CHAPTER ONE

1.0 INTRODUCTION

1.1 BACKGROUND

Africa's rich vegetation and diverse wildlife is unparalleled to any other on the globe. It is God's special gift to this continent and He has given man the freedom to utilise this gift in such a way that will serve and support his survival (as stated in Genesis 1:29 – 31 of the Holy Bible). Indeed every plant on the planet has the potential and capability of serving one purpose or another. Plants generally are used for food, beautification and medicine among others. The only limitation arises from the fact that man has not fully exploited these plants and this calls for the need to undertake research into these plants especially since the components of these organisms can serve the purpose of healing one disease or another (Ezekiel 47:12, Holy Bible). Plants are nature's gift to mankind in terms of providing us with food, oxygen, as well as shelter. Since time immemorial, they have served as the first line of defence used by our forefathers to fight diseases. Most orthodox drugs administered today were derived from plants (Ncube *et al*., 2008). For instance, the plant *Euphobia peplus* contains a compound called ingenol mebutate (Picato) which is used to treat skin cancer (Zarchi and Jemec, 2015). The common drug quinine was also derived from the bark of *Cinchona officinalis*, and this drug is widely prescribed for the treatment of malaria in countries that cannot afford the more expensive anti-malaria drugs (Reyburn *et al*., 2009). *Papaver somniferum*, a common plant has given rise to four important medicines namely, morphine, codeine, noscapine and papaverine. Morphine and codeine serve as painkillers with codeine additionally used as a remedy against cough. Noscapine is used against cough while papaverine was originally a remedy primarily used against vasopasm and more recently but occasionally used in the treatment of erectile dysfunction (Samuelson, 2004).

However, some types of plants have been under-utilized. Some examples are the ornamentals, primarily grown for beautification. Some, however, have no apparent use to mankind, as such are destroyed when they appear above the ground.

Among the various disease conditions that affect humans, cancers have proven to be one of the commonest and deadliest diseases that affect any individual irrespective of race or age (WHO, 2004). Cancer is a term used to describe a large group of diseases characterized by the uncontrolled proliferation and spread of abnormal cells (Hayflick, 1997). These cells spread and colonize other parts of the body via the lymphatic system or bloodstream. Its incidence and mortality has risen tremendously over the past decade causing the need for effective control measures (Ferlay *et al*., 2013). Recent statistics by the International Agency for Research on Cancer (IARC) indicates a changing trend in recorded incidence and mortality of which less developed countries now record the highest cases (Ferlay *et al*., 2013). This emerging trend could be attributed to the change in lifestyle of individuals from this part of the globe. The IARC thus calls for the need for protocols and measures that will ultimately curb this menace (Ferlay *et al*., 2013). Though there are several treatment options available, each comes with a high price tag often coupled with adverse side effects. Hence there is the need to exploit other remedies with possibly less known adverse effects and from readily accessible sources like plants. Plants could serve as a major source of bioactive compounds with potential efficacy against cancers (Talalay and Fahey, 2001).

1.2 PROBLEM STATEMENT

Several drugs like methotrexate, paclitaxel and fluorouracil have been extensively used in treating cancer (Martin *et al*., 2009; Ychou *et al*., 2011; FDA approved drugs for oncology, 2015). Most of the known drugs, although expensive, have been

reported to have adverse side effects on patients (Gill *et al*., 2006). These may range from loss of hair, nausea, fatigue, fever, low leukocyte count, to even death (Gill *et al*., 2006).

The high cost of treatment makes it impossible for effective treatment of cancer patients from less developed countries. Also, the problem of cancer cells becoming insensitive to chemotherapeutic agents and the acquisition of drug resistance through the expression of drug resistance genes is now a recent dilemma in curbing cancer (Shervington and Lu, 2008).

Considering the high cost of treatment coupled with the evolving paradigm of drug resistance to chemotherapeutic agents and the adverse side effects of efficient drugs, alternative drug interventions from readily accessible sources like plants need to be exploited (Awosika, 1993). Therefore, the aim of this research is to exploit the ornamental plant (*Codiaeum variegatum*) and weed (*Crotalaria retusa*) to assess their efficacy in the treatment of leukaemia, prostate, liver and breast cancers.

1.3 OBJECTIVES

1.3.1 General Objective

The main objective of this study was to evaluate the anti-proliferative potential of *Codiaeum variegatum* and *Crotalaria retusa* on leukaemia, prostate, liver and breast cancer cell lines.

1.3.2 Specific Objectives

This work was specifically to

1. Evaluate qualitatively, the phytochemical constituents of the hydroethanolic extracts of *Codiaeum variegatum* and *Crotalaria retusa* parts;
2. Evaluate the free radical scavenging activity of crude hydroethanolic extracts;
3. Evaluate the total phenolic content of the crude hydroethanolic extracts;

4. Evaluate the anti-proliferative activity of the 50% hydroethanolic extracts of the various parts of both plants on cancer and normal human cells;
5. Evaluate the anti-proliferative activity of fractions of the most active crude extracts;
6. Investigate the molecular process involved in cytotoxicity induction of the most active crude extract.

1.4 JUSTIFICATION

Ghana's data collection on cancer cases is rather inadequate. The first cancer registry, the Kumasi Cancer Registry, established in 2012 covers cancer cases in Kumasi. However, this proportionally (considering the whole country) rather small data presented by this registry suggests Ghana has a high incidence rate of 11.9 per 100,000 with about 70% of all cases detected in the late stages. Liver, prostate and head cancers topped the list of all cases in men while breast, cervical and liver cancers were the lead cancers in females (Laryea *et al*., 2014). Similar studies conducted by Wiredu and Armah (2006) reviewed all cancer related deaths occurring at the Korle-Bu Teaching Hospital, Accra, Ghana during a 10 year period and this revealed that breast cancer recorded the highest number of cancer deaths in females while liver cancer caused the highest mortalities in males from 1991 to 2000.

It is thus becoming imperative to obtain better drug interventions that will be more efficacious and at the same time have less known adverse side effects and be affordable to individuals diagnosed with this condition. This will also provide alternative chemotherapeutic agents that will address the problem of drug resistance of cancers.

From ethnomedicine, decoction of the stem bark of *C. variegatum* was and is still being used in the successful treatment of psoriasis (a chronic skin condition that

causes skin cells to grow too quickly) in the Fiji Islands (Cambie and Ash, 1994). The cytotoxicity of this part of the plant on cells has not been established scientifically and this study attempts to investigate this.

The seeds of *C. retusa* have been shown to contain a pyrrolizidine alkaloid called monocrotaline which is hepatotoxic (Nuhu *et al*., 2000). This compound has been suggested to have a potential for killing tumours since it is capable of killing lung cells (Schoental and Head, 1955). However, very little is known of its antiproliferative activity on cancerous cells.

Assessing the anticancer potential of these locally available plants brings us one step closer into discovering an alternative source of chemotherapy against cancer. This research could provide valuable leads to anticancer agents which will most likely have lesser adverse side effects and also could potentially be very affordable to Ghanaians living with the cancers investigated.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 CANCER

Cancer is a term used to describe a large group of diseases characterized by the uncontrolled proliferation and spread of abnormal cells. These cells spread and colonize other parts of the body via the lymphatic system or bloodstream (Goldsby *et al*., 2003). Generally all cancers begin with the damage or alteration of the DNA of a cell, thus transforming these normal cells into cancerous cells, with the loss of its ability to control its own division and the acquisition of some level of immortality. Though DNA alterations occur naturally in cells, most of these alterations are usually not harmful to the cell. However, the presence and effect of some exogenous factors (like tobacco and UV light in the case of lung and skin cancer) tend to increase the rate of DNA damage, thus predisposing the cell to becoming cancerous (Knudson, 2001; Croce, 2008).

2.1.1 Global Incidence of Cancer

Across the globe, the incidence as well as mortality of cancer continues to be a menace. In 2012, 14.1 million new cases were recorded compared to 12.7 million new cases recorded in 2008, indicating an 11% rise in incidence (Ferlay *et al*., 2013). A 7.8% increase in mortality has also been recorded since 2008 (Ferlay *et al*., 2013). In both scenarios more than half the total numbers were recorded in less developed countries. This changing trend is confirmatory of the emerging western lifestyles been adopted by individuals in less developed countries. Report by Blecher and Ross (2013) indicate that the smoking of tobacco (a major risk factor to cancer) will

increase from 6% to 14% in Africa by 2060 suggesting a gradual change in lifestyle among people in less developed countries. If this situation is left unattended, the incidence of cancer will rise to 19.3 million by 2025. Cancer global incidence and mortality could possibly be higher than was recorded by Ferlay *et al*. (2013). This is due to the fact that most countries have poor data collection systems on cancer and some countries even do not have registries to record diagnosed cancer cases. For instance, in 1966, the International Agency for Research on Cancer (IARC) and International Association of Cancer Registries' (IACR) publication of global cancer incidence from 1960 to 1962 covered thirty-two registries across twenty nine countries. The available global data on cancer has since then increased greatly and this is evident in the most recent publication which covers two hundred and ninety registries across sixty eight countries of which fourteen were African countries. Yet, this recent and most wide spread data ever collected covered only 14% of the world's population (Africa's data covered only 2% of its population). Ghana, specifically has never contributed to this global incidence recordings (Forman *et al*., 2014). This goes to prove that cancer incidence is probably underestimated.

There are several different types of cancers depending on the part of the body affected. Most cancers involve the cells that line the interior and exterior surfaces of organs. These are called carcinomas. Cancers of the lymphatic system affect the lymph nodes and lymph tissues (Dupler and Odle, 2005). These are called the lymphomas among which the Hodgkin and Non-Hodgkin lymphomas are most common. Leukaemia affect the blood cells most of which is recorded among children. A less known but rather fatal type of cancers are the sarcomas. These affect the mesodermal tissues which involve the bones, muscles and cartilages (Dupler and

Odle, 2005). This research specifically focuses on the breast, liver and prostate carcinomas as well as the blood cancer.

2.2 BREAST

The breast is a tissue that covers the pectoral muscles of the chest. It is found in all primates and contains the mammary glands which are responsible for the production of milk. It is composed of glandular, connective and fatty tissues which vary in amount among women (Drake *et al*., 2005). Its size largely depends on the amount of fat it contains and its firmness depends on the fat to connective tissue ratio. Women develop fairly larger breast than men and this covers the area from just below the clavicle to the armpit and extends across to the sternum (Grassley, 2002).

Each breast contains about 15 to 20 lobes that are made up of microscopic units called lobules which produce milk (Drake *et al*., 2005).. Each lobule is drained by 15 to 20 lactiferous ducts that converge and open at the nipple. The areola is the dark circular area around the nipple that contains tiny sweat glands which secrete moisture to lubricate the nipple during breast feeding. The predominant fatty tissue surrounds and protects the lobules and ducts and also gives the breast its conical shape (Drake *et al*., 2005).

2.2.1 Human breast conditions

The human breast plays very important roles in an individual's life, especially in females where it produces milk to help feed young animals. There are several breast conditions that are likely to develop through a mammal's life and such conditions hinder the normal function of the breast. These conditions can occur in either males or females but is most predominant in females partly due to the fluctuations in the sex hormones oestrogen and progesterone.

The breast tissue can be infected most commonly by *Staphylococcus aureus* and this could lead to inflammation and a condition called mastitis (Riordan and Nichols, 1990). Mastitis usually occurs during breast feeding, and affects about 10% of breastfeeding mothers (World Health Organization, 2000). Nonetheless, it can also affect non-breastfeeding individuals. Another condition that could also affect the physiology of the breast is the accumulation of calcium ions in the breast tissue. These ions can either be large in size (macrocalcification) or small in size (microcalcification) and usually detectable by means of a mammogram. Though calcium ions gets deposited in the breast, research has shown that the development of the condition does not depend on the amount of calcium in the diet but rather on past injury, inflammation or aging (US Department of Health and Human Services, 2013). It occurs in about half of women aged above 50 years and 10% of women under 50 years of age (US Department of Health and Human Services, 2013). Other breast conditions include fat necrosis, breast fibroadenoma, intraductal papilloma, gyneacomastia and the most deadly breast cancer.

2.2.2 Breast cancer

Breast cancer is one of the most common neoplasm and accounts for 25% of all cancers in females (Tavassoli and Devilee, 2003). Its aetiology involves multiple numbers of factors ranging from diet, genetics, reproductive health and associated hormonal imbalances. In general, research has revealed that it is most common in affluent societies, accounting for 26% of cancers diagnosed in females in such high economic areas (Parkin *et al*., 2001), hence much more prevalent in North America, Northern Europe and Australia. Such countries record 70 to 90 new cases per 100, 000 population each year (Parkin *et al*., 1997). Other low and middle income

countries which have become industrialized and adopting western lifestyles have increase in incidence and mortality (American Institute for Cancer Research, 1997).

The reproductive health of a woman can also pose some risk factors to developing breast cancer later in life. Women who remain nulliparous or even parous but have their deliveries after the age of thirty tend to be at a higher risk of breast cancer (Wohlfahrt and Melbye, 2001). Aside from the reproductive health of a woman, certain environmental factors like the consumption of alcohol, red and processed meat and the intake of oral contraceptives have all been associated with an increased breast cancer risk (Smith-Warner *et al*., 1998; Department of Health, 1998; IARC, 1999). Considering all these risk factors associated with this condition, it seems almost difficult for women to protect themselves from breast cancer, however, research has shown that just by increasing physical activity can reduce such risks by 20 to 40% irrespective of a woman's menopausal status (IARC, 2002).

The prognosis of the disease is very good if detected at an early stage since significant improvements in survival have been recorded in the Western countries since the late 1970s (Adami *et al*., 1989; Chu *et al*., 1996). However when it is detected at a late stage, chances of survival reduces to a minimum. The density of a breast has been identified as a major contributor to breast cancer mortality since it is the main reason why mammograms miss 20% of breast cancers, hence preventing their early detection (US Department of Health and Human Services, 2013). Having a dense breast is not abnormal since it depends on the relative percentage composition of available fatty tissue to the percentage composition of fibrous and glandular tissues. Women with very dense breast are usually diagnosed with breast cancer at a late stage due to the fact that mammograms hardly show cancers in dense

breasts, thus reducing their odds of survival. This is mostly common in young women since breast density decreases with age.

2.3 LIVER

During the ancient times, the liver was considered as the guardianship of the soul and sentiments because of its size and close association with the blood. The liver, among other organs, is one of the most important organs in the human body (Saladin, 2003). It is found in most vertebrates and in humans, it can be located in the upper right quadrant of the abdomen, just beneath the diaphragm. It is the largest organ and gland in the human, weighing between 1.43 to 1.67 kg in an adult. Its reddish brown colour is due to its great vascularity (Saladin, 2003).

Generally the liver carries out many roles in carbohydrate metabolism, lipid metabolism, digestion of fats, regulation of blood pressure, protein metabolism and most importantly detoxification of xenobiotics (Greenstein and Greenstein, 1996). Specifically, it carries out glycogenolysis, gluconeogenesis, glycogenesis, lipogenesis, cholesterol synthesis, albumin synthesis, angiotensinogen synthesis and the production of some important coagulation factors like fibrinogen among others. The liver also synthesizes essential amino acids through transamination reactions; it further synthesizes most of the blood plasma proteins (α and β globulins, prothrombin and several other clotting factors). However, it does not synthesize plasma enzymes, peptide hormones or γ globulins (Greenstein and Greenstein, 1996). The liver is the only organ in the human body that is capable of regenerating its lost tissues naturally. Nonetheless, when it is completely damaged it has to be replaced since no single man-made machine has been invented yet that can carry out all its

functions. It is thus imperative to restrain from factors like alcohol, viruses and certain drugs like excessive intake of paracetamol that pose great threats to the normal anatomy and physiology of the liver.

2.3.1 Liver Cancer

The aetiology of liver cancer still remains a mystery, however, most diagnosed cases are associated with damage or scarring of the liver (cirrhosis). As such any condition that contributes to cirrhosis has been cited as a major risk factor to liver cancer. Cancer of the liver can either originate from other organs elsewhere in the body and migrate to the liver or can originate from the liver itself. The latter is normally known as primary liver cancer while the former is called secondary liver cancer. Primary liver cancer is the sixth most common cancer and in 2012, it recorded 746,000 deaths making it the second highest cause of cancer deaths globally (Ferlay *et al*., 2013).

