ANTICANCER, ANTIOXIDANT AND PHYTOCHEMICAL EVALUATION OF *AGERATUM CONYZOIDES* LINN.

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DECLARATION

I hereby declare that this submission is my own work towards the MPhil and that, to the best of my knowledge, it contains no material previously published by another person nor material which has been accepted for the award of any other degree of the University, except where due acknowledgement has been made in the text.

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ABSTRACT

Cancer is still a major public health burden in many countries and despite the research strides made in respect of therapeutic interventions, challenges still abound in cancer treatment. Chemotherapy, the standard treatment method, is fraught with various shortcomings like drug resistance and undesirable side effects. Consequently, natural products present promising alternatives to cancer chemotherapy and hence finding new anticancer agents from natural sources will be critical in improving patient outcomes. *Ageratum conyzoides*, an annual herb is increasingly been used in folklore for the treatment of a wide range of diseases including cancer. The study was aimed at investigating the antioxidant and anticancer potentials as well as evaluating the phytochemical constituents of the aqueous and 50% hydroethanolic extracts and fractions of *Ageratum conyzoides*. The plant was subjected to decoction and ethanol extraction (cold maceration) under reduced pressure and further fractionation was carried out on the ethanolic leaf extract using different solvents. Total antioxidant potential of the crude aqueous and ethanolic extracts was investigated for free radical scavenging activity, for the presence of glutathione as well as total phenolic contents. The anticancer study was performed using the tetrazolium-basesd colorimetric, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, on leukemia, prostate and breast cancers, and prostate normal cell lines (Jurkat, LNCap, MCF-7, and PNT2), respectively, using the crude aqueous and ethanolic extracts as well as fractions of the ethanolic leaf extract. Curcumin was used as standard. The selective ability of each extract and fraction was also determined. The presence of tannins, flavonoids, alkaloids, terpenoids and saponins were qualitatively screened for in the crude aqueous and 50% hydroethanolic extracts. The results showed that the crude extracts scavenged 2,2- diphenyl-1-picryl hydrazyl (DPPH) free radical in a dose dependent manner compared to the positive control, butylated hydroxy toluene (BHT). Phenolic compounds were present, but glutathione was not detected in any of the crude extracts. The aqueous leaf extract was the strongest scavenger of DPPH, with 50% effective concentration (EC_{50}) of 0.091 ± 0.024 mg/ml. It also recorded the highest total phenol content, 1678.86 ± 40.67 mg/g equivalent of gallic acid. The anti-cancer results indicated that the ethanolic leaf extract had comparatively lower cytotoxic effects on the cell lines, with IC_{50} values of 15.08 ± 0.28, 304.22 ± 71.54 and 934.94 ± 105.91 µg/ml on Jurkat, LNCap and MCF-7 cells respectively. On the other hand, the chloroform and ethylacetate fractions exhibited stronger cytotoxic effects with IC_{50} values of 6.36 ± 1.56, 35.28 ± 6.46 and 74.27 ± 8.15 µg/ml for the former and 4.6 ± 0.12, 37.28 ± 2.09 and 67.38 ± 1.71 µg/ml for the latter in Jurkat, LNCap and MCF-7 cell lines respectively. All extracts and fractions were not cytotoxic to the PNT2 cell lines. The ethanolic leaf extract as well as the chloroform and ethylacetate fractions were strongly selective against Jurkat cell lines, with selectivity indices (SI) values of 66.33, 15.72 and 21.74 respectively. The presence of tannins, saponins and terpenoids were concentrated in the leaf and flower of the plant. The present investigation suggests that *Ageratum conyzoides* possesses remarkable antioxidant and anticancer properties and the chemical compounds detected could be responsible for these properties.

DEDICATION

I dedicate this work to the Almighty God whose abundant grace, mercies and divine blessings saw me through. I also dedicate my dissertation work to my family and many friends. A special feeling of gratitude to my loving parents, Edward and Christiana Acheampong whose words of encouragement and push for tenacity ring in my ears. My siblings Cecilia, Joyce, Eric, Samuel and Comfort have never left my side and are very special. I also dedicate this dissertation to my cherished one, Beatrice Serwaa Manu, who has supported me throughout the process. She has been my best cheerleader. I will always appreciate all relatives and friends who supported me in prayers. I dedicate this work and give special thanks to my friend Derrick Afful for helping me develop my technology skills, and for the many hours of proofreading.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER ONE

INTRODUCTION

1.1 INTRODUCTION

Cancer is a major public health problem globally. It is the second leading cause of death in the United States (Adebayo *et al*., 2010), with one in four deaths attributable to the disease (Jemal *et al*., 2014). Cancer is a group of diseases characterized by abnormal (uncontrolled) growth and spread of abnormal cells. The disease is caused by both internal factors (inherited genetic mutations, immune conditions and mutations that occur from metabolism) and external factors (nutritional deficiencies, infectious organisms, radiations and chemicals) that may act together or in sequence to initiate or promote its development (Sakarkar and Deshmukh, 2011). About 12.7 million cancer incidences and 7.6 million cancer mortalities were reported to have occurred based on GLOBOCAN estimates worldwide, with 56% of the cases and 64% of the deaths in the economically developing world (GLOBOCAN, 2008). Cancers of the lung, breast, colorectal, liver, stomach and prostate are among the most common cancers diagnosed worldwide (ACS, 2013).

In Ghana, studies conducted by Wiredu and Armah (2006) at the Korle-Bu Teaching Hospital (KBTH) on review of 3,659 cancer deaths over a ten year period showed that the commonest causes of cancer death in females were those of the breast, haematopoietic organs, liver and cervix malignancies while in males it was the malignancies of the liver, prostate, haematopoietic organs and that of stomach. Research in this area has become paramount as understanding of the disease mechanism and susceptibility of individuals to it, will go a long way to aid in the search for a cure.

Prevention, early detection and treatment remain at the forefront in the battle to reduce the burden of cancer. Treatment options available for cancers include; chemotherapy, surgery, hormone therapy, radiotherapy, cryotherapy, etc. Of these treatment methods, chemotherapy is considered the standard method and hence is widely used (Uma *et al.*, 2009; Dantu *et al.*, 2012). Notwithstanding this, cancer chemotherapy is still fraught with several challenges, the most notable being the resistance of most tumours to anticancer agents. Also the non-selective nature of some chemotherapeutic agents make them affect cancer and normal cells alike, often leading to undesirable side effects. These problems are also compounded by the high cost of western anticancer drugs and due to these, a large proportion of cancer patients have resorted to the use of Complementary and Alternative Medicine either as an adjunct or to completely supplant the use of chemotherapy (Olaku and White, 2011). Particularly, herbal medicines are widely used, with about 80% of cancer patients in developing countries (particularly African and Asian populations) relying on this method for cancer treatment (Gurib-Fakim, 2006). This has been the case as these medicinal plants are considered to be cheaper, safer and with less side effects (Olaku and White, 2011). The practice is based on accumulated knowledge passed on from one generation to another. However, recommendation of herbal formulations by herbalists lack safety and long term toxicity considerations, due to lack of education on the fact that active ingredients of plant extracts are chemicals similar to those in purified medications, and have the same potential to cause serious adverse effects.

Most of the new clinical applications of plant secondary metabolites and their derivatives have been towards combating cancer (Adebayo *et al*., 2010). Anticancer agents such as vincristine and vinblastine isolated from *Catharanthus roseus* have been used for the

treatment of various cancers, including that of breast and lung, advanced testicular, lymphomas, Kaposi's sarcoma and leukemias (Cragg and Newman, 2005) for more than 40 years (Van der Heijden *et al.*, 2004). Despite the high success of these and other anticancer agents in clinical use, their selectivity however remains a great challenge. To overcome this challenge, scientists have intensified the search for new anticancer agents from medicinal plants and other natural sources that have strict selectivity against cancer cells only.

In the cancer drug discovery program, a paradigm based on ethnobotanical and ethnopharmacological data is more economical and beneficial for identifying potential anticancer lead molecules than mass screening of plant species. Over five thousand plants are known to be used for medicinal purposes in Africa, but only a few have been described or studied (Taylor *et al.*, 2001). Many of them may contain some bioactive principles which remain to be fully discovered.

Ageratum conyzoides L. is an annual herb that belongs to the family and tribe of Asteraceae and Eupatoriae, respectively. It is commonly called Billygoat weed generally found in cultivated fields and other ecosystems such as pastures, grasslands, wastelands and even forest areas (Shirwaikar *et al.*, 2003). The plant is known to have originated from tropical America and now spread to various tropical and subtropical parts of the world (Nogueira *et al.*, 2010; Nasrin, 2013). The plant is also a common weed that grows in most part of Ghana. *A. conyzoides* has been used in folklore for the treatment of fever, pneumonia, cold, rheumatism, spasm, headache, and curing wounds (Ming, 1999; Shirwaikar *et al.*, 2003). Pharmacological investigations have also verified its antioxidative effect (Jagetia *et al.*, 2003; Adebayo *et al.*, 2010; Nasrin, 2013),

hepatoprotective effects (Ita *et al.*, 2009) and its property as a blood booster (Ita *et al.*, 2007). Phytochemical investigations conducted by Kamboj and Saluja, (2008), showed that *A. conyzoides* contains alkaloids, resins, saponins, tannins, glycosides and flavonoids. Many different bioactive principles have also been isolated and identified in *A. conyzoides*; such as kaempferol and glycoside (rhamnoside); quercetin, scutellarein, ageratochromene derivatives and alkane (Nyunaï *et al.*, 2010).

To date, only Adebayo and colleagues, (2010), and Adetutu *et al*., (2012), have evaluated and published the *in vitro* anticancer activity of this plant. Studies by Adebayo *et al*., (2010), described the *in vitro* anticancer activity of ethanol, petroleum ether, ethylacetate, n-butanol, and water extracts of *A. conyzoides* leaves against some cancer cell lines: Human non-small cell lung (A-549), human gastric (SGC-7901), human colon (HT-29), human glioma (U-251), human breast carcinoma (MDA-MB-231), human prostate carcinoma (DU-145), human hepatic carcinoma (BEL-7402), and mouse leukemia (P-388) cancer cell lines using the sulforhodamine B (SRB) assay. This group also reported the antioxidant (free radical scavenging) potential of this plant using the DPPH assay *in vitro*. Adetutu *et al*., (2012), also reported the *in vitro* cytotoxic effects of the ethanol extracts and fractions of the leaves of *A. conyzoides* on two human lung cancer cell lines (SK-LU 1 and SK-MES 1) and human skin fibroblast cell line (FS5 cells) using the MTT assay. Nasrin (2013), has also reported the free radical scavenging potential and the total phenolic content of the crude methanolic extract of *Ageratum conyzoides* stems using the DPPH assay and the Folin-Ciocalteau method respectively.

The present study were therefore aimed at investigating *in vitro* anticancer and antioxidant activities of crude aqueous and 50% hydroethanolic extracts of the stems,

flowers, leaves and whole plant of *Ageratum conyzoides* as well as evaluating the qualitative phytochemical constituents of these extracts.

1.2 STUDY OBJECTIVE

This study aimed at determining the anticancer and antioxidant effects of *A. conyzoides* aqueous and 50% hydroethanolic extracts as well as their phytochemical constituents.

1.2.1 Specific Objectives

This study specifically sought to:

a) Determine the anticancer activities of the aqueous and 50% hydroethanolic extracts of whole plant and parts of plant on different cell lines (Jurkat, LNCap, MCF7 and PNT2) *in vitro* using the MTT assay

b) Determine the anticancer activities of petroleum ether, ethylacetate, chloroform and hydroethanolic fractions of the 50% hydroethanolic leaf extracts of *A. conyzoides*.

c) Determine the antioxidant activity of the crude extracts using Folin-Ciocalteu method, DPPH and GSH assays.

d) Evaluate qualitative phytochemical constituents of the crude aqueous and 50% hydroethanolic extracts of this plant.

1.3 PROBLEM STATEMENT AND JUSTIFICATION

Even though cancer remains a major public health concern worldwide, the treatment options available, especially cancer chemotherapy, are very costly. Due to this, about 80% of the developing world (Olaku and White, 2011), especially some African

countries including Ghana, have resorted to use traditional medicine for treating cancer and other diseases. Comparing these to orthodox anticancer agents, the amount of information about the relative safety and long term toxicity of Ghanaian traditional medicine is limited. While most herbal medicines lack defined dose and potency data, they also benefit from containing many specific bioactive principles in their natural state, which possess a variety of influences on human biological processes (Taylor *et al.*, 2001). However, some of these unknown active principles are often toxic and among these are; lecithins, saponins, diterpenes, aristolochic acids, cyanogenic glycosides, etc. (Adewunmi and Ojewole, 2004). Over 5000 plants are known to be used for medicinal purposes in Africa, but only a few have been investigated (Taylor *et al*., 2001). The need to study popularly used but poorly understood medicinal plants with the view to assessing their therapeutic efficacy and establishing scientific basis cannot be over emphasized. In Kumasi, Ghana, an herbalist has suggested the folkloric use of the leaves and whole plant of *Ageratum conyzoides* in managing breast cancer (personal communications). But this is yet to be proven scientifically. To date, only Adebayo *et al*., (2010), and Adetutu *et al*., (2012), have evaluated and published the *in vitro* anticancer activity of this plant.

Also, cancer chemotherapy which is still considered the standard treatment method widely used (Dantu *et al.*, 2012; Uma *et al*., 2009) sometimes has peculiar problems, due to tumour resistance and its non-selective nature in clinical use. These agents affect both cancer and actively dividing normal cells. Therefore the need to isolate new anticancer lead molecules with strict selectivity against cancer cells only from natural products, especially medicinal plant sources, is essential.

With earlier studies and some herbalists suggesting anticancer activity of the plant, coupled with the need for new therapies, further research can be conducted to explore the plant's anticancer potential.

Antioxidants protect cells against the damaging effects of reactive oxygen species otherwise called free radicals. These include singlet oxygen, super oxide, peroxyl radicals, hydroxyl radicals and peroxynite and their presence induces oxidative stress leading to cellular damage (Mattson and Cheng, 2006; Doughari, 2012). These reactive oxygen species are responsible for initiating the multistage process including DNA damage in some cell lines which leads progressively to dysplastic cellular appearance, deregulated growth, and finally carcinoma (Atawodi, 2005). This induced damage caused by free radicals is thought to precede disease processes (Miller, 1996). Hence, there is the need for therapy with antioxidants (antiradicals or free-radical scavengers), that have the potential to prevent, delay or improve disease conditions (Delanty and Dichter, 2000). Antioxidants like ascorbic acid, gallic acid esters, butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA) and tertiary butylated hydroquinone, have been suspected to cause negative health effects at very high concentrations (Delanty and Dichter, 2000). Hence, strong restrictions have been placed on their application and there is a demand to substitute these with naturally occurring antioxidants. Also, antioxidants are often added to foods to prevent the radical chain reactions of oxidation, and they act by inhibiting the initiation and propagation step leading to the termination of the reaction and delay the oxidation process (Doughari, 2012). Due to safety concerns of synthetic compounds, food industries have focused on finding natural antioxidants to replace synthetic compounds (Doughari, 2012). In

addition, there is growing trend in consumer preferences for natural antioxidants, all of which has given more impetus to explore natural sources of antioxidants (Barlow, 1990).

Liu (2004), reported that phytochemicals act in synergy with chemotherapeutic drugs in overcoming cancer cell drug resistance and that the application of specific phytochemicals may allow the use of lower concentrations of drugs in cancer treatment with an increased efficacy. Traces of phytochemicals found in fruits and vegetables may potentiate the immune system against cancers (Trewavas and Stewart, 2003). Phytochemicals show biphasic dose responses on mammalian cells (Doughari, 2012). Though toxic at high concentrations, sub-toxic doses may induce adaptive stress response (Doughari, 2012). This includes the activation of signaling pathways that result in increased expression of genes encoding cytoprotective proteins. It is therefore suggested that hormetic mechanisms of action may underlie many of the health benefits of phytochemicals including their action against cancer drug resistance (Mattson, 2008).

Hence, the need for the present investigation of *A. conyzoides* for its anticancer, antioxidant and phytochemical constituents.

CHAPTER TWO

LITERATURE REVIEW

2.1 CANCER AS A PUBLIC HEALTH BURDEN

Globally, the trends in the cause and epidemiology of serious ill-health are changing. Non-communicable diseases such as cardiovascular disease and cancer are emerging as major public health burdens across all countries. Cancer is now the second leading cause of mortality globally (Thun *et al*., 2010) and amongst the top three leading causes of death in developing countries such as Ghana (Wiredu and Armah, 2006). It is a group of diseases characterized by abnormal (uncontrolled) growth and spread of abnormal cells (ACS, 2014). The spread of cancer cells to other tissues of the body is known as metastasis. If this spread is not controlled, it can lead to imminent patient death. The disease is caused by both internal factors (inherited genetic mutations, immune conditions and mutations that occur from metabolism) and external factors (nutritional deficiencies, infectious organisms, radiations and chemicals) that may act together or in sequence to initiate or promote its development (ACS, 2014). In 2008, about 12.7 million cancer incidences and 7.6 million cancer mortalities were reported to have occurred based on GLOBOCAN estimates worldwide, with 56% of the cases and 64% of the deaths in the economically developing world (Ferlay *et al*, 2008). This number is projected to be twenty six million new cancer cases and seventeen million cancer deaths per year by 2030 according to Thun *et al*., (2010) and International Agency for Research on Cancer (Ferlay *et al*, 2008).

This worldwide projected increase is as a consequence of growing and aging populations and will disproportionately affect economically developing countries, of which most

African countries are no exception (Bray *et al.*, 2013). According to United Nation's population estimates, the population of Africa between 2010 and 2030 is projected to increase from 1.03 billion to 1.52 billion (by 50% percent overall) and from 55 million to 105 million (by 90%) for those aged sixty years and above; consequently, this is the age at which cancer is mostly evident (Jemal *et al.*, 2011). Also, globalization of unhealthy Western lifestyles (particularly cigarette smoking and the adoption of diet high in hydrogenated fat and low in fibre content), obesity, family history of the disease (genetic predisposition), race, environmental exposures and physical inactivity are other risk factors that contribute to this disease in the low to middle income countries. Cancers of the lung, breast, colorectal, liver, stomach and prostate are among the most common cancers diagnosed worldwide (ACS, 2013).

2.2 GLOBAL CANCER SCENARIO

In 2012, the most common cancers diagnosed globally were those of the lung (1.8 million cases, 13.0% of the total), breast (1.7 million, 11.9%), and large bowel (1.4 million, 9.7%). The most common causes of cancer death were cancers of the lung (1.6 million, 19.4% of the total), liver (0.8 million, 9.1%), and stomach (0.7 million, 8.8%), (Stewart and Batist, 2014). More than 60% of the world's total cases occur in Africa, Asia, and Central and South America, and these regions account for about 70% of the world's cancer deaths, a situation that is made worse by the lack of early detection and access to treatment (IARC, 2013; Stewart and Batist, 2014). Figure 2-1 shows the worldwide distribution of cancer with estimated mortality among both males and females.

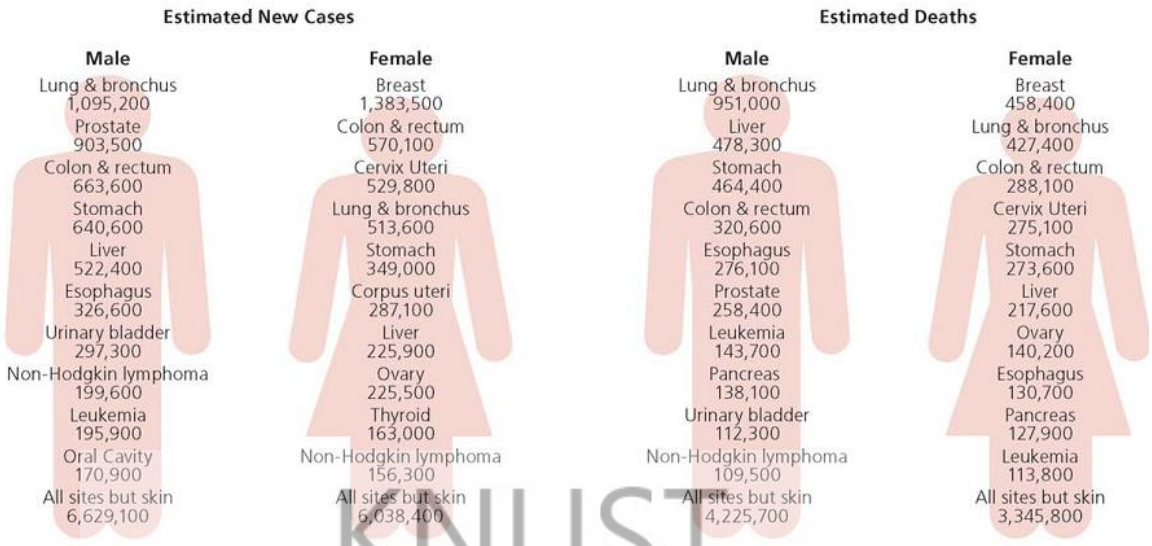


Figure 2-1 Estimated cancer incidences and mortalities globally for leading cancer sites (Source: ACS, 2013).

2.2.1 The cancer scenario in economically developed world

In economically developed world like the Western and Northern Europe, Australia/New Zealand, and North America, breast cancer is the most frequently diagnosed and the leading cause of cancer death in females, whiles in males lung cancer is preceded by prostate cancer as the most frequent diagnosed cancer whereas lung cancer is the leading cause of male mortality (Jemal *et al.*, 2011). Breast cancer represents 15% of new cases of all female cancers (ACS, 2013) while prostate cancer represents 15.3% of all cancers in men in the developed countries (Parkin *et al.*, 2001). These cancers are followed by colorectal and lung cancers in females and colorectal and lung cancers in males in a nonspecific rank order. Figure 2-2 below outlines the various distributions of the different cancer types among males and females.

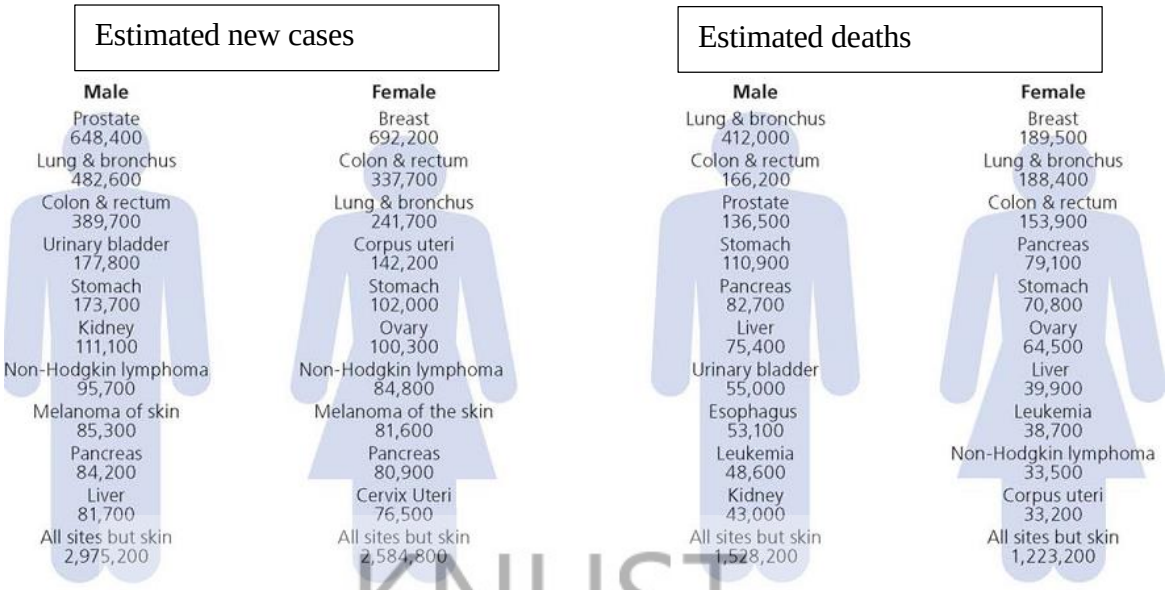


Figure 2-2 Estimated cancer incidences and mortalities for leading cancer sites in the developed world (Source: ACS, 2013).

