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Evaluation of the anti-proliferative effect, antioxidant and phytochemical constituents of *Ficus pumila* Linn.

By

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DECLARATION

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

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ABSTRACT

Plants, such as *Ficus pumila,* have been indispensable in treating and managing diverse forms of diseases including cancer. About 80% of the people living in developing countries depend on medicinal plants for their healthcare needs based on the knowledge of traditional use of medicinal plants. This study was aimed at evaluating the heavy metal content of the raw powder and extract of the plant, the phytochemical constituents, antioxidant effect by the use of DPPH assay, the total phenolic content using Folin Ciocalteu assay and the cytotoxic effect using the MTT Assay of *Ficus pumila* ethanolic extract, methanolic and hydro fractions on liver cancer cells (HepG2), Leukemic cell (Jurkat) and normal liver cells (Chang). FTIR analysis was also done to determine the functional groups of the active components of the *F. pumila* fractions. The structure of the compound of the most active fraction was determined using Gas Chromatography-Mass Spectrometry (GC-MS). Alkaloids, terpenoids, flavonoids, cardiac glycoside, saponins and tannins were present in the ethanolic extract of *F. pumila*. The heavy metal analysis revealed the presence of Iron in both the raw powder (1.97 ± 0.11 mg/l) and extract (0.92 ± 0.02 mg/L). Zinc was also detected in both the raw powder (1.19 ± 0.00 mg/l) and extract (0.6595 ± 0.02 mg/l). These levels were below the permissible level set by FAO/WHO. The results from the FTIR revealed the presence of alkynes, alkyl halides, aromatics and aliphatic amines common to all fractions and compounds such as Phenol, 2,4 –bis (1,1-Dimethylethyl) and Dodecane, 2,6, 10-trimethyl were detected in the samples by GC-MS. The DPPH assay also showed that all the fractions scavenged DPPH free radical in a dose dependent manner as compared to the positive control (Ascorbic acid). The hydro fraction had the strongest DPPH scavenging activity (EC_{50} =0.09 ± 0.00 mg/ml). Phenolic compounds were present in all the fractions with the hydro fraction having the highest level (16950 ± 331.95 mg GAE/100g). The MTT assay revealed that all the samples were cytotoxic against the cancer cell lines but only methanolic fraction was selective towards the Jurkat cell lines (Selectivity Index = 2.822) with IC_{50} value of 248.1 µg/ml. The most active fraction in terms of the antioxidant assay was the hydro fraction and with respect to the cytotoxicity assay was the methanolic fraction. This increase the prospect that this plant contains compound(s) which could serve as leads for novel anticancer drugs.

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CHAPTER ONE

1.0 INTRODUCTION

Communicable diseases have become a burden to most African countries. Notwithstanding this fact, it is also becoming very obvious that non-communicable diseases like cancers also require the attention of health care providers. The burden of cancer in Africa promises to increase over the coming years due to changes in diet and lifestyle (Jones, 1999).

The International Agency for Research on Cancer (IARC, 2014) reported that, about 715,000 new cancer cases and 542,000 cancer deaths occurred in 2008 in Africa and these numbers are projected to nearly double (1.28 million new cancer cases and 970,000 cancer deaths) by 2030. This could be because of the aging and growth of the population and could even be higher due to the adoption of certain behaviours and lifestyles associated with economic development, for example, physical inactivity, unhealthy diet and smoking (IARC, 2014).

In Africa, cancer continues to receive low public priority, despite this growing burden. This could be attributed to limited resources and other urgent health problems which include some communicable diseases like Ebola, HIV/AIDS and malaria (Rajesh *et al.,* 2011). It is obvious that the best way to "outwit" cancer is to prevent it altogether. A lot of researches have been made into this effect, with most of them still ongoing. Research organizations, for example, the American Cancer Society, remain committed to finding innovative ways to prevent the disease. Even though these researches have made contributions to our understanding of how to prevent cancer, deeper understanding on what factors prevent cancer is still being sought for. Over the next 20 years or even

more, researchers expect to learn more about how lifestyle, environment and genetic factors affect cancer risk (IARC, 2014).

The fact, however, remains that lots of people are suffering from cancer. Treatment options available for cancers include chemotherapy, surgery, hormone therapy, radiotherapy, cryotherapy, among others. The standard method of treating these people has been chemotherapy, which comes with its numerous side effects – nausea, toxicity, excessive hair loss, among others (WHO, 2009). Currently, researchers are looking for alternative ways of treating cancer with minimum or no side effects.

Plants have been indispensable in treating diverse forms of diseases including cancer. According to the World Health Organization, 80% of the people living in developing countries including Ghana depend on medicinal plants for their healthcare needs. These practices are solely based on the knowledge of traditional use of medicinal plants (WHO, 2002). *Ficus pumila* is one of such plants. Scientifically, a preliminary work on this plant revealed some anticancer potential using the crude ethanolic extract against leukemic cell lines (Larbie *et al*., 2015b). This current study sought to investigate the fractions of this plant for anticancer activity and to identify the active principle involved in its mode of action. When significant observations are made with this plant in this study, a further study could be conducted to investigate its activity *in vivo*.

1.1 PROBLEM STATEMENT

Curative surgery is the first option for patients with early-stage cancer while radiotherapy and chemotherapy have proven to be effective treatments for patients in the advanced stages. However, the curative effect of allopathic chemotherapeutic drugs

is limited, expensive and their side effects such as neurological and/or renal and cardiac toxicity are devastating.

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

Furthermore, antioxidants like ascorbic acid, gallic acid esters, butylated hydroxyl toluene (BHT), butylated hydroxy anisole (BHA) and tertiary butylated hydroquinone, have been suspected to cause negative health effects at very high concentrations (Delanty and Dichter, 2000). Hence, strong restrictions have been placed on their application and there is a demand to substitute these with naturally occurring antioxidants.

Traditional plant remedies have been used for the treatment of diseases like cancer for centuries, but only a few have been scientifically evaluated.

Ornamentals which are usually cultivated for aesthetic purposes are found to contain chemical agents that could offer therapeutic potential in cancer treatment. One of such plants is *Ficus pumila.* This plant has been reported safe for use in animals studies (Larbie *et al*., 2016).

1.2 STUDY OBJECTIVE

To evaluate the anticancer effect, antioxidant effect and phytochemical constituents of *Ficus pumila* Linn.

1.2.1 Specific Objectives

- To evaluate the *in vitro* cytotoxic effect of *F. pumila* 50% ethanolic extract on Jurkat (leukemia) and HepG2 (liver) cancer cell lines.
- To evaluate the antioxidant activity and total phenolic content of the 50% ethanolic extract, methanolic and hydro fractions of *F. pumila* using DPPH and Folin Ciocalteu assays respectively
- To determine the heavy metals content in the *F. pumila* raw powder and extract.
- To determine the structure of compounds of the most active fraction.

1.3 JUSTIFICATION

F. pumila is less expensive, readily available and less toxic than synthetic chemotherapeutic drugs hence the need to investigate the anticancer activities of the plant to serve as an alternative source of anticancer agents that will aid in the formulation of cheaper and safer anticancer drugs. Phytochemicals act in synergy with chemotherapeutic drugs in overcoming cancer cell drug resistance and that the application of specific phytochemicals may allow the use of lower concentrations of drugs in cancer treatment with an increased efficacy (Liu *et al*., 2001) and hence, the need for the present investigation of the plant for its antioxidant and phytochemical constituents.

The knowledge about the medicinal uses and phytochemical constituents of *F. pumila* can lead to conservation of the plants. The screening model adopted in the study could be extended to other potential plants in Africa, especially in Ghana which abound in rich diversity of plant sources.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 CANCER

Cancer is a group of diseases involving abnormal cell growth with the potential to invade or spread to other parts of the body. A balance is generally maintained in most organs and tissues where there is cell death and renewal of cells. So many forms of matured cells occur in the body with specific life span. When those cells die, new cells are brought forth or generated through proliferation and differentiation of the diverse forms of stem cells. From time to time, there arise some cells which do not respond to normal mechanisms of growth anymore. They produce a tumour or neoplasm, which expand to an appreciable size (Hejmadi, 2010).

Some tumours are incapable of indefinite growth; they do not invade the surrounding tissues that are healthy. These tumours are referred to as benign. On the other hand, there are other tumours that can continue to grow and invade healthy tissues; these kinds of tumours are referred to as malignant. Cancer is a malignant tumour (Pandey and Madhuri, 2009). Malignant tumour cells are able to move from a tumour and affect other healthy tissues by invading the blood vessels or lymphatic vessels which carry them to these healthy tissues. Once in these tissues, they continue to grow and expand. This process is referred to as metastasis. Several secondary tumours can be generated at different locations from one primary tumour (Alison, 2001).

2.1.1 Oncogene Theory

Oncogenes, also known as cancer-causing genes, can be found in transforming viruses and tissues. They have close relatives in the normal cells. They can be derived from genes in the cells that encode several growth-controlling proteins which are called c-

onc or proto-oncogenes. The gene products of c-onc are proteins whose function in the normal cells is known and include growth factor receptors and signal transducers (Shekar *et al*., 2013).

The oncogene theory states that, "when the proto-oncogenes (c-onc) are mutated or are activated by unusual mechanisms, they display increased expression or inappropriate expression of mutated forms of the gene product, thereby contributing to neoplastic transformation and development of cancer" (Todaro and Heubner, 1972). The transition of a proto-oncogene into an oncogene appears in many cases to accompany a change in the level of expression of a normal growth controlling protein. In human cancer, the oncogenes are aberrantly activated in several forms. These include lymphoma, sarcoma, carcinoma and leukaemia. The main activation mechanisms are chromosomal translocation, point mutation and gene amplification (Shekar *et al*., 2013).

2.2 GLOBAL CANCER TRENDS: A GROWING DISPARITY

2.2.1 Disparities in incidence

Globally, there is a variation in the causes and types of cancer. There are multiple and complex reasons why there is differences in the incidence, clinical characteristics, pathology and mortality of cancer in diverse geographical areas. So many factors or elements play a role in contributing to a higher or lower risk for specific cancers. These elements include genetics, background, environment, socio-cultural, lifestyles and behavioural factors. These factors may interact to decrease or increase these risks. The types and frequencies, as well as the outcome of cancer in these settings may be due to the large differences in the wealth between the developing and developed countries (Denny, 2005).

It should be noted that the rate of mortality and overall age-standardized incidence of cancer among the developing countries remain below those of the developed nations. Cancers associated with infectious diseases and with Western lifestyle provides the starkest demarcation in the incidence of cancer between the developing and developed countries, respectively (Liu *et al*., 2001). Tobacco may cause one out of three cancer deaths in the developed countries (Liu *et al*., 1998). Breast cancer, colorectal and prostate cancers are also evident with high incidence of malignancies associated with industrialized societies. Smoking, lack of exercise and diet, a lifestyle associated with developed countries, has been implicated in the high incidence of these cancer conditions (Jones, 1999).

2.2.2 Disparity in mortality

Cancer has become a leading cause of morbidity and mortality worldwide. In the year 2000, over ten million new cancer cases and seven million cancer deaths occurred; 53% incidence and 56% deaths from the developing countries. These numbers are projected to increase by 29% in the developed countries and 73% in the developing countries by the year 2020 due to the ageing, urbanization and change in dietary habits (Delanty and Ditcher, 2000). Figure 2.1 shows cancer death rates in developing and developed countries for males and females. This makes the future more alarming with respect to cancer cases. In the developing countries, the most frequent types of cancers include the lung, breast, stomach, colorectal and liver cancers (Denny, 2005). Globally, it is expected that cancer mortality will increase by 104% by 2020 Delanty and Ditcher, 2000). In the developing world, the rate of deaths will increase to about 5 times as compared to the established market economies. This disproportional mortality reflects

belated reactions by overburdened health care systems which are not equipped to deal with changing patterns of illness (IARC, 2014).

Mortality rates as a result of most forms of cancers have remained significantly greater in the developing nations as compared to that of the developed nations (IARC, 2014). This could be due to the lack of prevention or early detection. For instance, over 80% of new cases of cervical cancer will occur in the developing countries every year. This is the most common type of cancer in women globally and this leads to 250,000 deaths every year. Late diagnosis and inadequate treatment for advanced cancer also leads to mortality.

2.3 CAUSES OF CANCER IN DEVELOPING COUNTRIES

2.3.1 Infectious diseases

Over the last few decades, a lot of infectious diseases have been eliminated through the intervention of government and public health agencies (Parkin, 2006). It has however been, shown that infection is the cause of almost 25% of cancers in the developing nations as compared to less than 10% of cancers in the developed countries (Parkin, 2006). In the developing and developed nations, cervical cancers, stomach cancers and liver cancers have been shown to have the highest incidence and mortality burdens for neoplasms with infectious disease causes. Cervical cancers and colorectal cancers have been linked to the Human Papilloma virus (HPV) in both developing and developed countries (Parkin, 2006).

Even though these cancers caused by the oncogenic HPV have been attributed to developing and developed countries, their incidence is 5 times higher in the developing countries (Delanty and Ditcher, 2000). The absence of Pap cytologic screening (smear

test) results in the higher burden of cervical cancer in the developing economies. There are other co-factors that may also increase the risk of cervical cancer. These include early sexual life and male circumcision. There could be some regional disparities in the malignancy potential of some types of HPV. In Latin America and China, lesions induced by HPV31 and HPV58, respectively are more likely to advance to cervical carcinoma, when compared with Europe (Parkin, 2006).

Recent clinical trials of bivalent and tetravalent vaccines have shown that there is hope for prophylactic immunization against the HPV that causes cancer (Koutsky *et al*., 2002). Nonetheless, the promise of the HPV vaccine must be tempered by experience with diseases that are preventable by vaccine in the developing nations.

Long term infection with Hepatitis B Virus (HBV) has been a cause of hepatocellular carcinoma. Currently, around 90% of the world's liver cancer burden has been attributed to HBV which still occurs in the less developed countries although other vaccines against HBV which are effective and relatively inexpensive have been available for decades. It is not very clear as to when and what degree of upcoming HPV vaccines will be available out the developed nations and how effective they will be across a heterogeneous population (Parkin, 2006).

To decrease the burden of HPV infections and the outcomes of their potential malignancies, cytologic screening and prevention through the use of condom should be encouraged. Patients with Epstein Barr virus (EBV) infections are most likely to have nasopharyngeal cancers. This type of cancer has a higher incidence in the developing nations, as compared to the developed nations (Parkin, 2006). In the less developed countries, there are 10 to 60 times more cases of lymphoma and nasopharyngeal cancers which are attributed to EBV than the more developed countries (Parkin, 2006).

