IN VITRO ANTIPROLIFERATIVE ACTIVITY OF AQUEOUS ROOT BARK EXTRACT OF Cassia abbreviata (Holmes) Brenan

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DECLARATION

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

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DEDICATION

To the almighty God
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ABBREVIATIONS AND ACRONYMS

% Percentage
µL Micro litres
°C Degrees Celcius
AIDs Acquired Immunodeficiency Syndrome
ANOVA Analysis of Variance
APF Anaphase Promoting Factor
APL Acute Promyelocytic Leukemia
CO₂ Carbon (iv) Oxide
CRAE *Coptidis Rhizoma* Aqueous Extract
CTMDR Centre for Traditional Medicine and Drug Research
DMSO Dimethyl sulfoxide
DNA Deoxyribonucleic Acid
FDA Food and Drug Administration
HCC Hepatocellular Carcinoma
HIV Human Immunodeficiency Virus
KEMRI Kenya Medical Research Institute
MPF Mitosis Promoting Factor
MTT 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
ng Nanograms
OD Optical Density
P/S Penicillin/Streptomycin
RPMI Roswell Park Memorial Institute
SD Standard Deviation
WHO World Health Organization
ABSTRACT

Cancer is referred to as uncontrolled abnormal proliferation of body cells. It is a leading cause of death in developed world and second leading cause of death in developing world. Currently cancer treatment and management is a challenge due to complexity of the disease, toxicity of chemotherapy and unaffordability of treatment. Therefore, it is imperative to investigate complementary and alternative medicine for leads and development of anticancer drugs. *Cassia abbreviata* has traditionally been used for its ethnotherapeutic properties and pharmacological activities. It has been reported to possess antiproliferative activity, though up to date there is no scientific evidence to validate this claim. The main objective of this study was to determine the antiproliferative activity of aqueous root bark extract of *Cassia abbreviata* on hepatocellular carcinoma (HCC), Vero, and Macrophage cell lines in vitro. The antiproliferative effects of aqueous root bark extract of *Cassia abbreviata* was determined using MTT assay. The results showed that aqueous root bark extract of *Cassia abbreviata* has antiproliferative activity against HCC, Vero and Macrophage cell lines. The extract had the highest antiproliferative activity against HCC cell line with an IC$_{50}$ of 1.49µg/ml as compared to 81.08µg/ml and 128.38µg/ml in Macrophages and Vero cells respectively. The IC$_{50}$ observed on non-cancerous normal cells (Macrophages and vero cells) indicated that aqueous root bark extract of *Cassia abbreviata* had little antiproliferative effects on normal body cells hence regarded as safe. The extract contained flavonoids, phenols, tannins and saponins. In conclusion the antiproliferative activity of aqueous root bark extract of *Cassia abbreviata* observed could be attributed to the phytochemicals present in this plant extract. The results of this study, validates the claim that aqueous root bark extract of *Cassia abbreviata* has antiproliferative activity and justifies its use in herbal medicine.
CHAPTER ONE

INTRODUCTION

1.1 Background Information

Cancer is a leading cause of death in developed world and second cause of mortality in developing world. This is blamed on the rise in cancer-related lifestyles including smoking, lack of physical exercise, junk foods, environmental threats, costly medication and treatment. Cancer refers to a group of diseases that can affect any part of the body. It is a rapid growth of abnormal cells that grow beyond their normal cell division. It is also defined as a manifestation of malfunctions of immunity, as malignant cells escape recognition and elimination by the immune system (WHO, 2004; Jemal et al., 2011; Shurin, 2012; Piero and Joan, 2015). It involves a series of malignant diseases with multiple pathological stages such as cancer initiation, promotion and progression that involves multiple factors such as environmental, genetic, physical, chemical, psychological and biological factors (Weiss and McMichael, 2004). There are more than a hundred distinct types of malignancies including; lung, liver, stomach, throat, colorectal and breast malignancies amongst others (Cancer Facts and Figures, 2011).

Cancer pathology is featured by a rapid and uncontrolled cell proliferation, which form a tumor cell, metastasize throughout the body and initiate abnormal growth of neoplastic tissue on other sites in the body. Conventional anticancer drugs have pharmacological effects of antiproliferation, anti-inflammation, cytotoxicity, induction of differentiation, cell-cycle arrest and induction of apoptosis and/or autophagy, anti-invasion and anti-metastasis. However, they are characterized by challenges of drug
resistance, drug toxicity, severe side effects and unaffordability (Partridge et al., 2000; Kakde et al., 2011).

Before the advent of modern medicine and synthetic drug era, mankind relied on medicinal plants to fight threatening health conditions. Plants were used as sources of important products with medicinal and nutritional values in form of powder, teas, tincture, pastes and other formulations. Medicinal plants have served as sources of compounds for discovery and development of new therapeutic agents for many human ailments. There is an enormous unexplored reservoir of medicinal plants that require scientific evaluation and validation for use in herbal medicine and development of new therapeutic agents (Balunas and Kinghorn, 2005; McChesney et al., 2007).

Synthetic drug development has resulted to a number of drugs for the treatment of various human ailments. However, there are many diseases such as AIDS, cancer, asthma, diabetes mellitus, and hypertension, with no effective and permanent cure available in modern regimen of synthetic drugs. This is due to limitations in their use by severe side effects, toxicity, unaffordability and drug resistance. Therefore, it is imperative to research and evaluate alternative sources for efficacious, affordable, safer medicinal agents to produce permanent cure for chronic ailments (Ashok, 2011; Chauhan, 2014). Herbal medicine provides affordable alternative therapeutic agents than synthetic drugs. Natural products derived drugs tend to have fewer or no side effects (Muregi et al., 2007).
Herbal medicine has been extensively used as an alternative and complementary medicine for treatment of cancer. There is an elevated interest in the search of molecules isolated from plant sources with activity to inhibit abnormal cell proliferation (Feng et al., 2011). As an example, berberine isolated from Coptis genus has shown anticancer activity (Tang et al., 2009). Coptidis rhizoma aqueous extract (CRAE) has been found to have anticancer activity on nasopharyngeal carcinoma and hepatocellular carcinoma (Wang et al., 2010). Moringa oleifera has been used as a food substance and medicine for treatment of various diseases since ancient times. Moringa oleifera have been used traditionally in treatment of cancer. Its leaves extract have been confirmed to significantly induce apoptosis and inhibits tumor cell growth in human lung cancer cells (Jung, 2014). Treatment of cancer cells with Moringa oleifera extract significantly reduces cancer cell proliferation. Research has demonstrated anticancer effects of ethanolic extracts of Urtica membranacea, Artemisia monosperma, and Origanum dayi on human tumor cells (Solowey et al., 2014). Research has also demonstrated that ethanolic extract of Consolida orientalis, Ferula assa-foetida and Coronilla varia have anticancer activity on Hela and a cervical cancer cell lines (Nemati et al., 2013).

There is large number of medicinal plants used in traditional medicine for cancer treatment and only few have been investigated for their pharmacological effects and bioactivities. Therefore, it is important to bio-screen medicinal plants for anticancer activity since they are regarded as safe, affordable, have fewer side effects and no claimed side effects (Pezzuto, 1997; Wilmah, 2011; Kigen et al., 2013).
Cassia abbreviata is an important medicinal plant used traditionally in the treatment of various human ailments among them snakebites, bilharzias, skin diseases, cough, pneumonia, fever, gonorrhea, abdominal pains and headaches. In addition C. abbreviata extracts are used in treatment of water fever and heart diseases (Barakanye, 1998; Erasto, 2003; Erasto and Majinda, 2011). The medical effects of extracts of C. abbreviata is attributed to its phytochemical compounds most of which are phenolics, antioxidants and anthraquinones. In Kenya, C. abbreviata leaf decoction is taken to treat malaria (Keter and Mutiso, 2012).

Cassia abbreviata is used to treat skin rashes caused by human immunodeficiency virus (HIV) and acquired immune deficiency syndrome (AIDS) infections (Kisangau et al., 2011). C. abbreviata also possess antidiabetic, antiviral, antihelminthic, antimalarial, antibacterial and antifungal and antioxidant activity (Mongalo and Mafoko, 2013). Folklore information indicates that this plant is used in management of neoplasm. However, there is no documented scientific evidence for its antiproliferative activity. It is against this background that this study was designed to scientifically evaluate the unconfirmed antineoplastic claim of Cassia abbreviata.

1.2 Problem Statement and Justification

Cancer has become severe global menace causing significant morbidity and mortality in Kenya and it has become the leading cause of death worldwide. In 2012, there were 14.1 million new cancer cases, 8.2 million cancer deaths and 32.6 million people living
with cancer globally (Gravanis et al., 2014). Cancer is ranked third as a cause of death after infectious and cardiovascular and other diseases in Kenya. It usually causes 7% of total national mortality every year. It is estimated that the annual incidence of cancer is about 28,000 cases and the annual mortality to be over 22,000 in Kenya and 7 million globally (Ministry of Health, Kenya, 2011; Piero and Joan, 2015).

The various strategies employed to treat and control cancer have been ineffective in eradication of the disease. More so, the available cancer drugs are unaffordable, toxic and have severe side effects. Therefore, it is imperative to explore new vistas and avenues that are cost effective and affordable to the local patients of cancer.

A significant possible approach to improve the efficacy of anticancer drugs while decreasing toxicities, side effect and drug resistance is to embrace alternative and complementary medicine (Wang et al., 2013). Use of herbal medicine as source of therapeutic and templates for development of new drugs is in record and medicinal plants are a decent source of clinically important anticancer agents.

_Cassia abbreviata_ has been traditionally claimed to have antiproliferative and medicinal properties. However, there is no scientific evidence available to confirm this activity. It has a rich repertoire of phytochemicals which defines its medicinal effects. Communities have been using the plant in folklore without validation. There is a need to validate the potential of _Cassia abbreviata_ folk medicinal use. It is also imperative to determine phytochemical composition of aqueous extract of _Cassia abbreviata._
1.3 Research Questions

i. What is the effect of aqueous root bark extract of *Cassia abbreviata* on hepatocellular carcinoma (HCC), vero and macrophage cell lines *in vitro*?

ii. What is the qualitative phytochemical composition of aqueous root bark extract of *Cassia abbreviata*?

1.4 Objectives

1.4.1 Main Objective

To determine qualitative phytochemical composition and *in vitro* antiproliferative activity of aqueous root bark extract of *Cassia abbreviata*.

1.4.2 Specific Objectives

i. To determine the antiproliferative effect of aqueous root bark extract of *Cassia abbreviata* on hepatocellular carcinoma, macrophage and vero cell lines *in vitro*

ii. To determine qualitative phytochemical composition of aqueous root bark extract of *Cassia abbreviata*
CHAPTER TWO
LITERATURE REVIEW

2.1 The Biology of Cell Proliferation

Cell proliferation is the process that results in an increase in the number of cells and is responsible for cell growth and replacement of dead or worn out cells. It is defined as the balance between cell division and cell loss that occur through differentiation or cell death. It is one of the fundamental biological processes of life. It requires energy, nutrients and biosynthetic activity to duplicate all cell components during each step through the cell cycle (DeBerardinis et al., 2008).

The eukaryotic cell cycle consists of four coordinated processes: cell expansion, DNA replication, dispersal of the duplicated chromosomes to daughter cells and cytokinesis. It is divided into two basic parts: interphase and mitosis. The interphase is divided into; G\textsubscript{1} phase that prepares the cell for division by protein synthesis and precursors for DNA synthesis. S phase, the phase that DNA is duplicated. G\textsubscript{2} phase in which the cells grow and synthesize proteins in preparation for mitosis. Stages of mitosis are: Prophase, Metaphase, Anaphase and Telophase. The last phase, cytokinesis, involves division of the cytoplasm and separation of cells (Cooper, 2000; Golias et al., 2004).