2.2.2 The cancer scenario in economically developing world

Breast cancer is now also the leading cause of cancer death among females in the economically developing world like Botswana, Bolivia and Vietnam, a shift from the results from previous decade where the most common cause of cancer death was cervical cancer. The mortality burden for lung cancer among females is as high as the burden for cervical cancer, with each accounting for 11% of the total female cancer deaths. In males, lung cancer is responsible for the highest incidence and mortality rates (Jemal *et al.*, 2011). Even though the overall cancer incidence rates in the developing world are half those seen in the developed world in both sexes, the overall cancer mortality rates are generally similar.

In Africa, prostate cancer has the highest incidence (39,500 cases), followed by liver cancer (34,600 cases), Kaposi sarcoma (22,400 cases) with leukemia been the lowest (11,200 cases), whiles liver cancer accounted for the highest mortality (33,800 deaths),

followed by cancers of the prostate (28,000 deaths), lung (19,400 deaths) with leukemia recording the lowest mortality (10,600 deaths) in males (ACS, 2007). In females, breast cancer has the highest incidence (92,600 cases), followed by cancers of the cervix (80,400 cases), liver (16,900 cases) with leukemia been the lowest (8,300 cases) whiles cancer of the cervix accounted for the highest mortality (53,300 deaths), followed by cancers of the breast (50,000 deaths), liver (16,600 deaths) with leukemia accounting for lowest mortality (7,800 deaths) (ACS, 2007).

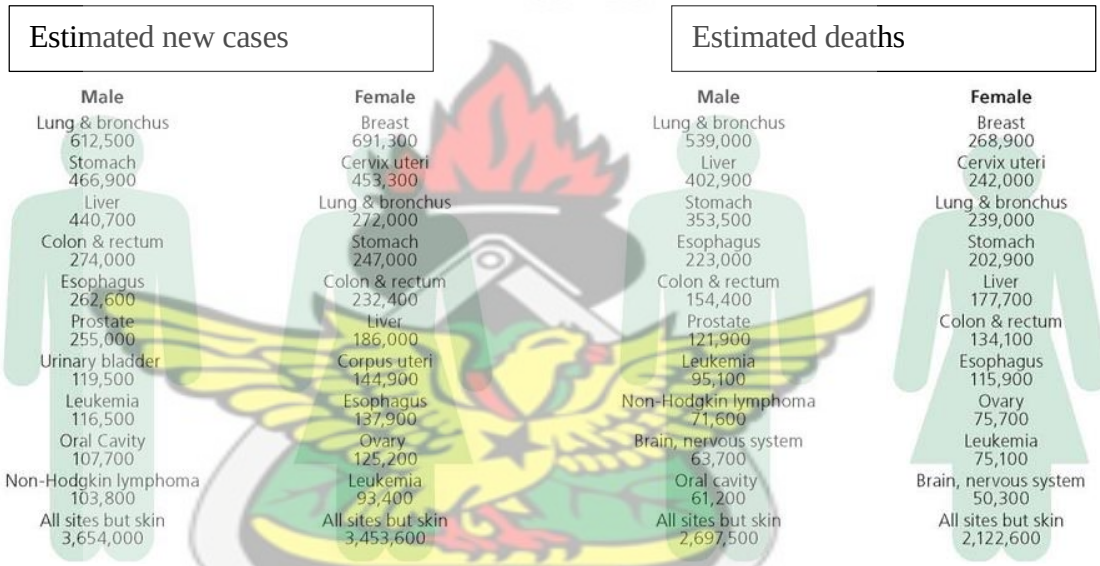


Figure 2-3 Estimated cancer incidences and mortalities for leading cancer sites in the developing world (Source: ACS, 2013).

2.2.2.1 The cancer situation in Ghana

Cancers like other non-communicable diseases were not a public health concern in developing nations such as Ghana until recently (Wiredu and Armah, 2006). However with the advancement of education, the economy has become associated with problems related to the lifestyle changes. This has led to dietary changes leading to obesity, poor nutrition, physical inactivity and excessive use of alcohol amongst the populace.

In Ghana, cancers accounted for 2.6% of all admissions and 5.6 % of all deaths at the Korle-Bu Teaching Hospital (KBTH) in 1996 alone, according to the review by Wiredu and Armah (2006). The studies conducted by Wiredu and Armah (2006) also reviewed a total of 3,659 cancer deaths over a ten year period (1991-2000). From the study, it was reported that the commonest cause of cancer death in females was that of the breast, haematopoietic organs, liver and cervix malignancies with a mean age of 46.5 years whiles that in males was the malignancies of the liver, prostate, haematopoietic organs and that of stomach with a mean age of 47.8 years.

Table 2-1 Age-Standardized Cancer Ratio (ASCR) for the various male and female cancer types responsible for patient mortalities at the Korle-Bu Teaching Hospital (KBTH) from 1991-2000

Females	
Cancer Types	**Age-Standardized Cancer Ratio (ASCR)**
Breast	17.24%
Haematopoietic organs	14.69%
Liver	10.97%
Cervix	8.47%
Males	
Cancer Types	**Age-Standardized Cancer Ratio (ASCR)**
Liver	21.15%
Prostate	17.35%
Haematopoietic organs	15.57%
Stomach	7.26%

Source: Wiredu and Armah, 2006

Although this study carried out at the Korle-Bu Teaching Hospital has reported a reliable data on the relative contributions of the various cancers to disease burden, lack of organized nationwide statistics has made it difficult to establish the true prevalence of the various cancers in Ghana.

2.3 TREATMENT OPTIONS FOR CANCER

Prevention, early detection and treatment remain at the forefront in the battle to reduce the burden of cancer. A lot of treatment options are available for cancer, but the major forms widely used include cancer chemotherapy, surgery oncology and radiation oncology.

2.3.1 Radiation oncology (therapy)

Radiation oncology (also called radiotherapy) uses high-energy X-rays to kill cancer cells. Radiation is very effective in killing fast-growing cells like breast cancer. Radiotherapy is very specific and only affects the area that is being treated, and can be given externally or internally. With external beam radiotherapy, the radiation comes from special machines which aim X-rays directly at the tumour or the tumour site after surgery. It is also aimed at a small area of normal tissue around the tumour just in case any cancer cells have spread. In the case of internal radiotherapy, either solid radioactive material close to or inside the tumour (brachytherapy) or as a radioactive liquid, is given either by mouth or as an injection into a vein (Irish Cancer Society, 2011)

2.3.2 Cancer surgery

This is a type of treatment where the body is operated on to remove cancer cells. Today, surgeries that involve less cutting (less invasive) often can be done to remove tumors

while saving as much normal tissue and function as possible. Surgery offers the greatest chance for cure for many types of cancer when the tumour has not spread to other parts of the body. Different kinds of surgery can be performed depending on the nature and stage of the cancer. Preventive (prophylactic) surgery is done to remove body tissue that is likely to become cancerous, even though there are no signs of cancer at the time of the surgery. Staging surgery is also done to find out how much cancer there is and how far it has spread. Moreover, curative surgery is usually done when cancer is found in only one area or region of the body, and it's likely that all of the cancer can be removed. In this case, curative surgery can be the main treatment (ACS, 2013).

2.3.3 Cancer chemotherapy

This is a treatment using anticancer (also called cytotoxic or antineoplastic) drugs which aims to destroy cancer cells. Chemotherapy differs from surgery or radiation in that it is used as a systemic treatment because the whole body is exposed to the drugs. This means the drugs travel throughout the body to reach cancer cells wherever they are. This therapy may be the only treatment used or it may be given along with other treatments. It may be used as adjuvant therapy or neoadjuvant therapy. After surgery or radiotherapy to remove the cancer, there may still be some cancer cells left behind that cannot be seen. When drugs are used to kill those unseen cancer cells, it's called adjuvant chemotherapy. Also, giving chemotherapy first can shrink a large cancerous tumor, making it easier to remove with surgery or radiotherapy. This application of chemotherapy to cancer is referred to as neoadjuvant therapy (ACS, 2013).

2.3.3.1 Chemotherapeutic agents used in cancer therapy

Chemotherapy agents such as alkylating agents, mitotic and topoisomerase inhibitors are used to treat different types of cancers and may be used in combination (ACS, 2014).

Alkylating agents, such as melphalan, busulfan and cisplatin, directly damage DNA to prevent cancer cells from reproducing, and are used to treat different cancers, including leukemia, lymphoma, sarcoma and multiple myeloma, as well as cancers of the breast, ovary and lung, (ACS, 2014). Topoisomerase inhibitors, interfere with topoisomerase enzymes, such as topotecan (topoisomerase I inhibitor) and etoposide (topoisomerase II inhibitor) are used to treat certain leukemias, as well as lung, ovarian and gastrointestinal cancers (ACS, 2014). Mitotic inhibitors such as docetaxel, vincristine and vinblastine stop mitosis and are used to treat several cancers including lung, breast, myelomas, lymphomas and leukemias (ACS, 2014).

Studies have shown that some chemotherapeutic agents inhibit cancer cells through apoptosis signalling. Research conducted by Arends and Wyllie (1991) and Mesner *et al.* (1997), demonstrated that anticancer agents, including chemotherapeutic agents, hormones, and various biologicals, induce apoptosis in malignant cells *in vitro*. The implicated cancer chemotherapeutic agents included; topotecan, campthotecin, etoposide, teniposide, methotrexate, cisplatin, cytarabine, colcemid and cycloheximide (Kaufmann and Earnshaw, 2000). Also, Li *et al*., (1994), demonstrated that cytarabine, mitoxantrone, etoposide, paclitaxel, and topotecan caused a marked increase in the number of apoptotic blasts during serial examination of peripheral blood mononuclear cells from acute leukemia patients undergoing induction therapy. Moreover, characteristic apoptotic changes have also been described in solid tumors after treatment of mice with various cytotoxic drugs, including cytarabine, 5-fluorouracil (5FU), fludarabine, doxorubicin, cyclophosphamide, cisplatin, etoposide, dactinomycin, and camptothecin as reviewed by Kaufmann and Earshaw, (2000).

2.3.3.1.1 Pathways targeted by Chemotherapy

The selective proteolytic cleavage of various intracellular polypeptides leads to many of the hallmarks observed as cells undergo apoptosis, including the commonly observed internucleosomal DNA degradation or fragmentation and the characteristic morphological alterations, chromatin condensation (Earnshaw *et al*., 1999). Inter-nucleosomal DNA degradation or fragmentation results from protease mediated cleavage of a nuclease inhibitor called inhibitor of caspase-activated DNase (ICAD). This releases a unique endonuclease called caspase-activated DNase (CAD) (Enari *et al*., 1998). One of the early morphological changes, chromatin condensation, results from the action of a number of polypeptides including the apoptosis inducing factor (a flavoprotein released from mitochondria) (Susin *et al*., 1999) and topoisomerase II (Kaufmann and Earnshaw, 2000) as well as two protease activated factors CAD (*Liu et al*., 1998; Samejima *et al*., 1998). Polypeptides that normally contribute to structural integrity of the nucleus, specifically the lamins and nuclear/mitotic apparatus protein undergo cleavage reflecting subsequent fragmentation of the nucleus (Earnshaw *et al*., 1999). Most of the proteolytic cleavages during apoptosis result from the action of a unique family of cysteine dependent proteases called caspases (Earnshaw *et al*., 1999). Six (caspases-3, -6, -7, -8, -9, and -10) out of the twelve known human caspases are definitely involved in apoptosis in various model systems (Kaufmann and Earnshaw, 1999). Caspases-3, -6, and -7 are the major effector or downstream caspases, that are responsible for most of the cleavages that disassemble the cell, while caspases-8 and -9 are the major initiator or upstream caspases initiating the proteolytic cascades (Kaufmann and Earnshaw, 2000).

Apoptosis occurs through two main pathways (Shoyama *et al*., 2013). The first, referred to as the extrinsic or cytoplasmic pathway, is triggered through the Fas death receptor, a member of the tumour necrosis factor (TNF) receptor superfamily (Ghobrial *et al*., 2005). The second pathway is the intrinsic or mitochondrial pathway that when stimulated leads to the release of cytochrome-c from the mitochondria and activation of the death signal (Khan *et al*., 2007). Both pathways converge to a final common pathway involving the activation of a cascade of proteases called caspases that cleave regulatory and structural molecules, culminating in the death of the cell (Ghobrial *et al*., 2005). Caspases-9 (intrinsic), -8 and -10 (extrinsic) activations, cleave downstream procaspase-3 to active caspase-3, which subsequently cleaves poly (ADP-ribose) polymerase (PARP) (Gomes *et al*., 2010). Also, cell cycle arrest at various checkpoints results in activation of intracellular pathways which culminates in apoptosis (Stewart and Pietenpol, 2002).

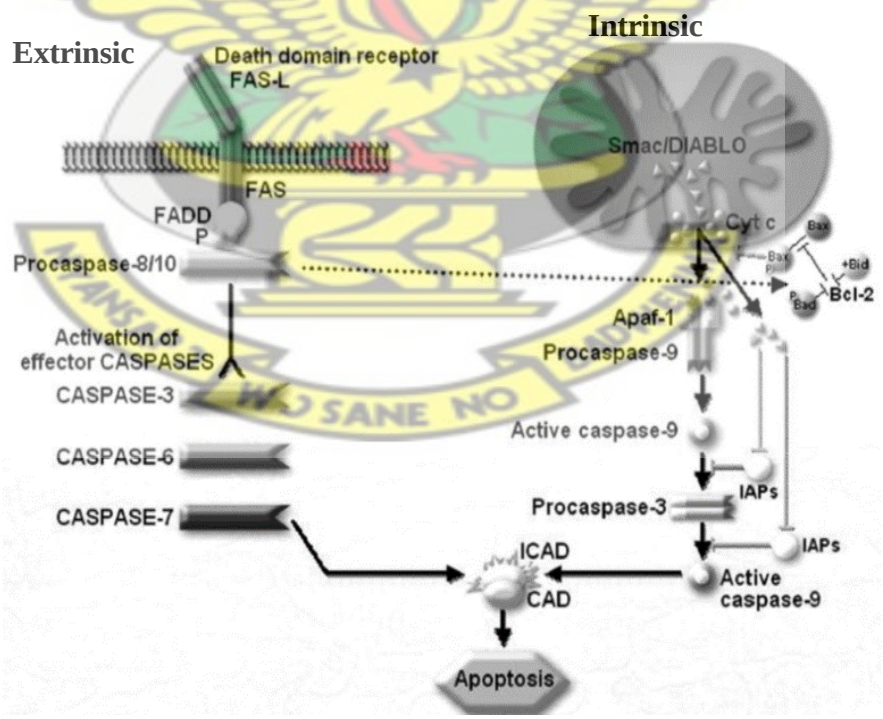


Figure 2-4 Extrinsic and intrinsic apoptotic pathways (Source: Ghobrial *et al*., 2005).

Some chemotherapeutic agents primarily target the apoptotic machinery. These are classified as those agents that target the extrinsic pathway, the intrinsic pathway, the common pathway (through caspases), or the proteins regulating apoptosis (Ghobrial *et al*., 2005). Preclinical testing of TRAIL (an extrinsic pathway inducer) in combination with conventional chemotherapeutic agents such as doxorubicin has demonstrated significant inhibition of tumour growth in a prostate cancer *in vivo* (Ghobrial *et al*., 2005). Also, TRAIL was found to induce apoptosis in human hepatocytes and in human brain cells *in vitro* (Ghobrial *et al*., 2005). However, it did not lead to apoptosis in the brain of animals in preclinical studies (Ghobrial *et al*., 2005). Arsenic trioxide (intrinsic pathway target) targets the PML-RAR α protein (Chou *et al*, 2005). At higher concentrations, it induces apoptosis of the leukemic cells by disruption of the mitochondrial inner membrane potential, leading to inhibition of Bcl-2 expression and elevation of caspase-3 (Chou *et al*, 2005).

2.3.3.1.2 Targeted therapy

Despite the promising results obtained by standard chemotherapy, majority of patients still die of metastatic disease. Modern advancements in molecular biology has prompted scientist to evaluate several molecules in metastatic disease. Currently, targeted therapies are been considered. Targeted anticancer therapy refers to treatments that selectively interfere with molecules considered to be important in neoplastic transformation, cell proliferation, invasion and metastasis, and/or tumour-related angiogenesis (Faivre *et al*., 2006). Targeted therapy spares normal cells and, therefore, is usually better tolerated. The greater target selectivity should translate into a wider therapeutic window that better enables the use of sufficient drug dosages to accomplish the therapeutic goals of tumour eradication or control while minimizing the risk of

resistance (Faivre *et al*., 2006). A key focus of these therapies has been signalling pathways important in malignant cell transformation and proliferation (Blay *et al*, 2005). In particular, targeted therapies that have been recently approved for the treatment of solid tumours inhibit growth receptors, principally, receptor tyrosine kinase (RTKs) or the signals that are generated following their activation (Tibes *et al*, 2005). These agents include monoclonal antibodies that bind to the extracellular domain of RTKs, thereby preventing the endogenous ligand from binding and activating the receptor, and small-molecule tyrosine kinase inhibitors (TKIs) that compete with adenosine triphosphate (ATP) for binding within the intracellular domain of RTKs. Another recent approach to molecularly targeted anticancer therapy involves the use of a monoclonal antibody directed against vascular endothelial growth factor (VEGF) (Hurwitz *et al*., 2004), the ligand for VEGF receptor (VEGFR), which has been associated with tumour-related angiogenesis (Ismaili *et al*., 2011). Overexpression of VEGF receptor on endothelial cells, and several receptors such as the epidermal growth factor receptor (EGFR), the platelet derived growth factor receptor (PDGFR), and the fibroblast growth factor receptor (FGFR), on tumour cells, have also informed researchers to evaluate the efficacy and safety of new molecules targeting signalling pathways controlled by these proteins (Ismaili *et al*., 2011).

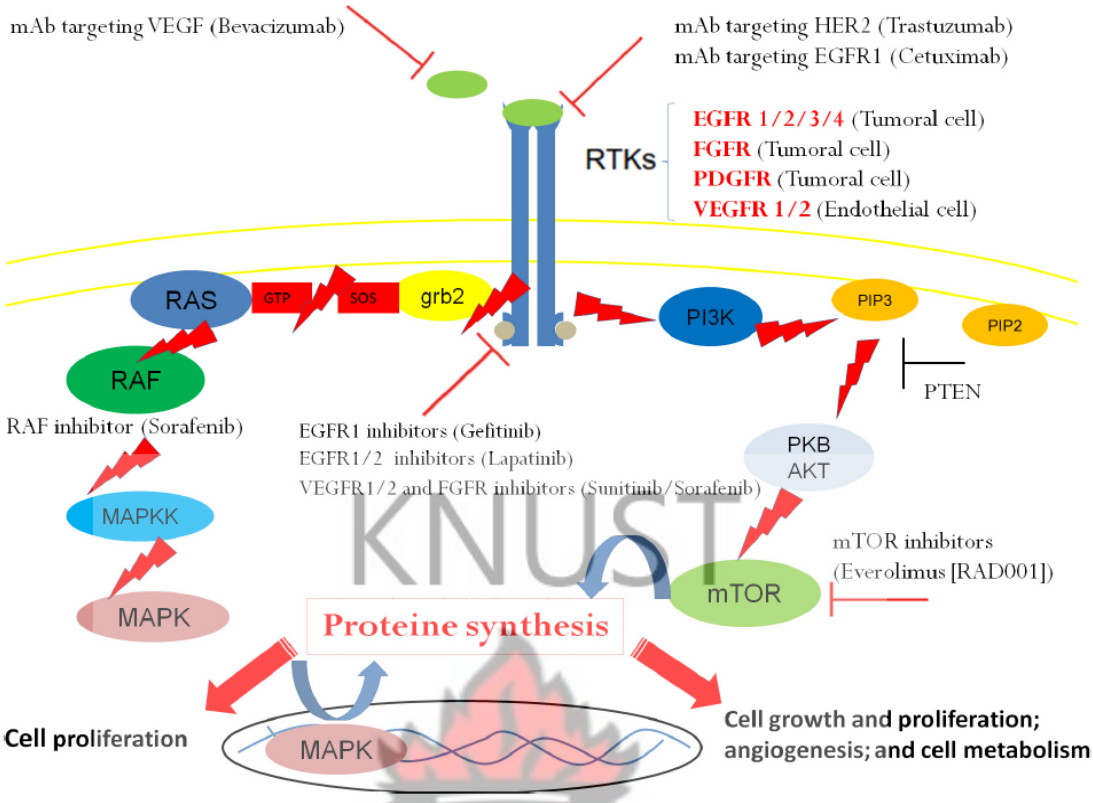


Figure 2-5 Deregulated signalling pathways and targeted therapy in bladder cancer. [Abbreviations: EGFR, Epithelial Growth Factor Receptor; VEGFR, Vascular Endothelial Growth Factor R; FGFR: Fibroblast Growth Factor Receptor; mTOR: mammalian Target of Rapamycin (Source: Ismaili *et al*., 2011)].

Sunitinib is a small molecule acting as a multi target intracellular tyrosine kinases inhibitor by inhibiting multiple receptors (EGFR, VERFR-1/2, C-KIT, PDGFR a/b) and the FLT3 and RET kinases (Ismaili *et al*., 2011). This drug is used as front-line treatment of metastatic renal cell carcinoma and in the second line treatment of GIST (gastrointestinal stromal tumours) after failure of imatinib (Ismaili *et al*., 2011). It has been tested in bladder cancer as single agent, in combination with chemotherapy, and showed an interesting antitumor activity (Gallagher *et al*., 2010). Bevacizumab is a humanized monoclonal antibody (mAb) targeting the VEGF (Vascular Endothelial Factor) which has been approved by FDA in combination with chemotherapy as a

standard treatment in first line and second line in different metastatic tumours (Ismaili *et al*., 2011).

2.3.3.2 Challenges of cancer chemotherapy

Chemotherapy is still considered the standard cancer treatment method and it is widely used (Dantu *et al.*, 2012; Uma *et al*., 2009). Also, it is the major treatment option available in Ghana with the first line drug being Geldanamycin and its analogs which act by mechanisms such as apoptosis, angiogenesis etc. (Bedin *et al.*, 2004). Like most cancer drugs, this drug has associated problems like hepatotoxicity. Although Ghana has a National Health Insurance Scheme which covers some cancer treatment, this is woefully inadequate as most of the drugs are expensive for the average Ghanaian and even for their family income to support. To make matters worse, these drugs are not readily available at most pharmacies in the country and sometimes even at the points of prescription. Also, cancer chemotherapy sometimes fail because cancer cells develop resistance to them. Moreover, most cancer chemotherapeutic agents aimed at eliminating the cancer cells and preventing tumour metastasis are often not selective because the toxic agents affect both rapidly or actively dividing normal cells and cancer cells. This leads to numerous side effects like the loss of hair (alopecia), nausea and vomiting, bone marrow suppression resulting in neutropenia, anaemia and thrombocytopenia which are very undesirable for most patients (ACS, 2013). Recently, there has been increased search for anticancer agents from natural products that are selective against cancer cells only. According to Newman *et al.* (2000), over 50 % of the drugs in clinical trials for anticancer properties were isolated from natural sources or are related to them. The utilization of natural product in this regard, especially those from medicinal plants, cannot be overemphasized, as these can be sources for the discovery of new anticancer lead molecules.

2.3.3.3 Carcinogenesis and signalling pathway targeted by antioxidant

Reactive oxygen species (ROS) and electrophiles cause damage to DNA resulting in malignancy development. To nullify this effect, higher animals have developed defense mechanisms, including antioxidant proteins and phase II detoxification enzymes (Lee and Johnson, 2004). Studies designed to identify the regulatory element for the defense mechanism genes (of antioxidant proteins and phase II detoxification enzymes) have revealed a central role of the antioxidant responsive element (ARE) (Lee and Johnson, 2004). The antioxidant responsive element (ARE) is a *cis*-acting regulatory element (enhancer sequence), which is found in promoter regions of genes encoding phase II detoxification enzymes and antioxidant proteins such as NAD(P)H: quinone oxidoreductase 1, glutathione S-transferases, and glutamate-cysteine ligase (Lee and Johnson, 2004).