HIV/AIDs pandemic has led to widespread outgrowth of Kaposi sarcoma, which is caused by human herpes virus 8 (HHV8) (Orem *et al*., 2004). There have been noted increases in the incidence of all HIV-related cancers that are caused by viruses in many less developed countries, particularly the Sub-Saharan African countries. These HIV-related cancers include squamous cell carcinoma conjunctiva (HPV), cervical and colorectal cancer (HPV), Kaposi sarcoma (HHV8), hepatocellular carcinoma (HBV) and non-Hodgkin's lymphoma (EBV) (Orem *et al*., 2004). Cancers of the bile duct and urinary bladder (relatively uncommon) are associated with liver flukes and Schistosoma, respectively; they occur only in developing countries (Parkin, 2006).

Infectious diseases are significant because of their association with increased economic cancer burden. There is evidence to suggest that economic burden of cancer may be significant even though few studies exist on this subject matter. It has also been found that the economic cancer burden increases substantially when the effect of the infectious diseases and its relationship to cancer is considered (Orem *et al*., 2004).

2.3.2 Environmental and dietary factors

Oncogenesis involves both genetic and environmental factors and hence, it is often considered as multifactorial. There are other influences from the environment that may be carcinogenic alone, in addition to the infectious agents, or in combination with one or more other predisposing factors. There are several environmental factors that cluster in specific geographic regions. Oral cancers, for instance, as a result of widespread betel nut and tobacco chewing practices, are extremely prevalent in regions such as India and South-East Asia. Lung cancer is also highly prevalent at regions such as China and Asia, where there is abundance of indoor air pollution or radon and smoking (Rastogi *et al*., 2004).

Stomach cancer continues to be a highly prevailing malignancy in the developing nations as compared with Europe and North America. Even though China, Ecuador, Chile and Costa Rica are striking examples, there is a high prevalence of stomach cancer in some developed countries such as Korea and Japan. Though the reasons for this observation or pattern are unclear, it is likely to be associated with the conservation of food with refrigerator, a more varied diet and smoking. Rastogi *et al*. (2004) reported that, *Helicobacter pylori* infection has been associated with about two to three times increased risk of cancer which include carcinoma of the stomach and gastric lymphoma, with convincing evidence.

According to Parkin (2006), *Helicobacter pylori* infection's overall prevalence is 74% in developing nations and 58% in the developed nations. Gastric cancer can result from highly salted foods and this is supported by the observation that people in some countries where there is consumption of large amounts of salted foods in spite of the availability of refrigerators, like Japan and Korea, have high prevalence of neoplasm. Like most malignancies, there is the likelihood that combinations of some factors, which include diet and environment, have a strong influence on gastric cancers (Rastogi *et al*., 2004).

2.3.3 Effects of tobacco

Tobacco use, both smoking and chewing, poses the greatest threat, among all other environmental factors, and contributes to cancer prevalence and mortality considerably in the developing countries and are associated with increased risk of cancer (IARC, 2014). In the developing country, this risk is increasing. From estimation, 8.8% of deaths (about 4.9 million) and 4.1% of disability-adjusted life years (DALYs; about 59.1 million) is caused by the use of tobacco worldwide (IARC, 2014). By comparing

these estimates for the year 2000 with those for the year 1990, a rapid evolution of the epidemic of tobacco can be illustrated. At least, there are one million more deaths which are attributed to the use of tobacco, and this rise has being most observed in developing countries according to WHO (2002).

Over the last part of the 20th century, enormous increases in smoking have been observed in developing countries, most especially with the males (IARC, 2014). The rate of smoking remains relatively high in some or most transition countries. In some industrialized countries, this contrasts with steady but slower decreases, especially among the men. In some high-income countries, the use of tobacco prevalence has declined even though it is on the rise in some low and middle-income countries, chiefly among young people and women (IARC, 2014).

Liu *et al*. (1998) reported that, the estimates of the impact on the health of smokers in China and India have shown a considerable increased risk of morbidity and mortality among the smokers. Tobacco accounts for a substantial proportion of mortality, especially in populations where smoking has been common for many years, which is illustrated by the estimates of smoking-attributable deaths in the industrialized nations. Numerous studies in diverse populations have documented the adverse effects of tobacco on the oral health (Reibel, 2003; *Liu et al*., 1998). These studies showed that smokers have a considerably higher risk of oral cancer than the non-smokers (Reibel, 2003).

Chewing and smoking cigarette are both hazardous even though smoking cigarette causes the majority of the adverse health effects of tobacco. Areca (betel) nut use comes with adverse side effects. These include oral and oropharyngeal cancer, gum disease, oral premalignant lesions and conditions (oral leukoplakia submucous fibrosis)

and addiction (Reibel, 2003). A lot of studies have demonstrated that, the chewing of tobacco which contains areka nut is highly prevalent among the adults even though this practice starts at their young age (Reibel, 2003; Kanavos, 2006; Liu *et al*., 1998). There is a rising concern as many users develop dependency on the use of tobacco, with regards to its consequences for the oral health (Kanavos, 2006).

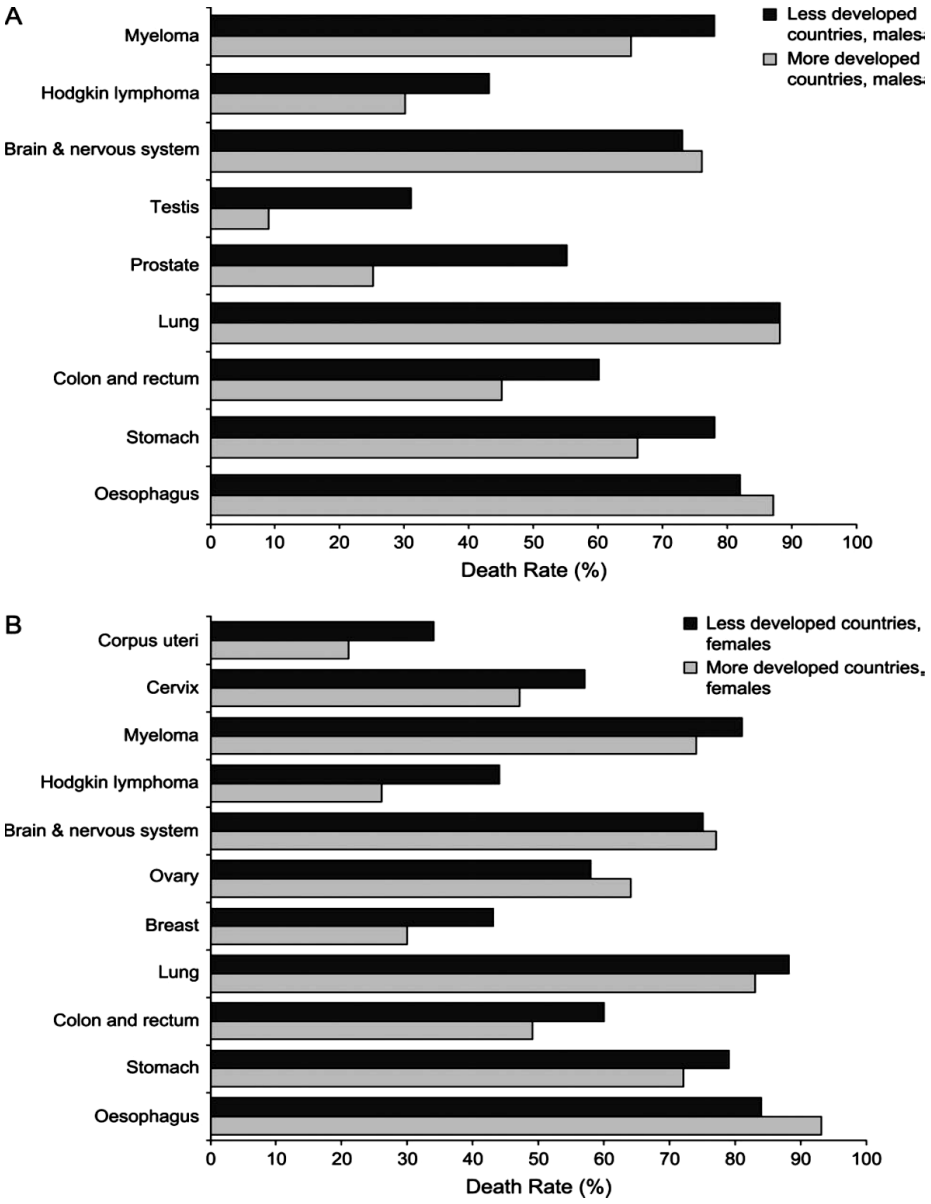

Figure 2.1: Cancer death rates in developing and developed countries for males (A) and females (B) (Kanavos, 2006)

The table 2.1 below shows top ten sites of cancers that were recorded in Kumasi according Laryea *et al*. (2014)

Table 2.1: Top ten sites of cancers recorded in Kumasi (both sexes)

Site	Frequency	Percentage
Breast	61	24.1
Cervix	52	20.6
Ovary	20	7.9
Liver	16	6.3
Prostate	10	4.0
Endometrium	8	3.2
Stomach	5	2.0
Lung	4	1.6
Kidney	3	1.2
Urinary bladder	3	1.2
Others	71	28.1
Total	253	100.0

(Laryea et al., 2014)

2.4 IMMUNODIAGNOSIS OF CANCER

The immunodiagnosis of cancers has two separate goals:

i. The immunological detection of antigens specific to tumour cells and

ii. The assessment of the host's immune response to the tumour.

Immunodiagnosis is based on immunologic X-reactivity, and immunological methods may be used to detect tumour antigens and other markers in cases where tumour antigens exhibit similarities from individual to individual.

If there is X-reactivity, antibody or lymphocytes from individuals with the same type of tumour would be expected to react with the X-reactive tumour antigens regardless of the individual from which they have been derived (Toru *et al*., 2001).

2.5 IMMUNOLOGIC DETECTION OF TUMOUR ANTIGENS

Tumour markers may be cytoplasmic, cell surface or secreted products that are different in nature and/or quantity from those produced by their normal counterparts (Taketa *et al*., 1990). The use of monoclonal antibodies has greatly enhanced the specificity of immunodiagnosis of tumour cells and their products. Some of the most widely used and reliable procedures are:

2.5.1. Detection of myeloma proteins produced by plasma cell tumours

Abnormally high concentration in serum of monoclonal immunoglobulins of a certain isotype, or the presence of light chains of these Igs (Bence-Jones proteins) in the urine, is indicative of plasma cell tumours. The concentration of these myeloma proteins in the blood or urine is a reflection of the mass of the tumour. Therefore, the effectiveness and duration of therapy may be monitored by measurement of the concentration of myeloma proteins in the serum and urine.

2.5.2. Detection of Alpha-Fetoprotein (AFP)

AFP is a major protein in foetal serum. After birth, the level of AFP falls to approximately 20 mg/ml. Levels of AFP are elevated in patients with liver cancer, but they are also elevated in non-cancerous hepatic disorders such as cirrhosis and hepatitis. However, concentrations of AFP of 500-1000 ng/ml are generally indicative of the presence of a tumour that is producing AFP, and monitoring AFP levels is indicative of repression or progression of the tumour (Taketa *et al*., 1990).

2.5.3. Carcinoembryonic Antigen

Carcinoembryonic antigen (CEA) is a term applied to a glycoprotein produced normally by cells that line the Gastrointestinal Tract (GIT), in particular the colon. If the cells become malignant, the CEA is released into the blood instead of the colon (Moertel *et al*., 1993). Concentrations in the blood of CEA exceeding 2.5 ng/ml are generally indicative of malignancy, and monitoring CEA levels is helpful in monitoring tumour growth or regression. In some cases, higher than normal levels of CEA in blood may be due to non-cancerous diseases, such cirrhosis of the liver or inflammatory diseases of the intestinal tract and lung.

2.5.4. Detection of prostate-specific antigen

Prostate-specific antigen (PSA) is currently widely used for screening and early detection of prostate cancer. Levels above 8-10 ng/ml blood are suggestive of prostate cancer (Han *et al*., 2003). Confirmatory tests are required since prostatitis and benign prostate hypertrophy also may result in the release into the bloodstream of the PSA derived from glandular prostate epithelium. The test is particularly useful for monitoring significant increases or decrease of blood levels of PSA that correlate with increase or decrease in tumour size.

There are other ‘markers’ associated with malignancies, such as enzymes and hormones that can be detected by immunologic methods. The immunologic detection of tumours has recently been vastly improved by the availability of highly specific, anti-tumour monoclonal antibodies. These antibodies are currently gaining use not only in the detection of antigens and products associated with the presence of tumour cells but also for their efficacy in the localization and imaging of tumours.

Injection of radio-labelled tumour specific antibodies (radioimmuno-conjugates) into the tumour-bearing individual permits visualization by computer-assisted tomography (CAT) of radiolabelled antibodies attached to the tumour. This method allows the detection of small metastases as well as the primary tumour mass (Han *et al*., 2003).

2.5.5. Cancer Antigen – 125

Diagnosing and monitoring therapy for ovarian cancer involves the immunodiagnostic measurement of serum cancer antigen 125 (CA-125) levels. Circulating levels of CA-125 also increases during peritoneal inflammatory processes (Melina *et al*., 2003).

2.5.6. Other markers

B72.3 is a monoclonal antibody that recognizes all carcinomas in humans (pancarcinoma antigen). This reagent is being used in tumour localization studies to find occult tumour deposits (Slingluff, 1996).

2.6 PREVENTION OF CANCER

A combination of genetic and environmental factors results in cancer. One of the effective means of preventing cancer is by changing any lifestyle that predisposes a person to carcinogens found in the environment. According to Pandey and Madhuri (2009), people who live a lifestyle that minimizes their exposure to sunlight, tobacco and pollution can greatly reduce their risk of cancer development. Foods that contain antioxidants and some other nutrients may help to prevent cancer, especially diet with large amounts of colourful fruits and vegetables, as recommended by the National Cancer Institute in 1993. These foods contain some phytochemicals and some vitamins such as Vitamin A, C and E as well as other antioxidants. These help to prevent cancer

according to Aditya *et al*. (2013). Cancer can be prevented by eating diets which are very rich in fruits and vegetables. These diets are not only shown to reduce the risk of cardiovascular disease, diabetes and obesity but also, they help to protect the body against cancer. Pandey and Madhuri (2009) reported that, some vaccines also offer some promise of cancer prevention.

Hepatitis B has been found to be associated with liver cancer. Hepatitis B vaccine was the first vaccine that was used to prevent cancer. There is a vaccine for Hepatitis B which can prevent hepatitis B as well as any cancer that follows its infection (Saslow *et al*., 2007). Test results from papillomavirus vaccine showed that the Human Papilloma virus type 16 affected almost 20% of adults, in the year 2002. This infection does not lead to cancer, but there has been some association of these papillomavirus infection with cervical cancer (Aditya *et al*., 2013). In a study done in the United States, papillomavirus vaccine was administered to 1200 women and in the space of 18 months, there was elevated levels of antibodies to the virus, as produced by the vaccine. Additionally, the vaccine prevented both infections from papillomavirus and any precancerous lesions in all the women. However, 41 infections and 9 precancerous lesions were found in the control group which consisted of 1200 women who were not administered with the vaccine (Aditya *et al*., 2013).