Malignant tumours can form in both mature and immature hepatocytes, bile duct and blood vessels within the liver. Hepatocellular carcinoma is a cancer formed by matured hepatocytes and it is the most commonly recorded primary liver cancer in both males and females (Parkin *et al*., 1999). However, there exists a vast variation in geographical incidence usually due to the different degree of exposure to its risk factors. Countries with high cases of smoking and alcohol abuse record high cases of hepatocellular carcinoma. Exposure to hepatitis B and C virus as well as aflatoxin can also increase a person's risk to hepatocellular carcinoma (Peers *et al*., 1987; Qiao *et al*., 1988, Chuang *et al*., 2009)). Hepatitis B and C viral infections are the most studied viral factors in hepatocellular carcinoma and it accounts for 80% of all hepatocellular carcinoma cases (Rosen, 2011; Arzumanyan *et al*., 2013). Research

has shown that the viruses are able to alter gene methylation thereby affecting gene expression and promoting or repressing cellular signal transduction pathways causing the cells to avoid apoptosis (Kanai *et al*., 1996). Aflatoxin B1 specifically cause hepatocellular carcinoma by causing a genetic mutation in the p53 gene resulting in the non-expression of this tumour suppressor gene (Bressac *et al*., 1991). When such tumours develop in immature hepatocytes, they are referred to as hepatoblastoma. Although this is a rare form of primary liver cancer, it mostly develops in children, accounting for about 1% of all cancers in children. Up to 90% of hepatoblastoma has a male predominance ratio of 1.5:1 to 2:1 with no racial or ethnic preference (Tanimura *et al*., 1998). The chances of a child developing this condition have been shown to be very high in infants with birth weights less than 1.5 kg.

Other forms of liver cancer include cholangiocarcinoma, intrahepatic cholangiocarcinoma, bile duct cystadenocarcinoma and the rare combined hepatocellular and cholangiocarcinoma.

2.4 PROSTATE

Found only in males, the prostate is a walnut-sized exocrine gland that is located just beneath the urinary bladder and in front of the rectum (Leissner and Tisell, 1979). It forms part of the male reproductive system of most mammals but there exist considerable differences in its anatomy and physiology among species. The main function of the prostate gland is to secrete a milky, slightly alkaline fluid that nourishes and protects sperm (Risbridger and Taylor, 2006). Its activity is regulated by dihydrotestosterone which has testosterone as its precursor molecule (Risbridger and Taylor, 2006). Pathologically, the prostate can become infected by bacteria

leading to a condition called prostatitis (Anderson *et al*., 2006). In older men it can become enlarged causing great pain during urination, a condition called benign prostatic hypertrophy. The cells of the prostate gland can also lose its ability to control its division and apoptosis and this eventually leads to prostate cancer, one of the most common forms of cancer in males (Risbridger and Taylor, 2006).

2.4.1 Prostate Cancer

Prostate cancer is one of the most common forms of cancer in males. Currently, data available suggests that it is the sixth most common cancer in the world (with respect to the number of new cases recorded) (Parkin, 2001). In Ghana, research revealed that prostate cancer was the second most common cause of cancer death from 1991 to 2000 among males (Wiredu and Armah, 2006). Its high prevalence rate is due to the fact that many men are able to survive long enough after diagnosis; with an estimated 1.5 million survivors with the condition for five years as at 2000 (Eble *et al*., 2004).

The risk of prostate cancer increases steeply with age, with about 75% of all cases occurring in men aged 65 years or above (Cook *et al*., 1969). Geographically, it presents with a distribution bias within different populations around the world. It has a low incidence in Asian and North African populations as compared to Australian, the Scandinavian countries, North and South America and Sub-Saharan Africa, thus suggesting a genetic risk factor among populations (Brawley, 1997). Its mortality rates also follow a similar geographical distribution with the U.S.A having a 26-fold increase in mortality relative to China (Brawley, 1997). Aside from age as a major risk factor, the environment also contributes to the development of this cancer since migrants from less prevalent regions of the world living in high prevalent regions

tend to become very susceptible to the condition (Bouchardy *et al*., 1991). However, despite extensive research on prostate cancer, its environmental risk factors are not well understood. Few studies have revealed a strong positive association of the condition with red meat intake and a not too convincing protective effect of fruits and vegetables against prostate cancer (Kolonel, 1996).

2.5 *CROTALARIA RETUSA* L.

Crotalaria retusa is a typical plant primarily categorised as a weed. On 1st May 1753, Carl von Linnaeus published the first paper on *C. retusa*, describing its morphology and proposing a classification system for the plant (Linnaeus, 1753). It is a legume that has the capability of accumulating monocrotaline, an important toxicant with a wide degree of toxicity in animals (World Health Organization, 1988). When cattle, chicken and bees feed on monocrotaline containing plants, they pass this alkaloid into milk, egg and honey. This is mostly crucial in the case of honey in which high levels (up to 1 mg/kg) can build up over time, causing a risk to those who consume large amounts of it. However monocrotaline does not build up in body tissues and thus cannot be found in meat. Due to the risks to humans who consume these animal products, the World Health Organization (1988) has called for extensive research into such plants that contain this alkaloid to assess their risks as well as usefulness to life.

2.5.1 Classification

Native to Africa, the weed *C. retusa* L., commonly called the devil bean or rattle box, is one of the numerous numbers of weeds in the Fabaceae family. The Fabaceae family is the third largest family of organisms on the planet just behind Asteraceae and Orchidaceae (Judd *et al*., 2002). It comprises of approximately 19,400 species

and 730 genera accounting for the great morphology and chemical diversity, thus its vast use and importance to mankind (Waterman, 1994). The largest genera in Fabaceae are Astragalus (over 2400 species), Acacia (over 950 species), Indigofera (around 700 species), Crotalaria (around 700 species) and Mimosa (around 500 species) which contains about 9.4% of all flowering plant species (Magallón and Sanderson, 2001). All the members of this family have the capability of fixing atmospheric nitrogen for protein synthesis hence have very high protein content of about 20 to 40% dry weight in the seeds and leaves depending on the species (Simpson and Ogorzaly, 1986).

C. retusa belongs to the genus Crotalaria which comprises of about 550 to 600 species worldwide, at least 500 of which have been identified to have originated from Africa (Polhill, 1982). Crotalaria belongs to the subfamily Faboideae which basically refers to rattlepods. The name Crotalaria was derived from the Greek meaning "castanet" and has the same root as the name for rattle snakes, *Crotalus*. This is because when the pods are fully matured, they turn dark-brown to black whiles the seeds in the pod become loose, causing it to make rattle sounds when shaken, hence its name the rattle box (Polhill, 1982).

Table 2.1 Taxonomic classification of *Crotalaria retusa*

Kingdom	Plantae
Phylum	Magnoliophyta
Class	Magnoliopsida
Order	Fabales
Family	Fabaceae
Genus	Crotalaria
Species	retusa

C. retusa is commonly known to the people of China as “diao qun cao”, and to the people of Venezuela as “maraquita” (Steyermark and Huber, 1978).

2.5.2 Morphology

C. retusa is a dicot, has an erect, ridged stem with short appressed hairs and capable of growing to a height of 1.3 m (Duffa, 1995). It bears long, hairless, greenish, cylindrical pods (about 3 to 4 cm long) which contain its seeds (approximately 23 seeds per pod).

The seeds are smooth and brown in colour and measure up to 4.5 mm in length. Its leaves are simple, 3.3- 9.2 cm long, 1- 3.8 cm wide with rounded (occasionally pointed) blunt tips. The flowers of *Crotalaria retusa* are 1.7 to 2.4 cm long with yellow coloured petals which bear fine purple lines near the base (Duffa, 1995). It flourishes and develops its yellow coloured flowers between August to September.

Figure 2.1: *Crotalaria retusa* L.

2.5.3 Cultivation

It is propagated by its seeds. Like all other legumes, this annual herb grows very well in fresh to moist sand-containing soils with preference for sunny atmospheric conditions, though it can tolerate temperatures down to -7 ^{o}C.

Figure 2.2: Seeds of *Crotalaria retusa* L.

2.5.4 Chemical Constituents

Phytochemical studies performed so far on the devil bean suggest that, its seed contain about 4.96% of the pyrrolizidine alkaloid, monocrotaline (Nuhu *et al*., 2000). Monocrotaline is an important antifeedant that protects the plant from being eaten by livestock since it is highly hepatotoxic (Maia *et al*., 2013). Clinical signs exhibited in goat showed acute centrilobular necrosis of the liver after dosing the animals with *C. retusa* for 10 to 11 days (Maia *et al*., 2013). The extent of damage to the organs was not limited to only the liver since significant toxic features were found in the lungs, kidneys and intestines when this experiment was conducted in Wistar rats (Nuhu *et*

al., 2000). In 1955, Schoental and Head published a paper which confirmed the hepatotoxicity of this alkaloid in rat (Schoental and Head, 1955).

Other phytochemicals like monocrotaline-N-oxide, necine, retronecine, retusamine, retusamine-N-oxide, retusin, isatinecine and indican have all been isolated from various parts of the plant.

2.5.5 Traditional Uses

Several members of the Crotalaria genus have been extensively used in the treatment of malaria, dysentery, black-water fever, anthrax, dropsy and chronic cough (Watt and Breyer-Brandwijk, 1932).

In Asian traditional medicine, decoction of the leaves and flowers were used to soothe cold (Oliver, 1959). They were and are still reported to be eaten as vegetables (Oliver, 1959). The leaves alone could also be mixed with those of *Crotalaria quinquefolia* and administered internally or externally against fever, scabies, lung diseases and impetigo (Nuhu *et al*., 2009). Studies conducted by Nuhu *et al*., (2009) revealed that the plant was not unpopular among traditional medicine practitioners in Zaria, Northern Nigeria. The flowers were squashed and potash added to it and cooked in a soup for drinking to remedy amenorrhea in women (Nuhu *et al*., 2009). In other instances, the powdered plant could be mixed with roasted black caraway and taken in small quantities by natives who suffered from stomach colic and flatulence (Nuhu *et al*., 2009). These traditional healers reportedly utilized decoction of the whole plant in alleviating cardiac problems. The ethno-medicinal uses of this plant cannot be over emphasized. The research further revealed that natives from Zaria used it to effectively drive away snakes by simply sprinkling the seeds in a

flame to produce smoke and even employed it in the making of love enchantments by reciting 'Qur'anic' verses on them (Nuhu *et al*., 2009).

Though the saponification value of the oil derived from the seeds renders it non-edible, whole seeds of the plant are roasted and eaten by natives in Vietnam. The oil however can be used in making hair shampoos, skin creams, paints and shoe polishes (Umerie *et al*., 2010). The oil also has a fairly high acid number value (10.36 mgKOH/g) indicating that very little purification is required to prolong its shelf-life. In ethno-medicine, these antispasmodic seeds were used for the treatment of fever and as a vermifuge for expelling parasitic worms from the body (Oliver, 1959). The seeds were also eaten raw to act as an analgesic to reduce the excruciating pain of a scorpion sting. This could be attributed to the fact that, the most abundant mineral in the seed is magnesium (50.82 mg/100g) which primarily helps in maintaining electrical potentials in nerves (Aremu *et al*., 2012).

2.5.6 Agricultural Uses

Being a legume, it is capable of fixing nitrogen into the soil using *Rhizobium* in its root nodules. In agriculture, it is grown by farmers as cover crops for controlling nematodes on farms (Silva *et al*., 1989). They can also be used in green manuring and are seldom used as ornamentals. In an attempt to increase okro yield, researchers from the University of Cape Coast, Ghana, used petroleum ether extract of *Crotalaria retusa* L. to successfully control the flea beetle, *Podagrica uniformis* L. which feed on the leaves of okro plant (Wie-Addo *et al*., 2010).

C. retusa L. serves as a host plant for the larvae of some butterfly species including *Endoclita sericeus*, *Etiella zinckenella* and *Utetheisa ornatrix*. They are used as food

by the larvae of these Lepidoptera species which incorporate the toxic alkaloids and utilize it in securing their defenses against predators (Eisner, 2003).

The uses of the devil- bean are not limited to agriculture and medicine only but encompass even biotechnology where it has been used in remediating soils contaminated with crude oil (Osam *et al*., 2011).

Crotalaria retusa L. is one of the few legumes that have been abandoned, as such is gradually becoming an unwanted plant on farms. Considering the numerous benefits of this plant outlined in this review, this plant could be promising in helping to improve health, agriculture and biotechnology.

2.6 *CODIAEUM VARIEGATUM* (L.) A. JUSS

2.6.1 Morphology

Codiaeum variegatum L. belongs to the family Euphorbiaceae and its native to India, Philippines, Sri Lanka, Thailand, Indonesia, Malaysia and some other Pacific Islands (Stamps and Osborne, 2003). It is a common perennial plant that grows very well in the tropics. It is an ornamental capable of growing to about 600 cm but usually maintained around 60 cm to 90 cm (Stamps and Osborne, 2003). It is commonly called garden croton or Joseph's coat due to its beautiful variegated leaves which are often either leathery or shiny and vary greatly in shape and colour. The blade length of its leaves often measures between 4 to 8 inches. The colours identified with the leaves of croton ranges from yellow, crimson, scarlet, brown, cream, red, orange, purple, pink, indigo, violet to white (Stamps and Osborne, 2003). The leaves are typically green with bands, streaks, patches or blotches of any one or more of the colours listed above (Stamps and Osborne, 2003). These various structural features

produce different variations in plant appearance thus yielding a wide range of varieties. There are over 200 varieties worldwide some of which include the Sunny Star, Petra, Gold Dust, Mona Lisa, Broad Spotted Guinea, Curly Boy, Frank Brown, Eleanor Roosevelt, Sunray, Punctatum, and Mrs Iceton among others (Ogunwenmo *et al*., 2007).

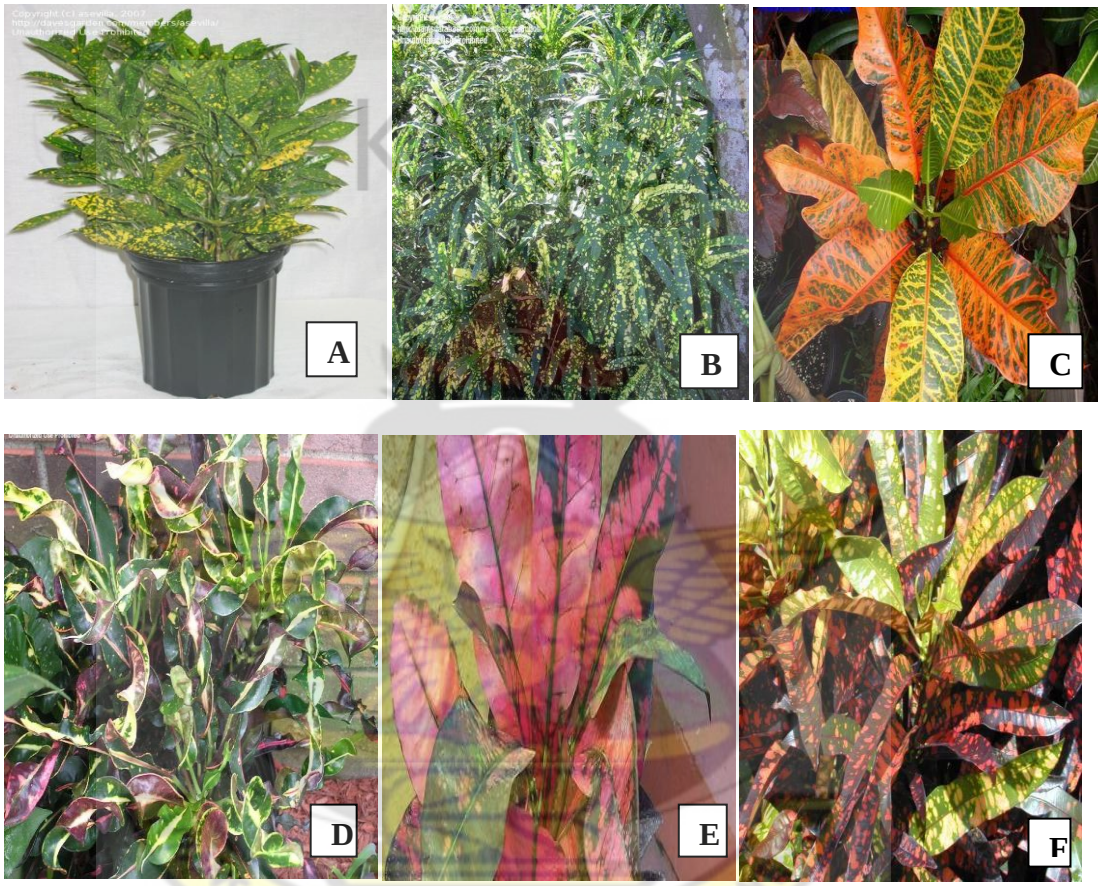


Figure 2.3: Common varieties of *Codiaeum variegatum*; Gold Dust (A), Eleanor Roosevelt (B), Frank Brown (C), Curly Boy (D), Mrs Iceton (E) and Franklin Roosevelt (F).