Many proteins have been suggested to regulate the ARE, but the underlying ARE activation mechanism began to be elucidated with the identification of nuclear erythroid 2-related factor 2 (Nrf2) (Lee and Johnson, 2004). Nrf2, a basic leucine zipper transcription factor, was identified as a binding protein of locus control region of ß-globin gene. Nrf2 during ARE-driven gene expression, is sequestered in the cytoplasm by Keap1 and ARE activation signals (that is, protein kinase pathways and electrophiles), dissociates from Keap1 during oxidative stress and translocates into the nucleus, followed by dimerization with a Maf (musculoaponeurotic fibrosarcoma) protein, binding to ARE, and transcription of the gene (Ma *et al*., 2004).

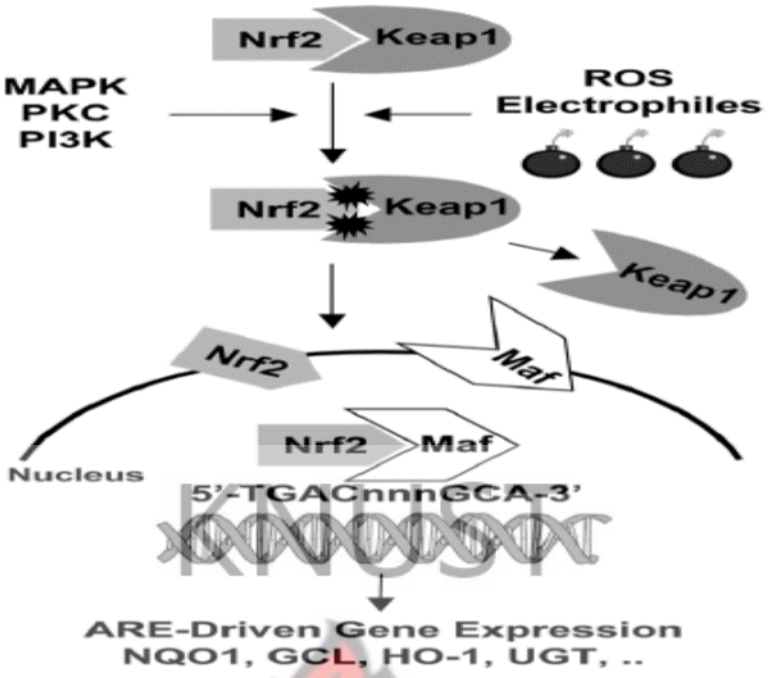


Figure 2-6 ARE-driven gene expression by Nrf2 (Source: Lee and Johnson, 2004)

One large group of these target genes is the phase II detoxification and antioxidant genes. By inducing these genes through the Nrf2/ARE pathway, chemopreventive agents could increase the detoxification of procarcinogens or carcinogens and protect normal cells from the DNA/ protein damage caused by electrophiles and reactive oxygen intermediates, thereby decreasing the incidence of tumour initiation and reducing the risk of cancer (Hu *et al*., 2006). The role of Nrf2 in preventing tumorigenesis is further supported by studies in which Nrf2 knockout mice were much more susceptible to carcinogen-induced carcinogenesis and failed to respond to certain cancer chemopreventive agents which were effective in Nrf2 wild-type mice (Hu *et al*., 2006). Some studies have also suggested that Nrf2 might be involved in apoptosis signalling pathways. Overexpression of cleaved Nrf2 (C-terminal fragment) induced apoptosis in HeLa cells and caspase-3 (-like) proteases cleave Nrf2, inferring cleaved Nrf2 might have some role in the induction of apoptosis (Ohtsubo *et al*., 1999). In addition, Kotlo *et al*. (2003), reported that overexpression of Nrf2 protects cells from Fas-induced

apoptosis, signifying an important role of Nrf2 in anti-apoptotic pathways. Work done by Li *et al*. (2002), identified the ARE-driven genes including NQO1 that were responsible for protecting IMR-32 human neuroblastoma cells from H_2O_2-induced apoptosis.

Moreover, the function of Nrf2 and its downstream target genes have been shown to be important for protection against oxidative stress-induced cellular damage. Chan and Kan (1999), showed that knockout ($Nrf2^{-/-}$) mice were extremely susceptible to the antioxidant, butylated hydroxytoluene (BHT), with the same doses of BHT tolerated by wild-type mice ($Nrf2^{+/+}$). Enomoto *et al*. (2001), also demonstrated that administration of acetaminophen induced more severe centrilobular hepatocellular necrosis in $Nrf2^{-/-}$ mice compared with $Nrf2^{+/+}$ mice. Studies by Lee *et al*. (2003a), on oligonucleotide microarray analysis revealed that, Nrf2 regulates the orchestrated gene expression of detoxification enzymes, antioxidant proteins, anti-inflammatory proteins, calcium homeostasis protein, and signaling molecules. For instance, Nrf2 coordinately up-regulates genes which are involved in maintenance (that is, synthesis and regeneration) and utilization of glutathione. This orchestrated up-regulation of ARE-driven genes by Nrf2 appeared to be very efficient in increasing cellular detoxification and antioxidant capacity, implying an important role for Nrf2-ARE pathway as a cellular antioxidant defense.

Since the Nrf2-ARE pathway acts as a master regulator of many protective genes, it may serve as a therapeutic target for carcinogenesis, in which oxidative stress is involved.

2.4 MEDICINAL PLANTS AS IMPORTANCE SOURCE OF NATURAL PRODUCT FOR ANTICANCER LEAD MOLECULES

In recent times, medicinal plants occupy an important position for being the paramount sources of drug discovery compounds. Medicinal plants have been indispensable in treating diverse forms of disease including cancer since antiquity (Balunas and Kinghorn, 2005). About 80% of the people living in the rural areas resort to medicinal plants as a primary treatment option for diseases according to World Health Organization (WHO, 2008; www.who.int/mediacentre/factsheets/fs 134/en/). The treatment option is preferred because many of the users consider them to be safe and come with less side effects. These practices are solely based on accumulated knowledge over centuries through traditional use of medicinal plants.

Natural products are formulated to generate different types of effective drugs to enhance anticancer activities. However, according to Fennell *et al.* (2004), it has been reported that many plants used as food or as traditional medicine are potentially toxic, mutagenic and carcinogenic. It has also been shown in studies conducted by Isnard Bagnis *et al.*, (2004), that the use of traditional herbal remedies has been implicated in 35% of all cases of acute renal failure in Africa. Despite these drawbacks, the high costs that come with undergoing chemotherapeutic treatment has meant that many patients in developing countries cannot afford treatment in this manner. Many therefore rely on the use of herbal remedies for treatment of cancer based on anecdotal evidence notwithstanding the fact that the efficacy, toxicity and safety of many of these plants have yet to be confirmed scientifically. Proper understanding of the complex synergistic interaction of various constituents of anticancer plants would help in formulating the design of

anticancer agents to attack the cancerous cells without harming the normal cells of the body (Larkin, 1983; Saxe, 1987; Narah *et al.*, 2012).

2.4.1 Anticancer properties of medicinal plants

In Africa, plants have been used for many centuries, and in most parts of the world today, medicinal plants are used for the treatment of different types of diseases including cancers (Adebayo *et al.*, 2010). About 35,000 plant samples have been collected from twenty countries, however only a few have been screened for anticancer activity by the National Cancer Institute (Shoeb, 2006; Chanda and Nagani, 2013). Between 1940 and 2002, the anticancer drugs available had 40% of its composition to be either natural products or their derivatives, with another 8% considered natural product mimics (Newman *et al.*, 2003).

There are four major classes of anticancer agents from medicinal plants which are currently in clinical use. These are grouped as Vinca alkaloids (otherwise called *Catharanthus* alkaloids), taxanes, camptothecins and epipodophyllotoxins. For example, vincristine and vinblastine which are vinca alkaloids were (Figure 2-7) isolated from *Catharanthus roseus* (L.) G. Don (Apocynaceae) (formerly *Vinca rosea* L.) and have been used clinically for more than 40 years (Van Der Heijden and colleagues, 2004). These antincancer drugs are primarily used either alone or in combination with other chemotherapeutic drugs for the treatment various cancers, including breast and lung cancers, advanced testicular cancer, lymphomas, Kaposi's sarcoma and leukemias (Cragg and Newmann, 2005).

The mechanism of Vinca alkaloids and several of their semi-synthetic derivatives is the arresting of metaphase stage of mitosis which occurs by binding specifically to tubulin resulting in its depolymerization (Okouneva *et al.*, 2003). Podophyllotoxin was isolated from the resin of *Podophyllum pelatum* L. (Berberidaceae) but was found to be too toxic in mice therefore derivatives were made with the first clinically approved drug being etoposide (Gordaliza *et al.*, 2004). Epipodophyllotoxin is an isomer of podophyllotoxin, which was isolated as the active antitumor agent from the roots of Podophyllum species, *Podophyllum peltatum* Linnaeus and *Podophyllum emodi* Wallich (Berberidaceae) (Stahelin, 1973; Shoeb, 2006). Etoposide and teniposide are two semi-synthetic derivatives of epipodophyllotoxin and are used in the treatment of lymphomas, bronchial and testicular cancers (Cragg and Newman, 2005). The mechanism of action for epipodophyllotoxins is through binding tubulin, causing DNA strand breaks during the gap two (G_2) phase of cell cycle by reversibly inhibiting DNA topoisomerase II (Gordaliza *et al.*, 2004).

Homoharringtonine, isolated from the Chinese tree *Cephalotaxus harringtonia* var. drupacea (Sieb and Zucc.) (Cephalotaxaceae), is another plant-derived agent in clinical use (Powell *et al.*, 1970; Itokawa *et al.*, 2008). A racemic mixture of harringtonine and homoharringtonine has been used successfully in China for the treatment of acute myelogenous leukemia and chronic myelogenous leukemia (Kantarjian *et al.*, 1996; Cragg and Newman, 2005). Paclitaxel (Figure 2-7), which was clinically introduced to the United States market in early 1990s, was originally isolated from *Taxus brevifolia* Nutt. (Taxaceae), (Oberlies and Kroll, 2004). Paclitaxel is significantly active against ovarian cancer, advanced breast cancer, small and non-small cell lung cancer (Rowinsky, 1997). The taxanes (paclitaxel and other derivatives) act by binding to

tubulin without allowing depolymerization of tubulin assembly (Horwitz, 2004). Camptothecin (Figure 2-7) was isolated from *Camptotheca acuminate* Decne. (Nyssaceae). This was initially not interesting as it showed unacceptable myelosuppression (Newman *et al*., 2003). However, interest in this isolate was rekindled when it was found to act by selective inhibition of topoisomerase I, which is involved in cleavage and reassembly of DNA (Newman *et al.*, 2003). Both taxanes and the camptothecins (Figure 2-7) accounted for approximately a third of the global antincancer market in 2002. Elliptinium , a derivative of ellipticine, isolated from a Fijian medicinal plant *Bleekeria vitensis* A.C. Sm., is marketed in France for the treatment of breast cancer (Cragg and Newman, 2005; Shoeb, 2006).

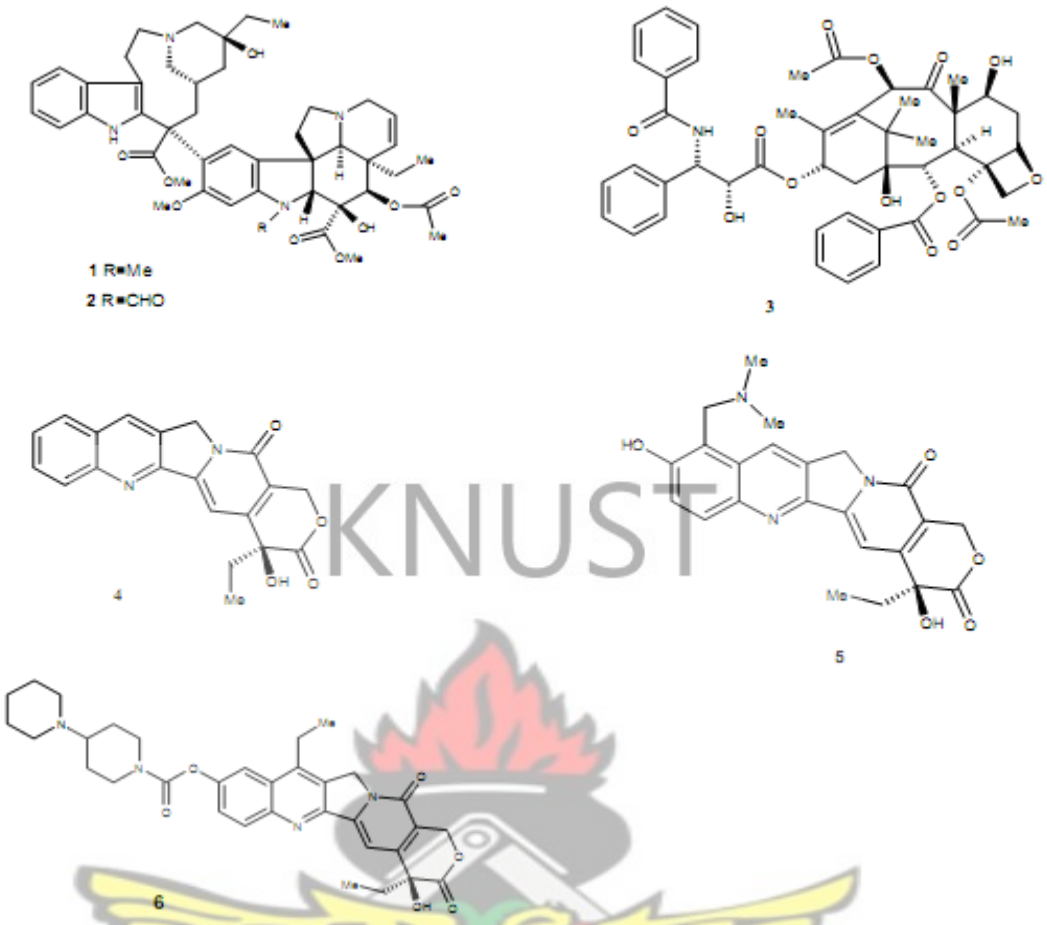


Figure 2-7 Plant-derived anticancer agent in clinical use: vinblastine (1), vincristine (2), paclitaxel (3), camptothecin (4), topotecan (5) and irinotecan (6). Source: Shoeb, 2006.

Numerous derivatives of all four classes of compounds have been synthesized, with some currently in clinical use. All of these natural products have led to significant biological discoveries related to their special mechanisms of action (Balunas and Kinghorn, 2005). More than 300,000 higher plants exist on the surface of the planet; therefore active research into medicinal plants will hopefully help isolate more bioactive principles that can inhibit the growth of cancer cells but not normal cells and the molecular mechanisms of action will also be elucidated.

Curcumin, a historically acknowledged component of the Ayurvedic, Unani, and Siddha medicine, and indigenous to Southern and Southeastern tropical Asia, has extensively been reported to having promising anticancer activities (Ravindran *et al*., 2009). As the major chemical component of turmeric (*Curcuma longa*), in vitro cell culture and in vivo animal studies have suggested its effectiveness to treating numerous types of cancers: breast, colon, kidney, liver, leukemia, prostate, rhabdomyosarcoma, and melanoma (Reuter *et al*., 2008). It has been reported to be cytotoxic to Jurkat cells and induce a caspase mediated apoptosis in these cells (Gopal *et al*., 2014). Its anti-leukemic property has been attributed to its ability to regulate the expression of both pro and anti-apoptotic proteins e.g. Bad, Bim, Caspase-3, p27, Bak, Bcl-2, Bax, Mcl-1, PARP (Reuter *et al*., 2008). Quercetin, isolated from methanol extract of *Asparagus cochinchinensis* (Lour.) Merr tuber exhibited strong cytotoxicity against the HeLa, human cervical cancer cell line with IC_{50} = 5.78 ± 0.36 µg/ml, followed by lung cancer cell line (NCI–H460), with IC_{50}= 12.57 ± 1.19 µg/ml and liver cancer cell line (Hep-G2) with IC_{50} = 20.58 ± 0.85 µg/ml (Son and Anh, 2013). The anticancer activity of quercetin against breast cancer cell line (MCF-7), was recorded (IC_{50} = 31.04 ± 3.14 µg/ml) using the sulfurhodamine B assay *in vitro* (Le Son and Anh, 2013). Out of the ten compounds isolated from ethanolic extracts of *Saxifraga stolonifera* (L.) Meeb, four of them showed anticancer activities on human gastric carcinoma cell line BGC-823 (Narah *et al*., 2012). Chen *et al*., (2008), also studied the effects of extracts from *S. stolonifera* on human gastric carcinoma cell line (BGC-823) *in vitro*, using the MTT assay at a dose range of 5-100 µM. They found that the inhibitory effects of gallic acid, quercetin and b-sitosterol were concentration dependent. Among the isolates, quercetin exhibited the highest cytotoxic effect on BGC-823 cells, with growth inhibition ratio of 39.3% after 72 hours of treatment at 100 µM, while the other compounds exhibited lower growth inhibition

ratios, ranging from 6.6-22.5% the same after 72 hours treatment. Also, studies conducted by Jain and Jain (2011) showed that alcoholic extract of *Sesbania grandiflora* exhibited a prominent inhibitory effect against MCF-7 (IC_{50}= 7.00 ± 0.08 µg/ml) and HL-60 (IC_{50}= 18.50 ± 0.6 µg/ml) under *in vitro* conditions using the MTT assay.

2.4.2 Medicinal Plants with Antioxidant Properties

During normal metabolic functions, highly reactive compounds called free radicals are generated in the body. These however, may also be introduced from the environment and/or dietary xenobiotics (Bandyopadhyay *et al.*, 1999). These molecules are inherently unstable as they possess unpaired electrons and hence become highly reactive. Environmental pollutants, radiation, chemicals, toxins, fatty foods as well as physical stress generates free radicals that cause depletion of immune system antioxidants, change in gene expression and induce the formation of abnormal proteins (Bandyopadhyay *et al.*, 1999).

Free radicals are electrically charged molecules, thus, they have an unpaired electron, which causes them to seek out and capture electrons from other substances in order to neutralize themselves (Bandyopadhyay *et al.*, 1999). Although the initial attack causes the free radical to become neutralized, another free radical is formed in the process, causing a chain reaction to occur. Until subsequent free radicals are deactivated, many free radical reactions can occur within seconds of the initial reaction. Reactive oxygen species (ROS) is a term that encompasses all highly reactive, oxygen containing molecules, including free radicals. Types of ROS include the hydroxyl radical, the superoxide anion radical, hydrogen peroxide, singlet oxygen, nitric oxide radical, hypochlorite radical, and various lipid peroxides. Reactive oxygen species normally

exist in all aerobic cells in balance with biochemical antioxidants (Bandyopadhyay *et al.*, 1999). When this balance is disrupted because of excess ROS, antioxidant depletion, or both, it can lead to oxidative stress. All ROS can cause cellular/tissue damage by reacting with lipids in cellular membrane, nucleotides in DNA and sulphydryl groups in proteins (Knight, 1995; Ahsan *et al.*, 2003). These have been implicated in a number of diseases including cancer, cardiovascular, diabetes, cataracts, inflammatory processes, asthma, muscular degeneration and other disease processes.

In carcinogenesis, ROS are responsible for initiating the multistage process starting with DNA damage and accumulation of genetic events in some cell lines which leads to progressively dysplastic cellular appearance, deregulated growth, and finally carcinoma (Atawodi, 2005). The mechanism of free damage includes the induction of peroxidation of polyunsaturated fatty acids by reactive oxygen species in cell membrane lipid bilayer, which causes a chain reaction of lipid peroxidation, thus damaging the cell membrane and causing further oxidation of membrane biomolecules (lipids and proteins). The content of the cell including its DNA become damaged. This induced damage caused by free radicals is what is thought to precede these disease processes (Miller, 1996). Hence, there is the need of therapy with antioxidants (antiradicals or free-radical scavengers), that have the potential to prevent, delay or improve these disease conditions (Delanty and Dichter, 2000). These antioxidant systems are contributed mainly by enzyme and non-enzymatic systems. Recently, antioxidants like ascorbic acid, gallic acid esters, butylated hydroxy toluene (BHT), butylated hydroxy anisole (BHA) and tertiary butylated hydroquinone, have been suspected to act as pro-oxidant effect at very high concentration. Hence, strong restrictions have been placed on their application and there is a demand to substitute these with naturally occurring antioxidants. Also, these

synthetic antioxidants show low solubility and moderate antioxidant activity (Barlow, 1990). There has been an increase of interest in the therapeutic potential of natural products, especially medicinal plant sources, as antioxidants in reducing free radical induced cellular/tissue injury.

This has led to the commercial exploitation of traditionally used and well known natural antioxidants from fruits, vegetables, and tea either as antioxidant or as nutritional supplements (Schuler, 1990). Studies conducted by Son and Anh (2008), showed that quercetin isolated from methanolic extract of *Asparagus cochinchinensis* (Lour.) Merr tuber had a strong antioxidant activity with an IC_{50} value of 14.52 ±2.12 µg/ml as compared to ascorbic acid (IC_{50} value of 10.49±2µg/ml) using the 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay. From studies conducted on the leaves of *Ageratum houstonianum* Mill. (Asteraceae), a closely related species of *Ageratum conyzoides*, using three solvents systems, hexane, methanol and ethylacetate, it was found that ethylacetate extract could scavenge oxidants with high percentage inhibition, followed by methanol and lastly hexane with % inhibition of DPPH free radical values of 88.26 ±0.35, 82.62±0.89 and 72.82±0.41 respectively (Tennyson *et al.*, 2012). This level of DPPH free radical scavenging was greater in the ethylacetate extract than that found in positive controls such as Butylated hydroxytoluene (BHT) and ascorbic acid, with % inhibition of DPPH free radical values of 74.49±0.39 and 85.12±1.08 respectively (Tennyson *et al.*, 2012). Antioxidant activity of plant species might be due their phenolic compounds (Pourmorad *et al.*, 2006), glutathione content (both are non-enzymatic) or enzyme systems (catalase, superoxide dismutase, peroxidase, glutathione reductases and transferases, etc.).

2.4.2.1 Glutathione (GSH) as an antioxidant

A tripeptide glutathione is an abundant compound in plant tissues. The tripeptide is made up of three amino acids: cysteine, glutamic acid and glycine bearing the name γ-glutamylcysteinylglycine. It has been found virtually in all cell compartments: cytosol, mitochondria, endoplasmic recticulum (ER) and vacuole (Jimenez *et al.*, 1998), where it executes multiple functions. Glutathione is the main storage form of sulfur, and acts as potent detoxifier of xenobiotics through GSH-conjugation, and can serve as precursor of phytochelatins (May *et al.*, 1998; Blokhina *et al.*, 2003). Functioning of glutathione as an antioxidant under oxidative stress condition has received much attention during the last two decades (Blokhina *et al.*, 2003). A central nucleophilic cysteine is responsible for the high reductive potential of glutathione. It scavenges cytotoxic hydrogen peroxide, and reacts non-enzymatically with other reactive oxygen species like hydroxyl radical, singlet oxygen and superoxide radical (Larson, 1988; Blokhina *et al.*, 2003).

In addition to neutralizing free radicals, glutathione is responsible for maintaining the reduced form of other antioxidants, thus maintaining the antioxidant activity of other antioxidants (Blokhina *et al.*, 2003). One basic function of glutathione is to maintain the sulfhydryl groups of proteins in the reduced state (Kałuzny *et al.*, 1995; Ball *et al.*, 2004). Together with glutathione peroxidase, glutathione reduces hydrogen peroxide and lipid peroxide, which is accompanied by the formation of glutathione disulphide, which is reduced by NADPH in a reaction catalysed by glutathione reductase (Dringen, 2000). This property is of great biological importance, in that it allows the maintenance of the cellular redox environment under normal conditions and upon the onset of stress, and

provides the basis for glutathione stress signalling. It is also able to regenerate vitamins C and E back to their active forms, via the ascorbate-glutathione cycles (Dringen, 2000).