2.2 Control of Cell Proliferation

Cell proliferation is strictly controlled by many regulatory mechanisms at different points during the cell cycle. Severe genetic damage can occur if cells proceed to the next phase of the cell cycle before the previous phase is correctly completed. For
instance, when S-phase cell is induced to undergo mitosis by fusion to a cell in mitosis, the Mitosis Promoting Factor (MPF) forces the chromosomes to condense. Since the replicating chromosomes are fragmented by this process, the premature entry into mitosis results in unreplicated DNA. To forestall such consequences, transition from one cell cycle phase to another occurs in an orderly and is regulated by different check/restriction point (Cooper, 2000).

There are four restriction/check points that control cell cycle; $G_1$ checkpoint causes cell cycle arrest if there is DNA damaged by genotoxicity such as radiation or chemical modification. The S phase checkpoint ensures that cells that have not replicated their entire chromosomes do not enter mitosis. Similarly, the $G_2$ checkpoint prevents the initiation of mitosis before completion of S phase. This checkpoint ensures that incomplete replicated DNA is not distributed to daughter cells. Also the genome is replicated once in a cell cycle by preventing cells from re-entering S phase. Restriction in $G_2$ allows repair of DNA double-strand breaks before mitosis. Mitotic Spindle Arrest checkpoint monitors the presence of improper mitotic spindle assembling. It inhibits activation of the Anaphase Promoting Complex (APC) polyubiquitination system that leads to degradation of the anaphase inhibitor. As a result of this arrest, MPF activity remains high, chromosomes remain condensed and the nuclear envelope does not reform (Cooper, 2000; Lodish, 2000).
2.3 Defective Regulation of Cell Proliferation

Controls or checkpoints exist in the cell cycle to ensure an orderly sequence of events in the cell cycle. Fundamental changes in the genetic control of cell division result in an unrestrained cell proliferation. Mutations in genes controlling cell proliferation occur in two classes of genes; proto-oncogenes and tumour suppressor genes. In cell cycle, the products of proto-oncogenes act at different levels in the pathways that stimulate cell proliferation. Activation of proto-oncogenes to oncogenes promotes tumor growth. Oncogenes are formed genetic changes to Proto-oncogenes.

The processes involved in development of oncogenes include gene mutations, gene amplification and chromosome rearrangement. Genetic mutation such as base substitutions, deletion and insertions in protein regulatory regions result in production of mutates proteins that remain uncontrollably active. Point mutations are the most mode of activation of Ras genes to oncogenes (Carlson et al., 1994; Pedraza-Fariña, 2006). Gene amplification involves expansion of gene copy number within the cell genome. This occurs through redundant amplification of whole genome that results in double mini chromosomes and homogeneous staining regions. These regions contain more gene copy numbers which confer selective cell growth resulting to neoplasm.

Gene amplification has been reported in proto-oncogene of myc, erb B, and ras families a many human tumors such as ovarian and breast cancers. Chromosome rearrangement involves chromosome translocation and inversions. This mechanism results mostly to hematological neoplasia through gene activation and gene fusion. In gene activation
during chromosome rearrangement a proto-oncogene is moved close to immunoglobin gene loci this. The proto-oncogene therefore is under regulatory elements of the immunoglobin gene. This phenomenon leads to deregulation of the proto-oncogene conferring neoplastic cell transformation. Gene fusion occurs when chromosomes breaks fall in the loci of two genes which give rise to composite structure with the head of one gene and the tail of the other. Both genes give rise to an oncoprotein with neoplasia transforming potential (Rodenhuis et al., 1997; Falini and Mason, 2002; Pierotti et al., 2003). Tumor suppressor gene prevents activation of proto-oncogenes to oncogenes. Mutated tumor suppressor genes and proto-oncogenes result to cancer (Vermeulen et al., 2003). Over expression, point mutation and gene translocation of proto-oncogene leads to cancer. Gain of function of proto-oncogene or loss of function of tumor suppressor genes through mutations results to tumor cells (Lodish et al., 2000).

Ras gene is a proto-oncogene that encodes for an intracellular signal-transduction protein for cell survival and proliferation. The mutant ras gene encodes an oncoprotein that provides an excessive or uncontrolled growth-promoting signal that promote cell proliferation indefinitely. Mutations that permanently activate ras gene have been found in 20-25% of human tumors and up to 90% in some types of cancer such as pancreatic cancer (Lodish et al., 2000; Downward, 2003). Therefore, ras pathway like other pathways can be a drug targets for development of anticancer agents.

Tumor-suppressor protein p53 is one of the proteins encoded by tumour-suppressor gene. The protein is an important cell cycle check-point regulator at G1/S and G2/M.
The role of p53 is to arrest cell cycle in G₁ after genotoxicity to allow DNA repair before replication and cell division. In response to severe DNA damage, p53 induce apoptotic cell death pathway. Mutations in p53 gene are present in more than half of all human tumours, showing its important role in conserving the integrity of normal cell cycle. Cells with defective p53 gene grow to tumour cells (Golias et al., 2004).

2.4 Cancer Metastasis

Cancer Metastasis is a process by which cancer cells spread and migrate from the original site to other parts of the body through the bloodstream, the lymphatic system and invasion resulting to metastatic tumors (Nguyen and Massague, 2007; Klein, 2008; Chiang and Massague, 2008). A tumor is considered metastatic when it has the capacity to spread beyond its original site and invade other tissue in the body. The steps involving metastases of cancer include; separation of cancer cells from the primary tumor, invasion to the other tissues and basement membranes, entry and survival in the circulation, lymphatic tissues, peritoneal space and colonizing distant target organs (Kumar et al., 2014).

The routes for spread of cancer cells include; Hematological route that involve spread of cancer cells through the blood stream to distant organs in the body, lymphatic route that allows spread of cancer cell to lymphoid tissues and to other parts of the body and transcoelomic routes that involve spread of malignancy into body cavities through penetrating the surfaces of pleural, peritoneal and pericardial cavities. Metastasis of
cancer complicates cancer treatment and increases the rate of patients death (Kumar et al., 2014).

2.5 Hepatocellular Carcinoma (HCC)

Liver cancer is the third most common cause of death in the world. Hepatocellular carcinoma (HCC) accounts for 95% of the liver cancers. It is an epithelial cancer emanating from hepatocytes (Giannelli et al., 2001; Wang et al., 2011). The incidence of HCC is rising in many countries with an estimated population of new cases of over 500,000 annually and an incidence of between 2.5 and 7% of patients with liver cirrhosis yearly (Montalto et al., 2002). Due to a high rate of relapse, HCC has a very poor prognosis, with a 5-year survival rate of 3–5%. The available HCC drugs are unaffordable, toxic and have severe side effects. Thus it is important to develop a new agent which effectively treats HCC with less toxicity, minimal or no side effects and prevents recurrence (Colombo et al., 1989; Yamanaka et al., 1990; Wang et al., 2011). Long-term prognosis of patients with HCC is poor, and survival is mainly affected by the occurrence of metastases (Yamanaka et al., 1990; Zhang et al., 2014).

2.6 Conventional Treatment of Cancer

2.6.1 Chemotherapeutic Drugs

Chemotherapeutic agents target the process of cell division because tumor cells are more likely to be dividing more rapidly than normal cells. However, their activity is not specific and they are associated with significant toxicity and severe side effects. Some therapeutic agents are discussed here below;
2.6.1.1 Alkylating Agents

Alkylating agents are highly reactive compounds that are dose-dependent and non-specific drugs that target the cell cycle. Most alkylating agents are bipolar having two groups capable of reacting with DNA. Their mode of action is through replacement of a hydrogen atom from the DNA molecule by an alkyl radical (R-CH$_2$-CH$^+$) through a covalent linkage. Alkylation results to cleavages in DNA and in cross links of DNA double helix, interfering with DNA replication and transcription. The cell eventually dies because it cannot replicate and triggers apoptosis (Payne and Miles, 2008).

Clinically useful alkylating agents are categorized in five classes namely; nitrosoureas (carmustine, lomustine and semustine), ethylene derivatives (thiotepa and mitomycin) alkyl alkane sulfonates (busulfan), triazine derivatives (dacarbazine) and nitrogen mustard derivatives (ifosfamide and cyclophosphamide) (Payne and Miles, 2008). Experiments have demonstrated that alkylation is the cause for the main toxic effects of alkylating agents. Alkylating agents have different pharmacological effects that include mutagenesis, interference with mitosis, immunosuppression and carcinogenesis (Damia and D’Incalci, 1998).

2.6.1.2 Antimetabolites

Antimetabolites directly interact with special enzymes, leading to the inhibition of the enzyme or subsequent synthesis of an aberrant molecule that cannot function normally. They are structural analogues to normal metabolites required for the synthesis of purine
and pyrimidine. Upon introduction of an antimetabolite into the cell to substitute a physiological substance such as vitamins, nucleosides or amino acids, they result in inhibition of the cell processes. Some antimetabolites can be incorporated directly into DNA or RNA altering the process of replication, transcription and protein synthesis. Most antimetabolites are phase-specific and act during the S-phase of the cell when the DNA is being synthesized (Payne and Miles, 2008).

There are three main classes of antimetabolites namely; Folic acid antagonists for example Methotrexate which competitively inhibits dihydrofolate reductase responsible for the formation of tetrahydrofolate from dihydrofolate, Pyrimidine analogues such as flouurracil. They are analogues to pyrimidine altering nucleic acid synthesis. They inhibit enzymes involved in DNA biosynthesis leading to eventual cell death (Kaye, 1998; Baba and Câtoi, 2007; Payne and Miles, 2008).

2.6.1.3 Topoisomerase Inhibitors

Topoisomerase enzymes are excellent molecular enzymes that play a critical role in DNA topology and chromatid segregation. Topoisomerases enzymes are necessary due to the large size of eukaryotic DNA, double helix structure of DNA and association with histones. For DNA replication, transcription and recombination, double helical structure of the DNA must be relaxed. During DNA replication, the two strands of the DNA are unlinked by topoisomerase. During transcription, the translocation of RNA polymerase generates supercoiling tension in the DNA that should be relaxed. The
unwinding of DNA is catalyzed by topoisomerases by creating transient breaks in the DNA (Champoux, 2001; Nitiss, 2009).

There are two types of topoisomerase enzyme namely; Topoisomerase I that binds to DNA molecule, cuts one strand and generates a covalent phosphoester bond between the cleaved phosphate on the DNA and a tyrosine residue in the enzyme. Topoisomerase II cuts both strands of a double-stranded DNA, pass another portion of the duplex by the generated cut, and religate the cut region. Topoisomerase inhibitors (TI) are designed to target topoisomerase enzymes because of their critical activity on replication and transcription of genetic information. Topoisomerase inhibitors act during G2 phase of cell cycle preventing cells from entering mitosis (Pommier, 2006; Pommier et al., 2010).

Topoisomerase inhibitors are grouped into two broad classes; Topoisomerase I inhibitor such as camptothecin, Irinotecan and topotecan. Topoisomerase I inhibitor binds to the topoisomerase I–DNA complex making it stable thus inhibiting DNA replication. Topoisomerase II inhibitor such as etoposide and vespid binds to topoisomerase II-DNA complex stabilizing it thus preventing DNA replication (Froelich-Ammon et al., 1995; Guichard and Danks, 1999; Berk et al., 2000; Wang et al., 2002; Hurwitz et al., 2004; Pommier, 2006; Pommier et al., 2010).
2.6.1.4 Antitumor Antibiotics

Antitumor antibiotics are natural products derived cytotoxic agents that act by formation of stable complexes with the nucleic acids inhibiting DNA and RNA synthesis. Clinically useful antitumor antibiotics in practice include; Bleomycin, Actinomycin-D and Antracycline antibiotics (Payne and Miles, 2008).

