparine L. Journal of International Pharmaceutical Research. 37*(5), 387-389.

Lin, Y., Shi, R., Wang, X. and Shen, H.-M. (2008). Luteolin, a flavonoid with potential for cancer prevention and therapy. *Current Cancer Drug Targets. 8*(7), 634-646.

Luo, J., Shen, W. L. and Montell, C. (2017). TRPA1 mediates sensation of the rate of temperature change in Drosophila larvae. *Nature Neuroscience. 20*(1), 34-41

Mahmood, A., Mahmood, A. and Tabassum, A. (2011). Ethnomedicinal survey of plants from District Sialkot, Pakistan. *Journal of Applied Pharmaceutical Science.* 3, 212-220.

Maragathavalli, S., Brindha, S., Kaviyarasi, N., Annadurai, B. and Gangwar, S. (2012).Antimicrobial activity in leaf extract of neem (*Azadirachta indica Linn*.). *InternationalJournal of Science and Nature. 3*(1), 110-113.

Massad, L. S., Seaberg, E. C., Watts, D. H., Minkoff, H., Levine, A. M., Henry, D. and Hessol, N. A. (2009). Long-term incidence of cervical cancer in women with human immunodeficiency virus. *Cancer. 115*(3), 524-530.

Mbatchi, S., Mbatchi, B., Banzouzi, J., Bansimba, T., Ntandou, G. N., Ouamba, J. M. and Benoit-Vical, F. (2006). *In vitro* antiplasmodial activity of 18 plants used in Congo Brazzaville traditional medicine. *Journal of Ethnopharmacology. 104*(1), 168-174.

Meletiadis, J., Pournaras, S., Roilides, E. and Walsh, T. J. (2010). Defining fractional inhibitory concentration index cutoffs for additive interactions based on self-drug additive combinations, Monte Carlo simulation analysis, and *in vitro-in vivo* correlation data for antifungal drug combinations against *Aspergillus fumigatus*. *Antimicrobial Agents and Chemotherapy. 54*(2), 602-609.

Mir, A. H., Sexena, M. and Malla, M. Y. (2013). An acute oral toxicity study of methanolic extract from *Tridex procumbens* in Sprague Dawley's Rats as per OECD guidelines *Asian Journal of Plant Science and Research. 3*(1), 16-20.

Mishra, N., Dubey, A., Mishra, R. and Barik, N. (2010). Study on antioxidant activity of common dry fruits. *Food and Chemical Toxicology. 48*(12), 3316-3320.

Mokkhasmit, M., Swatdimongkol, K. and Satrawaha, P. (1971). Study on toxicity of Thai medicinal plants. *Bulletin of the Department of Medical Scienses. 12*(2/4), 36-65.

Molassiotis, A., Fernadez-Ortega, P., Pud, D., Ozden, G., Scott, J. A., Panteli, V. and Patiraki, E. (2005). Use of complementary and alternative medicine in cancer patients:a European survey. *Annal Oncology. 16*(4), 655-663.

Molyneux, P. (2004). The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. *Songklanakarin Journal of Science and Technology. 26*(2), 211-219.

Morgan, R. A. and Boyerinas, B. (2016). Genetic Modification of T Cells. *Biomedicines. 4*(2), 9.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods. 65*(1-2), 55-63.

Moudi, M., Go, R., Yien, C. Y. S. and Nazre, M. (2013). *Vinca alkaloids. International Journal of Preventive Medicine. 4*(11).

Muddukrishnaiah, K. and Singh, S. (2015). Antimicrobial, Synergistic Activity and Antioxidant Studies on Multidrug Resistance Human Pathogen using Crude Extract of *Azadirachta indica* Leaf and *Withania somnifera* Rhizome. *Journal of Plant Pathology and Microbiology.* 3, 1-3.

Nabende, P. N. and Namukhosi, P. (2015). Safety and anti-proliferative activity of *Prunus africana, Warburgia stuhlmannii* and *Maytenus senegalensis* extracts in breast and colon cancer cell lines. *European Journal of Medicinal Plants. 5*(4), 366.

Nahak, G. and Sahu, R. (2011). Evaluation of antioxidant activity of flower and seed oil of *Azadirachta indica A. juss. Journal of Applied and Natural Science. 3*(1), 78-81.

Nassr-Allah, A. A., Aboul-Enein, A. M., Aboul-Enein, K. M., Lightfoot, D. A., Cocchetto and El-Shemy, H. A. (2009). Anti-cancer and anti-oxidant

activity of some Egyptian medicinal plants. *Journal of Medicinal Plants Research. 3*(10), 799-808.

Nelson, P. S. and Montgomery, B. (2003). Unconventional therapy for prostate cancer:good, bad or questionable? *Nature Reviews Cancer. 3*(11), 845-858.

Nisbet, A. J. (2000). Azadirachtin from the neem tree *Azadirachta indica*: its action against insects. *Anais da Sociedade Entomológica do Brasil. 29*(4), 615-632.

Njagi, S., Lagat, R., Mawia, A., Arika,W. and Wambua, F. (2016). *In vitro*

Antiproliferative Activity of Aqueous Root Bark Extract of *Cassia abbreviata* (Holmes) Brenan. *Journal of Cancer Science and Therapy.* 8, 114-121.

Ntekim, A. I. and Folasire, A. M. (2010). CD4 count and anti-retroviral therapy for HIV positive patients with cancer in Nigeria-a pilot study. *Clinical Medicine Insights. Oncology.* 4, 61.