2.4.2.2 Phenolic compounds as antioxidants

Phenolic compounds are a diverse group of secondary metabolites abundant in the tissues of most plants (Grace and Logan, 2000; Blokhina *et al.*, 2003). Phenolics are commonly found in both edible and nonedible plants, and they have been reported to have multiple biological effects, including antioxidant activity. Phenolic compounds include flavonoids, tannins, lignin and hydroxycinnamate. The structure of polyphenols makes them ideal for scavenging free radicals, and they have been shown to be more effective antioxidants *in vitro* than ascorbate and tocopherols. Polyphenol antioxidant properties arise from their high reactivity as electron or hydrogen donors, and from the ability of the polyphenol-derived radical to stabilize and delocalize the unpaired electron (chain-breaking function), and from their ability to chelate transition metal ions (termination of Fenton reaction) (Rice-Evans *et al.*, 1997). Another mechanism underlying the antioxidant properties of phenolic compounds is the ability of flavonoids to alter peroxidation kinetics by modification of the lipid packing order and to decrease fluidity of the membranes (Arora *et al.*, 2000). These changes could sterically hinder the diffusion of free radicals and restrict peroxidative reactions.

It has also been shown recently that phenolics can be involved in the hydrogen peroxide scavenging cascade in plant cells (Takahama and Oniki, 1997). Many phenolics, including flavonoids, have been suggested to play a preventive role in the development of cancer and heart diseases. Medicinal plants such Bog-rosemary, heather, meadowsweet, and willow herb, have been shown to accumulate large amounts of phenolic compounds (Kähkönen *et al.*, 1999). The amount of total phenolics are as

follows; bog-rosemary (32.8 mg/g GAE), heather (36.0 mg/g GAE), meadowsweet 942.1 mg/g GAE), and willow herb (32.2 mg/g GAE) (Kähkönen *et al.*, 1999). Although the total phenolic content of these medicinal plants generally did not correlate significantly with their antioxidant activities, phenolics have been suggested as responsible for the antioxidant activities of *Dioscorea bulbifera, Eriobotrya japonica, Tussilago farfara and Ephedra sinica* out of 56 chinese medicinal plants, with high correlation between antioxidant capacity and total phenolics (Li *et al.*, 2012).

2.4.3 Phytochemical constituents of medicinal plants

Medicinal plants are sources of herbal and synthetic drugs. A lot of medicinal plants, if not all, have active components which are responsible for the bioactivities they exhibit. Most of the activities reported above can be attributed to secondary metabolites (phytochemical constituents) that are bioactive and present in the plant. Secondary metabolites are organic compounds that are not directly involved in the normal growth, development and reproduction of the plant (Macias *et al.*, 2007) and thought to result from the evolutionary defense put up by plants (Becerra, 2007). Examples include alkaloids, tannins, terpenoids, flavonoids, saponins, phenolics, coumarins, chromenes, glycosides and steroids. These secondary metabolites are hugely responsible for the anticancer and antioxidant properties exhibited by most medicinal plants.

2.4.3.1 Flavonoids

These are water soluble polyphenolic molecules containing 15 carbon atoms, yellow in colour and ubiquitous in nature. Collectively, they are known as vitamin P or citrin. Flavonoids are secondary metabolites characterised by flavan nucleus (Figure 2-8) (Heim *et al.*, 2002) and C_6-C_8-C_6 carbon-skeleton (Tsuchiya, 2010). These are a group of structurally related compounds with a chromane-type skeleton having phenyl

substituent in C_2-C_3 position (de Rijke *et al.*, 2006). Over 4,000 flavonoids have been identified, many of which occurs in fruits, beverages and vegetables (soy products, grapes coffee, tea). Flavonoids and their derivatives with the strongest antioxidant potential include kaempferol, luteolin, epicatechin, quercetin and delflina. These also have five subgroups, viz; flavones, flavonoids, flavanones, flavanols and anthocyanidins (Nijveldt *et al.*, 2001).

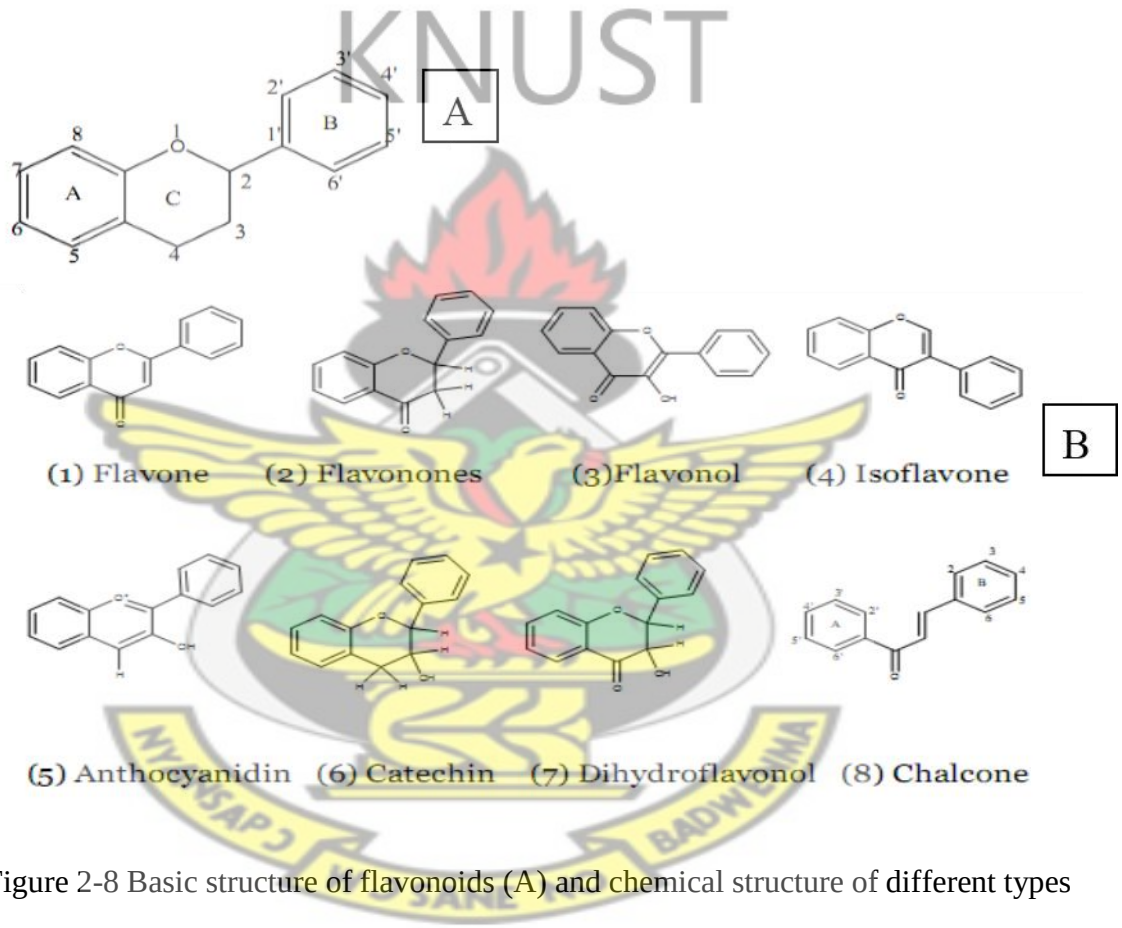


Figure 2-8 Basic structure of flavonoids (A) and chemical structure of different types of flavonoids (B), (Kumar *et al.* (2011).

Flavones (Figure 2-8) are characterized by a planar structure because of a double bond in the central aromatic ring. An example is quercetin which is abundant in onions, apples, broccoli and berries. Flavonoid (Figure 2-8) as a subgroup has been identified as a potent hypolipidemic agent in some studies (Narender *et al.*, 2006) and those from medicinal

plants have been shown to possess a high antioxidant potential due to their hydroxyl groups and they protect more efficiently against free radical related diseases like arteriosclerosis (Vaya *et al.*, 2003). This supports the idea of flavonoids been involved in scavenging of oxygen derived free radicals (Nijveldt *et al.*, 2001). The inhibitory effect of black tea polyphenols on aromatase activities has been investigated. The polyphenols present in black tea inhibited the proliferation of MCF-7 cells in *in vivo* models (Way *et al.*, 2004).

2.4.3.2 Alkaloids

They are a group of naturally occurring compounds containing heterocyclic nitrogen. They are also derived from a variety of sources, including plants, marine organisms and microbes through complex biosynthetic pathways. Structurally, alkaloids are a diverse class of basic nitrogen containing compounds with more than 12,000 structures elucidated from plant sources (Wink, 2003). Alkaloids may be used as anticancers agents, analgesics and antimalarials, and for treating other medical complaints like hypertension and central nervous system disorders (Rathbone and Bruce, 2002). They are classified based on their primary metabolite resulting in purine alkaloids (theobromine and caffeine), monoterpenoid indole alkaloids (vincristine and vinblastine), tropane alkaloids (cocaine) and benzylisoquinoline alkaloids (morphine). According to Marasco and Schmidt-Dannert (2007), purine alkaloids are produced from adenine or guanine, tropane alkaloids are produced from ornithine, isoquinoline alkaloids are synthesized from tyrosine, and monoterpenoid indole alkaloids are derived from tryptophan.

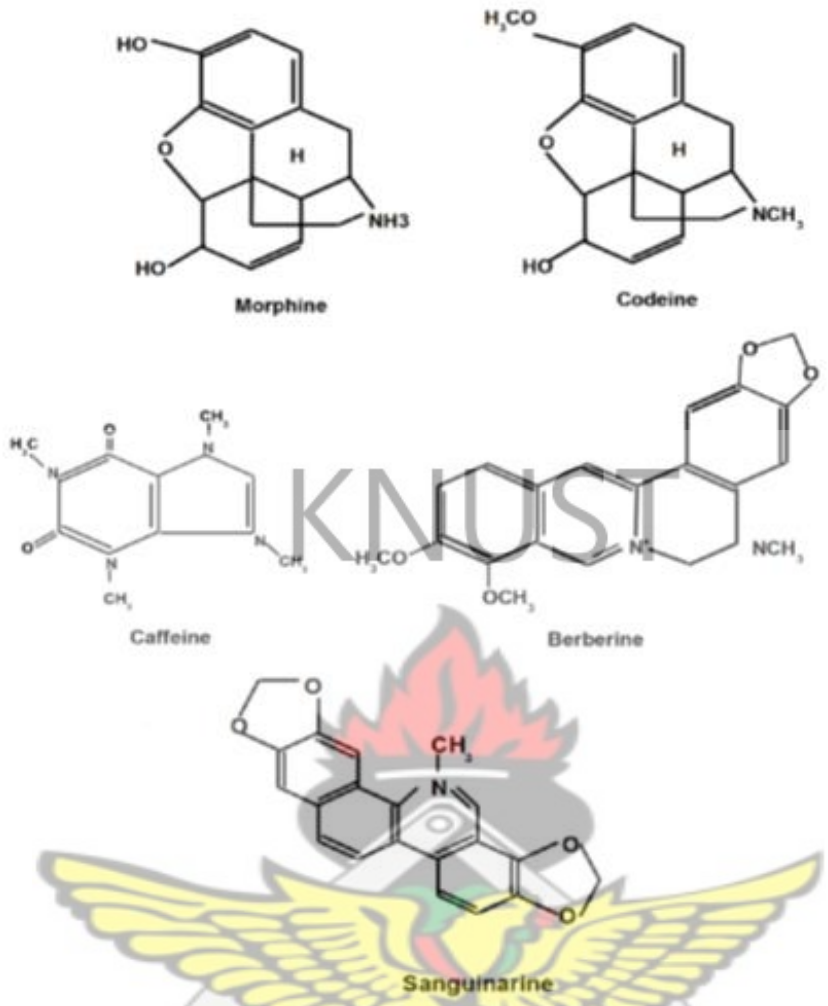


Figure 2-9 Examples of basic structures of some pharmacologically important plant derived alkaloids (Source: Doughari, 2012).

2.4.3.3 Tannins

These are astringent, bitter taste polyphenolic compounds of plant origin that binds and precipitates proteins and various other organic compounds like alkaloids (Haslam, 1989). There are two distinct types, hydrolysable tannins (polyesters of gallic acid) and condensed tannins (flavone). Hydrolysable tannins (Figure 2-9) are esters of gallic and ellagic acids that consist of polyols such as sugars and phenolics such as catechin. These are also more susceptible to enzyme and non-enzymatic hydrolysis than proanthocyanidins, and usually are more soluble in water. Further classification of

hydrolysable tannins is based on the products of hydrolysis as gallotannins yield gallic acid and ellagitannins yield ellagic acid and glucose (Roberts, 1990). Proanthocyanidins, commonly referred to as condensed tannins (Figure 2-9), are polymers of flavan-3-ols linked through an interflavan carbon bond that is not susceptible to hydrolysis. The nomenclature for proanthocyanindin is derived from the acid catalyzed oxidation reaction that produces the red anthocyanidins upon heating proanthocyanidin in acidic alcohol solutions. From the respective proanthocyanindins, procyanidin and prodelphinidin, the corresponding most common anthocyanidins produced are cyanidin and delphinidin (Reed, 1995).

The antinutritive effects of tannins are associated in their ability to bind dietary proteins, polymers (cellulose, hemicellulose and pectin) and minerals thus hindering their digestion. Tannins can also impair digestive processes by complexing with enzymes and endogenous proteins (McSweeney *et al.*, 2001).

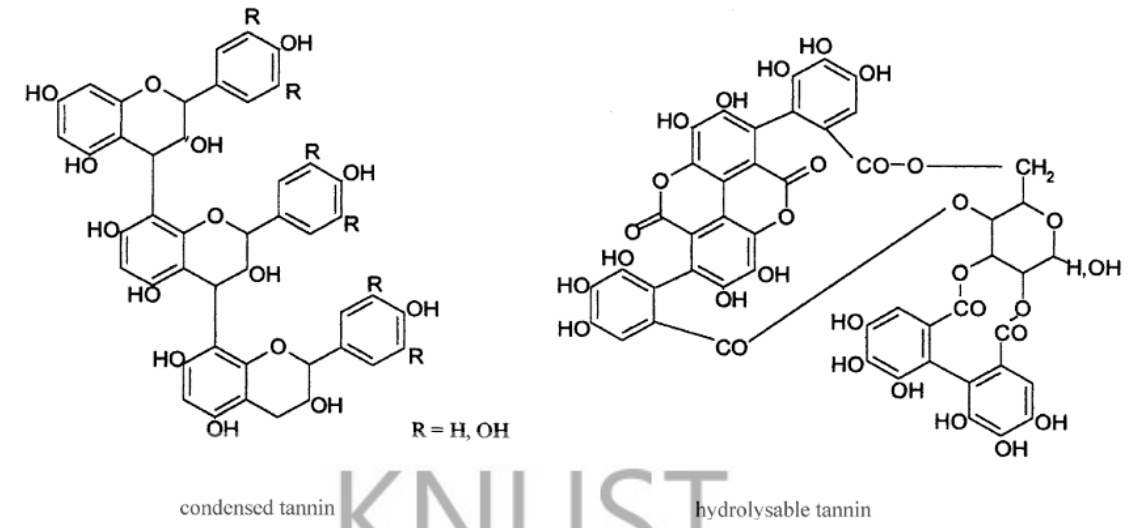


Figure 2-10 Chemical structure of condensed and hydrolysable tannins (Spurce: McSweeney *et al.*, 2001).

2.4.3.4 Saponins

They are naturally occurring surface glycosides with foaming characterisctics. They are mainly produced by plants and can be synthesized by lower marine animals and some bacteria (Yoshiki *et al.*, 1998). The name saponin was derived from its ability to form stable soap-like foams in aqueous solutions (Francis *et al.*, 2002). The foaming ability is caused by a combination of the hydrophobic aglycone and the hydrophilic sugar part. Structurally, saponin consists of an aglycone (sapogenin) and one or two complex sugar moieties. The most common sugar residues are hexoses (glucose, galactose), pentoses (arabinose, xylose), methylpentoses (furanose, quinovose, rhamnose), and uronic acids (glucuronic acid, galacturonic acid). The variability of aglycone structure, the nature of the side chains and the position of the attachment of these moieties on the aglycone result in the complexity of the saponins structure.

The triterpenoid and steroid (Figure 2-11) saponins are the two types of saponins that have been identified. If only one sugar complex is linked to the aglycone, the saponine is called monodesmoidic. The triterpenoid saponins can be found in many legumes (soybean, beans, and cowpea) whiles the steroid saponins are found in medicinal plants (Fenwick and Oakenfull, 1983). Oats, gingseng and aubergine are examples of plants from which saponins have been isolated. Both triterpene and steroidal aglycones have a number of different substituents (H, -COOH, CH_3). Saponin isolates have been shown to specifically inhibit the growth of cancer cells *in vitro* (De Marino *et al.*, 1998) and to have antioxidant properties (Yoshiki *et al.*, 1998). Studies have also shown that saponins can cause apoptosis of leukemia cells by inducing mitotic arrest (Yoshiki *et al.*, 1998).

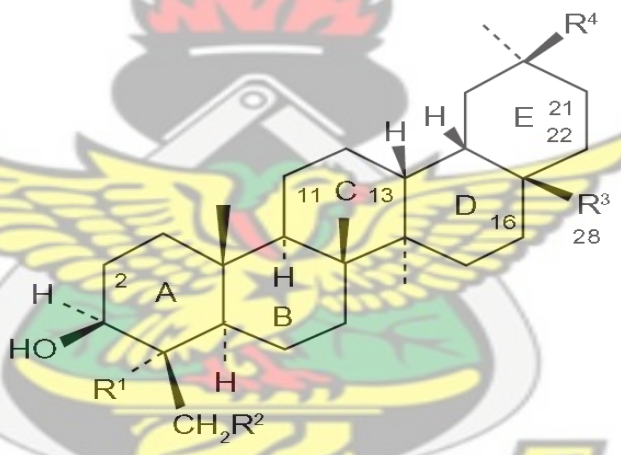


Figure 2-11 Core structure of triterpenoid sapogenins with arrangement of rings ABCDE (Source: Thakur *et al.*, 2011).

2.4.3.5 Terpenoids

These constitute the largest class of natural products and represent a subclass of the prenyllipids (terpenes, prenylquinones, and sterols). They are the oldest group of small molecular products synthesized by plants and are probably the most widespread group of natural products. Terpenoids can be described as modified terpenes, where methyl groups are moved or removed, or oxygen atoms added. They have unsaturated molecules

composed of linked isoprene units, generally having the formula $(C_5H_8)n$. Based on the number of building blocks, terpenoids are classified into several classes, such as monoterpenes (e.g., carvone, geraniol, d-limonene, and perillyl alcohol), diterpenes (e.g. retinol and trans-retinoic acid), triterpenes (e.g., betulinic acid, lupeol, oleanic acid, and ursolic acid), and tetraterpenes (e.g., α-carotene, β-carotene, lutein, and lycopene) (Rabi and Bishayee, 2009). Monoterpenoids represent a structurally diverse class of compounds and may be categorised into nearly 35 varying structural analogues. However, the most commonly occurring structural variations are of the following types, namely, myrcene (acyclic), p-menthane (monocyclic) and a-pine (bicyclic) (Templeton, 1969).

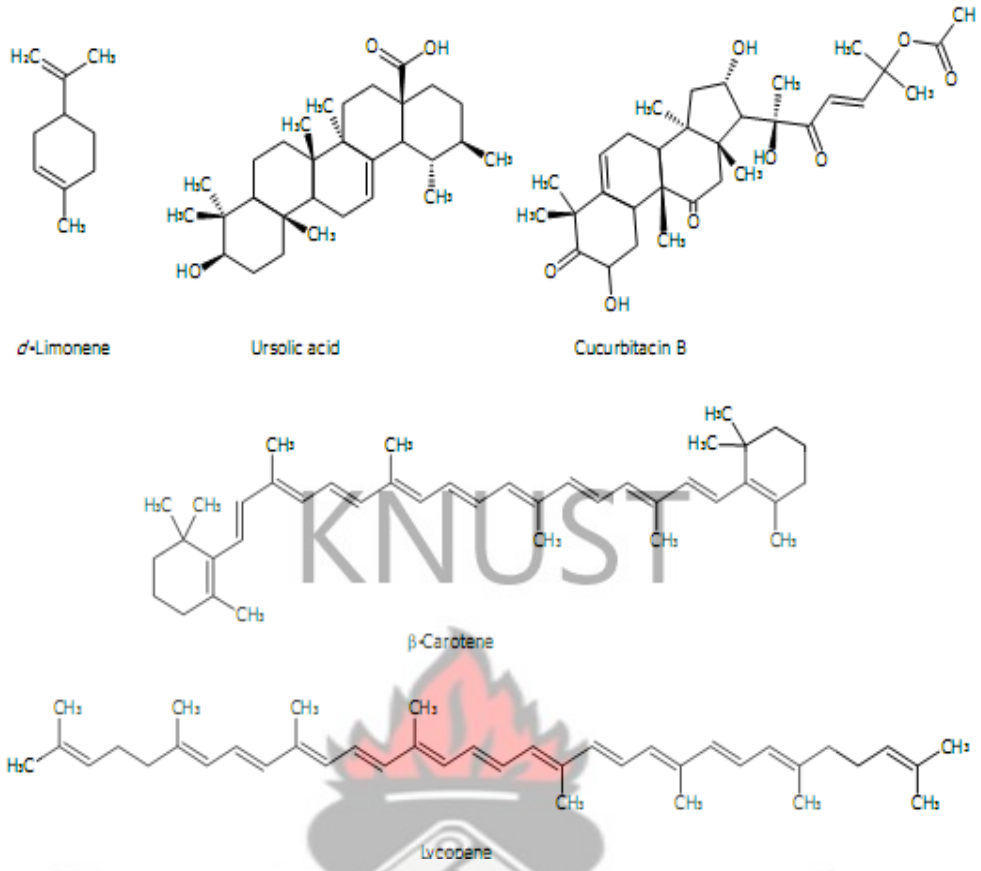


Figure 2-12 Chemical structures of some naturally occurring terpenoids. (Source: Thoppil and Bishayee, 2011).

It has been found that a large number of monoterpenoid derivatives belonging to these categories invariably occur naturally in the purest optically active form. Sesquiterpenoids are found to be extensively distributed in nature and represent the most abundantly prevailing class of terpenoids. Abscisic acid, b-cadinene, b-caryophyllene among others constitutes examples of sesquiterpenoids. Diterpenoid represent a broad class of non-volatile C_{20} compounds that have been essentially obtained from geranyl pyrophosphate. It has been observed that they mostly originate from the plant or fungal sources, but they are invariably formed by certain insects as well as marine organisms. Triterpenoids are found to share commonly the acyclic precursor squalene (C_{30}). Triterpenoids may be categorized into two major groups, namely, the tetracyclic and the

pentacyclic compounds; the former ones of the steriodal types with C-27 carbon atoms present in the skeleton while the latter are of the triterpenoid types with C-30 carbon atoms. Examples of terpenoids include; loganin, morroniside, Urosolic acid, betulinic acid, oleanolic acid, etc (Figure 2-12).

Terpenoids have been found to be useful in the prevention and therapy of several diseases, including cancer, and also to have antimicrobial, antifungal, antiparasitic, antiviral, anti-allergenic, anti-spasmodic, anti-hyperglycemic, anti-inflammatory, and immunomodulatory properties (Wagner and Elmadfa, 2003; Sultana and Ata, 2008; Rabi and Bishayee, 2009). A sesquiterpene (α-bisabolbol) was found to induce cytotoxicity in HepG2 cells and apoptosis, through both the intrinsic and extrinsic pathways, was identified as the mechanism of action exhibited by this natural compound (Chen *et al.*, 2010). Recently, several phase I/II clinical trials have been initiated to evaluate the chemopreventive as well as the anticancer efficacy of a number of triterpenoids (Liby *et al.*, 2007; Fulda, 2009; Thoppil and Bishayee, 2011).

2.5 *AGERATUM CONYZOIDES* LINNAEUS

The name *Ageratum* is derived from the Greek "*a geras*," meaning non-aging, referring to the longevity of the flowers or the whole plant. The specific epithet "conyzoides" resembles *Inula helenium* which bears the Greek "*kónyz*" from which the name was derived (Ming, 1999).

Latifolium and *conyzoides* are two subspecies classified by Johnson (1971). Subspecies *latifolium* is found in all the Americas and subspecies *conyzoides* has a pantropical distribution. The basic chromosome number is 2n = 20 but natural tetraploids are found.

