DECLARATION

I hereby declare that this is the product of my own research undertaken under supervision and has neither been presented in whole nor in part for another degree elsewhere. I am solely responsible for any residual flaws in the work.

Signature…….. Date: ……/……/…….

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DECLARATION BY SUPERVISORS

We hereby declare that the principal work and presentation of the thesis were supervised by us in accordance with guidelines on supervision of thesis laid down by the University of Ghana.

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(Prof. (Mrs.) Regina Appiah-Opong)

ABSTRACT

Background: Prostate cancer is the second highest cancer type among men in Africa. It brings financial and healthcare burden to a number of patients in Ghana. It accounted for about 30% of all cancers recorded in males in the year 2000 at the Korle-Bu Teaching Hospital, Accra - Ghana. Thus, there is a need for other therapeutic agents (interventions) *Momordica chrantia* (MCwp) and *Fleurya aestuans* (FAwp) plants are known to possess anticancer, anti-diabetic and anti-inflammatory properties. However, there is a dearth of information on the efficacy of both plants in the management of prostate cancer.

Aim: This study aimed at determining direct cytotoxic, anti-oxidant and pro-apoptotic activities of aqueous extracts of the leaves of MCwp and FAwp on PC3 and LNCaP prostate cancer cell lines.

Methodology: Aqueous extracts of the leaves of MCwp and FAwp were prepared from pulverized samples, with 11% and 14.96% yields, respectively. PC3 and LNCaP prostate cell lines were cultured in RPMI media substituted with 10% FBS and 1% penicillin-streptomycin. Morphological cellular changes were observed at 3, 6 and 24 h post-treatment with extracts and control compounds (ursolic acid and curcumin) using a Phase Contrast light microscope at 20X magnification. Cytotoxic activities of increasing concentrations of the extracts were ascertained using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on the two prostate cancer cell lines, PC3 and LNCaP. The pro-apoptotic activity of the extracts were assessed using the DNA laddering assay. The JC-1 mitochondrial assay was also used to assess the intof rinsic pro-apoptotic pathway. The antioxidant properties of the extracts were evaluated using 2, 2 diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and reducing power assay. Total phenolic content of the extracts were determined by Folin-Ciocalteu reagent method.

Results: *Morphological changes of cells post-treatment*: Ursolic acid (UA) and curcumin (positive controls caused apoptosis with ursolic acid showing more apoptotic cells in both cell lines at 24h post treatment.. FAwp and MCwp apoptotic activity was highest at 6 h post treatment

MTT Assay: FAwp was more cytotoxic to PC3 cells compared to MCwp. Curcumin had the least IC_{50} value of 2.10 µg/ml, and UA had IC_{50} value of 9.90 µg/ml. FAwp showed a concentration-dependent reduction in percentage mean cell viability with an IC_{50} value of 20.39 µg/ml, while that of MCwp was 38.22 µg/ml. The IC_{50} values for curcumin and ursolic acid in LNCaP-treated cells, with a value of 7.611 µg/ml and 34.44 µg/ml, respectively, whilst that of MCwp was 75.75 µg/ml. FAwp exhibited least cytotoxic against LNCaP (>100 µg/ml).

Mitochondrial membrane potential assay: MCwp had the least JC-1 monomer at 24 h for both PC3 and LNCaP. FAwp exhibited highest pro-apoptotic activity at 24 h post-treatment on PC3.

DNA fragmentation: Apoptotic bodies were formed in both cells treated with FAwp and MCwp.

Antioxidant capacity (DPPH Assay): FAwp and MCwp had an EC_{50} values > 5 mg/ml.

Total phenolics/Gallic acid assay: The Total phenolic content of FAwp was 1.97 mg/100g GAE. Whiles that of MCwp was 3.622 mg/100g GAE.

Conclusion: Both FAwp and MCwp had direct cytotoxic activities on PC3 and LNCaP prostate cell lines. They had anti-oxidant and apoptotic activity.

DEDICATION

To my unborn children: the sky is only the limit. See your vision as your gift. Hold no man to it.

They do not have your eyes.

To my nieces and nephews, never give up on your dreams, the whole world awaits your explosion.

What God puts in your hearts is your gift to the world.

Mummy loves you unconditionally.

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ABBREVIATIONS

ANOVA ……Analysis of Variance

BHT……...Butylated Hydroxytoluene

BMI……Body Mass Index

DPPH ……2, 2 diphenyl-1-picrylhydrazyl

DMSO ……..Dimethyl Sulfoxide

DUI45…….Prostate cancer cell line

FAwp ……*Fleurya aestuans*

FBS……... Fetal Bovine Serum

GAE……. Gallic acid Equivalent

GLOBOCAN……Global Burden of Cancer Study

GWAS…….. ...Genome-wide associated study

HHV 8…… ..Human Herpes Simplex Virus-8IARC…….International Agency for Research on Cancer

LNCaP……..Prostate cancer cell line

MCwp ……*Momordica charantia*

MTT …….....3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

PC3……...Prostate cancer cell line

RPMI ……...Rosewell Park Memorial Institute

UA……Ursolic acid

WHO……World Health Organization

VEGF……Vacular endothelial growth factors

CHAPTER ONE

INTRODUCTION

1.0 Background To The Study

Cancer accords serious health, financial and social burden on both developed and developing countries. Increase in life expectancy, as well as lifestyle modification, including factors such as smoking, obesity, sedentary lifestyles and preference to birthing at an older age (attributable to education), which are related to economic development and urbanization tend to be risk factors for the development of cancers. Global Burden of Cancer Study (GLOBOCAN) estimates the global incidence and mortalities of cancer to be about 14.1 million and 8.2 million, respectively in 2012 (Ferlay et al., 204515). The most prevalent cancer globally is cancer of the lung, overtaking breast cancer in females. Colorectal cancer in both sexes and prostate cancer in men, however, are the major causes of death from cancer worldwide. According to Ferlay *et al.* (2013), less developed countries have the highest incidence of cancers with infective etiology compared to developed countries. A subsequent study by the same authors revealed cervical cancer as the leading cause of cancer-related deaths in females in developing countries, with prostate cancer being first among males in developed countries (Ferlay *et al*., 2015). Developed countries have the burden of almost twice incidence of cancer in both sexes but with a mortality rate of only 8% to 15% higher compared to developing countries (Parkin *et al.,* 2010).

Reports from a recent study indicates that the incidence of prostate cancer is highest in the African Caribbean's, African Americans, Caucasians but low in Asians (Parkin *et al*., 2010). The global incidence of prostate cancer is dynamic, being the 15th most common cancer, and constituting 11.7% of new prostate cancer cases, 5% in developing countries and 19% in developed countries.

It is next to liver cancer in Western and African men (Parkin *et al.*, 2010). The estimated 5-year survival rates of prostate cancer vary from 80 – 92% in USA to <40% in Denmark, Poland and Algeria (Glover et *al.*, 1998; Hsing, 2000; Crawford, 2003). In Western countries, diagnosis is usually at <50 years and median age of diagnosis is 68 years (Parkin *et al.*, 2003; Parkin *et al.*, 2010). The incidence of prostate cancer is second highest to hepatocellular carcinoma in men in Africa (Parkin *et al.*, 2003). A retrospective study of the frequency and pattern of genitourinary cancers between 1980 and 1990 at Korle-Bu Teaching Hospital in Ghana revealed that prostate cancer accounted for 349 (81.4 %) out of 479 cases of cancers of the genitourinary challenges in men (Klufio *et al*., 1995). Another retrospective study conducted in the same hospital for all cancers between 1991 and 2000 also identified prostate cancer to be 17.35% of 659 cancer cases recorded in females and males and approximately 31.8 % of all cancers in men (Wiredu and Armah, 2006).

Over the years, radiotherapy, chemotherapy and surgery have been the approaches to cancer treatment and management. However, some of these treatment options are sometimes sophisticated and expensive (Mathenge *et al*., 2007). Other challenges with cancer management include illiteracy, stress on the cancer patient, and issues of drug resistance which contribute to low compliance to known treatment modalities (Akabue *et al*., 1982). Due to high cost and unavailability of needed medications, 80% of people in developing countries resort to herbal medicines, which are readily available alternative sources of medicine for their general wellbeing (Busmann *et al*., 2006, Cassady *et al*.,1980)). In addition, plants also serve as important, cheap, easily accessible and readily available sources of raw materials for the development of orthodox drugs (Anely *et al*., 2007). However, the safety of most of these medicines has not been extensively studied.

The etiology of carcinogenesis is postulated to include genetic and epigenetic mutations (caused spontaneously and by UV radiation, chemicals, etc), free radical formation, infections, dysregulation of apoptosis, as well as suppression of transcription of tumour suppressor genes. Therefore, exploration of these mechanisms is used in generating anti-cancer medications.