Oaks Jr, S. C., Shope, R. E. and Lederberg, J. (1992). *Emerging infections: microbial threats to health in the United States*: National Academies Press.

Oberlies, N. H. and Kroll, D. J. (2004). Camptothecin and Taxol: Historic Achievements in Natural Products Research. *Journal of Natural Products. 67*(2), 129-135.

Ochwang'i, D. O., Kimwele, C. N., Oduma, J. A., Gathumbi, P. K., Mbaria, J. M. and Kiama, S. G. (2014). Medicinal plants used in treatment and management of cancer in Kakamega County, Kenya. *Journal of Ethnopharmacology. 151*(3), 1040-1055.

Odida, M., Sandin, S., Mirembe, F., Kleter, B., Quint, W. and Weiderpass, E. (2011). HPV types, HIV and invasive cervical carcinoma risk in Kampala, Uganda: a case-control study. *Infect Agent Cancer, 6*(1), 8.

O'Dwyer, P. J., Stevenson, J. P. and Johnson, S. W. (1999). Clinical status of cisplatin, carboplatin, and other platinum-based antitumor drugs. *Cisplatin: Chemistry and Biochemistry of a Leading Anticancer Drug*. 31-69.

Okpanyi, S. and Ezeukwu, G. (1981). Anti-inflammatory and antipyretic activities of *Azadirachta indica*. *Planta Medica. 41*(01), 34-39.

Othman, F., Motalleb, G., Peng, S. L. T., Rahmat, A., Fakurazi, S. and Pei, C. P. (2011). Extract of *Azadirachta indica* (Neem) leaf induces apoptosis in 4T1 breast cancer BALB/c mice. *Cell Journal (Yakhteh). 13*(2).

Palombo, E. A. (2006). Phytochemicals from traditional medicinal plants used in the treatment of diarrhoea: modes of action and effects on intestinal function. *Phytotherapy Research. 20*(9), 717-724.

Pandey, G., Verma, K. and Singh, M. (2014). Evaluation of phytochemical, antibacterial and free radical scavenging properties of *Azadirachta indica* (neem) leaves. *International Journal of Pharmacy and Pharmaceutical Sciences. 6*(2), 444-447

Paritala, V., Chiruvella, K. K., Thammineni, C., Ghanta, R. G. and Mohammed, A. (2015). Phytochemicals and antimicrobial potentials of mahogany family. *Revista Brasileira de Farmacognosia. 25*(1), 61-83.

Patel, S., Gheewala, N., Suthar, A. and Shah, A. (2009). *In-vitro* cytotoxicity activity of *Solanum nigrum* extract against Hela cell line and Vero cell line. *International Journal of Pharmacy and Pharmaceutical Sciences. 1*(1), 38-46.

Pearson, N. J., Johnson, L. L. and Nahin, R. L. (2006). Insomnia, trouble sleeping, and complementary and alternative medicine: Analysis of the 2002 national health interview survey data. *Archives of internal medicine. 166*(16), 1775-1782.

Pietta, P.G. (2000). Flavonoids as antioxidants. *Journal of Natural Products. 63*(7), 1035-1042.

Platt, R. (1955). Clonal ageing and cancer. *The Lancet. 265*(6869), 867.

Prasanna Krishna, V. (2011). Evaluation of Acute and Chronic Toxicity studies of Ethanolic extract of *Ficus glomerata L. International Journal of Pharmacy Development and Technology. 1*(2), 66-70.

Poppendieck, H.-H. and Kubitzki, K. (1981). Systematics and evolution of seed plants *Progress in Botany/Fortschritte der Botanik* (pp188-235): Springer.

Prashanth, G. and Krishnaiah, G. (2014). Chemical composition of the leaves of *Azadirachta indica Linn* (Neem). *International Journal of Advancement in Engineering and Technology, Management and Applied Science*. 1, 21-31.

Puff, C. (1975). Leaf flavonoids of *Galium sect. Aparinoides* (*Rubiaceae*). *Plant Systematics and Evolution. 124*(1), 57-66

Raghavendra, M. and Reddy, A. (2011). Acute and chronic toxicity studies of ethanolic extract of *Rumex vesciscarius L.* in experimental animals. *Journal of Science.* 1, 16-20.

Rajkumar, U., Reddy, M., Rao, S., Radhika, K. and Shanmugam, M. (2011). Evaluation of growth, carcass, immune response and stress parameters in naked neck chicken and their normal siblings under tropical winter and summer temperatures. *Asian-Australasian Journal of Animal Sciences. 24*(4), 509-516.

Richardson, B. E., Beckett, K., Nowak, S. J. and Baylies, M. K. (2007). SCAR/WAVE and Arp2/3 are crucial for cytoskeletal remodeling at the site of myoblast fusion. *Development. 134*(24), 4357-4367.

Rozhok, A. I. and DeGregori, J. (2016). The evolution of lifespan and age-dependent cancer risk. *Trends in Cancer. 2*(10), 552-560

Ruch, R. J., Cheng, S. j. and Klaunig, J. E. (1989). Prevention of cytotoxicity and inhibition of intercellular communication by antioxidant catechins isolated from Chinese green tea. *Carcinogenesis. 10*(6), 1003-1008.

Saidi, H., Nyaim, E. O., Githaiga, J. W. and Karuri, D. (2008). CRC surgery trends in Kenya, 1993–2005. *World Journal of Surgery. 32*(2), 217-223.