A. conyzoides subspecies *latifolium* is diploid and *A. conyzoides* subspecies *conyzoides* is tetraploid (Ming, 1999).

Most taxa of this annual herb are found in Mexico, Central America, the Caribbean, and Florida. *Ageratum conyzoides* now is found in several countries in tropical and sub-tropical regions, including Brazil, Southeast Asia, South China, India, West Africa, Australia and South America (Baker *et al.*, 1965; Okunade, 2002; Kong, 2006; Lorenzi, 2008; Adebayo *et al.*, 2010).

Ageratum conyzoides Linnaeus, commonly called the Billy goat weed or White weed or Goat weed, belongs to the family Asteraceae and Tribe Eupatoriae. Herbs are the majority in this family while trees and shrubs are comparatively rare. The taxonomic system for the classification of *Ageratum conyzoides* is described in Table 2-2.

It is an erect herbaceous annual herb which grows up to 30 to 80 cm tall; stem and leaves of this plant are covered fully with fine white hairs. The leaves are ovate in shape and grow up to 7.5 cm long. Its leaves are opposite, pubescent with long petioles and include glandular trichomes (Ming 1999; Okunade, 2002). The flowers are arranged as acorymb in terminal inflorescence which are white to purple in colour. The fruits are achene and easily dispersed by air; seeds are photoblastic and often lost within 12 months. The seeds germinate between 20-25ºC; it prefers a moist and well-drained soil but may tolerate dry conditions (Ming, 1999; Okunade, 2002; Singh *et al.*, 2013).

Table 2-2 Taxonomic classification of *Ageratum conyzoides*

Kingdom	Plantae-Plants
Subkingdom	Tracheobionta-Vascular Plants
Superdivision	Spermatophyta-Seed plants
Division	Magnoliophyta-Flowering plants
Class	Magnoliopsida-Dicotyledons
Subclass	Asteridae
Order	Asterales
Family	Asteraceae-Aster family
Genus	Ageratum L.-whiteweed
Species	*Ageratum conyzoides* L.-tropical whiteweed

Figure 2-13 *Ageratum conyzoides* whole plant

The plant thrives in any garden soil and is very common in waste places and on ruined sites (Shirwaikar *et al.*, 2003). It has a peculiar odour likened in Australia to that of a male goat and hence its name 'goatweed' or 'billy goat weed'. It is traditionally called "yaa kankan" by the Ashanti's in the Ashanti region of Ghana. Also, among the Akuapim's in Eastern Ghana, it is known as "guakuro". These species appear highly adaptable to different ecological conditions and has great morphological variation (Hu and Kong, 2002).

2.5.1 Medicinal uses of Ageratum conyzoides

A. conyzoides is reported to have a wide range of medicinal uses in several countries of the world, although applications vary from one region to another. Traditionally, this herb has been used in various parts of the world like Africa, Asia and South America as folk medicine. Traditional communities in India use this specie as a bactericide, anti-dysentery and anti-lithic (Borthakur *et al.*, 1987). In Brazil, its aqueous extract has been extensively used to treat colic, cold, fevers, diarrhoea, rheumatism and spasms (Siqueira-Jaccoud, 1961;Marques Neto *et al.*, 1988; Oliveira *et al.*, 1993). Also, in Cameroon and Congo, its traditional use is to treat fever, rheumatism, headache and colic (Bioka *et al.*, 1993; Menut *et al.*, 1993). In Central Africa it is used to treat pneumonia, but the common use is to cure wounds and burns (Durodola, 1977). Likewise, the plant is used by the Fipa in South Africa as application to fresh wounds (Watt and Breyer-Brandwijk, 2006). Oladejo *et al.*, (2003) has reported wound healing enhancing action of the plant. Other folk remedies include anti-itch, sleeping sickness, and mouthwash for toothache, antitusive, vermifuge, tonic and killing lice (Kapur, 1993). Haemostatic, anti-inflammatory, and analgesic properties of plant extracts (alcoholic and methanolic) have also been reported (Bamidele *et al.*, 2010; Rahman *et al.*, 2012). The plant extract is also

found to have antioxidant and insecticidal activities (Nour *et al.*, 2010; Singh *et al.*, 2013).

2.5.2 Bioactive Principles isolated from A. conyzoides

This plant has been reported to contain many bioactive compounds which responsible for its diverse biological properties. The bioactive principles mostly isolated were from the essential oil of the plant. These include:

2.5.2.1 Flavonoids

This plant is a rich source of polyoxygenated flavonoids. A total of 21 polyoxygenated flavonoids have been reported from the species which include 119 polymethoxylated flavones namely scutellarein-5,6,7,1-tetrahydroxyflavones, quercetin, quercetin-3-rhamnopiranoside, kaemferol, 14 polymethoxy flavones (noteworthy, are the tricin derivatives, 3'4'5'-oxygenated flavones which occur in good yields in this plant, (González *et al.*, 1991)) eupalestin and kaemferol-3,7-diglucopyranoside (Nair *et al.*, 1977). A novel isoflavone glucoside, 5,7, 2, 19-tetrahydroxy-6,3-di-(3,3-dimethylallyl)-isoflavone and 5-O-α-rhamnopyranosyl-(1,19)-L-rhamnopyranoside was isolated from the stems (Gill *et al.*, 1978; Singh *et al.*, 2013).

2.5.2.2 Alkaloids

Only two alkaloids have been isolated from this plant. These are lycopsamine and echinatine which are example of pyrrolizidine alkaloids (Okunade, 2002). Pyrrolizidine alkaloids (PA) are widely distributed in Asteraceae family, particularly in tribes Senecioneae and Eupatorieae (Singh *et al.*, 2013). Also, some alkaloids found in *Ageratum* species include caffeic acid, phytol, 2-(2'-methylethyl) 5,6-dimethyoxybenzofuran, 2-(2-methylprop-2- enyl)-2-methyl-6,7-dimethoxychromane-

4-one, 2-(1'-oxo- 2'methypropyl)-2- methylpropyl)-2-methyl-6,7- dimethoxychromene and 3-(2'-methylpropyl)- 2-methyl-6,8-demothoxychrom-4-one (Singh *et al.*, 2013).

2.5.2.3 Triterpenes and sterols

The triterpene friedeline and sterols like beta-sitosterol and stigmasterol (major constituents) and brassicasterol (minor sterol) have been isolated from oil in the leaves and stems of this plant (Okunade, 2002).

2.5.2.4 Monoterpenes and sesquiterpenes

A total of 51 constituents have been reported from the GC-MS analysis of an oil sample of the plant collected from Nigeria (Singh *et al.*, 2013). Different kinds of mono-and sesquiterpenes have also been isolated from oil obtained from the leaves of this plant in other countries (Okunade, 2002).

2.5.2.5 Coumarin, benzofuran, chromone and chromene

Precocene I (7-methoxy-2,2dimethlchromene) ranging from 30 to 93% have been isolated from oil of this plant (Singh *et al*., 2013). Precocene II (ageratochromene, 6, 7-dimethoxy derivative) ranging from 0.7% to 55% while cumarine (5.01%) and transcaryophyllene (3.02%) are the most common components of essential oil of *A. conyzoides*. Other related compounds like 6-(1-hydroxyethyl)-7-methoxy–2,2-dimethylchromene; 6-(1-ethoxyethyl)-7-methoxy-2,2- dimethylchromene, 3-(2-methylpropyl)-2-methyl-6,8-demothoxychrom-4-one have also been isolated (Nair *et al.*, 1977). A yield of 1.219% of coumarin and a minor amount of benzofuran and its derivatives were obtained from the plants essential oil (Singh *et al.*, 2013).

2.5.3 Anticancer studies conducted on Ageratum conyzoides

Literature search has revealed that, only Adebayo *et al.*, (2010), and Adetutu *et al*., (2012), have evaluated and published the *in vitro* anticancer activity of this plant. The studies conducted by Adebayo *et al.*, (2010), described the *in vitro* anticancer activity of ethanol, petroleum ether, ethylacetate, n-butanol, and water extracts of *A. conyzoides* against the following cancer cell lines: Human non-small cell lung cancer (A-549), human gastric cancer (SGC-7901), human colon cancer (HT-29), human glioma cancer (U-251), human breast carcinoma (MDA-MB-231), human prostate carcinoma (DU-145), human hepatic carcinoma (BEL-7402), and mouse leukemia (P-388) cancer cell lines using the sulphorhodamine B (SRB) assay. The results showed the following; ethanol extract showed an IC_{50} value of 1.73 µg/ml in P- 388 cell line, while petroleum ether fraction had IC_{50} values of 14.06, 13.77 and 0.71 µg/ml in A-549 (human non-small cell lung cancer cell line), SGC-7901(human gastric cell line) and P-388 (mouse leukemic cell line) respectively. Similarly, ethyl acetate fraction had IC_{50} values of 0.68, 9.97, 14.88 and 0.0003 µg/ml in A-549, DU-145, SGC- 7901 and P-388 cells, respectively. However, n-butanol and water fractions had no significant inhibitory effect (IC_{50} values) on the cancer cell lines compared to standard anticancer drugs, taxol and hydroxy-camptothecin.

Adetutu *et al*., (2012), also reported the *in vitro* cytotoxic effects of the ethanol extracts as well as chloroform, ethylacetate and petroleum ether fractions of the leaves of *A. conyzoides* on two human lung cancer cell lines (SK-LU 1 and SK-MES 1) and human skin fibroblast cell line (FS5 cells) using the MTT assay. Their findings showed the following; Crude ethanol extract had IC_{50} values of 38.5 ± 0.60, 15.0 ± 0.15 and 0.25 ± 0.01 µg/ml on the SK-MES1, SK-LU1 and FS5 cells respectively. The petroleum ether fraction had IC50 values of 18 ± 0.40, 13.0 ± 0.11 and 0.50 ± 0.02 µg/ml respectively

on the SK-MES1, SK-LU1 and FS5 cell lines. The chloroform and ethylacetate fractions also recorded IC_{50} values of 13.0 ± 0.20, 14.0 ± 0.23 and 0.5 ± 0.02 µg/ml, and 10 ± 0.20, 13.0 ± 0.30 and 0.20 ± 0.02 µg/ml on SK-MES1, SK-LU1 and FS5 cell lines.

2.5.4 Antioxidant works on A. conyzoides

Adebayo *et al.*, (2010), showed that extracts and fractions (ethanol, petroleum ether, ethylacetate, n-butanol, and water) of the leaves of *A. conyzoides* possess antioxidant activity using the *in vitro* 1, 1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging assay. The preliminary result of the effect of extracts of *A. conyzoides* on the DPPH scavenging activity showed that the water extract, n-butanol extract, and ethylacetate extract have 87.80%, 85.40%, 72.56%, and 60.87% activity respectively while the petroleum ether extract had a rather weak percentage activity of 7.84. Kaempferol isolated from the ethylacetate extract of *A. conyzoides* rapidly scavenged DPPH at an EC_{50} value of 130.07 ± 17.36 g/ kg and was highly comparable to the standard control (vitamin C) with an EC_{50} value of 127.13 ± 8.56 g/kg. Studies conducted by Nasrin, (2013), on crude methanolic extract of the *Ageratum conyzoides* stems showed that, the percentage scavenging of DPPH free radical of the extract was found to be concentration dependent with IC_{50} value 46.01 ± 2.23µg/ml while IC_{50} value of standard ascorbic acid was found to be 29.56 ± 0.11µg/ml. Also, the total phenolics content was found to be 38.125+2.01mg/ml equivalent of gallic acid (Nasrin, 2013).

CHAPTER THREE

MATERIALS AND METHODS

3.1 REAGENTS AND CANCER CELL LINES

All reagents used for extraction such as ethanol, petroleum ether, chloroform and ethylacetate were of analytical grade (Sigma-Aldrich, Illnois, USA). Rose Park Memorial Institute (RPMI)-1640 and Dulbecco modified Eagle's culture media, fetal bovine serum (FBS), penicillin streptomycin L-glutamine (PSG), curcumin, 2,2-diphenyl-1-picryl hydrazyl free radical reagent, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye, dimethyl sulphoxide (DMSO), butylated hydroxyl toluene (BHT), glutathione (GSH), glutathione standard and buffer (pH 8.0), O-phthaldialdehyde (OPT), gallic acid, Folin-Ciocalteau reagent, sodium carbonate solution, phosphate buffered saline (PBS) and trypan blue were all purchased from Sigma-Aldrich (Illnois, USA).

Human leukaemia-immortalized T lymphocyte (Jurkat), human androgen-sensitive prostate carcinoma (LNCap), human hormone-responsive breast carcinoma (MCF7) and prostate normal (PNT2) cell lines were obtained from the Clinical Pathology Department, Noguchi Memorial Institute for Medical Research (NMIMR) Cell Bank where the *in vitro* work was performed. These cell lines were selected to study the effect of extracts on the prevalent cancers (leukemia, breast and prostate) in Ghana.

3.2 PLANT MATERIAL

Plants were handpicked from the land around Senior Staff Club House, on the Kwame Nkrumah University of Science and Technology (KNUST) campus, in the early

morning's hours between 6:00 am – 9:00 am, in October, 2013. The plant samples were authenticated at the Department of Herbal Medicine, KNUST, Kumasi by a taxonomist (Dr. George Sam), and a voucher specimen (KNUST/HMI/2014/WP005) deposited at the department's herbarium for reference purpose.

Preparation of the plant samples was done by sorting them into four components: whole plant, stem, leaves, and flowers. The whole plant and stem components were chopped into pieces. All four components were washed separately with water, three times, and air-dried at room temperature for two weeks at the Biochemistry Annex (Forig), KNUST-Kumasi. The dried samples were separately pulverized using a hammer mill (Christy Lab Mill, England) at the Department of Pharmaceutics, College of Health Sciences, KNUST, Kumasi.

Figure 3-1 Pulverized samples of whole plant and parts of *A. conyzoides* (A) Whole plant, (B) Leaf, (C) Flower and (D) Stem

3.3 CRUDE EXTRACT PREPARATION

3.3.1 Aqueous

A mass of 100 g of the resulting powders of whole plant, leaves and stem samples were decocted with 1000 mL (1 L) of distilled water by heating on a hot plate at 80 ºC for one hour (decoction). The samples were centrifuged (Homef LC-30 centrifuge, LH Wagneningen-04065) for 40 minutes at a speed of 1106 ×g at room temperature in 50 mL eppendorf tubes. The supernatants were taken up and the pellets were subsequently resuspended with the same volume of distilled water and the extraction done for a second time. All the supernatants (of the two centrifugations), totalling a volume of two liters (2 L) were combined and frozen in -20 ºC freezer. The frozen samples were lyophilized using a vacuum freeze dryer (YK-118, Taiwan) at the Zip Unit, Crop Research Institute,

Fumesua. For the flower sample, a mass of 50 g of the resulting pulverized powder was decocted with 500 mL of distilled water and the above aqueous extraction procedures followed.

3.3.2 Hydroethanolic (50%)

A mass of 100 g of the resulting powders of whole plant, leaves and stem samples were macerated with 1000 ml of 50% hydroethanol while shaking on an Orbital shaker (Gallenkamp, England) for 24 hours at room temperature. The supernatant was filtered by centrifugation (Homef LC-30 centrifuge, LH Wagneningen-04065) for 20 minutes at a speed of 1106 ×g in 50 mL centrifuge tubes at room temperature. A volume of 1000 mL of 50% hydroethanol was subsequently used to resuspend the pellets and the extraction was done for a second time. Both supernatants were pooled together (of the two centrifugations) and concentrated using a rotary evaporator (Buchi Rotavapor R-205, Switzerland) at 50 ºC. About 50 mL of the concentrate which remained out of a total volume of one liter (1 L), was frozen at -20 ºC. The frozen samples were lyophilized using a vacuum freeze dryer (YK-118, Taiwan).

For the flower sample, a mass of 50 g of the resulting pulverized powder was macerated with 500 mL of 50% hydroethanol and the above ethanolic extraction procedures followed.

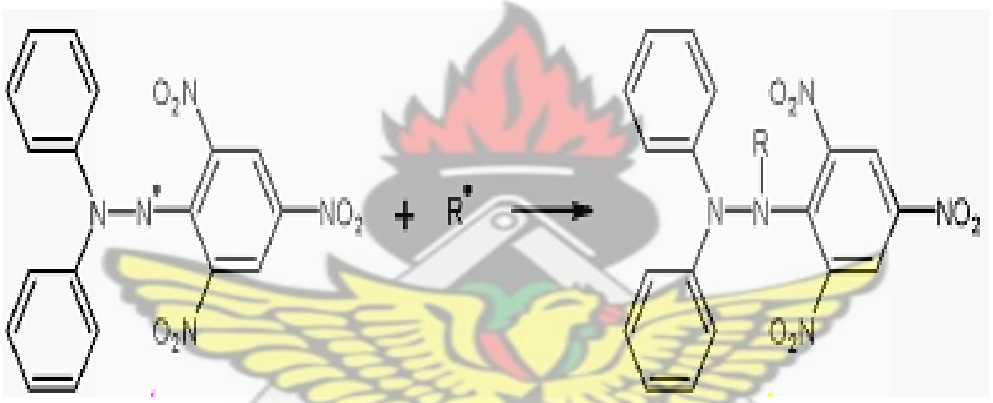
3.4 *IN VITRO* ASSESSMENT OF THE BIOLOGICAL ACTIVITIES OF CRUDE EXTRACTS AND FRACTIONS OF *A. CONYZOIDES*

3.4.1 Total antioxidant activity (2, 2- diphenyl-1-picryl hydrazyl-DPPH) Assay

3.4.1.1 Principle

When an antioxidant compound or extract that can donate hydrogen reacts with DPPH (a stable N centered radical purple in colour), it reduces the DPPH to yellow colour. This colour change can be measured at 517 nm using a UV/Vis light spectrophotomer.

$$Z^{\bullet} + AH = ZH + A^{\bullet}$$



3.4.1.2 Procedure

Stock solutions of the aqueous (4) and ethanolic (4) extracts were prepared by dissolving 10 mg of each of the freeze-dried samples in 1 ml of distilled water and 50% hydroethanol solvents respectively. Also, stock solutions of 10 mM of standard (butylated hydroxyl toluene, BHT) and 0.5 mM of DPPH were prepared by dissolving 2.2 mg of BHT and 3 mg of DPPH in 1mL and 15 mL absolute methanol respectively. The solutions were then vortexed until complete dissolution was achieved. The DPPH solution was immediately kept in the dark as it photo-bleaches in light.

The effects of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) antiradical or antioxidant potentials of the extracts were determined according to the procedures described by Brand-Williams *et al.*, (1995) with slight modifications as follows: in a 96 well plate, the extracts were serially diluted in distilled water (for aqueous samples) or 50% hydroethanol (for hdroethanolic extracts) to obtain a concentration range of 0.156–10 mg/ml, except aqueous leaf extract which was prepared at range of 0 - 2.2 mg/ml. The reaction mixture was made up of 100 μL of 0.5 mM 2, 2-diphenyl-1-picrylhydrazyl radical (DPPH), and 100 μL of each concentration of the test sample. For positive control or standard, butylated hydroxy toluene (BHT) was used at a concentration range of 0.156–10 mM in methanol. The solvents, methanol, distilled water and 50% hydroethanol were used as blanks. Triplicate experiments were performed. The plates were covered with aluminum foil, shaken gently and kept in the dark for 20 minutes after which the absorbance was read on a Tecan-PC infinite M200 Pro Plate reader (Austria), at the absorbance wavelength of 517 nm. Percentage scavenging activity was determined by;

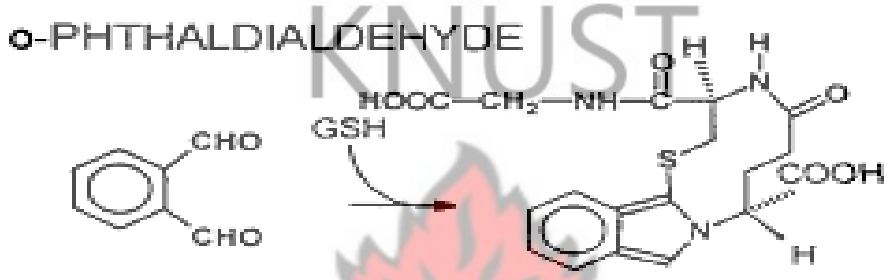
$$\% \text{ Scavenging} = \frac{[\text{Absorbance of blank } (OD_0) - \text{Absorbance of test } (OD_1)]}{\text{Absorbance of blank } (OD_0)} \times 100$$

The mean percentage antioxidant activity for the triplicate experiment was plotted for the standard and samples and their effective concentration at 50% (EC_{50}) values, which is the amount of antioxidant necessary to decrease the initial DPPH concentration by 50%, were determined by nonlinear regression analysis.

3.4.2 Glutathione (GSH) Assay

3.4.2.1 Principle

The reactive dye O-phthaldialdehyde forms fluorescent adducts with GSH. The emission of the resulting adduct is green-shifted and this fluorescence can be spectrophotometrically measured at a wavelength of 412 nm.



3.4.2.2 Procedure

Stock solutions of the aqueous (total of 4) and ethanolic (total of 4) extracts were prepared by dissolving 10 mg of each of the freeze-dried samples in 1 ml of distilled water and fifty percent (50%) hydroethanol solvents respectively. Stock solutions of 1 mg/mL of O-phthaldialdehyde (OPT) and 0.1 mM of glutathione (GSH) standard were prepared by dissolving 1 mg of OPT in 1 mL absolute ethanol and 0.0031 g of GSH standard in absolute ethanol. The solutions were then vortexed until complete dissolution was achieved.

The presence of glutathione in the extracts was determined according to procedures described by Brand-Williams *et al*., (1995), with slight modifications. A two fold serial dilution was carried out on the GSH standard to obtain six different concentrations 0.1, 0.05, 0.025, 0.0125, 0.00625, and 0.003125 mM. A methanol blank, that is, absolute

methanol without GSH was also prepared. A two fold serial dilution was also carried out on each extract to obtain three different concentrations, 10, 5, 2.5 mg/ml. Water and 50% hydroethanol solution (without extracts) were also prepared as blanks. A GSH buffer of pH 8.0 comprising of 0.1 M sodium dihydrogen phosphate dehydrate and 5 mM ethylene diamine tetra acetate was used.

Aliquots of 180 µL of the GSH buffer of pH 8.0 were pipetted into 96-well plate. A volume of 10 µL of the two fold serial dilutions of both GSH standard (in absolute methanol), concentration range of 0.003-0.1 mM, and extracts (in distilled water or 50% hydroethanol) were then added. The blanks were also added. This was followed by the addition of 10 µL of 0.0075mM OPT. This was incubated in the dark at room temperature for 15 minutes and fluorescence was read at 412 nm using microplate spectrophotometer (Tecan Infinite M200 Pro plate reader, Austria). Triplicate experiments were performed. A graph of fluorescence against concentration was plotted for the GSH standard. The concentration of GSH in each sample was determined using the GSH standard plot.

3.4.3 Total Phenol Content Determination

3.4.3.1 Principle

All phenolic compounds contained in a mixture of antioxidant compounds or extract are oxidized by Folin-Ciocalteu reagent. This reagent is formed from a mixture of phosphotungstic acid, $H_3PW_{12}O_{40}$, and phosphomolybdic acid, $H_3PMo_{12}O_{40}$, which, after oxidation of the phenols, is reduced to a mixture of blue oxides of tungstate, (W_8O_{23}), and molybdate, (Mo_8O_{23}), The blue coloration produced has a maximum

absorption in the region of 750 nm, and is proportional to the total quantity of phenolic compounds originally present (Muchuweti *et al.*, 2007).

3.4.3.2 Procedure

Stock solutions of the aqueous (4) and ethanolic (4) extracts were prepared by dissolving 10 mg of each of the freeze-dried samples in 1 ml of distilled water and fifty percent (50%) hydroethanol solvents, respectively. A stock solution of 5 mg/mL of standard (gallic acid) was prepared by dissolving 50 mg of it in 1 mL absolute ethanol. This was then diluted in 10mL distilled water to obtain the 5 mg/mL stock solution.

The method described by Ghasemi *et al.*, (2009) with slight modification was adopted for the determination of the total phenolic content of the extracts. A two fold serial dilution was carried out on the gallic acid standard to obtain six different concentrations 5, 2.5, 1.25, 0.625, 0.3125 and 0.15625 mM. An ethanol blank, that is, absolute ethanol without gallic acid, was also prepared. A two fold serial dilution was also carried out on each extract to obtain three different concentrations (10, 5, 2.5 mg/mL). Water and 50% hydroethanol without extracts, were also prepared as blanks.

A volume of 10 µL of each sample and gallic acid dilutions were aliquoted into a 2.0 mL eppendorf tube. Aliquots of 790 µL of distilled water were then added and this was followed by the addition of 50 µL of Folin-Ciocalteau reagent. The mixture was mixed thoroughly by vortexing for five seconds. This was followed by incubation of the tubes in darkness at room temperature for eight minutes. Afterwards, a volume of 150 µL of 7% sodium carbonate solution was added to each tube, mixed thoroughly by vortexing for five seconds and further incubation of the tubes in darkness at room temperature was done for two hours. After the two hour incubation, a volume of 200 µL of each extract

and gallic acid standard dilutions were aliquoted into wells on a 96-well plate in triplicate and absorbance read at 750 nm using microplate spectrophotometer (Tecan Infinite M200 Pro plate reader, Austria). A graph of absorbance against concentration was plotted for the gallic acid standard. The concentration of phenolics in each sample was determined using the gallic acid standard plot and the gallic acid equivalence for each extract also calculated.

3.4.4 Cell culture

Cells were cultured as described by Ham *et al*., (2012) with slight modifications. Jurkat, LNCap, and PNT2 cells were cultured in RPMI 1640 medium. MCF7 cell line was cultured in D-MEM medium. All culture media were supplemented with 1% PSG and 10% FBS. The cells were maintained in an incubator with 5% CO_2 concentration at 37 °C and passaged on reaching about 80% confluence.

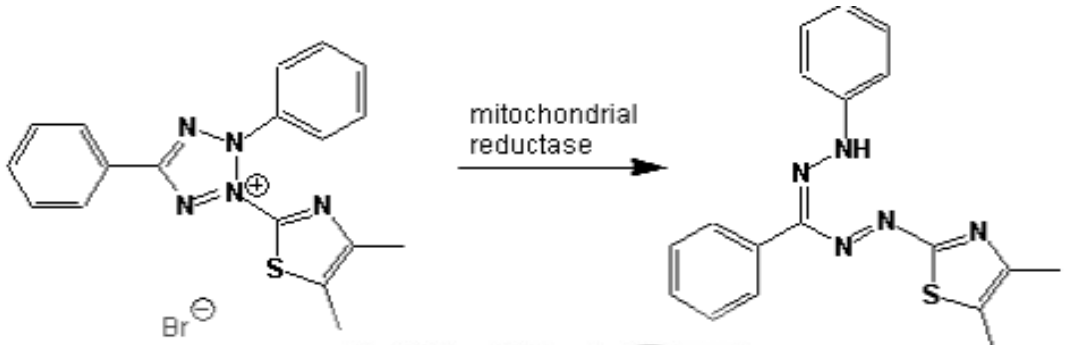
3.4.5 Cell viability Assay

The tetrazolium-based Colorimetric Assay (MTT) was used to determine the cytotoxicity of *A. conyzoides* on the cancer and normal cell lines.

3.4.5.1 Principle

The assay is based on the capacity of the cellular mitochondrial reductase enzyme in living cells to reduce the yellow water-soluble substrate 3-(4, 5-dimethylthiazol- 2yl)-2, 5-diphenyl tetrazolium bromide (MTT) into a purple formazan crystals which is soluble in acidified isopropanol. Since reduction of MTT can only occur in metabolically active

cells the level of activity is a measure of the viability of the cells. The colour change from yellow to purple can be measured at 570 nm using a spectrophotometer.



3.4.5.2 Procedure

Stock solutions of the both aqueous (leaves, stem, whole plant and flower) and hydroethanolic (leaves, stem, whole plant and flower) extracts were prepared by dissolving 50 mg of each of the freeze-dried samples in 1 ml of distilled water and 1 mL of 50% hydroethanol respectively. The solutions were vortexed and filter sterilized into cryotubes in a biosafety cabinet through 0.45 µm pore filters before storage at -20 °C until use.

The procedures described by Ayisi *et al.*, (2011) were followed. The MTT assay was adopted for the measurement of cytotoxic activity. Dilutions of the 50 mg/mL stock of each plant extract was made in distilled water for the aqueous extracts and in 50% hydroethanol for the ethanolic extracts, to obtain a final concentrations of 1 mg/mL. Subsequently, a twofold serial dilution was made of each extract to obtain four concentrations of 0.0625, 0.125, 0.25 and 0.5 mg/mL.

Suspension cells (Jurkat) in culture flask were then transferred into 50 mL tubes, spun down and pellets resuspended. A hemocytometer was used to count the viable cells and

the cell suspension was diluted with a media containing 5% FBS in order to obtain final density of 1x10^5 cells/ml. A volume of 100 µL (1 × 10^5 cells/mL) of cells was seeded into wells of 96-well plate. The cells were immediately treated with 10 µL of each extract dilutions in triplicates and incubated in an incubator with 5% CO_2 concentration at 37 °C for 72 hours (3 days). The final concentrations were in the range of 62.5-1000 µg/ml. The monolayer cells (LNCap, MCF7 and PNT2) were detached with trypsin and single cell suspensions were made using culture media. A hemocytometer was used to count the viable cells and the cell suspension was diluted with a media containing 5% FBS in order to obtain final density of 1x10^5 cells/ml. The 96-well plates at plating density of 10,000 cells/well were seeded with 100 µL per well of cell suspension and incubated for cell attachment at 37°C, 5% CO_2 in a humidified incubator for 24 hr. After this, 10 µL of each extract dilutions (dose range of 625-10,000 µg/ml) were added to the cells in triplicate. The final concentrations were in the range of 62.5-1000 µg/ml at 1% ethanol. This was also followed by 72 hours (3 days) of incubation. Afterwards, a volume of 20 µL of 2.5 mg/mL MTT solution was added to each well on the 96-well plate and incubated in a humidified 5% CO_2 incubator at 37 °C for further four (4) hours. Curcumin (dose range of 10-100µg/mL in 1% DMSO) was used as a positive control in all assays. Acidified isopropanol (150 µL), was added to each well to stop the reaction and the plates were incubated in the dark at room temperature overnight, before reading the absorbance at 570 nm using microplate spectrophotometer (Tecan Infinite M200 Pro plate reader, Austria). A colour control plate was also setup for each extract including the positive control, curcumin. Contents of wells in this plate were made up of 10 µL of sample or control in 100µL of media. The plate was incubated as describe above and absorbances were read at 570 nm using microplate spectrophotometer (Tecan Infinite M200 Pro plate reader, Austria). The percentage cell viability was determined by,

% Cell Viability = $[(ODT_0\text{-}ODT_1)/(ODU_0\text{-}ODU_1)] \times 100$

where ODT_0 is the average absorbance of wells treated with test extracts or curcumin (standard compound) for all cell lines; ODT_1 is the average absorbance of wells with curcumin or test extract control; ODU_0 is the average absorbance of wells with untreated cells (negative control) for all cell lines; ODU_1 is the average absorbance of wells containing blank (culture media only).

The mean percentage cell viability obtained from triplicate determinations at each concentration was plotted as a dose response curve using Microsoft Excel and the inhibition concentration at 50% (IC_{50}) values, that is, concentration of *A. conyzoides* extracts or standard compound inducing 50% of cancer cell growth, determined from the dose response curve by nonlinear regression analysis.

The selectivity index (SI) was determined by ratio between IC_{50} of each extract (and standard drug) on normal prostate PNT2 cell line and IC_{50} of the extract (and standard drug) on cancerous cell lines. SI value indicates selectivity of the sample to the cell lines tested. Samples with an SI greater than 2 were considered to have a high selectivity towards cancerous cells (Badisa *et al*., 2009).

$$\text{Selectivity index (SI)} = \frac{IC_{50} \text{ of the extract/standard drug on normal prostate PNT2 cell}}{IC_{50} \text{ of the extract/standard drug on cancerous cell lines}}$$

Where cancerous cell lines used are Jurkat, LNCap and MCF-7.

3.5 BIOASSAY-GUIDED FRACTIONATION ASSAY

The crude extract that strongly inhibited cancer cell growth (yielding IC_{50} value less than 20 µg/ml) and selective (selectivity indices, SI, greater than 2) against all the cancer cell lines was fractionated using different solvents. The fractions were then used to screen the cell lines (Jurkat, LNCap, PNT2 and MCF-7) for anticancer activity. The 50% crude hydroethanolic leaf extract had the lowest IC_{50} values and highest selectivity indices on all cancer cell lines.

3.5.1 Fractionation of 50% hydroethanolic leaf Extract

Fractionation of the ethanolic leaf extracts of *A. conyzoides* was carried out in a separating funnel using solvents of increasing polarity, petroleum ether, chloroform and ethylacetate. A mass of 15g of crude ethanolic leaf extract (50%) was dissolved in 150 mL of 50% hydroethanol solvent and was successively partitioned with petroleum ether, then with chloroform and finally with ethylacetate, each having a volume of 300 mL, to obtain petroleum ether, chloroform and ethylacetate fractions. This was done from two to three times as polarity increased. The remaining portion was designated as hydroethanolic fraction. The Petroleum ether, chloroform, ethylacetate and hydroethanolic fractions were then concentrated under reduced pressure using a rotary evaporator (Buchi Rotavapor R-205, Switzerland) at temperatures of 65ºC, 69ºC, 77ºC and 79ºC respectively. The petroleum ether, chloroform and ethylacetate were air dried at room temperature (25ºC) while the 50% hydroethanol fraction was lyophilized using a vacuum freeze dryer (Labconco, England). The fractions were then stored in a -20ºC freezer until use.

Stock solutions of the fractions were prepared by dissolving 50 mg of each sample in 1 ml of dimethyl sulphoxide (DMSO). The mixture were vortexed and filter sterilized through 0.45µm millipore filters into cryotubes in a biosafety cabinet before storage at -20°C until use.

Dilution of the 50 mg/mL stock of each fraction was made in dimethyl sulphoxide (DMSO), to obtain a final concentration of 0.1 mg/mL (100 µg/ml). Subsequently, a two-fold serial dilution was made of each extract in 10% DMSO to obtain four concentrations at 0.00625, 0.0125, 0.025 and 0.05 mg/mL. These dilutions were used for cell treatment and a final concentration range of 6.25-100 µg/ml at 1% DMSO obtained.

The above method used for the *in vitro* MTT assay and the SI determination on the crude plant extracts were also adopted for the fractions.

3.6 PHYTOCHEMICAL EVALUATION OF THE CRUDE EXTRACT

The methods described by Ayoola *et al.*, (2008), with slight modification were adopted for the qualitative phytochemical evaluation of the crude extracts. Briefly, the methods were as follows:

3.6.1 Terpenoids

A volume of 1ml of chloroform was added to 10 mg of each extract and standard, urosolic acid and 1ml of concentrated sulphuric acid was subsequently added to each. A reddish brown colour at the interface was indicative of the presence of terpenoids.

3.6.2 Saponins

A volume of 1ml of distilled water was added to 10 mg of each plant extract and shaken vigorously for 1 minute. A stable persistent froth indicated the presence of saponins.

3.6.3 Tannins

A mass of 10 mg of each extract and standard, gallic acid, was boiled with 2 ml of distilled water. Afterwards, the boiled extracts were centrifuged at 1106 ×g to obtain supernatant and three drops of 0.1% $FeCl_3$ added to each supernatant. A brownish green or blue black colouration indicated the presence of tannins.

3.6.4 Alkaloids

Ten milligrams of standard quinidine, and crude plant extracts were dissolved in 2 ml of acid alcohol. Afterwards, the solution was boiled for three minutes and centrifuged 1106 ×g to obtain supernatant. A volume of 1 ml of dilute ammonia was added to the supernatant. Subsequently, 2 ml of chloroform was added and shaken gently to extract alkaloidal base. The chloroform portion was separated and extracted with 2 ml of acetic acid. A reddish brown precipitate indicated the presence of alkaloids after adding four drops of Draggendorf's reagent to each extract and standard.

3.6.5 Flavonoids

A volume of 2 ml of dilute ammonia was added to portions of aqueous supernatant of each plant extract and standard, quercetin. Subsequently, 1 ml of sulphuric acid (concentrated) was added. A yellow colouration that disappeared on standing indicated the presence of flavonoids.

3.7 STATISTICAL ANALYSIS

Microsoft Excel Version 2.4.0.0 was used for the calculation and plotting of mean and S.D estimates in the graph. Mean EC_{50} and IC_{50} values were compared by one way ANOVA using GraphPad Version 6.1 and values with $p < 0.05$ was considered statistically significant.

CHAPTER FOUR

RESULTS

4.1 *IN VITRO* ASSESSMENT OF THE BIOLOGICAL ACTIVITIES OF CRUDE EXTRACTS AND FRACTIONS OF *A. CONYZOIDES*

4.1.1 In vitro DPPH scavenging activity

The ability of the test extracts to donate hydrogen atoms or electrons were measured spectrophotometrically. All extracts reduced DPPH to diphenylpicrylhydrazine, and diminished the absorbance at 517 nm. The results of the effect of the extracts of *A. conyzoides* on the DPPH scavenging activity showed that aqueous extracts scavenge DPPH radical more strongly compared to the ethanolic extracts (Figure 4-1), even though all followed a concentration dependent pattern compared to the positive control (BHT).

Based on the DPPH scavenging potential as determined above, the extracts that showed the most scavenging capabilities (aqueous flower, leaf, whole plant, as well as hydroethanolic whole plant) were compared (Figure 4-2). The hydroethanolic whole plant extract scavenged DPPH radical stronger than the aqueous stem extract but was slightly weak compared to aqueous flower, leaf and whole plant extracts.

EC_{50} value is the effective concentration required to scavenge 50% of the DPPH radical and was extrapolated from the % antioxidant activity against concentration plot. The strongest antioxidant effect was found in the aqueous leaf extract with EC_{50} value of 0.091 ± 0.024 mg/ml. The lowest effect was shown in the ethanolic stem extract with EC_{50} value of 3.545 ± 0.096 mg/ml. Generally, the difference between the various EC_{50}

values for the different extracts were statistically significant (p = 0.0003 for the aqueous extracts and p < 0.0001 for the hydroethanolic extracts) (Table 4-1).

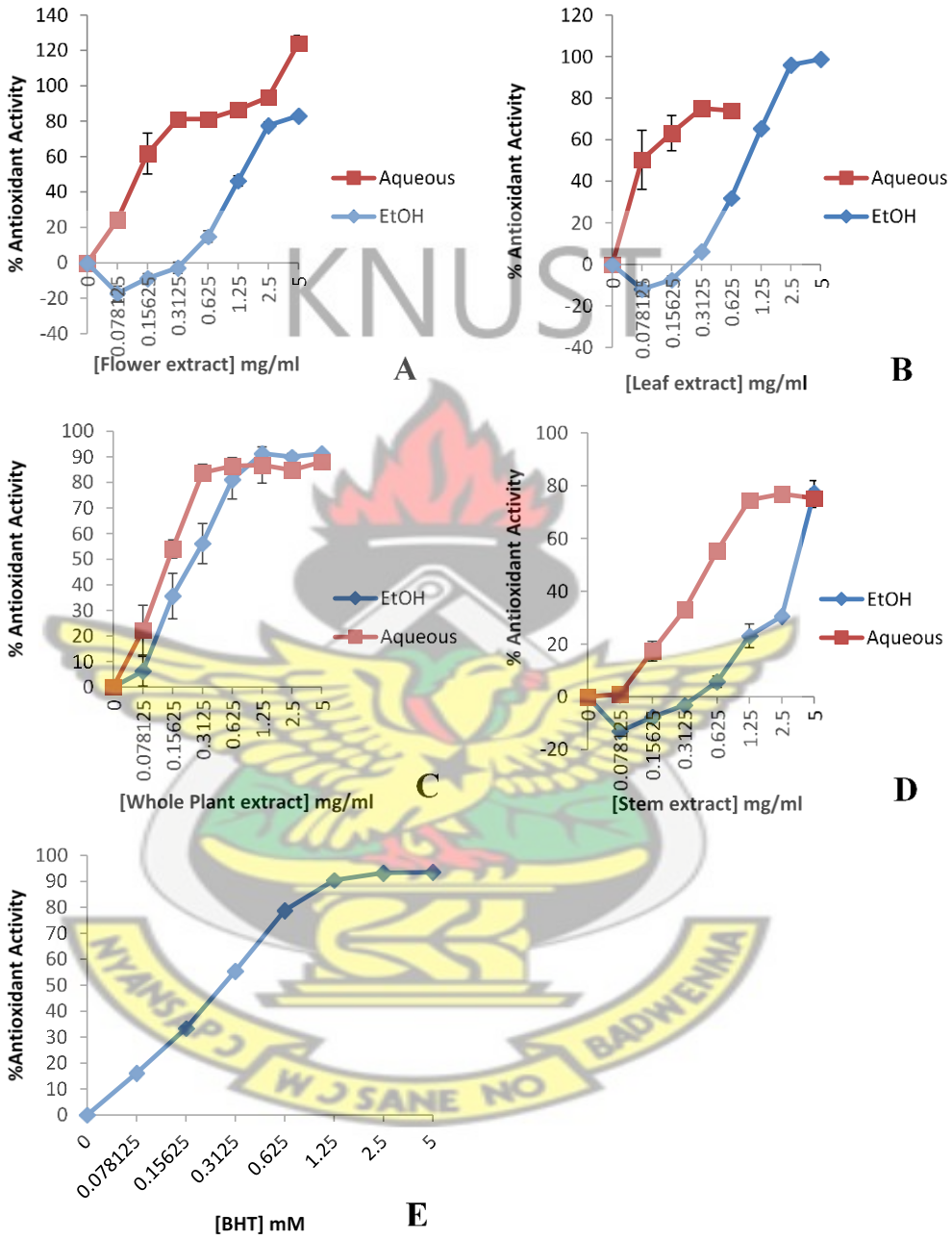


Figure 4-1 DPPH scavenging activity of 50% hydroethanolic and aqueous extracts of *A. conyzoides* flower (A), leaf (B), whole plant (C), stem (D) compared to butylated hydroxytoluene (standard compound) (E).

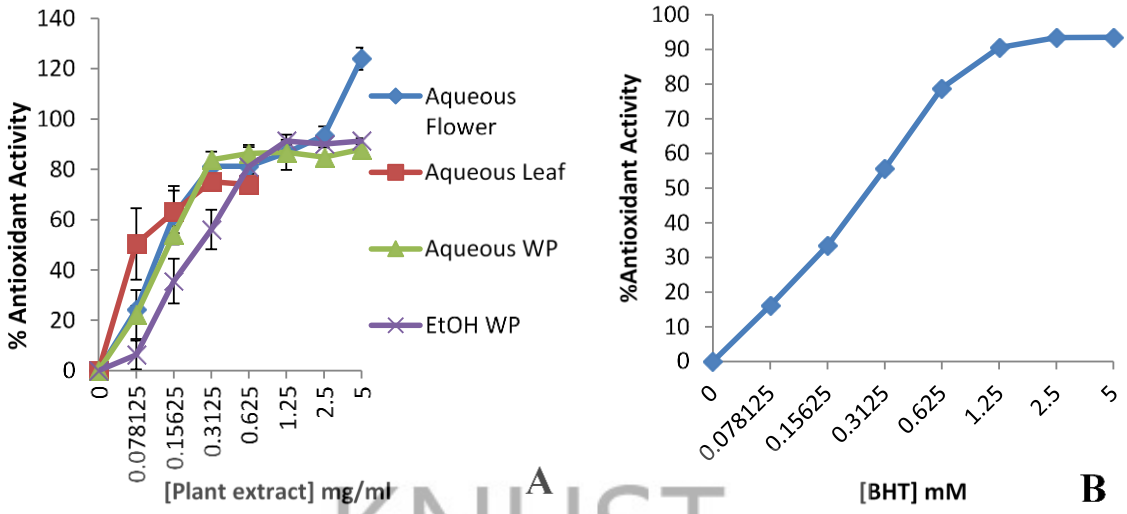


Figure 4-2 DPPH scavenging activity of hydroethanolic whole plant and aqueous extracts of *A. conyzoides* (flower, leaf, whole plant) (A) compared to butylated hydroxytoluene (standard compound) (B)

Table 4-1 Comparison of EC_{50} values of DPPH scavenging activities of aqueous and hydroethanolic extracts of *Ageratum conyzoides*

Sample/standard	EC_{50} values (mg/ml)				
	Flower	Stem	Leaves	Whole Plant	P-value
	n= 3	n= 3	n= 3	n= 3	
Aqueous	0.138 ± 0.018	0.592 ± 0.024	0.091 ± 0.024	0.163 ± 0.026	0.0003
Hydroethanolic	1.363 ± 0.082	3.552 ± 0.056	0.961 ± 0.012	0.262 ± 0.036	< 0.0001
BHT	0.060 ± 0.000				

Values are mean ± standard deviation; n=3 for each sample, (P = 0.003 or p < 0.0001 represent significant differences in DPPH scavenging activity of the different group of extracts compared to the positive control, BHT)

4.1.2 Total phenol content

The content of total phenols in the test extract was measured by Folin-Ciocalteau's reagent at an absorbance of 750 nm. Comparing the aqueous and ethanolic extracts, the total phenol contents did not differ significantly from each other with the aqueous leaf extract having the highest concentration (16.02 mg/ml) and the aqueous stem extract having the lowest concentration (4.08 mg/ml). In terms of gallic acid equivalent, the total phenol content in the aqueous leaf and stem extracts was found to be 1678.86 ± 40.67 and 407.59 ± 4.49, respectively, GAEs mg/g plant extract in crude extracts of *A. conyzoides* (Table 4-2).

Table 4-2 Total phenolic contents of aqueous and hydroethanolic extracts of *A. conyzoides*

Sample	Flower	Stem	Leaves	Whole Plant	P-value
	n= 3	n= 3	n= 3	n= 3	
Aqueous	1290.80 ± 36.34	407.59 ± 4.49	1678.86 ± 40.67	1197.14 ± 42.45	< 0.0001
Hydrothanolic	1388.18 ± 40.67	472.88 ± 19.52	1602.36 ± 36.68	1583.33 ±55.52	< 0.0001

Tabulated values are mean ± standard deviation; n=3 for each sample (P < 0.0003 represent significant differences in total phenol content among the different group of extracts).

4.1.3 Presence of glutathione

The presence of glutathione in the test extracts was determined by O-phthaldialdehyde reagent and fluorescence measured at a wavelength of 412 nm. Glutathione was not detected in any of the extracts tested.

4.1.4 In Vitro Cytotoxicity assessment

The cytotoxic effect of the crude extracts as well as fractions of ethanolic leaf extracts of *A. conyzoides* was measured on all four cell lines used in this study [three human cancer cell lines (Jurkat) leukemia, (LNCap) prostate, (MCF-7) breast and (PNT2) normal human prostate]. Curcumin was used as a positive control. Compared to the aqueous extracts, all the 50% hydroethanolic extracts, except the stem, exhibited strong cytotoxic effect on the cancer cell lines, with highest effect observed on human leukemia (Jurkat), and lowest effect on the breast cancer (MCF-7). Nonetheless, the effect of all the extracts was observed to follow a concentration response pattern compared to curcumin, the positive control (Figures 4-4 to 4-9).

The results as expressed by IC_{50} values (ug/ml) are presented in Table 4-3. Of the four 50% hydroethanolic crude extracts, the leaf extract was the most cytotoxic to the three cancer cell lines within the working concentration range tested. The highest and lowest activities were on Jurkat and MCF-7 cell lines, respectively. The hydroethanolic leaf extract gave IC_{50} values of 15.08 ± 0.28 ug/ml (Jurkat), 304.22 ± 71.54 ug/ml (LNCap) and 934.94 ± 105.91 ug/ml (MCF-7). The hydroethanolic flower extract was also cytotoxic against Jurkat and LNCap cell lines, recording IC_{50} values of 58.45 ± 8.32 ug/ml and 696.08 ± 49.02 ug/ml respectively. None of the extracts was cytotoxic against normal cell line (PNT2).

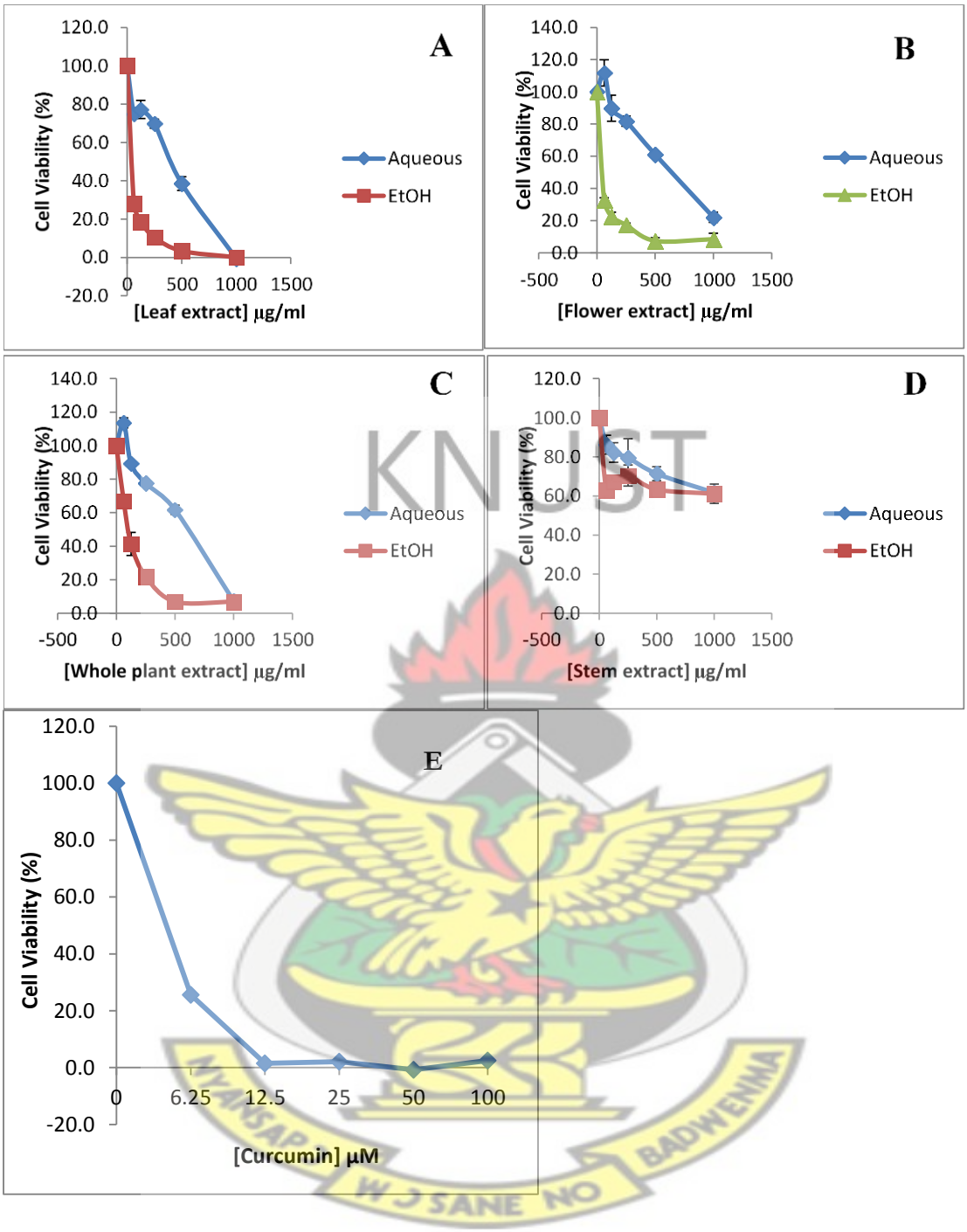


Figure 4-3 Cell viability curves showing cytotoxicity of aqueous and 50% hydroethanol extracts of *A. conyzoides* leaf (A), flower (B), whole plant (C), stem (D) and curcumin (E), against human leukemia (Jurkat) cell line

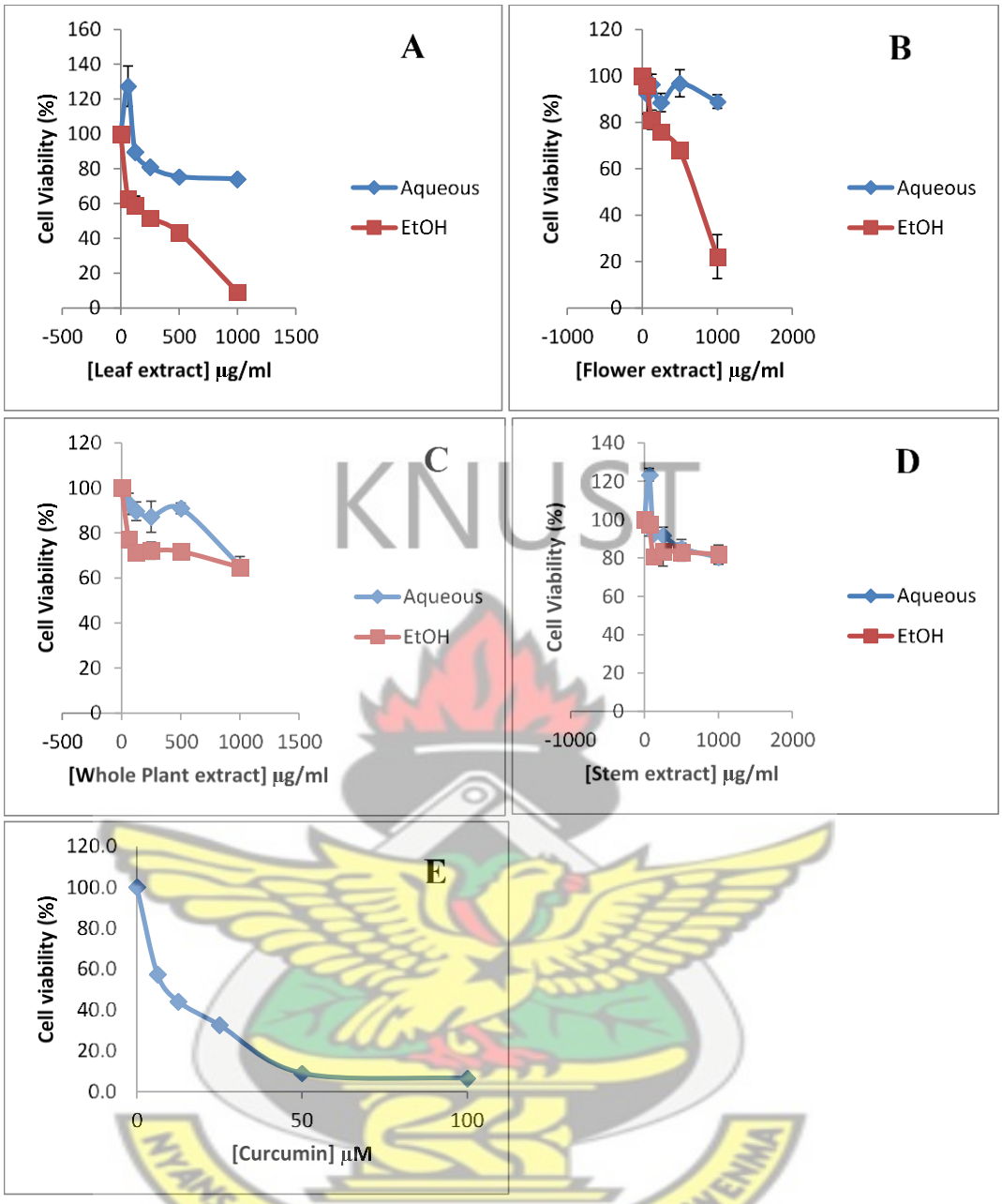


Figure 4-4 Cell viability curves showing cytotoxicity of aqueous and 50% hydroethanol extracts of *A. conyzoides* leaf (A), flower (B), whole plant (C), stem (D) and curcumin (E), against human prostate cancer (LNCap) cell line

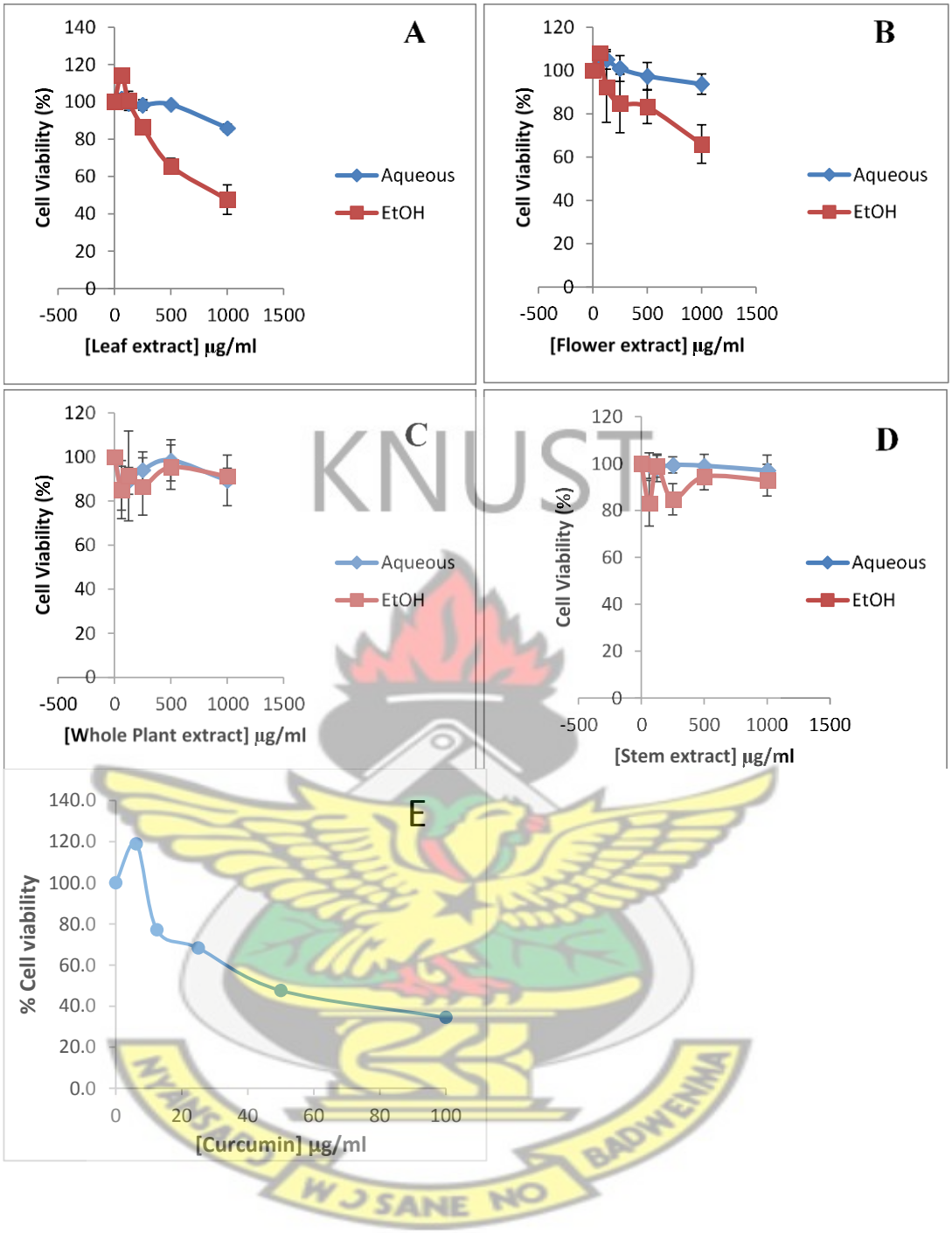


Figure 4-5 Cell viability curves showing cytotoxicity of aqueous and 50% hydroethanol extracts of *A. conyzoides* leaf (A), flower (B), whole plant (C), stem (D) and curcumin (E), against human breast cancer (MCF-7) cell line

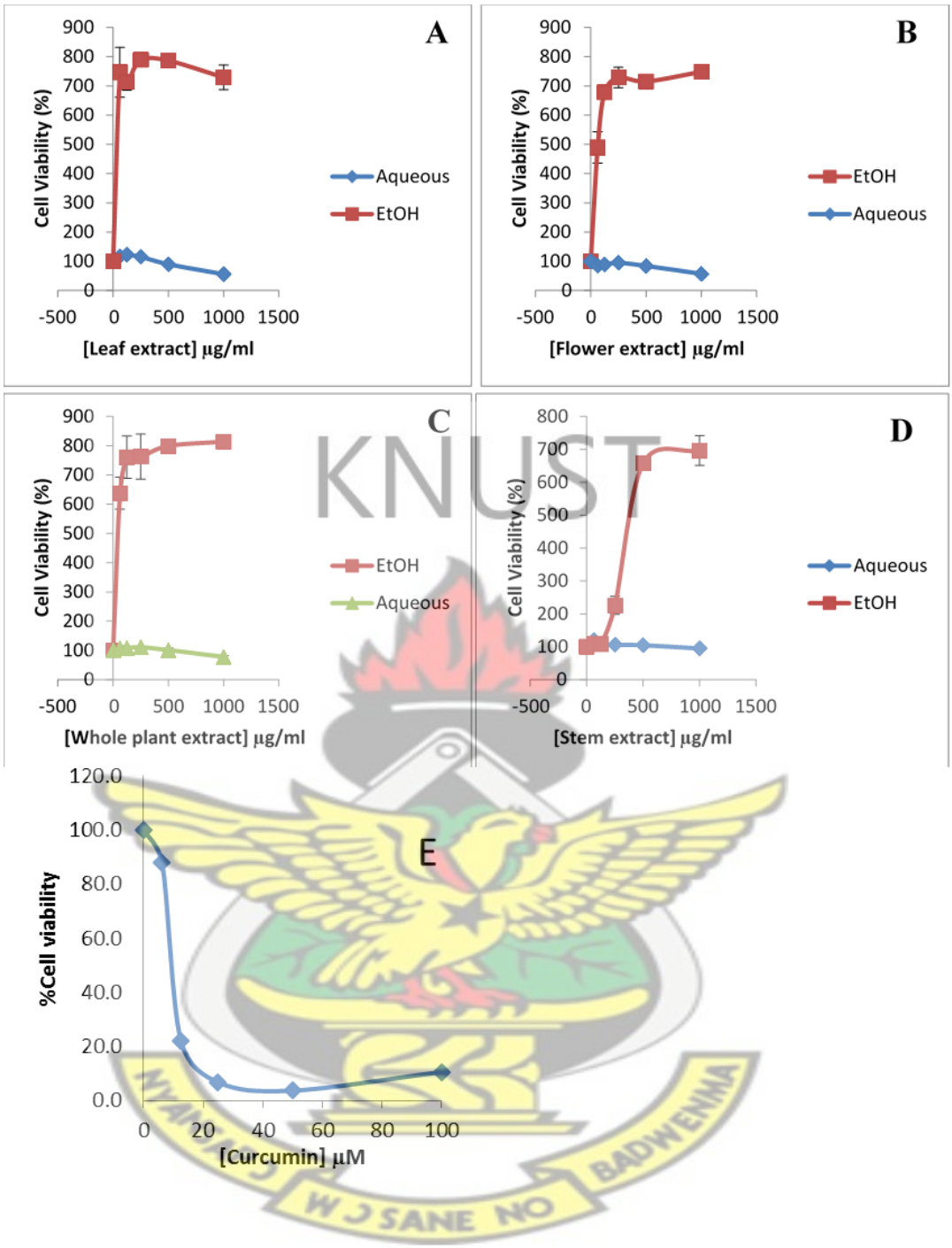


Figure 4-6 Cell viability curves showing cytotoxicity of aqueous and 50% hydroethanol extracts of *A. conyzoides* leaf (A), flower (B), whole plant (C), stem (D) and curcumin (E), against human prostate normal (PNT2) cell line

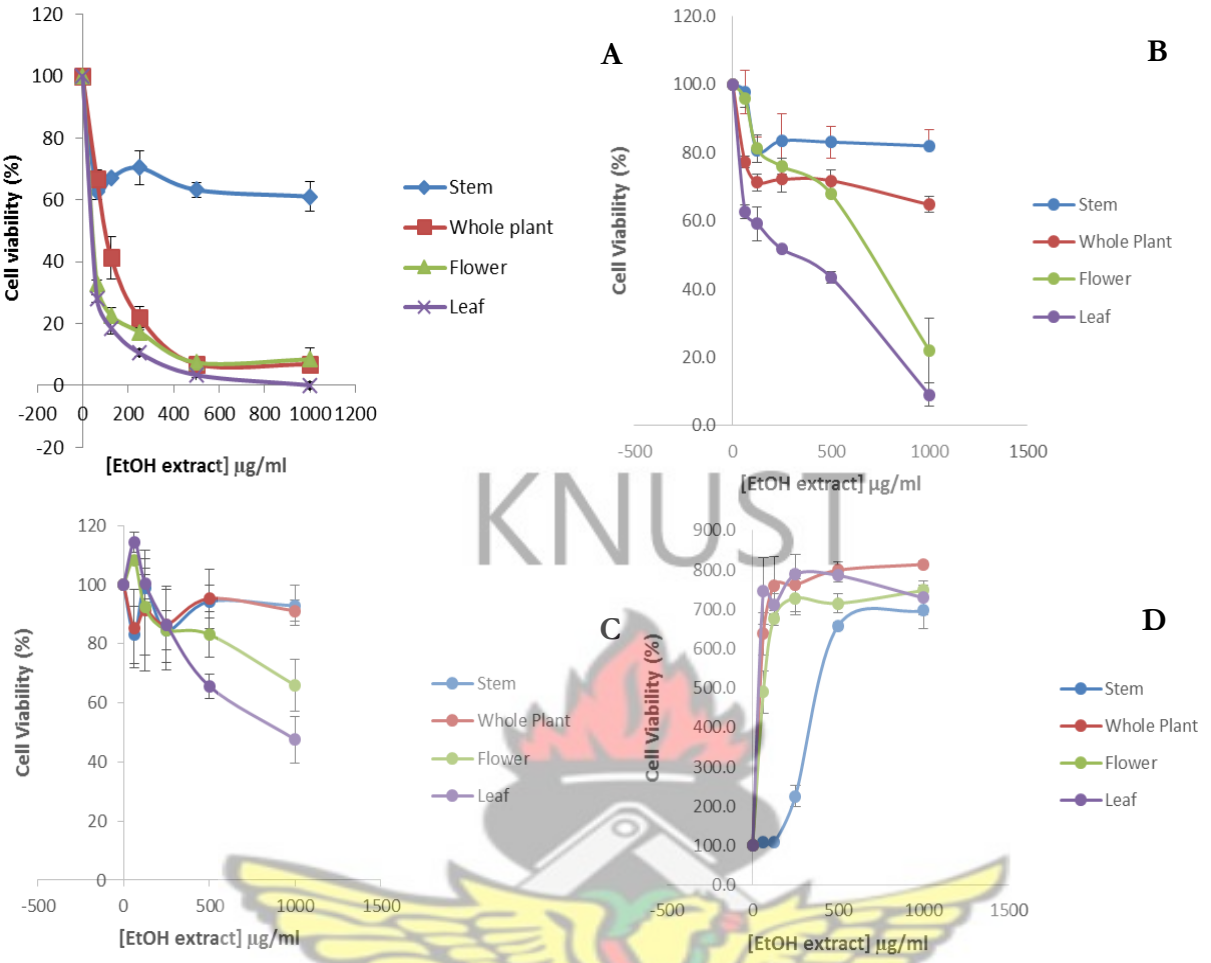


Figure 4-7 Cell viability curves showing cytotoxicity of the four 50% hydroethanol extracts of *A. conyzoides* (leaf, flower, whole plant and stem) against human leukemic (Jurkat) (A), human prostatic cancer(LNCap) (B), human breast cancer (MCF-7) (C) and human normal prostate (PNT2) (D).

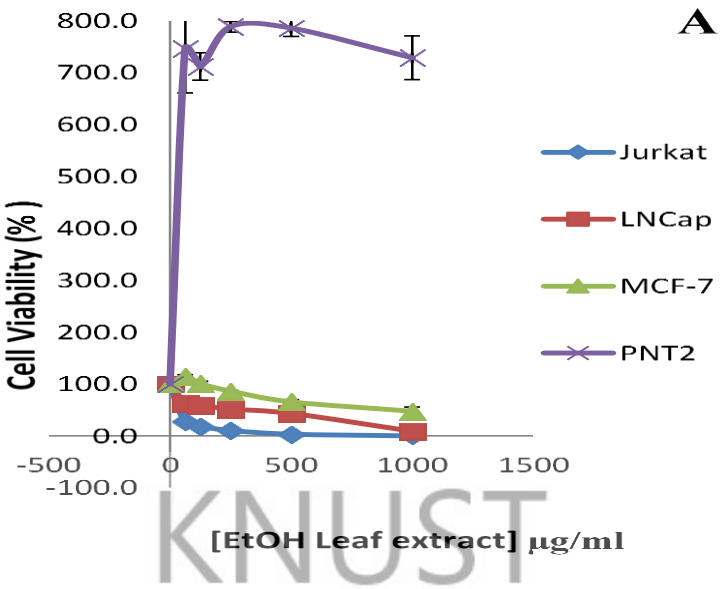


Figure 4-8 Cell viability curves showing cytotoxicity of human leukemia (Jurkat), human prostate cancer (LNCap), human breast cancer (MCF-7) and normal human prostate (PNT2) cells lines against 50% hydroethanolic extracts of *A. conyzoides* leaf (A).

Table 4-3 Cytotoxic activities (IC_{50} values) of aqueous and hydroethanolic extracts of *Ageratum conyzoides* tested on selected cell lines compared to curcumin.

Cell line	Sample	IC_{50} values (µg/ml)					IC_{50}Value(µM)
		Flower (n=3)	Stem (n=3)	Leaves (n=3)	Whole Plant (n=3)	P-value	Curcumin (n=3)
Jurkat	**Aqueous**	642.31 ± 19.84	>1000	408.15 ± 23.25	608.21 ± 17.96	< 0.0001	
	Hydroethanolic	58.45 ± 8.32	>1000	15.08 ± 0.28	103.92 ±14.93	< 0.0001	4.23 ± 0.31
LNCap	**Aqueous**	>1000	>1000	>1000	>1000	1.0000	
	Hydroethanolic	696.08 ± 49.02	>1000	304.22 ± 71.54	>1000	< 0.0001	9.73 ± 0.03
MCF-7	**Aqueous**	>1000	>1000	>1000	>1000	1.0000	
	Hydroethanolic	>1000	>1000	934.94 ± 105.91	>1000	0.1013	38.5 ± 0.76
PNT2	**Aqueous**	>1000	>1000	>1000	>1000	1.0000	
	Hydroethanolic	>1000	>1000	>1000	>1000	1.0000	9.85 ± 0.39

Values are means ± standard deviation of three replicates

The four fractions of the 50% hydroethanolic leaf extract were significantly cytotoxic with the exception of hydroethanolic fraction which did not show any activity against LNcap and MCF-7 cell lines within the working dose range of 0-100 ug/ml. Similarly, the petroleum ether fraction did not show any cytotoxic activity against MCF-7 cell line (Figures 4-10 and 4-11). The chloroform and ethylacetate fractions were the most cytotoxic against the three cancer cell lines (Table 4-4). IC_{50} values recorded for the chloroform fraction on respective cancer cell lines were 6.36 ± 1.56 ug/ml (Jurkat), 35.28 ± 6.46 ug/ml (LNCap) and 74.27 ± 8.15 ug/ml. Also, IC_{50} values recorded for the ethylacetate fraction on selected cancer cell lines were 4.6 ± 0.12 ug/ml (Jurkat), 37.28 ± 2.09 ug/ml (LNCap) and 67.38 ± 1.71 ug/ml (MCF-7). However, none of the fractions was cytotoxic against the PNT2 cell line.