Genital warts which is also caused by this strain of virus, can also be prevented by the vaccine. It is obvious that virus-associated cancers can be fought against using some of these vaccines (Future I/II Study Group. 2010).

2.6.1 Tumour Immunoprophylaxis

Immunization against an oncogenic virus would be expected to provide prophylaxis against the virus and hence against the subsequent induction of tumour virus. This approach has been successful in the protection of chickens against Marek and a significant degree of protection against feline leukaemia has been achieved by immunizing cats with the oncogenic virus (Hardy *et al*., 1973). Vaccines against HPV are commercially available for the prevention of cervical cancer in women (Saslow *et al*., 2007).

There are reports of effective immunization against transplantable (transferable) animal tumours as immunogens:

i. Sublethal doses of live tumour cells

ii. Tumour cells in which replication has been blocked

iii. Tumour cells with enzymatically or chemical modified surface membranes

iv. Extract of antigens from the surface tumour cells (either unmodified or chemically modified).

Despite these reported successes in the protection of experimental animals against transplantable tumours, the efficacy of immunoprophylaxis for protection of humans and animals against spontaneous tumours has not been sufficiently evaluated. This lack of complete study relates to the need for appropriate immunogens and the danger of inducing the production of immunological elements that may, in fact, enhance metastasis and thus be detrimental to the host (Provinciali and Smorlesi, 2005).

2.7 TREATMENT REGIMES OF CANCER AND EFFECTS

In a case where the cancerous cells have not spread enough to invade other tissues, surgery is the most effective option to deal with such a situation. Radiotherapy and combination of anticancer drugs are other ways of killing or destroying the cancerous cells. In this case, the drugs used are designed with substances that are radioactive. When they are sent into the body, they are able to locate the cancerous cells and thereby destroy then by disrupting their DNA synthesis, which in turn affect their cell division (Mauceri *et al*., 1998). This kind of treatment comes with a disadvantage. This occurs when the drugs are not able to differentiate the cancerous cells from the normal body cells. When this happens, the drugs destroy the cancerous cells together with the normal body cells.

In the advanced stages of cancer, other drugs such as alkylating agents, among others, are used in cancer treatment (chemotherapy). There are a lots of sides effects experienced by the patients undergoing chemotherapy. Among these side effects is anaemia which is due to the fact that the functions of the bone marrow of patients have been affected by the drugs. There are other side effects which include vomiting, hair loss, and fatigue (Alison, 2001; Pandey and Madhuri, 2006).

The pathogenesis of several diseases like cancer, reperfusion disorder, atherosclerosis and diabetes mellitus have been associated to these ROS and free radicals. Their deleterious effects have been demonstrated to be prevented by the appropriate consumption of foods which contain antioxidants like herbs and vegetables (Leong *et al.*, 2008). Intake of natural antioxidants has therefore been shown to reduce the risk from these diseases (Liu *et al*., 2001). It should however be noted that, these antioxidant, although, are very essential for survival, they also come with some

damaging effects. Hence, complex systems of various types of antioxidants such as vitamin C and glutathione are maintained in animals and plants (Sirisha *et al*., 2010).

In the field of drug discovery and development, the use of natural products has played significant roles especially as agents against cancer and infectious diseases. The United States Food and Drugs Administration has approved 62% of cancer drugs of plants or natural origin (Balunas and Kinghorn, 2005).

2.8 FREE RADICALS AND ANTIOXIDANTS

2.8.1 Free radicals

Free radicals can be defined as chemicals that are highly reactive and have the potential to harm living cells. When an atom of molecule gains or loses an electron, it leads to the creation of free radicals. Even though they are naturally formed in the body and contribute to several normal cellular processes, they can be harmful to the body and can destroy all the major components of the cells including DNA, proteins and cell membranes, at high concentrations (Diplock *et al.*, 1998).

Cancer development as well as other health conditions may result from the damage caused by these free radicals, particularly the damage caused to DNA (Valko *et al.*, 2007). The exposure of the body to ionization radiation and other environmental toxins can lead to the abnormal high concentrations of these free radicals in the body. An electron of an atom or a molecule can be lost when ionization radiation hits the atom or molecule and this can lead to the formation of a free radical. The mechanism by which ionization radiation destroys cell is the production of abnormally high levels of free radicals. The production of more free radicals can be stimulated in the body cells by exposure to some environmental toxins which may include cigarette smoke, some metals and high oxygen atmospheres. These may contain high levels of free radicals.

Oxygen-containing free radicals are the most common types of free radicals that are produced in the living tissue. They can also be referred to as reactive oxygen species (ROS) (Valko *et al.,* 2007).

2.8.2 Antioxidants

Free radicals can be neutralized by chemicals called antioxidants, also known as free radical scavengers, thereby preventing them from causing any damage (Patterson *et al.*, 1997). Some antioxidants can be synthesized by the body to neutralize free radicals and these are referred to as endogenous antioxidants. Examples are glutathione, alpha-lipoic acid, coenzyme Q, ferritin, uric acid, bilirubin, catalase, among other. The rest of the antioxidants needed by the body is provided from exogenous or external sources, primarily through diet, known as the dietary antioxidants. Examples of rich sources of dietary antioxidants are fruits, vegetables, grains and dietary supplements (Bouayed and Bohn, 2010). Beta-carotene, vitamins A, C and E and lycopene are specific examples of dietary antioxidants.

Phenolics, also known as phenolic acids are metabolites from plants that are widely spread through the plant kingdom. Their protective role against oxidative damage diseases such as stroke, cancers and heart diseases, has led to the recent interests. Phenolic compounds are very crucial for plant growth and reproduction. When plants are injured, phenolic compounds are produced as a response for defending or protecting these plants from pathogens (Cai *et al*., 2004). In recent years, the role of phenolic compounds as antioxidants and their possible usage as a natural antioxidant in processed foods have reached a new high level according to Huang *et al.* (2009). Phenolics have been suggested as responsible for the antioxidant activities of

Dioscorea bulbifera, Eriobotrya japonica, Tussilago farfara and Ephedra sinica with high correlation between antioxidant capacity and total phenolics (Li *et al.,* 2012).

2.9 PHYTOCHEMICALS

Phytochemicals refer to plant chemicals that are non-nutritive and can protect the body against diseases. They are not required by the body to sustain life and hence are known to be non-essential nutrients. Even though these chemicals are produced by plants to protect the plant itself from diseases, it has been demonstrated that they can also prevent diseases in humans. A lot of phytochemicals exist in nature. Examples include tannins, saponins, flavonone, isoflavones, lycopene, among others (Wadood *et al*., 2013)

2.9.1 How phytochemicals work

Each phytochemical works in a different way. Some of these possible ways include:

As antioxidant: Most phytochemicals help protect the cells against free radicals and oxidative damage, and help to minimize the risk of developing cancer. Examples of some of these phytochemicals which provide antioxidant activity include carotenoids found in fruits and carrots, flavonoids found in some fruits and vegetables, allyl sulfides found in leeks, onions and garlic, polyphenols found in grapes and tea, among others (Youdim and Joseph, 2001).

Hormonal action: Phytochemicals, such as isoflavones in soy, help to reduce the risk of osteoporosis and menopausal symptoms. They do so by imitating the human estrogens (Virgili and Marino, 2008).

Enzyme stimulation: Phytochemicals like indoles (commonly found in cabbages), help stimulate the enzymes that produce the estrogen less effectively. They could even reduce the risk for the development of breast cancer (Ohno *et al*., 2002).

Interference with DNA replication: Cancer cell multiplication can be prevented by some phytochemicals such as saponins (found in beans) by interfering with the cell DNA replication. Capsaicin is another phytochemical which offers protection of DNA from carcinogens. They are mostly found in hot peppers (Kelly *et al*., 2001).

Physical action: Some phytochemicals provide an anti-adhesion property by binding to the human cell walls and thus preventing pathogens from adhering to the cell walls. Example is proanthocyanidins found in cranberry, which help to reduce urinary tract infection risks and improve dental health (Wadood *et al*., 2013).

2.10 MEDICINAL PLANTS AS ANTICANCER AGENTS

Medicinal plants have been known to be rich sources of drug discovery compounds. They have been essential in managing several forms of ailments which include cancer (Balunas and Kinghorn, 2005). According to the World Health Organization in 2009, four out of five people in the rural areas rely on medicinal plant for the treatment of diseases. They prefer this kind of treatment because they consider medicinal plants as safe with no or minimal side effects. These practices are based on the ethno-knowledge of these plants. Natural products are developed to produce several types of drugs which are effective to enhance anticancer activities. However, Fennell *et al*. (2004) reported that several plants that are used as food or traditional medicine could be toxic, carcinogenic or mutagenic. Studies have also shown that the use of traditional herbal

treatments has been implicated in about 35% of all renal failure cases in Africa (Isnard *et al*., 2004).

Chemotherapy comes with high cost and this means that several cancer patients in the developing nations cannot afford this treatment, therefore they rely on these herbal medicines for their treatment. The efficacy, toxicity and safety of many of these plants have not been scientifically evaluated. Narah *et al*. (2012) reported that a complete understanding of the complex synergistic interaction of the various anticancer plants' constituents would be helpful in formulating the design of anticancer agents which can attack the cancerous cells without damaging the normal body cells.

Between 1940 and 2002, the anticancer drugs available had 40% of its composition to be either natural products or their derivatives, with another 8% considered natural product mimics (Newman *et al.*, 2003).

2.10.1 Mechanistic action of anticancer agents

There are four major classes of anticancer agents from medicinal plants which are currently in clinical use. These are grouped as Vinca alkaloids (otherwise called *Catharanthus* alkaloids), taxanes, camptothecins and epipodophyllotoxins. For example, vincristine and vinblastine which are vinca alkaloids were isolated from *Catharanthus roseus* (L.) G. Don (Apocynaceae) (formerly *Vinca rosea* L.) and have been used clinically for more than 40 years. These antincancer drugs are primarily used either alone or in combination with other chemotherapeutic drugs for the treatment various cancers, including breast and lung cancers, advanced testicular cancer, lymphomas, Kaposi's sarcoma and leukemias (Cragg and Newmann, 2005). The mechanism of Vinca alkaloids and several of their semi-synthetic derivatives is the arresting of metaphase stage of mitosis which occurs by binding specifically to tubulin

resulting in its depolymerization (Okouneva *et al.*, 2003). Podophyllotoxin was isolated from the resin of *Podophyllum pelatum* L. (Berberidaceae) but was found to be too toxic in mice therefore derivatives were made with the first clinically approved drug being etoposide (Gordaliza *et al.*, 2004). Epipodophyllotoxin is an isomer of podophyllotoxin, which was isolated as the active antitumor agent from the roots of Podophyllum species, *Podophyllum peltatum* Linnaeus and *Podophyllum emodi* Wallich (Berberidaceae) (Stahelin, 1973). Etoposide and teniposide are two semi-synthetic derivatives of epipodophyllotoxin and are used in the treatment of lymphomas, bronchial and testicular cancers (Cragg and Newman, 2005). The mechanism of action for epipodophyllotoxins is through binding tubulin, causing DNA strand breaks during the gap two (G**2**) phase of cell cycle by reversibly inhibiting DNA topoisomerase II (Gordaliza *et al.*, 2004). Homoharringtonine, isolated from the Chinese tree *Cephalotaxus harringtonia* var. drupacea (Sieb and Zucc.) (Cephalotaxaceae), is another plant-derived agent in clinical use (Itokawa *et al.*, 2008). A racemic mixture of harringtonine and homoharringtonine has been used successfully in China for the treatment of acute myelogenous leukemia and chronic myelogenous leukemia (Cragg and Newman, 2005).

2.10.1 Anticancer activities of some plants

More than 300,000 higher plants exist on the surface of the planet; therefore active research into medicinal plants will hopefully help isolate more bioactive principles that can inhibit the growth of cancer cells but not normal cells and the molecular mechanisms of action will also be elucidated. Curcumin, a historically acknowledged component of the Ayurvedic, Unani, and Siddha medicine, and indigenous to Southern and Southeastern tropical Asia, has extensively been reported to having promising

anticancer activities (Ravindran *et al*., 2009). As the major chemical component of turmeric (*Curcuma longa*), *in vitro* cell culture and *in vivo* animal studies have suggested its effectiveness to treating numerous types of cancers: breast, colon, kidney, liver, leukemia, prostate, rhabdomyosarcoma, and melanoma (Reuter *et al*., 2008). It has been reported to be cytotoxic to Jurkat cells and induce a caspase mediated apoptosis in these cells. Its anti-leukemic property has been attributed to its ability to regulate the expression of both pro and anti-apoptotic proteins e.g. Bad, Bim, Caspase-3, p27, Bak, Bcl-2, Bax, Mcl-1, PARP (Reuter *et al*., 2008). Quercetin, isolated from methanol extract of *Asparagus cochinchinensis* (Lour.) Merr tuber exhibited strong cytotoxicity against the HeLa, human cervical cancer cell line with IC50 = 5.78 ± 0.36 µg/ml, followed by lung cancer cell line (NCI–H460), with IC50= 12.57 ± 1.19 µg/ml and liver cancer cell line (Hep-G2) with IC50 = 20.58 ± 0.85 µg/ml (Son and Anh, 2013). The anticancer activity of quercetin against breast cancer cell line (MCF-7), was recorded (IC50 = 31.04 ± 3.14 µg/ml) using the sulfurhodamine B assay *in vitro* (Le Son and Anh, 2013*).* Out of the ten compounds isolated from ethanolic extracts of *Saxifraga stolonifera* (L.) Meeb, four of them showed anticancer activities on human gastric carcinoma cell line BGC-823 (Narah *et al.*, 2012). Chen *et al.*, (2008), also studied the effects of extracts from *S. stolonifera* on human gastric carcinoma cell line (BGC-823) *in vitro*, using the MTT assay at a dose range of 5-100 µM. They found that the inhibitory effects of gallic acid, quercetin and b-sitosterol were concentration dependent. Among the isolates, quercetin exhibited the highest cytotoxic effect on BGC-823 cells, with growth inhibition ratio of 39.3% after 72 hours of treatment at 100 µM, while the other compounds exhibited lower growth inhibition 33 ratios, ranging from 6.6-22.5% the same after 72 hours treatment. Also, studies conducted by Jain and Jain (2011) showed that alcoholic extract of *Sesbania*

grandiflora exhibited a prominent inhibitory effect against MCF-7 (IC50= 7.00 ± 0.08 µg/ml) and HL-60 (IC50= 18.50 ± 0.6 µg/ml) under *in vitro* conditions using the MTT assay.

