IN VITRO ANTI-PROLIFERATIVE ACTIVITY OF SELECTED PLANT EXTRACTS AGAINST CERVICAL AND PROSTATE CANCER CELL LINES

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DECLARATION

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

Signature……. Date……...

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

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DEDICATION

I wish to dedicate this work to my dear Mum; Mrs. Nancy M. Kimani, Dad; Mr. George S. Kimani, my brothers and sister for their tireless belief in me.

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All glory belongs to God for this far I have come.

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LIST OF ABBREVIATIONS AND ACRONYMS

ACS	American Cancer Society
ATCC	American Type Culture Collection
Bax	BCL2-Associated X Protein
BcL-2	B-cell lymphoma 2
BcL-XL	B-cell lymphoma-extra large
CC_{50}	Cytotoxic concentration at 50%
CDK2	Cyclin-dependent kinase 2
CDK4	Cyclin-dependent kinase 4
CDKN1A	**C**yclin-dependent kinase inhibitor 1A
cDNA	Complementary deoxyribonucleic acid
DHT	Dihydrotestosterone
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic acid
EBV	Epstein Barr virus
E2F	E2 factor
EBRT	External Beam Radiotherapy
HBV	Hepatitis B virus
HPV	Human Papillomavirus
IC_{50}	Inhibition concentration at 50%
KEMRI	Kenya Medical Research Institute
MEM	Minimum Essential Medium
MOH	Ministry of Health

MOMS	Ministry of Medical Services
MOPHS	Ministry of Public Health and Sanitation
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide
NFκB	Nuclear Factor κapper B
p53	Tumor protein 53
pRb	Retinoblastoma Protein
PBS	Phosphate Buffered Saline
PI3K	Phosphoinositide 3-kinase
PIP2	Phosphatidylinositol (4, 5)-bisphosphate
PIP3	Phosphatidylinositol (3, 4,5)-triphosphate
PKB	Protein Kinase B
PTEN	Phosphatase and Tensin Homologue
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
pRb	Retinoblastoma protein
RAF	Rapidly Accelerated Fibrosarcoma
SI	Selective index
WHO	World Health Organization
µl	Microliter
IARC	International Agency for Research on Cancer
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
FBS	Fetal bovine serum

ABSTRACT

Prostate and cervical cancers are among the leading cancer in men and women respectively. The World Health Organization ranks cervical cancer as the fourth most common cancer among women globally. The numbers are estimated to be 604,000 new cases and 342,000 deaths in 2020. From this, about 90% of the new cases and deaths worldwide in 2020 occurred in low and middle-income countries in the world (WHO, 2022). Current conventional cancer therapies like chemotherapy and radiotherapy present severe side effects and in many developing countries are inaccessible to many cancer patients. Traditional plant-based medicines have long been used to treat various diseases as the plants are readily available. However, the inadequate understanding of the therapeutic mechanisms at a cellular level has contributed to the growing difficulty of bringing these drugs to conventional use. Using the MTT assay method, this study investigated the anti-proliferative activity of methanol and water extracts from four plant species- namely *Aloe secundiflora*, *Maytenus obscura*, *Vernonia zanzibarensis* and *Dichrostachys cinerea*, against prostate cancer cells (Du145 and 22RV1), cervical cancer cells (HeLa) and African green monkey (Vero) cell lines. The extracts studied suppressed the growth of the cancer cells in a dose-dependent manner at concentrations of 1.37 µg/ml to 1000 µg/ml. Extracts of *V. zanzibarensis* reported low antiproliferative activity against DU145, 22RV1 and HeLa cell lines while the aqueous extract of *M. obscura* stem bark and *A. Secundiflora* had moderate activity on DU145, 22RV1 and HeLa. The methanol extract of *D. cinerea* stem bark had the highest anti-proliferative activity with an IC50 of 8.04 ± 2.8 µg/ml against the 22RV1 cells and a low cytotoxic effect against the Vero cells with CC50 of 812.1 ± 12.72µg/ml. This study shows that the *D. cinerea* stem bark has anti-proliferative activity with low cytotoxicity to normal cells. These results suggest that the *D. cinerea* stem bark extract anti-proliferative activity can be linked to its ability to up-regulate the expression of tumor suppressor gene p53. This was after analysis of p53 gene modulation by the *D. cinerea* stem bark extract which showed that the extract upregulated the gene expression in Du145 cells by 1.76 folds. The conclusion of this study details the survey literature of ethno medicinal uses and validates phytochemical profile for management of cancer after an investigative focus to the synergistic pharmacological effect in killing the cancer cells.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Cancer poses a major disease burden worldwide, with considerable geographic variations in incidence, mortality, and survival (Bray, et al., 2018) (Sung, et al., 2020). As of 2020 there were an estimated 19.3 million new cancer cases and almost 10.0 million cancer deaths with the global burden of cancer expected to be 28.4 million cases in 2040, a 47% rise from 2020 (Sung, et al., 2020). Incidences of cancer in African countries are grossly underestimated due to inadequate or lack of efficient cancer registries, poor medical infrastructure with limited access to diagnostic services, and a lack of adequate vital records (Fadlelmola, 2016). However, Cancer death rates in Africa are projected to exceed the global average by 30% in the next 20 years with the incidence and mortality patterns varied across the regions (Hamdi, et al., 2021). In Kenya, Cancer is the second leading cause of non-communicable diseases mortality with an increase of deaths related to cancer of about 16%, from 2012 to 2018 and the incidence of cancer expected to rise by more than 120% over the next two decades (Macharia, Mureithi, & Anzala, 2019). The five most common cancers in Kenya are breast, cervical, prostate, esophageal, and colorectal. The leading cause of cancer death in Kenya is cervical, followed by breast, esophageal, colorectal, and prostate cancers (WHO, 2021).

Globally, prostate cancer is the fourth most common cancer in both sexes combined and the second most common cancer in men with an estimated 1.1 million men worldwide diagnosed with prostate cancer in 2012, accounting for 15% of the cancers diagnosed in men (Ferlay, et al., 2013). However, in Africa, prostate cancer is the leading cancer among men. The mainstay treatment of advanced prostate cancer is focused on suppression of Intra prostatic testosterone and dihydrotestosterone (DHT) actions (Fang, Merrick, & Wallner, 2010). However, after an initial response, therapy-resistant clones can appear and result in cancer progression and metastasis with high mortality (Damber & Aus,

2008). Surgery (radical hysterectomy) is often the first treatment option for solid tumors but in many cases, the simple excision with extensive resection does not solve the problem of recurrence and metastasis (Tohme, Simmons, & Tsung, 2017). First-line Docetaxel and prednisone chemotherapy drugs are given (usually for a minimum of six cycles) to patients with metastatic disease to provide disease control and palliation but have not demonstrated significant improvement in overall survival (Hotte & Saad , 2010). In case of limited monetary resources, 5-Floro uracil(5-FU) with or without cisplatin is the recommended treatment (Sara, et al., 2018).

Cervical cancer is the fourth most common cancer in women worldwide, with an estimated 604,000 new cases and 342,000 deaths in 2020 (WHO, 2022). It is largely a preventable disease using vaccines for preventive care and early screen for proper management. Gynecological pap smear screening has had an important impact on the reduction in cervical cancer mortality over the years while WHO recommends vaccination for girls aged between 9-13 years since this is the most cost-effective public health measure against cervical cancer (WHO, 2019). However, cervical cancer screening coverage in Kenya for women, age 18 to 69, is only 3.2% and in contrast to the developed nations, 80% of cases are very advanced at presentation thus prognosis is usually poor (MOMS & MOPHS, 2012). Two types of Human Papillomavirus (HPV)-vaccines are approved and allowed for use in Kenya. However, HPV vaccination was for a long time not part of the national immunization scheme and the cost implication had marked the uptake of the vaccine to be low (ICRH, 2013). There is also a global disinformation campaign against vaccines leading to rising concerns among the general population hence lower uptake of vaccines even and especially in developed countries. In addition, these vaccines are not effective against all HPV types that can cause cervical cancer, neither do they treat pre-existing HPV infection nor HPV-associated disease (ICRH, 2013). The stage of cancer is always the most important factor in choosing treatment. In cervical cancer that has not spread beyond the cervix, procedures such as conization, hysteroscopy, bilateral salpingo-oophorectomy and exenteration is used (ASCO, 2020). On the other hand, prostate cancer regimens include both food and drug administration. These regimens

are always to supplement the latest treatment strategies. Some of the regimes include; goserelin, histrelin, leuprodile and flutamide (haymarket, 2020).

Use of natural plant therapeutics is as old as human civilization and in many regions of the world is still the primary modality of health care. The Ebers Papyrus, which represents the history of Egyptian medicine dating from 1500 BC, documents over 700 drugs (Cragg & Newman, 2001). The Chinese Materia Medica, which describes more than 600 medicinal plants, has been well documented with the first record dating from about 1100 BC (Cragg & Newman, 2001). Despite the potential of plants to provide useful pharmaceutical agents, the field is still poorly studied. Medicinal plants represent a vast potential source for anticancer compounds by boosting the actions and reducing the toxicity of conventional chemotherapeutic drugs (El-Mesery, Al-Gayyar, Salem, Darweish, & El-Mowafy, 2009). Natural products have historically been a rich source of lead molecules in drug discovery; however, they have largely been ignored (Koehn, 2008). The inadequate understanding of the pathological and therapeutic mechanisms at a cellular level has contributed to the growing difficulty of bringing new drugs to market (Sorger & Schoeberl, 2012). The missing link between basic science and useful therapeutics is the quantitative, multifactorial understanding of networks that operate within and between cells and of the changes that drugs induce in these networks (Berger & Iyengar, 2009). A network-based approach is expected to offer important insights into the relationship between plants based active products and anticancer targets (Koiri & Mehrotra, 2018). Analysis of biological networks has contributed to the genesis of systems pharmacology, improved global understanding of drug targets, suggested new drug targets and provided a deeper understanding of the therapeutic use of phytochemicals (Berger & Iyengar, 2009).

The growth and development of healthy cells depend on fine regulation of growth-promoting- oncogenes and growth-inhibiting - tumor suppressor genes pathways. Oncogenes and tumor suppressor genes encode the proteins that regulate cell division/cell cycle, as well as for the repair of damaged DNA and cell programmed death- apoptosis. Mutations within these genes have been implicated in the onset of cancer (Hanahan &

Weinberg, 2011). Example is the p53 which is implicated in cancer, it is always converted from a tumor suppressor gene into a dominant transforming oncogene that can immortalize primary cells and bring about full transformation in combination with an activated RAS family gene. The critical role of *p53* is evident by the fact that it is mutated in over 50% of all human cancers suggesting that expression of *p53* mutants may confer a selective advantage in the development of tumors (Rivlin, Brosh, Oren, & Rotter, 2011).

The aim of this study was to extract the methanol and aqueous extracts of *Aloe secundiflora, Maytenus obscura, Vernonia zanzibarensis and Dichrostachys cinerea;* leaves and barks, and identify the active extracts against prostate cancer (Du-145, 22RV1) cell lines and cervical cancer (HeLa) cell lines. The main reason for using the plants and cell lines was to gain more insight into the potential mechanism of anti-proliferative action by evaluating the expression levels of the gene coding for *p53,* which plays a pleotropic role in cell proliferation and apoptotic regulation.

1.2 Problem Statement

Cancer ranks third among the main causes of death in Kenya after infectious and cardiovascular diseases where it accounts for approximately 18,000 deaths annually, with up to 60% of fatalities occurring among people who are in the most productive years of their life (Bray, et al., 2018) Men are commonly diagnosed with prostate or esophageal cancer while most women are most frequently affected by breast and cervical cancer (Hyuna Sung,Jacques Ferlay, et all, 2021). The control of cancer in Kenya is hampered by inadequate and expensive medication, limited specialized human resource capacity, late presentation, and diagnosis as well as a low awareness on cancer prevention and control in the population (Wambalaba, Son, Wambalaba, Nyong'o, & Nyong'o, 2019).

WHO recommends the vaccination for girls aged between 9-13 years since this is the most cost-effective public health measure against cervical cancer (WHO, 2019). However, vaccines don't protect against all HPV types that can cause cervical cancer, neither do they treat pre-existing HPV infection or HPV-associated disease (ICRH, 2013). There is

also a growing challenge among most parents, who are against the vaccination of their children. Leading to a low uptake of the vaccines.

Chemotherapy and radiotherapy treatment have provided an alternative of killing fast-growing cancer cells to avoid them multiplying than most cells in the body (Mayo Clinic, 2022). However, they are often limited by toxicity to non-targeted tissues and unfavorable side effects. Other options involve the use of immunotherapy, but experiments and practical results have proved that immunotherapy alone has an inconsistent efficacy (Wen, Thisted, Rowley, & Schreiber, 2012).

In addition, with only a handful of public hospital equipped to handle the management of cancer and a high cost involved, cancer management remains elusive for most patients in Kenya (Ngutu & Nyamongo, 2015).

1.3 Justification

The lack of diversity in the modes of treatment for cancer together with the robust economic input required for cancer treatment has led to a shift in focus from conventional medicine to traditional medicine. Chemotherapy, surgeries, and radiotherapy are still major conventional cancer therapies. However, major drawbacks can be pinpointed to these strategies despite major milestones gained in the fight against cancer. Therefore, the major need for the development novel therapies and treatment plans with higher efficacies. This will not only minimize the financial burden for the patients as well as the related side effects of some of the conventional methods of cancer treatment.

Medicinal plants offer an alternative to conventional treatment as the raw material can be locally sourced hence bring down the cost of the medication. This is because, plants have the potential to synthesize extremely complex molecular structure, some with anti-tumor activity. Currently, many cytotoxic chemotherapeutic agents in use are originally purified from herbs such as vincristine and vinblastine (from *Catharanthus roseus*) and taxol (derived from *Taxus brevifolia* (Greenwell & Rahman, 2015). Medicinal plants have also been used in cancer treatment, an example is, Egyptian plant *Moringa peregrina* that

demonstrates cytotoxic activities against breast cancer (El-Alfy TS, Ezzat SM, Hegazy AK, Amer AM, Kamel GM, 2011). Extensive screening procedures are required to identify the bioactive plant extracts and their potential mechanism of action to be elucidated for a better understanding of their potential anti-tumor activity. This then forms a basis for the present study.

1.4 Objectives

1.4.1 Main Objective

To investigate the antiproliferative activity of *Aloe secundiflora, Maytenus obscura, Vernonia zanzibarensis and Dichrostachys cinerea* using cervical and prostate cells and determine the regulation of *p53* gene by the most bioactive plant extract.

1.4.2 Specific Objectives

i. To determine the activity of extracts of *A. secundiflora, M. obscura, V. zanzibarensis and D. cinerea* against prostate and cervical cancer cell lines.
ii. To determine cytotoxicity levels of the bioactive *plant extracts* on Vero cell lines.
iii. To evaluate the mRNA expression levels of the *p53* gene in the cells treated with the bioactive plant extracts using qRT-PCR.