Bleomycin is a family of glycopeptide-derived antibiotics isolated from species of *Streptomyces* (Takita et al., 1972). Bleomycins are highly active in the G₂ and M phases of the cell cycle. They react with the DNA and cause an oxidative cleavage through production of free radicals (Boger and Cai, 1999). Bleomycin activity is limited due to altered cellular uptake, damage to normal cell DNA causing DNA lesions and inactivation by cellular enzymes (Akiyama et al. 1981; Ramotar and Wang, 2003).

D actinomycins are polypeptide antineoplastic antibiotics isolated from species of *Streptomyces* genus (Waksman and Woodruff, 1940; Hollstein, 1974). They inhibit cell proliferation nonspecifically through formation of stable complexes with double-stranded DNA by intercalating with deoxyguanosine and deoxycytosine residues. Actinomycin D activity also results to single-strand DNA breaks. Their activity results in ultimate reduction of DNA, RNA and protein synthesis. They are useful in the treatment of embryonic rhabdomyosarcoma, testicular tumors and pancreatic cancer (Gilman, 1990; Kleeff et al., 2000; Payne and Miles, 2008).
Antracyclines are another class of anticancer antibiotics isolated from *Streptomyces peucetius* discovered in 1960s. They play a critical role in cancer treatment since they are among the most effective anticancer treatments and have a broad spectrum of activity against many types of neoplastic diseases (Marco *et al.*, 1980; Peng *et al.*, 2005).

Antracycline mode of action involves inhibition of DNA and RNA synthesis by intercalation between basepairs. This results to generation of free radicals that cause damage to cell membrane, proteins and DNA. They also inhibit topoisomerase enzyme preventing relaxation of supercoiled blocking DNA replication and transcription (Takimoto *et al.*, 2008; Pommier *et al.*, 2010; Pang *et al.*, 2013). Some of the antracyclines in clinical use include; doxorubicin and daunorubicin. A limitation in application of antracycline is severe cardiotoxicity that result to cardiomyopathy and congestive heart failure (Hortobágyi, 1997; Minotti *et al.*, 2004; Volkova and Russe, 2011; Zhang *et al.*, 2012).

### 2.6.1.5 Metals

Application of metals and metal compounds in medicine has been in existence for several years throughout human history. Metals are important components of cellular systems and they play critical roles in biochemical processes in living organisms. Metals versatility in their activity is as a result of excellent characteristics such as, redox activity, reactivity to organic substrates and variable coordination modes. Alteration of some of essential metals during normal biological reactions has resulted in development
of many pathological conditions such as cancer (Orvig et al., 1999; Yaman et al., 2007). Therefore, due to their critical essential role in biological systems metals and metal compound have been researched with the objective of developing anticancer metallodrugs. Such metals in clinical application include;

2.6.1.5.1 Platinum Agents

Platinum anticancer agents include; cisplatin, oxaliplatin and carboplatin. Cisplatin is an organic metal which mechanism of action involves loss of a chloride ion after diffusing into the cell. This enables Cisplatin to cross-link with the DNA resulting to DNA intra- and interstrand eventually resulting in inhibition of DNA replication, RNA and protein synthesis. Cisplatin has been used to treat cervical, ovarian, non-small cell lung carcinoma, head and neck and testicular cancers. Its clinical use is hindered by increased toxicity such as ototoxicity, nephrotoxicity and resistance (Jamieson and Lippard, 1999; Galanski et al., 2003; Galanski et al., 2005; Kelland, 2007; App et al., 2015).

Oxaliplatin is a chemotherapeutic platinum anticancer drug used in management of colon cancer (Ehrsson et al., 2002; App et al., 2015). Oxaliplatin consist of a complex of a platinum atom, oxalate and diaminocyclohexane (DACH) group. It forms highly reactive complexes of platinum that inhibit DNA replication and transcription (Jamieson and Lippard, 1999; Wiseman et al., 1999; Graham et al., 2004). In vivo and in vitro studies have demonstrated that oxaliplatin has broader spectrum of
antiproliferative activity on several tumor models including colon cancer and safer than
cisplatin and carboplatin (Raymond et al., 1998; Di Francesco et al., 2002).

Carboplatin is a platinum-based antitumor agent structurally similar to cisplatin, but
have organic carboxylate group instead of chlorine as in cisplatin. The carboxylate
group results to increased water solubility, slower excretion and breakdown. It is less
nephrotoxic and neurotoxic, but causes severe toxicity due to myelosuppression. It used
in management of ovarian carcinoma, head and neck, lung, endometrial, bladder,
esophageal, breast and cervical cancers, central nervous system or germ cell tumors;
osteogenic sarcoma (Wheate et al., 2010; App et al., 2015).

2.6.1.5.2 Arsenic
Arsenic is a naturally occurring metal found in conjunction with many minerals such
sulfur and as a pure elemental crystal. It has been used since ancient time to treat a wide
variety of human diseases. In Chinese traditional medicine arsenic is used to treat
cancer and other conditions (Zhu et al., 2002; Gielen and Tiekink, 2005). Arsenic has
been previously used to treat hematological malignancies and is currently approved for
treatment of refractory promyelocytic leukemia (Berenson and Yeh, 2006).

The antitumor mechanism of action of arsenic is apoptosis via the induction of p53-
dependent G1 or G2/M cell cycle arrest (Li et al., 2002; Liu et al., 2003). Despite the
application of arsenic in cancer management there are reports of drug resistance,
cardiotoxicity and APL differentiation syndrome (Huang et al., 2002; Kang, 2003).
There are other various metals such as iron, vanadium, gold and titanium, involved in cancer treatment whose discussion cannot be exhausted in this forum.

There has been an impressive progress in development of anticancer chemotherapy drugs but the limitation in safety profile and efficacy of chemotherapeutic anticancer agents cannot be ignored. Anticancer chemotherapeutic agents affect rapidly dividing cells without specifically targeting cancer cells. Further they only affect cell ability to divide and have minimal effect on other effects of tumour progression such as metastases and tissue invasion. Additionally, anticancer chemotherapy has notable severe side effects such as myelosuppression, alopecia, mucositis, neurotoxicity, nephrotoxicity, skin irritation, anemia, infertility, nausea and vomiting (Dunton, 2002; Remesh, 2012; Dy and Adjei, 2013; Jung, 2014). Therefore, there is a need to research and develop new anticancer drugs with minimal limitation of application such as less toxicity, highly selective, less severe side effects and affordability.

2.6.2 Surgery
Cancer surgery is a procedure of cancer management that involves removal of tumors and surrounding tissue through invasive incision in the body. Historically surgical removal of localized tumors has been the first line of cancer treatment. Through technological improvements surgery procedures for destroying tumors have been significantly improved. Technological progress made in ultrasound, magnetic resonance imaging, computerized tomography and positron emission tomography have replaced most invasive surgical operation operations (Subotic, 2012).
Surgery objective is to either remove the tumor or the entire organ depending on the level of spread though it is limited in case of vital body organs. Surgical procedures for cancer treatment include prostatectomy for prostate cancer, mastectomy for breast cancer and lung cancer surgery for non-small cell lung cancer. Tumor excision is limited by contradictions such as recurrence and metastasis (Sabel et al., 2006; Sudhakar, 2009; Subotic, 2012).

2.6.3 Radiotherapy

Cancer radiotherapy is a treatment measure that involves use of ionizing radiation to kill cancer cells. Radiation therapy is commonly applied to the cancerous tumor because it has the ability to inhibit cell proliferation and kill cancer cells. Ionizing radiation mode of action is by damaging the DNA of cancerous tissue leading to cell death. The DNA damage is caused by one of two types of energies namely photon and charged particle that causes direct or indirect ionization of the atoms that make up the DNA molecule (Harrison et al., 2002).

There are several radiation therapies in application that include; Conformal proton beam therapy used to kill tumor cells instead of X-rays, Stereotactic surgery and Stereotactic therapy that is used to treat common brain tumors, Intra-operative radiation therapy used for adjacent tissue after tumor has been removed surgically. Radiotherapy is limited by the fact that ionizing radiations have got long-term effects of causing
cancer in non-cancerous cells. It is associated with severe side effects such as vomiting, oedema and infertility (Harrison et al., 2002; Gutfeld et al., 2002; Sudhakar, 2009).

2.7 Economics of Maladies

The cost of treatment of cancer is high and unaffordable for patients worldwide. A case study in Canada reflects one month of a cancer treatment and management could cost approximately 10 million Kenya Shillings per patient. According to the study, chemotherapy contribute to more than 31% of the costs, other costs being distributed among hospitalization (27%), Physician services (12%), diagnostic tests (2%), radiotherapy (5%), home care (5%), and prescription drugs (5%) (Oliveira and Krahn, 2014). The implication of prohibiting cancer drug prices is one of the calamities that have befallen cancer victims. There is need to investigate alternative affordable, less toxic, sources of treatment to cancer.

2.8 Herbal Management of Maladies

Herbal medicine has been an important segment of human health care in the developing world, particularly Africa. The dependence of Africa on herbal medicine has been due to affordability, accessibility, local availability and cultural acceptance by the local society. Furthermore, the available modern facilities of medicine such as hospitals, equipment and medical personnel are limited and not available to local communities making herbal medicine a lifesaving alternative. Effectiveness of some herbal treatment to conditions such as malaria is well documented (Fennell et al., 2004; Bickii et al., 2007). According to World Health Organization (WHO), 80% of the population in some
developing countries uses herbal medicine (WHO, 2002). In Kenya, many communities believe in the potency and healing efficacy of herbal medicine, even when modern medicine is accessible. In various complicated medical conditions such as chronic infections like HIV/AIDS, cancers, infertility, diabetes and hypertension people often combine both traditional and modern medicine (Kigen et al., 2013).

Based on recent scientific research and evidence of therapeutic activity of medicinal plants, herbal therapies have been recommended as alternative treatments for cancer (Risberg et al., 1998). Several studies have demonstrated that extracts from several herbal plants have anticancer potential and could inhibit cancer cell proliferation in vitro or in vivo (Bonham et al., 2002; Vicker et al., 2002). It has been recognized that different components in herbs can have synergistic activity and buffering toxic effects. Extracts from a mixture of herbs pooled together might have more therapeutic or preventive effect than a single extract. Herbal therapy has been prescribed in various countries over ages as a remedy for various diseases including infectious and malignant diseases. Therefore, herbs have been defined as an important natural and valuable source of anticancer drugs discovery (Hu et al., 2002).

Medicinal plants have been a source of many drugs currently used in conventional medicine. Digoxin, vinblastine and taxol isolated initially from Pacific Yew (Taxus brevifolia), artemesin and nabilone are all from plant sources (Kummalue, 2005). Drug discovery from medicinal plants involve a multidisciplinary approach combining botanical, biological, phytochemistry and molecular techniques. Development and
discovery of Drug from medicinal plants has continued to provide important leads to various human ailments such as cancer, HIV/AIDS, Alzheimer's, malaria, and pain (Balunas and Kinghorn, 2005). Currently, approximately 60 % of antihypertensive and anticancer drugs in the market are from plant sources (Kigen et al., 2013). Therefore, medicinal plants have become a significant field of biotechnology research and development of new therapeutics.