Table 2.2: Cytotoxic activities of some selected Ghanaian medicinal plants

Plant	Part used	Cell Line	IC_{50}	Reference
Adenia lobata	Root	DLD-1 MCF 7	170 ± 3.5 mg/ml 230 ± 1.2 mg/ml	Ayim *et al*., 2007
Croton membranaceus	Root	DLD-1 MCF 7	16.0 ±1.0 mg/ml 17.4 ±1.6 mg/ml	Ayim *et al*., 2007
Zanthoxylum xanthoxyloides	Bark	DLD-1 MCF 7	16 ± 1.8 mg/ml 43.3 ± 3.1 mg/ml	Ayim *et al*., 2007
Ageratum conyzoides	Leaves	Jurkat LNCap	15.08 ± 0.28 µg/ml 304.22 ± 71.54 µg/ml	Acheampong *et al*., 2015
Codiaeum variegatum	Stem bark	Jurkat MCF 7 PC 3 WRL 68	59.71 ±12.20 µg/ml 35.55 ± 1.50 µg/ml 52.54 ± 1.88 µg/ml 49.37 ± 2.7 µg/ml	Anim *et al*., 2016
Amaranthus viridis	Leaves	Jurkat HL60	111.41 µg/ml 122.5 µg/ml	Larbie *et al*., 2014
Ficus asperifolia	Leaves	DLD-1 MCF 7	45 ±1.0 µg/ml 49 ± 3.2 µg/ml	Ayim *et al*., 2007

2.11. FICUS PUMILA LINN.

Synthetic chemicals used for medicines tend to pose a great deal of side effects aside their high costs (Hoareau and DaSilver, 1999). The demand for drugs is exceptionally high in recent times as diseases emerge and/or remerge. Ficus has one of the largest genus of about 800 different species (Zhang *et al.*, 2011). These plants contain certain phytochemicals that accounts for their active biological activities (Edeoga *et al.*, 2005).

2.11.1 Classification

Kingdom:	Plantae
Phylum:	Tracheophyta
Class:	Magnoliospida
Order:	Rosales
Family:	Moraceae
Genus:	Ficus
Species:	pumila

Figure 2.2: *Ficus pumila* plant

The genus *Ficus* belongs to the Moraceae family and is called the fig genus. It mostly grows in tropical areas due to its ability to withstand drought. Plants in this genus are of significant importance due to their numerous medicinal properties and the phytochemicals present in them (El- Hawary *et al.*, 2012). They are used in the treatment of skin diseases, dysentery, leprosy, enlargement of liver and spleen, lung, gonorrhoea, heart diseases, asthma, piles, etc. (Kaur, 2012). Notable species amongst them are *Ficus pumila* L., *Ficus carica* L., *Ficus bengalensis* L. *and Ficus elastica* ex Hornem (EL- Hawary *et al.*, 2012).

2.11.2 Description

Ficus pumila Linn.is a creeping ornamental plant. It is synonymous to *F. repens* Hort., *F. hanceana* Maxim., *F. scandens* Lam., *F. stipulate* Thunb. (Wunderlin and Hansen, 2000) and is commonly known as the creeping fig, climbing fig, creeping rubber plant, because of its creeping nature. It is said to be native to Vietnam, East Asia, south China, New Zealand Nepal, Western Australia, Taiwan, India and Japan (Kaur, 2012). The dense, rapid growth of small, dark green, alternate heart shaped and leathery leaves on slender stems makes creeping fig a favourite vine to grow on walls where it gives a beautiful design in its early stages of growth. The leaves are about 3- 6 cm long and 2-3 cm wide (Kaur, 2012). It needs no support to adhere to a wall. With time, the twigs grow into larger matured leaves on its woody, hairy stem. It also makes a low, dense ground cover only one or two inches high (Gilman, 1999). It grows to a height of 4m/13.1ft and spread of 3m/9.8ft. (www.plantdatabase.ie/ficus_pumila).

2.11.3 Cultivation

F. pumila is a common ornamental plant grown in tropical areas around the world. The plant grows as a vine and can adhere to rock, concrete, and other surfaces by means of a rubbery substance which exudes from aerial roots. It is often planted along rock walls, on sides of buildings, and on other trees (Starr *et al*., 2003).

2.11.4 Invasiveness

A plant is said to be invasive if it is non-native to the environment under consideration and its introduction is likely to cause, or harm human health or the environment. Most plants are invasive due to the rapid nature of their growth (Abbey, 2003). *Ficus pumila*

has been shown to be invasive because it grows at a very fast rate and if allowed, will grow to undesired places (Starr *et al*., 2003).

2.11.5 Medicinal value

Besides phenolic acid compounds, flavonoid glycosides with antioxidant properties have been isolated from the 50% aqueous ethanolic leaf extract of *Ficus pumila*. The flavonoid glycosides which were isolated and identified are rutin, apigenin 6-neohesperidose, kaempferol 3-robinobioside and kaempferol 3- rutinoside. Among these compounds, rutin exhibited the strongest antioxidant activity. These results show that *Ficus pumila* leaves serve as a good natural source of antioxidants and can be used in the treatment of diseases such as cancer (Sirisha *et al*., 2010).

According to Muhammed *et al*. (2013) the leaves of *F. pumila*, has anti-diarrhoeal properties and can be used as a novel lead compound for anti-diarrhoeal drug development. Traditionally, the leaves of *F. pumila* are consumed in Okinawan (in Japan) to treat various diseases including dizziness and neuralgia (Leong *et al.*, 2008). According to Yuri, the fruits of *F. pumila* are used in traditional Chinese medicine for the treatment of breast cancer and attributed its cytotoxicity to the presence of sesquiterpenoids found in them. According to Ashraf *et al*. (2012), the leaves of *F. pumila* show hypolipidemic and hypoglycaemic abilities in streptozotocin-induced diabetic rats. The plant is also said to exhibit anti-inflammatory, anti-ulcer, antihypertensive and antioxidant activities and also has analgesic properties (Liao *et al.*, 2012).

According to Liao *et al.* (2012), the leaves and stems of *F. pumila* folklorically are used in the treatment of various ailments like oedema, throat pain, postpartum disorders,

rheumatoid arthritis, tonic medicament and abdominal pains. The plant contains certain flavonoids like rutin, luteolin, and apigenin which accounts for the anti-inflammatory properties in the plant. However, in their study, the methanol extract of the plant was found not to be acutely toxic due to the fact that the LD_{50} value of FPMeOH was greater than 10 g/kg in the mice.

Ficus pumila is used in Chinesse and Indian ethnomedicine for the treatment of haemorrhoids, piles, diarrhoea, gastrointestinal problems as well as uterine problems (Kaur, 2012). According to Kaur (2012), leaves of *F. pumila* have antimutagenic, antimicrobial and antioxidant activities. The leaves are also used in traditional medicine for the treatment of skin infections, injuries, tuberculosis, high blood pressure and diabetes. However, the leaves and fruits together, are used in treating swellings, fever and even as detoxifiers in traditional Chinese medicine, according to Kaur (2012). In his work, the phytochemical screening of the methanolic extract of the leaves revealed the presence of steroids, tannins, carbohydrates, flavonoids, tritepenoids and phenolic compounds.

Proper understanding of the complex synergistic interaction of various constituents of anticancer plants would help in formulating the design of anticancer agents to attack the cancerous cells without harming the normal cells of the body (Narah *et al.*, 2012)

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 CELL LINES AND REAGENTS

The cell lines for the study included Jurkat, HepG2 and Chang cells and were obtained from the Cell Bank of the Noguchi Memorial Institute for Medical Research (NMIMR), Legon. Culture media used included Dulbecco's Minimum Essential Medium (DMEM), Roswell Park Memorial Institute Medium (RPMI), Foetal Bovine Serum (FBS), supplemented with antibiotics (Penicillin, Streptomycin and Glutamine), Phosphate buffered saline, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye, acidified isopropanol, trypan blue solution, absolute ethanol, ethylacetate, methanol, 2, 2- diphenyl-1-picrylhydrazyl (DPPH), Folin Ciocalteu, and Sodium Carbonate were of analytical grade and were purchased from Sigma Aldrich (USA). All experiments were conducted at the Department of Biochemistry and Biotechnology, Central Laboratory, KNUST-Kumasi and the Clinical Pathology Laboratory of NMIMR, Legon.

3.2 PLANT MATERIAL

Ficus pumila plant (with voucher number KNUST/HM1/2014/L093) was collected near the Bomso Clinic, Kumasi and the leaves were separated from the stems. The leaves were washed, air-dried, pulverized and packaged in zip-locks and stored at room temperature.

3.3 PREPARATION OF PLANT EXTRACTS AND FRACTIONATION

The 50% ethanolic extraction of the leaves was carried out by suspending 6000 grams of the powder of the leaves in 6000 ml of 50% ethanol in distilled water (50:50 v/v).

The extraction was done by cold maceration for 24 hours at room temperature on a shaker. The extract was then filtered through cotton wool, concentrated using a rotary evaporator and freeze-dried to obtain the *F. pumila* ethanolic leaf (FPL) extract.

FPL extract was sequentially extracted with solvents of increasing polarity starting with petroleum ether and followed by methanol. This was done by suspending 60 g of the ethanolic crude extract in 400 ml of the petroleum ether in a separating funnel. The mixture was shaken and left for 48 hours at room temperature after which the liquid portion was separated from the solid residue. This step was repeated for methanol. The remaining residue was designated hydro fraction. For each fractionation step, extraction was performed twice with 400 ml of solvent. The Petroleum ether, methanol and hydro fractions were then concentrated by air drying and freeze-drying for aqueous fraction.

3.3.1 Determination of extract yield

The percentage yield was obtained using the formula; Yield = $(W_2\text{-}W_1/W_0)$ x 100%; where W_2 is the weight of the extract and the container, W_1 the weight of the container alone and W_0 the weight of the initial dried sample.

3.4 HEAVY METAL ANALYSIS

About 1 g of each powdered sample (raw powder and extract) was weighed into 50 ml digestion tube, 1 ml H_2O, 2 mL HCl, 5 mL of 1:1 HNO_3:$HClO_4$ and 2 ml H_2SO_4 were added. Samples were allowed to stand for about 20 minutes at room temperature to enable the foam that formed to settle. They were then heated in a digestion block on a hot plate for about 2 hours at a temperature of 150 ^oC. The digested samples were allowed to cool and diluted to 50 ml. Blanks were prepared alongside. The samples

were then stored for analysis by the Atomic Absorption Spectrophotometer. Metals analysed included lead, copper, cadmium, nickel, zinc and iron.

3.5 TOTAL PHENOLIC CONTENT DETERMINATION

3.5.1 Principle

The total phenolic content assay is on the basis that all phenolic compounds contained in a mixture of antioxidant compounds or extract are oxidized by Folin-Ciocalteu reagent. This reagent is formed from a mixture of phosphotungstic acid, $H_3PW_{12}O_{40}$, and phosphomolybdic acid, $H_3PMo_{12}O_{40}$, which, after oxidation of the phenols, is reduced to a mixture of blue oxides of tungstate (W_8O_{23}), and molybdate (Mo_8O_{23}). The blue colouration produced has a maximum absorption in the region of 750 nm, and is proportional to the total quantity of phenolic compounds originally present.

3.5.2 Procedure

Stock solutions of the ethanolic crude extract, methanolic and hydro fractions were prepared by dissolving 10 mg of each of the dried samples in 1 ml of the respective solvent and filtering. 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 9 mL distilled water to obtain the 5 mg/ml stock solution.

Two-fold serial dilutions were carried out on the gallic acid standard to obtain six different concentrations 5, 2.5, 1.25, 0.625, 0.3125 and 0.15625 mg/ml. An ethanol blank was also prepared. A two-fold serial dilution was also carried out on the samples to obtain three different concentrations of extract and fractions (10, 5, 2.5 mg/ml). Distilled water without extracts, were also prepared as blanks.

A volume of 10 µl of each sample dilutions and gallic acid dilutions were aliquoted into a 2.0 mL eppendorf tube. Aliquots of 790 µl of distilled water were then added and this was followed by the addition of 50 µl of Folin-Ciocalteu reagent. The mixture was mixed thoroughly by vortexing for five seconds. This was followed by incubation of the tubes in darkness at room temperature for eight minutes. Afterwards, a volume of 150 µl of 7% sodium carbonate solution was added to each tube, mixed thoroughly by vortexing for five seconds and further incubation of the tubes in darkness at room temperature was done for two hours. After the two-hour incubation, a volume of 200 µL of each extract and gallic acid were aliquoted into wells on a 96-well plate in triplicate and absorbance read at 750 nm using microplate spectrophotometer (Synergy H1, USA). A graph of absorbance against concentration was plotted for the gallic acid standard. The concentration of phenolics in each of the samples was determined using the gallic acid standard plot and the gallic acid equivalence for each fraction also calculated.

3.6 IN VITRO ANTIOXIDANT ASSAY

3.6.1 Pricinple

When an antioxidant compound or extract that can donate hydrogen reacts with DPPH (a stable N centred radical purple in colour), it reduces the DPPH to yellow colour. This colour change can be measured at 517 nm using a UV/Vis light spectrophotomer. The antioxidant effect was evaluated by calculating the EC_{50} (Effective concentration at 50%) value, which is the concentration of the fraction that can scavenge 50% of the free radicals. The smaller, or the closer this value is to zero, the better the antioxidant effect of the fraction or compound (Larbie *et al*., 2015b).

3.6.2 Procedure

The stock solutions of the ethanolic extract, methanolic and hydro fractions were used for the determination of antioxidant activity as described by Brand-Williams *et al.* (1995). Also, stock solutions of 10 mM of standard (Ascorbic acid) and 0.5 mM of DPPH were prepared by dissolving 0.176 mg of Ascorbic acid and 3 mg of DPPH in 1mL of distilled water and 15 mL absolute methanol respectively. The solutions were then vortexed until complete dissolution was achieved. The DPPH solution was immediately kept in the dark as it photo-bleaches in light.

In 1.5 mL eppendorf tubes, the samples were serially diluted in distilled water to obtain a concentration range of 0.156–10 mg/ml. Hundred microliters of each concentration of the test sample was transferred into a 96 well plate. This was followed by the addition of 100 μL of 0.5 mM DPPH. For positive control or standard, Ascorbic acid was used at a concentration range of 0.156–10 mM in distilled water. Distilled water was used as blank. Triplicate experiments were performed. The plates were covered with aluminum foil, shaken gently and kept in the dark for 20 minutes after which an absorbance was read on the plate reader at the absorbance wavelength of 517 nm. Percentage scavenging activity was determined by;

$$\% \text{ Scavenging} = \frac{[\text{Absorbance of blank (OD}\mathbf{0}\text{) - Absorbance of test (OD}\mathbf{1}\text{)}]}{\text{Absorbance of blank (OD}\mathbf{0}\text{)}} \times 100$$

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

3.7 FTIR SPECTROSCOPIC ANALYSIS

FTIR analysis was performed on each fraction using PerkinElmer Spectrophotometer system from USA, which was used to detect the characteristic peaks and their functional groups. The peak values of the FTIR were recorded and compared to standard values.