Momordica charantia (MCwp; bitter lemon) is one of the medicinal plants used to treat cancer in some developing countries (Rajapaksa, 2017). A recent study conducted in Kandy General Hospital Cancer Unit in Sri Lanka reported effective treatment of cancer patients with bitter gourd seeds through its cytotoxic property (Rajapaksa, 2017). While this plant has also shown some potential anti-cancer activity in laboratory experiments (Craig, 2013), further studies are required to recommend its use. Also, no study has reported the activity of this plant on prostate cancer.

Fleurya aestuans (FAwp) is another medicinal plant that has been used to treat different types of cancer (Ruffo *et al*., 2002). It is a perennial and annual herb which sometimes grows as a climber, and is native to Africa, is one of the medicinal species with active compounds against various types of cancers (Ruffo *et al*., 2002). Its leaves are claimed by traditional medicine practitioners to possess therapeutic potentials for treatment of conditions including asthma, hypertension, rheumatism, rickets and wound healing (Friis *et al.*, 1993). The content of biotic agents in the leaves show medicinal values to man and on the other hand it is edible as fodder to piggery (Anely *et al*., 2007). These plants grow in Ghana and have proved to be of immense medicinal value. Preliminary studies done in our laboratory have demonstrated anti-cancer properties of these plants on selected cancer cell lines (unpublished data).

This study aimed at investigating the anti-cancer properties of the aqueous extracts of MCwp and FAwp against prostate cancer cell lines.

1.1 Problem Statement

Prostate cancer is the most commonly diagnosed cancer in men globally. An estimated 161,360 men in the United States are projected to be diagnosed with the disease (Prostate Cancer: Statistics, 2017). The risk of prostate cancer is 74% more common in black adult men than in non-Hispanic white men, for unknown reasons (Glover et *al.*, 1998). Diagnosis is commonly at an early stage of the disease (92%); confined disease or nearby organ (stage I and II prostate cancer). Men with distant organ metastasis have a 5-year survival rate of 29%. Prostate cancer is notably the third leading cause of cancer death in men in the United States of America. Prostate cancer is expected be responsible for 26,730 deaths which have been estimated to occur from this disease in 2017 (Prostate Cancer: Statistics, 2017). The prevalence of this condition among Africans including Ghanaian men, the survival rate and the associated mortality with metastasis in the presence of available intervention makes the search for other readily available and more effective anticancer agents necessary. Known interventions include but not limited to: preventive lifestyle, early screening and detection, chemotherapy, radiotherapy and surgery. These and their limitations will be discussed further in ensuing chapters.

The overall financial, morbidity, risk of mortality and burden on the already scanty Ghanaian health work force makes the need to get more easily accessible, less toxic, less expensive options to treatment an urgent health need. According to the latest WHO data published in 2015, life expectancy in Ghana is 61.0yrs for males and 63.9yrs for females, and total life expectancy is 62.4yrs, which gives Ghana a world life expectancy ranking of 149 out of 192 countries. It is

therefore important to research and produce drugs with a high efficacy aim at destroying prostate cancer cell lines in order to save more lives.

1.2 Justification

Cancer and its treatment cause enormous financial, social and mental burden to society, affecting patients, care takers as well as dependents on a whole. The demand on the healthcare system, as far as available human resource, division of labour, financial/monetary allocation to tackle the morbidity and mortality posed by the cancer burden, makes it a primary health concern. Therefore, it needs a lot of investment in education, prevention, early diagnosis, availability and cost of treatment. The cost of investigation and management of cancer does not only pose a threat on the national budget as well as international organizations, but also on the family, healthcare providers and society. Mariotto *et al*. (2011) reported that $137.4 billion was used in cancer care in the United States in 2010. This financial burden is, however, expected to escalate with lifestyle modification to a more sedentary 'careless' lifestyle, modernization, urbanization, the discovery of new, sophisticated, and expensive anticancer drugs and modes of treatment, an improved life expectancy, as well as an upsurge in anticancer drug resistance (Bradley *et al.*, 2008; Warren *et al.*, 2008; Mariotto *et al.*, 2011). Drug resistance through complex molecular mechanisms, lack of compliance due to intolerable side effects, and treatment failure also support the need for studies into more commonly available and affordable alternatives of cancer treatment with more tolerable side effects.

1.3 Aim

To investigate the antioxidant activity, cytotoxic and apoptotic properties of the aqueous whole plant extracts of *M. charantia* and *F. aestuans* against PC3 and LNCaP.

1.4 Specific Objectives

1. To assess the cytotoxic activity of aqueous fractions of *Momordica charantia* (MCwp) and *Fleurya aestuans* (FAwp) ~~on~~ PC3 and LNCaP prostate cell lines using the tetrazolium-based colorimetric (MTT) assay.
2. To determine the pro-apoptotic activity of the fractions ~~on~~ the prostate cell lines using the DNA fragmentation (laddering) assay, microscopy and the JC-1 mitochondrion membrane potentiation assay.
3. To evaluate the antioxidant activities of the aqueous extracts of FAwp and MCwp using 2, 2- diphenyl-1-picryl hydrazyl (DPPH) free radical scavenging assay.
4. To assess the total phenolic content for FAwp and MCwp using Folin Ciocalteu reagent method.

CHAPTER TWO

LITERATURE REVIEW

2.1 The Global Cancer Burden

Cancer-related death is now more prevalent in developing countries compared to developed ones (65% compared to 57%) globally (WHO, 2014). Lung cancer is the most common cause of cancer-related death in all sexes globally, overtaking breast cancer as the number one cause of cancer-related death in females in developing countries (Ferlay *et al*., 2013).

Colorectal cancer in both sexes and prostate cancer in men continue to be the leading causes of cancer death in more developed countries. Developing countries have the burden of cancer death with infective pathology being commonest (liver and stomach cancer in men and cervical cancer in women). Developed countries have the burden of an almost twice incidence of cancer in both sexes but with a mortality rate of only 8 to 15% higher compared to developing countries (Ferlay *et al*., 2014). This conflict shows the effect of demarcation in the types of cancers present, treatment options, early diagnosis, availability and affordability of treatment, the level of illiteracy and lifestyle found in these areas.

Cancer is associated with modifiable risk factors that include smoking of tobacco, for liver, stomach, lung and colorectal cancers, sedentary lifestyle, for colorectal and breast cancers, and infections, prominently for cervical, stomach and liver cancer (Forman *et al*., 2013). Most cancers can be prevented through education on the avoidance or minimization of risk factors, early screening and vaccination (Curado *et al*., 2007). Cancer-related death burden has been estimated to worsen globally with a globally ageing population, this estimate further affects the quality of

life of developing countries where at least 82% of the world's population resides (Forman *et al*., 2013). Urbanization comes with lifestyle changes such as less healthy diet, physical inactivity, alcoholism and smoking which increase cancer prevalence. Career influenced change in reproductive pattern to a later age at birthing and lower parity preference, even further worsens this cancer burden in poverty endemic, less industrialized countries. (Forman *et al*., 2013)

2.1.1 Incidence and mortality rates for cancers

Although the incidence of cancer is much higher in developed countries, the distribution is not uniform globally (figure 2.1). Under-developed countries have a higher rate of the infection-related cancers such as cancer of the cervix (100% caused by infections globally), liver (77%) and gastrointestinal tract (75%). Female breast cancer, prostate and colorectal, and lung cancers are found more commonly in developed countries. This distribution may be attributable to education, quality of life, economic stability and even the implementation of avoidable risk factors, screening and availability of vaccination (Ferlay *et al*., 2015). Western Europe has almost double the cancer incidence compared to Eastern Africa.

2.2.2 Prostatic conditions

Prostatitis is an inflammation of the prostate gland causing tender (painful) swelling of the prostate. It is usually of bacterial origin and can be treated using antibiotics. Chronic nonbacterial prostatitis also results in inflammation and pain in the lower urinary tract. Benign prostatic hypertrophy is common after 50 years and may present with obstructive urinary tract symptoms. Prostate cancer is cancer of the prostate (Powell, 2007).

2.2.3 Cancer prevalence

Figure 2.2 shows the global incidence and mortality of prostate cancer. Incidence is lowest in China 1.9% (9/100,000), White Americans/Caucasians 1.4–2.4% (161.4/100,000), African Americans 2.2–5.1% (255.5/100,000), African Caribbean men 5 – 10.4% (281/100,000). In Africa, epidemiological studies have found low Prostate Specific Antigen testing as reported from Nigeria, Ghana, Uganda, Tanzania, Senegal with related late presentation and low percentage of Organ confined disease (T1 – T2) but high percentage of locally advanced (30%) and metastatic prostate cancer (55%) cases (Crawford, 2003; Hsing, 2000; Glover *et al.*, 1998).

Inflammation, Prostatitis and increased BMI (Cooperberg *et al*., 2004; Cooperberg *et al* 2005; Ritchey *et al*., 2005; Mutetwa *et al*., 2010; Gann, 2002; Laponite *et al*., 2008).