In therapeutic evaluation of folk medicinal products, there has been shift of paradigm towards research based evidence of activity. There is a synchrony of the strengths of traditional systems of medicine and that of the modern idealism. The paradigm has shifted toward research based medical evaluation, standardization of herbal medicinal products and clinical trials to provide valid evidence for clinical efficacy (Thyagarajan et al., 2002). Herbal medicine regimen such as Ayurveda, Chinese medicine, Kampo medicine, and homeopathy are gaining popularity as effective strategies for treatment of cancer. The increased popularity of herbal therapy is as a result of a number of scientific proofs of anticancer activity at laboratory and clinical trial levels.

In Asian continent, regions and countries such as Taiwan, China, Japan, Hong Kong, and Korea, some herbal formulations are regarded as drugs in their statutory drug regulations (Samuelson, 1999; Saito, 2000; Liang, 2004; Zheng, 2015). In USA, FDA policy recognizes products derived from herbal medicines as dietary supplements and botanical drug products (Gahche et al., 2011; Food and Drug Administration, 2012).
Plant-derived compounds are an important source of useful anti-cancer agents currently in clinical practice such as vincristine, vinblastine, camptothecin and its derivatives, irinotecan, topotecan and etoposide and paclitaxel (Jacobs et al., 2004; Oberlies and Krol, 2004; Cragg and Newman, 2005). The interest in search of therapeutics from natural sources such as plant has increased and intensified to provide therapy to ever demanding health conditions and increased lifestyle diseases. The realm of modern science and conventional medicine has accepted herbal medicine as a source of bioactive and therapeutic agents (Kirira et al., 2006). Plants have a great diversity of bioactive compounds which makes them an excellent source of different types of therapeutic agents. A significant number of therapeutic drugs have been produced from medicinal plants (Mahmood et al., 2012). It is expected that herbs still serve as sources of new drug leads, given their therapeutic potential and diversity (Batista et al., 2009).

Several plant-derived anticancer drugs have been introduced into world markets as commercial products. Some of these herbal therapies have been released as patented dietary supplements and botanical drugs. Such substances include triptolide isolated from Tripterygium wilfordii which has been patented as an apoptosis modulator used for treatment of various cancers including Colon cancer, breast cancer, lung cancer and prostate cancer. A pharmaceutical composition containing Manassantin A and Eepimanassantin A extracted from Saururus chinensis has been patented for management of various cancers. An anticancer composition comprising of sesquiterpenes isolated from Resina ferulae is commercially used for treatment of lung and ovary cancers. Berberine isolated from genus Berberis plants has been patented as
an anticancer agent against lung cancer (Hahn et al., 2004; Ryu et al., 2004; Maung et al., 2007; Dai et al., 2010). Therefore, there is a beacon of hope in herbal medicines against cancer.

2.9 Methods for Determination of Cell Proliferation

Assays to determine cell proliferation are utilized to determine number of viable cell in a cell culture. Such assays have been utilized in various studies to determine antiproliferative activity of various substances against cell growth. The methods are as discussed below.

2.9.1 Real-Time Luciferase Assay

This is a method that determines real time cell density by use of a marine shrimp derived luciferase enzyme and a pro-substrate. The pro-substrate is a cell permeable substance that is not a direct substrate for the luciferase but its metabolized by the active cells. The luciferase and pro-substrate are added to the culture medium together and incubated. The pro-substrate is then metabolized into a substrate that diffuse out of the cells upon which it is acted on by the luciferase enzyme to produce to generate a luminescent signal whose intensity is spectrophotometrically. The enzyme is normally stable in the media for approximately 72 hour which allows monitoring of the luminescent signal over time. The amount of the signal with cell death and is directly proportional to viable cells and inversely proportional to death cells. The advantage of real time luciferase assay is that it can be multiplexed with other cellular assay and can
give the number of viable cells at a given time otherwise its costly (Duellman et al., 2015; Heart et al., 2016).

2.9.2 ATP Assay

Adenosine triphosphate (ATP) is an energy molecule produced by metabolically active cell. ATP is an excellent biomarker for cell viability. It is released from cell when integrity of the cell membrane is compromised and endogenous ATPases rapidly metabolize any ATP available in the cytoplasm. Reagents for detection of ATP includes a lysis buffer to break the cell membrane, ATPase inhibitors to inhibit ATP digestion by ATPase, luciferin substrate and luciferase enzyme. ATP provides energy required for conversion of luciferin to oxyluciferin with an emission of light. The intensity of the light produced is determine through spectrophotometric technics.

\[
\text{ATP} + \text{d-Luciferin} + \text{O2} \rightarrow \text{Oxyluciferin} + \text{AMP} + \text{PPi} + \text{CO2} + \text{Light} \ (560\text{nm})
\]

When the amount of ATP is limiting the intensity of light produced is low suggesting low cell viability. It is one the most rapid and sensitive methods for detection of cell viability though it is limited by reproducibility of pipetting replicate samples and it is costly. Care should be taken to prevent digestion of ATP by cytoplasm ATPase which would suggest false cell death (Kurbacher et al., 1998; Auld et al., 2009).

2.9.3 Lactate Dehydrogenase Assay

Lactate dehydrogenase is an oxidative enzyme produced by nearly all cell in the body especially during injury therefore, it is a critical biomarker of cell death. Cell release
LDH into the media once the integrity of cell membrane is compromised. Released LDH converts lactate to pyruvate with reduction of NAD+ to NADH. 

![Chemical reaction diagram]

The NADH produced is detected calorimetrically at 450 nm. The amount of the NADH is directly proportional to the amount of LDH released from the damaged cells hence inversely proportion cell viability (Sigma-aldrich, 2016). Another substrate that substitutes pyruvate as substrate in the same reaction is resazurin which converted into resorufin that produce fluorescence. The fluorescent resorufin produced is directly proportional to the amount of LDH produced hence the level of cell death. The assay is quick and sensitive and it does not damage viable cells though it might be overshadowed by cellular artifacts (Abe and Matsuki, 2000; D'Alessandro et al., 2013; Promega, 2016).
2.9.4 Protease Viability Marker Assay

Constitutive and conserved proteases have been useful as biomarkers for cell viability. Glycylphenylalanyl-aminofluorocoumarin (GF-AFC) a cell permeable protease substrate to detect precisely protease activity restricted to viable cell. Once the substrate penetrates the cell is metabolized by aminopeptidase to aminofluorocoumarin by removal of gly and phe amino acids.

When the cells die, aminopeptidases rapidly disappears hence no protease activity hence making it a discriminating marker for cell viability. Aminofluorocoumarin fluoresces producing a signal equivalent to the number of viable cell. The method is non-toxic to cells making it suitable for multiplexing with other cellular assays though it’s costly (Iversen et al., 2012; Promega, 2016).

2.9.5 MTT Tetrazolium Assay

MTT tetrazolium assay depend on the ability of mitochondria succinate dehydrogenase to convert MTT substrate to formazan that is detected spectrophotometrically. Dead cell cannot convert metabolize MTT substrate to formazan there will be little or no calorimetric signal.
During metabolic metabolism of MTT, formazan accumulates as an insoluble substance in the cell and the media. Then it is solubilized by addition of Dimethylsulfate turning purple coloured. The calorimetric signal is measured spectrophotometrically. The amount of formazan produced is directly proportion the number of viable cell. The limitation of formazan is that it cannot be multiplexed since its toxic to cell over long period of incubation. Otherwise, it has an adequate performance and inexpensive. This technology has been popularly applied and remains common in research laboratories as seen in many published articles (Berridge et al., 2005; Riss et al., 2015). Therefore this method was adopted for this study.

2.10 Anticancer Associated Phytochemicals

Global cancer burden is on the rise and therefore there is a demand for new effective therapies to manage and treat cancer. Research on new anticancer drugs is shifting towards plant derived natural products. Phytochemicals are plant secondary metabolites useful for plant process and growth which are being investigated for development of
new clinical anticancer drugs. Such metabolite includes flavonoids, tannins, saponins, alkaloids among others.

Classes of flavonoids include flavones, isoflavones, flavanols flavanones, flavanonols and flavonols. Flavanoids from a wide variety of plant extracts have demonstrated anticancer activities. Gastric cancer chemopreventive activity of licorice extract which is one of the most common herbal remedy in Kampo medicine has been attributed to flavonoids present in the extract (Fukai et al., 2002). Dryopteris erythrosora cytotoxicity activity against human lung cancer cells has been associated to the flavonoids present in the plant (Coa et al., 2013). Flavonoids purified from Litchi chinensis leaf have been shown to have anticancer activities against other human cancers cancer cell lines including hepatoma (Hep-G2), cervical carcinoma (Hela) and breast cancer (MCF-7) (Wen et al., 2014). The flavonoids extracted from Antiproliferative activity of Stem bark extracts of Erythrina suberosa against human leukemia cancer cell lines has been attributed to the flavonoids contents of the extract (Kumar et al., 2013).

Tannins are polyphenolic compounds present in plant extract that have been associated with anticancer properties various medicinal plants. Aqueous tannin fractions of Calycogonium squamulosum, Cornus Canadensis, Rubus odaratus and Lespedeza capita have demonstrated antitumor activity against murine breast carcinoma cell line (Fong et al., 2006). Musaceas tannin fractions have been shown to exhibit antitumor breast and prostate cancer cell lines (Kazi et al., 2003). Total Punica granatum Tannin
extract have been shown to have anti-proliferative activity human oral, colon and prostate cancer cell lines (Seeram et al., 2005). Tannins fractions of Eugenia jambos have shown anticancer activity against human promyelocytic leukemia cell line with less cytotoxicity to normal cell lines of human lymphocytes and Chang liver cells (Yang et al., 2000).

Saponins have been usually associated with anticancer properties of various medicinal plants. Balanites aegyptiaca antiproliferative activity has been exhibited against against MCF-7 human breast and human colon cancer cell line and the activity was attributed to the saponins compounds present in the extract (Beit-Yannai et al., 2011). Astragalus propinquus saponins have demonstrates antitumor activity against HT-29 human colon cancer cell lines (Tin et al., 2007). Extracts of Aralia elata leaves have exhibited significant cytotoxic potential against human leukemia cancer and human lung carcinoma cell lines which was attributed to triterpene saponins confirmed present in the extract (Zhang et al., 2012). Fractions of saponin from Panax notoginseng leaves extract exhibited antiproliferative activity against human pancreatic cancer, human hepatocellular cancer and human gastric adenocarcinoma cell lines (Qian et al., 2014). Total saponin content of Pinguicula villosa extract was determined to have antitumor activity against mouse melanoma, human cervical cancer and mouse lymphocytic leukemia cell lines (Guo and Gao, 2013). Therefore, owing to the evidence that these plant phytochemicals have demonstrated anticancer potential there is a likelihood of a promising future of anticancer therapies developed from plant source.
2.11 Mechanism of Activity of Anticancer Associated Phytochemicals

Development of cancer involves tumor initiation, promotion and progression. Medicinal plants and various anticancer drugs act by modulating these processes of carcinogenesis. Cancer initiation can be halted by modulation of trans-membrane transport system, modulation of metabolism, inhibition of reactive oxygen species (ROS) and nitrogen species, protection of DNA structure, and control of gene expression. Cancer promotion and initiation can be limited by blocking gene toxicity, encouraging anti-inflammatory and antioxidant activity, blocking proteases and tumor cell growth, modulation of cell signaling pathways, modulation of the immune system and hormonal status (De Flora and Ferguson, 2005; Fernando and Rupasinghe, 2013).