3.8 GAS CHROMATOGRAPHY – MASS SPECTROMETRY ANALYSIS

GC-MS analysis of the methanolic and hydro fractions, were performed using a PerkinElmer GC Clarus 580 and a Gas Chromatograph interfaced to a Mass Spectrometer PerkinElmer (Clarus SQ 8 S) equipped with Elite-5MS (5% diphenyl/95% dimethyl poly siloxane) fused with a capillary column (30 × 0.25 μm ID × 0.25 μm DF). For GC-MS detection, an electron ionization system with ionizing energy of 70 eV was used. Helium gas (99.999%) was used as the carrier gas at constant flow rate of 1ml/min and an injection volume of 2 μl was employed (split ratio of 10:1); Injector temperature 80°C; Ion-source temperature 250°C. The oven temperature was programmed from 110°C (isothermal for 2 min.), with an increase of 10 °C/min, to 200°C, then 5 °C/min to 250°C, ending with a 9 min isothermal at 280°C. Mass spectra were taken at 70 eV; a scan interval of 0.5 seconds and fragments from 45 to 450 Da. Total GC running time was noted (29 minutes).

The mass-detector used in this analysis was Turbo-Mass, and the software adopted to handle mass spectra and chromatograms was a Turbo-Mass ver-6.1.0. The components were identified based on comparison of their relative retention time and mass spectra with those of NIST Library data. The results were confirmed by the comparison of the compounds elution and order with their relative retention indices on non-polar phases

reported in the literature. The name, molecular weight and chemical formula of the components of the test material were ascertained.

3.9 PHYTOCHEMICAL ANALYSIS

The crude ethanaolic extract was screened for the presence of alkaloids, tannins, terpenoids, saponins and flavonoids according to the methods described by Ayoola *et al*. (2008) with slight modifications.

3.9.1 Terpenoids

One millilitre of absolute chloroform was added to 10 mg of each extract and standard, ursolic acid, and 1 ml of 0.1M sulphuric acid was subsequently added. A reddish-brown colour at the interface was indicative of the presence of terpenoids.

3.9.2 Saponins

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

3.9.3 Tannins

Ten milligrams of each extract and standard, gallic acid, was boiled with 2 ml of distilled water. The boiled extract was centrifuged to obtain supernatant to which three drops of 0.1% $FeCl_3$ was added to each supernatant. A blue-black colouration indicates the presence of tannins.

3.9.4 Alkaloids

Ten milligrams of standard, quinidine, and crude ethanolic plant extracts were dissolved in 2 ml of acid alcohol (Concentrated HCl in 70% Ethanol) (v/v). The solution was boiled for three minutes and centrifuged to obtain supernatant. One millilitre of dilute ammonia was added to the supernatant. Subsequently, 2 ml of absolute chloroform was added and shaken gently to extract alkaloidal base. The chloroform fraction was then extracted with 2 ml of acetic acid. After adding four drops of Draggendorf's reagent to each extract and standard, a reddish-brown precipitate indicated the presence of alkaloids.

3.9.5 Flavonoids

Two milliliters of dilute ammonia was added to 2 ml portions of aqueous supernatant of each plant extracts and standard, quercetin. Subsequently, 1ml of 0.1 M sulphuric acid was added to the mixture. A yellow colouration that disappears on standing for 5 minutes indicated the presence of flavonoids.

3.9.6 Cardiac Glycoside

One ml aqueous supernatant of the crude extract and its fractions was added to 1 ml of diluted hydrochloric acid and was boiled for 10 minutes in a water bath. Filtrate after boiling was obtained using benzene and 1 ml of ammonia solution added subsequently. Glycoside was indicated through the appearance of red colour in the ammonia layer.

3.9.7 Sterols

A mass of 0.5 g each of extracts was added to 2 ml of acetic anhydride, afterwards 2ml of sulphuric acid was also added. The presence of sterol was indicated by a blue colouration.

3.10 IN VITRO CYTOTOXICITY (MTT) ASSAY

3.10.1 Principle

The MTT assay was used to determine the cytotoxicity of the extract and fractions on the principle that, the mitochondria of metabolically active living cells produce the enzyme mitochondrial reductase which can reduce the yellow water-soluble substrate, MTT, into a purple formazan crystal. This crystal is soluble in acidified isopropanol or dimethyl sulfoxide (DMSO). The purple colour change can be measured at 570 nm with the use of a spectrophotometer, which gives a measure of cell viability.

3.10.2 Procedure

The *in vitro* cytotoxicity of the fractions was performed on cancer (liver - HepG2 and leukemic - Jurkat) and normal (liver – Chang) cell lines. Cells in exponential growth were seeded into 96-well plates at a concentration of 10^4 cells/well. The cells were then treated with various concentrations of the fractions of *F. pumila* at a concentration range of 0–1000 µg/ml (in 1% DMSO). Negative control (untreated) experiment was included. Culture medium was used as blank. A colour control plate was also setup for each test extract. After 72 hours of incubation at 37ºC, under 5% CO_2, in humidified atmosphere, 20 µL of 2.5 mg/mL of MTT was added to each well and the plates were kept in the dark for 4 hours. Subsequently, 150 µL of acidified isopropanol was added to stop the reaction and solubilize the formazan crystals formed. Absorbance readings

were taken at 570 nm on a microplate reader after overnight incubation of the plates in the dark. Triplicate experiments were performed. Dose response curves were plotted as percentages of cell viability against concentration.

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

where ODT_0 is the average absorbance of wells treated with test extracts for all cell lines; ODT_1 is the average absorbance of wells with curcumin or test extract control; ODU_0 is the average absorbance of wells with untreated cells (negative control) for all cell lines; ODU_1 is the average absorbance of wells containing blank (culture media only). The inhibition concentration at 50% (IC_{50}) values, that is, concentration of test substance that caused 50% inhibition of various cell lines were determined from the dose response curves by nonlinear regression analysis. The selectivity index (SI), a measure of cytotoxic selectivity, was calculated for the samples. This is the ratio of the IC_{50} values of each crude extract or fraction in the normal cell line (Chang liver) to IC_{50} values in the cancer cell lines (HepG2 and Jurkat). Samples with SI greater than 2 were considered to have a good selectivity towards cancer cells.

3.11 DATA ANALYSIS

Microsoft Excel Version 2010 was used for the calculation and plotting of mean and S.D estimates in the graph. Mean EC50 and IC50 values were compared by one way ANOVA using SPSS Version 16.0 and values with $p < 0.05$ were considered statistically significant.

CHAPTER FOUR

4.0 RESULTS

4.1 EXTRACT YIELD

Six thousand grams of *Ficus pumila* dry powder yielded 89 g of crude ethanolic extract which is 1.483% yield. Sequential fractionation was done using 60 g of the crude ethanolic extract. In the fractions, methanolic fraction gave a yield of 6.43% (3.809 g). However, petroleum ether gave an insignificant yield. The remaining residue after the entire fractionation step – the hydro fraction, was 56.191 g (93.66% yield).

4.2 HEAVY METAL ANALYSIS

Table 4.1: Heavy metal content in F. pumila raw powder and extract

	Concentration in mg/l		
Heavy Metal	**Blank**	***F. pumila* Raw**	***F. pumila* Ethanolic Extract**
Iron (Fe)	BDL	1.97 ± 0.11	0.92 ± 0.02
Copper (Cu)	BDL	BDL	BDL
Zinc (Zn)	BDL	1.19 ± 0.00	0.66 ± 0.02
Cadmium (Cd)	BDL	BDL	BDL
Nickel (Ni)	BDL	BDL	BDL
Lead (Pb)	BDL	BDL	BDL

- BDL means below detection limit
- Detection Limit – 0.00001mg/l

Heavy metal analysis by Atomic Absorption Spectrophotometry (AAS) revealed that *Ficus pumila* raw powder contained 1.97 mg/l of Iron and 0.19 mg/l of Zinc as shown in table 4.1. The extract also contained 0.92 mg/l of Iron and 0.66 mg/l of Zinc. Out of the six heavy metals analyzed in both the raw and the extracts of *F. pumila*, the levels of the rest of the metals; Copper, Cadmium, Nickel and Lead, were below detection

limit (0.00001mg/l). The content of iron and zinc were within acceptable limits as accepted by the WHO.

4.3 TOTAL PHENOLIC CONTENT

A linear plot of Gallic acid standard produced a straight line (y = 0.2435x + 0.0413, R^2=0.9967) as shown in Figure 4.1. The total phenolic content of the three fractions were extrapolated from the Gallic acid standard curve and was estimated as gallic acid equivalent (GAE)**;** [Phenolic]mg GAE/100g as shown in figure 4.2.

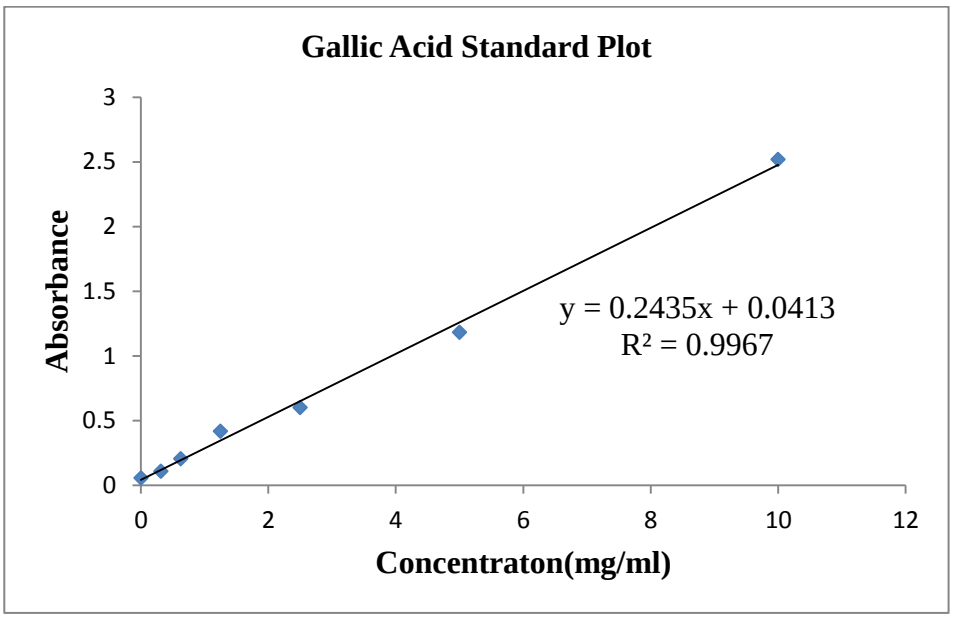

Figure 4.1: Standard concentration curve of absorbance against concentration of Standard Gallic Acid

The hydro fraction recorded the highest total phenolic content (0.85 ± 0.02 mg/ml). This was followed by the crude ethanolic extract (0.69 ± 0.00 mg/ml) and finally, methanolic fraction (0.57 ± 0.04 mg/ml). In terms of Gallic acid equivalence, the total phenolic content in the hydro fraction was found to be 16950 ± 331.95 mg GAE/100 g.

The crude ethanolic extract and methanolic fraction recorded Gallic acid equivalence of 13883.6 ± 47.42 and 11310.1± 716.041 mg GAE/100 g, respectively ($p < 0.001$).

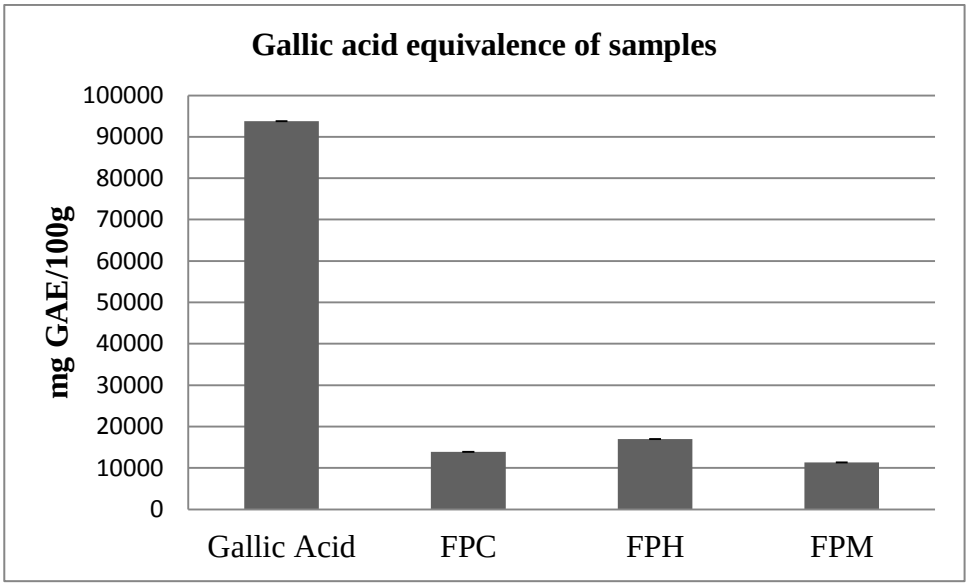

Figure 4.2: Total phenolic contents (mg/ml) in the various F. pumila fractions against Gallic acid standard.

FPC- *Ficus pumila* ethanolic crude extract, FPH - *Ficus pumila* hydro fraction, FPM – *Ficus pumila* methanolic fraction. Each bar represents a mean ± standard deviation, n=3, $p<0.0001$ between extracts and Gallic acid.

4.4 IN VITRO ANTIOXIDANT ASSAY

Table 4.2 shows the comparison of the EC_{50} values of DPPH scavenging activities of the extract and fractions of *Ficus pumila*.

Table 4.2: Antioxidant activities of ascorbic acid and extracts of F. pumila

Sample/Standard	EC_{50} Value (mg/ml) n=3	P value
Ascorbic Acid (Standard)	0.0852 ± 0.0037	
Crude ethanolic extract	0.1722 ± 0.0319	0.016
Hydro fraction	0.0900 ± 0.0006	0.996
Methanolic fraction	0.4170 ± 0.0420	0.000

All fractions reduced DPPH to diphenylpicrylhydrazine, and diminished the absorbance at 517 nm. The results showed that the hydro fraction scavenge DPPH radical more strongly (EC_{50} = 0.09 ± 0.0006 mg/ml) compared to the other fractions, with the methanolic fraction having the least antioxidant activity (EC_{50} = 0.417 ± 0.042 mg/ml), and all followed a concentration dependent pattern compared to the positive control (Ascorbic acid). The EC_{50} value is the effective concentration at which 50% of free radicals are scavenged. The smaller the EC_{50} value, the better the antioxidant effect of the fraction. Generally, the difference between the various EC_{50} values for the different extract were statistically different ($p = 0.002$, $n = 3$).

4.5 FTIR SPECTROSCOPIC ANALYSIS

The Fourier Transform Infrared Spectroscopy (FTIR) analysis was done to determine the functional groups of the active components of *F. pumila* fractions based on peak value in the region of infrared radiation. The results of FTIR analysis of *F. pumila* is shown below.