Sannigrahi, S., Mazumder, U. K., Mondal, A., Pal, D., Mishra, S. L. and Roy, S. (2010). Flavonoids of *Enhydra fluctuans* exhibit anticancer activity against *Ehrlich's ascites carcinoma* in mice. *Natural Product Communications. 5*(8), 1239-1242

Scott, J. A., Kearney, N., Hummerston, S. and Molassiotis, A. (2005). Use of complementary and alternative medicine in patients with cancer: a UK survey. *European Journal of Oncology Nursing. 9*(2), 131-137.

S Akinboye, E. and Bakare, O. (2011). Biological activities of emetine. *The Open Natural Products Journal. 4*(1).

Sarmiento, W. C., Maramba, C. C. and Gonzales, M. L. M. (2011). An *in-vitro* study on the antibacterial effect of neem (*Azadirachta indica*) leaf extract on methicillin-sensitive and Methicillin-resistant *Staphylococcus aureus*. *Pediatric Infectious Disease Society of the Philippines. 12*(1), 40-45.

Shang, W., Qiao, J., Gu, C., Yin, W., Du, J., Wang, W. and Lu, W. (2011). Anticancer activity of an extract from needles and twigs of *Taxus cuspidata* and its synergistic effect as a cocktail with 5-fluorouracil. *BMC Complementary and Alternative Medicine. 11*(1), 123.

Sharma, C., Vas, A. J., Goala, P., Gheewala, T. M., Rizvi, T. A. and Hussain, A. (2014). Ethanolic neem (*Azadirachta indica*) leaf extract prevents growth of MCF-7 and HeLa cells and potentiates the therapeutic index of cisplatin. *Journal of Oncology.* 2014.

Shukla, D. P., Shah, K. P., Rawal, R. M. and Jain, N. K. (2016). Anticancer and Cytotoxic Potential of Turmeric (*Curcuma longa*), Neem (*Azadirachta indica*), Tulasi (*Ocimum sanctum*) and Ginger (*Zingiber officinale*) Extracts on HeLa Cell line. *International Journal of Life Sciences and Scientific Research. 2*(4).

Siddhuraju, P., Mohan, P. and Becker, K. (2002). Studies on the antioxidant activity of Indian Laburnum (*Cassia fistula L.*): a preliminary assessment of crude extracts from stem bark, leaves, flowers and fruit pulp. *Food Chemistry. 79*(1), 61-67.

Singh, N. and Rajini, P. (2004). Free radical scavenging activity of an aqueous extract of potato peel. *Food Chemistry. 85*(4), 611-616.

Sithisarn, P., Supabphol, R. and Gritsanapan, W. (2005). Antioxidant activity of Siamese neem tree (VP1209). *Journal of Ethnopharmacology. 99*(1), 109-112

Soerjomataram, I., Lortet-Tieulent, J., Parkin, D. M., Ferlay, J., Mathers, C., Forman, D. and Bray, F. (2012). Global burden of cancer in 2008: a systematic analysis of disability-adjusted life-years in 12 world regions. *The Lancet. 380*(9856), 1840-1850.

Sonnenschein, C., Soto, A. M., Rangarajan, A. and Kulkarni, P. (2014). Competing views on cancer. *Journal of Biosciences. 39*(2), 281-302.

Sofowora, A., Ogunbodede, E. and Onayade, A. (2013). The role and place of medicinal plants in the strategies for disease prevention. *African Journal of Traditional, Complementary and Alternative Medicines. 10*(5), 210-229.

Sisodiya, P. S. (2013). Plant derived anticancer agents: A review.

Soto, A. M. and Sonnenschein, C. (2004). The somatic mutation theory of cancer: growing problems with the paradigm? *Bioessays. 26*(10), 1097-1107.
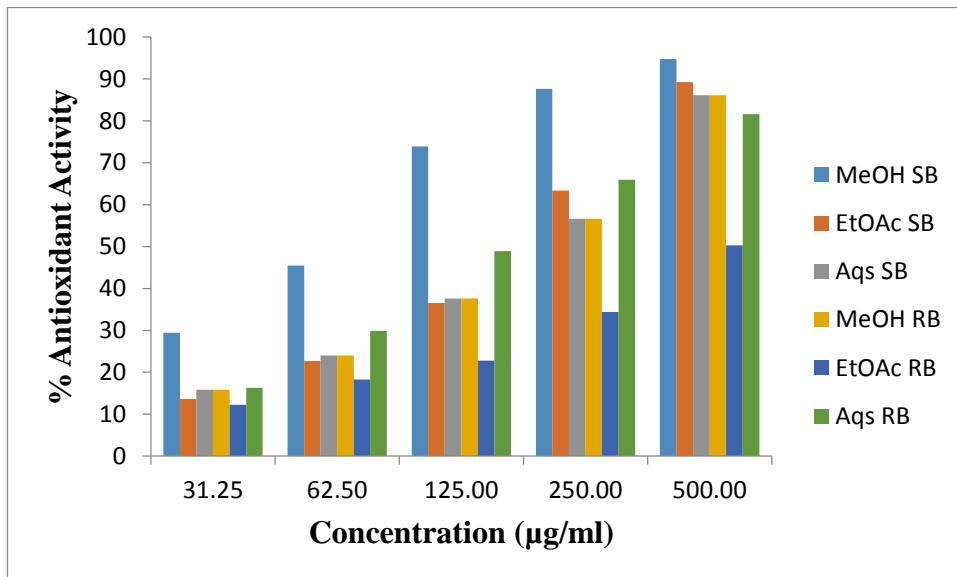
Soto, A. M. and Sonnenschein, C. (2011). The tissue organization field theory of cancer: a testable replacement for the somatic mutation theory. *Bioessays. 33*(5), 332-340.