In modulation of metabolism, phytochemicals such as flavonoids interact with phases I and II metabolizing enzymes to halt tumor initiation, promotion and progression. Procarcinogens are activated by Phase I metabolizing enzymes like cytochrome P450, to intermediates reactive species which interact with cellular nucleophiles eventually initiating cancer. Phase I metabolizing enzymes have been shown to be inhibited by phytochemical demonstrating protective activity against cellular damage. Phase II metabolizing enzymes are involved in detoxification, inactivation and excretion of carcinogens from the body. They include quinone reductase, glutathione-S-transferase and UDP-glucuronyl transferase. Phytochemical induce activity of phase II metabolizing enzymes demonstrating chemopreventive effects against carcinogenesis (Tsyrllov et al., 1994; Ren et al., 2003).
Cell cycle is strictly regulated at various check point to ensure no emergency of abnormal cell division. Carcinogenic substances induce indefinite cell division bypassing various cell cycle checkpoints. DNA synthesis occurs during the S phase of the cell cycle during interphase. Cell cycle G1/SandG2/M checkpoint ensures that the cell DNA is properly synthesis with no mutation or genetic errors. Plant phytochemicals have been found to induce cell cycle arrest at this checkpoint in tumor cell in presence of cell cycle deregulation (Casagrande and Darbon, 2001; Jung-achoi et al., 2001).

Angiogenesis is a physiological process that involves formation of new blood vessels. Tumor microenvironment is characterized with overexpression of angiogenic factors that promote aggressive formation of blood vessels in the tumor cell. This confers a competitive advantage for nutrients and oxygen to tumor cells facilitating accelerated proliferation of the tumor cells. Plant phytochemicals have been attributed to inhibit angiogenic promoters in tumors facilitating treatment of solid tumors (Weis and Cheresh, 2011; Birbrair et al., 2015).

Apoptosis is a physiological process described as programed cell death. It is an essential process that to eliminate worn out and damaged cells from the body hence balancing cell division. Apoptosis is characterized by morphological hallmarks that include chromatid condensation and nuclear fragmentation, membrane bebbing and loss of integrity. The biochemical events of apoptosis include activation of caspases proteins and DNA and protein breakdown. In cancer phenomenon, deregulation of apoptosis results in imbalance of cell division and cell loss. Apoptotic has been a target pathway
for development of various anticancer therapeutics (Wong, 2011). Phytochemical such as flavonoids and tannins have been associated to induce apoptosis in cancer treatment through reduction of reactive oxygen species (ROS), activation of caspase proteins, blocking of DNA topoisomerase I/II activity, modulation of cell signaling pathways among others (Wang et al., 1999; Wenzel et al., 2000; Bailey, 2000). Owing to various mechanisms of anticancer activities exemplified by various mechanisms of activity demonstrated by various phytochemicals medicinal plants possesses key ingredients for development of anticancer drugs. Therefore bio-screening of various plant extracts for anticancer activity and mechanism of activity is greatly emphasized.

2.12 Cassia abbreviata

2.13.1 Plant Description

*Cassia abbreviata* is a shrub tree belonging to the family of Fabaceae. It is known by various local names according to various communities namely Afrikaans (sambokpeul), Bemba (musambafwa), Somali (rabuya or domader), Swahili (mkakatika or mbaraka). It grows to a height of 3–10m in height. *Cassia abbreviata* is widespread from Somalia to South Africa at altitude 220–1520m above sea level. It grows in open woodland and wooded grassland, sometimes on river banks hillsides and termite mounds. Its leaves are evenly compound with a pair of leaflets per leaf. Flowers are pea-like, yellow coloured and appear at the same time with leaves. Fruits are up to 80cm long, thin and cylindrical in shape. Pods ripen nearly a year after flowering and release seeds once they are mature but remain on the tree for a long time (Orwa et al., 2009; Sepeke et al., 2013). The image of the plant is shown in figure 2.1.
Figure 2.1: *Cassia abbreviata*

2.13.2 Economic Importance

There are many economic benefits derived from this plant species. The wood is used for firewood and timber, while the bark and roots are used in traditional medicine to treat different ailments. Leaves and young branches provide fodder to wildlife, while pods are eaten by baboons and monkey. Seeds are eaten by birds as food (Sekepe et al., 2013).

2.13.3 Medicinal Uses

2.13.3.1 Roots

The root is ground into powder and mixed in water then drunk against abdominal pains, dysentery, fever, malaria, hernia, wounds, diarrhea, constipation, venereal diseases,
syphilis, impotency and snake bite and also used as aphrodisiac and arbotifacient (Chhabra et al., 1987; Gessler et al., 1995; Makundi et al., 2006; Wyk and Gericke, 2007; Muthaura et al., 2007; Ribeiro et al., 2010).

2.13.3.2 Stem Bark

Stem bark extract is taken orally to treat stomach ache and malaria. Infusion of roots, leaves and stem bark mixed is taken orally to treat stomach ache (Ribeiro et al., 2010). Bark and roots can be used to treat stomach ache of a mother during pregnancy, close fontanelle of newborn babies, treats dysentery, blood vomits, snake bites, bilharzia, hernia, post-partum pains and menstrual cycle pains (Mojeremane et al., 2005; Bruschi et al., 2011).

2.13.3.3 Leaves

Leaf decoction of Cassia abbreviata is taken orally to treat malaria (Keter and Mutiso, 2012). It is also used to treat skin rashes and wounds associated with human immunodeficiency virus (HIV) and acquired immune deficiency syndrome (AIDS) (Kisangau et al., 2011). Leaves, roots and bark are taken orally, nasally and anally to treat infertility, cough, vomiting, epilepsy, bilharzia, syphilis, gonorrhoea, jaundice and hernia (Augustino et al., 2011).
CHAPTER THREE
MATERIALS AND METHODS

3.1 Collection and Preparation of Plant Material

The *Cassia abbreviata* root bark used in this study was collected from Bura, Taita Taveta with help of local herbalist. The root bark was cleaned and transported to Kenya Medical Research Institute (KEMRI) for further processing. The plant samples were then provided to an acknowledged taxonomist for botanical verification and a voucher specimen deposited at the KEMRI Herbarium. The root bark was dried under shade at Kenya Medical Research Institute at room temperature. After drying it was ground to powder by an electric mill, weighed and packaged.

3.2 Aqueous Extraction

A weight of 100g of root bark powder of *Cassia abbreviata* was soaked in 1 litter of double distilled water and heated at 60°C in water bath for 6 hours for aqueous extraction. It was covered and left to cool under room temperature. The extract was decanted in a clean 1000ml conical flask and filtered with Whatman no. 1 filter paper into a clean sterile 1000ml conical flask. Decantation and filtration was repeated until the filtrate become clear. The filtrate was centrifuged at 3000 RPM for 5 minutes, freeze dried, weighed and stored in an air tight container under -20°C for future use in bioassay (Ngugi *et al.*, 2011; Kigondu *et al.*, 2011).
3.3 Determination of Antiproliferative Activity

3.3.1 Cell Lines

This study used HCC Hep G2, Macrophages Raw 264.7 and Vero E6 cell lines. All the cell lines were provided by Kenya Medical Research Institute (KEMRI), Centre for Traditional Medicine and Drug Research (CTMDR).

3.3.2 Cell Culture

The cell lines used in the study were maintained as monolayer cultures in RPMI 1640 supplemented with 10% heat inactivated fetal bovine serum (FBS), L-Glutamine, 100μg/ml penicillin and 100μg/ml streptomycin incubated at 37°C in a humidified incubator at 5% CO₂.

3.3.3 Cell Treatment

The obtained cell monolayer was treated with trypsin 0.25%-EDTA 10mM after they had reached 70-100% confluence. A volume of 5ml of growth media was added to neutralize the trypsin enzyme. The cell suspension was transferred to centrifuge tubes and centrifuged at 3000 rpm for 5 minutes. The supernatant was discarded and cells resuspended in 5ml culture media and cell density was determined using trypan blue exclusion assay. The cells were seeded independently in 96 well plate at 2x10⁴ cells per well at a volume of 100μl and incubated at 5% CO₂ humidified incubator at 37°C for 24 hours.
The treatment was done by adding 50μl of aqueous extract of *C. abbreviata* at 7 concentrations namely 1000μg, 333.33μg, 111.11μg, 37.04μg, 12.35μg, 4.12μg, 1.37μg and the last row of the 96 well plates was left untreated. Tamoxifen was used as the reference drug at the following concentrations 100μg, 33.33μg, 11.11μg, 3.70μg, 1.24μg, 0.46μg, 0.41μg. They were then incubated in 5% CO₂ humidified environment at 37°C for 48 h after which the MTT viability assay was carried out. The design is summarized in table 3.1.

### Table 3.1: Experimental Design

<table>
<thead>
<tr>
<th>Groups</th>
<th>Cassia abbreviata</th>
<th>Tamoxifen (Reference drug)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Negative control</td>
<td>Untreated cells</td>
<td>Untreated cells</td>
</tr>
<tr>
<td>Experimental Groups</td>
<td>Concentration (μg/ml)</td>
<td>Concentration (μg/ml)</td>
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<tr>
<td>I</td>
<td>1.37</td>
<td>0.41</td>
</tr>
<tr>
<td>II</td>
<td>4.12</td>
<td>0.46</td>
</tr>
<tr>
<td>III</td>
<td>12.35</td>
<td>1.24</td>
</tr>
<tr>
<td>IV</td>
<td>37.04</td>
<td>3.70</td>
</tr>
<tr>
<td>V</td>
<td>111.11</td>
<td>11.11</td>
</tr>
<tr>
<td>VI</td>
<td>333.33</td>
<td>33.33</td>
</tr>
<tr>
<td>VII</td>
<td>1000</td>
<td>100</td>
</tr>
</tbody>
</table>

#### 3.3.4 Viability MTT Test

##### 3.3.4.1 Principle

This is a colorimetric assay based on enzymatic activity of Mitochondria succinate dehydrogenase enzymes in living cells to reduce the yellow water soluble substrate 3- (4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into formazan. Formazan is an insoluble yellow colored product that is measured spectrophotometrically in an optical density reader (Mosmann, 1983; Patel *et al.*, 2009).
The activity of the enzyme to produce formazan is directly proportional to the level of cell viability and inversely proportional to the level of cell inhibition (Mantani et al., 2001; Berridge et al., 2005).

### 3.3.4.2 MTT Assay Procedure

The viability test was done by the tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye reduction assay (Mosmann, 1983; Omoregie et al., 2011). After 48 hours of incubation, the culture medium in plates was discarded and wells washed with phosphate buffered saline (PBS). A volume of 10μl of PBS solution containing 10μg/ml of MTT was pipetted into each well and the plates incubated for 3 hours. A volume of 10μl of Pure DMSO was added and incubated for 30 minutes. The plates were mildly shaken at room temperature and the Optical density (OD) determined at 560 nm using a microplate reader spectrophotometer.

### 3.3.4.3 Determination of Proliferation

The proliferation of the cells after treatment was calculated using the formula developed by Patel et al. (2009) and Awasare et al. (2012) as follows;

\[
Proliferation\ rate = \frac{At - Ab}{Ac - Ab}
\]

\[
Percentage\ viability = \frac{At - Ab}{Ac - Ab} \times 100
\]

\[
Percentage\ inhibition = 100 - \frac{At - Ab}{Ac - Ab} \times 100
\]
Where,

\[ At = \text{Absorbance value of test compound} \]
\[ Ab = \text{Absorbance value of blank} \]
\[ Ac = \text{Absorbance value of negative control (cells plus media)} \]

### 3.4 Qualitative Phytochemical Screening

Qualitative phytochemical screening of aqueous root bark extract of *Cassisa abbreviata* was performed as described by Algelaagbe and Osamudiamen (2009). Secondary metabolites tested for included; alkaloids, anthraquinones, flavonoids, phenols, saponins, and terpenoids.