2.3 Pathogenesis of Cancer

Cells of the body can undergo abnormal transformation through carcinogenesis, oncogenesis or tumorigenesis. Carcinogenesis is marked by molecular changes at the genetic, epigenetic and cellular levels with a probable change into a malignancy. There is a natural autoregulation between cell proliferation and programmed cell death. However, an imbalance usually results in cancer formation. According to somatic mutation theory (the prevailing accepted theory of carcinogenesis), mutations and epimutations in DNA that lead to cancer disrupt these orderly processes by disrupting the programmed regulation of the processes, upsetting the normal balance between proliferation and cell death (Fearon *et al*., 1900). This results in uncontrolled cell division and development by natural selection.

Variations in inherited genes may be a factor that predisposes individuals to cancer. In addition to genetic factors, environmental factors including carcinogens and radiation result in mutations that start the process of carcinogenesis. Furthermore, random mistakes in the replication of normal DNA may lead to cancer-causing mutations (Tomasetti *et al*., 2017). An interplay of different mutations of certain classes of genes are necessary for transformation into cancerous cell (Wood *et al*., 2007, Knudson, 2001). Uncontrolled mutations in genes that regulate cellular division, apoptosis, and DNA repair can lead to unprogrammed cell proliferation and consequently cancer. The six classic hallmarks of cancer include uncontrolled replication, lack of regulation of growth signals, absence of apoptosis, absence of response to antigrowth signals, sustained angiogenesis

and neo-vascularization, and the ability to permeate neighboring and distant tissues (metastasize) (Knudson, 2001). Other features of malignant cells/tissues include an enhancement in anabolic metabolism, avoidance of immunoresponse, as well as numerous stress phenotypic expressions. Carcinogenesis is generally a disease of atypical tissue growth regulation. For a typical cell to undergo atypical transformation there has to be significant alteration of genetic control and modification of genetic regulation of cellular differentiation and death (Croce, 2008). Epigenetic and genetic changes are recognized at various levels, ranging from entire chromosomal loss or gain to a single DNA nucleotide mutation, a microRNA activation or silencing that regulates the overt expression of 100 to 500 genes (Lim *et al*., 2005; Balaguer *et al*., 2010).

Oncogenes can be defined as an upregulation of normal genes or an alteration of normal genetic control of cells to have an atypical form and qualities. Both situations nurture the malignant phenotypic expression of cancerous cells (Croce, 2008). Tumor suppressor genes inhibit abnormal cell division, survival and the qualities of cancerous cells. These genes are downregulated by genes that promote cancer (ғigure 2.3). Finally, oncovirinae are viruses that house an oncogene and have the special property of growth tumor in the host tissue. This is called a viral transformation (Gschwind *et al*., 2004).

2.3.1 DNA Damage

DNA damage and epigenetic defects play a pivotal role in faulty repair of damaged DNA genes in carcinogenesis. It is the primary cause of cancer (Bernstein *et al*., 2013, Kastan, 2008*).* Over 60,000 novel original DNA damages occur averagely in every human cell day, resulting from various cellular functions. Also, exposure to exogenous agents such as tobacco smoke increases

DNA damage, which likely results in lung cancer formation (Cunningham *et al.*, 2011). UV light from solar radiation is notorious in melanoma formation (Kanavy *et al.*, 2011). Examples of microorganism facilitated cancer include *Helicobacter pylori* proliferation (colonizes the gastric mucosa), which has a high yield of reactive oxygen species that injure DNA and plays a role in gastric cancer (Handa *et al.*, 2011) and the *Aspergillus flamigatus* metabolite, aflatoxin, causes DNA damage and is implicated in liver cancer. Macrophages and neutrophils in an inflamed colonic epithelium are endogenous agents that also cause DNA damage, causing excess free radical production that initiate colon cancer (Katsurano *et al.*, 2012). Also, high levels of bile acids in high fat diet may also contributes to colon cancer (Bernstein *et al.*, 2011).

A delay in DNA repair mechanisms causes a build up of DNA damage and further increases the risk of cancer formation. Individuals with inherited deficiency of any of the 34 DNA repair genes have high risk of resulting DNA defects (e.g. p53 mutations) causing up to 100% chance of cancer in an individual's lifetime (Malkin, 2011) (figure 2.4). Most cancers are non-hereditary or "sporadic cancers". It is important to note that undefined hereditary component is found in 30% of sporadic cancers while 70% of them do not have any hereditary component (Lichtenstein *et al.*, 2000). Although it is often not practicable to establish the initial causative factors for most cancers, one causative factor may exist in a few cases. For example, the Human Herpes Simplex Virus-8 (HHV 8) causes all Kaposi's sarcomas. In contrast, lung cancer has many contributory factors, including tobacco use and radon gas. Tobacco smokers have a 14 times higher chance at developing lung cancer compared to non-smokers. There is a 93% chance of lung cancer diagnosis in a tobacco smoker as opposed to a 7% chance of lung cancer being caused by radon

gas or other non-tobacco causes (Cuozzo, 2007). This data makes it possible for researchers and health workers to reach significant milestones in the fight against lung cancer via prevention techniques as well as education. Compounds such as acrolein, formaldehyde, acrylonitrile, 1,3-butadiene, acetaldehyde, ethylene oxide and isoprene have been reported to exhibit most potent mutagenic effects at highest concentrations among the 5,000 compounds found in tobacco smoke (Cunningham *et al.,* 2011).

Molecular biology techniques are used to categorize mutations, epimutations and chromosomal aberrations within a cancerous growth to determine a prognosis. At least half of all tumors have defective p53 (a gene with several mechanisms of anticancer function) (Malkin, 2011). This defect has a correlation with poor prognosis, as there is an unlikely chance of damaged cells going into apoptosis. Mutations of telomerase extinct barriers further, thereby extending the number turnover for cellular carcinogenesis (Katsurano *et al*., 2012). There are genetic mutations geared towards neo-vascularization and metastasis. When cancer is formed, there is evolution, clone formation and sub clone production. There are spiral of activities that need active interventions for control.

2.4 Apoptosis

Apoptosis is defined as ~~of~~ programmed cell death in multicellular organisms (Green *et al*., 2011). Specific biochemical processes lead to the cellular morphological changes and eventually death. Blebbing, cell shrinkage, nuclear fragmentation, chromosomal DNA fragmentation, chromatin condensation and mRNA decay are among the changes that precede apoptosis (Figure 2.3.A and B). Between 50 and 70 billion cells are lost daily due to apoptosis in the average human adult. A healthy child between the ages of 8 and 14 undergoes about 20 billion to 30 billion cellular deaths

2.5 Oxidants, Antioxidants and Carcinogenesis

Free radicals including reactive oxygen species such as superoxide anions (O_2^{2-}), hydrogen peroxide (H_2O_2), and hydroxyl radical (^{-}OH), malondialdehyde (MDA) and nitric oxide (NO) are primarily or secondarily implicated in the interplay of carcinogenesis (Cerutti *et al*., 1988). DNA damage with mutagenic potential has ROS as the main contributory factor, especially in the dysregulation of tumour suppressor genes. Reactive Oxygen Metabolites (ROM) serves as initiator and/or promotor in the development of cancer (Crawfordd *et al*., 1989).

These free radicals are recognized in most incidence of sister chromatid exchanges (SCEs) and chromosome breaks and gaps (CBGs). Malondialdehyde (MDA) is a by-product of lipid peroxidation, which is involved in DNA adduct formations, believed to be responsible for carcinogenesis (Crawfordd *et al*., 1989). Nitric oxide, however, serves a dual role in cancer. In higher quantities, NO causes tumour destruction. However, lower levels tend to support carcinogenesis and metastases. Nitric oxide is notorious for causing single and double DNA strand breaks and destruction. Peroxynitrite ($OONO^{-}$) a metabolite of NO is a strong mutagen that can induce transversion mutations. Superoxide anion, hydrogen peroxide and hydroxyl radical (O_2^{2-}, H_2O_2, OH^{-})-induced lipid peroxidation can also be stimulated by nitric oxide. The deleterious effects of these reactions can be countered by endogenous and exogenous antioxidants.

There are enzymatic and non-enzymatic molecules that form the body's antioxidant defense system, effectively counteracting or inhibiting the damaging action and effects of free radicals. These antioxidants include superoxide dismutase (SOD), glutathione peroxidase (GPx), and

catalase (CAT). SOD converts O_2^{2-} to H_2O_2, which is then converted to H_2O with the help of GPx and CAT (Ray et *al*., 2002). Hydroxyl radical production is prevented by SOD. SOD has antipoliferative and anticarcinogenic actions. It also acts as an inhibitor at initiation and promotion/transformation level in carcinogenesis. Of the above-mentioned antioxidants, the most efficient enzyme is CAT (Ray et *al*., 2002). GPx and CAT deactivate many exogenous mutagens (Amstad *et al*, 1984). Levels of sister chromatid exchanges and chromosomal aberrations are reduced by CAT. Besides the antioxidants described above, vitamins such as vitamins A, E, and C, possess antioxidant properties and have many biological roles such as immune stimulation, nitrosamine formation, prevention and modification of the metabolic initiation of potential carcinogens. Antioxidants have the innate ability to prevent the genetic phenotypic expression caused by DNA damage via reactive oxygen species mechanisms. These confer anticancer properties on antioxidants (Ray et *al*., 2002).