Spinella, M. (2002). The importance of pharmacological synergy in psychoative herbal medicines.(Herbal synergy review). *Alternative Medicine Review. 7*(2), 130-138.

Stephens, P. J., Greenman, C. D., Fu, B., Yang, F., Bignell, G. R., Mudie, L. J. and

Stebbings, L. A. (2011). Massive genomic rearrangement acquired in a single catastrophic event during cancer development. *Cell. 144*(1), 27-40.

Subapriya, R. and Nagini, S. (2003). Ethanolic neem leaf extract protects against N-methyl-N'-nitro-N-nitrosoguanidine-induced gastric carcinogenesis in Wistar rats. *Asian Pacific Journal of Cancer Prevention. 4*(3), 215-224.

Sweeney, C. J., Roth, B. J., Kabbinavar, F. F., Vaughn, D. J., Arning, M., Curiel, R. E. and Kaufman, D. S. (2006). Phase II study of pemetrexed for second-line treatment of transitional cell cancer of the urothelium. *Journal of Clinical Oncology. 24*(21), 3451-3457.

Truant, T. and Bottorff, J. L. (1999). Decision making related to complementary therapies: a process of regaining control. *Patient Education and Counseling. 38*(2), 131-142.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M. and Telser, J. (2007).Free radicals and antioxidants in normal physiological functions and human disease. *The International Journal of Biochemistry & Cell Biology. 39*(1), 44-84.

van Vuuren, S. and Viljoen, A. (2011). Plant-based antimicrobial studies–methods and approaches to study the interaction between natural products. *Planta Medica. 77*(11), 1168-1182.

Vlase, L., Benedec, D., Hanganu, D., Damian, G., Csillag, I., Sevastre, B. and Tilea, I. (2014). Evaluation of antioxidant and antimicrobial activities and phenolic profile for *Hyssopus officinalis, Ocimum basilicum and Teucrium chamaedrys*. *Molecules. 19*(5), 5490-5507.

Vogelstein, B., Papadopoulos, N., Velculescu, V. E., Zhou, S., Diaz, L. A. and Kinzler, K. W. (2013). Cancer genome landscapes. *Science. 339*(6127), 1546-1558.

Wagner, H. (2011). Synergy research: approaching a new generation of phytopharmaceuticals. *Fitoterapia. 82*(1), 34-37.

Wang, X., Ge, J., Wang, K., Qian, J. and Zou, Y. (2006). Evaluation of MTT assay for measurement of emodin-induced cytotoxicity. *Assay and Drug Development Technologies. 4*(2), 203-207.

Webb, M. S., Johnstone, S., Morris, T. J., Kennedy, A., Gallagher, R., Harasym, N. and Dragowska, W. H. (2007). *In vitro and in vivo* characterization of a combination chemotherapy formulation consisting of vinorelbine and phosphatidylserine. *European Journal of Pharmaceutics and Biopharmaceutics. 65*(3), 289-299.

Weinberg, R. A. (2008). In retrospect: the chromosome trail. *Nature. 453*(7196), 725-725.

Wong, F. C., Woo, C. C., Hsu, A. and Tan, B. K. H. (2013). The anti-cancer activities of *Vernonia amygdalina* extract in human breast cancer cell lines are mediated through caspase-dependent and p53-independent pathways. *PloS One. 8*(10), 78021.

World Health Organization, W. H. (2000). Global strategy for the prevention and control of non-communicable diseases. *Geneva: World Health Organization*.

World Health Organization, W. H. (2014). Global status report on non-communicable Diseases 2014: World Health Organization.
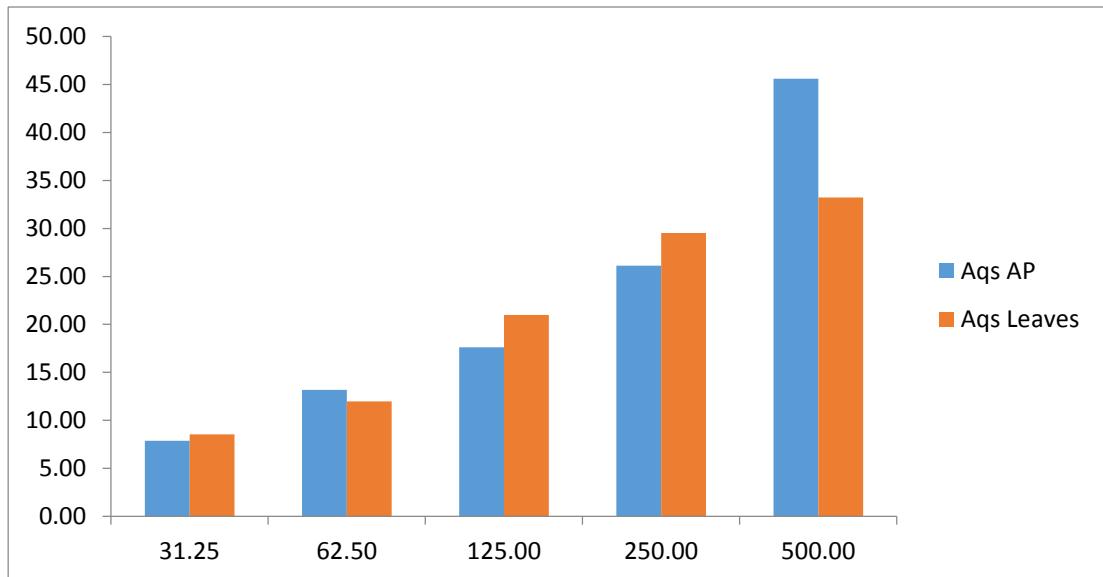
Yeap, S. K., Ho, W. Y., Beh, B. K., San Liang, W., Ky, H., Yousr, A. H. N. and Alitheen, N.B. (2010). *Vernonia amygdalina*, an ethnoveterinary and ethnomedical used green vegetable withmultiple bio-activities. *Journal of Medicinal Plants Research. 4*(25), 2787-2812.