Codiaeum variegatum develops dry brownish globular fruits, 9 mm in diameter, within which its seeds can be found. Its axillary flowers are small in size and long in shape and usually unisexual racemes (Esan *et al*., 2005). *C. variegatum* is known as "pokok pudding" in Malaysia; "kohson" in Thailand; "kalipayang" in the Philippines,

"ku kieng" in Vietnam; "biang ye mu" in China; "henyo-boku" in Japan and "sacasaca" in the Fiji islands (Buttner, 2001; Seidemann, 2005).

2.6.2 Classification

C. variegatum belongs to the family Euphorbiaceae which is a large family of flowering plants comprising of about 500 genera which are native to tropical Africa and the Indo-Malaysian region. There are over 5000 species of plants in this family with most of these species serving as treatment options for asthma, leukemia, and other forms of cancer and also used as a laxative and diuretic across the globe (Tohme and Tohme, 2007).

Table 2.2: Taxonomic classification of *Codiaeum variegatum*

Kingdom	Plantae
Division	Magnoliophyta
Class	Magnoliopsida
Order	Malpighiales
Family	Euphorbiaceae
Genus	Codiaeum
Species	variegatum

2.6.3 Cultivation

C. variegatum is propagated by means of stem cuttings, specifically cutting about 8 to 15 cm of the apex of the stem. Although the seeds can also be used as a means of propagation, this yields less stable offsprings that are often unidentical to the parent plant. As such propagation by stem cuttings is the most reliable and efficient way of sustaining a desired variety. When the stem cuttings are obtained they start to develop roots within 4 weeks provided they are kept at a temperature between 21 to 27 ^{0}C (Stamps and Osborne, 2003). However if this temperature is altered below this

range, rooting might take a much longer time. Prior to planting, the gardener should ensure that the soil is well aerated to allow proper movement of air and water and the temperature kept above 16 ^{O}C (Huxley, 1992).

This traditional means of propagating *C. variegatum* though effective is very slow and often unable to meet its high demands especially in shorter periods of time (Nasib *et al*., 2008). However a more convenient and fast method of propagation can be achieved *in vitro* using micropropagation. Micropropagation is a modern technique which is been used to improve yield of plants (Nasib *et al*., 2008). In this method, freshly grown shoot tips with at least 2 nodes is selected as the explant source and cultured in Murashige and Skoog media (Murashige and Skoog, 1962). The media is supplemented with BAP (6-benzyl amino purine) or IBA (indole-3-butyric acid) for shoot and root induction respectively. To further increase the yield, peptone and malt extracts can be added as adjuvants at 25 mg/L each (Nasib *et al*., 2008). The plants are then transferred to the green house after roots have developed.

C. variegatum blossoms beautifully and displays its diverse leaf colours when grown outside in warm environments. It however defoliates when kept at low temperatures. The 'Joseph's coat' is one of the few ornamentals that have a very high rate of transpiration, hence regular irrigation should be ensured. Nonetheless, too much irrigation can also lead to oedema which often manifests as blisters around the leaves (Huxley, 1992). This plant is a very resilient plant and capable of resisting insect and pest attack (Eileen and Turner, 2001). Unlike its leaves, very few insects attack its hard woody stem.

2.6.4 Uses

Garden croton produces a lot of secondary metabolites including but not limited to flavonoids, terpenes and alkaloids. Methanolic extracts of *C. variegatum* cv. *spirale* has been cited to yield five flavonoids; apigenin, vitexin, isovitexin, orientin and vicenin-2 alongside other phenolic acids known as caffeic and *p*-coumaric acids (Hassan *et al*., 2014). These phytochemicals are responsible for its pharmacological properties and hence its varied uses in the field of medicine. A cyanoglucoside identified as 2-(3,4,5)-trihydoxy-6-hydroxymethyltetrahydropyran-2-yloxymethylacrylonitrile isolated from its chromatographic fraction has also been found to display virucidal activity against influenza A virus without impairment of haemagglutination properties (Forero, 2008).

Aside from its main purpose in serving as an ornamental, the root decoction of *C. variegatum* is used to treat gastric ulcers. Bronson (2005) reported the presence of eczema on some gardeners as well as burns of the mouth after repeated exposure to the latex of its bark and root. All of its exudates have also been reported to cause skin irritation even though they may serve as purgative in both humans and domestic animals (Bronson, 2005). The leaves of croton have antibacterial and antiamoebic properties and can be crushed and drunk to cure diarrhoea (Moundipa *et al*., 2005). In indigenous Malaysian medicine, the plant is used as an anti-infective and an anti-cancer agent (Ali *et al*., 1996). Research conducted by Hassan *et al*. (2013) exposed the cytotoxicity effect of *C. variegatum* CV. *petra* leaves on human caucasian breast adenocarcinoma (MCF7), hepatocellular carcinoma (HepG2), colon cell line (HCT116) and lung carcinoma cell line (A549) with activities ranging from 17.3% to 98%. However, there is very little scientific evidence to support the anti-proliferative activity of this plant established by Hassan *et al*. (2013).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 CHEMICALS, REAGENTS AND CANCER CELL LINES

All solvents used for extraction such as ethanol, petroleum ether, chloroform and ethylacetate were of analytical grade and obtained from Sigma-Aldrich Company (Missouri, USA). Rose Park Memorial Institute (RPMI)-1640 and Dulbecco Modified Eagle's culture Media (DMEM), Fetal Bovine Serum (FBS), penicillin streptomycin L-glutamine (PSG), curcumin, 2,2- diphenyl-1-picryl hydrazyl free radical reagent (DPPH), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye, dimethyl sulphoxide (DMSO), butylated hydroxyl toluene (BHT), gallic acid, Folin-Ciocalteau reagent, sodium carbonate solution, phosphate buffered saline (PBS), proteinase K enzyme, lysis buffer, DNA loading buffer, DNA marker (ladder)-1kb, tris-acetate-EDTA (TAE) buffer, ethidium bromide, trypan blue, RNase enzyme and Agarose were purchased from Sigma-Aldrich Company (Missouri, USA).

Human leukaemia (Jurkat), human prostate carcinoma (PC3), human breast carcinoma (MCF7), human liver carcinoma (HepG2) and normal human liver (WRL68) cell lines which had being passaged for at most seven times were obtained from the Clinical Pathology Department, Noguchi Memorial Institute for Medical Research (NMIMR) where the *in vitro* studies were performed.

3.2 PLANT MATERIAL

C. retusa L. samples were handpicked from the Tech Credit Union Building environs (6°40'13.7"N 1°34'28.2"W), on the KNUST campus while *C. variegatum* cv. *gold dust* samples were also handpicked from the environs of the New Times Corporation

(5°34'10.5"N 0°13'20.5"W), North Industrial Area, Accra. Both samples were collected in the morning before 9:00 am in April, 2014. Specimens of both plants were sent to the Department of Herbal Medicine, Faculty of Pharmacy and Pharmaceutical Sciences, KNUST, Kumasi for authentication by a taxonomist and voucher specimens were deposited at the Herbarium for reference purpose. The voucher specimen numbers KNUST/HMI/2014/L092 and KNUST/HMI/2014/L094 were assigned to the *C. retusa* and *C. variegatum* specimens respectively. Preparation of *C. retusa* plant samples was done by sorting them into pods, flowers, leaves, seeds and stem while *C. variegatum* was sorted into stem bark and leaves. The stem bark and stem components were chopped into pieces. All the seven different components were washed separately with water three times and air dried at room temperature for three weeks. 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.

3.3 HYDROETHANOLIC (50%) EXTRACTION

3.3.1 *Crotalaria retusa*

A mass of 100 g of the resulting powder of leaves was macerated with 1000 mL of 50% ethanol while shaking on an Orbital shaker (Gallenkamp, England) for 24 h. The supernatant was filtered by centrifugation (Homef LC-30 centrifuge, LH Wagneningen-04065, Netherlands) for 20 minutes at a speed of 3000 rpm in 50 mL centrifuge tubes at room temperature. The extraction was repeated with a similar volume of 50% ethanol. All the supernatants of the two centrifugations were pooled together and concentrated using a rotary evaporator (Buchi Rotavapor R-205, Switzerland) at 50 ^{o}C The remaining aqueous extract was frozen at -20 ^{o}C. The

frozen samples were lyophilized for 72 hours using a vacuum freeze dryer (YK-118, Taiwan), at the ZIP Unit, Crop Research Institute, Fumesua.

For the flower and pod samples, a mass of 25 g of the resulting pulverized powder was macerated with 250 mL of 50% ethanol and the above extraction procedures were followed.

For the seed and stem samples, 16 g and 80 g of the resulting pulverized powder was weighed out and macerated with 160 mL and 800 mL of 50% ethanol, respectively and the above extraction procedures were followed.

3.3.2 *Codiaeum variegatum* cv. *gold dust*

For the leaves and stem bark samples, a mass of 135 g and 25 g of the resulting pulverized powder was weighed out and macerated with 1350 mL and 250 mL of 50% ethanol, respectively and the above extraction procedures were followed.

3.4 PHYTOCHEMICAL EVALUATION OF THE CRUDE EXTRACT

Phytochemical tests were performed on the hydroethanolic extracts using standard methods described by Harborne (1998), Trease and Evans (1989), and Sofowora (1993).

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

3.4.1 Terpenoids

A volume of 1 mL of chloroform was added to 10 mg of each extract and standard, urosolic acid and 1 mL of concentrated sulphuric acid (H_2SO_4) was subsequently added down the side of each tube to form a lower layer. A reddish brown colour at the interface was indicative of the presence of terpenoids.

3.4.2 Saponins

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

3.4.3 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 to obtain supernatants and three drops of 0.1% $FeCl_3$ added to each supernatant. A brownish green or blue black colouration indicated the presence of tannins.

3.4.4 Alkaloids

Ten milligrams of standard, quinidine, and crude plant extracts were dissolved in 2 mL of acid alcohol. Afterwards, the solution was boiled for 3 min and centrifuged to obtain the 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 the alkaloidal base. The chloroformic extract was then separated and the chloroform was evaporated off. The residue was then dissolved in 2 mL of acetic acid. A reddish brown precipitate observed after adding four drops of Draggendorf's reagent to each extract and standard indicated the presence of alkaloids.

3.4.5 Flavonoids

A volume of 1 mL of ethanol was added to 0.2 g of each plant extract and standard, quercetin. Magnesium turnings were then added to the ethanolic extracts. Subsequently, a few drops of concentrated sulphuric acid were added. A pink or magenta-red colouration observed indicated the presence of flavonoids.

3.4.6 Sterols

Chloroformic extracts of each sample were prepared by dissolving 10 mg of each extract in 1 mL of chloroform. To 1 mL of each extract in a test tube, acetic anhydride was first added and then concentrated sulphuric acid added carefully down the side of the tube for the formation of a lower layer. The formation of a bluish-green colour at the interface indicated the presence of sterols.

3.4.7 General Glycosides

A volume of 5 mL of 10% dilute HCl was added to 0.2 g of each extract in a test tube and heated over a water bath for 2 min. The mixture was then filtered through a Whatman filter paper grade number 2 and 2 to 5 drops of 20% NaOH was added to the filtrate. Subsequently, 1 mL of Fehling's solution A and B was added to the filtrate and heated again on a water bath for 2 min. The observation of a brick-red precipitate indicated the presence of glycosides.

3.4.8 Anthracene

A mass of 0.2 g of each extract was boiled with 2 mL of dilute H_2SO_4 and 2 mL of 5% $FeCl_3$ for 5 min. The mixture was then filtered while still hot and then allowed to cool. About 1mL of chloroform was subsequently added and the chloroform layer separated from the mixture. A volume of 0.5 mL dilute NH_3 was added and shaken.

The presence of anthracene was indicated by the formation of a rose to pink colouration.

3.5 *IN VITRO* ASSESSMENT OF THE BIOLOGICAL ACTIVITIES OF CRUDE EXTRACTS AND FRACTIONS OF *C. RETUSA* AND *C. VARIEGATUM*

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

3.5.1.1 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 a wavelength of 517 nm using a UV/Vis light spectrophotomer.

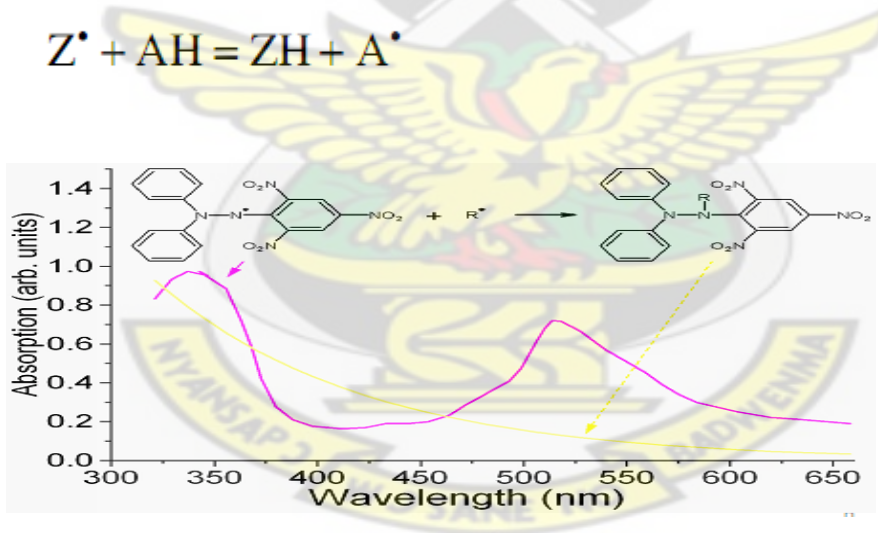


Figure 3.1 Absorption spectrum of DPPH

3.5.1.2 Procedure

Stock solutions of the hydroethanolic extracts were prepared by dissolving 20 mg of each of the freeze-dried samples in 1 mL of 50% ethanolic solution. Also, stock solutions of 4.4 mg/mL of standard (butylated hydroxyl toluene-BHT) and 0.5 mM of DPPH were prepared by dissolving 4.4 mg of BHT and 3.9 mg of DPPH in 1 mL

and 20 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 Ebrahimzadeh *et al*. (2009) with slight modifications as follows: on a 96-well plate, the extracts were serially diluted in 50% ethanol to obtain a concentration range of 0.027–20 mg/mL. The reaction mixture was made up of 100 μL of 0.5 mM DPPH, and 100 μL of each concentration of the test extracts. For positive control or standard, butylated hydroxy toluene (BHT) was used at a concentration range of 0.006–4.4 mg/mL in methanol. The solvents, methanol and 50% ethanol were used as blanks. Duplicate experiments were performed. The plates were covered with aluminum foil and subsequently shaken gently and kept in the dark for 20 min after which the absorbance was read (on a Tecan-PC infinite M200 Pro Plate reader, Austria), at a 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 scavenging (antioxidant activity) for the duplicate experiment was plotted for the standard and test samples and their effective concentrations at 50% (EC_{50}) values, which are the concentrations of antioxidant necessary to decrease the initial DPPH concentration by 50%, were determined by nonlinear regression analysis.