2.6 Diagnosis of Prostate Cancer

There are various methods used to assist in the diagnosis of prostate cancer. These include digital rectal exam and prostate-specific antigen test.

2.6.1 Digital Rectal Examination

A digital rectal examination (DRE) involves the clinical examination (palpation) of the prostate gland. The patient is examined in the bent over prone position and a gloved examining finger is inserted into the rectum. The gland is allocated in front of the rectum. The characteristics including a size estimation, edges, consistency and palpation of a dividing median groove are noted and interpreted. Further tests may be requested depending on the findings of this clinical assessment.

2.6.2 Prostate-Specific Antigen Test

It is a non-specific blood test done in cases of suspected prostate pathologies. It has a falsely high value after a DRE, in cases of prostatitis and even trauma to the prostate. In the absence of these, a value of 0-4ng/ml is accepted as normal. Any value above 4ng/ml ~~is~~ signifies a high probability of prostate pathology in the absence of trauma or DRE. It is used in conjunction with the clinical presentation and DRE findings to make a diagnosis of prostate cancer. These tests are screening tests with no direct correlation to mortality from the disease (Bunker *et al.*, 2002). Ultrasound and biopsy may be necessary after the first two primary tests are done to further make a care plan and categorize prostate cancer. Ultrasound (transrectal ultrasound) can be performed for a more objective assessment of the gland. During the procedure, a cigar-sized probe is inserted into the rectum to examine the gland. Prostatic biopsy is performed where a needle is used to harvest cells or tissue for histological and cytological assessment.

2.6.3 Determination of aggressive prostate cancer

A histological analysis of harvested tissue is necessary to determine the grade/level of aggressiveness of the cancer (Heyns *et al*., 2011). The Gleason's score is used for this purpose. Scoring ranges from 2 (nonaggressive cancer) to 10 (very aggressive cancer). A score of 2 to 4 indicates that the cells still look very much like normal cells and pose little danger of spreading quickly. A score of 8 to 10 stipulates that the cells have very few features of a normal cell and are likely to be aggressive. A score of 5 to 7 however indicates intermediate risk (Vallencien *et al*., 1999).

available options, risk of intervention to the patient, age, quality of life and cost (Cooperberg *et al.,* 2004; Cooperberg *et al.,* 2005).

2.7 General Therapeutic Modality Options in Cancer Treatment

Several treatment options exist. The multi-modal approach is employed. Treatment options are influenced by a personal preference or choice, availability, cost, quality of life and stage of the disease. The aim of treatment is to alleviate symptoms, improve the overall quality of life and prevent recurrence (Kantoff *et al*., 2010). Treatment options include; surgery, radiation, chemotherapy and hormonal therapy. Others include; biological therapy (Immunotherapy and biological response modifier therapy), gene therapy, virotherapy, nutritional therapy and spiritual support.

2.7.1 Surgical Intervention

Surgery remains one of the oldest cancer therapy modalities and the most effective treatment for many types of cancer. It serves both diagnostic as well as treatment options. It can also serve as a palliative function. One advantage of surgery as a cancer therapy modality is its tumor specificity, the emergence of tissue resistance is also not possible. In addition, surgery restores the normal anatomy of the body, as well as relieve some pressure symptoms. One main disadvantage of surgery as a therapeutic modality in cancer treatment is that it is usually unhelpful in established metastatic disease or for unrecognized metastases. Different types of surgery are used depending on the type of cancer, where it is located, and the goals of surgery.

2.7.1.1 Types of Surgery for Cancer Therapy

There are 4 types: conventional, laser, cryosurgery, microscopically controlled, and endoscopic surgery. With conventional approach, the surgery requires large cuts, called incisions, through skin, muscle and sometimes bone. It appears to be more invasive, requires prolonged recovery periods and more painful (Wang, 2008). Laparoscopic surgery involves the use of a thin light tube with an inbuilt camera attached to perform the intervention via small skin incisions. Laser surgery employs a narrow beam of high intensity light. Cryosurgery requires the use of liquid nitrogen to freeze and kill atypical cells. In microscopically controlled surgery the cancerous tissue is peeled off, a layer at a time until normal tissue is reached on histological examination. Endoscopy affords the examiner the opportunity to visualize as well as take samples for histology, debulk and even treat by excising the potential cancerous tissue. Radical prostatectomy involves the excision of the gland, some neighboring tissue and some lymph nodes. Radical prostatectomy can be classified as robot-assisted, laparoscopic, transurethral, transrectal or retropubic prostatectomy. Urinary incontinence and erectile dysfunction are the main side effects of this procedure (Weldon *et al*., 1988). Cryosurgery can also been employed.

2.7.2 Radiation Therapy

This utilizes high powered energy radiation to destroy cancerous cells thereby restricting their growth and systemic spread. Just like surgery, radiotherapy is a localized form of treatment and external physical intervention with a chemical target (DNA). However, since radiotherapy is not especially tumor-specific damage to normal tissue can occur during it application. It can be invaluable for debulking huge local tumor or reducing tumor size to allow surgery. There are two main forms of delivery; internal and external. In external radiotherapy, rays are delivered from

outside of the body. In internal radiation, or brachytherapy, a radioactive substance is implanted into or near the diseased organ. The use of external beam or brachytherapy may be employed (Zeleftsky *et al*., 2002) in treatment of prostate cancer. It is normal to undergo external radiotherapy five times a week for several weeks. Brachytherapy is done under ultrasound guidance with a gentle introduction of the radioactive agent. It is self-limiting and relatively safe to the body. Challenges posed by radiotherapy to patients include urinary tract irritation such as dysuria (painful urination), increased urinary frequency and urgency. Erectile dysfunction, diarrhea, and painful defecation, dyschezia, have also been recognized.

Generally, radiotherapy can be used as curative, limiting and palliative form of treatment. However, undesired side effects may accompany even the most precise therapy. Most of the undesired symptoms of radiotherapy may be alleviated with medications, diet, and various bodily exercises, and at times some of the effects fade when treatments are completed. Also, malignancies can be induced after radiotherapy. The efficiency of the radiation therapy is limited utility against extensive and systemic cancerous disease (UNSCEAR, 2000). Its effectiveness is also limited by tumor heterogeneity and selection for radiation-resistant variants. Some common type of electromagnetic radiation therapies: Proton therapy which is a form of radiotherapy treatment that utilizes the positively charged particle of an atom (proton) instead of X-rays to treat cancer. Protons are cytotoxic at very high energy levels. Though its efficiency is undoubted, access limit its use. X-Ray therapy includes photodynamic therapy, neutron capture therapy, laser-microwave therapy and radiowave therapy.

2.7.3 Chemotherapy

Chemotherapeutic agents are drugs (chemicals) that are expected to be toxic to cancer cells but harmless to the normal cells. Most chemotherapeutic agents interfere with cell proliferation and rely on rapid cell cycling and/or promotion of apoptosis for their relative selectivity against cancer cells. Tumors are most sensitive to chemotherapy when they are multiplying rapidly, primarily because of progression through the cell cycle. These metabolically active cells are thus susceptible to drugs that interfere with cell growth and multiplication (Brentjens *et al*., 2013). Most chemotherapeutic agents are classified based on the stage of the cell cycle it acts on: Cell cycle specific (CCS): Most effective in tumors with large proportion of proliferating (growth fraction) cells (G_1, S, G_2, M). DNA synthesis inhibitors (antimetabolites, folate antagonists), are S-phase specific. Microtubule poisons (taxanes, vinca alkaloids) interfere with spindle fibre formation during M phase. Figure 2.7, depicts the various levels of actions of CCS anticancer agents. Alkylating agents damage DNA and other cellular macromolecules during all phases of the cell cycle. Cell cycle non-specific drugs (CCNS) act independent of the cell cycle. They are particularly useful in low growth fraction solid tumors (G_O + cycling cells).

2.7.3.1. Modes of Anti-Cancer Drug Resistance

Modes of anticancer drug resistance include decreased ability to repair DNA defects, reduced cellular permeability, inactivation of glutathione synthase and deactivation of alkylating agents through conjugation reactions (catalyzed by glutathione S-transferase), deficient apoptosis, increased production of reactive oxygen species as well as defective antioxidant mechanisms (Brentjens *et al*., 2013).