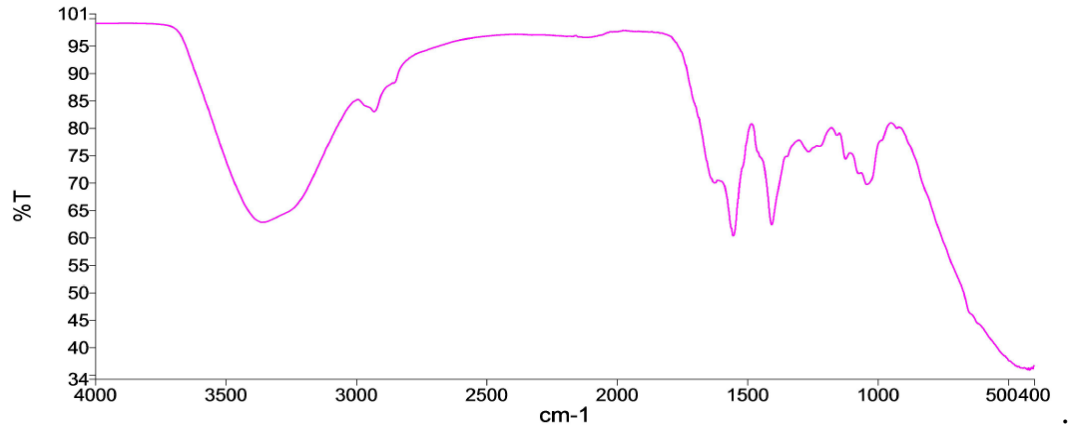

Figure 4.3: FT-IR Spectra of Methanolic Fraction of *F. pumila*.

The Tables 4.3 and 4.4 show the functional groups present in the *F. pumila* methanolic and hydro fractions, respectively, as well as the nature of their bonds. The frequencies and nature of the various peaks were used to identify the various functional groups by comparing the values with standard values (Appendix 1).

Table 4.3: FTIR Peak Values of Methanolic fraction of *F. pumila*

Peak	X(cm-1)	Y (%T)	Bond	Functional Group
1	3367.89	62.9	N–H stretch	1°, 2° amines, amides
2	2934.06	83.11	C–H stretch	Alkanes
3	2171.8	96.87	–C≡C– stretch	Alkynes
4	2122.98	96.68	–C≡C– stretch	Alkynes
5	1626.79	70.08	N–H bend	1° amines
6	1554.33	60.4	Unknown	Unknown
7	1407	62.4	C–C stretch (in–ring)	Aromatics
8	1266.73	75.82	C–H wag (–CH_2X)	Alkyl halides
9	1224.26	76.78	C–N stretch	Aliphatic amines
10	1156.46	78.71	C–H wag (–CH_2X)	Alkyl halides
11	1123.09	74.46	C–N stretch	Aliphatic amines
12	1072.14	71.82	C–N stretch	Aliphatic amines
13	1041.86	69.8	C–N stretch	Aliphatic amines
14	925.99	80.07	O–H bend	Carboxylic acids
15	439.13	36.14	Unknown	Unknown
16	423.39	35.87	Unknown	Unknown
17	415.96	35.84	Unknown	Unknown
18	403.88	36.03	Unknown	Unknown

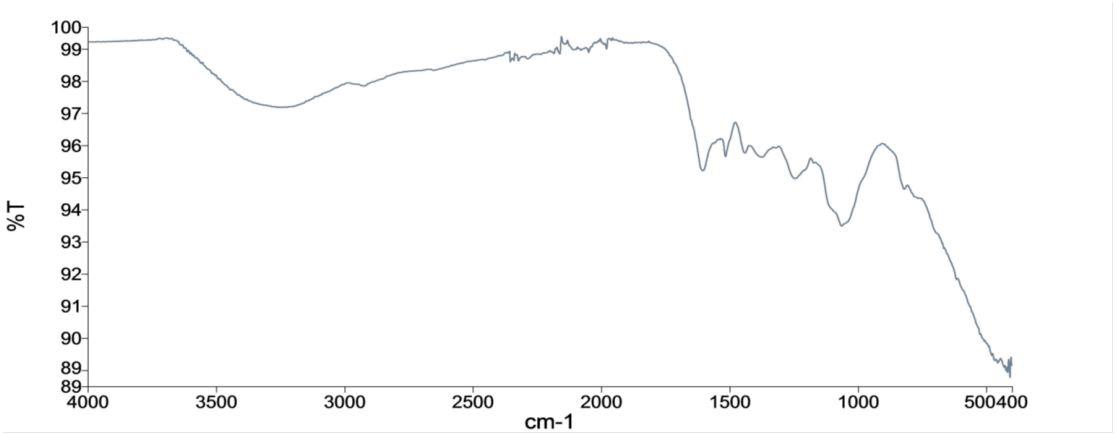

Figure 4.4: FT-IR Spectra of Hydro Fraction of F. pumila

Table 4.4 FTIR Peak Values of Hydro fraction of F. pumila

Peak	X(cm-1)	Y (%T)	Bond	Functional Group
1	3241.04	97.21	O–H stretch, H–bonded	Alcohols, phenols
2	2355.9	98.63	Unknown	Unknown
3	2323.94	98.66	Unknown	Unknown
4	2162.57	98.89	–C≡C– stretch	Alkynes
5	2143.91	99.18	–C≡C– stretch	Alkynes
6	2050.34	98.93	Unknown	Unknown
7	1980.25	99.04	Unknown	Unknown
8	1603.29	95.23	Unknown	Unknown
9	1516.13	95.68	N–O asymmetric stretch	Nitro compounds
10	1440.08	95.79	C–C stretch (in–ring)	Aromatics
11	1374.09	95.65	Unknown	Unknown
12	1243.56	94.99	C–N stretch	Aliphatic amines
13	1063.96	93.5	C–N stretch	Aliphatic amines
14	818.98	94.65	C–Cl stretch	Alkyl halides and Alkenes
15	454.99	89.22	Unknown	Unknown
16	416.33	88.93	Unknown	Unknown
17	406.93	88.77	Unknown	Unknown

4.6. IN VITRO CYTOTOXICITY ASSAY

4.6.1 MTT Assay

The anti-proliferative effect of the ethanolic extract (FPC) and fractions (FPM and FPH) were evaluated using the MTT assay against two cancer cell lines (Jurkat and

HepG2) and a normal cell line (Chang liver). The samples exhibited cytotoxic effect against the cancer cell lines as shown in the Figures 4.5, 4.6 and 4.7. Their cytotoxic effects were evaluated by calculating for the IC_{50} values and compared to the standard curcumin. The smaller this value, the stronger the cytotoxic effect.

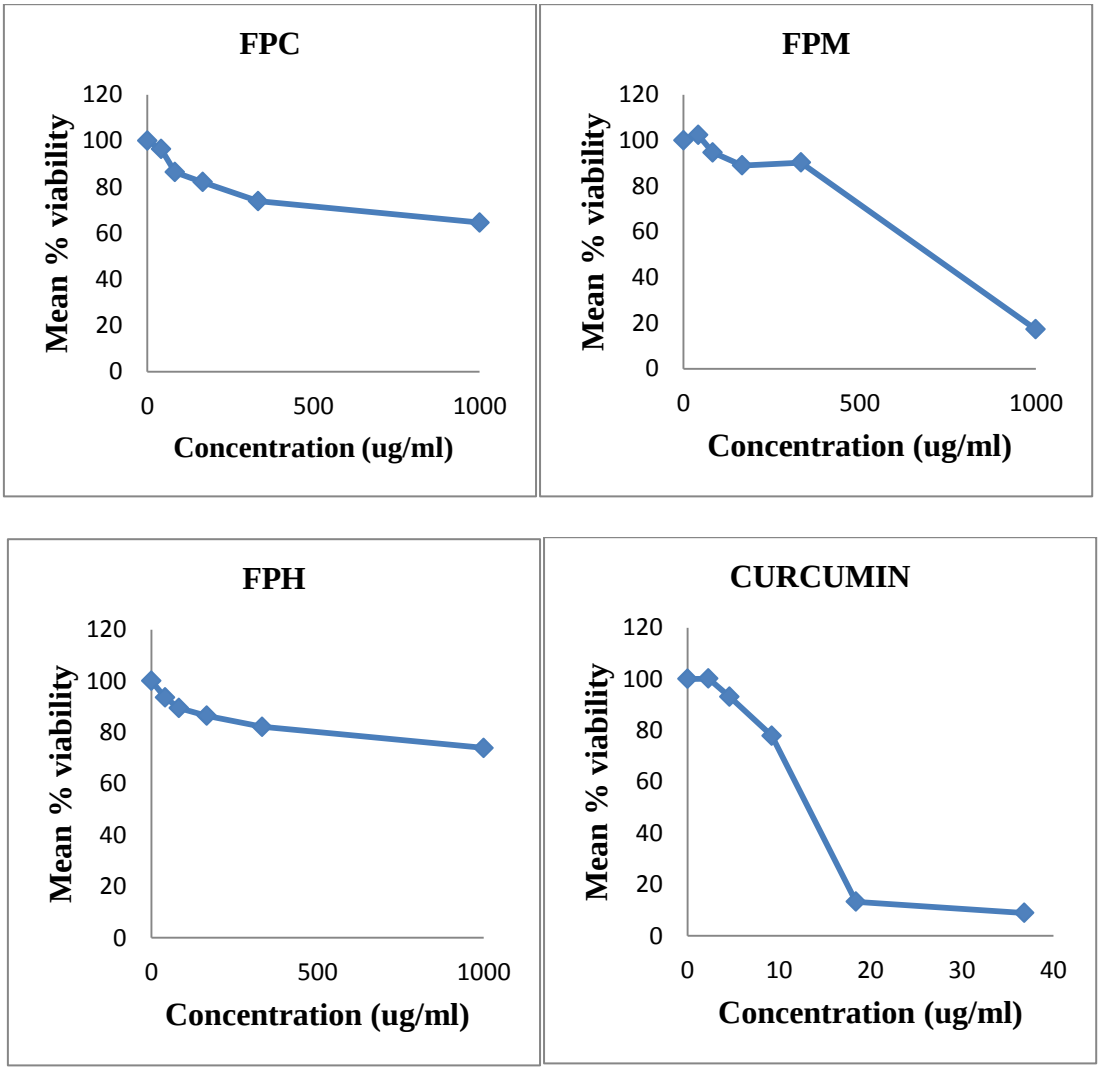

Figure 4.5: Cell viability curves showing cytotoxicity effect of various samples on Chang liver cell lines.

The ethanolic crude extract (FPC) and hydro fraction (FPH) were seen to decrease the cell viability to a lesser extent as concentration increased compared to standard curcumin as seen in Figure 4.5. Methanolic fraction (FPM), however decreased the

viability of the cells, hence killing 50% of the cells at a concentration of 700.19 µg/ml. The standard curcumin was also cytotoxic against the cells with IC_{50} value of 13.18 µg/ml. The difference between the IC_{50} values of the various samples were statistically significant ($p < 0.05$).

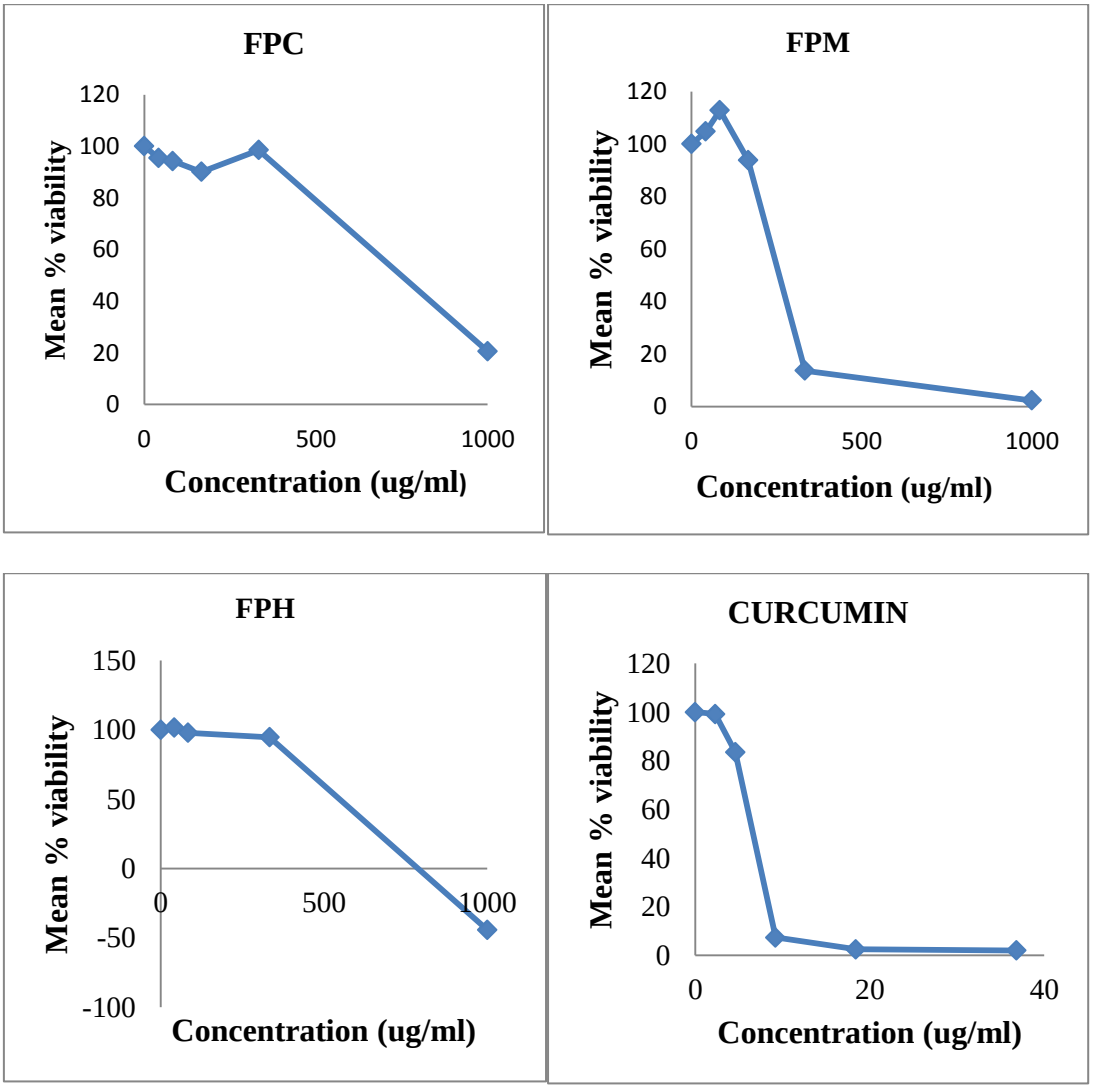

Figure 4.6: Cell viability curves showing cytotoxicity effect of various samples on Jurkat cell lines.

All the samples were cytotoxic against the Jurkat cell lines (Figure 4.6). The methanolic fraction showed the highest cytotoxicity, with IC_{50} of 248.10 ± 0.79 µg/ml.