3.5.2 Determination of Total Phenolic Content

3.5.2.1 Principle

All phenolic compounds contained in a compound 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 tungsten, W_8O_{23}, and molybdenum, Mo_8O_{23}. The blue colouration produced has a maximum absorption in the region of 760 nm, and is proportional to the total quantity of phenolic compounds originally present (Singleton *et al*., 1999).

3.5.2.2 Procedure

Stock solutions of the extracts were prepared by dissolving 20 mg of each of the freeze-dried samples in 1 mL of 50% ethanolic solution. 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 10 mL 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 phenolics content of the extracts. A two-fold serial dilution was carried out on the gallic acid standard to obtain seven different concentrations: 1, 0.5, 0.25, 0.125, 0.0625, 0.03125 and 0.0156 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. Ethanolic solution (50%), without extracts, was also prepared and used as blanks.

A volume of 10 µL of each sample and gallic acid dilutions were aliquoted into 2.0 mL eppendorf tubes separately. Aliquots of 790 µL of distilled water was then added which was followed by the addition of 50 µL of Folin-Ciocalteau reagent, and mixed thoroughly by vortexing for 5 s. This was followed by incubation of the tubes in darkness at room temperature for 5 min. Afterwards, a volume of 150 µL of 1.9 M sodium carbonate solution was added to each tube, mixed thoroughly by vortexing for 5 s and the tubes were incubated in darkness at room temperature for 2 h. After the 2 h incubation, a volume of 200 µL of each extract and gallic acid standard dilutions were aliquoted into wells on a 96-well plate in duplicate and absorbance read at a wavelength of 760 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.

3.5.3 Cell culture

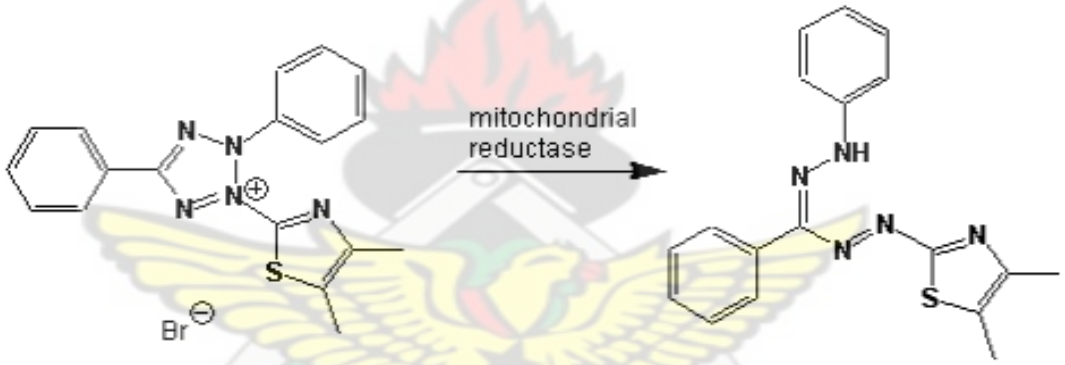
Cells were cultured as described by Ham *et al*. (2012) with slight modifications. The Jurkat cells were cultured in RPMI 1640 medium. MCF7, PC3, HepG2 and WRL68 cell lines were cultured in D-MEM medium. All culture media were supplemented with 1% PSG and 10% FBS. The cells were maintained in an incubator with 5% CO_2 concentration at 37 ^{o}C and passaged on reaching about 80% confluence.

3.5.4 Cell viability assay

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

3.5.4.1 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 (Berridge and Tan, 1993).



3.5.4.2 Procedure

Stock solutions of the extracts were prepared by dissolving 50 mg of each of the freeze-dried samples in 1 mL of 50% ethanol solution. The solutions were vortexed and filter sterilized into cryotubes in a biosafety cabinet through 0.45 µm millipore filters before storage at -20 ^{o}C until use.

The procedures described by Ayisi *et al*. (1991) were followed. The 3[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was adopted for the measurement of cell growth and viability. Dilution of the 50 mg/mL stock of each plant extract was made in 50% ethanol, to obtain a final concentration of 1 mg/mL.

Subsequently, a two-fold serial dilution was made of each extract to obtain four concentrations of 0.0625, 0.125, 0.25 and 0.5 mg/mL. The dilutions gave a dose range of 0.0625 – 1 mg/mL, thus 62.5-1000 µg/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 complete culture media in order to obtain final density of $1x10^5$ cells/mL. A volume of 100 µL (1×10^5 cells/mL) of cell suspension 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 5% CO_2 at 37 ^oC for 72 h.

The monolayer adhesive cells (PC3, HepG2, MCF7 and WRL68) were detached with 3 mL of 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 complete culture media in order to obtain final cell density of $1x10^5$ cells/mL. Cells were seeded in 96-well plates at plating density of $1x10^4$ cells/well with 100 µL/well of cell suspension and incubated for cell attachment at 37 ^oC with 5% CO_2. The cells were treated with serially diluted concentrations of the test samples after 24 h. Subsequently, 10 µL of each extract dilution was added to the cells in triplicates. This was also followed by 72 h of incubation. Curcumin was used as a positive control in all assays. A volume of 20 µL of 2.5 mg/mL MTT solution was added to each well on the 96-well plate and further incubated in a humidified 5% CO_2 incubator at 37 ^oC for 4 h. Acidified isopropanol (150 µL) was added to each well to stop the reaction and dissolve the formazan crystals formed, and the plates were incubated in the dark at room temperature overnight, before reading the absorbance at 570 nm using a microplate spectrophotometer (Tecan Infinite M200 Pro plate

reader, Austria). A colour control plate was also setup for each extract including the positive control, curcumin. This was made up of 10 µL of sample in 100 µL of media. The plate was incubated as described above and absorbances were read at 570 nm. The percentage cell survival (viability) was determined by the following formula:

$$\%cell\ viability = \left(\frac{Absorbance\ of\ treated\ cells - Absorbance\ of\ blank}{Absorbance\ of\ untreated\ cells - Absorbance\ of\ blank}\right) x100$$

The average cell percentage viability obtained from triplicate determinations at each concentration was plotted as a dose-response curve using Graph Pad Prism version 5.0 and the inhibition concentration at 50% (IC_{50}) values, that is, concentration of extracts or standard drug inducing 50% inhibition of cancer cells, determined from the dose- response curve by nonlinear regression analysis.

The selectivity index (SI) was determined from the ratio of IC_{50} values of each extract (or standard drug) on a normal human liver cell line (WRL 68) to IC_{50} value of the extract (or standard drug) on cancer cell lines. The SI value indicates selectivity of the sample to the cell lines tested. Samples with SI values greater than 2 were considered to have a good selectivity towards the cancer cell lines. The formula for calculation of SI is as follows:

$$S.I = \frac{IC_{50}\ of\ the\ test\ sample\ or\ standard\ on\ normal\ cells}{IC_{50}\ of\ the\ test\ sample\ or\ standard\ on\ cancer\ cells}$$

3.6 BIOACTIVITY GUIDED FRACTIONATION

The crude extract of each plant that showed strong activity (yielded the lowest IC_{50} values) against the cancer cell lines was fractionated using different solvents. The

fractions were then screened against the cell lines (Jurkat, HepG2 and MCF-7) for anticancer activity as earlier described. The 50% crude ethanolic extracts of *C. retusa* stem and *C. variegatum* stem bark had the lowest IC_{50} values and hence were selected for fractionation.

3.6.1 Fractionation of the 50% ethanolic extracts

Fractionation of the hydroethanolic stem extract of *C. retusa* and stem bark extract of *C. variegatum* was carried out separately in a separating funnel using solvents of increasing polarity, petroleum ether, chloroform and ethyl acetate. A mass of 2.5 g of crude hydroethanolic stem extract of *C. retusa* was dissolved in 25 mL of 50% ethanolic solution and was successively partitioned with petroleum ether, then with chloroform and finally with ethyl acetate, each having a volume of 50 mL, to obtain petroleum ether, chloroform and ethyl acetate fractions. This was done for two to three times as polarity increased. The remaining portion was designated as 50% ethanol fraction.

For the stem bark extract of *C. variegatum*, a mass of 1.5 g was dissolved in 15 mL of 50% ethanolic solution and was successively partitioned with petroleum ether, then chloroform and finally with ethyl acetate, each having a volume of 30 mL, to obtain petroleum ether, chloroform and ethyl acetate fractions. This was done for two to three times as polarity increased. The remaining portion was designated as hydroethanolic fraction.

The petroleum ether, chloroform, ethyl acetate and hydroethanolic fractions were then concentrated under reduced pressure using a rotary evaporator (Buchi Rotavapor R-205, Switzerland) at temperatures of 65 ^{o}C, 69 ^{o}C, 77 ^{o}C and 79 ^{o}C, respectively. The petroleum ether, chloroform and ethyl acetate were air-dried at room

temperature (25 ^{o}C) while the aqueous portion of the hydroethanolic fractions were frozen at -20 ^{o}C and lyophilized using a vacuum freeze dryer (Labconco, England). The dried fractions were then stored at -20 ^{o}C until use.

Stock solutions of the fractions were prepared by dissolving 10 mg of each sample in 1 mL of dimethyl sulphoxide (DMSO). The solutions were vortexed and filter sterilized through 0.45 µm millipore filters into cryotubes in a biosafety cabinet before storage at -20 ^{o}C.

Dilution of the 10 mg/mL stock solutions of each fraction was made in DMSO, to obtain final concentrations of 1 mg/mL. Subsequently, a two-fold serial dilution was made of each fraction in 10% DMSO to obtain four concentrations of 0.0625, 0.125, 0.25 and 0.5 mg/mL.

The above methods used for the *in vitro* MTT assay on the crude plant extracts were also adopted for the fractions and the SI determined as described earlier.

3.7 MOLECULAR MECHANISM STUDY

3.7.1 Nuclear Morphology Examination (Hoechst Staining)

MCF-7 cells were seeded at 1x10^{6} cells /mL in a total volume of 6 ml in sterile petri dishes and incubated for 24 h at 37 ^{o}C in 5% CO_2 to allow the cells to adhere to the dishes. The cells were then treated with two different concentrations (20 and 40 µg/mL) of the most active crude extract (*C. variegatum* stem bark) and standard urosolic acid (5.7 µg/mL) and then re-incubated for 24 h at 37 ^{o}C in 5% CO_2. The cells were scraped from the petri dishes with a cell lifter and transferred into 15 mL centrifuge tubes. Centrifugation was done at 1000 rpm for 5 min and the supernatant was discarded. The remaining cell pellets were re-suspended in 1 mL of phosphate

buffered saline (PBS). The cells were then transferred into 1.5 mL eppendorf tubes and centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the cell pellets were treated with 200 µL of 1% glutaraldehyde and then incubated at room temperature for 30 min. The centrifugation was subsequently repeated (as above) and the supernatant was removed. A volume of 50 µL of PBS and 8 µL of Hoechst solutions was finally added and mixed gently but uniformly. The samples were applied on microscope slides, covered with cover slips, mounted and examined on a fluorescent microscope (Olympus, U.S.A).

3.7.2 Flow cytometric analysis

MCF-7 (breast cancer) cells were seeded at $2x10^5$ cells /mL in a total volume of 3 mL in sterile petri dishes and incubated for 24 h at 37 ^oC in 5% CO_2. The cells were then treated with different concentrations (20 and 40 µg/mL) of the most active crude extract (*C. variegatum* stem bark) and standard curcumin and then re-incubated for 24 h at 37 ^oC in 5% CO_2. The cells were scraped from the petri dishes with a cell lifter and stirred gently and uniformly. A volume of 100 µL was aliquoted into wells in a 96 well plate. An equal volume of the Guava Nexin Reagent was aliquoted into each well, mixed thoroughly and incubated for 20 min. The plates were read in a flow cytometer (Guava Easycyte, Germany) (Vermes *et al*., 1995).

3.8 STATISTICAL ANALYSIS

Data were analyzed by one-way analysis of variance and the means assessed by Tukey's test at 5% level of significance ($p < 0.05$) using Graph Pad Prism version 5.0. Experimental results are presented as tables and graphs using Microsoft Excel 2007.

CHAPTER FOUR

4.0 RESULTS

4.1 PHYTOCHEMICAL SCREENING

All the crude extracts were tested to assess the phytochemical constituents present. Seven samples were analysed for the presence of general glycosides, anthracene glycosides, saponins, tannins, alkaloids, flavonoids, sterols and triterpenoids. The table below summarizes the phytochemicals present in the samples.

Table 4.1: Phytochemicals present in crude extracts

	C. variegatum		***C. retusa***				
	Stem bark	Leaf	Seed	Pod	Flower	Stem	Leaf
General glycoside	++	++	++	++	+++	++	++
Anthracene glycoside	-	-	-	-	-	-	-
Saponins	+++	-	+++	+++	+++	+++	+++
Tannins	+++	+++	+++	+++	+++	+++	+++
Alkaloids	++	+++	+++	++	++	++	++
Flavonoids	+++	+++	+++	+++	+++	+++	-
Sterols	+++	+++	+	+++	+++	++	++
Triterpenoids	-	+++	-	+++	-	-	-

The data show the intensities of observed colours or froths as compared to standards

+++ present at high concentration; ++ present in moderate concentration; + present in low concentration; - absent.

4.2 TOTAL PHENOLIC CONTENT

A total of seven different concentrations were prepared for the gallic acid with the highest concentration of 1 mg/mL. Figure 4.1 shows the standard curve constructed from the absorbance readings obtained from the various concentrations of gallic acid prepared.

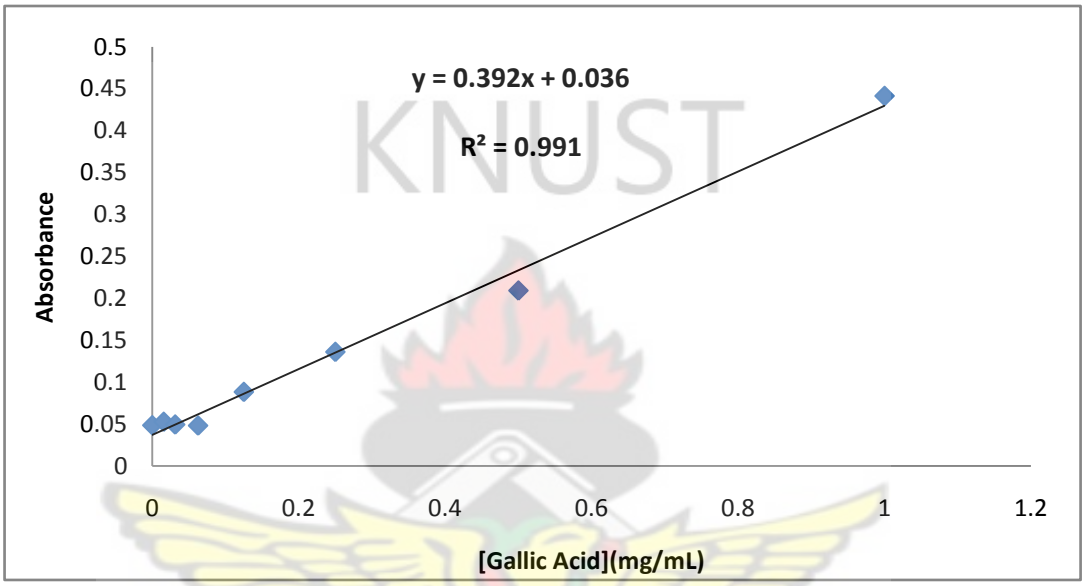


Figure 4.1: Standard calibration curve obtained from the various gallic acid concentrations

From the standard curve constructed, the total phenolic content of all the plant extracts were extrapolated. Out of the seven extracts tested, the stem bark of *C. variegatum* recorded the highest concentration of phenols (163.0±3.323 mg Gallic acid equivalent/g) while the leaf of the *C. retusa* plant recorded the highest concentration of phenols (67.35±1.153 mg Gallic acid equivalent/g) for the devil bean.