2.7.4. Hormone Therapy

Hormones are the body's chemical messengers produced in the endocrine glands such as the thyroid, pancreas, and ovaries in women and testicles in men. Hormones are known to inhibit, stop or kill the proliferation of cancerous cells. In hormonal treatment, activities of specific hormones in the body are modified to negatively influence the rate of cancerous cellular growth (Kantoff *et al*., 2010). Side effects of hormonal therapy is dependent on the cancer target. Breast cancer targeted therapy has been associated with menstrual irregularities, hot flashes, decreased libido, vaginal irritation, dryness and discharge and even mood swings. Social support, improvement in nutrition, natural remedies as well as spiritual support may be of significance to control these effects (Schlom, 2012).

Testosterone is the prostate-feeding hormone. This therapy is directed against the production of this hormone to reduce growth of the prostate cancer cells. Types of hormonal anti-prostate cancer therapies include anti-testosterone medication (Luteinizing hormone-releasing hormone (LH-RH)) agonists. This causes a negative feed-back inhibition of testicular production of testosterone. Examples include; leuprolide (Lupron, Eligard), goserelin (Zoladex), triptorelin (Trelstar) and histrelin (Vantas), ketoconazole and abiraterone (Zytiga). Medications targeting transport of androgens to the diseased prostate gland include bicalutamide (Casodex), flutamide, and nilutamide (Nilandron). Orchidectomy is the surgical removal of the testis (a source of androgens

in the body) and may be employed in early disease. Hormone therapy is used in men with advanced prostate cancer to shrink the cancer and slow the growth of tumors. At stage I and II of prostate cancer, hormone therapy is mainly used to debulk the tumour to enhance response to radiotherapy. It presents with an increase in BMI, reduced libido, hot flashes, osteoporosis as well as erectile dysfunction.

2.7.5 Biological Modifier Therapy - Immunotherapy

This mode of treatment entails the use of living organisms or the products of such organisms to kill cancerous cells. It employs vaccines, bacteria, cytokines, interferon, etc., to activate immune response and thereby inhibit carcinogenesis (Rivoltini *et al*., 2005). They may not directly attack cancerous cells but may produce and autoimmune response to cancerous cells. This approach can be potentially highly tumor-specific and it has been reported to be effective against disseminated disease including unrecognized micro-metastases (Brentjens *et al*., 2013; Sutlu and Alici, 2009). Reports indicate that they are probably of limited value against extensively advanced diseases. It can involve severe, life-threatening, treatment-limiting side-reactions, limited by tumor heterogeneity, selection for unresponsive variants, and emergence of immune-escape. Common types of immunotherapy include monoclonal antibodies, which are synthetically produced antibodies with an excellent characteristic of specific receptor interaction. It may be surface antigen binding specific. Some approved monoclonal antibodies with cell surface targets include rituximab-aims for the CD20 antigen located on non-Hodgkin lymphoma cells, and alemtuzumab, that aims for the CD52 antigen found on B-cell chronic lymphocytic leukemia (CLL) cells (Kantoff *et al*., 2010).

Cancer vaccines

Vaccines are given to generate an immune modulated response against cancer cells. Examples include sipuleucel-T for hormone refractory prostate cancer (Disis *et al*., 2009; Gulley *et al*., 2010) and Cervarix. The latter is a vaccination given to young ladies with no sexual exposure which has been introduced to prevent the incidence of cervical cancer.

Non-specific immunotherapies: they entail a non-specific immuno-stimulation using synthetically produced cytokines such as interleukins (IL) and interferons (INF). INF-α causes cytotoxicity as well as has antiproliferative functions and is employed in the treatment of melanoma, Kaposi sarcoma, and other hematologic cancers (Joshi *et al*., 2009). IL-2 stimulates the B cell antibody production. Aldesleukin, synthetic IL-2 is recommended for the treatment of metastatic renal cancers and metastatic melanoma (Jonasch and Haluska, 2000). In prostate cancer, the vaccine; sipuleucel-T (Provenge) is used (D'Amico *et al*., 2008).

2.7.6 Gene Therapy

Gene therapy although is still under investigations, tends to insert genetic material DNA or RNA into the diseased cells. The gene is usually transferred into the cancer cell by a vector. The most common types of carriers used in gene therapy are viruses due to their ability to invade cells and deliver genetic material. The viruses are attenuated so that they cannot cause serious diseases. Gene therapy is potentially highly tumor-specific. However, accessibility of cell targets is a major challenge for general application (Triozzi *et al*., 2011). There are several ways by which gene therapy are designed to function. Three main means are employed. Firstly, by boosting the immune response via improving the genetic expression of cytokines by the implantation of the genes into

the cancer cells. Secondly, by improving sensitivity to treatment as well as enhancing the anti-cancer treatments. Thirdly, the employment of "suicide" gene therapy which involves insertion of genes into the cancerous cells that allows the cells to convert drugs from an inactive form

(prodrugs) to an active form. The active toxic drug is activated only in the tumour. Normal cells, which do not express the suicide genes are thus not affected by the pro-drug (Waseem *et al.*, 2011).

2.7.6.1 Blocking Processes – Protection of Cancer Cells

This procedure involves the insertion of genetic material to block the expression of oncogenes that function to enhance tumor growth. These include short nucleic acid strands (RNA or DNA molecules) with sequences that are complementary to the gene's mRNA. They are then loaded into vectors or given to cells directly. These short synthetic single-stranded nucleic acid molecules, called oligonucleotides, can bind to the target mRNA, preventing synthesis of protein products or even causing its degradation. At times there can be substitution of altered tumor suppressor genes that produces a defective protein or fails to produce any protein with a normal version of the gene. This is because tumor suppressors genes (e.g., TP53) play a role in destroying cancer cells, restoring the normal function of these genes may inhibit cancer growth or promote cancer regression (Mosolits *et al*., 2005).

2.7.7 Virotherapy

This strategy involves using viruses such as oncolytic reovirus, vaccine virus, measles virus, mumps virus, adenovirus, etc. with a high turnover in the affected cells and invariably cause cytotoxicity (Finn, 2008). These viruses are tumour cell specific and can be engineered to enhancesuch an ability. However, virotherapy, exhibits lethal immune-responses in persons sensitized to viral vector (Hodi *et al*., 2010). Two main techniques are employed: transductional and transcriptional targeting.

2.7.7.1 Transductional Targeting

This involves engineering of viruses such as adenovirus to attach themselves to adaptor molecules on the surface of only cancer cells. They do not infect normal cells. These viruses then multiply in cancer cells and causes cancer cells to burst and disperse virus to infect other cancer cells (Grupp *et al.,* 2013).

2.7.7.2 Transcriptional Targeting

Some viruses are engineered to replicate under control of tumor promoter genes. Virus replicates only in cancer cells that have the tumor-specific promoter. For example, oncolytic viruses with the ability to produce specific cancer-associated antigen, such as EGFR or HER-2 may be engineered towards cancer cell specificity. The infected cancer cells bursts and disperse viral particles to infect other cancer cells (Finn, 2008; Liu *et al*., 2008).

2.7.8 Nutritional Therapy

The nutrition therapy helps restore digestive health, prevent malnutrition and provide dietary recommendations during cancer treatment. The goal of this therapy is to help cancer patients stay strong and nourished so they can continue with cancer treatment. This involves selection of the most suitable nourishing foods usually based on the person`s health history, disease type and treatment plan.

2.7.9 Spiritual Therapy

Spiritual support can be a fundamental part of treatment ~~at~~ cancer. Faith, hope and love in the presence of peaceful familiarity can help in the long run to boost the immune system, reduce the risk of depression and suicide, reduce hospital stay and improve the overall outcome.

and have also been used as disinfectants. Four major types of vinca alkaloids, vinblastine, vinorelbine, vincristine and vindesine, are used clinically as anticancer medications. However, only the first three alkaloids are approved for use in the United States and in many countries for the management of Leukemia and Lymohomas (Kufe *et al*., 2003). Notable side effects are; constipation, hair loss as well as nausea and vomiting.

2.9.2. Doxorubicin

Doxorubicin is an anti-cancer agent that is used to treat cancer of the bladder, breast, thyroid, lung, ovary, stomach, acute lymphocytic leukemia, Hodgkin's lymphoma, Kaposi's sarcoma and many more (Rossi, 2013). It is administered intravenously in combination with other anticancer agents. Its side effect include bone marrow suppression, hair loss, inflammation of the oral cavity, vomiting and rashes. Other deleterious side effects may include damage to the heart, damage to tissue at the site of injection, treatment-related leukemia, and anaphylactic reactions. Urine appears reddish brown following administration for a few days. Doxorubicin, an anthracycline and antitumor antibiotic, interferes with the function of DNA (Tacar *et al*., 2013). Several drugs have been designed in which doxorubicin have been incorporated. These include AC (adriamycin, cyclophosphamide), FAC (5-fluorouracil, adriamycin, cyclophosphamide), CHOP (cyclophosphamide, hydroxydaunorubicin, vincristine, prednisone), TAC (taxotere, AC), ABVD (Adriamycin, bleomycin, vinblastine, dacarbazine) (Brayfield *et al*., 2013).