This was followed by the hydro fraction with IC_{50} of 546.15 ± 6.98 µg/ml. The crude ethanolic extract gave an IC_{50} value of 749.07 ± 27.2 µg/ml. The standard curcumin was also cytotoxic against the cells with IC_{50} value of 6.61 ± 0.44 µg/ml. The difference between the IC_{50} values of the various samples were statistically significant ($p < 0.01$).

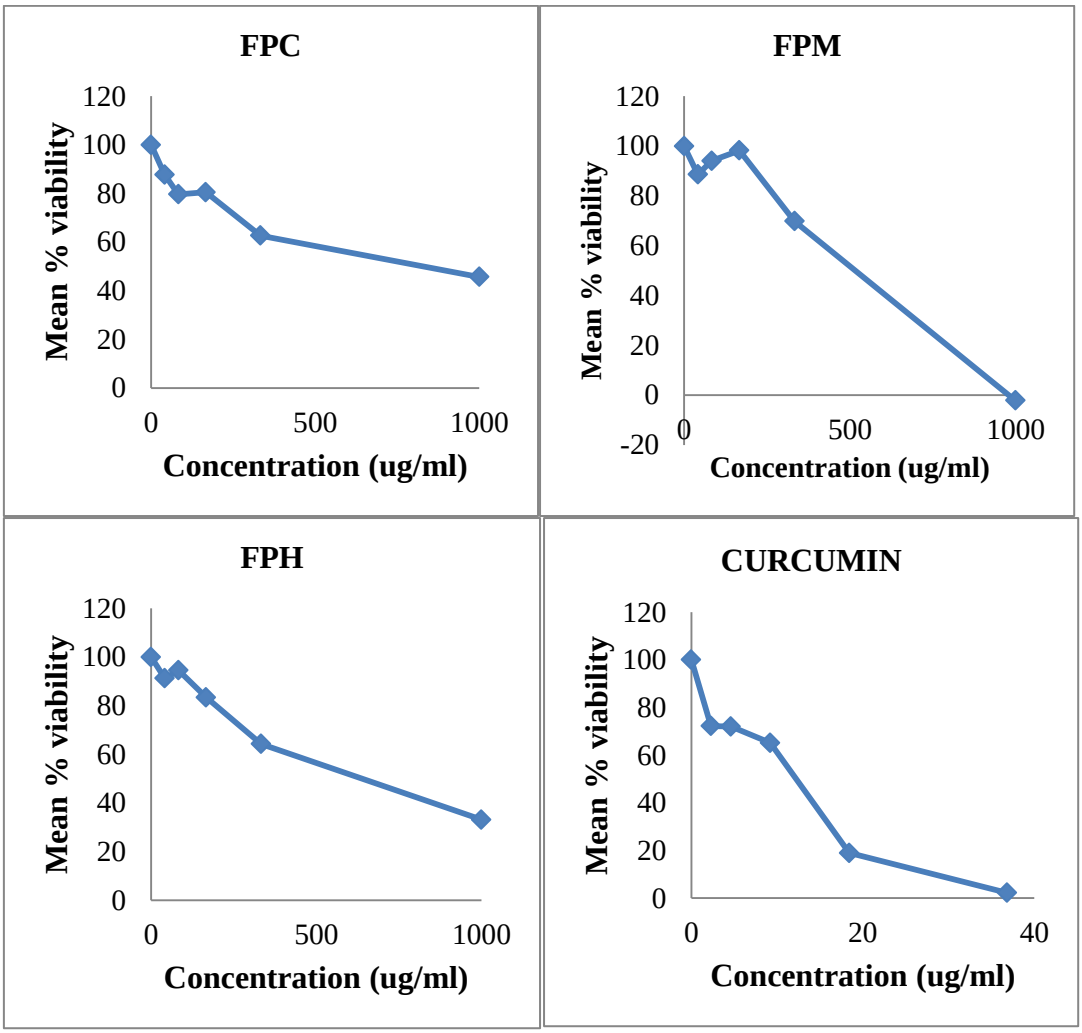

Figure 4.7: Cell viability curves showing cytotoxicity effect of various samples against HepG2 cell lines.

All the samples were cytotoxic against the HepG2 cell lines (Figure 4.7). The methanolic fraction showed the highest cytotoxicity, with IC_{50} of 515.86 ± 20.81

µg/ml. This was followed by the hydro fraction with IC_{50} of 621.77 ± 45.92 µg/ml. The crude ethanolic extract gave an IC_{50} value of 705.53 ± 65.47 µg/ml. The standard curcumin was also cytotoxic against the cells with IC_{50} value of 12.28 ± 0.70 µg/ml. The difference between the IC_{50} values of the various samples were statistically significant ($p < 0.01$).

Table 4.5: Comparison of IC50 values of samples tested on the various cell lines.

	IC_{50} (µg/ml)		
SAMPLE	**Chang**	**Jurkat**	**HepG2**
Crude ethanolic Extract	>1000	749.07 ± 27.2	705.53 ± 65.47
Hydro fraction	>1000	546.15 ±6.98	621.77 ± 45.92
Methanolic Fraction	700.19 ± 20.28	248.10 ± 0.79	515.86 ± 20.81
Curcumin	13.18 ± 0.35	6.61 ± 0.44	12.28 ± 0.70
P value	0.00	0.00	0.00

The p value represents the comparison between the IC_{50} values of one sample among the three cell lines.

4.6.2 Selectivity Indices

SI of the various samples against the cancer cell lines were calculated by dividing the IC_{50} value of the normal cells by that of the cancerous cells. The SI values of the various samples are shown in Table 4.6.

Table 4.6: Selectivity indices of the crude ethanolic extract, fractions and standard.

	Selectivity Index	
	JURKAT	**HEPG2**
Ethanolic Extract	1.335	1.417
Hydro Fraction	1.832	1.608
Methanolic Fraction	2.822	1.357
Curcumin	1.994	1.074

4.7 QUALITATIVE PHYTOCHEMICAL ANALYSIS

The phytochemicals present in the crude ethanolic extract of *F. pumila* are presented in Table 4.7.

Table 4.7 Phytochemical constituents of F. pumila crude ethanolic extract

Phytochemical	**Crude ethanolic extract**
Alkaloid	+
Terpenoids	+
Flavonoid	+
Cardiac glycoside	+
Sterols	-
Saponins	+
Tannins	+

4.8 GC-MS ANALYSIS OF FRACTIONS

The Gas chromatography mass spectrophotometric analysis was carried out on the *Ficus pumila* hydro fraction and methanolic fraction. Figures 4.12 and 4.13 show the total ion chromatogram for the fraction which sums up intensities of all mass spectral peaks.

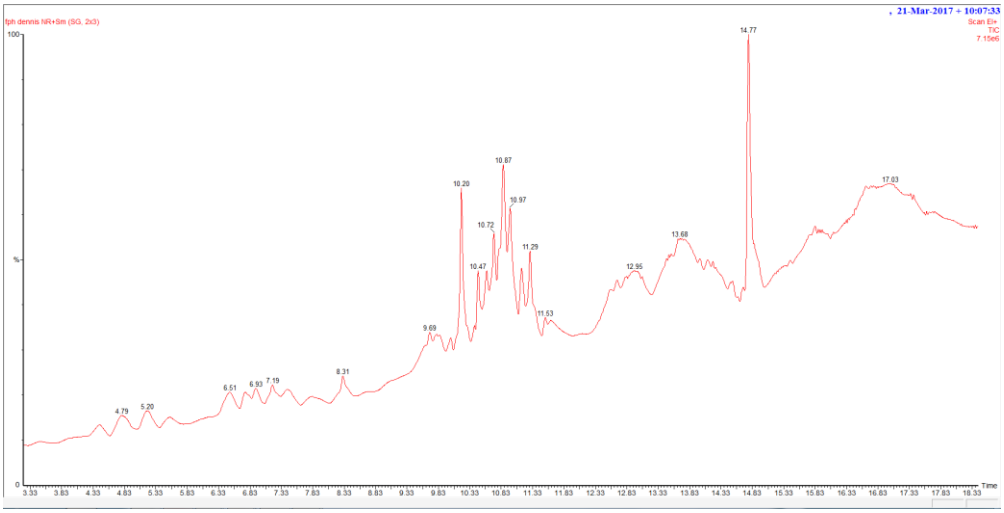

Figure 4.8: Total Ion Chromatogram (TIC) of *Ficus pumila* Hydro fraction

The active compounds with their retention time (RT), Molecular formula and Molecular weight (MW) in the hydro fraction of *F. pumila* are presented in Table 4.8. Six compounds were identified.

Table 4.8: Compounds present in the hydro fraction of *Ficus pumila*

Peak	RT	Compound name	Molecular formula	MW
1	8.31	Phenol, 2,4 -bis(1,1-Dimethylethyl)-	$C_{14}H_{22}O$	206
2	10.20	2,4,4,6,6,8,8-Heptamethyl-1-nonene	$C_{16}H_{32}$	224
3	10.47	1-Hexadecanol, 3,7,11,15-tetramethyl-	$C_{20}H_{42}O$	298
4	10.72	1-Decanol, 2-hexyl-	$C_{16}H_{34}O$	242
5	10.72	Nonadecylheptafluorobutyrate	$C_{23}H_{39}O_2F_7$	480
6	10.87	Heptacosylheptafluorobutyrate	$C_{31}H_{55}O_2F_7$	592
7	14.77	2,4,4,6,6,8,8-Heptamethyl-1-nonene	$C_{16}H_{32}$	224

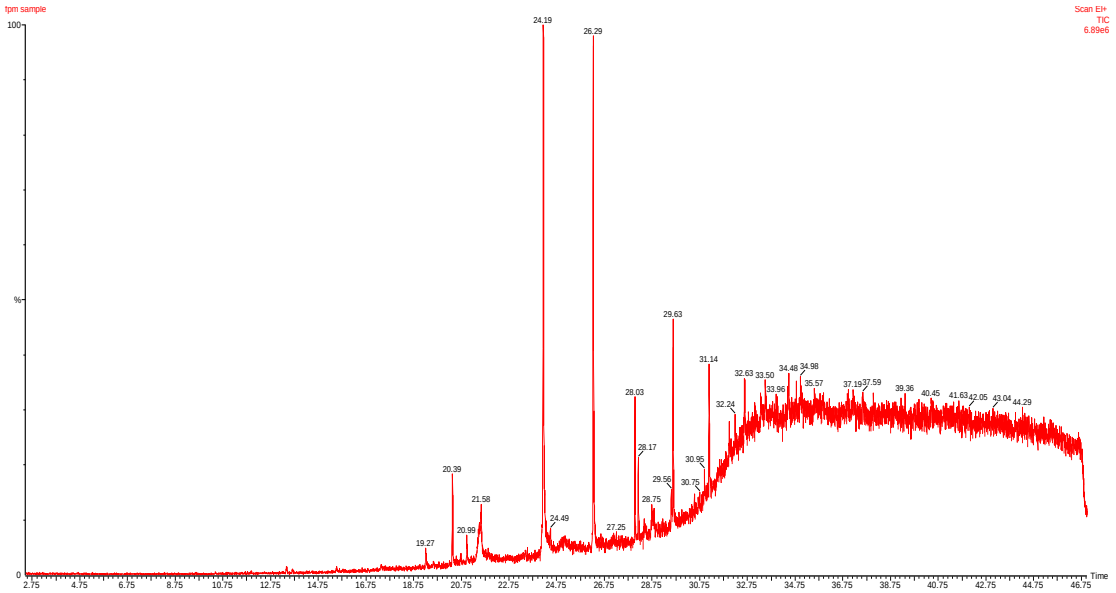

Figure 4.9: TIC of *Ficus pumila* methanolic fraction

Table 4.9: Compounds present in the *methanolic* fraction of *Ficus pumila*

Peak	RT	Compound name	Molecular formula	MW
1	19.27	Dodecane, 2, 6, 10- trimethyl-	$C_{15}H_{32}$	212
2	20.39	Benzene, 1,3-bis (1,1-dimethylethyl)-	$C_{14}H_{22}$	190
3	20.99	Sulforous acid, pentyl undecyl ester	$C_{16}H_{34}O_3S$	306

CHAPTER FIVE

5.0 DISCUSSION

The presence of heavy metals such as Iron (Fe), Copper (Cu), Zinc (Zn), Cadmium (Cd), Nickel (Ni) and Lead (Pb) in medicinal plants can pose some health risks on individuals, although some of these metals serve as micronutrients which are important for the proper functioning of some vital organs in the human body. Iron for instance, is a component of the haemoglobin and other compounds that are used in respiration (Lakshmi *et al*., 2013; Annan *et al*., 2010). Copper and zinc are also very essential in the body. They play important roles biochemically and physiologically in maintaining good health throughout life (Annan *et al*., 2013). Excessive concentrations of these metals in foods and medicines, however, is associated with several diseases, most especially diseases of the cardiovascular, nervous, renal and skeletal systems (Maobe *et al*., 2012). It is therefore vital to have good quality medicinal plants in order to protect consumers from contamination.

Even though a lot of phytochemical and bioactivity studies have been carried out on many medicinal plants in Ghana, not much has been reported on the heavy metal contents of these plants (Annan *et al*., 2013). This study therefore sought to establish the presence and quantity of six heavy metals (Iron, copper, zinc, nickel, cadmium and lead) in *F. pumila* raw powder and crude ethanolic extract. The results revealed the highest concentration of Iron in the raw powdered leaf sample (1.97 ± 0.11 mg/l) and the ethanolic extract (0.92 ± 0.02 mg/l) but at a lower concentration. Zinc was also detected in both the raw powder (0.19 ± 0.00 mg/l) and extract (0.66 ± 0.02) of *F. pumila* as indicated in the table 4.1. The permissible limit of iron set by FAO/WHO (1984) in edible plants is 20 ppm (20 mg/l) whiles that of zinc 27.4 mg/l, thus these levels in plant materials were below maximum permissible limits. This means that,

with respect to iron and zinc levels, the plant extract and raw powder will not cause any negative effect resulting from excess iron and zinc, when taken in as medicine.

The remaining heavy metals (copper, nickel, cadmium and lead) were all below the detection limit of the atomic absorption spectrophotometer. These values indicate that the *Ficus pumila* powder and ethanolic extract from the Bamso clinic can be consumed without any effect resulting from these heavy metals that were screened. *Ficus pumila* has also been reported safe for use according to the toxicity study conducted by Larbie *et al.* (2016). In this study, the median acute toxicity (LD_{50}) values of ethanolic extract was determined to be <5 g/kg body weight in mice. Observations after sub-chronic toxicity included hypolipidaemia, increased ALP at higher doses and normal creatine and urea levels. The extract did not produce any toxic effect on vital organs except decreases in uterus weight in female rats and hence, considered safe at moderate doses (Larbie *et al*., 2016).