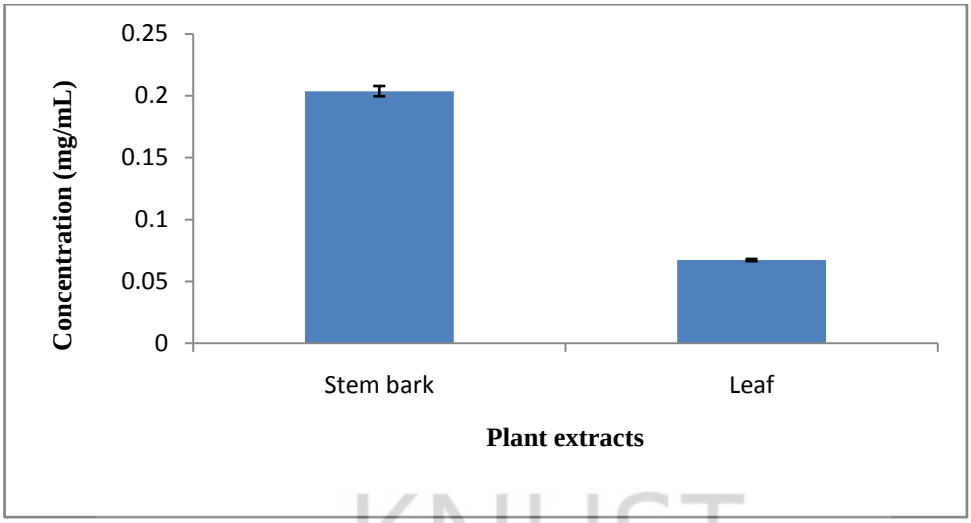


Figure 4.2: Total phenolic content of hydroethanolic extracts of *C. variegatum* in mg/mL

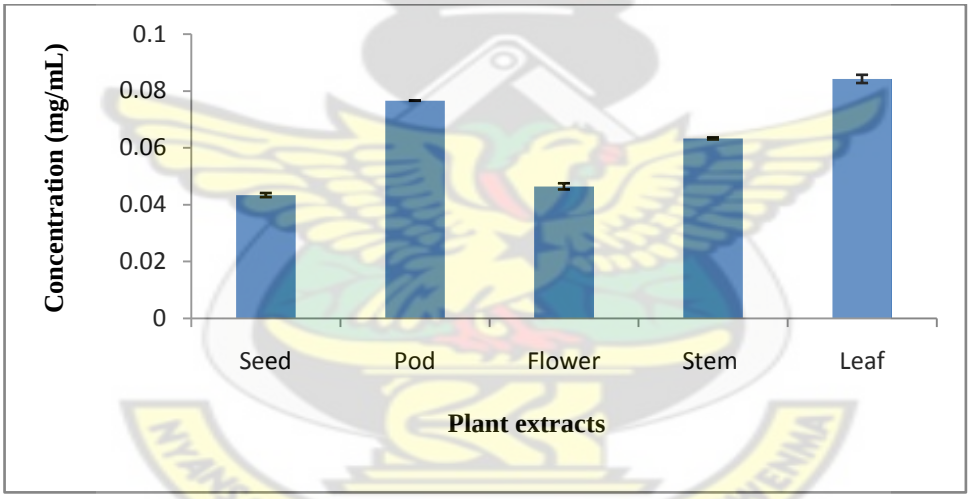


Figure 4.3: Total phenolic content of hydroethanolic extracts of *C. retusa* in mg/mL

The total phenolic content, as determined by Folin Ciocalteu method, are reported as gallic acid equivalents by reference to the standard curve ($y = 0.392x + 0.036, R^2 = 0.991$).

Table 4.2: Total phenolic content of hydroethanolic extracts

Plant	**Part**	**Total phenolic content (mg GAE/g)**	**P-value**
C. variegatum	Stem bark	163.0±3.323	0.0005
	Leaf	53.78±0.721	
C. retusa	Seed	34.70±0.573	< 0.0001
	Pod	61.22±0.0	
	Flower	37.15±0.870	
	Stem	50.62±0.290	
	Leaf	67.35±1.153	

Tabulated values are mean ± standard deviation of two replicates.

P-values compare the statistical difference between the calculated means using Tukey's test.

4.3 TOTAL ANTIOXIDANT ACTIVITY OF EXTRACTS

A total of seven concentrations were prepared for the standard, BHT as well as all the plant extracts with the highest concentration being 10 mg/mL and the lowest 0.0135 mg/mL. Figure 4.4 below represents graphs of the antioxidant activities of BHT and the samples analysed.

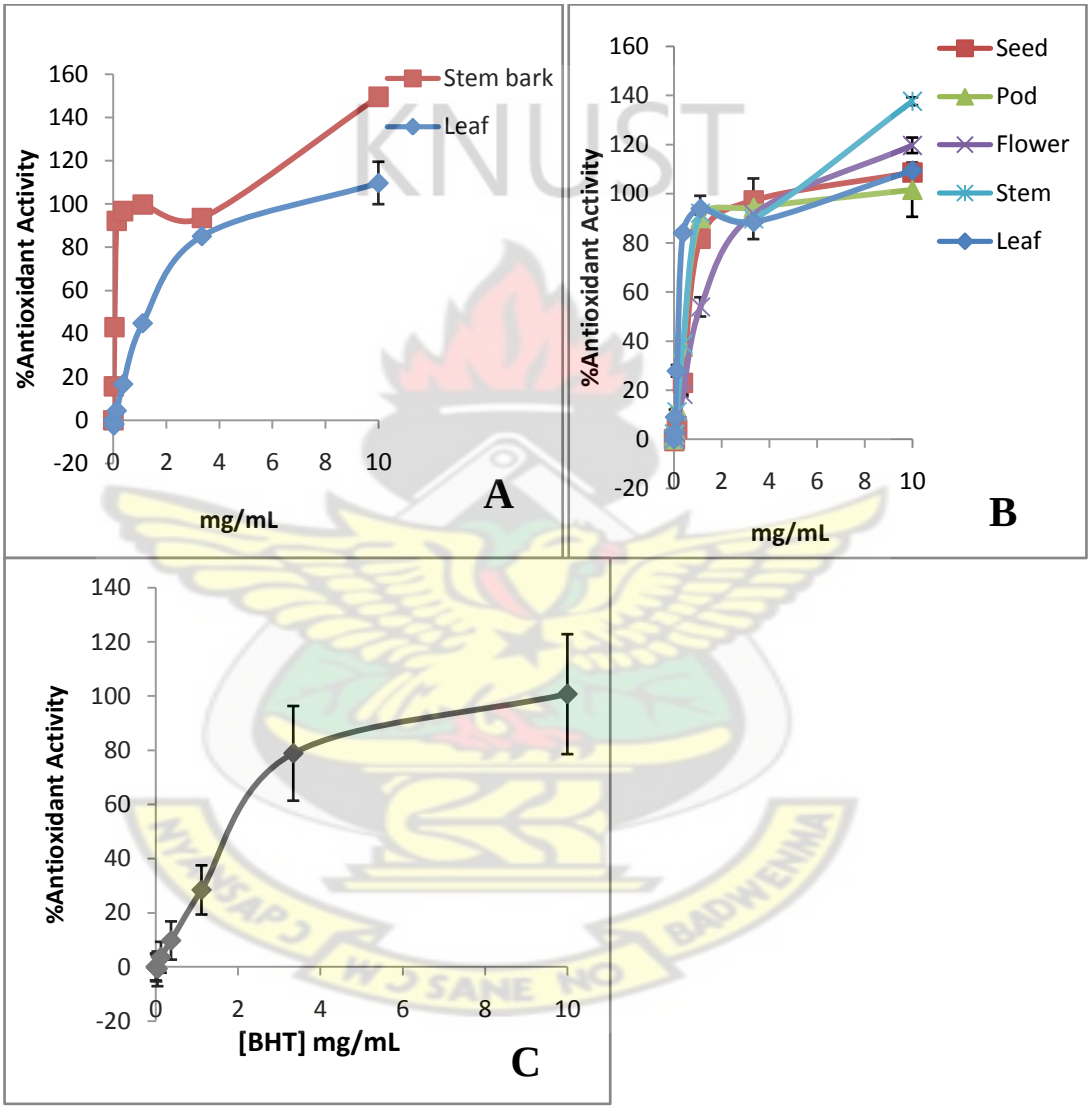


Figure 4.4: DPPH free radical scavenging activity of hydroethanolic crude extracts of *C. variegatum* (A), *C. retusa* (B) and butylatedhydroxytoluene (standard compound) (C)

The stem bark of *C. variegatum* was observed to exhibit the highest antioxidant activity due to its ability to scavenge 50% of the DPPH radical at a concentration of 0.053 mg/mL. Table 4.3 shows the concentration of the each extract and standard (BHT) required to scavenge 50% of DPPH.

Table 4.3: EC_{50} antioxidant values of hydroethanolic extracts of *C. variegatum*, *C. retusa* and BHT.

Plant	**Part/standard**	**Concentration (mg/mL)**	**P-value**
C. variegatum	Stem bark	0.053±0.004	0.0002
	Leaf	1.396±0.073	
	BHT	0.454±0.031	
C. retusa	Seed	0.710±0.018	< 0.0001
	Pod	0.543±0.013	
	Flower	1.029±0.033	
	Stem	0.525±0.003	
	Leaf	0.222±0.004	
	BHT	0.454±0.031	

Tabulated values are mean ± standard deviation of two replicates.

P-values compare the statistical difference between the calculated means using Tukey's test.

4.4 ANTIPROLIFERATIVE ACTIVITIES OF EXTRACTS

4.4.1 ANTIPROLIFERATIVE ACTIVITIES OF THE PLANT EXTRACTS ON JURKAT (LEUKEMIC) CELLS

Cultured Jurkat cells were treated with the standard compound curcumin as well as the individual plant extracts to evaluate the antiproliferative activity on the cells. A highest concentration of 100 µM and 1000 µg/mL were prepared for curcumin and the extracts, respectively.

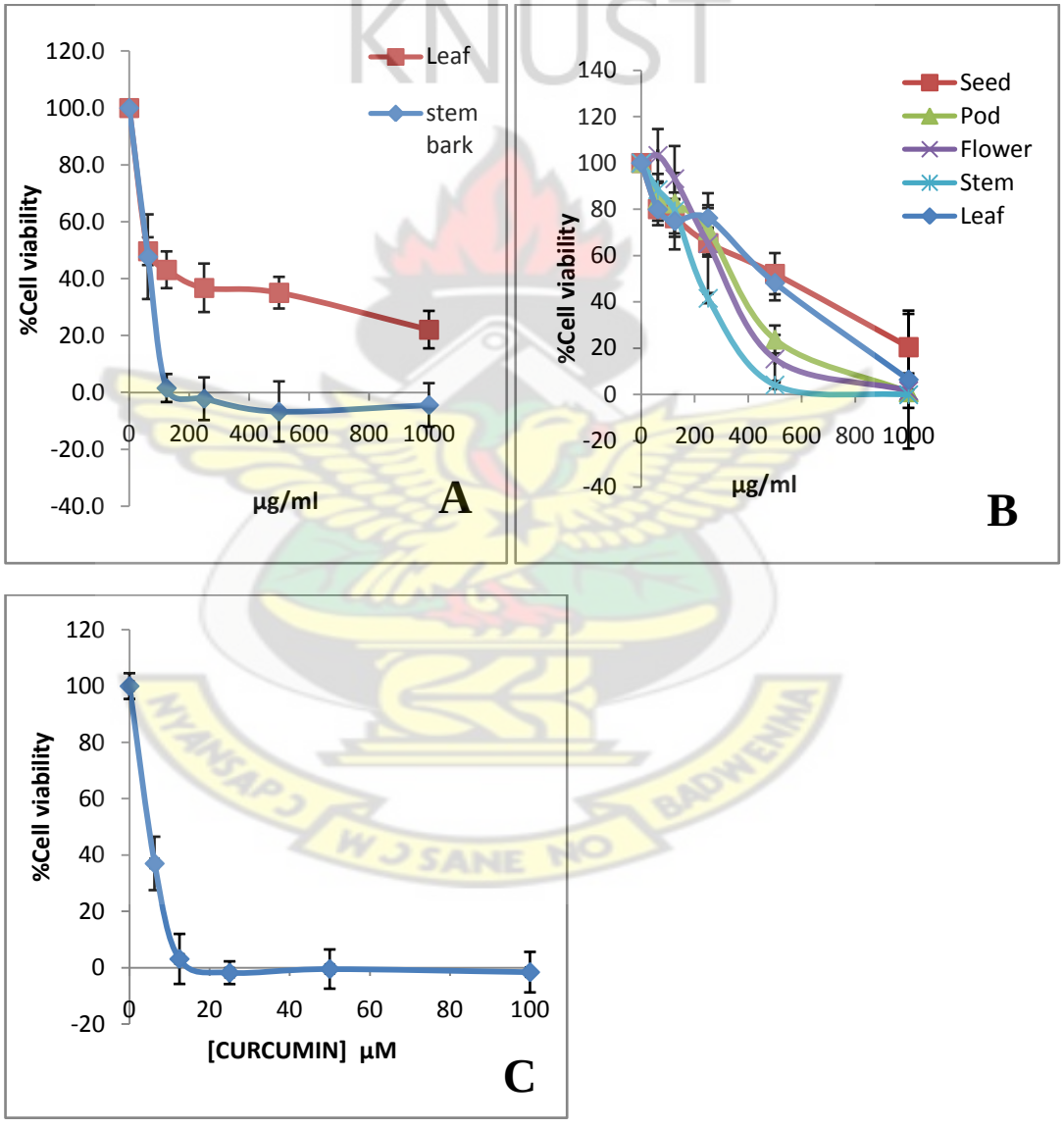


Figure 4.5: Cytotoxicity curves of *C. variegatum* (A), *C. retusa* (B) and curcumin (C) on human leukemic cancer (Jurkat) cell line.

4.4.2 ANTIPROLIFERATIVE ACTIVITIES OF THE PLANT EXTRACTS ON MCF 7 (BREAST CANCER) CELLS

Cultured MCF 7 cells were treated with curcumin as well as the individual plant extracts to evaluate the antiproliferative activity on the cells. A highest concentration of 100 µM and 1000 µg/mL were prepared for curcumin and the extracts, respectively.

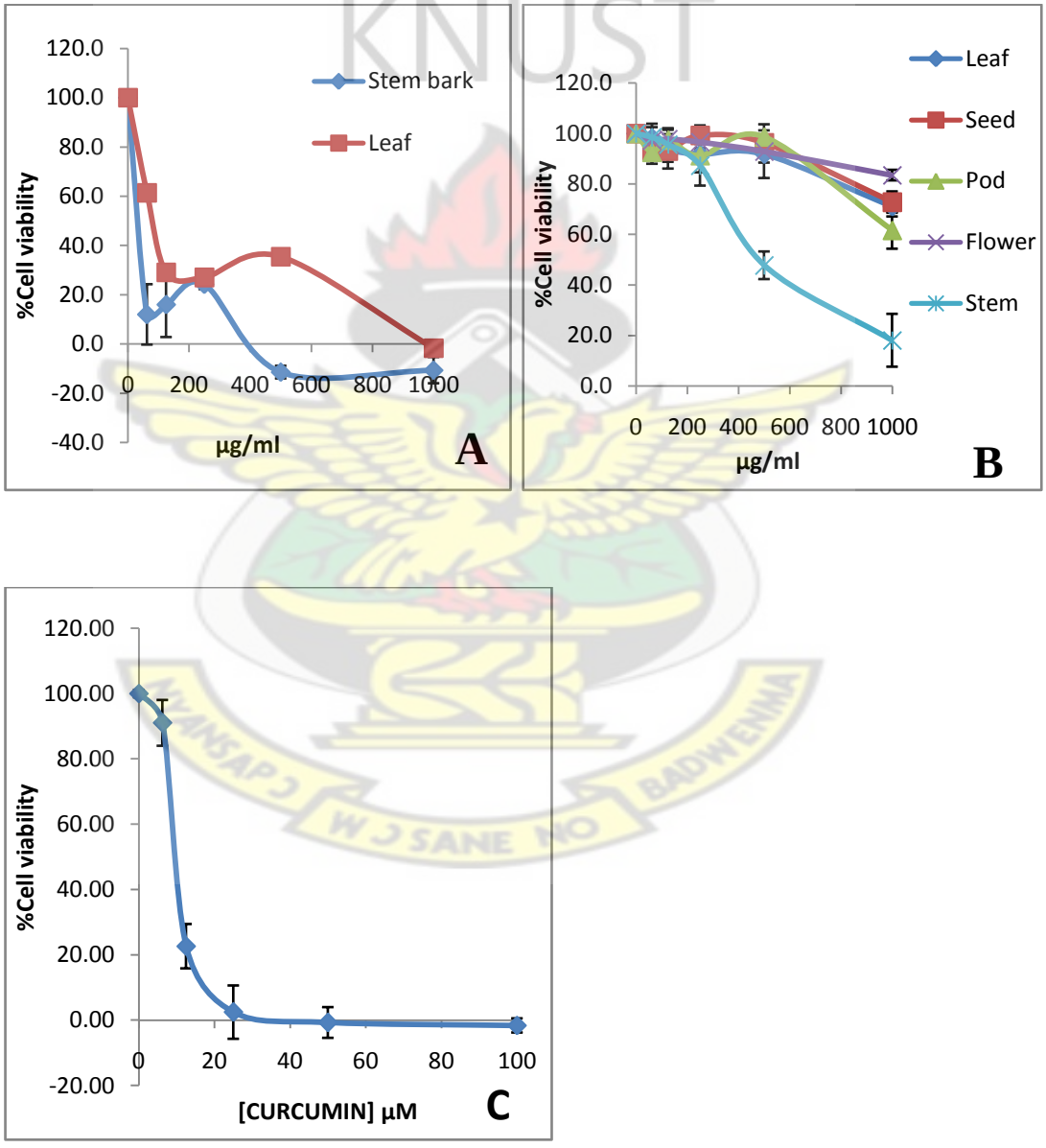


Figure 4.6: Cytotoxicity curves of *C. variegatum* (A), *C. retusa* (B) and curcumin (C) on breast cancer (MCF 7) cell line.