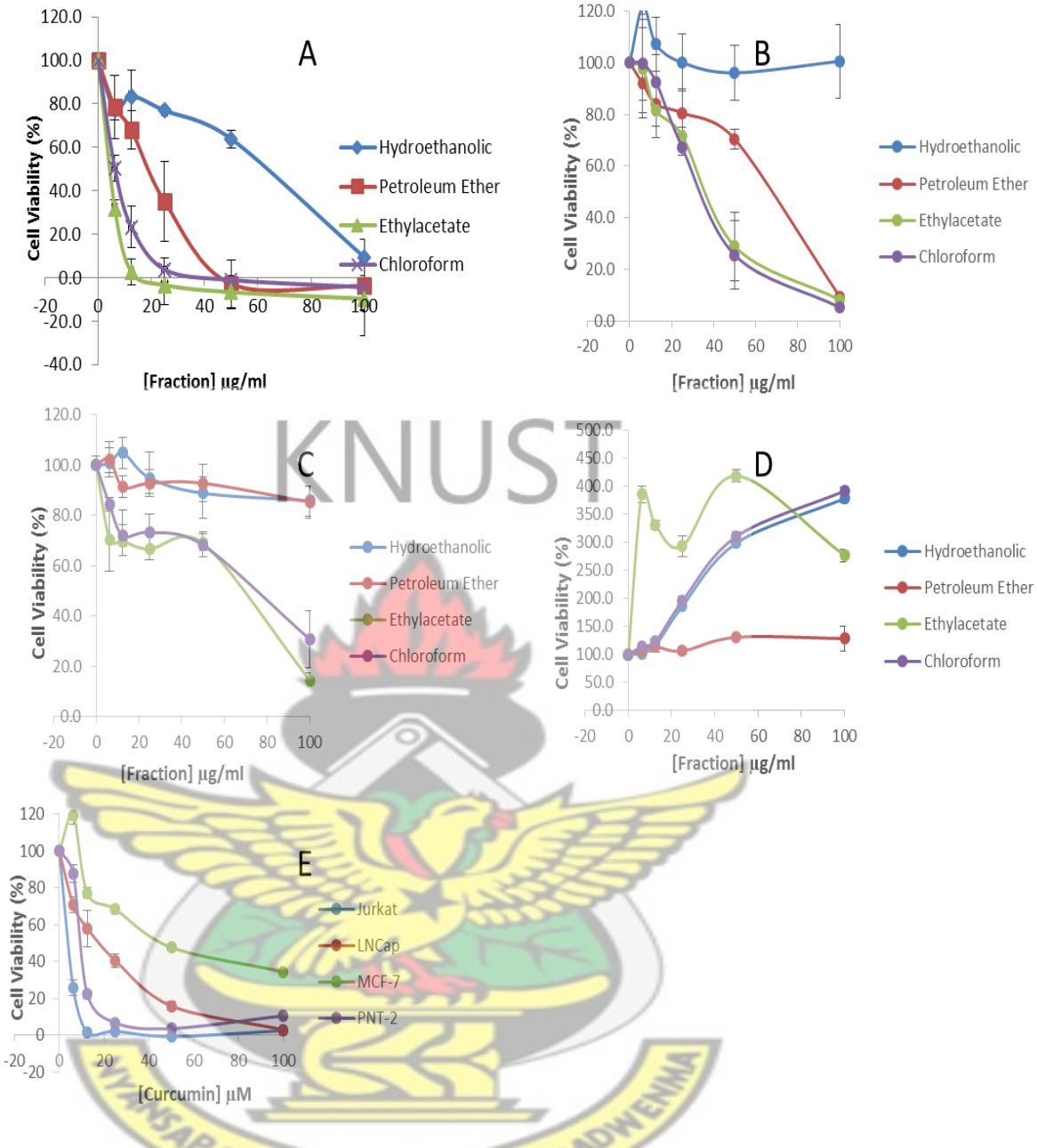


Figure 4-9 Cell viability curves showing cytotoxicity of fractions of 50% hydroethanolic leaf extract of *A. conyzoides* against human leukemic (Jurkat) (A), human prostatic cancer (LNCap) (B), human breast cancer (MCF-7) (C) and human prostate normal (PNT2) (D) cell lines compared to curcumin (E).

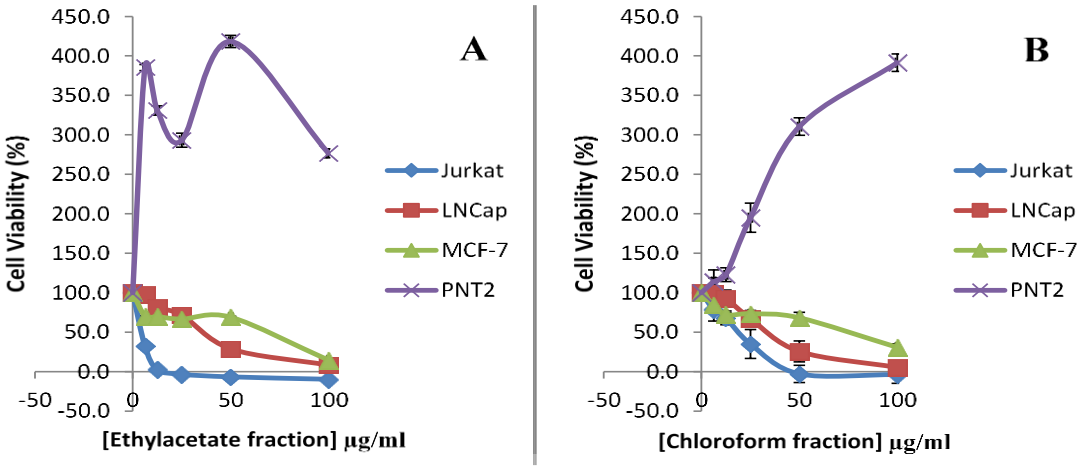


Figure 4-10 Cell viability curves showing cytotoxicity of human leukemia (Jurkat), human prostate cancer (LNCap), human breast cancer (MCF-7) and normal human prostate (PNT2) cells lines against fractions of 50% hydroethanolic leaf extract of *A. conyzoides*, ethylacetate (A), chloroform (B).

Table 4-4 Cytotoxic activity (IC_{50} values) of fractions of *Ageratum conyzoides* hydroethanolic leaf extract tested on respective cell lines compared to curcumin

Cell line	Sample	IC_{50} values (µg/ml)				P-value	IC_{50}Value(µM)
		Pet. Ether	Chloroform	Ethylacetate	Hydroethanolic		Curcumin
Jurkat	**Fraction**	19.32 ± 1.34	6.36 ± 1.56	4.6 ± 0.12	62.75 ± 7.05	< 0.0001	4.23 ± 0.31
LNCap	**Fraction**	66.74 ± 1.53	35.28 ± 6.46	37.28 ± 2.09	>100	< 0.0001	9.73 ± 0.03
MCF-7	**Fraction**	>100	74.27 ± 8.15	67.38 ± 1.71	>100	< 0.0001	38.5 ± 0.76
PNT2	**Fraction**	>100	>100	>100	>100	1.0000	9.85 ± 0.39

Values are means ± standard deviation of three replicates

4.1.5 Selectivity Indices

Selectivity of the cytotoxic activity of the extracts were determined by comparing the IC_{50} values of each extract against cancer cell lines (Jurkat, LNCap and MCF-7) with that of the normal human prostate (PNT2) cells. The results were expressed as a selectivity index (SI) (Table 4-5). The SI greater than 2 was considered highly selective. All the hydroethanolic extracts except the stem, and the aqueous leaf extracts showed greater selectivity against the Jurkat cell lines compared to the positive control, curcumin. The results presented as SI values can be found in table (4-5). Hydroethanolic leaf extract showed the highest selectivity, SI value of 66.33, whiles the aqueous leaf extract showed the lowest, SI value of 2.45. Also, only the hydroethanolic leaf extract showed good selectivity against LNCap cell line, recording an SI value of 3.29. None of the crude extracts had selective cytotoxicity against the MCF-7 cell line.

Table 4-5 Selectivity of aqueous and hydroethanolic extracts of *A. conyzoides* for selected cancer cell lines compared to curcumin.

Cell line	Sample	Flower	Stem	Leaves	Whole Plant	Curcumin
Jurkat	Aqueous	1.56	1.00	2.45	1.60	
	Hydrothanolic	17.11	1.00	66.33	9.60	2.33
LNCap	Aqueous	1.00	1.00	1.00	1.00	
	Hydrothanolic	1.44	1.00	3.29	1.00	1.01
MCF-7	Aqueous	1.00	1.00	1.00	1.00	
	Hydrothanolic	1.00	1.00	1.05	1.00	0.26

In addition, all fractions of the ethanolic leaf extract except hydroethanolic showed stronger selectivity for the Jurkat cell line compared to curcumin, positive control. The SI values recorded for petroleum ether, chloroform and ethylacetate were 5.18, 15.72 and 21.74, respectively, (Table 4-6). Only the ethylacetate and chloroform showed good selective ability against the LNCap cell line compared to curcumin. All fractions and positive control showed poor selectivity against the MCF-7 cell line.

Table 4-6 Selectivity of fractions of *Ageratum conyzoides* hydroethanolic leaf extract for selected cancer cell lines compared to curcumin.

Cell line	**Fractions**				
	Pet. Ether	**Chloroform**	**Ethylacetate**	**Hydroethanolic**	**Curcumin**
Jurkat	5.18	15.72	21.74	1.6	2.33
LNCap	1.50	2.83	2.65	1.00	1.01
MCF-7	1.00	1.35	1.48	1.00	0.26

4.2 QUALITATIVE PHYTOCHEMICAL SCREENING

Comparing the aqueous and 50% hydroethanolic extracts of the various plant parts, the presence of the various phytochemicals (terpenoids, alkaloids, flavonoids, tannins and saponins) did not differ from each other (Table 4-7). The various phytochemicals were found to be most abundant in the leaf and flower extracts of *Ageratum conyzoides*. However, the presence of flavonoids and alkaloids could not be detected in any of the extracts (Table 4-7).

Table 4-7 Phytochemical screening of aqueous and hydroethanolic extracts of *Ageratum conyzoides*

Phytochemical	Extracts	Leaf	Flower	Whole plant	Stem	Standard
Terpenoids (Urosolic acid)	**Aqueous**	+++	+++	++	+++	+++
	Ethanolic	+++	+++	++	+	+++
Alkaloids (Quinidine)	**Aqueous**	-	-	-	-	+++
	Ethanolic	-	-	-	-	+++
Tannins (Gallic Acid)	**Aqueous**	+++	+++	++	+	+++
	Ethanolic	+++	+	+++	+	+++
Saponins	**Aqueous**	+++	+++	+	++	
	Ethanolic	+	+++	+	+	
Flavonoids (Quercetin)	**Aqueous**	-	-	-	-	+++
	Ethanolic	-	-	-	-	+++

+++ Means present in high concentration; ++ Means present in moderate concentration; + Means present in low concentration;

– Means Absent

Compounds in parenthesis were standards used for the tests.

CHAPTER FIVE

DISCUSSIONS

5.1 ANTIOXIDANT ACTIVITY

Free radicals, particularly, reactive oxygen species are ubiquitously present in most aerobic environments as animals and plants alike require oxygen for energy metabolism. Nonetheless, excess generation of these free radicals could cause among other things the depletion of immune system antioxidants, damage to DNA and alteration of gene expression, and induction of abnormal proteins, processes which have been implicated in the genesis of many disorders in humans particularly cancer. Antioxidants can protect the body by preventing the formation of free radicals through interruption of free radicals attack, or by scavenging the reactive metabolites or converting them to less reactive molecules (Hegde and Joshi, 2009).

The DPPH test is the oldest indirect method for determining the antioxidant activity based on the ability of the stable free radical 2, 2-diphenyl-1-picrylhydrazyl to react with hydrogen donors including phenols. DPPH, an N centered radical, has a characteristic absorbance at 517 nm, which decreases with the scavenging of the proton radical (Jao and Ko, 2002). Another feature of antioxidants is the presence of total phenolic compounds. These include tannins, flavonoids, hydroxycinnamate and lignin. Total phenol content is measured by Folin-Ciocalteu method at an absorbance of 750 nm (Ghasemi *et al*., 2009). Moreover, glutathione (a tripeptide), as an antioxidant is responsible for neutralizing free radicals and maintaining other antioxidants in their reduced state. Glutathione content is measured by O-phthaldialdehyde (OPT) method which results in a characteristic fluorescence at 412 nm (Hedley and Chow, 1994).

Polyphenolic phytochemicals including flavonoids and phenols have been reported in *A. conyzoides* which has been suggested to contribute to its antioxidant activity (Kähkönen *et al.*, 1999). Phenolic compounds have been suggested to induce the cellular antioxidant system by approximately 50% cellular glutathione concentration increment. Flavonoids are significant in the modulation of γ-glutamylcysteine synthase in both cellular antioxidant defenses and detoxification of xenobiotics (Muchuweti *et al.*, 2007). A host of studies have reported a positive correlation between free radical scavenging activities and total phenolic content to various plant products. Studies conducted by Lu and Yeap Foo, (2000) and Siriwardhana *et al.*, (2003) for instance reported a high correlation between DPPH scavenging potential and total phenolic content. In another study, radical scavenging activity increased with increasing phenolic compound content (Oki *et al.*, 2002). Although such a relationship was not necessarily established, this study still observed that both the aqueous and hydroethanolic leaf, flower, stem, and whole plant extracts of *Ageratum conyzoides* exhibited antioxidant effects (that is, free radical scavenging on DPPH, and total phenolic content). Among the extracts, the aqueous leaf extract showed the strongest DPPH scavenging potential, and the weakest effect was observed for the hydroethanolic stem extract. The aqueous flower and whole plant as well as the ethanolic whole plant extracts also scavenged DPPH radical strongly.

The presence of phenolic compounds was also observed in all extracts, with the aqueous leaf extract recording the highest total phenolic content, 1678.86 ± 40.67mg/g plant extract (GAE) in crude extracts of *A. conyzoides* whiles the aqueous stem extract recorded the lowest total phenolic content. However, the presence of GSH could not be quantified in any of the extracts worked on. This suggests that the antioxidant activity of the *A. conyzoides* could be partly attributed to the presence of the various phenolic

compounds in the extracts which may act through their ability to adsorb, neutralize and quench free radicals. Adebayo and colleagues, (2010), reported that the antioxidant activity of *A. conyzoides* resided in ethylacetate extract and also suggested that the bioactive compound responsible for this effect was kaemferol (a flavonol), an isolate from the ethyl acetate extract. Also Nasrin, (2013) reported free DPPH radical scavenging activity of the methanolic extract of *A. conyzoides* stem with an EC_{50} value of 46.01 ± 2.23 µg/ml and total phenolic content of 38.125+2.01 mg/g equivalent of gallic acid. These studies also suggest that the presence of phenolic compounds may be partly responsible for the observed antioxidant activity seen in *A. conyzoides*. The result of the present investigation has shown that *A. conyzoides* possesses antioxidant activity and this property is predominantly found in the aqueous leaf extract.

5.2 ANTICANCER ACTIVITY

Cancer is one of the most widespread diseases in humans and remains a great public health burden in both the developed and developing world. Although cancer chemotherapy remains the standard mode of treatment widely used (Dantu *et al*., 2012; Uma *et al*., 2009), it is still fraught with several challenges, the most notable being the resistance of most tumours to anticancer agents, as well as the non-selective nature of these chemotherapeutic agents, hence affecting cancer and normal cells alike. As a result, there is considerable scientific and commercial interest in the continuous discovery of new anticancer agents from natural product sources (Balunas and Kinghorn, 2005; Fouche *et al.*, 2008). Over 50% of drugs used in clinical trials for cancer therapy have been isolated from natural sources or are related to them (Newman and Cragg, 2007). A number of active compounds, some from medicinal plant sources which include flavonoids, diterpenoids, triterpenoids, and alkaloids, have been shown to

possess anticancer activity (Han *et al.*, 2008). These are responsible for the cancer preventive effects of plants and also contribute to several mechanisms of action proposed which include inhibition of mutagenesis by inhibiting metabolism, inhibition of DNA adduct formation, free-radical scavenging, and effects on cell proliferation and tumour growth through apoptosis signalling (Shukla and Kalra, 2007).

This study aimed at evaluating the *in vitro* anticancer activity of a common Ghanaian weed, *Ageratum conyzoides*, using the aqueous as well as hydroethanolic extracts of the plant, and its leaves, flowers, and stem. The plant has been used in folklore medicine for the treatment of a variety of ailments, and it has been shown to contain some phytochemicals such as alkaloids, resins, saponins, tannins, glycosides and flavonoids (Kamboj and Saluja, 2008). To date, only studies by Adebayo *et al*., (2010), and Adetutu *et al*., (2012), have validated the *in vitro* anticancer activity using the ethanolic leaf extract as well as fractions of the plant. Consequently, the *in vitro* anticancer effect of the flower, stem and whole plant of *A. conyzoides* have not yet been published. Results from this study demonstrates that the hydroethanolic leaf extract, as well as its ethylacetate and chloroform fractions of *A. conyzoides* have a more pronounced cytotoxic effect on leukemia, prostate and breast cancer cell lines (Jurkat, LNCap and MCF-7) than the other crude extracts and fractions used. Some of the crude extracts used for the anticancer assay exhibited cytotoxic effect on the selected cancer cells lines in a concentration dependent pattern when compared to that of the positive control, curcumin. However, comparing the aqueous and hydroethanolic extracts, it was observed that the hydroethanol extracts significantly inhibited the growth of the cancer cell lines. This suggests that ethanolic extract contains higher concentration of the active anticancer compound(s) in *A. conyzoides*. Also, comparing the cytotoxic activity of the

various hydroethanolic extracts, that is, of the leaf, stem, flower and whole plant, on the respective cancer cell lines, it was observed that the leaf extract had the most pronounced cytotoxic effect whiles the stem extract had the least effect. On the various cell lines, the hydroethanolic leaf extract showed the strongest cytotoxic effect on Jurkat cells, and weaker effects on LNCap and MCF-7 and a rather weak effect was observed in the hydroethanolic stem extract. However, the hydroethanolic flower extract also showed good cytotoxic effect on the leukemia (Jurkat) cell line and a weak effect on prostate cancer (LNCap) cells but was not cytotoxic to breast cancer (MCF-7) cell line. This also suggests that more of the active anticancer compound(s) were present in the ethanolic leaf extract of *A. conyzoides*.

When the cytotoxic effects of fractions of ethanolic leaf extract on the three cancer cell lines were compared, it was observed that the ethylacetate and chloroform fractions showed more pronounced effect than the petroleum ether and hydroethanolic fractions even though all followed a concentration dependent pattern with increasing concentration compared to the positive control, curcumin. Also, it was observed that the petroleum ether extract showed strong cytotoxic effect on the human leukemic cell line (Jurkat), compared to the less cytotoxic hydroethanolic fraction, within a similar concentration range. Similar patterns of activities were observed for the human prostate (LNCap) and human breast (MCF-7) cancer cell lines (Table 4-4). Adebayo and colleagues, (2010), reported that the petroleum ether and ethylacetate extracts of ethanolic leaf extract of *A. conyzoides* showed inhibitory activity on a wide range of cancer cell lines with ethylacetate extract having the greatest activity. Also, Adetutu *et al*., (2012) reported that the same fractions and chloroform of *A. conyzoides* was cytotoxic to two lung carcinoma (SK-MES 1 and SK-LU 1) cell lines. The findings from

these study are in agreement with their work. The huge amount of phenolics and flavonoids present in *A. conyzoides* might be responsible for its promising cytotoxic activity (Moreira *et al.*, 2007; Okwori *et al.*, 2008). Adebayo *et al.*, (2010) have also suggested that flavonoids may be responsible for the anticancer activity of *A. conyzoides*. The observed cytotoxic effect of *A. conyzoides* in this study could therefore be attributed to the presence of some of these secondary metabolites.

Interestingly, the inhibition of proliferation of normal prostate cells (PNT2) by the crude extracts and fractions after 72 h of incubation was very weak (IC_{50} values > 1000 µg/ml). This indicates that the crude extracts and fractions were not cytotoxic to the normal cells. It is important for an anticancer agent to exhibit cytotoxicity but such activity should be specific for cancer cells only (Lai *et al.*, 2008). If selectivity index, (SI), is greater than 2 (Badisa *et al*., 2009), substances are considered to have significant therapeutic effects. The crude ethanolic leaf, flower and whole plant extracts, and the aqueous leaf extract had significant selectivity indices (SI ≥ 2). However, the SI for the ethanolic extracts, flower, leaves and whole plant, were about 7-, 28- and 5-fold respectively higher than curcumin. The ethylacetate fraction was the most selective towards Jurkat cells followed by the chloroform and petroleum ether fractions with SI values about 9-, 7- and 2-fold higher than the positive control, curcumin. However, only the hydroethanolic leaf extract as well as chloroform and ethylacetate fractions were selective towards the LNCap cell line. This is in agreement with an earlier report by Adetutu *et al*., (2012), in which the crude ethanolic extracts of *A. conyzoides* and its petroleum ether, chloroform and ethyl acetate fractions did not show much inhibition to the skin fibroblast (FS5) cell line. None of the crude extracts and fractions was selective against the MCF-7 cell line. High selectivity in cytotoxic effect between cancer cell and normal cell lines increase the

prospects that this plant contain compound(s) which could serve as leads for novel anticancer drugs. It can be inferred from this study that, the leukemic cell line (Jurkat) is the most susceptible to *A. conyzoides* whiles the human breast cancer cell line is the most resistant to the plant.

This study has shown that *A. conyzoides* possess anticancer activity, and this property may reside in the chloroform and ethylacetate fractions of the hydroethanolic leaf extracts.

5.3 PHYTOCHEMICAL SCREENING

Phytochemicals act in many ways to assist the body in fighting against diseases and health problems. They are thought to combine with some biomolecules to neutralize activity of scavenging free radicals before they can cause damage within the body (Okaka and Okaka, 2001). In the present study phytochemical screening (Table 4-7) revealed the presence of tannins, saponins, and terpenoids but not alkaloids and flavonoids. Amadi *et al*., (2012), have suggested that pure isolated alkaloids and their derivatives from plant sources have been found to possess analgesic and bactericidal activities.

Okwu, (2005) has also reported on the ability of flavonoid to scavenge hydroxyl radicals, superoxide anions and lipid peroxy radicals. Tannins have astringent properties, and have been reported to hasten the healing of wounds and inflamed mucous membrane (Okwu and Ekeke, 2003). Saponin isolates have been shown to specifically inhibit the growth of cancer cells *in vitro* (De Marino *et al.*, 1998) and to have antioxidant properties (Yoshiki *et al*., 1998). Saponins may also exhibit cytotoxic effect and the growth

inhibition against a variety of cells making them have anti-inflammatory and anticancer properties (Amadi *et al.*, 2012). Terpenoids are known to fight malaria and cancer (Rice-Evan *et al*., 1995). The observed antioxidant and anticancer activities in this study could partly be attributed to the presence of tannins, saponins and terpenoids as well as phenolic compounds.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

Ageratum conyzoides possess many biological properties including antioxidant and anticancer properties. The aqueous leaf extract may contain the active principles that mediate the antioxidant properties of this plant as confirmed by this study. The anticancer properties of this plant have also been shown in the ethanolic and aqueous leaf, flower and whole plant extracts except that of the stem. This property appears to reside in the crude ethanolic leaf extract in particular, as well as the chloroform and ethylacetate fractions where the anticancer activities were maximal. Data from the present study suggest that the crude ethanolic leaf extract as well as fractions of *A. conyzoides* are highly selective towards the two cancer cell lines (Jurkat and LNCap) but not MCF-7 cell line, as compared to normal prostate cells (PNT2).These activities could be partly attributed to the presence of phytochemicals, tannins, saponins and terpenoids. Therefore, the plant could be considered useful in ethno-medicine for management of cancerous and oxidative stress related diseases. Further work is needed to unravel the active principles responsible for the anticancer and antioxidant properties and also ascertain the mechanism of actions of the active components.

6.2 RECOMMENDATIONS FOR FURTHER WORK

The crude ethanolic and aqueous extracts should be fractionated and the fractions be used to screen for antioxidant activity. The active principles responsible for the anticancer activities should be isolated from the various fractions and characterized.

Since apoptosis is a preferred mechanism for anticancer activity, effect of the active components on mechanisms that characterize apoptosis must be investigated using the cell lines tested. These include molecular mechanisms such as DNA fragmentation, morphological changes (chromatin condensation), cell cycle analysis, mitochondria membrane potential changes, detection of apoptotic caspases (3, 8, and 9), PARP cleavage, heat shock proteins 8 and 9 detection.

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