2.9.3. Other medicines

Topotecan is a semi-synthetic compound derived agent from extracts of *Camptotheca acuminate*. Etoposide as well as Teniposide have a similar chemical

structure like the toxin podophyllotoxin from *Podophyllum peltatum* (Cragg and Newman, 2005). Docetaxel and paclitaxel were extracted from *Taxus brevifolia* (Olaku *et al*., 2013). This group of drugs are notorious for their neurotoxic effects.

Orthodox anti-cancer chemotherapeutic agents have long been known to be manufactured from plants and even soil microbes. Considering issues of drug resistance, compliance, side effects, cost, availability, and even the increase in incidence of cancer makes the idea of readily available alternatives to chemotherapeutic agents that have the potential of also being less toxic and expensive imperative. *Momordica charantia* and *Fleurya aestuans* are two such plants used by traditional medical practitioners as anticancer remedies.

This study focuses on the cytotoxic mode(s) of action of aqueous extracts of the aqueous leafs of the afore mentioned plants on PC3 and LNCaP prostate cancer cell lines.

2.10. Medicinal Plants Used

2.10.1 Momordica charantia

Momordica charantia (MCwp) is of the kingdom Plantae and order Cucurbitales. The plant belongs to the family Cucurbitacceae, genus *Momordica*. Synonyms of the species *M. charantia* include bitter lemon, balsam pear and bitter gourd. The plant is widely distributed in the Caribbean, Asia and Africa. It is herbaceous, tendril-bearing vine that can grow up to 5m (16ft) in length. It bears simple, alternate leaves 4–12 cm (1.6–4.7 in) across, with 3 to 7 widely separated lobes (Ananya *et al*., 1998). The plant bears male and female flowers which appear yellow in colour. In the Northern Hemisphere, flowering period occurs from June to August, followed by fruiting period from September to November. The fruit is most often eaten green, or as it is

(Peng *et al*., 2011). The anti-diabetic effect of concentrated bitter lemon extracts is supported by numerous animal studies and small-scale human studies (Wang *et al*., 2011). A review revealed that MCwp, when consumed in raw or juice form, is efficient as an oral hypoglycaemic agent. The effectiveness at playing this role is highly dependent on mode of administration. More research is necessary to verify this effect (Bachok *et al*., 2014). The plant has shown a lot of promise in the treatment and management of dyslipedemia (Saad *et al.*, 2017). The administration of bitter lemons aborts oxidative stress and lipid peroxidation associated with hypercholesterolemia. This plant has been suggested to have anticancer properties against prostate cancer (Clafin *et al*., 1978).

2.10.1.2. Adverse effects of *M. charantia*

Fever, diarrhea, hypoglycemia, urinary incontinence, abdominal pain and chest pain are among some of the adverse effects of MCwp. Symptoms do not require treatment, as they are generally mild and can resolve with rest (Ooi *et al*., 2012).

2.10.2. *Fleurya aestuans*

The plant, *Fleurya aestuans* (Fawp), is of the order *Rosales*, family Urticaceae, genus *Laportea* and species *aestuans*. Studies have revealed that the leaves are used by traditional medicine practitioners to treat asthma, hypertension, rheumatism, rickets and wounds (Essiett *et al*., 2011). As a result, several types of drugs could be produced from these plants as antidote and antibiotic drugs (Essiett *et al*., 2011).

As a result, the observed IC_{50} value for (-)-epigallocatechin-3-gallate) EGCG was 2-fold higher using MTT and MTS compared to dyes quantifying ATP and DNA. In contrast, when cells were treated with apigenin MTT and MTS assays showed consistent results with ATP, DNA, or trypan blue assays. The MTT assay is a globally accepted method of assessing cell viability because it is readily available, easy to perform and produces reliable results.

2.11.2 Methods for Assessing Antioxidant Activity

The difference between antioxidant and anti-radical activity is explained by (Tirzitis *et al.*, 2010). According to the authors, anti-radical activity describes the characteristic of compounds to react and quench free radicals (in a single free radical reaction). Antioxidant activity, on the other hand, is the ability of a substance to block oxidation process. All tests using a stable free radical (eg. 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) and 2,2-diphenyl-1-picrylhydrazyl (DPPH)) give information on the radical scavenging or antiradical activity, although in many cases this activity does not correspond to the antioxidant activity. To obtain information about the real antioxidant activity with respect to lipids or food stabilization, it is necessary to carry out the studies on the real product (plant oil, lipoproteins, etc.). Numerous methods are used to evaluate antioxidant activities of natural compounds in foods or biological systems with conflicting results in some instances. Two free radicals commonly used to assess antioxidant activity *in vitro* are ABTS and DPPH. Both radicals are foreign to biological systems. ABTS assay measures the relative ability of an antioxidant to scavenge the ABTS generated in aqueous phase, as compared with a Trolox

(water soluble vitamin E analogue) standard. The ABTS (radical coloured) solution by hydrogen-donating antioxidant is measured by suppression of its characteristic long wave (734nm) absorption spectrum (Miller *et al*., 1999).

DPPH is a stable free radical with an absorption band at 515 nm. It loses this absorption when reduced by an antioxidant or a free radical species. The DPPH method is widely used to determine antiradical/antioxidant activity of purified phenolic compounds as well as natural plant extracts (Fukumoto *et al*, 2000). Most phenolic antioxidants react slowly with DPPH, reaching a steady state in 1-6 h or longer (Bondet *et al*, 1997). This suggests that antioxidant activity using DPPH should be evaluated over time. The method also has good repeatability and is used frequently, however, like ABTS, it has limited relevance if any, to biological systems. Particularly, colour interference of DPPH with samples that contain anthocyanins leads to underestimation of antioxidant activity (Arnao *et al*., 2000). Although ABTS has many seeming advantages over DPPH availability, cost, as well as frequency of its usage in similar investigations contributes to its use in this experiment.

Generally, natural antioxidants include polyphenols, flavanoids, vitamins and some volatile chemicals. There are two general types of antioxidant assays: One is lipid peroxidation-related assay, which includes thiobarbituric acid (TBA) assay, malonaldehyde/gas chromatography (MA/GC) assay, malonaldehyde/high-performance liquid chromatography (MA/HPLC) assay, beta carotene bleaching assay and conjugated diene assay. The other type of antioxidant assay is electron- or free radical scavenging-related assay. This type of assay includes DPPH assay, ABTS assay, ferric

reducing/antioxidant power (FRAP) assay, ferrrous oxidation-xylene orange (FOX) assay, ferric thiocyanate assay, and aldehyde/carboxylic acid (ACA) assay.

2.11.3 Determination of Total Phenolic Contents in the Plant Extracts

The concentration of phenolics in plant extracts is commonly determined using spectrophotometric method (Singleton *et al*., 1999). Gallic acid (also known as 3,4,5-trihydroxybenzoic acid) is a trihydroxybenzoic acid, a type of phenolic acid, found in gallnuts, sumac, witch hazel, tea leaves, oak bark, and other plants. The Gallic acid assay, a standard test used for determining the total phenolic content using the Folin-Ciocalteu assay has been used in numerous scientific experiments (Trinidad *et al*., 2008; Suman *et al*., 2014) compared two different colorimetric methods over three years and indicated that a ferrous ammonium sulfate indicator procedure was a good choice for their General Organic and Biochemistry laboratory. The more complex Folin-Ciocalteu reagent method, which was applied to solid foods, also performed well.

2.12 Apoptosis

The degree of apoptosis is measured using gel electrophoresis, light or electronic microscope and flow cytometry. Other popular measures include Caspase 3, p53, Immunochemistry (IHC), M30, Annexin V and the *in situ* 3-end labelling method (ISEL). The agarose gel electrophoresis assay has been used globally to determine the degree of apoptosis (Gong *et al*., 2014; Cohen *et al*., 1992; Walker *et al*., 1993; Ito *et al*., 1992). The agarose gel electrophoresis assay was used in this investigation because of its ease of use as well as availability.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Plant Samples

Freeze-dried FAwp and MCwp samples stored at -20^{o}C were obtained from the Department of Clinical Pathology, Noguchi Memorial Institute for Medical Research. The plants were previously harvested from the Botanical Gardens of the University of Ghana, Ghana with Geographical coordinates: N05.657235o W000.18751o) in September 2012 and verified by a Taxonomist at the University of Ghana Herbarium, where voucher specimen were kept.