CHAPTER TWO

LITERATURE REVIEW

2.1 Cancer

Cancer is the name given to a collection of related diseases characterized by the abnormal proliferation of cells in the body. These cells, that have lost the ability to control both cell proliferation & differentiation into a mature cell, grow out of control and form growths called tumors. Tumors can be malignant, which means they can break off and travel to distant places in the body through the blood or the lymph system and form new tumors far from the original tumor- a process known as metastasis. Unlike malignant tumors, benign tumors do metastasis to nearby tissues. Benign tumors can sometimes be quite large. However, when removed benign tumors usually don't grow back, whereas malignant tumors sometimes grow back (Hanahan & Weinberg, 2011).Their growth sometimes cause serious and life threatening symptoms such as benign tumors in the brain (National Cancer Institute, 2022). According to WHO definition, one defining feature of cancer is rapid creation of abnormal cells that grow beyond their usual boundaries, and which with time invade adjoining parts of the body and spread to other nearby organs; this process is always referred to as metastasis and they are the primary cause of death from cancer (WHO, 2021).

2.1.1 Carcinogenesis

The process of cancer formation is referred to as carcinogenesis or tumor genesis. This is a multi-step process that begins with initiation followed by promotion and progression (Basu, 2018). These processes involve a series of epigenetic and genetic alterations that can be inherited or acquired through somatic mutations during cell division or damage to DNA caused by certain biological (specific viruses), physical (ultraviolet light, x-rays) or chemicals such as asbestos, benzene, beryllium and Cadmium (Malarkey, Hoenerhoff, & Maronpot, 2013). The genetic changes that contribute to cancer tend to affect three main

types of genes: proto-oncogenes, tumor suppressor genes, and DNA repair genes. Mutations within the proto-oncogenes and tumor suppressor genes have been largely implicated in the onset of cancer (Hanahan & Weinberg, 2011). The hallmarks of cancer are in six biological capabilities acquired during the multistep development of human tumors. They are in organizing principle for rationalizing the complexities of neoplastic disease. Sustaining proliferative signaling, growth suppressors evasion, cell death resist, enabling replicative immortality, inducing angiogenesis, and activating invasion and metastasis summarizes the six. Proto-oncogenes encode proteins that function to stimulate cell division, inhibit cell differentiation, and halt cell death. Mutations in proto-oncogenes are typically dominant in nature, and a mutated version of a proto-oncogene is called an oncogene (Chial, 2008). Oncogenes typically exhibit either increased production of these proteins or the protein itself is altered in transcription, thus leading to increased cell division, decreased cell differentiation, and/or inhibition of cell death; taken together, these phenotypes define cancer cells (Chial, 2008).

In contrast to proto-oncogene and oncogene proteins, the proteins encoded by most tumor suppressor genes serve as the "stop" signals to restrain inappropriate cell growth and division, as well as to stimulate cell death to keep cells in proper balance (Hanahan & Weinberg, 2011). In this way, tumor suppressor genes act as "brakes" to stop cells in their tracks before they can take the road to cancer. Inactivation of tumor suppressor genes, therefore, leads to tumor development by eliminating negative regulatory proteins. One of the most mutated tumor suppressor genes is the *p53* gene, which is inactivated in more than 50% of human cancers and inheritance of a mutant *p53* allele predisposes humans to the Li-Fraumeni cancer syndrome, which is characterized by the development of tumors in early adulthood (Rivlin, Brosh, Oren, & Rotter, 2011). As such, *p53* has been described as “the guardian of the genome” referring to its role in conserving genomic stability by preventing genome mutation.

p53 (also known as protein 53 or tumor protein 53), is a tumor suppressor protein that in humans is encoded by the *p53* gene located on the short arm of chromosome 17

(17p13.1). The *p53* protein is located in the cytosol and once activated it translocate into the nucleus where it acts as a nuclear transcription factor. *p53* is activated by cellular stress signals such as DNA damage, hypoxia, oncogene expression, nutrient deprivation, and ribosomal dysfunction. Once activated *p53* restricts tumor development by transactivating numerous target genes involved in the induction of apoptosis, regulation of the cell cycle and DNA repair. Under normal conditions, *p53* is expressed at an extremely low level, which is caused by proteasomal degradation mediated largely by E3 ubiquitin-protein ligase MDM2 (Toshinori Ozaki , Akira Nakagawara, 2011). The *p53*- MDM2 complex dissociates in response to DNA damage and other stress signals**,** leading to the post-translational modifications of *p53* and its subsequent translocation into the cell nucleus as represented in Figure 2.1. Other tumor suppressor genes (*APC and MADR2*) are also frequently mutated in colon cancers (Sinauer Associates, 2000).

Under conditions of low-level stress, functionally active *p53* transactivate an appropriate set of growth inhibitory genes that mediate a DNA damage-induced checkpoint to halt the progression of the cell cycle. Among these growth-inhibitory genes, the small 165 amino acid protein p21 (also known as $p21^{WAF1/Cip1}$) mediates *p53*-dependent G1 to S- phase growth arrest (Hoeferlin, Oleinik, Krupenko, & Krupenko, 2011). The p21 protein inhibits cell cycle progression primarily through the inhibition of Cyclin-dependent kinase 2 (CDK2) activity, which is required not only for the phosphorylation of retinoblastoma protein (RB) with the consequent release and activation of E2f-dependent gene expression, but also for the firing of replication origins and for the activity of proteins directly involved in DNA synthesis (Grant & Cook, 2017). During this DNA damage-induced checkpoint, *p53* can activate several DNA repair genes contain *p53* response elements and their transcription is positively regulated by *p53*.

If the damage to DNA is irreparable, *p53* triggers its pro-apoptotic mechanism to avoid the proliferation of cells containing abnormal DNA (Speidel, 2015). *p53*-triggered apoptosis involves the transcriptional induction of components of both the extrinsic and intrinsic death pathways, including BAX, FAS, NOXA, and PUMA, among others, which

collaboratively promote cell death (Brady & Laura, 2010). In the receptor-dependent or extrinsic pathway, *p53* promotes the trimerization of Fas/APO-1 (CD95) or TNF receptor 1 which activate caspase 8, which in turn starts the apoptotic machinery. In the receptor-independent or intrinsic pathway activated *p53* induces the release of cytochrome C from the mitochondrial intermembrane space into the cytosol. The cytochrome C in the presence of ATP causes the oligomerization and activation of the Apoptotic protease activating factor 1 (Apaf-1) and caspase 9 which then activate caspase 3 and other effector caspases that trigger apoptosis (Jin & El-Deiry, 2005).

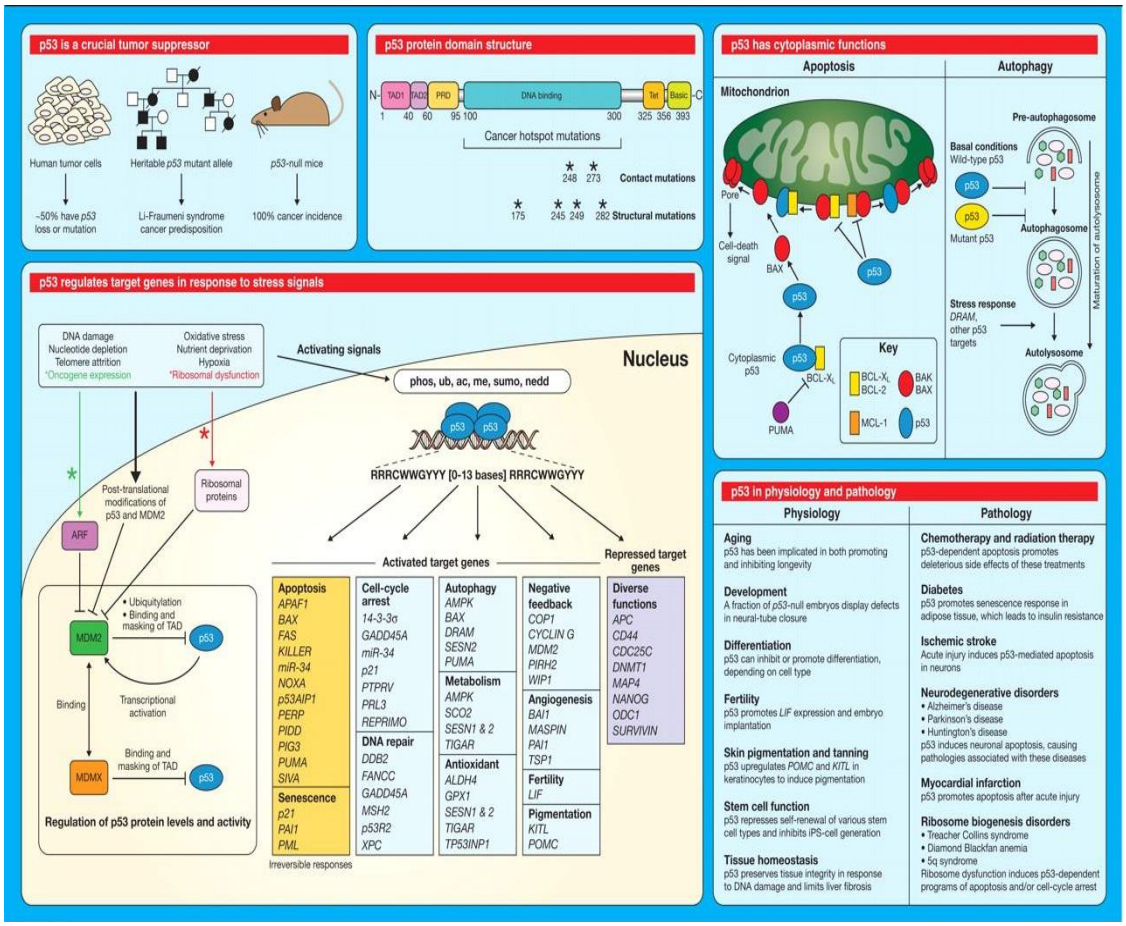

Figure 2.1: Molecular functions and mediators

2.1.2 Prostate Cancer

Prostate cancer is an adenocarcinoma of the prostate gland, that may be slow-growing, aggressively evolving and metastasizing predominantly in the bones and lymph nodes (Leslie, Soon-Sutton, & Siref, 2019). Globally, Prostate cancer is the second most common cancer in men with varied distribution across the world as shown in Figure 2.2. An estimated 1.3 million men worldwide diagnosed in 2018, accounting for 13.8% of the cancers diagnosed in men. However, prostate cancer is the leading cause of cancer death among men in Sub-Saharan Africa and the Caribbean (Bray, et al., 2018).

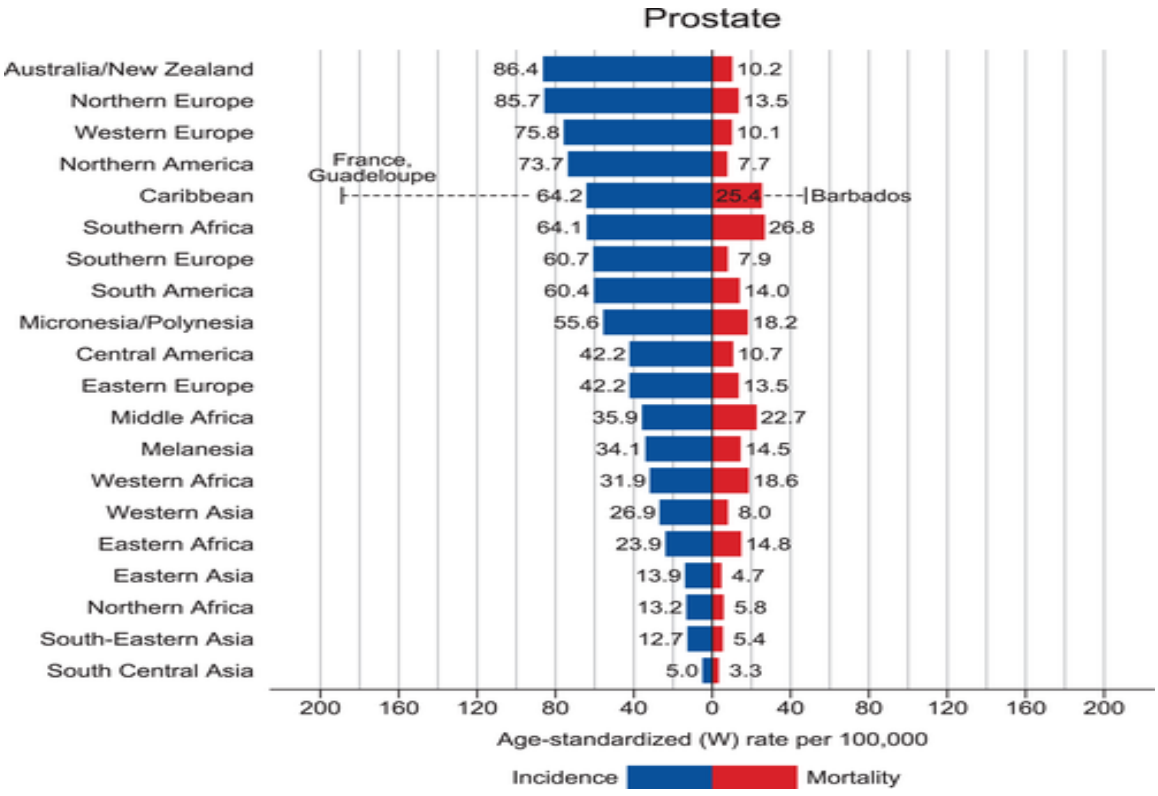

Figure 2.2: Bar chart of Region-Specific Incidence and Mortality Age-Standardized Rates for Cancers of the Prostate in 2018.

Source (Bray, et al., 2018).

In Kenya, PSA level testing, biopsy, and digital rectal examination are some of the methods used in ruling out prostate cancer (Bizna Club, 2021). In the early stages, the

majority of prostate cancers do not cause any symptoms and when symptoms occur, the signs can be quite similar to non-malignant prostatic diseases. Men with advanced prostate cancer may have local symptoms such as poor stream, frequency, and urgency of urination as well as symptoms from metastases, such as bone pain or lymphedema. Men with advanced prostate cancer may also often have systematic advanced cancer prostate symptoms, such as lethargy (due to anemia, uremia and non-specific effects), weight loss and cachexia (Gilkes, Camilla, & Ward, 2013).

Treatment of prostate cancer depends on the stage and level of the disease together with age and health status of the patient at the time of diagnosis. If prostate cancer is diagnosed, conventional treatment regimens include hormone therapy, surgical excision of the prostate (radical prostatectomy), or irradiation through external beam therapy or implantation of radioactive "seeds" (brachytherapy) (Leslie, Soon-Sutton, & Siref, 2019). In the case of advanced cancer, these regimens are usually followed or substituted with androgen deprivation therapy, which initially will reduce tumor burden and/or circulating PSA to low or undetectable levels, but ultimately the disease will recur in most cases (Fang, Merrick, & Wallner, 2010). This recurrent form of prostate cancer is s5known as androgen-independent, an essentially untreatable form of prostate cancer that ultimately progresses and metastasizes. In this aggressive type of prostate cancer, the administration of the most effective standard chemotherapeutic regimens, such as docetaxel, diethylstilbestrol, abiraterone acetate, flutamide, buserelin, leuprolide, goserelin, estramustine, and bicalutamide, only leads to a mean increase in survival time of two months (Fang, Merrick, & Wallner, 2010).