#### 3.4.1 Test for Saponins

One gram of powdered roots of the plant was shaken vigorously with warm water in a test tube. After shaking the test tube was allowed to stand. A persistent froth was observed which indicated presence of saponins.

#### 3.4.2 Test for Tannins

Weight of 1g of the plant extract was stirred in 2ml of distilled water and the filtered. Ferric chloride was then added to the filtrate. A green precipitate indicated presence of tannins.
3.4.3 Test for Alkaloids

Weight of 1g of the plant extract was heated gently in 10ml of 10% sulfuric acid for 5minutes. Resulting solution was tested for presence of alkaloids by adding 2 drops of Meyer's reagent. There was no white precipitate formed which indicated absence of alkaloids.

3.4.4 Test for Phenolics

Weight of 1g of plant extract was dissolved in 2ml of water and 2 drops of dilute ferric chloride solution was added. The formation of a red coloration evidenced presence of phenols.

3.4.5 Test for Flavonoids (Sodium hydroxide test)

Weight of 1g of the plant extract was dissolved in water, briefly warmed and filtered. Drop wise, 10% aqueous sodium hydroxide was added to 2ml of the filtrate. This produced a yellow coloration which turned colourless upon drop wise addition of dilute hydrochloric acid which indicated presence of flavonoids.

3.4.6 Test for Anthraquinones

Weight of 2g of the aqueous root bark extract of Cassia abbreviata was added to 5ml of benzene and filtered. A volume of 5ml 10% ammonium hydroxide was added and shaken. Violet colour in the ammoniacal phase was evidence of anthraquinones presence.
3.5 Data Management and Statistical Analysis

The data collected was both qualitative and quantitative. Data generated from *in vitro* antiproliferative potential of aqueous root bark extract of *Cassia abbreviata* experiments was quantitative data. It was stored in spreadsheets, subjected to descriptive statistics and values were expressed as Mean±SEM.

One-way ANOVA was used to compare means among treatment after which they were subjected to tukey’s post-hoc test for pairwise separation and comparison of means. The values of $P \leq 0.05$ were considered significant. A dose response curve was plotted and used to determine the IC$_{50}$ values by linear regression analysis. Unpaired t-test was used to compare between IC$_{50}$ values of aqueous root bark extract of *Cassia abbreviata* and Tamoxifen, the reference drug. Analysis of the data was done using Minitab statistical software version 17.0. The data was presented in graphs and tables.

The data generated from phytochemical screening of aqueous root bark extract of *Cassia abbreviata* was qualitative and was tabulated.

3.6 Ethical Considerations

This study did not involve human subjects or animals at any stage. However, all measures were undertaken to ensure that standard operating procedures were carried out to the letter and safety measures observed throughout the study.
CHAPTER FOUR

RESULTS

4.1 Determination of Antiproliferative Activity

4.1.1 Antiproliferative Activity of Aqueous Root Bark Extract of *Cassia abbreviata* on HCC Cell Line

Generally, aqueous root bark extract of *Cassia abbreviata* inhibited proliferation rate of the HCC cell line. Table 4.1 shows the effects of aqueous root back extract of *C. abbreviata* on proliferation rate of HCC cell line. There was a concentration dependent cell inhibition. As the concentration of aqueous root back extract of *C. abbreviata* increased, the rate of cell proliferation decreased (Table 4.1). Cell proliferation rate was low at the extract concentration of 1000µg/ml compared to untreated cells. The highest cell proliferation rate was observed in untreated cells which was significantly different from all treatments of HCC cells with the aqueous root bark extract of *Cassia abbreviata* \((p<0.05; \text{Table 4.1})\).

Similarly, as the concentration of aqueous root back extract of *C. abbreviata* increased the percentage inhibition increased and percentage cell viability decreased (Appendix I; Figure 4.1). Generally, it was observed that percentage cell inhibition was highest at higher concentration and low at lowest concentrations of the plant extract (Appendix 4.1).
Table 4.1: Effect of aqueous root bark extract of *Cassia abbreviata* on proliferation rate of HCC Cell Line

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Proliferation Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated cells</td>
<td>1.00±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.37</td>
<td>0.57±0.01&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4.12</td>
<td>0.44±0.03&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>12.35</td>
<td>0.34±0.07&lt;sup&gt;bcd&lt;/sup&gt;</td>
</tr>
<tr>
<td>37.04</td>
<td>0.20±0.04&lt;sup&gt;cde&lt;/sup&gt;</td>
</tr>
<tr>
<td>111.11</td>
<td>0.18±0.04&lt;sup&gt;cde&lt;/sup&gt;</td>
</tr>
<tr>
<td>333.33</td>
<td>0.14±0.06&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td>1000</td>
<td>0.06±0.04&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All values are expressed as Mean±SEM. Values followed by the same superscript are not significantly different (*P*<0.05).

Figure 4.1: Dose response curve for percentage inhibition and percentage viability for the effects of aqueous root bark extract of *Cassia abbreviata* on HCC cell line

Tamoxifen (reference drug) also showed antiproliferative activity against HCC cell line.

There was general observation that the inhibition of HCC proliferation by tamoxifen
was dose dependent. Increased tamoxifen concentration reduced the proliferation rate of HCC cells (Table 4.2). The highest proliferation rate was observed in untreated cell and lowest proliferation rate was observed in 100 µg/ml of tamoxifen. The proliferation rate in untreated cell was significantly different compared to proliferation rate at 100 µg/ml of tamoxifen (Table 4.2; P<0.05). The percentage cell inhibition increased and percentage cell viability decreased as the concentration as the concentration of the tamoxifen increased (Appendix II; Figure 4.2).

**Table 4.2: Effect of tamoxifen on proliferation rate of HCC cell line**

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Proliferation Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated Cells</td>
<td>1.00±0.00⁷</td>
</tr>
<tr>
<td>0.41</td>
<td>0.76±0.20ab</td>
</tr>
<tr>
<td>0.46</td>
<td>0.57±0.02abc</td>
</tr>
<tr>
<td>1.24</td>
<td>0.50±0.01abcd</td>
</tr>
<tr>
<td>3.70</td>
<td>0.45±0.03bcd</td>
</tr>
<tr>
<td>11.11</td>
<td>0.32±0.06bcd</td>
</tr>
<tr>
<td>33.33</td>
<td>0.11±0.09cd</td>
</tr>
<tr>
<td>100.00</td>
<td>0.08±0.09d</td>
</tr>
</tbody>
</table>

All values are expressed as Mean±SEM. Values followed by the same superscript are not significantly different (P>0.05).

**Figure 4.2: Dose response curve for percentage inhibition and percentage viability for effect of tamoxifen on HCC cell line**

\[
y = 11.596\ln(x) + 55.207 \quad R^2 = 0.9254
\]

\[
y = 23.877x^{-0.736} \quad R^2 = 0.9093
\]
4.1.2 Antiproliferative Activity of Aqueous Root Bark Extract of *Cassia abbreviata* on Vero Cell Line

Treatment of vero cells with aqueous root bark extract of *Cassia abbreviata* generally reduced proliferation rate of the vero cells (Table 4.3). The plant extract demonstrated a dose dependent manner of inhibition of proliferation rate of vero cells. As the concentration of plant extract increased from 1.37μg/ml to 1000μg/ml the proliferation rate decreased. The proliferation rate was lowest at 1000μg/ml and the highest at in untreated cell (Table 4.3). As the concentration increased, the proliferation rate reduced and proliferation rate increased as the concentration decreased (Table 4.3, Figure 4.5). There was a significant difference between proliferation rate at 1000μg/ml and untreated cells (p< 0.05; Table 4.3).

Percentage cell inhibition increased as the concentration of aqueous root bark extract of *Cassia abbreviata* increased while percentage cell viability increased as the concentration reduced (Appendix III; Figure 4.3). The highest percentage cell viability was observed in untreated cells and highest percentage cell inhibition was observed in 1000μg/ml (Appendix III; Figure 4.3).
Table 4.3: Effect of aqueous root bark extract of *Cassia abbreviata* on proliferation rate of vero cell line

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Proliferation Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated Cells</td>
<td>1.00±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.37</td>
<td>0.95± 0.00&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>4.12</td>
<td>0.67±0.05&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>12.35</td>
<td>0.62±0.02&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>37.04</td>
<td>0.60±0.02&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>111.11</td>
<td>0.58±0.02&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>333.33</td>
<td>0.47±0.01&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>1000</td>
<td>0.28±0.08&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All values are expressed as Mean±SEM. Values followed by the same superscript are not significantly different (*P*>0.05)

![Graph showing dose response curve for percentage inhibition and percentage viability for effect of *Cassia abbreviata* on vero cell line]

**Figure 4.3: Dose response curve for percentage inhibition and percentage viability for effect of *Cassia abbreviata* on vero cell line**

Table 4.4 shows the effects of tamoxifen of proliferation rate of vero cells. There was a concentration dependent inhibition of proliferation of vero cells by tamoxifen. As the concentration of tamoxifen increased the proliferation rate of vero cells decreased (Table 4.4). It was observed that the percentage cell inhibition increased as the
concentration of the drug increased and percentage cell viability decreased as the concentration of the tamoxifen increased (Appendix IV; Figure 4.4).

**Table 4.4: Effect of tamoxifen on proliferation rate of vero cell line**

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Proliferation Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated Cells</td>
<td>1.00±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.41</td>
<td>0.74±0.16&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.46</td>
<td>0.56±0.10&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.24</td>
<td>0.41±0.14&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>3.70</td>
<td>0.33±0.15&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>11.11</td>
<td>0.20±0.14&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>33.33</td>
<td>0.15±0.05&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>100.00</td>
<td>0.07±0.03&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All values are expressed as Mean±SEM. Values followed by the same superscript are not significantly different (P>0.05).

**Figure 4.4: Dose response curve for percentage inhibition and percentage viability for effect of tamoxifen on vero cell line**

**4.1.3 Antiproliferative Activity of Aqueous Root Bark Extract of *Cassia abbreviata* on Macrophage Cell Line**

Administration of aqueous root bark extract of *Cassia abbreviata* to macrophage generally limited proliferation rate of the macrophage cells. The results of effect of
aqueous root back extract of *C. abbreviata* on macrophage cell line are shown in table 4.5. The plant exhibited a dose dependent inhibition of proliferation rate of macrophage cell line. As the concentration of aqueous root bark extract of *Cassia abbreviata* enhanced, the proliferation rate of macrophage cells decreased (Table 4.5). At 1000µg/ml the proliferation rate was low compared to 1.37µg/ml of the plant extract. The proliferation rate was significantly different between treatments at 1000µg/ml of plant extract and untreated cells (p< 0.05; Table 4.5).

High percentage cell viability was observed at lower concentrations of the plant extract (Appendix V; Figure 4.5). As figure 4.5 shows, there was an increase in percentage cell inhibition and decrease in percentage cell viability as the plant extract concentration increased (Appendix V).

**Table 4.5: Effect of aqueous root bark extract of *Cassia abbreviata* on proliferation rate of macrophage cell line**

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Proliferation Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Untreated Cells</strong></td>
<td>1.00±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.37</td>
<td>0.85± 0.00&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>4.16</td>
<td>0.75±0.04&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>12.35</td>
<td>0.70±0.03&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>37.04</td>
<td>0.70±0.02&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>111.11</td>
<td>0.57±0.01&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>333.33</td>
<td>0.37±0.04&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td>1000</td>
<td>0.31±0.07&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All values are expressed as Mean±SEM. Values followed by the same superscript are not significantly different (*P*<0.05)
On the other hand, tamoxifen inhibited proliferation of macrophages in a concentration dependent manner. Increased concentration of tamoxifen reduced proliferation rate of Macrophage cells (Table 4.6). It was observed that the percentage cell inhibition increased as the concentration of the drug increased and percentage cell viability decreased as the concentration of the drug increased (Appendix VI; Figure 4.6).

Table 4.6: Effect of tamoxifen on proliferation rate of macrophage cell line

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>Proliferation Rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated Cells</td>
<td>1.00±0.00&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.41</td>
<td>0.93±0.05&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>0.46</td>
<td>0.79±0.07&lt;sup&gt;abc&lt;/sup&gt;</td>
</tr>
<tr>
<td>1.24</td>
<td>0.76±0.09&lt;sup&gt;ab&lt;/sup&gt;</td>
</tr>
<tr>
<td>3.70</td>
<td>0.62±0.02&lt;sup&gt;bc&lt;/sup&gt;</td>
</tr>
<tr>
<td>11.11</td>
<td>0.49±0.04&lt;sup&gt;cde&lt;/sup&gt;</td>
</tr>
<tr>
<td>33.33</td>
<td>0.38±0.08&lt;sup&gt;de&lt;/sup&gt;</td>
</tr>
<tr>
<td>100.00</td>
<td>0.19±0.09&lt;sup&gt;e&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

All values are expressed as Mean±SEM. Values followed by the same superscript are not significantly different (P>0.05)
Figure 4.6: Dose response curve for percentage inhibition and percentage viability for effects of tamoxifen on macrophage cell line

4.2 Determination of IC$_{50}$ of Aqueous Root Bark Extract of *Cassia abbreviata* against HCC, Macrophage and Vero Cell Lines

The IC$_{50}$ values were determined from drug-response curve plotted on MS Excel using the equation of the trend lines attached to the curve. Aqueous root bark extract of *Cassia abbreviata* had IC$_{50}$ of 1.49µg/ml, 81.08µg/ml, and 128.38µg/ml on HCC, macrophage and vero cell lines respectively. Tamoxifen had IC$_{50}$ values of 5.75µg/ml, 29.00µg/ml and 1.60µg/ml on HCC, macrophage and vero cell lines respectively (Table 4.7). Aqueous root bark extract of *Cassia abbreviata* had the highest IC$_{50}$ on HCC, followed by macrophage and vero cell lines. A similar trend was observed with the reference drug. Unpaired Student t-test was used to determine significant difference between the IC$_{50}$ of aqueous root bark extract of *Cassia abbreviata* and reference drug. The antiproliferative activity of the plant extract against HCC cell line was comparable.
to that of tamoxifen in HCC cell line ($P>0.05$; table 4.7). Tamoxifen had significantly higher antiproliferative effects on macrophage and vero cell lines than the aqueous root bark extract of *Cassia abbreviata* ($P>0.05$; Table 4.7).

Table 4.7: IC$_{50}$ values of aqueous root bark extract of *Cassia abbreviata* and tamoxifen on HCC, macrophage and vero Cell Lines.

<table>
<thead>
<tr>
<th>Cell lines</th>
<th>IC$_{50}$</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Cassia abbreviata</em></td>
<td>Tamoxifen</td>
</tr>
<tr>
<td>HCC</td>
<td>1.49$^a$</td>
</tr>
<tr>
<td>Macrophages</td>
<td>81.08$^a$</td>
</tr>
<tr>
<td>Vero E6</td>
<td>128.38$^a$</td>
</tr>
</tbody>
</table>

4.3 Qualitative Phytochemical Screening

A number of classes of compounds were identified in aqueous root bark extract of *Cassia Abbreviata*. As table 4.4 shows the aqueous root bark extract of *Cassia Abbreviata* contained flavonoids, saponins, anthraquinones, phenolics, tannins, saponins however alkaloids were absents.
Table 4. 8: Qualitative phytochemistry of aqueous root bark extract of *Cassia abbreviata*

<table>
<thead>
<tr>
<th>Phytochemicals</th>
<th>Results</th>
</tr>
</thead>
<tbody>
<tr>
<td>Flavonoids</td>
<td>+</td>
</tr>
<tr>
<td>Phenolics</td>
<td>+</td>
</tr>
<tr>
<td>Tannins</td>
<td>+</td>
</tr>
<tr>
<td>Saponins</td>
<td>+</td>
</tr>
<tr>
<td>Alkaloids</td>
<td>-</td>
</tr>
<tr>
<td>Anthraquinones</td>
<td>+</td>
</tr>
</tbody>
</table>

Present phytochemicals denoted by (+) sign; absent phytochemicals denoted by (-) sign
CHAPTER FIVE
DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 Discussion

This research investigated the antiproliferative activity of aqueous root bark extract of Cassia abbreviata on three cell lines namely; hepatocellular carcinoma (HCC), macrophage and vero cell lines. Generally, the aqueous root bark extract of Cassia abbreviata had antiproliferative activity against hepatocellular carcinoma, macrophages and vero cells. The plant extract demonstrated a concentration dependent antiproliferative activity on all the cell lines. A similar observation was made by Awasare et al. (2012) who demonstrated a concentration dependent inhibition of five cell lines namely; human leukemia (HL-60) cell line, human colon cancer (HT-29) cell line, human breast cancer (MCF-7) cell line, human skin cancer (A 431) cell line and human lung cancer (A 549) cell lines by oleanane type of triterpenoid saponin from stem bark extract of Manilkara zapota linn. Grbović et al. (2013) observed a dose dependent inhibition of Colon cancer (HCT-116) and breast cancer (MDA-MB-231) cell lines by methanolic extract of O. vulgare in vitro. Bhaumik et al. (2014) also observed a concentration dependent inhibition in in vitro evaluation of cytotoxic activity of fruits of Lagerstroemia speciosa L on Human Acute Monocytic Leukemia-HL-60 cell line and Human Colon Cancer- DLD-1 cell line.

That the aqueous root bark extract of Cassia abbreviata had antiproliferative activity against HCC, vero and macrophage cell lines, agrees with the effects of other medicinal plant on these cell lines. Similar work carried out by Kumar and Selvakumar (2015)
demonstrated antiproliferative activity of *Tabernaemontana divaricata* against HCC and vero cell lines. Rungrojtrakoo *et al.* (2012) undertook a study that demonstrated antiproliferative activity of Thai medicinal plants against various cancer cell lines including HCC and vero cell lines. Talib and Mahasneh, (2010) demonstrated antiproliferative activity of plant extracts used against cancer in traditional medicine against various cancer cell lines including HCC and vero cell lines.

An extract is defined to be highly active if it has IC$_{50}$ < 10µg/ml, moderately active if IC$_{50}$ is between 10µg/ml-50µg/ml and inactive if IC$_{50}$ > 50µg/ml (Mbatchi *et al*., 2006). In this study, the IC$_{50}$ for *Cassia abbreviata* against HCC was 1.49µg/ml. Therefore, aqueous root bark extract of *Cassia abbreviata* is considered highly antiproliferative against the cancer cell line. On the other hand, the plant extract had an IC$_{50}$ of 81.08µg/ml and 128.38µg/ml on macrophage and vero cell lines respectively. Its high IC$_{50}$ values for normal vero and macrophage cell lines indicate that it is a safe anticancer medicinal plant and is selectively inhibitory on cancer cells. According to Zirich *et al.* (2005) plants with IC$_{50}$ ≥ 20µg/ml on normal cells are recognized as safe. Inhibitory selectivity of medicinal plants with antiproliferative activity has been also demonstrated in previous studies. Gul *et al.* (2013) while determining antioxidant and antiproliferative activities of *Abrus precatorius* leaf extract, observed that the extract selectively inhibited proliferation of HCC cell lines among others while sparing macrophage cells. In a corresponding work, Patel *et al.* (2009) observed that *Solanum nigrum* extract had selective antiproliferative activity against hela cell line and moderately inhibitory against vero cell line. In addition, methanolic extracts of *Elaeis*
guineensis have shown selective antiproliferative effects against MCF-7 and Vero cell lines as demonstrated by (Vijayarathna and Sasidharan, 2012).

There is a need to develop cancer drugs that can potentially selectively target cancer cells and spare normal body cells by their inherent cellular difference. The discovery of such drugs with differential action will be very significant in cancer therapy to reduce cancer drugs toxicity and side effects (Janki and Piyush, 2013). Aqueous root bark extract of Cassia abbreviata demonstrated such selectivity.

The antiproliferative activity of aqueous root bark extract of Cassia abbreviata could be attributed to the phytochemicals present in the extract. Qualitative phytochemical screening in this study showed that the aqueous root bark extract of Cassia abbreviata contains saponins, phenolics, anthraquinones, flavonoids and tannins. Tannins have been reported to possess anticancer and antioxidant activities (Rivière et al., 2009; Yıldırım and Kutlu, 2015). A tannin compound, Cuphiin D1 (CD1), isolated from Cuphea hyssopifolia has been proven to exert antiproliferative effects against human promyelocytic leukemia HL-60 cell line (Wang et al., 2000). Corilagin, a tannin compound, isolated from a number of medicinal plants including Phyllanthus niruri has been shown to have antiproliferative effects against ovarian cancer cell lines (Jia et al., 2013). Pancreatic cancer cells and breast cancer cells proliferation has been inhibited by Ellitannin compound isolated from the Cistus ladanifer (Barrajon-Catalan et al., 2010).
Saponins have also been reported to show antioxidant and anticancer effects (Mithraja et al., 2011). It has been observed that triterpene saponins (saxifragifolin B and saxifragifolin D) extracted from *Androsace umbellate* extract inhibited cancer cell growth and induced apoptosis in multi-drug resistance and non-multidrug resistance tumor cell lines (Park et al., 2010). Hu et al. (2014) found that triterpene saponins extracted from *Nepenthes glandulifera* exhibited antiproliferative activity against human lung carcinoma A-549 cell line.

Flavonoids have been shown to possess antioxidant and anticancer properties (Middleton et al., 2000; Chahar et al., 2011). Flavopiridol is a flavonoid that has been shown to inhibit several cyclin dependent kinases blocking the cell cycle block. It has demonstrated anticancer activity in renal, colon, non-Hodgkin's lymphoma, prostate, colon and gastric carcinomas (Senderowicz, 2001). Quercetin, a flavonoid compound has been reported to have antiproliferative activity against many cancers namely hepatocellular carcinoma and neuroblastoma (Chandrappa et al., 2014; Priya et al., 2014). Therefore, it is likely that the phytochemicals identified as occurring in the aqueous root bark extract of *Cassia abbreviata* are responsible for its antiproliferative activity.

This study found that *Cassia abbreviata* had IC\textsubscript{50} values of 1.49µg/ml, 81.08µg/ml, and 128.38µg/ml on HCC, macrophage and vero cell lines respectively. *Abrus precatorius leaf extracts* was reported to have an IC\textsubscript{50} value of 27.03µg/ml on HCC cell line (Gul et al., 2013). An IC\textsubscript{50} value of 6.862µg/ml against vero cells was reported in a study to
determine *in vitro* cytotoxicity activity of *Solanum nigrum* extract (Patel *et al.*, 2009). *Elaeis guineensis* extract showed antiproliferative activity demonstrating an IC$_{50}$ value of 22.00µg/ml against vero cells (Vijayarantha and Sasidharan, 2012). *Ononis hirta* has been shown to inhibit the proliferation of different cancer cell lines and showed IC$_{50}$ values of 54.22µg/ml and 41.87µg/ml against HCC and Vero cell lines (Talib and Mahasneh, 2010). *Argemone gracilenta* methanolic extract has been shown to have antiproliferative activity against macrophage cells with IC$_{50}$ values of 64.45µg/ml (Leyva-Peralta *et al.*, 2015).

Rungrojtrakool *et al.* (2012) determined antiproliferative activity against various cancer cell lines of Thai herbal formula. His study reported that *Caesalpinia hymenocarpa* had 72.6µg/ml and 551.5µg/ml IC$_{50}$ values on HCC and vero cell lines respectively. Mbong *et al.* (2013) determined antiproliferative effect of hydroethanolic extracts of seeds of *Cola verticillata* and leaves of *Solanum scabrum*. The study reported that *Solanum scabrum* had an IC$_{50}$ value of 11.30µg/ml on human ovarian carcinoma cell line. Janki and Piyush, (2013) demonstrated anticancer and cytotoxic potential of *Triticum aestivum* extract on hela cell line. The study reported an IC$_{50}$ value of 156µg/ml against hela cell line. Grbović *et al.* (2013) determined *in vitro* antiproliferative activity of *Origanum vulgare* on human colon cancer and human breast cancer cell lines. The study reported an IC$_{50}$ value of 109µg/ml and 506µg/ml on human colon cancer and human breast cancer cell lines respectively. Itharat *et al.* (2003) determined *in vitro* antiproliferative activity of Thai medicinal plants used to treat cancer. The study reported that aqueous extract of *Dioscorea membranacea* had IC$_{50}$ values of 5.5µg/ml, 15.6µg/ml and
16.3μg/ml against breast, colon and lung cancer cell lines respectively. George et al. (2015) confirmed in vitro antiproliferative activity of the acetone extract of Rubus fairholmianus root against human colorectal cancer cells.

5.2 Conclusion

In conclusion, the present study has demonstrated antiproliferative activity of aqueous root bark extract of Cassia abbreviata in vitro. The significant antiproliferative activity of Cassia abbreviata against HCC cell line reflects that the plant is endowed with potent antiproliferative properties. The ability of Cassia abbreviata to have more antiproliferative effects on HCC cell line than in normal cell lines, as denoted by the IC50 values, is evident of selective inhibitory properties of the plant. The classes of phytochemicals confirmed in aqueous root bark extract of Cassia abbreviata have been recognized to have anticancer properties.

Therefore, Cassia abbreviata serves as a good bio-resource for generating a readily available herbal anticancer formulation. However, the antiproliferative mechanism of action of the studied extract is still obscure. This study, therefore, scientifically confirms and supports the traditional use of Cassia abbreviata for management of neoplasm. The research questions for this study have thus been answered.
5.3 Recommendations

From this study, the following recommendations can be made;

i. Aqueous root bark extract of *Cassia abbreviata* may be used for management of various for antineoplastic purposes.

ii. *Cassia abbreviata* can be used as a potential candidate for development of antineoplastic drugs to hepatocellular carcinoma.

5.4 Suggestions For Further Research

i. Elucidation of antiproliferative mechanism of *Cassia abbreviata*.

ii. Determination of *in vivo* antiproliferative activity of aqueous root bark extract of *Cassia abbreviata*.

iii. Determination of antiproliferative activity of other parts of the plant

iv. Isolation and identification of the antiproliferation compounds in *Cassia abbreviata* plant.
REFERENCES


containing ellagitannins show antioxidant and antimicrobial capacity, and cytotoxic activity against human cancer cells. *Food and Chemical Toxicology*, 48(8): 2273-2282.


Sigma-aldrich, 2016. lactate dehydrogenase kit, Online.


### APPENDICES

**Appendix I:** Percentage inhibition and percentage viability effect of aqueous root bark extract of *Cassia abbreviata* on HCC cell lines.

<table>
<thead>
<tr>
<th>Concentration (µg/ml)</th>
<th>% Viability</th>
<th>% Inhibition</th>
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</thead>
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<td>0</td>
</tr>
<tr>
<td>1.37</td>
<td>57</td>
<td>43</td>
</tr>
<tr>
<td>4.12</td>
<td>44</td>
<td>56</td>
</tr>
<tr>
<td>12.35</td>
<td>34</td>
<td>66</td>
</tr>
<tr>
<td>37.04</td>
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<td>80</td>
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<td>1000</td>
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**Appendix II:** Percentage inhibition and percentage viability for effect of aqueous root bark extract of tamoxifen on HCC cell line

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<th>% Viability</th>
<th>% Inhibition</th>
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</thead>
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<tr>
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</table>
Appendix III: Percentage inhibition and percentage viability for effect of aqueous root bark extract of *Cassia abbreviata* on vero cell lines.

<table>
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<th>Concentration (µg/ml)</th>
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<tr>
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Appendix IV: Percentage inhibition and percentage viability for effect of aqueous root bark extract of tamoxifen on vero cell line

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<th>% Inhibition</th>
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Appendix V: Percentage inhibition and percentage viability for effect of aqueous root bark extract of *Cassia abbreviata* on macrophage cell lines.

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<th>Concentration (µg/ml)</th>
<th>% Viability</th>
<th>% Inhibition</th>
</tr>
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<td>37</td>
<td>63</td>
</tr>
<tr>
<td>1000</td>
<td>31</td>
<td>69</td>
</tr>
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</table>

Appendix VI: Percentage inhibition and percentage viability for effect of aqueous root bark extract of tamoxifen on macrophage cell line

<table>
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<th>Concentration (µg/ml)</th>
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<th>% Inhibition</th>
</tr>
</thead>
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<td>51</td>
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<td>38</td>
<td>72</td>
</tr>
<tr>
<td>100.00</td>
<td>19</td>
<td>81</td>
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</table>
Appendix VIII: Reagents

The reagents used in this study included; RPMI1640, Fetal Bovine serum, Phenol red, L-glutamine, HEPES, Trypsin, Sodium hydrogen carbonate, Hand gloves (sterile), Dimethyl sulfoxide (DMSO), 5ml sterile graduated pipettes, MTT reagent, Penicillin/streptomycin, 96 well plate flat bottomed with cover, Culture flasks: T75, Ethanol analar, Trypan Blue Sigma, 25ml graduated sterile, pipettes, Eagles MEM medium, 24 well plate, Acetone and Pippete tips 20, 100, 200µl. These reagents were procured from Sigma Aldrich, Germany. Tamoxifen, streptomycin and penicillin were procured locally from local pharmacies.

Appendix VIII: Equipment

The following equipment were used in this study; CO2 Incubator (Sanyo, Japan), water bath (Sakura, Japan), Liophilizer (Edward, Britain), Electric Mill (Edward, Britain), Weighing Balance (Mettler AC 1000, Switzland), Inverted Microscope (Nikon, Japan), Biosafety Cabinet ClassII (Esco, Japan), Deep Freezer (Sanyo, Japan), ELISA plate reader (Labsystems, Japan) and Hemocytometer (Esco, Japan).
One-way ANOVA: Concentration versus macrophage responses

<table>
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<tr>
<th>Source</th>
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<th>Adj MS</th>
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<th>P-Value</th>
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<td>1.1348</td>
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Means

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<th>95% CI</th>
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<tbody>
<tr>
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<td>3</td>
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<td>(0.836, 1.164)</td>
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<tr>
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<tr>
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</tr>
<tr>
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<td>(0.144, 0.471)</td>
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Pooled StDev = 0.133804

One-way ANOVA: Concentration versus HCC Responses

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Means

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Pooled StDev = 0.0938969

One-way ANOVA: Concentration versus Vero responses

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Means

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### One-way ANOVA: Tamoxifen Concentration versus Macrophages responses

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#### Model Summary

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#### Means

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<th>95% CI</th>
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<td>0.0428</td>
<td>(0.4832, 0.7624)</td>
</tr>
<tr>
<td>11.111</td>
<td>3</td>
<td>0.4910</td>
<td>0.0651</td>
<td>(0.3514, 0.6306)</td>
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<tr>
<td>33.333</td>
<td>3</td>
<td>0.3817</td>
<td>0.1425</td>
<td>(0.2421, 0.5213)</td>
</tr>
<tr>
<td>100.000</td>
<td>3</td>
<td>0.1905</td>
<td>0.1574</td>
<td>(0.0510, 0.3301)</td>
</tr>
</tbody>
</table>

**Pooled StDev = 0.114042**

### One-way ANOVA: Tamoxifen Concentration versus Vero responses

<table>
<thead>
<tr>
<th>Source</th>
<th>DF</th>
<th>Adj SS</th>
<th>Adj MS</th>
<th>F-Value</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>conc</td>
<td>7</td>
<td>2.1309</td>
<td>0.30441</td>
<td>8.37</td>
<td>0.000</td>
</tr>
<tr>
<td>Error</td>
<td>16</td>
<td>0.5820</td>
<td>0.03638</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>23</td>
<td>2.7129</td>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

#### Model Summary

<table>
<thead>
<tr>
<th>S</th>
<th>R-sq</th>
<th>R-sq(adj)</th>
<th>R-sq(pred)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.190726</td>
<td>78.55%</td>
<td>69.16%</td>
<td>51.73%</td>
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</table>

#### Means

<table>
<thead>
<tr>
<th>conc</th>
<th>N</th>
<th>Mean</th>
<th>StDev</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.000</td>
<td>3</td>
<td>1.000</td>
<td>0.000</td>
<td>(0.767, 1.233)</td>
</tr>
<tr>
<td>1.371</td>
<td>3</td>
<td>0.744</td>
<td>0.273</td>
<td>(0.511, 0.977)</td>
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<tr>
<td>4.115</td>
<td>3</td>
<td>0.5589</td>
<td>0.1689</td>
<td>(0.3254, 0.7923)</td>
</tr>
<tr>
<td>12.345</td>
<td>3</td>
<td>0.408</td>
<td>0.239</td>
<td>(0.174, 0.641)</td>
</tr>
<tr>
<td>37.037</td>
<td>3</td>
<td>0.332</td>
<td>0.257</td>
<td>(0.099, 0.566)</td>
</tr>
<tr>
<td>111.111</td>
<td>3</td>
<td>0.202</td>
<td>0.238</td>
<td>(-0.031, 0.436)</td>
</tr>
<tr>
<td>333.333</td>
<td>3</td>
<td>0.1505</td>
<td>0.0778</td>
<td>(-0.0830, 0.3839)</td>
</tr>
<tr>
<td>1000.000</td>
<td>3</td>
<td>0.0691</td>
<td>0.0501</td>
<td>(-0.1643, 0.3026)</td>
</tr>
</tbody>
</table>

**Pooled StDev = 0.190726**
THIS IS TO CERTIFY THAT:
MR. NJAGI SHADRACK MWENDA
of KYENATTA UNIVERSITY, 16-60401 chogoria, has been permitted to conduct research in Nairobi County
on the topic: IN VITRO ANTIPROLIFERATIVE ACTIVITY OF AQUEOUS ROOT BARK EXTRACT OF CASSIA ABBREVIA
for the period ending: 13th June, 2017

CONDITIONS

1. You must report to the County Commissioner and the County Education Officer of the area before embarking on your research. Failure to do that may lead to the cancellation of your permit.
2. Government Officers will not be interviewed without prior appointment.
3. No questionnaire will be used unless it has been approved.
4. Excavation, filming and collection of biological specimens are subject to further permission from the relevant Government Ministries.
5. You are required to submit at least two (2) hard copies and one (1) soft copy of your final report.
6. The Government of Kenya reserves the right to modify the conditions of this permit including its cancellation without notice.

Serial No. A 9578

CONDITIONS: see back page