3.2 Cell Lines and Reagents

The cell lines used (LNCaP and PC3) were obtained from RIKEN BioResource Centre Cell Bank (Japan). Culture medium Rosewell Park Memorial Institute (RPMI), MTT dye, isopropanol, Hydrochloric acid (HCL), trypan blue solution, Butylated Hydroxytoluene (BHT) absolute ethanol, foetal bovine serum (FBS), antibiotics (penicillin and streptomycin), DPPH, curcumin, ursorlic acid and phosphate buffer saline were acquired from Sigma-Aldrich Company (St. Louis, MO, USA). The JC-1 Mitochondrial Membrane Potential Assay Kit was acquired from Wako Pure Chemical Industries (Osaka, Japan) and the Agarose gel Electrophoresis reagent was from EMD Millipore Cooperation (Hayward CA). RNase A and dimethyl sulfoxide (DMSO) were obtained from Wako Pure Chemical Industries (Osaka, Japan) and DNA marker and loading dye were purchased from Watson (Japan). Folin-Ciocalteu reagent and gallic acid were purchased from Sigma Chemical (Selangor, Malaysia).

3.3 Preparation of Test Solutions

The MCwp and FAwp were air-dried and pulverized separately. Pulverized samples were extracted with distilled water at 80°C for 1 h and centrifuged at 4500 rpm for 15 min at 25^{o}C. The supernatants were collected and pellets re-suspended in distilled water to repeat the extraction procedure. The two supernatants for each sample were pooled together, frozen at -20 ^{o}C and freeze-dried using a LABCONCO® freeze dryer. The percentage yields for FAwp and MCwp were 14.96 (0.3g) and 11.00 (0.22 g) respectively relative to a starting weight of 2 g each. Freeze dried samples were stored at -20 ^{o}C until analysis. The freeze dried materials, stored at -20 ^{o}C since 2012 were used for the current studies.

3.4 Cell Culture

The PC3 and LNCaP cells were cultured in RPMI-1640 medium, supplemented with 10% FBS and 1% penicillin–streptomycin solution. Cultured cells were maintained in a humidified incubator at 37°C in the presence of 5% CO_2.

3.5 Methods

3.5.1 Morphological Changes after Treatment

After culturing the PC3 and LNCaP cells, they were treated with FAwp, MCwp and the positive controls curcumin and ursolic acid, as well as cells devoid of any treatment (negative control). Cellular morphological changes were then observed at 3, 6 and 24 h using phase-contrast microscope at 20X magnification.

DPPH + A-H ⟶ DPPH-H + A. Absorbance decreases as DPPH radical binds to one electron. A discolouration occurs which is stoichiometrically related to the number of electrons gained (Lacine *et al*., 2013).

Methanolic solution of 100 µL of 0.5 mM DPPH was added to equivalent volumes of various concentrations of each extract (concentration range 0-5 mg/ml) and incubated for 20 min. The DPPH is light sensitive and photo-bleaches on exposure to light therefore the mixture was immediately kept in the dark at room temperature (25^{o}C). The blanks used in the assay were absolute methanol and water without the extracts or standard, BHT. After 20 min of incubation at room temperature, the absorbance was read at a wavelength of 517 nm (Tecan Infinite M200 Pro plate reader, Austria). The concentration that effectively reduced the free radical effect of DPPH by 50% (EC_{50}) was calculated from the following formula for each extract:

$$\% \text{ Antioxidant activity} = [(A_0 - A_1)/A_0 \times 100]$$

Where A_0 is the absorbance of negative control (methanol), and A_1 is the absorbance of test sample with DPPH. Triplicate experiments were performed. The mean percentage antioxidant activity was plotted against the concentration tested for positive control (BHT) and extracts and EC_{50} values were calculated. The concentration of antioxidants required to reduce initial DPPH concentration by 50% is referred to as EC_{50}.

3.5.4 Determination of Total Phenolics

Total phenolic content (TPC) was determined using the modified Folin-Ciocalteu colorimetry method as described by Larbie *et al*. (2015). Briefly, aliquot of 10 µL of sample were pipetted into Eppendorf tube. Seven hundred and ninety millilitres of distilled water was added, then 50 µL of Folin-Ciocalteu reagent and mix thoroughly by pipetting. Incubation was done at room temperature for about 8 min. A volume of 150 µL of sodium carbonate Na_2CO_3 (7.5%, w/v) solution was added, mixed and incubated at room temperature (25±1 ºC) for 2 h. The mixture was aliquoted into 96 well

plate, absorbance was then measured at a wavelength of 750 nm using, UV-visible spectrophotometer (Shimadzu UV-160A PC, Shimadzu Corporation, Kyoto, Japan).

The TPC was conveyed as mg gallic acid equivalent (GAE), per 100 g wet weight material. Gallic acid standards were prepared by diluting 100 mg of pure Gallic acid in one millilitre of absolute ethanol. A standard solution of gallic acid was prepared, and used as the reference curve.

3.5.5. Induction of Apoptosis

Observation of cell morphological changes

Morphology of cultured LNCaP and PC3 was examined following treatment of the cells with aqueous extracts of MCwp and FAwp. Whole cells were considered viable whereas the presence of fragments (apoptotic bodies) was indicative of apoptosis. LNCaP and PC3 cells were seeded into 6 cm petri dishes at a density of $1x10^6$ cells. Cells were treated with specific concentrations of the leaf extracts from MCwp and FAwp. The cells were incubated for 24 h at 37°C. Ursolic acid was used as positive control. Cells were then examined under a phase-contrast microscope (magnification 20X) to determine the degree of cell fragmentation and blebbing.

3.5.6 DNA Fragmentation Analysis

DNA fragmentation and agarose gel electrophoresis were performed as described by Uto *et al,* (2013). Briefly, LNCaP and PC3 cells ($1x10^4$ cells/well) were treated with leaf extracts of MCwp and FAwp at concentrations close to the IC_{50} values obtained with the MTT assay. Usorlic acid and curcumin were used as positive controls. The treated cells were washed in ice-cold phosphate buffer saline (PBS) and harvested by centrifugation. The pellets were re-suspended in cell lysis buffer (50 mM Tris at pH 8.0, 10 mM EDTA, 0.5% sodium dodecyl sulphate (SDS) with 0.2 mg/ml RNase A). After incubation for 30 min at 50°C, 0.1 mg/ml proteinase K was added and the cells were incubated overnight.

DNA fragments were isolated and separated on 2% agarose gel, and finally visualized under UV illumination after staining with ethidium bromide.

3.5.7. JC-1 (Mitochondrion Membrane Potential Determination) Assay

Effect of aqueous extracts of MCwp and FAwp on mitochondrial membrane potential LNCaP and PC3 cells, seeded at a density of $1x10^6$ cells/6 ml into 6 cm petri dishes, were treated with 5 µg/ml aqueous extracts of FAwp and MCwp for 24 h. Ursolic acid and curcumin were used as positive control compounds. Staining of the cells was done using 5,5',6,6'-tetrachloro-1,1',3,3'-tetraethylbenzimidazolylcarbocyanine iodide (JC-1) solution (a sensitive mitochondrial membrane potential probe) and fluorescence readings were done using a microplate reader (Tecan Infinite M200, Austria) at wavelengths of 560 nm excitation and 595 nm emission for red J-aggregate. Green JC-1 monomers were detected at 485 nm excitation and 535 nm emission.

3.6. Statistical Analysis

Percentage cell viability was calculated using the formula of Ayisi *et al*., (2011) and plotted against extract concentrations to calculate the IC50 values. Analyses of antioxidant actions were performed using non-linear regression analysis with GraphPad Prism 5.0 (GraphPad software, Carlifornia, USA) and Microsoft Excel 8.0 (Microsoftware Inc. San Diego, CA, USA). Data are presented as ± standard error of the mean (SEM). Significant differences between groups were determined using One-way and Two-way ANOVA. P-values less than 0.05 was considered statistically significant.

4.2 Cytotoxic Activity

Results on treatment with aqueous fractions of MCwp and FAwp on PC3 and LNCaP prostate cell lines are shown in figures 4.2.1 and 4.2.2, respectively.

4.2.1 MTT Assay (PC3 Cells)

Figure 4.2.1a represents MTT assay results on cytotoxic effects of FAwp and MCwp extracts and controls on viability of PC3 cells. FAwp-treated cells had more cytotoxic activity than MCwp treated cells. The FAwp graph exhibited less cytotoxic activity with IC_{50} value of 20.39 µg/ml ($P \leq 0.001$) compared to the value for curcumin. The IC_{50} value of MCwp was higher than FAwp at 38.22 µg/ml ($P \leq 0.001$), indicating that the latter had stronger cytotoxic activity. The high cytotoxic activity of curcumin was evidenced by a decline in percentage cell viability with the lowest IC_{50} value of 2.09 µg/ml. Ursolic acid curve exhibited a similar concentration-dependent reduction in mean percentage cell viability, with a higher IC_{50} value of 9.90 µg/ml compared to that of curcumin. Curcumin-treated cells had the least cellular viability compared to cells treated with FAwp, MCwp and ursolic acid, and these were all in a dose-dependent fashion. There was significant cytotoxic activity of positive controls as expected.