4.4.3 ANTIPROLIFERATIVE ACTIVITIES OF THE PLANT EXTRACTS ON PC 3 (PROSTATE CANCER) CELLS

Cultured PC 3 cells were treated with curcumin as well as the individual plant extracts to evaluate the antiproliferative activity on the cells. A highest concentration of 100 µM and 1000 µg/mL were prepared for curcumin and the extracts, respectively.

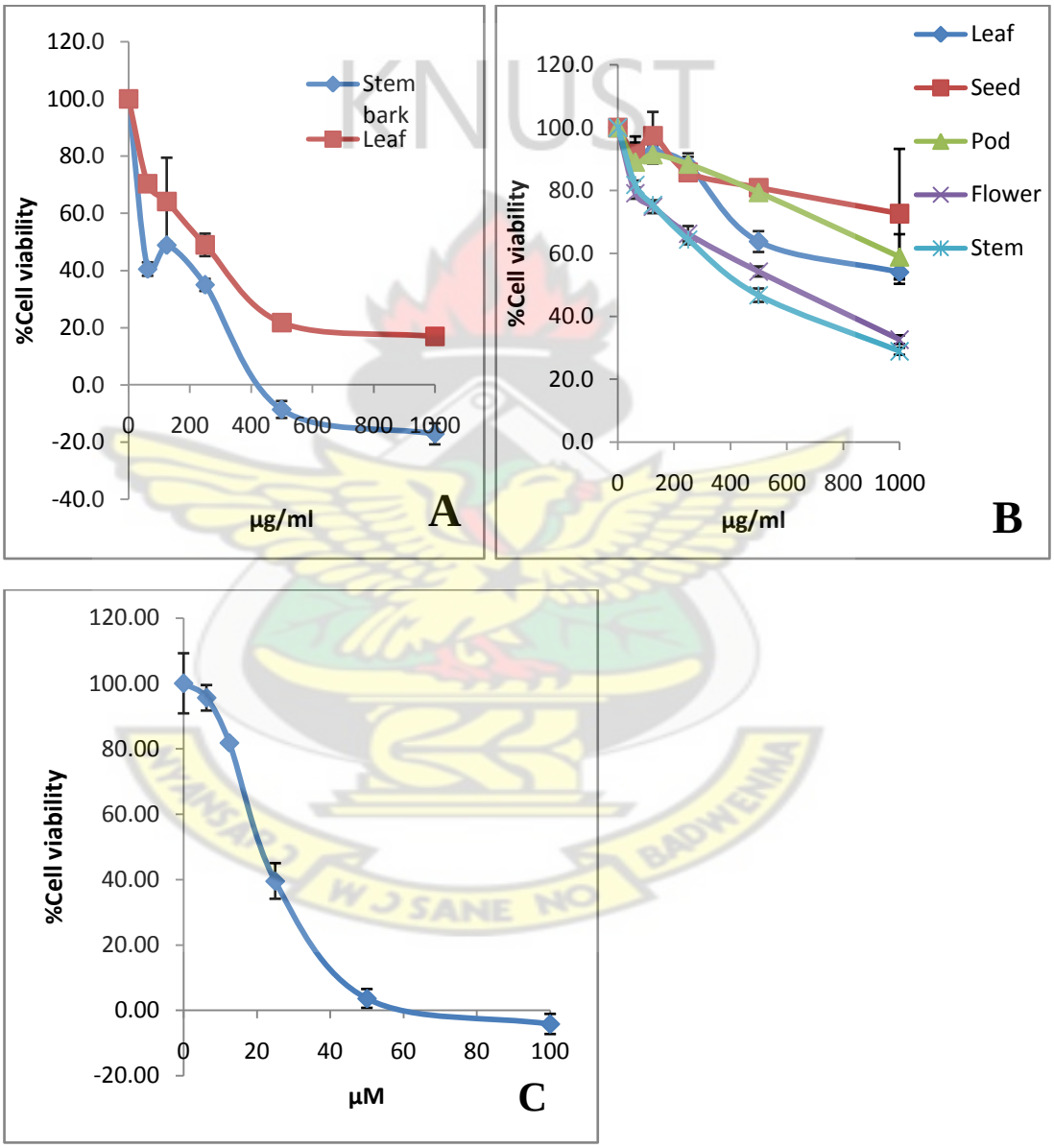


Figure 4.7: Cytotoxicity curves of *C. variegatum* (A), *C. retusa* (B) and curcumin (C) on prostate cancer (PC 3) cell line.

4.4.4 ANTIPROLIFERATIVE ACTIVITIES OF THE PLANT EXTRACTS ON WRL 68 (NORMAL HUMAN LIVER) CELLS

Cultured WRL 68 cells were treated with curcumin as well as the individual plant extracts to evaluate the antiproliferative activity on the cells. A highest concentration of 100 µM and 1000 µg/mL were prepared for curcumin and the extracts, respectively.

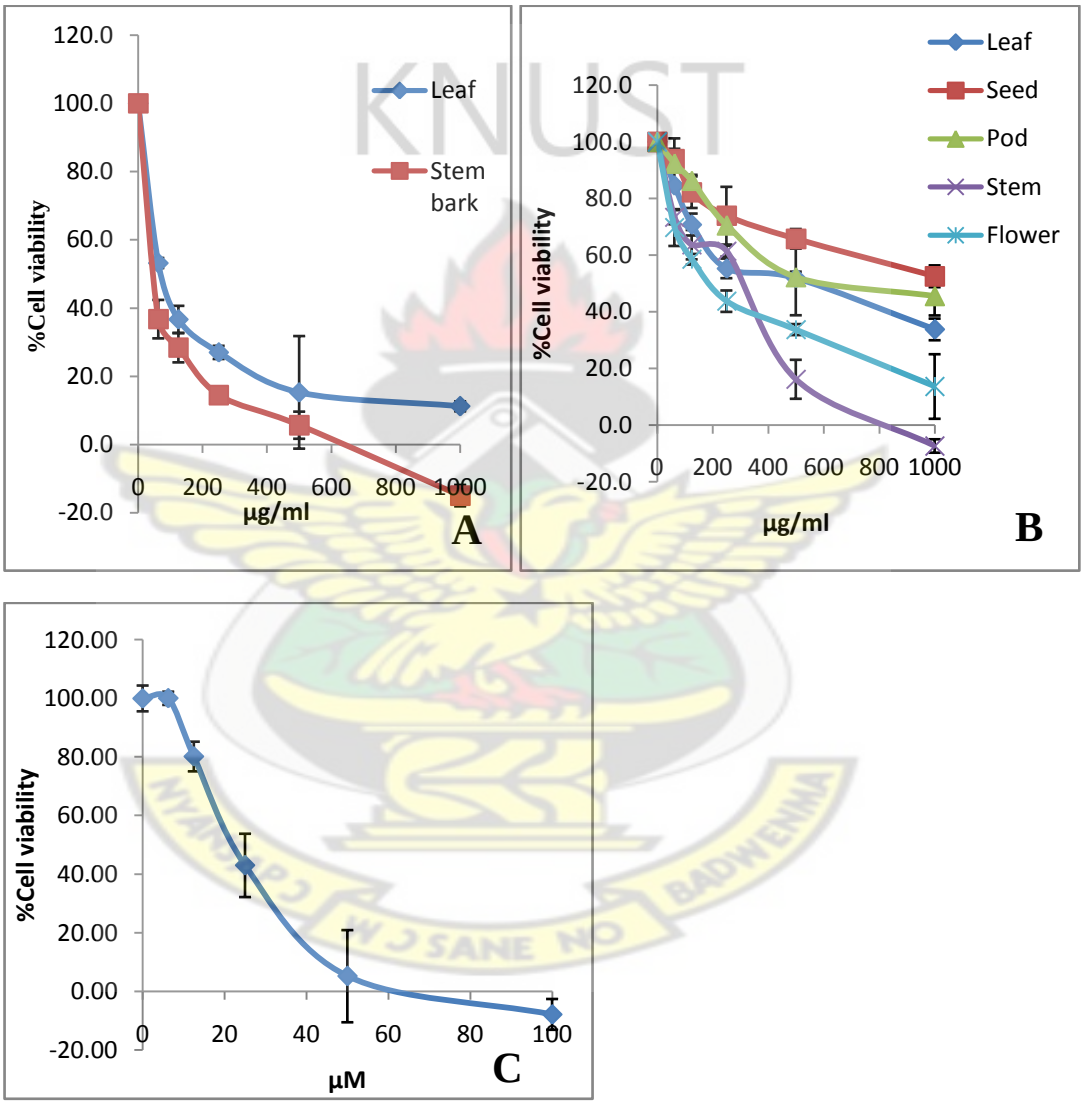


Figure 4.8: Cytotoxicity curves of *C. variegatum* (A), *C. retusa* (B) and curcumin (C) on normal human liver (WRL 68) cell line.

Tables 4.4 and 4.5 show the concentration of each extract and standard required to kill 50% of the selected cell lines.

Table 4.4: Cytotoxic activities of *C. variegatum* crude extracts

	IC_{50} values (µg/mL)			
Cell Line	**Stem bark**	**Leaf**	**Curcumin**	**P-value**
Jurkat	59.71 ± 12.20	62.03 ± 8.49	1.84 ± 0.16	0.0002
MCF 7	35.55 ± 1.50	84.44 ± 1.53	3.65 ± 0.08	< 0.0001
PC 3	52.54 ± 1.88	211.20 ± 77.09	8.10 ± 0.82	0.0032
WRL 68	49.37 ± 2.7	74.55 ± 4.8	8.35 ± 0.40	< 0.0001

Tabulated values represent mean ± standard deviation of three replicates.

P-values compare the statistical difference between the calculated means using Tukey's test.

For each plant extract tested n=3

Table 4.5: Cytotoxic activities of *C. retusa* crude extracts

		Jurkat	**MCF 7**	**PC 3**	**WRL 68**
IC_{50} values (µg/mL)	**Seed**	532.12±100.34	>1000	>1000	>1000
	Pod	360.99±33.09	>1000	>1000	671.64±283.39
	Flower	326.15±65.76	>1000	599.07±21.20	197.28±11.45
	Stem	221.97±4.45	486.08±17.78	453.51±23.75	313.05±8.57
	Leaf	483.10±52.53	>1000	>1000	552.49±41.55
	Curcumin	1.84 ± 0.16	3.65 ± 0.08	8.10 ± 0.82	8.35 ± 0.40
	P-value	< 0.0001	< 0.0001	< 0.0001	0.0003

Tabulated values represent mean ± standard deviation of three replicates

P-values compare the statistical difference between the calculated means using Tukey's test

For each plant extract tested n=3

4.5 SELECTIVITY INDICES OF CRUDE EXTRACTS

The selectivity indices of extracts was assessed as their abilities to specifically target and inhibit proliferation of only cancer cells without affecting normal cells. Extracts with selectivity indices (SI) greater than 2 are considered to have good selectivity against specific cancer cells.

Table 4.6 Selective ability of hydroethanolic extracts of *C. variegatum* and *C. retusa* towards selected cancer cell lines

		Jurkat	**MCF 7**	**PC 3**
	Curcumin	4.53	2.25	1.03
C. variegatum	**Stem bark**	0.83	1.39	0.94
	Leaf	1.20	0.88	0.31
C. retusa	**Seed**	N/A	N/A	N/A
	Pod	1.86	N/A	N/A
	Flower	0.60	N/A	0.33
	Stem	1.41	0.64	0.69
	Leaf	1.14	N/A	N/A

4.6 MOLECULAR MECHANISM OF ACTION OF *C. VARIEGATUM* STEM BARK

Unlike necrosis, apoptosis is a highly regulated type of cell death that follows rather coordinated set of pathways that is often characterised by nuclear condensation, chromosomal DNA fragmentation and the formation of cell fragments called apoptotic bodies. Phagocytic cells then engulf these bodies and quickly remove them before their contents are spilled out onto surrounding cells to cause damage (Bruce *et al*., 2008). Cells that are not undergoing cell death often possess a nucleus with the normal roughly spherical morphology.

The most active crude extract among all the extracts tested, *C. variegatum* stem bark, was analysed to elucidate its molecular mechanism of action. The effect of this

selected crude extract on the nuclear morphology of breast cancer cells (MCF 7) and its specific mode of action was analysed using the Hoechst staining and the flow cytometry. Concentrations of 20 and 40 µg/mL were prepared to assess the mechanism of action of the extract. For the Hoechst staining and flow cytometry, urosolic acid and curcumin at concentrations of 5.7 µg/mL and 3.7 µg/mL respectively, were used as standards.

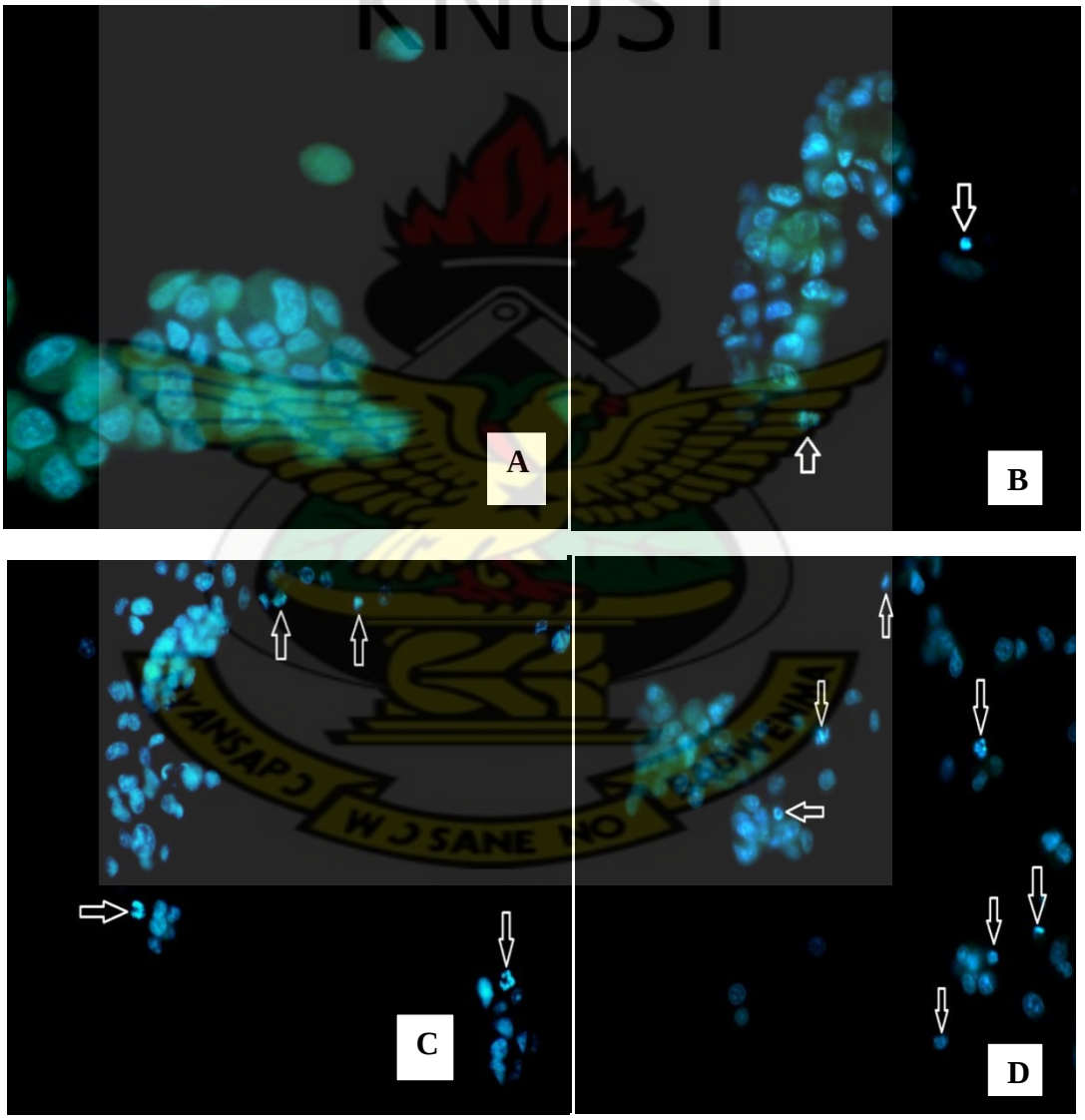


Figure 4.9: Nuclear morphology of MCF 7 cells after 24 h of incubation without any treatment, control (A), treatment with 20 µg/mL (B), 40 µg/mL of *C. variegatum* stem bark extract (C) and urosolic acid (standard) (D). Arrows point to nuclei that have fragmented/shrunk

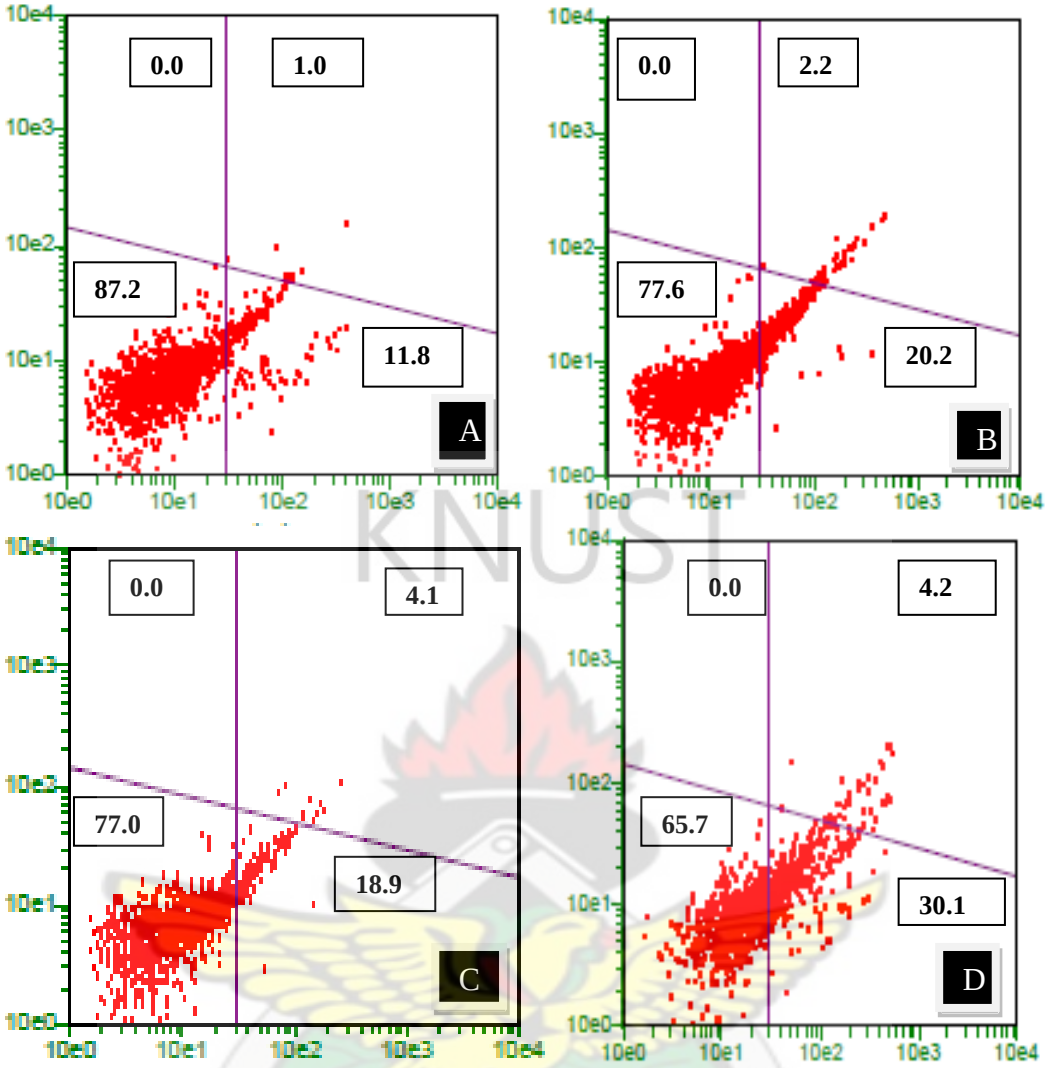


Figure 4.10: Flow cytometric evaluation of apoptotic effects on MCF 7 cells after 24 h of incubation without any treatment, control (A) and treatment with 20 µg/mL (B), 40 µg/mL (C) of *C. variegatum* stem bark extract and curcumin (standard) (D).

Inserted values represent percentage of cells of two replicates. Upper left quadrant represents nuclear debris, upper right quadrant represents cells in late apoptotic stage, lower left quadrant represents non apoptotic cells and lower right quadrant represents cells in early stage apoptosis.

4.7 ANTIPROLIFERATIVE ACTIVITIES OF FRACTIONS

4.7.1 ANTIPROLIFERATIVE ACTIVITIES OF FRACTIONS ON MCF 7 (BREAST CANCER) CELLS

Fractions prepared from *C. variegatum* stem bark and *C. retusa* stem were analysed for their antiproliferative effect on breast cancer cells. Curcumin which was used as the standard compound elicited the greatest antiproliferative activity on this cell line with an IC_{50} value of 2.93±0.62 µg/mL.

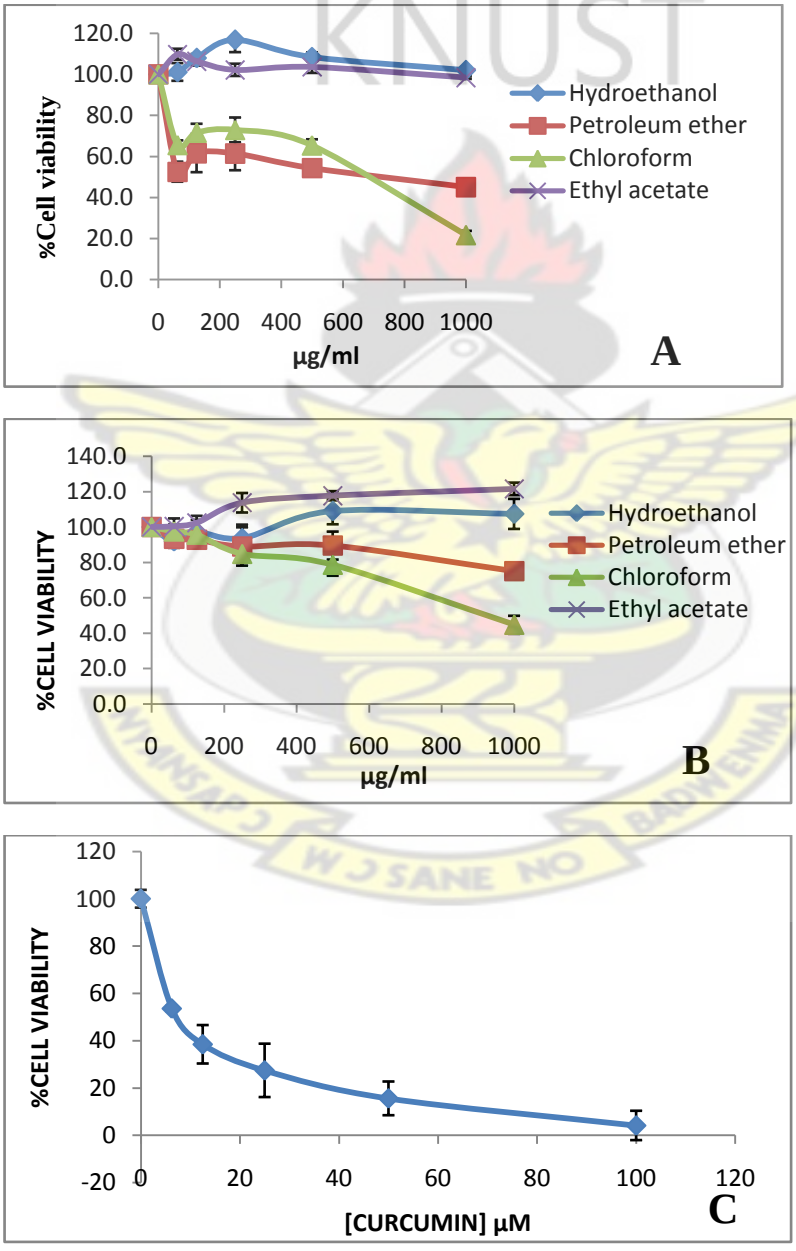


Figure 4.11: Cytotoxicity curves of *C. variegatum* stem bark (A), *C. retusa* stem (B) fractions and curcumin (C) on breast cancer (MCF 7) cell line.

4.7.2 ANTIPROLIFERATIVE ACTIVITIES OF FRACTIONS ON JURKAT (LEUKEMIC) CELLS

Fractions prepared from *C. variegatum* stem bark and *C. retusa* stem were analysed for their antiproliferative effect on leukemic cells. Curcumin, used as the standard elicited the greatest antiproliferative activity on this cell line with an IC_{50} value of 18.99±0.16 µg/mL

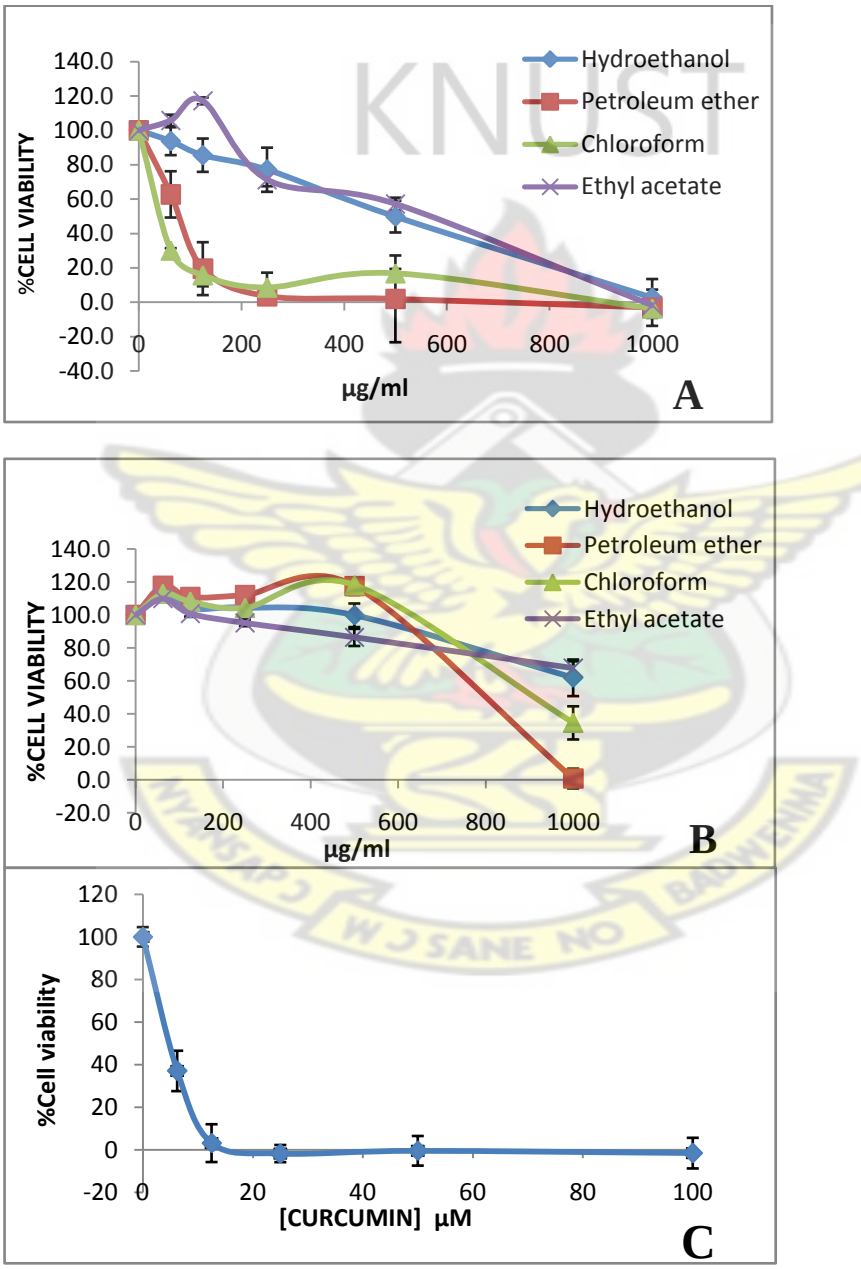


Figure 4.12: Cytotoxicity curves of *C. variegatum* stem bark (A), *C. retusa* stem (B) fractions and curcumin (C) on leukemic (Jurkat) cell line.

4.7.3 ANTIPROLIFERATIVE ACTIVITIES OF FRACTIONS ON HepG2 (LIVER CANCER) CELLS

Fractions prepared from *C. variegatum* stem bark and *C. retusa* stem were analysed for their antiproliferative effect on leukemic cells. Curcumin used as the standard elicited the greatest antiproliferative activity on the cell line with an IC_{50} value of 24.08±1.62 µg/mL.

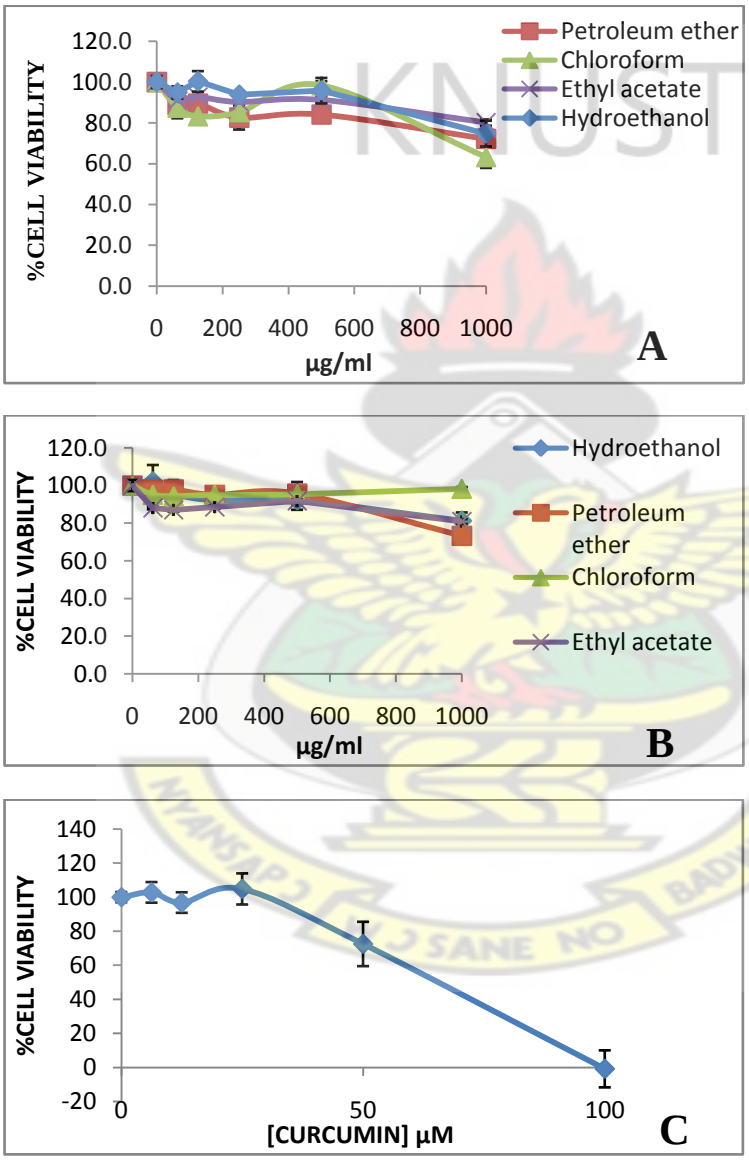


Figure 4.13: Cytotoxicity curves of *C. variegatum* stem bark (A), *C. retusa* stem (B) fractions and curcumin (C) on liver cancer (HepG2) cell line.

From the cytotoxicity curves obtained, the concentration of each extract required to kill 50% of the cells was calculated and these are presented in tables 4.8 and 4.9.

Table 4.7 Cytotoxic activities of fractions of *C. variegatum* stem bark

		Jurkat	**MCF 7**	**HepG2**
IC_{50} values (µg/mL)	**Petroleum ether**	80.87 ±13.90	736.56±183.7	>1000
	Chloroform	44.71 ±0.44	675.80±33.06	>1000
	Ethyl acetate	560.27 ± 22.16	>1000	>1000
	Hydroethanol	498.17±4.74	>1000	>1000
	Curcumin	1.90±0.16	2.93±0.62	24.08±1.62
	P-value	< 0.0001	0.003	-

Tabulated values represent mean ± standard deviation of three replicates

P-values compare the statistical difference between the calculated means using Tukey's test.

For each fraction n=3

Table 4.8 Cytotoxic activities of fractions of *C. retusa* stem

		Jurkat	**MCF 7**	**HepG2**
IC_{50} values (µg/mL)	**Petroleum ether**	789.45±15.98	>1000	>1000
	Chloroform	907.78±4.14	923.30±45.50	>1000
	Ethyl acetate	>1000	>1000	>1000
	Hydroethanol	>1000	>1000	>1000
	Curcumin	1.90±0.16	2.93±0.62	24.08±1.62
	P-value	< 0.0001	< 0.0001	-

Tabulated values represent mean ± standard deviation of three replicates

P-values compare the statistical difference between the calculated means using Tukey's test.

For each fraction n=3

CHAPTER FIVE

5.0 DISCUSSION

Medicinal plants are of great importance to the general health of individuals and communities. It is also an immense source of medicines in pharmacognosy. The medicinal value of these plants lies greatly in their wide range of phytochemical compounds. The presence and levels (marked by intensity of colour) of each phytochemical was evaluated in all samples. In all, the presence of saponins, general glycosides, anthracene glycosides, tannins, alkaloids, flavonoids, sterols and triterpenoids was evaluated. The crude extracts were assayed for their phytochemical constituents and it was observed that all the samples analysed possessed tannins, alkaloids and sterols at varying levels or concentrations.

Earlier studies have shown the presence of alkaloids, sterols and flavonoids in the leaf of *C. retusa* (Dhole *et al*., 2012). The presence of alkaloids and sterols in the leaf of this plant was confirmed in this research. However, flavonoids were absent from the leaves tested for, a contradiction to that reported earlier by Dhole *et al*. (2012). All the crude extracts of the various parts of the plant considered expressed a rather high level of saponins and tannins, with alkaloids and sterols varying in concentration.

Research by Ogunwenmo *et al*. (2007) proved a difference in the phytochemical constituents among various *C. variegatum* varieties. The presence of alkaloids, saponins, and tannins were reported by Ogunwenmo *et al*. (2007) to be varying in levels among varieties of this plant. With respect to the *C. variegatum* cv gold dust tested in this research, both its stem bark and leaf tested positive for general glycosides, tannins, alkaloids, flavonoids and sterols. However, saponins were absent

from the crude extract of *C. variegatum* leaves, (probably due to the difference in variety as compared to that used by Ogunwenmo *et al.,* 2007) while its stem bark showed no traces of triterpenoids. The presence of alkaloids, saponins and tannins in such high concentrations could render this plant antibacterial and antiamoebic and this could be related to its use in the treatment of diarrhoea (Moundipa *et al*., 2005).

Phytochemicals such as phenols and polyphenolic compounds like flavonoids are generally present in medicinal plants and these compounds have been shown to possess good antioxidant activities (van Acker *et al*., 1996). The model of scavenging the stable DPPH radical is a widely used method to evaluate the free radical scavenging ability of various samples, thus assessing the antioxidant potential of these samples (Lee *et al*., 2003). All the samples analysed showed an increase in antioxidant activity with increasing concentrations, thus exhibiting a concentration dependent pattern of free radical scavenging ability. The stem bark extract of *C. variegatum* recorded the strongest antioxidant activity. With respect to *C. retusa*, the leaf was shown to possess the strongest antioxidant activity. Both the leaf of the *C. retusa* and the stem bark of the *C. variegatum* appeared to have stronger antioxidant activities as compared with the standard (BHT) (p-value = 0.0002 and <0.0001 respectively at 95% confidence level).

Several researches have revealed the strong relationship between the total phenolic content of plants and their antioxidant potential and this study confirms such findings (Kiselova *et al.,* 2006; Klimczak *et al.,*2007; Kedage *et al.,* 2007; Jayaprakasha *et al.,* 2008). Members of the Euphorbiaceae plant family possess strong antioxidant activities which are greatly associated with the presence of phenolic compounds (Shahwar *et al*., 2010). For instance, analysis of the leaf extract of *C. variegatum* cv spiral and royal-like by HPLC-DAD showed that ellagic acid, a phenolic compound

may be responsible for its antioxidant activity (Saffoon *et al*., 2014). Findings from this research proved that both the leaf of the *C. retusa* and the stem bark of the *C. variegatum* recorded the highest concentration of total phenolics among all the parts of each plant considered. This goes to support Saffoon *et al*. (2014) and suggest that the observed antioxidant activity could be partly attributed to the high levels of total phenolics present in the samples since these two parts also recorded the highest radical scavenging ability.

The total phenolic content of *C. variegatum* stem bark extract was significantly higher than its leaf (p-value = 0.0005). Although a number of researches have been done on the DPPH free radical scavenging ability of *C. variegatum*, all of these researches evaluated this parameter on its leaf (Saffoon *et al*., 2014; Hassan *et al*., 2014). This research reveals that the stem bark of *C. variegatum* is an even stronger free radical scavenger than its leaf, thus requiring a shift in focus to this part of the plant. However, further study on the stem bark of *C. variegatum* is needed to reveal its active principle.

An earlier study revealed that *C. retusa* possessed the strongest antioxidant activity compared with other Crotalaria species (Devendra *et al*., 2012). Results from this research showed that *C. retusa* indeed has great antioxidant activity, predominantly found in its leaf. With respect to *C. retusa*, there was a highly significant difference between the recorded total phenolic contents of the various parts considered and the antioxidant activities measured (p-value < 0.0001). Devendra *et al*. (2012) indicated that compounds isolated from ethanolic extracts of Crotalaria species possess pharmacological properties and potential to develop natural compound-based pharmaceutical products. These findings confirm the presence of potential compounds with the ability to scavenge free radical and potential anti-carcinogenic,

inflammatory agents because of their high antioxidant effect (Stavric, 1993; Elangovan *et al*., 1994; Martin *et al*., 2002).

The antiproliferative effect of both plants on selected cancer and normal cell lines was also assessed using the MTT Assay. A previous study on *C. variegatum* cv petra established the cytotoxicity of the leaves of this plant on human caucasian breast adenocarcinoma (MCF7), hepatocellular carcinoma (HepG2), colon cell line (HCT116) and lung carcinoma cell line (A549) with activities ranging from 17.3% to 98% (Hassan *et al*., 2013). All the parts of both plants showed various levels of cytotoxicity against Jurkat cells, with the stem bark of *C. variegatum* and stem of *C. retusa* being the most cytotoxic parts of both plants. However, the cytotoxic effect of the standard (curcumin) was significantly higher than that of both plant extracts (p-values = 0.0002 and < 0.0001 respectively). A similar trend was observed with respect to breast (MCF 7) and prostate (PC 3) cancer cell lines in which the stem bark of *C. variegatum* and stem of *C. retusa* were the most cytotoxic parts of both plants. This suggests that the stem bark of *C. variegatum* could possibly be a better source of bioactive compounds for chemotherapy than was previously established (as its leaf) by Hassan *et al*. (2013).

In elucidating the cytotoxic effect of both plants on the normal human liver cell line (WRL 68), it was observed that the stem bark of *C. variegatum* was the most toxic part of *C. variegatum*. The seed of *C. retusa* has been reported by Maia *et al*. (2013), to have high levels of the hepatotoxic alkaloid, monocrotaline, which requires bioactivation to become toxic to hepatocytes (John *et al*., 2005). Thus, the flower (other than the seed) of *C. retusa* was the most hepatotoxic part of this plant, even though the seed has the highest levels of monocrotaline. These findings suggest that

indeed, monocrotaline, though hepatotoxic would require bioactivation (John *et al*., 2005), hence its reduced toxicity *in vitro*. The flower could possibly contain certain compounds that would not require bioactivation consequently, its observed toxicity *in vitro*. In all the assays, there were significant differences between the recorded IC_{50} of the extracts of both plants.

Hassan *et al*. (2013) further purified pure compounds from the leaf extract of *C. variegatum* and reported an increase in cytotoxicity of one of the isolated compounds, hemiargyrine, towards HepG2. A similar work done on *C. retusa* by Srinivas *et al*. (2014) yielded monocrotaline, which was much more toxic towards VERO (kidney) and HeLa (cervical) cancer cells. Thus it is possible that further isolation of a pure compound from the stem bark of *C. variegatum* is likely to improve the observed cytotoxicity on the cancer cell lines. This could serve as a lead to identifying and developing a potential chemotherapeutic agent against cancer. None of the extracts showed good selectivity against the cancer cell lines with respect to the normal human liver cells. This suggests that even the most active part of both plants could in one way or the other cause damage to the liver and probably other organs of the body when this medicine is administered. However, further studies will be needed to eliminate the toxic components of the extracts and isolate the active principle.

Apoptosis is an important homeostatic mechanism that balances cell division and cell death, thus maintaining the appropriate cell number in the body. Disturbances of apoptosis in cancer cells have been studied in detail, and induction of apoptosis was one of the strategies for anticancer drug development (Martin and Green, 1995; Hu and Kavanagh, 2003). The mode of cytotoxicity induction by the *C. variegatum* stem bark was determined by the Hoechst staining to probably be, apoptosis, due to the

nuclear fragmentation of the MCF 7 cells at 20 and 40 µg/mL. The findings of the Hoechst staining showed that there were significant morphological changes in nuclear chromatin similar to the changes observed in the apoptotic mechanism of action by other members of the Euphorbiaceae family on MCF 7 cells (Aslanturk and Celik, 2013). It was observed that this extract was apoptotic at 20 and 40 µg/mL with a majority of these cancer cells being non-apoptotic (normal) and the remaining being in their early and late stages of apoptosis. Data generated from the flow cytometric assay indicated and confirmed a dose-dependent and apoptotic mode of cytotoxicity for *C. variegatum* stem bark. Emerging evidence has demonstrated that, the anticancer activities of certain chemotherapeutic agents involved in the induction of apoptosis have no side effects on normal tissues, and are thus regarded as the preferred method of treating cancer (Xiao, 2007). Hence, the apoptotic nature of *C. variegatum* renders it a good candidate for chemoptherapy.

The most active part of the two plants (*C. variegatum* stem bark and *C. retusa* stem) was further fractionated to obtain petroleum ether, chloroform, ethyl acetate and hydroethanolic fractions. These were also analysed to verify if the fractions could elicit an increase in cytotoxic activity. The fractions were tested against MCF 7, Jurkat and HepG2 cell lines.

Curcumin was cytotoxic to all the cells tested with a strongest activity of 2.93±0.62 µg/mL against MCF 7 compared to the fractions. There was also significant difference between the IC_{50} of the samples analysed.

With the exception of the chloroform extract of *C. variegatum* stem bark which recorded an increase in cytotoxicity towards Jurkat cells compared to the extract, all the other fractions were relatively less toxic against the cancer cells as compared to

their crude extracts. This could be attributed to the fact that the active molecules in the extracts worked in a synergistic manner (or the activity of the active compound was complemented by another compound) and individually was not that effective. On the other hand, it is possible that the solvent used for fractionation was unsuitable for the purpose, therefore, the active principle(s) remained in the aqueous fraction.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

This study provides information on the phytochemical constituents, antioxidant and antiproliferative effect of *C. variegatum* and *C. retusa*. From the study the presence of general glycosides, saponins, tannins, alkaloids, flavonoids, sterols and triterpenoids were detected in various parts of the two plants at varying concentrations.

The stem bark of *C. variegatum* showed great antioxidant activity in scavenging free radicals as compared to the leaf extract of this plant and exhibited an immense concentration of total phenolic content as compared to its leaf.

The leaf of *C. retusa* also showed a strong antioxidant activity and possessed a high total phenolic content as compared to its pod, stem, flower and seed in decreasing order of total phenolic content.

All the extracts demonstrated various levels of antiproliferative activity in a concentration-dependent manner. The stem bark and leaf of *C. variegatum* though showed poor selectivity were effective in killing the breast and prostate cancer cells in addition to the leukemic cells suggesting that both parts could be used in treating these types of cancers. However, only the stem of *C. retusa* proved to be effective against all the three cancer cell types.

Though the stem bark of *C. variegatum* and stem of *C. retusa* showed interesting anticancer activities amongst all the parts of both plants analysed, both parts were very toxic to the normal human liver cells (WRL 68), suggesting a possible health

risk to individuals who take the preparations of this extract for the purpose of alleviating illnesses especially on chronic users.

The petroleum ether, chloroform, ethyl acetate and hydroethanolic fractions obtained from the two most active parts of the two plants elicited a relatively poor anticancer activity as compared to their respective crude extracts. However, only the chloroform fraction of the *C. variegatum* stem bark recorded an improvement in cytotoxicity against Jurkat as compared to the crude extract. The findings of this research suggest that both plants have good anticancer activities and could be lead to promising medicines against cancer.

In addition to the anticancer evaluation, the molecular studies conducted on the *C. variegatum* stem bark crude extract using the Hoechst staining and flow cytometry revealed an apoptotic mechanism of action.

6.2 RECOMMENDATIONS

This research has established the concentrations at which each extract is most effective against the selected cancer cell lines, however, it is necessary to conduct further studies on these extracts focusing on the antiproliferative effect over time, thus a time-dependent assay.

Secondly, further purification studies need to be conducted to isolate and characterize compounds in the chloroform fraction of *C. variegatum* stem bark since it showed an increase in cytotoxicity against the Jurkat cell line. This could possibly lead to the discovery of an active compound(s) that can be further studied to determine its molecular mechanism of action against Jurkat and if possible serve as a remedy against leukaemia.

Furthermore, it is imperative that this research is repeated in animal models like rats to determine if this observed *in vitro* anticancer activity will be replicated *in vivo*. This will serve as an index of its potential efficacy and toxicity in humans.

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