Although the specific underlying mechanisms of prostate carcinogenesis have not been unraveled yet, its proposed that a multistep process involving a series of genetic, epigenetic and environmental factors, including several susceptibility genes for inherited prostate cancer, ethnicity and family history, as well as different dietary and lifestyle factors are involved (Koochekpour, 2011). The progressive accumulation of genetic alterations is postulated to facilitate cellular transformation from normal prostate

epithelium to prostatic intraepithelial neoplasia, invasive neoplasia, and castration resistance. These genetic and epigenetic alterations may occur over many years hence increasing the risk of developing prostate cancer as a man gets older and nearly 50% of all new prostate cancers are diagnosed in men over 70 years old (Miah & Catto, 2014). These genetic and epigenetic alterations lead the normal epithelial cells into malignant cells by escaping the normal regulatory control by *p53* as well as other regulatory factors and the promotion of growth by pro-proliferative factors as shown in figure 2.3. Reports of *p53* alterations in prostate cancer specimens indicate that primary untreated prostate cancer has a lower degree of *p53* activation than more advanced disease, ranging from 0% to 79% (Testa, Castelli, & Pelosi, 2019). *p53* protein expression is an independent prognostic marker for disease-free survival after radical prostatectomy Furthermore, utilizing an adenovirus vector to transfect normal *p53* into prostate cancer cell lines, demonstrates increased apoptosis (Pisters, et al., 2004). This suggests that the upregulation of *p53* expression would then help to mitigate the effects of the genetic and epigenetic alterations of prostate epithelial cells. A focus on the difficulty and serendipity for the establishment of a new cell line can be understood by taking into consideration the extreme differences such as growth factor dependence, oxygen percentage and interaction with the stroma and immune cells that exist between the in vivo and in vitro microenvironment (Drexler H.G., Matsuo Y., MacLeod R.A.F., 2000).

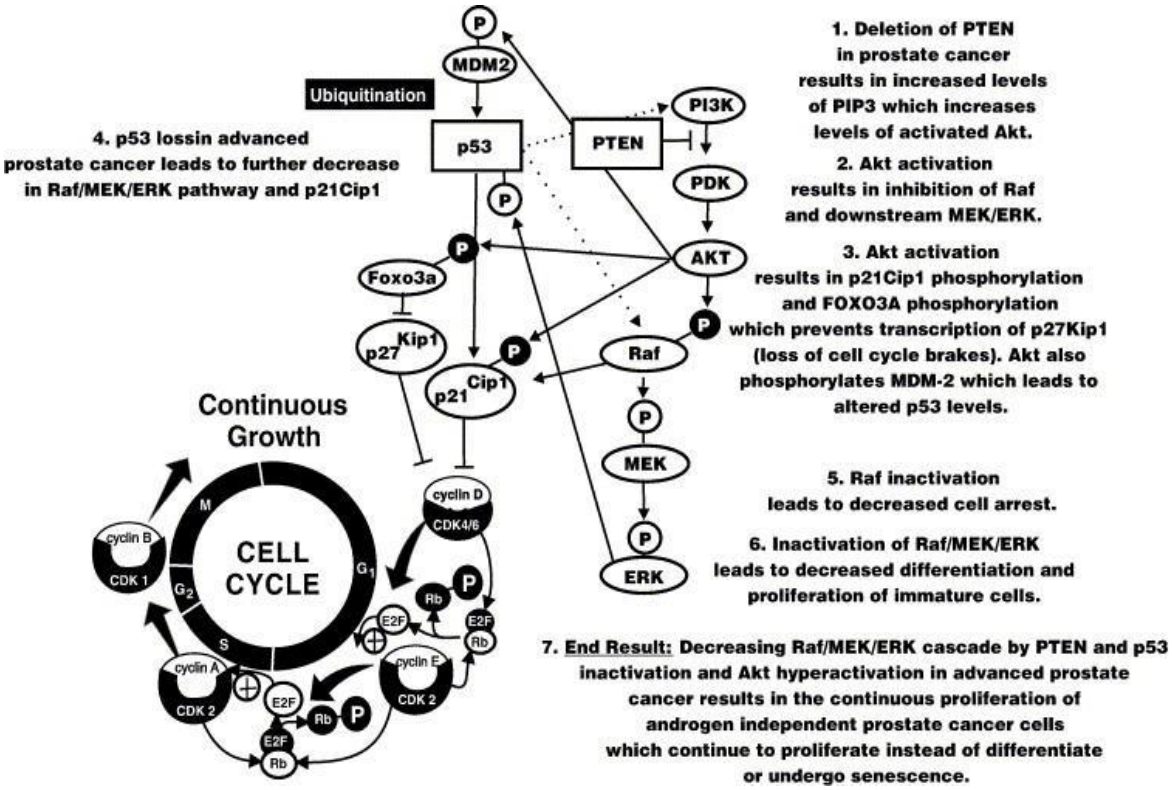

Figure 2.3: Schematic representation of pathways between p53, Raf/MEK/ERK and PI3K/Akt

Source *(McCubrey, et al., 2007)*.

2.1.3 Cervical Cancer

Cervical cancer, just like any other cancer occurs in different stages, changes, and phases. These changes may take many years before becoming invasive cancer, hence providing ample opportunity for detection and treatment. Unfortunately, although preventable using prophylactic HPV vaccines, there are still a large numbers of women who die of cervical cancer in many developing countries. The global cancer observatory through the International agency for Research on Cancer (IARC) shows that in 2020, there were 5,236 (19.7%) new cases of cervical cancer in Kenya across women of all ages (Ferlay, et al., 2021).

Cervical cancer is induced by persistent or chronic infection with one or more of the high-risk (cancer-causing or oncogenic) types of human papillomavirus (HPV) (Tulay & Serakinci, 2016). After entering cervical epithelial cells, high-risk HPV infection interferes with their normal functions, leading to changes characteristic of pre-cancer (also called dysplasia). The HPV type, immune status, co-infection with other sexually transmitted agents, tobacco smoking, parity and young age at first birth are some of the factors that are implicated in leading HPV infection to persist and progress to cancer. Seventy percent of all cervical cancer cases reported throughout the world are caused by two types of HPV subtypes: 16 and 18 (Tulay & Serakinci, 2016). Another four high-risk HPV types – 31, 33, 45 and 58 – are less commonly found to be associated with cervical cancer, with particular types being more prevalent than others in certain geographical areas (WHO, 2019).

The mechanism by which high-risk HPV causes cervical cancer has been linked to the inactivation of *p53* function hence leading to the unregulated growth of cells (Lehoux, D'Abramo, & Archambault, 2009). The transformation process of cervical cells is associated with the expression of the high-risk HPV oncogenic proteins E6 and E7 as shown in figure 2.4. High-risk HPV-derived E7 override the growth-inhibitory activities of cyclin-dependent kinase inhibitors, including the pocket protein family members, pRb, p107, and p130 and the cyclin-dependent kinase inhibitors $p21^{CIP1}$ and $p27^{Kip1,}$ leading to the maintenance of a replication-competent cellular milieu even after differentiation of host epithelial cells (Lehoux, D'Abramo, & Archambault, 2009). This situation triggers a cellular defense mechanism "trophic sentinel response" against the aberrant cellular and/or viral DNA synthesis in differentiated keratinocytes presumably lacking environmental mitogen stimulation. *p53* mediates the process of eliminating the transformed cells through cell-type-specific abortive processes including cell death, differentiation, and senescence (El Mzibri, et al., 2011). To circumvent this trophic sentinel response triggered by E7 expression, high-risk HPV-derived E6 protein forms a trimeric complex comprising E6, *p53*, and the cellular ubiquitination enzyme E6-AP,

hence stimulating the degradation of the *p53* protein (Lehoux, D'Abramo, & Archambault, 2009).

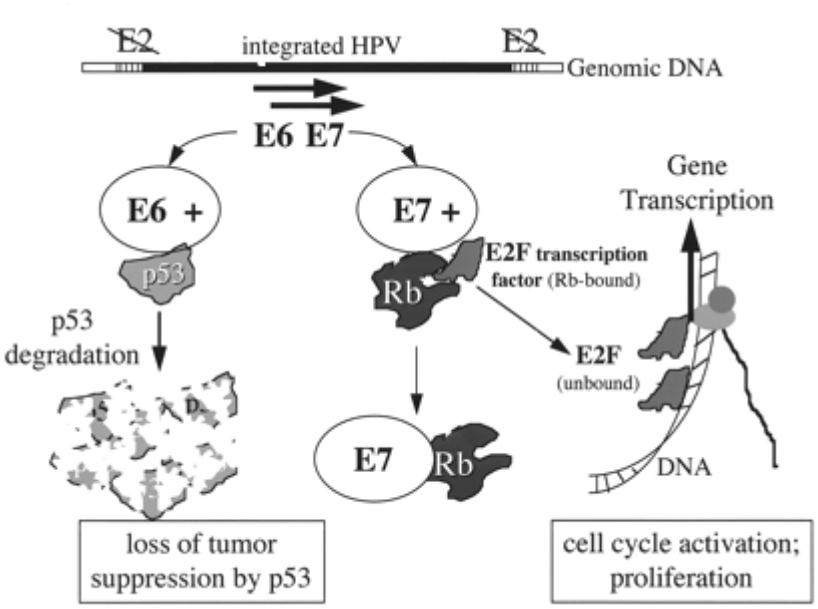

Figure 2.4: Schematic representation of the Degradation of p53

(El Mzibri, et al., 2011)

2.2 Conventional Cancer Treatment

The principal methods of cancer treatment include chemotherapy, radiotherapy, and surgery. Surgery may be used alongside other forms of treatment, but it usually precedes other forms of treatment in benign tumors (Liauw, Connell, & Weichselbaum, 2013). In theory, benign tumors can be cured if entirely removed by surgery, however complete surgical removal is usually impossible and the cancers tend to metastasize to other sites in the body (Liauw, Connell, & Weichselbaum, 2013). Radiotherapy is the use of ionizing radiation of local-regional toxicity to kill cancer cells. Radiation therapy can be administered externally via external beam radiotherapy (EBRT) or internally via brachytherapy (Moding, Kastan, & Kirsch, 2013). Particularly, it affects the rapidly dividing cells of mucosa, causing urinary discomfort and blood loss. Later toxic effects

result from damage to the more slowly proliferating cells such as fibroblasts, endothelial, or parenchymal stem cells causing chronic fibrosis and vascular damage (Chang, Lasley, Das, Mendonca, & Dynlacht, 2014). Other undesired side effects, such as immunosuppression, bone necrosis, lung fibrosis and skin de-vascularization are seen with all types of conventional therapies (Huang, Ju, Chang, Reddy, & Velmurugan, 2017)

Chemotherapy is a systemic treatment with drugs whose mechanisms of action causes cell death or prevent cell growth, generally through inhibiting microtubule function such as anti-microtubule agents, inhibiting protein function such as topoisomerase II inhibitors, inhibiting DNA synthesis such as alkylating agents and antimetabolites, or work through multiple phases of the cell cycle such antitumor antibiotics (Falzone, Salomone, & Libra, 2018). Most forms of chemotherapy drugs target rapidly dividing cells at various stages in their cell cycle. Although these drugs are not specific to cancer cells, some degree of specificity may come from the inability of many cancer cells to repair DNA damage, while normal cells generally can (Bagnyukova, et al., 2010). However, chemotherapy has the potential to harm healthy tissue, especially those tissues that have a high replacement rate (e.g. intestinal lining and immune system cells) (Mishra, et al., 2013). Various kinds of toxicities may occur as a result of chemotherapeutic treatments. For example, 5-fluorouracil, a common chemotherapeutic agent, is known to cause myelotoxicity and cardiotoxicity (Desai, et al., 2008). Other widely used chemo drugs have been known to cause cardiotoxicity, renal toxicity, myelotoxicity, bladder toxicity in the form of hemorrhagic cystitis, immunosuppression, alopecia and at high doses (Huang, Ju, Chang, Reddy, & Velmurugan, 2017).

2.3 Medicinal Plants

The connection between man and his search for drugs in nature spans decades of years with ample evidence from various sources: written documents, preserved monuments, and even original plant medicines (Petrovska, 2012). Prior to the introduction of aspirin derived from *Spiraea ulmaria*, crude extracts from the plant were already prescribed for fever and swelling in Egyptian papyri and recommended by the Greek Hippocrates for

pain and fever (Pan, et al., 2014). Until the advent of iatrochemistry in 16th century, plants had been the source of treatment and prophylaxis (Petrovska, 2012). Recently the decreasing efficacy of synthetic drugs and the increasing contraindications of their usage make the usage of natural drugs topical again with greater interest in screening for plants to be used in cancer prevention and treatment.

Conventional therapies such as chemotherapy and radiotherapy for the management of cancer have several side effects due to their lack of specificity and are limited in rural settings. Further, the sturdy resistance of cancerous cells to cytotoxic and antineoplastic drugs has presented a fresh challenge, giving unsatisfactory ministration outcomes and capricious resistance to antineoplastic agents. Coupled with the prohibitive costs, unavailability of allopathic drugs, and chronic poverty in Kenya, there is a need to fold back on home grown solutions, exploring flora and fauna. Kenya is rich in biological diversity and around 7000 species of plants have so far been recorded. Plants are regaining shape and emerging as an integral part of the ethnomedical approach for the management of diseases in Kenya. Over 5,000 phytochemicals such as phenolics, carotenoids, glucosinolates, terpenoids, and alkaloids from over 3,000 plant species have been reported to be key actors in cancer therapy (Omara, et al., 2020). Plants have a long history of use in the treatment of cancer and continue to be a major source of new drugs (Greenwell & Rahman, 2015). The scientific literature is rich in epidemiological studies that indicate a significantly lower incidence in the occurrence of cancers in populations that consume a high level of natural herbal products (Grosso, et al., 2013). Many natural products or synthetic analogs are still widely used clinically, for example, the so-called vinca alkaloids, vinblastine, and vincristine, isolated from the Madagascar periwinkle (Cragg, 2016). There is evidence to suggest that oncologists, although often unfamiliar with Complementary and alternative medicine, may take a less negative view of medicinal plant use today than they did previously (Buckner, Lafrenie, Dénommée, Caswell, & Want, 2018)

This study focused on the potential antiproliferative potential of various methanolic and water extracts from four plant species namely *Aloe secundiflora*, *Maytenus obscura*, *Vernonia zanzibarensis,* and *Dichrostachys cinerea.*

2.3.1 *Maytenus Obscura*

2.3.1.1 Description

Maytenus obscura (A. Rich.) Cuf. (*M. obscura*) belongs to the genus *Maytenus* which is distributed worldwide, particularly in riverine forest, drier forest margins and evergreen bushland of the subtropical and tropical regions of Africa, China, Brazil, Paraguay, Uruguay, and Argentina and in southern regions of Saudi Arabia (Mohamed & Perwez, 2014). The plant grows to 2.5-9 m tall and has elliptic serrate leaves while flowers are greenish cream and the fruits are ovoid-shaped (Mohamed & Perwez, 2014) .

The plant is known locally in Kenya as Muthuthi/Mutanda in Kikuyu, Muthunthi in Kamba, Kwanget/ Chepkwatet in Sebei and Abukwa in Tugen (Orwa, Mutua, Kindt, Jamnadass, & Simons, Agroforestree Database: a tree reference and selection guide. Version 4, 2009). Generally, *M. obscura* is one of the most frequently used species of the *Maytenus Molina* genus in the African traditional medicine (along with *M. senegalensis*, *M. peduncularis*, *M. putterlichwides*, *M. serrata* and *M. endata*) (Novinger, 2011).

Figure 2.5: Maytenus obscura in its natural habitat

2.3.1.2 Medicinal Use

Species belonging to the Maytenus genus are widely used in folk medicine such as antiseptic, treatment of respiratory ailments, infertility, inflammation, and antiulcer (Mohamed & Perwez, 2014). In Tropical and Southwestern Africa, *M. heterophylla* leaves are also employed in the treatment of dysmenorrhea and *M. senegalensis* leaves are used to treat toothaches, in India (Da Silva, Serrano, & Silva, 2011). The root decoction of M obscura is used as a laxative for cleaning the digestive system, juice from the leaves is given in opium poisoning as a de-addiction aid. It is also used in the palliative care of rheumatism, gout, leucoderma, paralysis, asthma and as a stimulant in nerve tonic (Deodhar and Shinde, 2015). This provides a theoretical basis for the clinical use of *Maytenus* compound as a therapeutic drug and thus further investigations through clinical trials is required.

2.3.1.3 Chemical Compounds

Phytochemical studies done on *Maytenus* obscura found that the methanol extract of the *M. obscura* leaf had phenolics, terpenoids and saponins which were associated with *in vivo* anti-inflammatory activities (Maina, et al., 2015). In another study that the ant nociceptive activity of *Maytenus obscura* (A.Rich.) at 50 mg/kg body weight in the early phase was demonstrated to be almost similar to the standard diclofenac group (Maina, et al., 2015). Studies done indicate that *Maytenus* compound exhibits significant anti-hepatocellular carcinoma effects both in vitro and in vivo by suppressing the activation of the EGFR-PI3K-AKT signaling (Zhao, et al., 2021)

2.3.1.4 Biological Activity

Maytenus, has been used for treating asthma, digestive tract illnesses and rheumatoid diseases. The diversity of the biological and pharmacological activities attributed to *Maytenus* is always associated with the presence of large numbers of chemical substances in these species, the most important of which are triterpenes, sesquiterpenes, and alkaloids.

Among the three, certain triterpenes have been demonstrated to be effective anticancer agents (Zhao, et al., 2021).

2.3.3 *Dichrostachys Cinerea*

2.3.3.1 Description

Dichrostachys cinerea (L) Wight & Arn, also called Sickle Bush or Chinese Lantern tree or Marabou thorn or Kalahari Christmas tree is a semi-deciduous shrub or small tree with an open crown; it can grow up to 8 meters tall, with occasional specimens to 12 meters. The plant is known in kiswahili as mvunja shoka, mkingiri, msigino or mkulagembe. Its native range includes parts of the eastern belt of Africa; Ethiopia, Uganda, Kenya and parts of west Africa Cameroon, Ghana, Nigeria among others (Orwa, Mutua, Kindt, Jamnadass, & Simons, 2009).

Figure 2.7: Dichrostachys cinerea in its natural habitat.

2.3.3.2 Chemical Compounds

Phytochemicals present in the stem and roots of *D. cinerea* included alkaloids, saponins and tannins, with roots containing the major share. Steroids and cyan glycoside were more present in the stem. This was after the application of chromatographic and spectroscopic

techniques to the aqueous alcoholic leaves extract of *Dichrostachys cinerea*. The results led to the isolation and identification of phenolic compounds: apigenin-7-O-apiosyl (1-2) glucoside and chrysoeriol-7-O-apiosyl (1-2) glucoside (El-Sharawy, Elkhateeb, Marzouk, & El-Ansari, 2017).

2.3.3.3 Medicinal Use

Traditionally, infusions of *D. cinerea* stem barks are used to treat rheumatism, dysentery, headaches, toothaches, and elephantiasis. Leaves and root infusions are taken for diabetes, burns, scabies, tuberculosis, leprosy, gonorrhea, boils, sore eyes, epilepsy, syphilis, coughs, anthelmintic, purgative, as a diuretic and laxative (Orwa, Mutua, Kindt, Jamnadass, & Simons, 2009).

2.3.3.4 Biological Activity

Infusions from the leaves or roots are also taken for the treatment of snakebites, scorpion stings as a local anesthesia as well as a contraceptive for women (Orwa, Mutua, Kindt, Jamnadass, & Simons, 2009). Studies on network pharmacology, and molecular docking technology of *D. cinerea* sub fractions for enzyme inhibitions and neuropharmacological activities are required and could accelerate the process of pharmaceutical development of the plant. *D. cinerea* has demonstrated both in vitro and in vivo biological activities. In vitro, biological activities exhibited include enzyme inhibition, antibacterial, anti-fungal, and antimalarial activities. In vivo activities demonstrated by *D. cinerea* include anti-inflammatory, antidiarrheal, anti-analgesic, hepatoprotective, anti-urolithiatic, anti-lice, anti-dandruff, and neuropharmacological activities.

2.3.4 *Vernonia Zanzibarensis*

2.3.4.1 Description

Vernonia zanzibarensis Less also known as; Mtukutu or Mtumbaku mwitu (kiswahili), chiwasa (Digo), Mgekwa (shambaa), is a woody herb or shrub, sometimes scandent, 1-4

meters tall (Orwa, Mutua, Kindt, Jamnadass, & Simons, 2009). Young stems are green with rusty tomentum of simple hairs while older stems are brown bark (Robinson, Keeley, Skvarla, & Chan, 2014).

2.3.4.2 Chemical Compounds

The extract of Vernonia amygdalina leaves contains a variety of substances including terpenoids, saponins, flavonoids, steroids, tannins, anthraquinone, phenols, alkaloids The phytochemical studies revealed the plant is enriched with proteins, fats, fibres, amino acids, minerals, vitamins, and carbohydrates (Ngo, 2021).

2.3.4.3 Medical Use

Traditionally made infusion of the plant is used to treat pimples, skin rashes and stomach pain (Orwa, Mutua, Kindt, Jamnadass, & Simons, 2009). However, only one study has reported anticancer activity of silver nanoparticles in MCF-7 cells and thus several studies need to be done to validate the same (Julia Joseph, Kang Zi Khor, Emmanuel Jairaj Moses, 2021). *Vernonia z* has been wildly used both as food and medicinal plants in Asia and West Africa due to its pharmacological effects, that is antioxidant, anti-diabetes, anti-inflammatory, anticancer and anti-malaria.

2.3.4.4 Biological Activity

Scientific studies have shown that the plant have different pharmacological properties including anthelmintic, anti-infection, antimicrobial, anticancer, and antispasmodic properties. Plant leaves are effective in controlling a number of chronic diseases, including glucose and lipid regulatory properties in animals and humans. In Kenya, commonly cited plant species were *Microglossa pyrifolia*, *Prunus africana* , *Cyphostemma serpens* , *Catharanthus roseus* and *Aloe volkensii* (Dominic O Ochwang'i et al, 2013).

2.3.2 *Aloe Secundiflora*

2.3.2.1 Description

Aloe secundiflora is an evergreen, succulent, perennial plant that produces a dense rosette of about 20 spear-shaped leaves that can be 30 - 75cm long and 8 - 30 cm wide at the base (Fern, 2018) (Figure 2.6). The plant can be stem-less, or with a short stem up to 30cm long; it is usually solitary, but sometimes suckers grow to form small groups (Ngumbau, et al., 2020). The plant is well adapted to dry semi-arid conditions of grassland and open woodland on sandy soil at elevations from 600 - 2,000 meters found in the tropics and subtropics of East Africa; Sudan, Ethiopia, Kenya, Rwanda and Tanzania (Fern, 2018).

Figure 2.6: Aloe secundiflora in its natural habitat.

2.3.2.2 Medicinal Use

Plants from the genus *Aloe* (family Aloaceae) have a long history of medicinal use in the treatment of a wide variety of medical disorders and conditions. In Kenya, the plant is locally known as Mugwa Nugu (Kikuyu), Eshikhaka (Luhya), Kiluma (Kamba) and Ogaka (Luo) (Bjorå, Wabuyele, Grace, Nordal, & Newton, 2015). Herbalists from the Lake Victoria region have traditionally used *Aloe secundiflora* to treat ailments including chest problems, polio, malaria and stomachache but with no knowledge of the scientific

base of their activities (Mariita, et al., 2011). The leaf sap is drunk as an appetizer and anti-emetic. Diluted leaf sap is drunk as a cure for malaria, typhoid fever, diarrhea, edema, swollen diaphragm, nosebleeds, headache, pneumonia, chest pain and a disinfectant (Newton, 2006).

2.3.2.3 Chemical Compounds

The plant constitutes phenolic compounds such as anthraquinone and chromone. The main components in the exudates from the leaf include anthrones aloenin, aloenin B, aloin A, and other aloin-derivatives (Salehi, et al., 2018). The *Aloe secundiflora is* noted to have a low content of aliphatic compounds and polysaccharides. However, they have a high percentage of anthrones in exudates Aloin present is an anthraquinone in *Aloe secundiflora* that has been proposed as a potential therapeutic option from the cancer treatment (Salehi, et al., 2018).

2.3.2.4 Biological Activity

The aloin content inhibits the endothelial growth factor (VEGF) secretion in cancer cells. VEGF is known for inducing tumors. It also inhibits tumor and angiogenesis (Salehi, et al., 2018). There are also some reports on the antimicrobial effects on pure components and obtained from *Aloe* species. It has been shown that *Aloe* leaf gel can inhibit the growth of two Gram-positive bacteria, *Shigella flexneri* and *Streptococcus progenies* (Barandozi, 2013).

CHAPTER THREE

MATERIALS AND METHODOLOGY

3.1 Study Design

The study was a laboratory based experimental study

3.2 Study Site

Plant parts of *M. obscura*, *A. secundiflora*, *D. cinerea* and *V. zanzibarensis* were collected from natural reserves in Kibwezi, Kenya and identified by a plant taxonomist

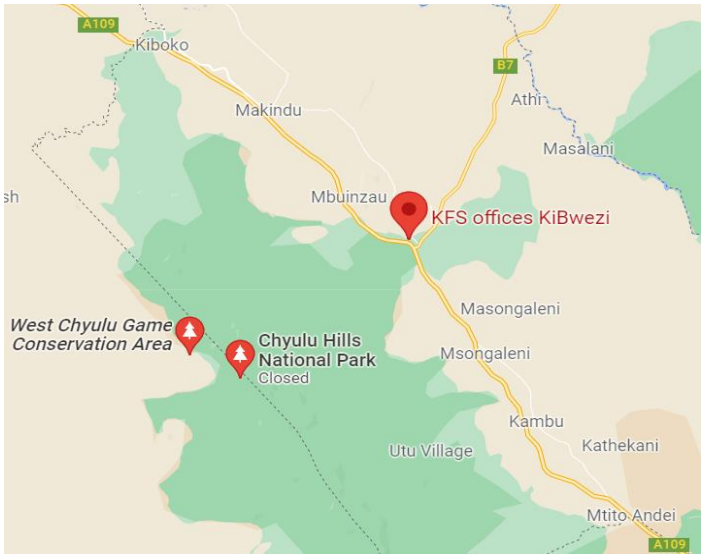

Figure 3.2: Map of the plant material collection site

The Kibwezi Forest Reserve, is part of Tsavo Conservation Area and is one of Kenya's last remaining groundwater woodlands served by the Underground Umani springs which passes through the forest and spring out in Kibwezi town (Mugambi, Kagendo, Kweyu, & Mbuvi, 2020). The forest is located in Kibwezi Sub County of Makueni County Kenya at the periphery of Chyulu ecosystem and covers an area of 5849 hectares.

Table 3.2: *M. obscura, A. secundiflora, D. cinerea* , and *V.* zanzibarensis plant parts that were collected and identified from natural reserves in Kibwezi, Kenya.

Botanical Name	Family	Voucher Number	Location	GPS Information	Parts used
A. secundiflora	Asphodelaceae	PGK/2014/015	Kibwezi	2°54'57.53"S 37°86'99.95"E	Leaves
D. cinerea	Fabaceae	PGK/2014/004	Kibwezi	2°55'53.67"S 37°76'99.90"E	Leaves & stem
V. zanzibarensis	Compositae	PGK/2014/012	Kibwezi	2°44'67.79"S 37°56'99.90"E	Leaves & stem
M. obscura	Celastraceae	PGK/2014/006	Kibwezi	2°54'57.69"S 37°86'99.90"E	Leaves & stem

3.3 Study Design

This was a Laboratory based experimental study carried out at Centre for Traditional Medicine and Drug Research (CTMDR) laboratories at Kenya Medical Research Institute (KEMRI) Nairobi, Kenya.

3.4. 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/021/3151 (Appendix 1).

3.5 Plant Material Preparation

After washing the plant parts, the selected parts were dried and ground using a laboratory mill (Christy and Norris Ltd., Chelmsford, England), weighed, packaged in brown bags and stored for further use.

3.5.1 Water Extraction

The water extraction fractions were prepared using distilled water as the solvent. Briefly, 100 ml of distilled water was added into individual flasks containing 50 grams of a plant part. The flasks were capped with cotton wool and placed into a water bath at 60°C for 2 hours. After filtering the mixture into the round bottom containers, the contents were frozen in dry ice. The frozen extracts were lyophilized in a freeze dryer machine (Edwards freeze dryer Modulyo) for 48 hours. The final product weighed and stored in a -20 °C freezer for the subsequent assays. The percentage yield of the extract was calculated using the below formular.

Plant extract Yield (%) = (W1 × 100)/W2

where

W1 = Weight of the extract residue obtained after solvent removal

W2 = Weight of dried plant material

3.5.2 Methanol Extraction

For each plant part, 50 grams of the dried-ground material was weighed and put in a conical flask. A volume of 200 ml of methanol was added to the plant material in a conical flask and capped with cotton wool to prevent evaporation of the methanol. After 24 hours the methanol containing the extract was filtered using Whatman filter paper no.1(Sigma Aldrich). The filtrate was evaporated (concentrated) to dryness under reduced pressure using a rotatory evaporator (Buchi Rotavapor R-114) with a water bath temperature set to 60°C, the cooling water temperature at 20°C and the boiling point for the solvent was adjusted to 40°C. Fresh methanol (100 ml) was added to the macerate and extracted for 48 hours. The mixture was then filtered and mixed with the previous extract after solvent evaporation. The extracts obtained were labeled and stored in a -20 °C freezer for further assays. The percentage yield of the extract was calculated using the formular above (Equation 3.4).

3.5.3 Preparation Of Test Extracts

Ten milligrams of the test extracts were dissolved in 100 µl DMSO (Dimethyl Sulfoxide) and topped up with 900 µl of PBS to make a stock solution with a final concentration of 10 mg/ml. Before using the stock solution, the mixture was filter-sterilized using 0.22 µm Millex® syringe driven filter unit. The stock solution was kept at 4°C fridge in a dark-colored Eppendorf tub

3.6 Cell Culture

3.6.1 Reagents

The study used the following cancer cell lines: human prostate carcinoma epithelial cell line; 22RV1 (ATCC®CRL-2505™) and DU145 (ATCC®HTB-81™), human cervical carcinoma cell line; Hela (ATCC®CCL-2™) and African Green Monkey Kidney Epithelial Cell line; Vero (ATCC®CCL-81™) cells. ATCC-formulated Eagle's Minimum

Essential Medium (MEM), Catalog No. 30-2003, RPMI-1640 medium and fetal calf serum (Gibco Laboratories, Grand Island, NY, USA). The MTT (3-(4,5-dimethyl thiazolyl)-2, 5-diphenyl-tetrazolium bromide), dimethyl sulfoxide (DMSO) and other chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

The base medium for the Vero, DU145 and Hela cell line was ATCC-formulated Eagle's Minimum Essential Medium (MEM), Catalog No. 30-2003. To make the complete growth medium MEM(GM-MEM),the following components were added to the base medium: heat-activated fetal bovine serum (FBS) (GIBCO, Invitrogen) to a final concentration of 10%, 1%streptomycin/penicillin (pen strep) solution (GIBCO, Invitrogen), 1.5% sodium carbonate (Na_2CO_3), 1% HEPES solution (GIBCO, Invitrogen) and 1% L-glutamine (GIBCO, Invitrogen).To make complete maintenance media MEM (MM-MEM), the following components were added to the base medium: heat-activated FBS to a final concentration of 2%, 1%streptomycin/penicillin (pen strep) solution, 2.5% Na_2CO_3, 1% HEPES solution and 1% L-glutamine.

The base media for 22RV1 cells was RPMI 1640 (Roswell park memorial institute 1640) medium (GIBCO, Invitrogen), that is already been supplemented by the supplier with glucose (4.5 g/l), L glutamine (1 mM) and sodium pyruvate (0.110 g/l). To make the complete growth medium RPMI (GM-RPMI) was augmented with 10% heat-activated FBS, 1% Pen-Strep, 1.5% $Na_2CO_3$and 1% HEPES solution. To make complete maintenance media RPMI (MM RPMI), the following components were added to the base medium: heat-activated FBS to a final concentration of 2%, 1% Pen-strep solution, 2.5% $Na_2CO_3$and 1% HEPES solution.

3.6.2 Cell Culture and Maintenance

22RV1 cells were grown aseptically in 25 cm^3 tissue culture flasks (Corning Inc) containing GM-RPMI warmed at room temperature for 2-3 minutes. Du145, Hela, and VERO cell lines were grown aseptically in 75 cm^3 tissue culture flasks (Corning Inc) containing GM-MEM pre-warmed at room temperature for 2-3 minutes. The tissue culture

flasks, containing cells with GM, were placed in a CO_2 incubator (Snijder Scientific) containing a 5% CO_2 atmosphere and a temperature of 37^{o}C. The flasks were monitored macroscopically and microscopically using an inverted light microscope (Nikon). They were monitored macroscopically to check on the color change and the turbidity of the media in the flasks to ensure that contaminated flasks were thrown away. This also ensured monitoring on whether there was contamination of the incubator and associated culture flasks. They were monitored microscopically to check on the growth of the cells. The cells were said to have formed a monolayer when they had reached the exponential growth phase and were over 80% confluent.

3.6.3 Trypsinization and Sub-culturing

Once the cells in the cell culture flask had obtained 80-90 confluence of the media in the culture flask had turned acidic the cells were subcultured. The media in the flasks was poured out and the excess media washed twice using Phosphate Buffered Saline minus (PBS-). An aliquot of 0.5 ml filter-sterilized trypsin solution (0.25% trypsin with 0.1% EDTA (ethylene diamine tetra-acetic acid) (1:1 v/v) and 0.1% glucose) (Highveld Biological) was thereafter added to the flask and gently swirled until the trypsin solution turned turbid, as an indication that the cells had detached from the flask. To stop the trypsin process, 10 ml of growth media was added into the flask and using a pipette and pipette ball the cells were purged to break the clumps. The flasks were then viewed under the inverted microscope (Nikon) to ensure that the cells were floating in the solution and that they were healthy for sub-culturing. Based on the split ratio of the cell line, the required volume of cells to be passaged was picked from the re-suspended cells and pipette into a new culture flask. This suspension was then topped up with fresh growth media up to a volume of 25 ml. the new cell culture was then placed into the CO_2 incubator (Snijder Scientific) and the cells observed periodically to check for growth and contamination.

Cell enumeration was carried out using the trypan blue dye exclusion method of Freshney (1987) on a Neubauer counting chamber (hemocytometer). The chamber was first cleaned

with 70% ethanol. The Neubauer chamber contains a grid of 4 sets of 16 squares. A cell suspension was prepared from the culture flask following the trypsin process explained above and to which 10 ml Maintenance Media had been added to stop the reaction between trypsin and membrane components of the cells. Approximately 1 ml of the suspension was placed into an Eppendorf tube then 100 µl of cell suspension from the Eppendorf tube was placed into another Eppendorf tube to which 300 µl of trypan blue dye was added and mixed properly using a pipette to give a mixture ratio of 1:3. A coverslip was placed over the chamber and 50 µl of cell suspension with trypan blue dye, was run on the edge of the grooves using a Gilson tip. The chamber was thereafter observed under the phase-contrast microscope (Nikon).

Only healthy cells with a bright appearance were counted. The cells that were blue were ignored because they had taken up trypan blue dye and were therefore considered dead according to (Freshney, 1987). The following equation was used to obtain the cell count:

- Total cell count/ml = (cells counted in four corners /4) × dilution factor × chamber factor

  Where: dilution factor = 1:3

  Chamber factor= 10^4

- Volume to pick from the neat cell suspension= volume required/ volume in the neat cell suspension

The volume picked was topped up with growth media to 1 ml

3.7 Antiproliferative Assay

3.7.1 Inhibition Concentration

This study used the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) dye reduction assay to evaluate the cell viability. The MTT assay has become the gold standard for determination of cell viability and proliferation since its development by Mosmann in the 1980′s (Mosmann, 1983). This colorimetric assay utilizes the enzymatic activity of mitochondrial succinate dehydrogenase enzyme, found in living cells, to reduces the yellow water-soluble MTT to formazan insoluble, purple-colored crystals that are measured using a spectrophotometer at 450 nm (Riss, et al., 2016). The activity of the enzymes to produce formazan is directly proportional to the level of cell viability and inversely proportional to the level of cell inhibition (WST-8 patent) (Riss, et al., 2016).

DU145, 22RV1 and HeLa cells were maintained as a monolayer culture in their respective cell culture media supplemented with 10% fetal bovine serum and incubated at 37 °C in a humidified incubator at 5 % CO_2. The three types of cells were seeded in 96-well plates to a final volume 100 µl media containing 2.4×10^4 cells per well, enumerated using the trypan blue dye exclusion method. The plates were incubated for 24 hours, then the extracts were added in row H and serially diluted up to row B in their respective wells at concentrations of 1000µg/ml, 333.33µg/ml, 111.11µg/ml, 37.04µg/ml, 12.35µg/ml and 4.12µg/ml. Row A, of the 96 well plates were left untreated to serve as a negative cell control. After 48 hours of incubation, the culture medium in the plates was discarded, followed by washing step using phosphate-buffered saline (PBS). A volume of 10 µl of PBS solution containing five µg/ml MTT dye (5 mg of MTT, dissolved in 1 ml serum-free PBS) was added to all the wells and incubated for another 4 hours after which excess dye was removed by gentle aspiration and washing with PBS followed by solubilizing the formazan crystals in 100µl of neat DMSO to improve the simplicity and sensitivity of the assay. The plates were read using a scanning multiwell spectrophotometer (Multiskan Ex

lab systems) at 450 nm. The percentage inhibition of the cells was calculated using the formular previously described (Patel, Suthar, & Patel, 2009)

Proliferation rate=(At-Ab)/(Ac-Ab)

Percentage viability=(At-Ab)/(Ac-Ab) X 100

Percentage inhibition=100-(At-Ab)/(Ac-Ab) x100

Where,

At= Absorbance value of test compound

Ab= Absorbance value of blank

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

3.7.2 Cytotoxicity Concentration

VERO cells were maintained as a monolayer culture in MEM cell culture media supplemented with 10% fetal bovine serum and incubated at 37 °C in a humidified incubator at 5 % CO_2. The cells were then seeded in 96-well plates to a final volume 100 µl media containing 2.4×10^4 cells per well, enumerated using the trypan blue dye exclusion method. The plates were incubated for 24 hours, then the selected extracts and the standard drug were added in separate wells in row H and serially diluted up to row B in their respective wells at concentrations of 1000µg/ml, 333.33µg/ml, 111.11µg/ml, 37.04µg/ml, 12.35µg/ml and 4.12µg/ml. Row A, of the 96 well plates were left untreated to serve as a negative cell control. After 48 hours of incubation, the culture medium in the plates was discarded, followed by washing step using phosphate-buffered saline (PBS). A volume of 10 µl of PBS solution containing five µg/ml MTT dye (5 mg of MTT, dissolved in 1 ml serum-free PBS) was added to all the wells and incubated for another 4 hours after which excess dye was removed by gentle aspiration and washing with PBS followed by solubilizing the formazan crystals in 100µl of neat DMSO to improve the simplicity and sensitivity of the assay. The plates were read using a scanning multiwell

spectrophotometer (Multiskan Ex lab systems) at 450 nm. The percentage inhibition of the cells was calculated using the formula above.

3.7.3 Selectivity Index

To compare the amount of the drug that caused 50% inhibitory effect to the normal cells (CC_{50}) to the amount that causes death to the cancer cells (IC_{50}), the selective index was calculated using

Selective Index (SI) = CC_{50} / IC_{50}.

Where: CC_{50}= inhibition concentration of extract in VERO cells

IC_{50} = Inhibition concentration of extract in test cells.

Samples with SI values greater than 3 were considered to have high selective between normal cells and cancer cells (Mahavorasirikul, Viyanant, Chaijaroenkul, Itharat, & Na-Bangchang, 2010)

3.8 RNA Extraction

Total RNA, from DU145 cells pretreated with IC_{50} concentration of *D. cinerea* Aq stem bark extract, was extracted using the PureLink®RNA Mini kit (Life Technologies) according to the manufacture instructions. Briefly Du145 cells, in a T25 cell culture flask, non-treated control and pretreated cells with an IC_{50} concentration (25 µg/ml) of *D. cinerea* aqueous stem bark extract in growth media MEM were incubated in a CO2 incubator (5% CO2) for 24 hours. The growth media was then poured and the cell monolayer was washed with PBS and trypsinized using 0.5 ml Lysis buffer with 2-mercaptoethanol. The flask was then gently vortexed to dislodge the cells from the flask. the lysate was transferred into an RNAse free Eppendorf tube and the sample homogenized by centrifuging it at 12,000 x g for 2 minutes. the RNA was precipitated using 70% ethanol at a ratio of 1:1 of the cell lysates. the mixture was vortexed thoroughly to mix. A volume of 700 µl of the cell homogenate was transferred into a spin cartridge

with a collection tube and the setup was centrifuged at 12,000 x g for 15 seconds at room temperature. the flow-through was discarded and the process repeated until the entire sample was processed to filter off the ethanol and trap the nucleic material in the silicon matric of the spin cartridge. The nucleic material was bound to the silicon matric by adding 700 µl of wash buffer I into the spin column with the collection tube and the setup centrifuged at 12,000 x g for 15 seconds at room temperature. The collection tubes with the flow-through were discarded and the spin cartridge placed into a new collection tube. The contaminants were taken care of by adding 500 µl of wash buffer II into the spin cartridge with the collection tube and the set up centrifuged at 12,000 x g for 15 seconds at room temperature. this process was repeated once. the spin cartridge with the collection tube was then centrifuged at 12,000 x g for 2 minutes at room temperature to dry the membrane with bound RNA. The collection tubes with the flow-through were discarded and the spin cartridge inserted into a recovery tube. The RNA was eluted using 100µl of RNase free water pipetted into the center of the spin cartridge. this setup was incubated at room temperature for 1 minute then it was centrifuged at 12,000 x g for 2 minutes at room temperature. The quality and quantity of total RNA was assessed by spectrophotometer (Nano Drop 2000, USA) and samples were stored at −80∘C until use.

3.9 cDNA Synthesis and Real Time qRT-PCR

The housekeeping gene *GAPDH* and T*P53* primer sequences used for RT-QPCR were as follows

Table 3.2*: GAPDH* and *p53* Forward and Reverse Primer Sequences for Real-time qPCR

Primer		Sequence	PCR Tm, °C
p53	Forward	5′-ATGATGATCTGGATCCACCAA-3′	56.47
	Reverse	5′-TTGGTGGATCCAGATCATCAT- 3′	56.47
GAPDH	Forward	5'-TCATGAAGTGTGTGACGTGGACATC-3'	60.1
	Reverse	5'-CAGGAGGAGCAATGATCTTGATCT-3'	58.4

The performances of all primer pairs were tested by primer concentration to determine the optimal reaction conditions. Thermal cycler conditions were 15 min at 50∘C for cDNA synthesis, 10 min at 95∘C followed by 40 cycles of 15 s at 95∘C to denature the DNA, and 1 min at 60∘C to anneal and extend the template. Melting curve analysis was performed to ascertain specificity by continuous acquisition from 60∘C to 95∘C with a temperature transient rate of 0.1∘C/Sec. All reactions were performed in triplicate in a Quant Studio™ 5 Real-Time PCR System (Thermo Scientific) and syber green dye used as the dye of the reaction. The value obtained for the T*P53* target gene expression was normalized to *GAPDH* and analyzed by the relative gene expression $2^{-\Delta\Delta CT}$ method as shown below: (Livak & Schmittgen, 2001)

Fold change = $2^{-\Delta\Delta CT}$

Where:

$\Delta\Delta Ct = \Delta Ct$ (treated sample) – ΔCt (untreated sample)

ΔCt = Ct (gene of interest) – Ct (housekeeping gene)

CHAPTER FOUR

RESULTS

4.1 Antiproliferative Assay

4.1.1 Inhibition Concentration

A total of 12 extracts from 4 plant species: *Aloe secundiflora* (leaves), *Maytenus obscura* (stem and leaves), *Vernonia zanzibarensis* (stem and leaves), *Dichrostachys cinerea* (stem and leaves) were extracted using aqueous (water) and organic (methanol) solvents (Appendix 2). Each extract was inoculated into 3 cell lines (DU145, 22RV1 and HeLa), in triplicates at various concentrations. Cytotoxic activity was demonstrated by the decrease of the metabolic activity of the cells using the MTT assay and the data was analyzed using GraphPad Prism software. Plant extracts with IC_{50}< 1 µg/ml were interplated as having high active, those with IC_{50}; 1-10 µg/ml as active and the extracts with IC_{50}; 10- 100 µg/ml as moderate activity. Extracts with IC_{50}> 100 µg/ml were considered inactive (Wang, Ge, Wang, Qian, & Zou, 2006). The results obtained were indicated in Table 4.1. The methanol extract of *D. cinerea* stem bark was found to have the most anti-proliferative activity among the 12 extracts with an IC_{50} of 8.04 ± 2.02 µg/ml on 22RV1 cells. In addition, the extract had moderate activity of 32.53 ± 5.71 µg/ml on the HeLa cells. Other extracts with moderate activity were *D. cinerea* aqueous stem bark extract on DU145 cells (22.75 ± 4.12µg/ml) and HeLa cells (83.75 ± 13.87µg/m) and *A. secundiflora* methanol extract on HeLa cells (23.19 ± 3.22µg/m). The aqueous extract of *M. obscura* stem bark had moderate activity on DU145, 22RV1 and HeLa with IC_{50} of 25.03 ± 3.14, 30.88 ± 2.55, and 23.11 ± 3.08 µg/ml respectively. While most of the extracts from *V. zanziberensis* showed no activity with IC_{50} greater than 100 µg/ml on all cell lines, the *V. zanziberensis* methanol stem bark extract had moderate activity on DU145, 22RV1 and HeLa with IC_{50} values of 92.53 ± 6.29, 73.37 ± 14.52 and 81.31 ± 10.56 µg/ml respectively. The methanol and aqueous extracts of *D. cinerea* leaves and the aqueous extract of *M. obscura* leaves all had IC_{50} greater than 100µg/ml, therefore

considered inactive against the cancer cells. The standard drug showed a high activity against the three cell lines; DU145, 22RV1 and the HeLa with IC_{50} of 25.03 ± 1.7, 9.22 ± 1.42 and 14.23 ± 1.72 µg/ml respectively.

Table 4.1: Inhibition concentration of selected plant part extract using the MTT assay on DU145, HeLa and 22Rv1 cell lines.

EXTRACT	DU145	22RV1	HeLa
D. cinerea water leaf extract	132.37 ± 76.81	225.39± 51.79	98.86 ± 36.10
D. cinerea water stem bark extract	22.75 ± 4.20	113.26± 23.83	83.75± 13.70
D. cinerea water leaf extract	147.24± 27.08	302.64 ±19.4	96.74± 31.66
D. cinerea methanol stem bark extract	104.17± 18.30	8.04 ± 2.8	32.53 ± 5.11
V. zan water leaf extract	258.26± 84.16	259.27±85.73	94.85±28.73
V. zan water stem bark extract	153.74±73.84	292.11 ± 92.3	287.83±28.42
V. zanzibarensis water leaf extract	92.53 ± 26.29	73.37 ± 42.52	81.31 ± 10.56
V. zanzibarensis methanol stem bark extract	193.22± 82.50	242.71±29.07	281.4 ± 65.23
A. secundiflora water leaf extract	246.28± 72.25	317.14± 51.15	67.05 ± 18.65
A. secundiflora methanol leaf extract	139.27± 42.85	90.42 ± 25.24	23.19 ± 3.20
M.obscura water leaf extract	159.62± 13.73	240.18± 97.91	89.21 ± 9.14
M.obscura water stem bark extract	25.03 ± 9.31	30.88 ± 10.65	23.11 ± 3.08

4.1.2 Cytotoxic Concentration

Cytotoxicity can be defined as the toxicity caused due to the action of chemotherapeutical agents on living cells. The tests are normally very important in nanoparticles as the assist in the determination of the proposed biomedical use. This involves dyes such as Alamar blue, Tryspan blue and Coomassie blue (Mukherjee, 2019). Based on inhibition concentration results above, the most bioactive extracts, *D. cinerea* methanol SB extract, *D. cinerea water SB extract* and *M. obscura* stem bark were selected to be analyzed for their 50 % cytotoxic concentration (CC_{50}) in the Vero cell line. Investigation of comparative activity of these extracts and the standard drug 5-Fluorouracil against DU145, 22RV1, HeLa and Vero cell lines indicate difference in responsiveness with a

dose-dependent cytotoxic effect (Table 4.3 and Appendix 1). Results were interpreted as plant extracts with IC_{50}< 1 µg/ml were interpolated as having high active, those with IC_{50}; 1-10 µg/ml as active and the extracts with IC_{50}; 10- 100 µg/ml as moderate activity. Extracts with IC_{50}> 100 µg/ml were considered inactive (Wang, Ge, Wang, Qian, & Zou, 2006). Extracts with the least activity against the Vero cells were most desirable. Plant extracts with high CC_{50} were *D. cinerea* water SB extract having the highest CC_{50} (812.1 ± 12.72µg/ml) and the *D. cinerea* methanol SB extract (200.4 ± 7.54µg/ml) The standard drug also had a CC_{50} greater than the maximum concentration used in the experiment.

Apart from the methanol extract of *D. cinerea* stem bark against the DU145 all the test samples had selective index greater than 3. Among the plant extracts, the aqueous extract of *D. cinerea* stem bark had the greatest SI (35.7) on the DU145, followed by the methanol extract of *D. cinerea* stem bark (24.93) on the 22RV1 (Table 4.2). The standard drug had very high SI in all the three cell lines.

Table 4.2: Anti-proliferative activity and Selectivity index of selected plant extracts and a reference drug (5-Fluorouracil) against DU145, 22Rv1, HeLa and Vero cell line.

EXTRACT	DU145		22RV1	HeLa		Vero	
	IC50	SI	IC50	SI	IC50	SI	CC50
D.cinerea stem bark water extract	22.75 ±4.12	35.69	113.26± 12.83	7.17	83.75± 13.87	9.69	812.10±12.72
D.cinerea methanol stem bark extract	104.17± 10.31	1.92	8.04± 2.02	24.93	32.53±5.71	6.16	200.40±7.54
M.obscura stem bark water extract	25.03± 3.14	7.87	30.88 ± 2.55	6.38	23.11±3.08	8.52	196.93±5.31
5-Fluorouracil	25.03±1.7	>39.95	9.22 ± 1.42	108.45	14.23±1.72	70.27	>1000

The results are presented as anti-proliferative activity at 50% mean ± SD (n=3).

4.2 RNA Extraction

Total RNA, from DU145 cells pretreated with IC_{50} concentration of *D. cinerea* Aq stem bark extract, was extracted using the PureLink®RNA Mini kit (Life Technologies) according to the manufacture instructions. A NanoDrop™ 2000 Spectrophotometer (Thermo Scientific) was used to determine the purity and quantity of RNA extracted from DU145 cells treated with IC_{50} cytotoxic concentration (25 µg/ml) of the *D. cinerea* water SB extract and a control of 0.1 % DMSO treated cell culture. The results from the RNA quantification were as shown (Appendix 3). The extracted RNA from the control and the treated cell was found to have a high purity with A260/A280 ratio of 2.1. Very pure RNA has an A260/A280 ratio of ~2.1. Anything higher than 1.8 is considered to be of acceptable purity, and a ratio of <1.8 indicates potential contamination by DNA, proteins phenol or other compounds that absorb light at 280 nm. The A260/A230 ratio of the RNA extracted was above 2.1. A ratio between 2.0 - 2.2 is considered to have pure RNA. A low A260/A280 ratio is likely due contamination with the wash solutions, chaotropic salts, phenols or protein.

4.3 Expression Assay

The *p53* gene modulation effects of IC_{50} cytotoxic concentration (25 µg/ml) of the *D. cinerea* water SB extractueous extract on the DU145 was investigated by qRT-PCR. A 0.1 % DMSO treated cell culture was used as a control for the test. The *GAPDH* gene was used as internal control and the cycle threshold time was found as shown below.

Table 4.3: qRT-PCR p53 gene expression for the treated vs untreated cells

Sample	**Genes**	
	Target (*p53*)	**Reference (*GAPDH*)**
Calibrator (untreated cells)	20.76	9.68
Test cells (D. cinera treated)	24.52	13.68

Gene expressions were shown as fold changes which are calculated by using $2^{-\Delta\Delta Ct}$ method as shown below.

Step 1: Normalise the Ct value of the target genes to the Ct values of the reference gene

- ΔCt (calibrator) = Ct (Target, cal) - Ct (reference, test)
    - ΔCt (calibrator) = 20.75 – 8.53
    - ΔCt (calibrator) = 12.22

- ΔCt (test) = Ct (Target, test) - Ct (reference, test)
    - ΔCt (test) = 24.52 – 13.68
    - ΔCt (test) = (test) = 10.84

Step II: Normalise ΔCt value of the test sample to the ΔCt of the calibrator

- ΔΔCt = ΔCt (test) - ΔCt (Calibrator)
- ΔΔCt = 10.84 – 12.22
- ΔΔCt = - 1.38

Step III: Calculate the fold difference in expression

$2^{-\Delta\Delta Ct}$

- Where ΔΔCt = - 0.23
- $2^{-\Delta\Delta Ct}$ = 2^1.38
- $2^{-\Delta\Delta Ct}$ = 2.6

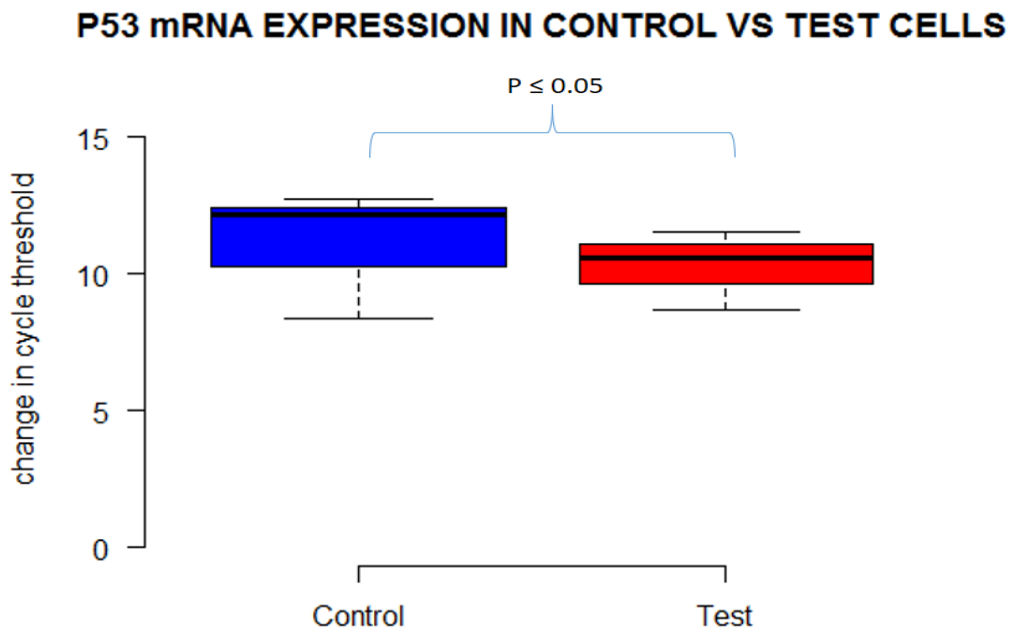

Figure 4.2: Box plot showing the change in the cycle threshold of *p53* expression

The control cells took more cycles to reach the threshold cutoff point as compared to the test cells indicating that the test cells expressed the *p53* gene. This was supported by the $2^{-\Delta\Delta Ct}$ method of gene expression analysis which indicates that *p53* was upregulated by 2.6 folds. The experiment was run in triplicates and data presented as mean ± SEM, n = 3, paired T-test value of p = 0.02 was obtained.

CHAPTER FIVE

DISCUSSION

5.1 Determination of Anti-Proliferative Activity

In recent times there has been a great interest in the search for novel medicinal products as the number of chronic conditions such as cancer develops resistance to conventional drugs. Cytotoxicity experiments are a crucial part of a modern pharmaceutical preclinical phase of drug discovery to assess a certain chemical compound's effects on a given human cell line. The tetrazolium-based MTT cytotoxicity assay is a cytotoxicity experiment for evaluating toxicity of exogenous inoculums in a time-dependent manner and it has long been regarded as the gold standard for determination of cell viability and proliferation since its development by Mosmann in the 1980′s.This study used the MTT assay to investigate the anti-proliferative potential of the aqueous and methanolic extracts of *A. secundiflora, M. obscura, V. zanzibarensis* and *D. cinerea* plant parts against two prostate cancer cell lines; DU145 and 22RV1, and one human cervical cancer cell lines; HeLa. To evaluate their potential effect on normal cells, the extracts were exposed to Vero cells. Vero cells have been recommended for cytotoxicity studies as a representative of normal cells (Kirkpatrick, 1998). A conventional drug; 5-Fluorouracil, was incorporated in the study as a standard.

The present study found that the *D. cinerea* stem bark had the highest anti-proliferative activity among the 12 plant extracts, against 22RV1 cells. However, the organic extract of the *D. cinerea* stem bark had the highest selectivity index of 1.92 against the DU145 cells. The selectivity index of a compound is a widely accepted parameter used to express a compound's in vitro efficacy in inhibition studies. Theoretically, the higher the SI ratio, the more effective and safe a drug would be during *in vivo* treatment since the drug kills the normal cells at high concentration while killing the cancer cells at low concentration. Phytochemical analysis conducted on *D. cinerea* stem bark extracts

revealed the presence of tannins, alkaloids, flavonoids, steroids and saponins (Neondo, Mbithe, Njenga, & Muthuri, 2012). Alkaloids have been associated with medicinal uses for centuries and one of their common biological properties is their cytotoxicity (Hussain, et al., 2018). Flavonoids have been proposed to initiate and promote stages of the anticancer including influences on development and hormonal activities (Kumar & Pandey, 2013). *In vivo* study on the methanol extract of *D. cinerea* (Mimoseae) leaves indicate significant hepatoprotective activity with a high LD_{50} (Babu, Krishna, Maruthi, Shankarmurthy, & Babu, 2011). Ethanol extract of aerial parts of *Dichrostachys cinerea* have also exhibited good inhibitory activity on brain tumor cell line and liver carcinoma cell line at IC_{50} of 2.41µg and 4.3µg respectively (Zeid, Hifnawy, & Mohammed, 2008)

This study observed no anti-proliferative activity of *V. zanzibarensis* plant extracts. Phytochemical screening of the extracts of vernonia species revealed the presence of steroids/terpenes, saponins, flavonoids, alkaloids, tannins and glycosides (Aliyu, Musa, Abdullahi, Ibrahimi, & Oyewale, 2011). The Vernolide-A (C21H28O7), a class of sesquiterpene lactone present in the plant *Vernonia cinerea* L. (Asteraceae) has been demonstrated to have anticancer activity (Toyang & Verpoorte, 2013). Other studied of the genus- Vernonia and have shown interesting bioactivity in antiplasmodial, antileishmanial, antischistosomal, antibacterial and anti-inflammatory assays (Dogra & Kumar, 2015)

Compounds isolated from Maytenus genus include mayteine and maytansine alkaloids that are documented as having antiproliferative activity (Da Silva, Serrano, & Silva, 2011). In the current study, the extracts of *M. obscura* had moderate antiproliferative activity against DU145, 22RV1, and HeLa. Other species of the genus Maytenus have reported antitumor activity such as *M. emarginata, M. jelskii* and *M.cuzcoina* showed effective antitumor activity (Da Silva, Serrano, & Silva, 2011). Derivatives isolated from *M. rigida* have been demonstrated to inhibit Vascular endothelial growth factor-induced Kaposi's sarcoma cell proliferation by 50% (Martucciello, et al., 2010)

A. Secundiflora showed moderate anti-proliferative activity against three cancer cell lines; DU145, 22RV1, and HeLa. Studies of the leaf exudate of *Aloe secundiflora* have revealed that it comprises a mixture of phenolic compounds, mainly anthrones (aloenin, aloenin B, isobarbaloin, barbaloin and other aloin derivatives), chromones and phenylpyrones with a low content of polysaccharides and aliphatic compounds (Waihenya, et al., 2003). Other studies have shown anti-cancer effect of aloe-emodin, an anthraquinone compound present in the leaves of *Aloe vera* (Lin & Uen, 2010)

5.2 *p53* Gene Expression.

Analysis of the molecular mechanism underlying the anti-proliferative activity of the plant extract was done with a focus on the expression of *p53* gene in DU145 cells treated with *D. cinerea* stem bark water extract that had the highest selectivity index against the DU145 cells. The *p53* gene was chosen for analysis as a potential molecular target of the extract due to its pleiotropic role in control of apoptosis, DNA repair, and senescence. The expression of *p53* was analyzed using $2^{-\Delta\Delta Ct}$ method to quantify relative changes RNA expression in the control group versus the test group.The expression of the *p53* gene in test cells was found to be elevated by 2.6 fold. Given that tumor suppressors such as *p53* are frequently downregulated or mutated with loss-of-function, upregulating or restoring their normal functions to treat cancer holds tremendous therapeutic potential. The upregulation of *p53* gene in cancer cells can be a vital key to the regulation of the cancer cells as this may promote the death of irreversibly damaged cells as well as seek to repair the cells undergoing active transition. The results of this study indicate that the aqueous extract of *D. cinerea* stem bark promotes the upregulation of *p53* which correlates with decreased cell growth as evidenced in the cell's decreased ability to breakdown the MTT dye in the study. In other studies, the increased expression of *p53* in cancer cells correlated with enhancement of apoptosis and cell cycle arrest as observed in *Gleditsia sinensis* thorns- used as a medicinal herb, which showed a decrease in cell growth and an increase in cell cycle arrest during the G2/M-phase arrest with increased *p53* levels

CHAPTER SIX

CONCLUSIONS

This research demonstrated the anti-proliferative activity of extracts from *Aloe secundiflora, Maytenus obscura, Vernonia zanzibarensis* and *Dichrostachys cinerea* against Hela, DU-145 and 22RV1 cell lines. The phytochemicals present in the tested extracts were probably responsible for the observed anti-tumor activity. Analysis of the most bioactive extract against Vero cell line demonstrate its potential to be a good candidate for use in prostate cancer treatment after the isolation and determination of the active phytochemical in the plant extract. These results rationalize the ethno-medicinal use of the medicinal plants as the water extract of *D. cinerea* stem bark demonstrated a 2.6-fold upregulation of *p53* and thus potentially increases apoptosis in Du145 cells treated with the extract as compared to non-treated Du145 cells.

RECOMMENDATIONS

Anti-proliferative activity of medicinal plants is usually attributed to the phytonutrients they contain. The study indicates that the *D. cinerea* extract had the most promising activity among the four plant extracts. This activity can be further enhanced by isolating/purifying the active ingredient to reduce the potential antagonistic effect of the crude extract. It should be noted that the results of this research to do not rule out the potential of all the extracts to inhibit cancer cell and be consider for further scientific studies in different cell lines and animal models. Plants of the same species may contain different concentrations of phytochemicals owing to their different environmental conditions (soil, climate, exposure to stress) which may lead to their synthesis of varying concentrations of phytochemicals (Altemimi A. , Lakhssassi, Baharlouei, Watson, & Lightfoot, 2017). Research on these plants needs to be conducted from a wider geographical area. Furthermore, isolation and characterization of bioactive compound(s) may promise the discovery of new and valuable drugs candidate to tackle various human diseases.

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APPENDICES

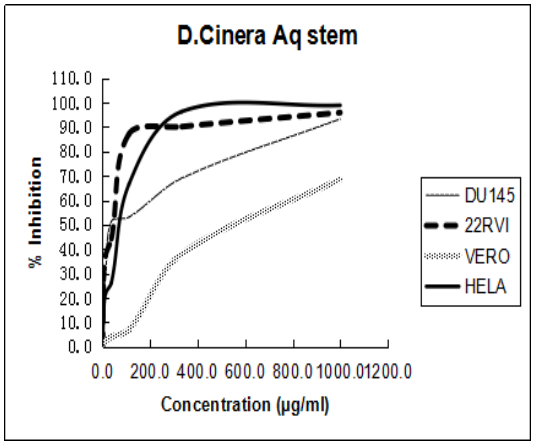
Appendix I: M. obscura, A. secundiflora, *D. cinerea* , and V. zanzibarensis plant parts that were collected and identification from a natural reserve in Kibwezi, Kenya.

Botanical Name	Family	Voucher number	Location	GPS Information	Parts used
A.secundiflora	Asphodelaceae	PGK/2014/015	Kibwezi	2°54'57.53"S 37°86'99.95" E	Leaves
D. cinerea	Fabaceae	PGK/2014/004	Kibwezi	2°55'53.67"S 37°76'99.90" E	Leaves & stem
V.zanzibarensis	Compositae	PGK/2014/012	Kibwezi	2°44'67.79"S 37°56'99.90" E	Leaves & stem
M. obscura	Celastraceae	PGK/2014/006	Kibwezi	2°54'57.69"S 37°86'99.90" E	Leaves & stem

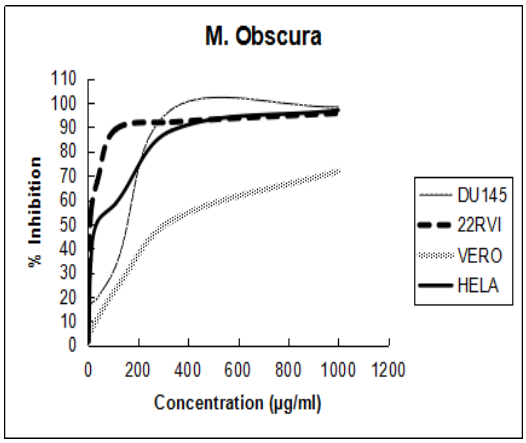
Appendix II: Selected plant species and their organic and aqueous extract yields in grams

Plant	Plant part	Extraction solvent	Weight before extraction	Weight after extraction (Grams)	Percentage Yeild
D cinerea	leaves	Water	50	0.8	1.6
		Methanol	50	1.25	2.5
	stem	Water	50	0.7	1.4
		Methanol	100	1.83	1.83
V zanzibarensis	leaves	Water	50	0.7	1.4
		Methanol	50	1.25	2.5
	stem	Water	50	0.8	1.6
		Methanol	100	1.5	1.5
Aloe secundiflora	leaves	Water	25	0.325	1.3
		Methanol	25	0.225	0.9
Maytenus obscura	leaves	Water	50	0.85	1.7
	stem	Water	100	2.5	2.5

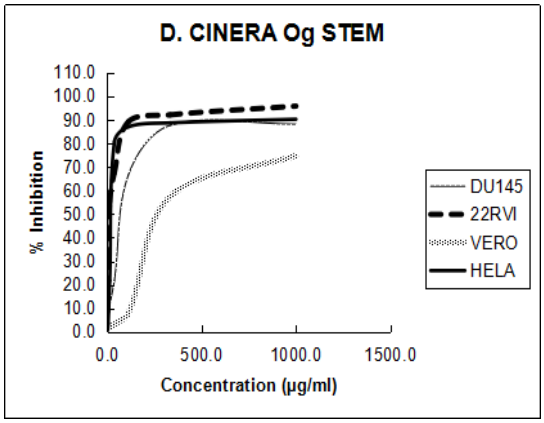
Appendix III: Dose response curves of DU145, 22RV1, VERO and HeLa cells treated with selected plant extracts.



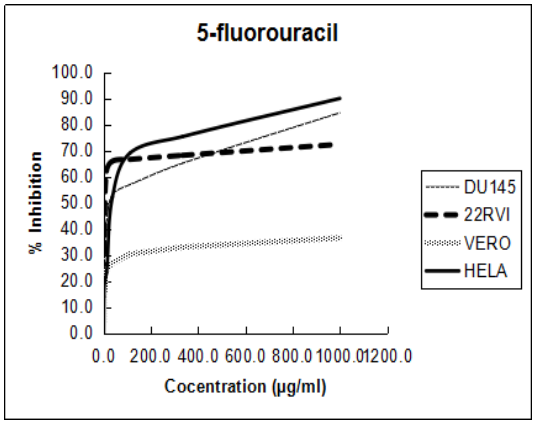
a)



b)



c)



d)

Appendix IV: Quantity and purity of RNA extracted from DU145 cells treated with IC50 cytotoxic concentration (25 µg/ml) of the *D. cinerea* water SB extract extract and a control of 0.1 % DMSO treated cell culture.

	RNA concentration (µg/µl ± sd)	**A260/280**	**A260/230**
Treated	256.7± 4.1	2.10	2.10
Control	175.2 ± 2.5	2.10	2.11

Appendix V: The *p53* and housekeeping gene *GAPDH* cycle threshold values of the triplicate runs of the Du145 cells treated with *D. cinerea* stem bark water extract (Test) vs the non-treated Du145 cells (Control)

	Control		**Test**	
	p53 target	*GAPDH*	*p53* target	*GAPDH*
	20.79	8.65	24.45	13.61
	22.24	9.49	24.74	12.41
	19.25	10.9	24.44	15.02
Average Ct	20.76	9.68	24.54	13.68

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In Vitro Anti-Proliferative Activity of Selected Plant Extracts Against Cervical and Prostate Cancer Cell Lines

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Abstract

Prostate and cervical cancers are among the leading cancers in men and women respectively. In 2012 the world health organization reported an estimated 266,000 deaths from cervical cancer worldwide, while 1.1 million men worldwide were diagnosed with prostate cancer. The current conventional cancer therapies, chemotherapy and radiotherapy, present severe side effects and in many developing countries are inaccessible to many cancer patients. The alternative traditional medicine offers the much-needed hope. This study investigated the anti-proliferative activity of methanolic and water extracts from four plant species namely *Aloe secundiflora*, *Maytenus obscura*, *Vernonia zanzibarensis* and *Dichrostachys cinerea* using prostate cancer cells (DU145 and 22Rv1), cervical cancer cells (HeLa) and African green monkey cells (Vero) cell lines using the MTT assay. All extracts suppressed the growth of the cancer cells in a dose-dependent manner at concentrations of 1.37 µg/ml to 1000 µg/ml. The methanol extract of *D. cinerea* stem bark had the highest anti proliferative activity among the plant extracts studied with an IC50 of 8.04 ± 2.02 µg/ml against the 22Rv1 cells and a low cytotoxic effect against the Vero cells with CC50 of 812.1 ± 12.72 µg/ml. The study indicates that the methanol extract of *D. cinerea* stem bark has potential anti-proliferative activity with low cytotoxicity to normal cells. Our results validate the ethnomedicinal use of these plants for management of cancer. The active elements in the extracts studied here need to be isolated and purified to investigate the synergy and additive pharmacological effect in killing cancer cells.

Keywords: Cancer; Anti proliferation; Extract; Cytotoxicity; DU145; Vero; 22Rv1; HELA; IC50

Introduction

Cancer is a significant disease worldwide with considerable geographical variations in incidence, mortality and survival. As of 2012, there were 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living with cancer worldwide [1].

Surgery is often the first treatment option. However, the simple excision with extensive resection does not prevent future recurrence and metastasis [2]. Chemotherapy and radiotherapy are alternative measures of cancer management. However, they are often limited by the toxicity to non-target tissues in the body [3]. The alternative traditional medicines offer the much-needed hope. Plants hold a vast cheap potential source for new anticancer compounds with the reduced toxicity of conventional chemotherapeutic drugs [4].

Herbalists from the Lake Victoria region of Kenya have traditionally used *A. secundiflora* to treat various ailments such as chest problems, polio, malaria and stomach ache but with little scientific knowledge on their modes of action [5]. Plants of the genus *Maytenus* have antiseptic, antiasthmatic, fertility-regulating agents and antiulcer properties [6]. The *M. buchananii* contain the compound Maytansine, a highly potent microtubule inhibitor [7]. The traditional healers from Nairobi use *M. obscura*, and *M. buchananii* extracts for the palliative care of cancer patients discharged from the hospitals [8]. In Kenya and Tanzania, the *Vernonia zanzibarensis* extracts are used to treat pimples, skin rashes and stomach pains [9]. Extracts from *Dichrostachys cinerea* bark and leaves are active against pathogens causing diseases such as dysentery, elephantiasis, gonorrhoea and boils [10].

We hypothesized that the methanolic and water extracts from *Aloe secundiflora*, *Maytenus obscura*, *Vernonia zanzibarensis* and *Dichrostachys cinerea* have anti-proliferative activity. The extracts *in vitro* anti-proliferative activity against DU145 and 22Rv1 (Prostate cancer), HeLa (cervical cancer) and Vero (African green monkey), cancer cells was investigated using the MTT assay. In this study we found significant anti-proliferative activity of the organic extract of *D. cinerea* stem bark against the 22Rv1 extracts.

Materials and Methods

Reagents

The study used the following cancer cell lines: human prostate carcinoma epithelial cell line; 22Rv1 (ATCC®CRL-2505TM) and DU-145 (ATCC®HTB-81TM), human cervical carcinoma cell line; Hela (ATCC®CCL-2TM) and African Green Monkey Kidney Epithelial Cell line; Vero (ATCC®CCL-81TM) cells. ATCC-formulated Eagle's Minimum Essential Medium (MEM), RPMI-1640 medium and fetal calf serum (Gibco Laboratories Grand Island, NY, USA). The MTT (3-(4,5-dimethyl thiazolyl)-2, 5-diphenyl-tetrazolium bromide), dimethyl

sulfoxide (DMSO) and other chemicals used were purchased from Sigma Chemical Co. (St. Louis, MO, USA).

Collection and preparation of plant material

Plant material of *M. obscura*, *A. secundiflora*, *D. cinerea* and *V. zanzibarensis* were collected from a natural reserve in Kibwezi, Kenya and identified by a plant taxonomist. After washing the plant parts, the selected components were dried and ground using a laboratory mill (Christy and Norris Ltd., Chelmsford, England), weighed, packaged in brown bags and stored for further use.

Aqueous extraction

The aqueous fractions were prepared using distilled water as the solvent. Briefly, 100 ml of distilled water was added into individual flasks containing 50 grams of the plant part. The flasks were capped with cotton wool and placed into a water bath at 60°C for 2 hours. After filtering the mixture into the round bottom flask, the contents were frozen in dry ice. The frozen extracts were lyophilized in a freeze dryer machine (Edwards freeze dryer Modulyo) for 48 hours. The final product weighed and stored in a -20°C freezer for the subsequent assays.

Organic solvent extraction

For each plant part, 50 grams of the dried-ground material were weighed and put in a conical flask. A volume of 200 ml of methanol was added to the plant material in a conical flask and capped with cotton wool to prevent evaporation of the methanol. The methanol containing the extract was filtered after incubating the mixture at room temperature for 24 hours. The filtrate was evaporated (concentrated) to dryness under reduced pressure using a rotatory evaporator set at 60°C. Fresh methanol (100 ml) was added to the macerate and extracted for 48 hours. The mixture was then filtered and mixed with the previous extract after solvent evaporation. The extracts obtained were labelled and stored in a -20°C freezer for further assays.

Preparation of test extract

10 mg of the test extracts were dissolved in 100 µl DMSO and topped up with 900 µl of PBS to make a stock solution with a final concentration of 10 mg/ml. Before using the stock solution, the mixture was filter sterilized using 0.22 µm Millex® syringe driven filter unit. The stock solution was kept at 4°C fridge in a dark-colored Eppendorf tube.

Anti-proliferative assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) dye reduction assay (Sigma, USA) was used to evaluate the cell viability. This colorimetric assay utilizes the enzymatic activity of mitochondrial succinate dehydrogenase enzyme found in living cells. The enzyme reduces the yellow water-soluble MTT to formazan insoluble purple colored crystals that are measured using a spectrophotometer [11,12]. The activity of the enzymes to produce formazan is directly proportional to the level of cell viability and inversely proportional to the level of cell inhibition (WST-8 patent) [13].

Cells were maintained as a monolayer culture in their respective cell culture media supplemented with 10% fetal bovine serum and incubated at 37°C in a humidified incubator at 5% CO_2. The cells were seeded in 96-well plates to a final volume 100 µl media containing 2.4 × 10^4 cells per well, enumerated using the trypan blue dye exclusion method. The plates were incubated for 24 hours, then the extracts were added in row H and serially diluted up to row B in their respective wells at concentrations of 1000 µg/ml, 333.33 µg/ml, 111.11 µg/ml, 37.04 µg/ml, 12.35 µg/ml and 4.12 µg/ml. Rows A, of the 96 well plates were left untreated to serve as a negative cell control. After 48 hours of incubation, the culture medium in the plates was discarded, followed by washing step using phosphate buffered saline (PBS). A volume of 10 µl of PBS solution containing 5 mg/ml MTT dye (5 mg of MTT, dissolved in 1 ml serum-free PBS) was added to all the wells and incubated for another 4 hours after which 100 µl of 100% DMSO was added. The plates were read using a scanning multiwell spectrophotometer (Multiskan Ex lab systems) at 562 nm and 690 nm as reference. The proliferation rate of the cells was calculated using the formula by Patel [12].

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

$$Percentageviability = \frac{At - Ab}{Ac - Ab} \times 100$$

$$Percentageinhibition = 100 - \frac{At - Ab}{Ac - Ab} \times 100$$

Where,

At = Absorbance value of test compound

Ab = Absorbance value of blank

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

Results and Discussion

Plant products have been used in traditional medicine to manage a variety of diseases including cancer. Despite the potential of plants to provide useful pharmaceutical agents, the medicinal potential of various herbal drugs is not yet scientifically studied. In recent times there has been a great interest in the search for novel medicinal plant products as the number of chronic diseases such as cancer develop resistance to conventional drugs. Plants represent a vast potential source for anticancer compounds that can boost the activity of traditional chemotherapeutic drugs [4].

This study reports for the first time the anti-proliferative potential of four plant species namely *A. secundiflora*, *M. obscura*, *V. zanzibarensis* and *D. cinerea*. Methanolic and aqueous extracts obtained from the four plants were tested for their anti-proliferative potential against two prostate cancer cell lines; DU145 and 22Rv1, and one human cervical cancer cell lines; HeLa. The cytotoxicity of extracts is evaluated using the Vero cells as a representative of the normal cells [14,15]. Thus, we evaluated the effect of our extracts on Vero cells with 5-Fluorouracil as a reference drug.

Total of 12 extracts from 4 plant species: *Aloe secundiflora* (leaves), *Maytenus obscura* (stem and leaves), *Vernonia zanzibarensis* (stem and leaves), *Dichrostachys cinerea* (stem and leaves) were extracted using aqueous (water) and organic (methanol) solvents. Extracts at different concentrations in a triplicate experiment per selected extract were inoculated into the three cell lines (DU145, 22Rv1 and HeLa) (Table 1). We interpreted the results as described according to Wang [16] plant extracts with IC50< 1 µg/ml described as highly active, those with IC50; 1-10 µg/ml as active and the extracts with IC50; 10-100

µg/ml as moderately active. Extracts with IC50>100 µg/ml were considered inactive.

The methanol extract of *D. cinerea* stem bark was found to have the highest anti-proliferative activity among the 12 extracts with an IC50 of 8.04 ± 2.02 µg/ml on 22Rv1 cells. This activity was higher than the reference drug (9.22 ± 1.42 µg/ml) against the 22Rv1 cells (Table 2). Other extracts with moderate activity were *D. cinerea* aqueous stem bark extract on DU145 cells (22.75 ± 4.12 µg/ml) with high anti-proliferative activity than the reference drug (25.03 ± 1.7 µg/ml). Traditionally *D. cinerea* stem bark is used for the treatment of dysentery, headaches, toothaches and elephantiasis. The leaves of *D. cinerea* are used in the treatment of epilepsy, as a diuretic and laxative [10].

Plants of the genus *Maytenus* are used in South America to prepare infusions or decoctions as anti-inflammatory and analgesic remedies for topical and oral administration. Compounds isolated from the *Maytenus* genus include mayteine and maytansine alkaloids that are documented as having anti-proliferative activity [17]. In the current study the aqueous extract of M. obscura stem bark had moderate activity on DU145, 22Rv1 and HeLa with IC50 of 25.03 ± 3.14, 30.88 ± 2.55, and 23.11 ± 3.08 µg/ml respectively. The aqueous extract of M. obscura leaves had less activity as compared to the stem bark extract, with an IC50 of 159.62 ± 13.73, 240.18 ± 17.91 and 89.21 ± 9.14 µg/ml on DU145, 22Rv1 and HeLa respectively.

Extract	DU145	22Rv1	HeLa
D. cinerea Aq Leaves	132.37 ± 6.81	225.39 ± 21.79	98.86 ± 6.31
D. cinerea Aq stem bark	22.75 ± 4.12	113.26 ± 12.83	83.75 ± 13.87
D. cinerea Og leaves	147.24 ± 22.08	302.64 ± 19.43	96.74 ± 11.66
D. cinerea Og stem bark	104.17 ± 10.31	8.04 ± 2.02	32.53 ± 5.71
V. zan Aq Leaves	258.26 ± 14.16	259.27 ± 20.73	94.85 ± 18.73
V. zan Aq stem bark	153.74 ± 13.84	292.11 ± 22.37	287.83 ± 28.42
V. zan Og Leaves	92.53 ± 6.29	73.37 ± 14.52	81.31 ± 10.56
V. zan Og stem bark	193.22 ± 12.50	242.71 ± 19.07	281.4 ± 25.23
A. sec Aq	246.28 ± 12.25	317.14 ± 2.15	67.05 ± 8.65
A. sec Og	139.27 ± 22.85	90.42 ± 5.24	23.19 ± 3.22
M. obscura Aq Leaves	159.62 ± 13.73	240.18 ± 17.91	89.21 ± 9.14
M. obscura Aq stem bark	25.03 ± 3.14	30.88 ± 2.55	23.11 ± 3.08

Key: Aq: Aqueous extract, Og: Methanol extract, Zan: *Zanzibarensis*, Sec: *Secundiflora*

Table 1: Inhibition concentration 50% (IC50) of extracts of *Aloe secundiflora*, *Maytenus obscura*, *Vernonia zanzibarensis* and *Dichrostachys cinerea* extracts inoculated in DU145, HeLa and 22Rv1 cell lines. The results presented as Inhibition concentration at 50% mean ± SD (n=3).

While most of the extracts from *V. zanzibarensis* showed no activity with IC50 greater than 100 µg/ml on all cell lines, the methanol extract of the stem bark had moderate activity on DU145, 22Rv1 and HeLa with IC50 values of 92.53 ± 6.29, 73.37 ± 14.52 and 81.31 ± 10.56 µg/ml respectively. Traditionally, a decoction of the *V. zanzibarensis* roots are used as a remedy for pain in the spleen or kidneys, relieve strangulated hernia and for chest diseases while the leaves are powdered, mixed with hot water and drunk twice a day for coughs [18,19].

For the *A. secundiflora* only the leaves were used for this study. Traditionally the diluted leaf sap is drunk as a cure for malaria, typhoid fever, diarrhea, oedema, swollen diaphragm, nose bleeding, headache, pneumonia, chest pain and as a disinfectant. The significant components of the leaf exudate of *Aloe secundiflora* are the anthrones aloenin, aloenin B, aloin A (barbaloin) and other aloin-derivatives [20]. Barbaloin possesses anti-inflammatory, antibacterial, antiviral and anticancer activities mediated by the anti-oxidative capacity [21-25]. In the current study both the methanolic and aqueous extracts of the *A. Secundiflora* showed moderate anti-proliferative activity against the three cancer cell lines; DU145, 22Rv1 and HeLa.

We evaluated the 50% cytotoxic concentration (CC50) of extracts of *D. cinerea* stem bark Og, *D. cinerea* stem bark Aq and *M. obscura* stem bark Aq, against the Vero cell line. The relative activity of the extracts and the reference drug 5-Fluorouracil against DU145, 22Rv1, HeLa and Vero cell lines indicate a dose-dependent cytotoxic effect (Figure 1).

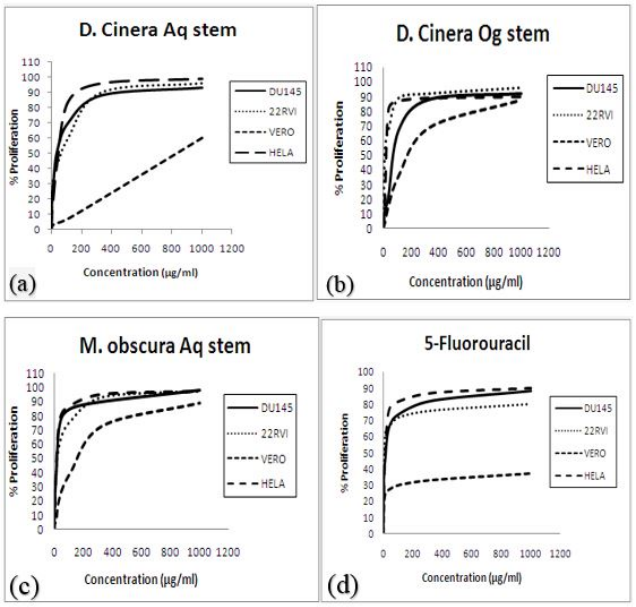

Figure 1: The dose-response curves showing the percentage inhibition of DU145, HeLa, 22Rv1 and Vero cell lines against the concentration of extracts from; *Dichrostachys cinerea* Aq stem **(a)**, *Maytenus obscura* Aq stem bark **(b)**, *Dichrostachys cinerea* Og stem **(c)**, and a reference drug, 5- Fluorouracil **(d)**.

Extracts with the least activity against the Vero cells were described to have the least toxicity to normal cells. The aqueous extract of *D. cinerea* stem bark had low toxicity to Vero cell; with CC50 of 812.1 ± 12.72 µg/ml, followed by the methanol extract of *D. cinerea* stem bark (200.4 ± 7.54 µg/ml). This study indicates that the *D. cinerea* stem bark has potential anti-proliferative activity with low cytotoxicity to normal cells. Phytochemical analysis conducted on *D. Cinerea* leaves, and stem bark extracts reveal the presence of tannins, Alkaloid, flavonoids, steroids and saponins (Johnstone et al., 2012). Several alkaloids have

been shown to have *in vivo* and *in vitro* anti-proliferative activity, while others such as Camptothecin and Vinblastine, have already been successfully developed into anticancer drugs [26]. Flavonoids have been proposed to prevent the initiation stages of carcinogenesis by inhibiting ornithine decarboxylase induced by tumor promoters causing a subsequent decrease in polyamine and inhibition of DNA and protein synthesis [27]. *In vivo* study on the methanolic extract of *D. cinerea* leaves indicate significant hepatoprotective activity with a high LD50 [28].

The selectivity index is a comparison of the amount of a test compound that causes 50% inhibitory effect to the standard cells (CC50) to the amount that causes death to the cancer cells (IC50). The selective index was calculated using the formulae: Selective Index (SI)=CC50/IC50. Samples with SI value greater than three were considered to be highly selective to cancer cells against normal cells [29]. All the test samples apart from the methanol extract of *D. cinerea* stem bark (1.92) had a selective index greater than 3 (Table 2). Among the plant extracts, the aqueous extract of *D. cinerea* stem bark had the most significant SI (35.7) on the DU145, followed by the methanol extract of *D. cinerea* stem bark (24.93) on the 22Rv1. The reference drug had SI>30 in all the three cell lines [30-37].

Extract	DU145		22Rv1		HeLa		Vero
	IC50	SI	IC50	SI	IC50	SI	CC50
D. cinerea stem bark Aq	22.75 ± 4.12	35.7	113.26 ± 12.83	7.17	83.75 ± 13.87	9.7	812.10 ± 12.72
D. cinerea stem bark Og	104.17 ± 10.31	1.92	8.04 ± 2.02	24.93	32.53 ± 5.71	6.16	200.40 ± 7.54
M. obscura stem bark Aq	25.03 ± 3.14	7.87	30.88 ± 2.55	6.38	23.11 ± 3.08	8.52	196.93 ± 5.31
5-Fluorouracil	25.03 ± 1.7	39.95	9.22 ± 1.42	108.45	14.23 ± 1.72	70.27	>1000
Key: Aq: Aqueous extract, Og: Methanol extract							

Table 2: Anti-proliferative activity and selectivity index of *Dichrostachys cinerea* Aq stem, *Dichrostachys cinerea* Og stem, *Maytenus obscura* Aq stem bark extracts and a reference drug against DU145, 22Rv1, HeLa and Vero cell line. The results are presented as anti-proliferative activity at 50% mean ± SD (n=3).

Conclusion

The present study supports the ethno-medicinal use of medicinal plants: *D. cinerea*, *A. secundiflora* and *M. obscura* in prostate cancer cell lines (Du145, 22Rv1) and cervical cancer cell line (HeLa) used in this study [38-43]. This study suggests wisdom in the use of these plants for further investigation in developing efficient, specific and non-toxic anticancer drugs that are affordable for developing countries.

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