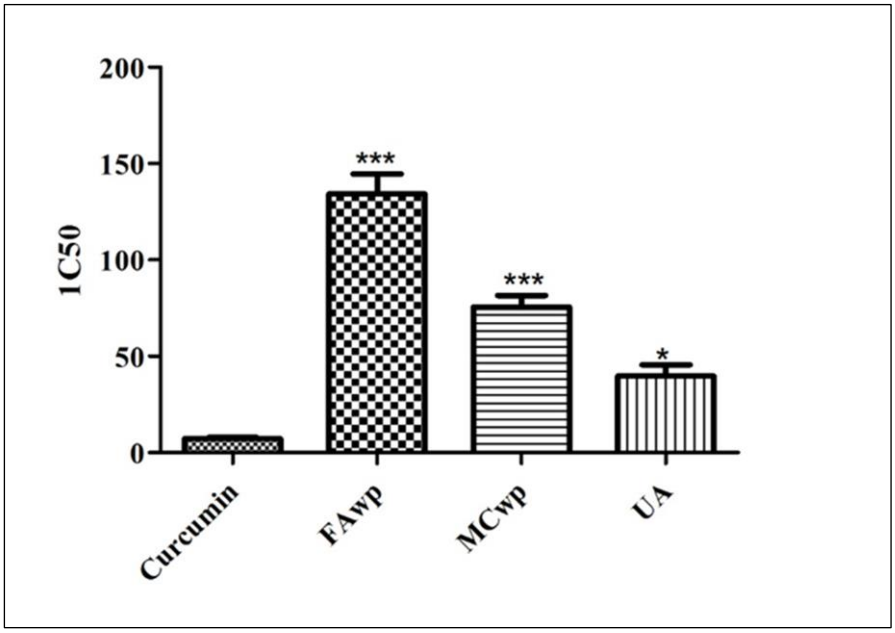

Figure 4.2.2b. IC50 Values For Percentage Viability of LNcap Cells After Treatment With FAwp, MCwp,Curcumin And Ursolic Acid.

Values are IC50 ± SEM (n = 3). P values ≤ 0.001 are denoted by the symbol *** compared to curcumin (one-way ANOVA and Bonferroni's post hoc test).

4.3 Pro-Apoptotic Activity

To determine pro-apoptotic activity of the fractions of MCwp and FAwp on the prostate cell lines, the DNA fragmentation assay and JC-1 assay were employed.

4.3.1 DNA Fragmentation (Laddering) Assay

Figure 4.3.1 (A and B) represents pro-apoptotic activity of the fractions of MCwp and FAwp on PC3 and LNCaP cell lines using DNA fragmentation assay. The results show a weak apoptotic band in the MCwp-treated PC3 cells compared to those treated with ursolic acid. However, FAwp-treated PC3 and LNCaP cells were fragmented in a manner similar to positive controls.

CHAPTER FIVE

DISCUSSION OF FINDINGS, CONCLUSION AND RECOMMENDATIONS

5.0 Discussion of Findings

The purpose of the study was to investigate specific mechanisms of action of aqueous extracts of FAwp and MCwp against prostate cancer cell lines (PC3 and LNCaP). The results obtained suggest that aqueous extracts of FAwp and MCwp exert anti-cancer against against both prostate cancer cell lines through pro-apoptotic action. This finding supports results from previous studies in which aqueous extract of MCwp exhibited anticancer activity through inhibition of DNA as well as RNA and cellular protein synthesis (Claflin *et al*., 1978; Licastro *et al*., 1980; Tsao *et al*., 1990; Zhu *et al*., 1990; Terenzi *et al*., 1999; Chang *et a*l., 2008). Other previous studies have suggested that *M. charantia* has anticancer activities against human leukemia, colon, liver, breast and cervical cancer (Alshehri, 2016; Brennan et al., 2012; Fang *et al.,* 2012a; Fang *et al.,* 2012b; Weng *et al.,* 2013; Yung *et al.,* 2015). *M. charantia* leaf was also reported to suppress rat prostate cancer progression *in vitro* and *in vivo* (Pitchakarn et al., 2010). However, the effect of the plant on human prostate cancer cells has not yet been reported. The extracts also demonstrated direct cytotoxic activity and antioxidant activities (Okekere *et al.,* 2014).

In determining pro-apoptotic activity of the fractions on the prostate cell lines in the present study, there was apoptotic body formation observed in both FAwp- and MCwp-treated LNCaP and PC3 cell lines, though a weak band was observed in the MCwp-treated-PC3 treated cells. Also, both FAwp and MCwp induced apoptosis by decreasing J-aggregates and increasing JC-1 monomers, indicating apoptosis via intrinsic (mitochondrial) pathway. The study also revealed that IC5O values were statistically insignificant (Suffness and Pezzuto, 1990) except curcumin on LNCaP cell line. This is because curcumin is a standard anti-cancer agent with established

direct cytotoxic activity (Suffness and Pezzuto, 1990; Kenfield *et al*., 2011). Both FAwp and MCwp did not exhibited weak antiproliferative property against LNCaP. FAwp, however, showed good antiproliferative property against PC3. The activity of MCwp against PC3 was not statically significant. A previous study by Pitchakarn *et al*. (2012) also investigated the effects of MCwp on PC3. The authors reported that cancer cell growth inhibition was mainly through the arrest of G1 phase of the cell cycle, resulting in a decrease in the proliferating-cell nuclear antigen, cyclin-dependent kinases (Cdk2 and Cdk4), and decreased levels of cyclins (D1 and E). Moreover, addition of 1% and 5% of the leaf extract of MCwp resulted in 63% and 57% inhibition of PC3 xenograft growth in the absence of any adverse effect on host body weight. The cytotoxic activity of MCwp observed in the present study may be via the same mechanism as observed by Pitchakarn *et al*. (2012) and perhaps via other potential but unidentified mechanisms. Furthermore, MCwp has been found to have apoptotic and direct inhibition of cell growth through G1 cell cycle phase arrest on androgen-dependent LNCaP (Pitchakarn *et al*., 2011). Apoptosis was measured by an increase in poly (ADP-ribose) polymerase and cleavage of caspase-3, all attributable to a reduction in the levels of apoptosis-inhibiting protein, survivin, and potentiation of Bax/Bcl-2 and Bad/Bcl-xL proteins. A decrease of p53 levels by RNA interference suggested that inhibition of LNCaP cellular growth by the leaf extract of MCwp partly through p53-dependent cell cycle arrest and apoptotic pathways (Pitchakarn *et al*., 2011). The observation that MCwp possesses pro-apoptotic activity against LNCaP in the present study supports the finding by Pitchakarn *et al*. (2011).

On the other hand no reports were found on anticancer properties of FAwp, however earlier studies have shown that traditional medicine practitioners use the leaves to treat asthma, hypertension, rickets, rheumatism and wounds (Essiett *et al*., 2011).

Triperpenoids from *M. charantia* were also found to induce apoptosis

Investigation of the antioxidant property of both plants showed that hydro-ethanolic leaf extract of FAwp had a high scavenging activity against DPPH. However, aqueous extracts of both plants had rather weak antioxidant activities compared with the positive control. This difference could be due to the different solvents used in the extraction which may have extracted /isolated different active compounds or chemicals. The present study also found that the total phenolic content of FAwp at 5.0 mg/ml was significantly lower than that of MCwp. This suggests some antioxidant activity of FAwp and MCwp, as some components of phenol (e.g. flavanoids) have direct anti-oxidant quantity. The finding also suggests that MCwp has almost twice the antioxidant activity of FAwp. A recent study also investigated the antioxidant potentials of FAwp and chemopreventive potentials on urinary inflammatory markers using albino rats. The rats were fed with 10%, 30% and 50% FAwp, resulting in a reduction in nitric oxide levels compared to control ($P < 0.05$) while concentration of polyphenols in urine increased significantly with simultaneous increased percentage of FAwp-supplemented diet (Okereke *et al*., 2014). This study confirmed an earlier study in which Oteng, (2016) reported antioxidant, anti-inflammatory and wound-healing activity of FAwp.

5.1 Conclusion

In conclusion, *Fleurya aestuans* and *Momordica charantia* contained phenolic substances and had weak antioxidant activity *in vitro*. The plant extracts demonstrated cytotoxic and apoptotic properties against PC3 and LNCaP cells.

5.2 Recommendations

Future studies should consider using in vivo studies to further ascertain the above findings. Future studies should identify and isolate the pure chemical constituents of the plants used which are responsible for the cytotoxicity, apoptotic and antioxidant activities. It will be necessary to use Western blot technique to determine the expression of apoptotic proteins (e.g. caspase 3). In

addition, it will be worthwhile to repeat this study using prostate cancer cell lines derived from non-Caucasian men, including Ghanaian men since prostate cancer is prevalent among blacks and non-caucasians. Furthermore, the JC-1 mitochondrion membrane potential assay should be done on these cell lines using these plants with results observed at 3h post-treatment to provide information at this time in point.

Methanolic and Ethanolic solvents can be explored in future to assess especially, the antioxidant property of FAwp and MCwp.

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