APPENDICIES
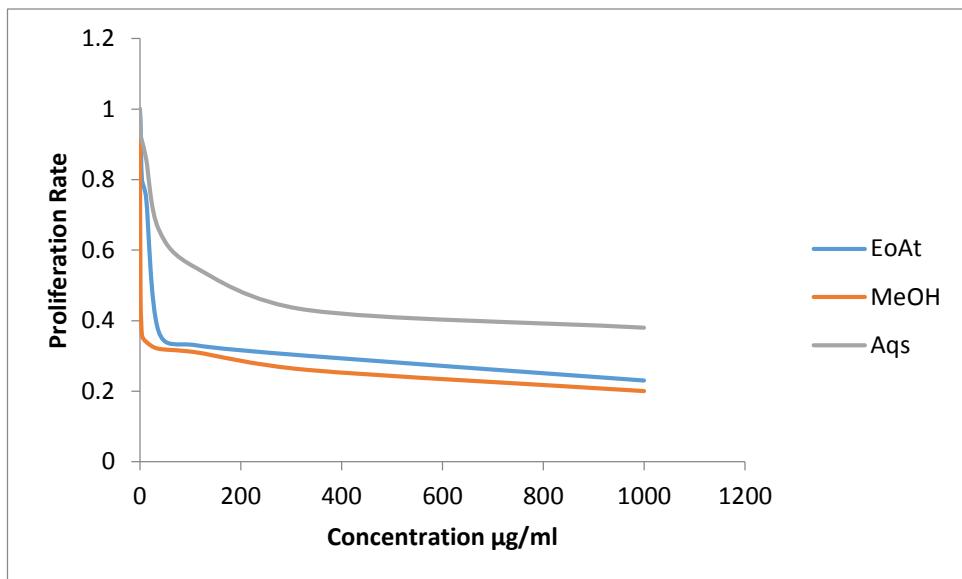
## Appendix 1: Percentage inhibition of *A. indica* RB and SB extracts
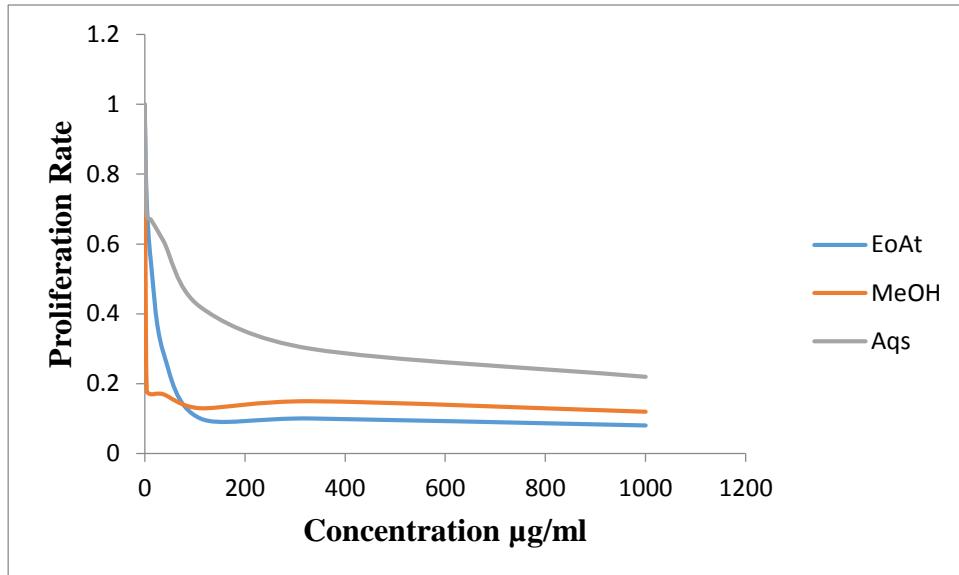
**Appendix 2: Percentage inhibition of *V. amygdalina* extracts**
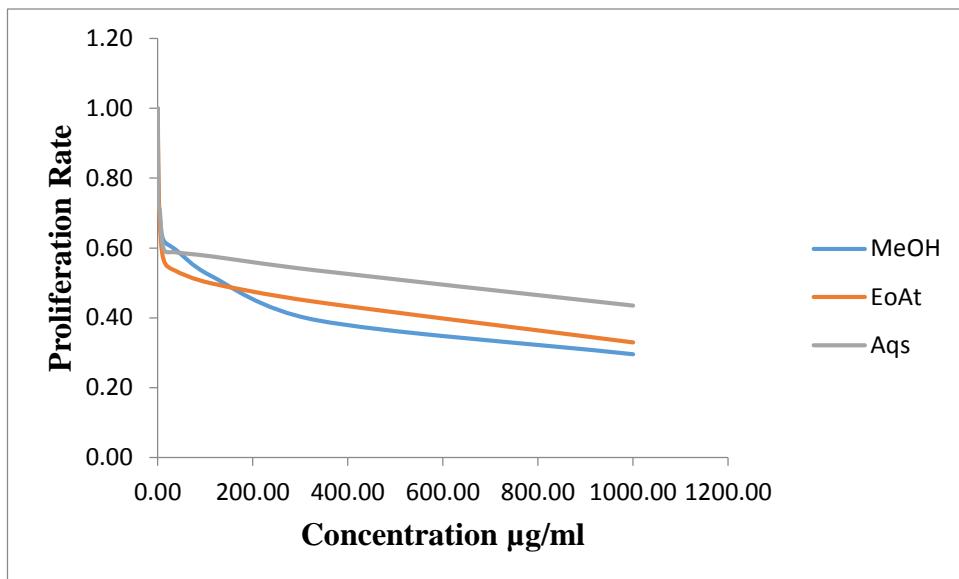
Appendix 3: Effects of *A. indica* SB extract on DU145 cell line proliferation rate
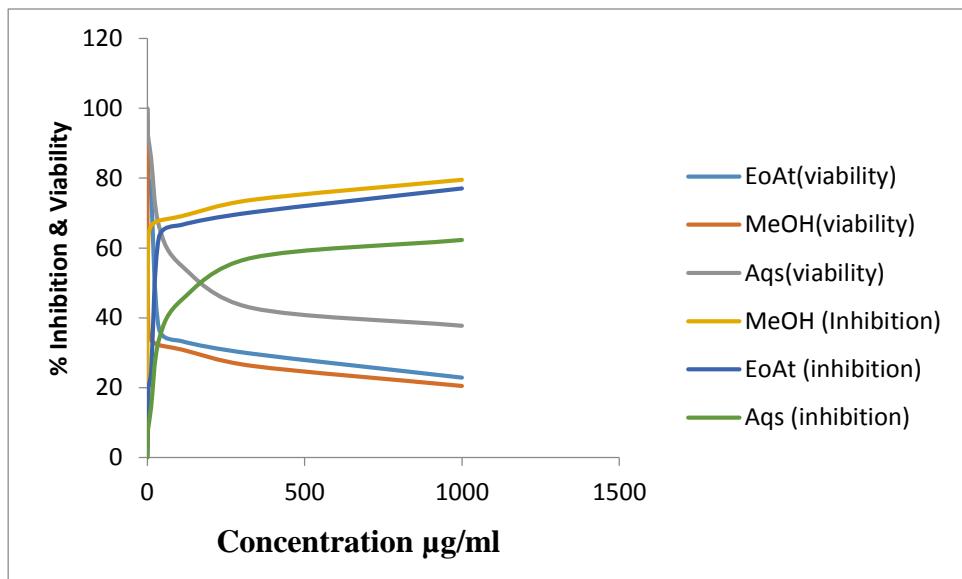
Appendix 4: Effects of *A. indica* SB extract on Hela cell line proliferation rate
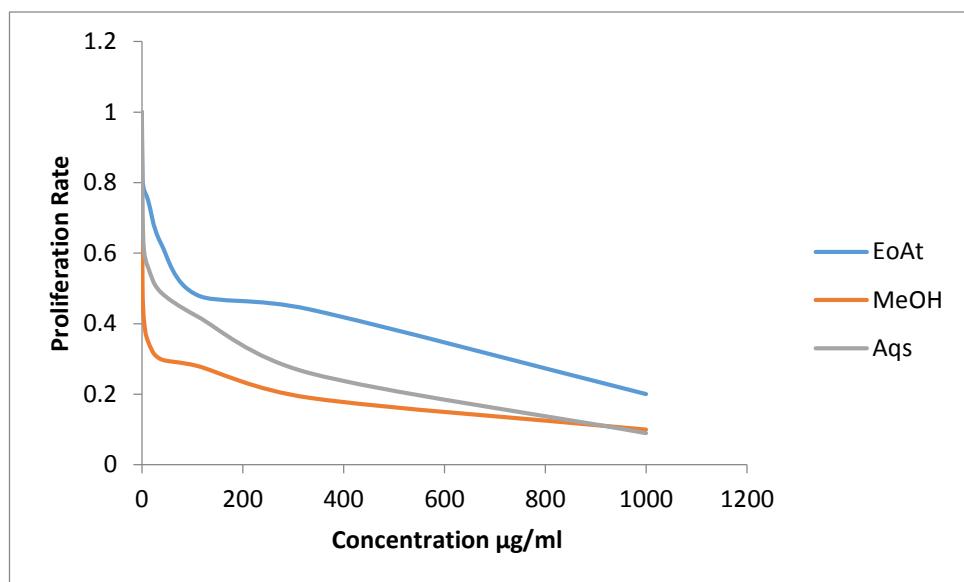
Appendix 5: Effects of *A. indica* SB extract on Vero cell line proliferation rate
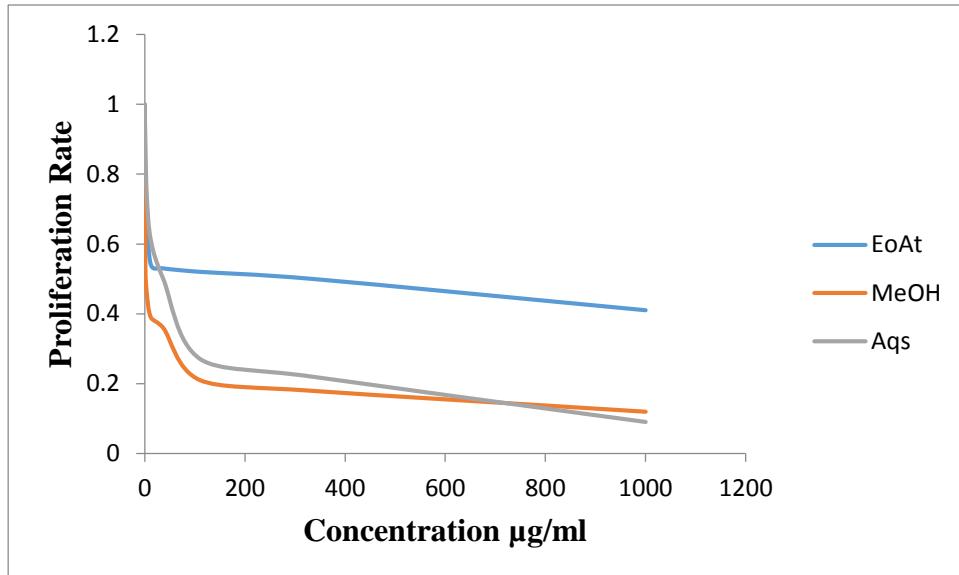
Appendix 6: *A. indica* **effects on percentage inhibition and viability of the cell lines**
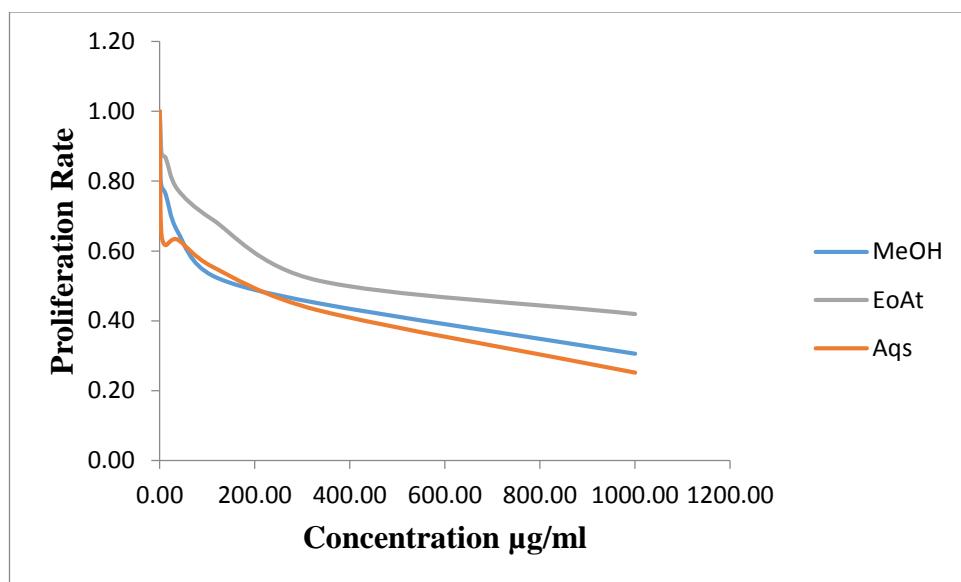
Appendix 7: Effects of *A. indica* RB extract on DU145 cell line proliferation rate
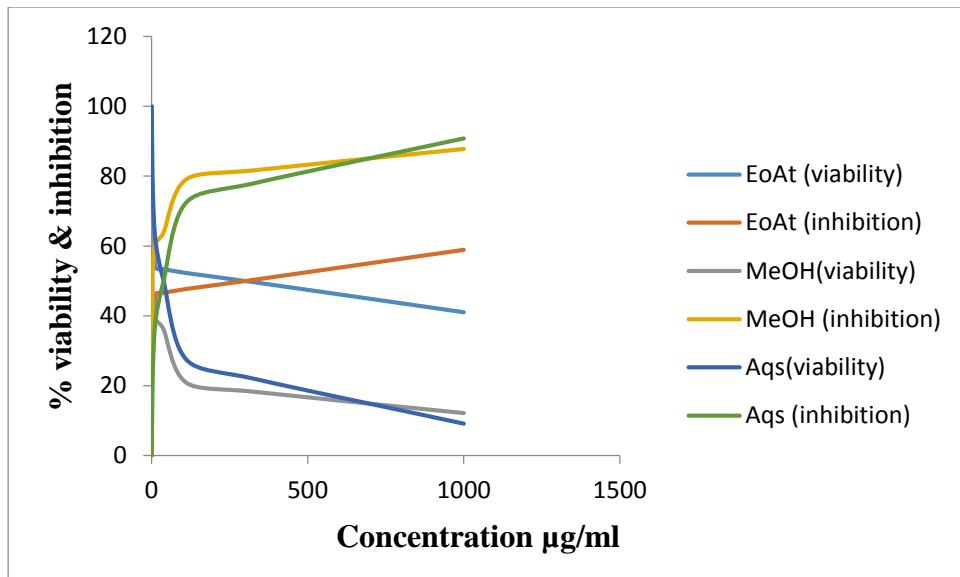
Appendix 8: Effects of *A. indica* RB extract on Hela cell line proliferation rate
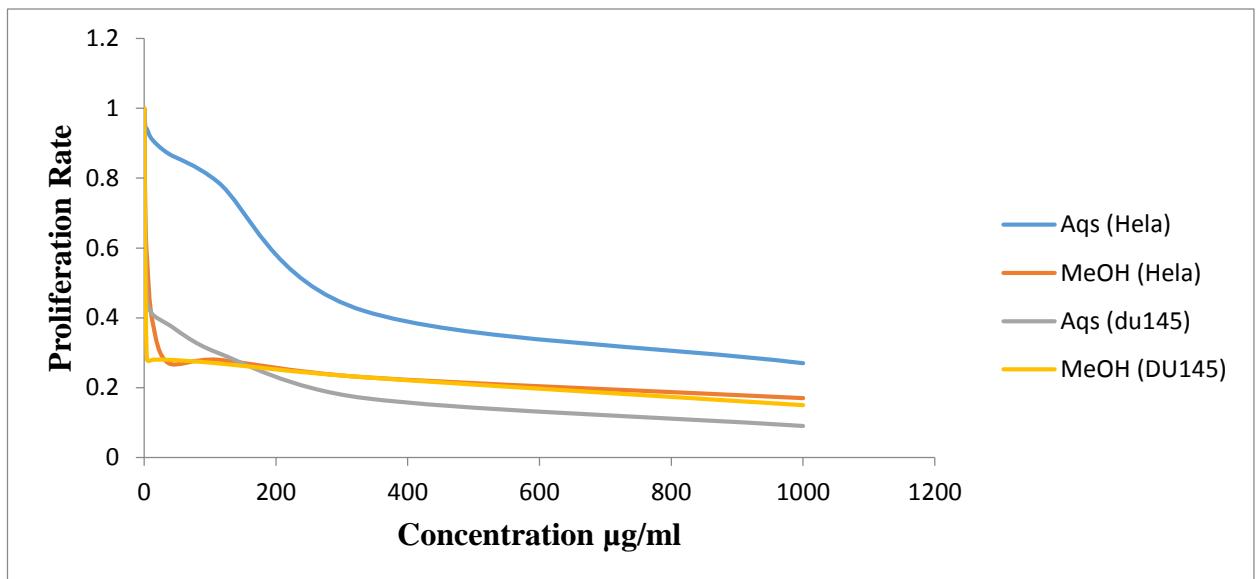
Appendix 9: Effects of *A. indica* RB extract on Vero cell line proliferation rate
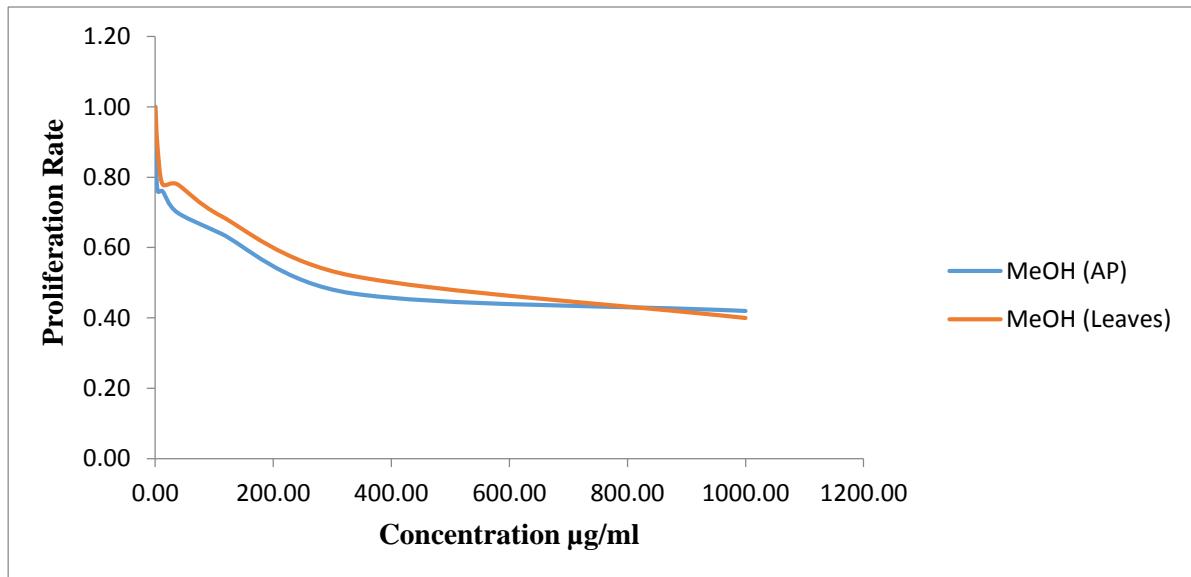
Appendix 10: *A. indica* **effects on % inhibition and viability of the cell lines**

Appendix 11: Effects of *V. amygdalina* on Hela and DU145 cells proliferation rate

Appendix 12: Effects of *V. amygdalina* on Vero cell line proliferation rate

Appendix 13: *V. amygdalina* **effects on % inhibition and viability rate**