At low or moderate concentrations, reactive oxygen species (ROS) and reactive nitrogen species (RNS) play vital roles in the physiological functions of the human body, despite their harmful effects. Some of these roles include signal transduction pathway, defence against infectious agents, smooth muscle relaxation and cell growth (Phang *et al*., 2011). When these free radicals accumulate in the body, however, they lead to a phenomenon known as oxidative stress. Oxidative stress refers to an imbalance between antioxidants and oxidants in the favour of the oxidants, which potentially leads to the damage of cells (Sirisha *et al*., 2010). The occurrence of oxidative stress eventually leads to numerous deteriorating effects to the cellular biomolecules which include lipid peroxidation, DNA damage, protein degradation, tissue injury, among others. Free radicals are therefore known for their contribution to

diverse forms of diseases such as arthritis, atherosclerosis, neurodegenerative disorders and aging (Shekhar and Anju, 2014).

Additionally, Ficus species have also been reported as rich sources of naturally occurring antioxidants and play very vital roles in preventing several diseases that are related to oxidative stress such as cardiovascular diseases and cancer. Due to their strong antioxidant properties, Ficus species are also known to diffuse the toxic free radicals such as Hydroxyl radicals and hence, can be used in nutraceutical and biopharmaceutical industries and can also be used as a possible food additive (Sirisha *et al*., 2010). Preliminary work done on the antioxidant effect of *Ficus pumila* ethanolic crude extract revealed strong antioxidant effect (EC_{50}= 0.07 mg/ml; Larbie *et al*., 2015b). This current study sought to evaluate the antioxidant effect of *Ficus pumila* fractions compared to standard, L-Ascorbic acid.

The results of the DPPH assay showed that, *F. pumila* hydro fraction had the highest antioxidant activity with EC_{50} value of 0.09 ± 0.00 mg/ml. There was difference between the EC_{50} values of the hydro fraction and the standard Ascorbic acid (EC_{50}= 0.0852 ± 0.0037 mg/ml). *F. pumila* methanolic fraction had the lowest antioxidant effect (EC_{50}= 0.42 ± 0.04 mg/ml) among the three fractions. Even though methanolic fraction had the least EC_{50} value, it still possessed good antioxidant activity. Results from the antioxidant activity suggest that, the extract and fractions could play very vital role as health protecting factors. They could do so by scavenging free radicals from the body. There is scientific evidence that suggests that antioxidants reduce the risk for chronic diseases which includes heart diseases and cancer (Badu *et al*., 2012) and hence *Ficus pumila* could help in the prevention of these diseases.

The total phenolic content was carried out based on the absorbance values of the various solutions of the fractions which reacted with Folin–Ciocalteu reagent and compared with the standard solutions of gallic acid. The data from the total phenolic content support the key role of phenolic compounds in the antioxidant activity of the plant. Phenolic compounds were present in all the fractions. Hydro fraction recorded the highest total phenolic contents and methanolic fraction recorded the least. There are lots of studies that have reported a positive correlation between total phenolic contents of various plants extracts and their antioxidant activities. For instance, Hossain *et al.* (2011) reported a positive relationship between antioxidant potential and the amount of phenolic compounds. Another study conducted by Larbie *et al*. (2015b) also confirms this correlation. This current study also confirms the fact that hydro fraction, which had the highest total phenolic content, had the highest antioxidant activity with the least EC_{50} value. This was followed by the crude ethanolic extract and then methanolic fraction with respect to the EC_{50} values calculated.

Fruits and vegetables are the predominant sources of antioxidant vitamins (Vitamin A, C and E), which act as free radical scavengers, making these foods essential to human health. However, more than 80% of the total antioxidant activity in fruits and vegetables come from the ingredients other than antioxidant vitamins, indicating the presence of other potentially important antioxidants in these foods. Examples of these ingredients are total phenols, flavonoids, alkaloids, glutathione, among others. Therefore, the presence of some of these ingredients could give rise to the antioxidant effect observed in the fractions and not only due to the presence of the phenolic compounds in the fractions.

Fourier Transform Infrared (FTIR) Spectroscopy is a high resolution analytical technique which is used in the identification of bioactive chemical constituents of compounds. It is also used to reveal the structure of compounds. In this technique, molecules such as organic compounds show absorption in a characteristic range of frequency mainly in the range of 4000-400 cm^{-1} which play a key role in the study of these compounds (Ashokkumar and Ramaswamy, 2014). FTIR is perhaps the most powerful tool used for the identification of the functional groups and the types of chemical bonds present in compounds. The wavelength of light that is absorbed is characteristic of the chemical bond which is observed in the annotated spectrum. The chemical bond in a molecule can be determined by the interpretation of the infrared absorption spectrum (Rani *et al*., 2016; Vijayarekha and Sengottaiyan, 2016).

The Figures 4.7 and 4.8 show the FTIR spectrum of the various fractions and their interpretations are shown in the Tables 4.3 and 4.4. The FTIR spectroscopic studies of *F. pumila* methanolic fraction revealed 18 characteristic peak values with various functional compounds in the fractions. This analysis revealed the presence of primary and secondary amides, alkanes, alkynes, alkyl halides, aromatics, aliphatics amines and carboxylic acid. Alcohols, phenols, alkynes, alkenes, aromatics, nitro compounds, aliphatic amines and alkyl halides were also shown in the hydro fraction of *F. pumila*, which revealed 17 peaks. Alcohols and phenols were only observed in the hydro fraction, with a very high transmittance value, and this shows the relatively higher total phenolic contents observed in this fraction by the Folin-Ciocalteu assay. However, aromatic compounds were observed in the methanolic fraction, but with a relatively lower transmittance value. These aromatic compounds could include phenols and this could account for the presence of the total phenols observed in the Folin-Ciocalteu assay of these fractions.

This current work sought to determine the cytotoxicity effect of the crude ethanolic extract of *Ficus pumila* as well as its fractions – methanolic and hydro, with curcumin as standard on cancer and normal cells. The results revealed that the crude extract and hydro fraction had no cytotoxic effect on the normal cells – Chang cell. However, the methanolic fraction and standard curcumin decreased the viability of the normal cells with IC_{50} values 700.19 ± 20.28 and 13.18 ± 0.35 µg/ml, respectively. Comparing the methanolic fraction to the curcumin, it was observed that the curcumin was highly toxic to the normal cells than the methanolic fraction. All the samples showed some level of cytotoxicity on the cancerous cell lines. The methanolic fraction gave an IC_{50} value of 248.10 ± 0.79 µg/ml while the hydro fraction also gave an IC_{50} value of 546.15 ± 6.98 µg/ml. This shows that though the two fractions are moderately cytotoxic against the Jurkat cells, the methanolic fraction had a better activity. Comparing the cytotoxicity effect of the crude ethanolic extract and fractions against the standard curcumin, it was observed that the differences were statistically significant.

A similar trend was observed with the HepG2 cells. All the samples were cytotoxic against the HepG2 cell lines. The methanolic fraction recorded the highest cytotoxicity against the HepG2 cells (IC_{50} = 515.86 ± 20.81 µg/ml); hydro (621.77 ± 45.92 µg/ml) and crude ethanolic extract (705.53 ± 65.47 µg/ml). All the samples had statistically significant cytotoxicity effect on the three cell lines as shown in Table 4.5.

Overall, the samples (crude ethanolic extract and fractions) had varying cytotoxic effects on the cancer cell lines. However, to be named as an anticancer agent, the sample should show good selectivity (SI > 2) for cancer cells only (Acheampong *et al*., 2015). This SI value means that the sample is twice more cytotoxic to the cancer cell line as compared with the normal cell line. From the results, it was observed that only

the methanolic fraction was selective towards the Jurkat cell line (SI = 2.822). The remaining fraction had an SI value less than 2 and hence can be said to be non-selective towards the cancer cell lines, even though they all exhibited cytotoxic effect on the cancerous cell lines.

From the result, the Jurkat cell line showed the highest sensitivity toward all the samples. Ethanolic extract of *F. pumila* was shown to possess anti-proliferative effect against Jurkat, CEM and HL-60 leukemic cell lines in a preliminary work done by Larbie *et al*. (2015b). This study has confirmed the anti-leukemic effect of the crude ethanolic extract and has also revealed that this property is inherent in the methanolic fraction. Phytochemicals present in *F. pumila* could be the contributing factor for the observed cytotoxic activity. According to Bhandari *et al*. (2017), phytochemicals such as flavonoids, steroids and terpenoids have been shown to have anti-cancer potential and these phytochemicals could be abundant in the methanolic fraction.

The GC-MS analysis revealed a variety of compounds with some medicinal values. For instance, Phenol-2,4-bis (1,1-dimethylethyl) that was found in the hydro fraction of *F. pumila*, possesses some therapeutic effect like anti-arthritic, anti-inflammatory and anti-hyperlipidemic activities as reported by Vinjamuri and Sharad (2017). 1-Hexadecanol-3,7,11,15-tetramethyl- which was present in *F. pumila* hydro fraction also possesses anti-mycobacterial activity (Rajab *et al*., 1998). These activities could be as a result of their antioxidant activities, hence the higher antioxidant activity observed in the hydro fraction in the DPPH assay. The methanolic fraction contained Dodecane, 2, 6, 10- trimethyl-, Benzene, 1,3-bis (1,1-dimethylethyl)- and Sulforous acid, which could be responsible for the cytotoxic activity observed.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

Ficus pumila crude ethanolic extract and raw powder contain iron and zinc whose concentrations are below the permissible level proposed by FAO/WHO for medicinal plants and hence, with respect to iron and zinc levels, the plant extract and raw powder will not cause any negative effect resulting from excess iron and zinc, when taken in as medicine. *Ficus pumila* crude ethanolic extract also contain phytochemicals like tannins, flavonoids, terpenoids, alkaloids and saponins which provides it with medicinal properties. The hydro fraction of *F. pumila* had the highest total phenolic content and antioxidant effect, and hence, can be used to prevent the occurrence of diseases associated with oxidative stress like cardiovascular diseases and cancers. The methanolic fraction had the highest cytotoxicity effect and was highly selective for Jurkat cell lines. This cytotoxic selectivity between the Jurkat cells and normal (Chang liver) cell lines increase the prospect that this plant contains compound(s) which could serve as leads for novel anticancer drugs. The most active fraction in terms of the antioxidant assay was the hydro fraction and with respect to the cytotoxicity assay was the methanolic fraction.

6.2 RECOMMENDATION

The mechanism of action of the active fraction – methanolic fraction, should be determined by the use of Hoechst staining to determine the apoptotic activity and the use of flow cytometry to determine the effect of the sample on the cell cycle distribution. Further work should be done using of different solvent fractions of the *Ficus pumila*.

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APPENDIX

1. FOURIER TRANSFORM INFRARED SPECTROPHOTOMETRY

1. A Table of Characteristic IR Absorptions

Frequency, cm-1	Bond	Functional group
3640–3610 (s, sh)	O–H stretch, free hydroxyl	alcohols, phenols
3500–3200 (s,b)	O–H stretch, H–bonded	alcohols, phenols
3400–3250 (m)	N–H stretch	1°, 2° amines, amides
3300–2500 (m)	O–H stretch	carboxylic acids
3330–3270 (n, s)	–C≡C–H: C–H stretch	alkynes (terminal)
3100–3000 (s)	C–H stretch	aromatics
3100–3000 (m)	=C–H stretch	alkenes
3000–2850 (m)	C–H stretch	alkanes
2830–2695 (m)	H–C=O: C–H stretch	aldehydes
2260–2210 (v)	C≡N stretch	nitriles
2260–2100 (w)	–C≡C– stretch	alkynes
1760–1665 (s)	C=O stretch	carbonyls (general)
1760–1690 (s)	C=O stretch	carboxylic acids
1750–1735 (s)	C=O stretch	esters, saturated aliphatic
1740–1720 (s)	C=O stretch	aldehydes, saturated aliphatic
1730–1715 (s)	C=O stretch	α, β–unsaturated esters
1715 (s)	C=O stretch	ketones, saturated aliphatic
1710–1665 (s)	C=O stretch	α,β–unsaturated aldehydes, ketones
1680–1640 (m)	–C=C– stretch	alkenes
1650–1580 (m)	N–H bend	1° amines
1600–1585 (m)	C–C stretch (in–ring)	aromatics
1550–1475 (s)	N–O asymmetric stretch	nitro compounds
1500–1400 (m)	C–C stretch (in–ring)	aromatics
1470–1450 (m)	C–H bend	alkanes
1370–1350 (m)	C–H rock	alkanes
1360–1290 (m)	N–O symmetric stretch	nitro compounds
1335–1250 (s)	C–N stretch	aromatic amines
1320–1000 (s)	C–O stretch	alcohols, carboxylic acids, esters, ethers
1300–1150 (m)	C–H wag (–CH2X)	alkyl halides
1250–1020 (m)	C–N stretch	aliphatic amines
1000–650 (s)	=C–H bend	alkenes
950–910 (m)	O–H bend	carboxylic acids
910–665 (s, b)	N–H wag	1°, 2° amines

900–675 (s)	C–H “oop”	aromatics
850–550 (m)	C–Cl stretch	alkyl halides
725–720 (m)	C–H rock	alkanes
700–610 (b, s)	–C≡C–H: C–H bend	alkynes
690–515 (m)	C–Br stretch	alkyl halides

2. PROPERTIES OF CELL LINES USED IN THE CYTOTOXICTY ASSAY

A. CHANG CELLS

Tissue: Liver

Morphology: Epithelial

Disease: Used as normal (slow growing cancer cells)

Organism: Homo sapiens

Genes expressed: The cells produce a C type retrovirus.The cells are positive for keratin by immunoperoxidase staining.

Growth Properties: Monolayer, adherent, cells pile up at high density

Growth Medium: DMEM supplemented with 2 mM L-glutamine, 1% Non-Essential Amino Acids,1mM sodium pyruvate and 10% fetal bovine serum

B. JURKAT CELLS

Tissue: Peripheral blood, Blood

Morphology : Lymphoblast

Cell Type T lymphocyte

Disease acute T cell leukemia

Organism Homo sapiens

Genes expressed: Interleukin-2 (interleukin 2, IL-2), CD3; Homo sapiens, expressed

Growth Properties:	Suspension
Growth Medium:	RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum

C. HEPG2 CELLS

Tissue:	Liver
Morphology:	Epithelial
Cell Type:	Human liver carcinoma cells
Disease:	Hepatocellular carcinoma
Organism:	Homo sapiens
Genes expressed:	Alpha fetoprotein (alpha-fetoprotein); hepatitis B surface antigen (HBsAg); albumin; alpha2 macroglobulin (alpha-2-macroglobulin); alpha1 antitrypsin (alpha-1-antitrypsin); transferrin;, alpha1 antichymotrypsin (alpha-1-antichymotrypsin); haptoglobin; ceruloplasmin; plasminogen; complement (C3, C4); C3 activator; fibrinogen; alpha1 acid glycoprotein (alpha-1 acid glycoprotein);, alpha2 HS glycoprotein (alpha-2-HS-glycoprotein); beta lipoprotein (beta-lipoprotein); retinol binding protein (retinol-binding protein)
Growth Properties:	Adherent
Growth Medium:	RPMI 1640 medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum