IN VITRO ANTIOXIDANT AND ANTICANCER PROPERTIES OF EXTRACTS OF CITRUS LIMON (LEMON)

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DECLARATION

I, EMMANUEL ROTIMI SAWYERR, of the Department of Animal Biology and Conservation Science, of the University of Ghana (UG), do hereby declare that this research study is my own work, with the exception of references that have been acknowledged. I carried out this work at the Department of Clinical Pathology, Noguchi Memorial Institute for Medical Research (NMIMR) under the supervision of Prof. Dominic Edoh of the Department of Animal Biology and Conservation Science (UG) and Prof. (Mrs.) Regina Appiah-Opong of the Department of Clinical Pathology, NMIMR, UG.

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DEDICATION

This work is dedicated to God Almighty for His grace, guidance and protection throughout my MPhil studies. I also dedicate this work to my dear father Dr. E.H. Olu-Sawyerr, who has been of tremendous support throughout my course of study, my late mother Thelma Sawyerr, my sister Ruth Tobi Sawyerr and my best friend Fred Sitsofe Buame, who have all been very encouraging and supportive.
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ABBREVIATIONS

A
AD: Anno domini
ADT: Androgen deprivation therapy
AIDS: Acquired Immunodeficiency Syndrome
AqR: Aqueous Rind
AqP: Aqueous Pulp
AqS: Aqueous Seed
AqJ: Aqueous Juice
APDN: Applied DNA Sciences Incorporated

B
Bcl-x(L): B-cell lymphoma-extra large
BHA: Butylated hydroxyanisol
BHT: Butylated Hydroxytoluene

C
CAM: Complementary and Alternative Medicines
CDK4: Cyclin-dependent kinase 4

D
DCIS: Ductal carcinoma in situ
DMSO: Dimethyl sulphoxide
DNA: Deoxyribonucleic acid
DPPH: 2,2-diphenyl-1-picryl-hydrazyl hydrate

DXT: Deep X-Ray therapy

E

EAR: Ethyl Acetate Rind

EAP: Ethyl Acetate Pulp

EAS: Ethyl Acetate Seed

EAJ: Ethyl Acetate Juice

EBV: Epstein-Barr Virus

EC50: Effective concentrations at 50%

EolR: Ethanolic Rind

EolP: Ethanolic Pulp

EolS: Ethanolic Seed

EolJ: Ethanolic Juice

ER: Estrogen Receptor

F

FBS: Fetal Bovine Serum

G

GAE: Gallic acid equivalent

GST: Glutathione S-transferase
H

H⁻: Hydride

\( \text{H}_2\text{O}_2 \): Hydrogen peroxide

HBV: Hepatitis B virus

HCL: Hydrochloric acid

HER2: Human Epidermal Growth Factor Receptor 2

HIV: Human Immunodeficiency Virus

\( \text{HO}_2^- \): Hydroperoxyl

HPV: Human Papillomavirus

HTLV-I: Human T-lymphotropic virus 1

I

IARC: International Agency for Research on Cancer

IC: Inhibitory concentration

L

LMIC: Low and middle-income countries

LOO⁻: Lipid peroxyy

M

MCF7: Human hormone-responsive breast carcinoma

MTT: 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide
N
N/A: Not applicable
NADH: Nicotinamide adenine dinucleotide
NADPH: Nicotinamide adenine dinucleotide phosphate
NCD: Non-Communicable Disease
NHL: Non-Hodgkin’s Lymphoma
NMIMR: Noguchi Memorial Institute for Medical Research
NO-: Nitric oxide
NO2-: Nitrogen dioxide

O
O2−: Superoxide
OD: Optical density
OH-: Hydroxyl

P
PBCR: Population Based Cancer Registries
PBS: Phosphate buffered saline
PC3: Prostate Cancer Cell Line
PCR3: Human prostate
PER: Petroleum Ether Rind
PEP: Petroleum Ether Pulp
PES: Petroleum Ether Seed
PEJ: Petroleum Ether Juice
PG: Propyl gallate

PNT2: Human normal prostate

PR: Progesterone Receptor

PSA: Prostate-Specific Antigen

PSG: Penicillin streptomycin L-glutamine

R

RNA: Ribonucleic acid

RO-: Alkoxy

RO2-: Peroxy

ROO-: Peroxy

ROS: Reactive oxygen species

RPMI: Rose Park Memorial Institute

S

SD: Standard deviation

SI: Selectivity index

SOD: Superoxide dismutase

T

TB: Tuberculosis

TBHQ: Tertiary butyl hydroquinone

TFC: Total flavonoid content

TGF-β1: Transforming growth factor beta 1
TPC: Total phenolic content

U

UICC: Union for International Cancer Control

UV: Ultraviolet

W

WHO: World Health Organization
ABSTRACT

Cancer is a disease of health concern globally, arising from uncontrolled proliferation of cells. Most drugs used in chemotherapy are derived from plants. Citrus limon (lemon) has been shown to possess significant amounts of anticancer agents. The purpose of this study was to evaluate the antioxidant and anticancer properties of extracts of C. limon. Aqueous extracts of C. limon were prepared using distilled water by heating, filtration and centrifugation. Organic extracts were prepared by cold maceration and rotary evaporation. Antioxidant activity of the extracts was determined using 2, 2-diphenyl-1-picrylhydrazyl (DPPH), Folin Ciocalteau and total flavonoids (Aluminium Chloride) assays. Cytotoxic activities of extracts on prostate cancer (PC3), breast cancer (MCF-7) and normal prostate (PNT2) cell lines were evaluated using the 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) assay. Rind extracts showed high total phenols and flavonoids, ethyl acetate rind extract having the highest total phenols (6415.268 ± 0.0095mg Gallic acid equivalent/100g extract) and petroleum ether rind extract having the highest flavonoid content (2603.025 ± 0.0121mg Quercetin equivalent /100g extract). Rind extracts also showed high total antioxidant content with the ethyl acetate rind extract having strongest activity (EC$_{50}$ = 0.4 ± 0.02mg/ml) followed by the ethanolic seed extract (EC$_{50}$ = 1.0 ± 0.1mg/ml). Ethyl acetate rind extract exhibited strongest cytotoxic activity against PC3 cells (IC$_{50}$ = 99.1 ± 8.4mg/ml) and petroleum ether rind extract showed strongest cytotoxic activity against MCF-7 cells with an IC$_{50}$ of 384.6 ± 17.1mg/ml. The ethyl acetate rind extract was found to be the most promising anticancer agent for prostate cancer with a selectivity index of 3.70. The results obtained lend support to earlier reports on the antioxidant and anticancer properties of C. limon.
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CHAPTER ONE

1.0 INTRODUCTION

Cancer arises as a result of uncontrolled proliferation of cells. Normal cells have certain signals such as growth, growth inhibitory and apoptotic signals which control growth, proliferation and programmed cell death but cancer cells develop a means of functioning independent of these signals. The spread of cancerous cells, if unchecked could be fatal; about 90% of cancer-related deaths are as a result of the spread to vital organs, a process known as metastasis (Hejmadi, 2010).

Out of the 12.7 million new cancer cases in 2008 worldwide, 5.6 million occurred in economically developed countries and 7.1 million in economically developing countries (American Cancer Society, 2011). Cancer deaths in 2008 were estimated at about 7.6 million, 2.8 million of which were in economically developed countries with 4.8 million in economically developing countries. There are predictions that by the year 2030, cancer incidence will be 21.4 million new cancer cases globally and deaths are expected to be 13.2 million (American Cancer Society, 2011). The acquisition of quality data on cancer especially in developing countries has been a challenge over the years. This has mainly been due to the lack of Population Based Cancer Registries (PBCR) in Africa observed from the little representation of African countries in the report on global cancer estimates (Curado et al., 2007). The African Cancer Registry Network (AFCRN) is however currently seeking to set up more PBCRs in Africa as they are very useful in informing decisions centered at cancer prevention as well as control programmes (Laryea et al., 2012).

*C. limon* (Lemon) of the family Rutaceae, is an oval citrus fruit with a smooth porous surface that grows on small thorny trees. They are known to be a source of alkaloids, which possess anticancer properties. They also are a good source of flavonoids, which function as antioxidants and have the ability to scavenge free radicals (Mohanapriya, Ramaswamy, & Rajendran, 2013). Citrus
flavonoids have been shown to possess the ability to inhibit abnormal cell proliferation (Duthie & Crozier, 2000). The peel of citrus fruits also contains flavonoid glycosides, coumarins, and volatile oils which are all good anticancer agents. The fiber of citrus fruits also contains bioactive compounds, such as polyphenols, and vitamin C, which studies have proven to be the cure for scurvy (Mohanapriya, Ramaswamy, & Rajendran, 2013).

Polyphenols are a large class of polyhydroxylated chemicals found in plants, that have common structures. They can be categorized into three main subclasses namely, flavonoids, phenolic acids and the stilbenoids, flavonoids being the most isolated compounds among the three. It is estimated that about 8,000 polyphenolic compounds have been isolated and described (Alexis Biochemicals, 2008). The term “polyphenols” is a collective term for several sub-groups of phenolic compounds, however, use of the term “polyphenols” has been quite confusing and though the name suggests its chemical structure, there still exists some ambiguity to it. Studies show that polyphenolic subgroups differ widely in stability, bioavailability and physiological functions with regards to human health (Tsao, 2010). Over the years, research has been able to establish a relationship between the long-term consumption of polyphenol-rich plant-based diet and protective advantage over carcinogenesis, cardiovascular diseases, diabetes, osteoporosis and neurodegenerative diseases (Rizvi & Pandey, 2009).

This could be due to the fact that polyphenols are strong antioxidants that have the ability to work synergistically with antioxidant vitamins and enzymes in a manner so as to provide defense against oxidative stress by reactive oxygen species (ROS) in the body. Though most of the research on polyphenols has been done in vitro, there is increasing evidence that suggests that they may also function outside antioxidant mechanisms in vivo (Tsao, 2010).
Studies show that citrus fruits are an important source of numerous bioactive compounds. Naringin and hesperidin are citrus flavonoids and are the most common glycosidic flavanones that are available in citrus plants, (Abeysinghe et al., 2007) while caffeic, chlorogenic, ferulic, sinapic and p-coumaric acids are the most bioavailable phenolic acids found in citrus plants (Tokusoglu & Hall, 2011). In a study to determine the phenolic content and antioxidant activity of lemon, orange, mandarin, white grapefruit and red grapefruit, phenolic content was generally higher in juice extracts, but another study by Ghasemi et al. (2009) showed the highest content to be found in the peels, (orange), suggesting that the observed differences may be due to diversity of types of extraction methods, solvents used, as well as differences in origin of the samples (Fejzić & Ćavar, 2014).

This study was aimed at investigating the antioxidant and anticancer properties of crude lemon extracts.

1.1 PROBLEM STATEMENT

There are 14 million new cases of cancer each year around the world, and in 2015, about 8.8 million people died from cancer, representing one-sixth of all world deaths (WHO, 2017). In 2011, cancer deaths were higher than deaths due to coronary heart disease or stroke. With an ever-increasing cancer burden mostly in low and middle-income countries (LMIC), projections show an estimated 20 million new cancer cases from the year 2025 onwards (Ferlay & Soerjomataram, 2014). The most frequent cases of cancer in the developed countries in the year 2008 were prostate, lung, breast and colorectal cancer. In economically developing countries, the most frequently diagnosed were lung, stomach, liver, breast and cervical cancers (American Cancer Society, 2011).

In Ghana, 16,600 cases of cancer occur annually, the most common being cancers of the prostate and liver, and Non-Hodgkin’s Lymphoma (NHL) in men and cancers of the breast, cervix and
liver in women (Ministry of Health, 2011). About 12,700 cancer deaths were recorded in the country in the year 2008; 5,800 occurred in men and 6,900 occurred in women.

Breast cancer is a major issue of public health concern in Ghana, having the highest incidence and mortality among cancers in women (WHO, 2008). It accounts for 16% of all cancers in the country (Clegg-Lamptey & Hodasi, 2007). Prostate cancer (13.2%) was also found to be the second leading type of cancer among men, following liver cancer (21.1%) (Laryea et al., 2012). There is low cancer awareness in Ghana and so there is often late stage presentation at hospitals. More than half of women with breast cancer report to hospitals with advanced disease; averagely, 8 months or more after first observing changes in their breasts, making treatment difficult (Clegg-Lamptey & Hodasi, 2007).

Types of cancer treatment include surgery, radiation therapy and systemic therapy. Cancer treatment may be administered singly or in combination depending on the type of cancer and the patient’s age and health. Chemotherapy, however, presents major side effects including anaemia, bleeding or clotting, immune suppression, nausea, hair loss, memory loss, infertility and excruciating pain among others (American Cancer Society, 2016). Inasmuch as chemotherapy is the primary cancer control mechanism, challenges with dosage selection leading to cytotoxicity to normal cells, coupled with rapid drug metabolism, drug resistance and harmful side effects, have limited the effectiveness of chemotherapy thus far. Research is gradually being directed towards the use of complementary and alternative medicines (CAM) due to claims that they have anticancer potential with no side-effects although a world of information remains unknown in this field (Mondal et al., 2014).
1.2 JUSTIFICATION

Cancer continues to be a disease of major concern worldwide, killing more people than HIV/AIDS, TB and malaria combined (Ministry of Health, 2011). In 2008, 12,700 people died of cancer in Ghana. The many deaths could be as a result of late stage diagnosis due to little cancer awareness. Alternative forms of treatment which are less damaging and cost-effective are vital in the hope of curbing the ever-rising trends of cancer incidence and deaths in the country and eventually on a global scale.

Research shows that increasing fruit and vegetable intake by one to two servings a day may reduce the risk of cardiovascular disease by 30%. The biologically active components of fruits and vegetables responsible for this protective effect are active micronutrients and phytochemicals (Silalahi, 2002). For many years, plants have been a very important source of natural products which have tremendous health benefits through their use in drug production. This has driven research directed at harnessing the pharmaceutical potential of citrus fruits (Ortuno et al., 2006).

Research shows that citrus fruits possess some antioxidant, antimicrobial, antifungal, anticancer, anti-inflammatory, antityphoid, antiulcer and hepatoprotective activities (Tomar, Mall, & Rai, 2013). In a study to determine the pharmacological importance of citrus fruits, Aleksandra et al. (2013) reported that citrus fruits (Sampion cultivar and white grape peels, and the seeds of the Idared cultivar and orange) have high antioxidant potential. Citrus fruits are also a rich source of dietary fibre, which contains pectin, which affects several digestive and metabolic activities and is implicated in the prevention of colon cancer (Silalahi, 2002). Furthermore, a study by Suzawa et al., (2014) on the in vivo carcinogenic property of a formulated citrus peel extract in mice showed that Gold Lotion (GL), an extract of multiple varieties of citrus peels may prove to be a potent
anticancer agent, especially against skin, colon and prostate cancer, due to its high content of flavonoids, a high percentage of which were polymethoxylflavones.

*C. limon* is an important medicinal citrus plant of the family Rutaceae. It has a number of health benefits such as preventing kidney stones, balancing the pH, purifying the blood, preventing osteoporosis and scurvy, and possesses antiasthma and anticancer properties (Chaturvedi & Shrivastava, 2016). *C. limon* is a good source of the antioxidant, vitamin C. *C. limon* and their peels are also a source of chlorogenic acid, didymin, diosmin, eriocitrin, hesperidin, hesperetin, limonene, limonin and γ-terpinene, and all of these have been shown to possess some anticancer properties (Charles, 2009). Due to the numerous beneficial biological properties of *C. limon*, people have claimed that it is effective in curing all types of cancers and is even 10,000 times stronger than chemotherapy, but no scientific evidence exists to this effect (Novas, 2017).

However, in an *in vitro* study on the phytochemical content, antioxidant, anticancer, immunomodulatory, and antigenotoxic activities of Lemon, Grapefruit, and Mandarin peels, lemon peels were shown to have the highest of Total Polyphenol Content and the second highest Total Flavonoid Content (Diab, 2016). Lemon peels also possessed the strongest antioxidant activity and were found to have some immune-stimulatory activity. Furthermore, in a study involving 43 women with newly diagnosed operable breast cancer, each taking 2 grams of limonene, which is a terpene, most concentrated in the rind of *C. limon*, daily for two to six weeks before surgery, limonene was found to preferentially concentrate in the breast tissue and resulted in a 22% reduction in breast tumour cyclin D1 expression (Miller & Lang, 2013).

The numerous health benefits of bioactive compounds in plants have led to their increased use in the pharmaceutical industry (Ighodaro, 2012). A good measure of research into possible
chemotherapy drugs is oriented at harnessing the potency of naturally occurring bioactive compounds for cancer treatment.

1.3 HYPOTHESIS

*C. limon* extracts have antioxidant, anti-prostate and anti-breast cancer activities.

1.4 GENERAL OBJECTIVE

To investigate the *in vitro* antioxidant, anti-prostate and anti-breast cancer properties of *C. limon* extracts.

1.5 SPECIFIC OBJECTIVES

1. To determine the cytotoxic activities of *C. limon* extracts on prostate (PC3) and breast cancer (MCF-7) cell lines *in vitro* using the tetrazolium-based colorimetric assay.

2. To determine the total antioxidant activity of *C. limon* extracts – petroleum ether, ethyl acetate, ethanol and aqueous extracts using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical scavenging assay.

3. To determine the total phenolic content of *C. limon* extracts using the Folin Ciocalteau assay.

4. To determine the total flavonoid content of *C. limon* extracts using a spectrophotometric-based assay.
CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 CANCER

Cancer arises from a series of molecular events that change the normal fundamental biological characteristics of cells. Normal cells possess control systems that prevent overgrowth of cells as well as invasion of other tissues. These normal cellular control systems are disabled in cancer cells therefore, such altered cells would continue to undergo division and growth even in the midst of signals that would normally disrupt cell growth after a certain point. These altered cells consequently would no longer need special signals to induce cell growth and division. As growth of such cells progresses, they begin to develop certain characteristics which include changes in cell structure, production of new enzymes and reduced cell adhesion. These changes are hereditary, and so may result in the cell and its progeny dividing and growing uncontrollably even in the presence of normal cells that possess characteristic inhibitory properties to abnormal growth of nearby cells. These changes result in cancerous cells spreading and invading other tissues (Does, Thiel, & Johnson, 2003).

Mutations in protein-encoding genes that regulate cell division are what give rise to abnormalities observed in cancer cells (Does, Thiel, & Johnson, 2003). After a while, more genes become mutated, usually due to malfunctioning of the genes that synthesize proteins that would normally repair damaged DNA because these malfunctioning genes are themselves mutated. As a result, there is increase in mutations in the cell, causing more abnormalities in that cell and consequently, the daughter cells. Mutations of such cells may sometimes result in cell death, but other times, coupled with other alterations, such abnormal cells might have a selective advantage over normal cells, allowing them to multiply more rapidly than these normal cells. This increased growth has
become the feature that characterizes most cancer cells, which, in the process, gain functions that are usually repressed in normal healthy cells. Abnormal cells of this nature, if they remain in their original location, are considered benign. However, if they become invasive, are termed malignant. Cancer cells from malignant tumors often metastasize, resulting in these cancer cells being introduced to other locations of the body where new tumors could result (Does, Thiel, & Johnson, 2003). Metastatic cancer has proven to be fatal, and in fact, about 90% of cancer-related deaths are due to tumors that are metastatic (Hejmadi, 2010).

Carcinogenesis and its advancement depend on both external factors in the environment such as tobacco, chemicals, radiation and infectious organisms, as well as internal factors, that is, those inherent in cells such as mutations, both inherited and metabolic, hormones and immune conditions (Hejmadi, 2010). These factors may act together or in sequence to produce abnormalities in cell behavior and excessive proliferation. There would then be a resultant growth and expansion in cell mass which would in turn affect surrounding normal tissues, for example, in the brain, and can also spread to other sites in the body (metastasis). However, mutations resulting in most cancers can take several months to progress to a stage where the cancer becomes detectable. DNA mutations occur at a frequency of 1 in every 20 million per gene per cell division, and each individual has an average of about $10^{16}$ cells formed in their lifetime, with about 10 million cells being replaced every second (Hejmadi, 2010). It should therefore follow that the frequencies of cancer should be similar in all parts of the world, however, this is not so. The incidence of cancer varies dramatically across the globe, and so by inference, it follows that the factors that result in cancer are not just hereditary, but environmental as well. This means that either people in certain parts of the world carry a large number of cancer-susceptible genes or the environments that certain populations inhabit contribute majorly to their development of cancer (Hejmadi, 2010).
The factors that account for the geographic differences in types and burden of cancer include variations in magnitude of major risk factors, accessibility to medical care such as cancer screening, availability and quality of treatment, and age structure (Hejmadi, 2010). In 2008, infection was directly linked to two of four leading cancers in men (stomach and liver cancer) and women (cervical and stomach cancer) in developing countries (Hejmadi, 2010). Frequency of the most common cancer diagnoses and deaths also varies with geographic disposition. In 10 out of 21 world areas, breast cancer was the most common cause of cancer deaths among women, while cervical and lung cancers led in causes of cancer deaths in the other 11 world areas (Hejmadi, 2010). In 2008, the most common cancer site in males in majority of developed countries was the prostate, except in Japan where the most common was the stomach. In Eastern Europe and Asia, it was the lungs. Africa had the greatest variation in common cancer sites, including prostate, lung, liver, esophagus, bladder, Kaposi sarcoma and Non-Hodgkin lymphoma (American Cancer Society, 2011). In females around the globe, the most common sites were breast and the cervix, with the exception of China (lung), South Korea (thyroid), and Vietnam and Mongolia (liver) (American Cancer Society, 2011).

2.1.1 CAUSES OF CANCER

The body comprises different cell types, including skin, muscle and blood cells. These divide continuously in our body, forming new cells. An error could occur in the making of a cell, causing it to become a cancer cell and resultantly, its inability to die after a certain period creating a buildup of abnormal cells (Cancer Support Community, 2014). This can progress into a mass called a tumor, and can crowd out normal healthy cells, an example being leukemia.
Genes are distributed unequally around the world but this alone cannot explain the differences in cancer incidence observed in different parts of the world. The risk of developing cancer is more environmental in nature, with about 90% of all cancers resulting from environmental factors (Hejmadi, 2010).

An English physician named John Hill was the first to link lifestyle to cancer through a study showing the effects of tobacco snuff on development of nasal cancer (Hejmadi, 2010). Sir Percival Pott, in the 18th century also linked scrotal cancer to poor hygiene. In 1950, epidemiological studies showed a strong relationship between tobacco smoking and lung cancer, and since then, about 170,000 throat and mouth cancer deaths are recorded annually as a result of tobacco and alcohol consumption in the USA alone (Hejmadi, 2010). Over half a million deaths from cancer are expected annually as a result of lifestyle choices such as obesity, sedentary living and diet (low in vegetables and fiber and high in salt, nitrate and fat) (American Cancer Society, 2017). Infectious agents such as Hepatitis B virus (HBV), Human Papillomavirus (HPV), Human Immunodeficiency Virus (HIV), bacteria such as *Helicobacter pylori* and parasites such as *Schistosoma haematobium* also account for increase in risk of getting cancer (American Cancer Society, 2017). Cancer may also arise from exposure to certain cancer-causing agents – carcinogens and mutagens. These can be found in food, drink, in the air, in chemicals and sunlight. Epithelial cells cover the skin and line the respiratory and alimentary tracts. These cells metabolize carcinogens, and so about 90% of cancers originate from epithelial carcinomas (Hejmadi, 2010).

Age also plays a role in the risk of developing cancer. Although persons of any age can develop cancer, about 60% of new cases and two-thirds of cancer deaths occur in persons above 65 years. Several theories to why cancer prevalence is higher among the elderly exist, and these may include
decreased immunity with advancement in age, lifetime exposure to carcinogens, accumulation of random genetic mutations as well as hormonal alterations (Hejmadi, 2010).

2.1.2 TYPES OF CANCER

Cancers can be grouped into categories depending on their origin. The main categories are as follows.

- Carcinoma, which is the most common kind and is generally characterized by the site in the body where the cancer starts, such as the lung, breast, or colon.
- Sarcoma, found in supporting tissues like bone, muscle and fat.
- Leukemia, which is cancer of the blood, usually starting in the bone marrow where there is abnormal production of blood cells.
- Lymphoma, which starts in immune cells within the lymphatic system.
- Central nervous system cancers, which begin in the brain or spinal cord.

There are several types of cancer within these groups and so it is important to know the type of cancer as well as its stage before making treatment decisions (Cancer Support Community, 2014).

2.2 THE PROSTATE

The name ‘prostate’ is of Greek origin, from the word, ‘prohistani’, which means ‘to stand in front of’. It is an exocrine sex gland in males that secretes an alkaline fluid constituting about a third’s the volume of seminal fluid. Prostatic fluid composition and secretion play a major role in sperm function and male fertility (Sharma, Gupta, & Dhole, 2017). The prostate gland is located near the
rectum and its main role is to make fluid that protects, feeds sperm and helps them flow along the ducts of the male reproductive system (Andrology Australia, 2013).

Prostate carcinogenesis happens when there is a build-up of somatic genetic and epigenetic changes that result in the inactivation of tumour-suppressor genes and caretaker genes, as well as activation of oncogenes (De Marzo et al., 2007).

The determination of the serum Prostate-Specific Antigen PSA level is currently used for diagnosis of prostate cancer (Miki & Kamoi, 2011). Though prostate-specific, it is however not prostate cancer-specific. The size of the prostate and the presence of benign prostatic hyperplasia and prostatitis may also influence levels of PSA in the body (Miki & Kamoi, 2011).

2.3 PROSTATE CANCER

In the year 2012, prostate cancer was the second most diagnosed cancer with about 15% of all male cancer cases worldwide being of the prostate (World Cancer Report, 2014). It was the sixth leading cause of cancer death in males (Jemal et al., 2011). In 2010, prostate cancer resulted in 256,000 deaths, a significant increase from 156,000 deaths recorded in the year 1990 (Lozano et al., 2010). There are major variations in prostate cancer figures around the globe. It is observed to be least common in South and East Asia, and more common in Europe, North America, Australia and New Zealand (Prostate Cancer Statistics, 2013).

The prostate gland has been estimated to be about the size of a walnut, but it is normal to observe a gradual increase in size as men grow older. This may sometimes present problems such as difficulties in urinating, however, these may not always be symptoms of prostate cancer (Prostate Cancer Foundation of Australia, 2014). The projected risk of a man at the age of 50 to be diagnosed with prostate cancer is estimated to be around 10%. Over 65% of all prostate cancer cases are in men above the age of 65 years (Abbvie Inc, 2013). Symptoms of prostate cancer may not arise in
the early stages, but later on as the disease progresses. They may include frequent urination, difficulty in urinating usually accompanied with discomfort, bloody urine or semen and pains in the lower back, upper thighs and (or) hips. These symptoms may, however, not be a concrete diagnosis of the disease (Prostate Cancer Foundation of Australia, 2014).

There are four stages of prostate cancer (Stage I through to Stage IV) based on how far the cancer has grown and spread (Lavery, Kirby, & Chowdhury, 2016). The Prostate-Specific Antigen, which is a protein produced by the fluid-producing cells that line the inner walls of the glands in the prostate can give an indication of the stage of the cancer and can be used to monitor treatment (Lavery et al., 2016). Digital Rectal Examination, which involves using a finger to feel the rectal wall for the prostate, is also used for prostate cancer screening. PSA levels can be measured from blood sample because some of it enters the bloodstream. The larger the prostate, the more PSA it can make and so higher PSA values may indicate prostate cancer or any other condition that results in enlargement of the prostate (Lavery et al., 2016).

The cause of prostate cancer remains quite elusive, however, certain factors are known to predispose a person to prostate cancer. These include age, obesity and family history (Mustafa et al., 2016). Diet and lifestyle have also been majorly implicated in the risk of getting prostate cancer (Mustafa et al., 2016). Treatment regimens for prostate cancer depend on the size and site of the tumor, extent of spread and general health of the patient. At an early stage of diagnosis, active surveillance is usually recommended. This involves close monitoring of the cancer with active treatment only when it starts to spread or cause pain or discomfort. Treatment options include surgery or radiation therapy. For later stages, hormone therapy, or androgen deprivation therapy (ADT), may be given before surgery or radiation therapy given after surgery (Lavery et al., 2016). Chemotherapy and immunotherapy may also be used.
2.4 BREAST CANCER

Breast tissues are common to both men and women. In women, breasts are made up of milk glands consisting of lobules, which produce milk and ducts which transport milk to the nipples. Testosterone in men suppresses the development of lobules at puberty. Certain supportive fibrous and fatty tissues in the breasts are, however, common to both men and women. Breast cancer develops when cells lining the lobules or ducts begin to grow abnormally, and they have the potential for spreading to other parts of the body (Cancer Council, 2016). Types of breast cancer include non-invasive breast cancer, where the cancer cells are contained within the ducts of the breast, also known as ductal carcinoma in situ (DCIS), and invasive breast cancer where the cancer spreads from ducts or lobules to surrounding tissues and even lymph nodes in the armpits sometimes. Some other types of breast cancer include locally advanced breast cancer, secondary breast cancer, inflammatory breast cancer and Paget’s disease of the nipple (Cancer Council, 2016).

Breast cancer is the most common type of cancer among females in the western part of the world, with a lifetime risk of the order of 1/10 (Sigurdur, 2001). It is frequently fatal and is ranked second in causes of cancer death in the Eastern Mediterranean. Each year, over 1.2 million women are diagnosed with breast cancer all over the world (World Health Organisation, 2006). Over 500,000 women die from the disease annually (Roche, 2016). In Ghana, breast cancer incidence is the highest among cancer types in women accounting for 15.4% of all malignancies (Clegg-Lamptey & Hodasi, 2007). Data from the Korle-Bu Teaching Hospital Cancer Register from the year 1972 to 1975 showed that breast cancer accounted for 7.5% of all cancers in Ghana being the fourth in incidence after liver and cervical cancer, and Burkitt lymphoma (Edmund, Naaeder, Tettey, & Gyasi, 2013). Breast cancer in Ghana is more recently common in young women, most of whom
present late with large and advanced tumors resulting in low survival rates (Quayson, Wiredu, Adjei, & Anim, 2014). The average age of breast cancer incidence in the country is 48 years with about 67% having lymph node metastasis. Male breast cancer, though existent, is generally not common, and in Ghana, there is a 2.9% incidence of breast cancer in males. There has generally been no improvement in the stage at which patients present with breast cancer in the past 30 years (Quayson et al., 2014).

A number of factors increase the risk of getting breast cancer. Risk of acquiring the condition increases with increasing age as majority of breast cancer cases occur in women above the age of 50 years (Roche, 2016). Research also shows that women with family history as well as women who have previously had benign breast tumor have an increased risk of developing breast cancer in the future (Roche, 2016). A late first pregnancy, that is after 35 years of age, may also predispose a woman to the development of breast cancer. An extended menstrual life and the use of hormone replacement therapy after menopause may also increase risk of developing breast cancer as certain breast cancer types may be dependent on hormonal levels in the body. Lifestyle also plays a major role as a risk factor. Obesity, especially after the menopause, lethargy, high fat diets and high alcohol intake can greatly increase an individual’s risk of getting breast cancer (Roche, 2016).

In most cases, breast cancer treatment is done using a multidisciplinary approach that combines a number of treatment types to achieve an effective overall treatment regimen. This combination is dependent on a number of factors, including the stage and subtype of the tumor, whether the cancer is metastatic, whether or not the individual has reached menopause, age, general health, the tumor’s hormone receptor (Estrogen Receptor [ER], Progesterone Receptor [PR]) and Human Epidermal Growth Factor Receptor 2 (HER2) status, and the presence of known mutations in inherited breast cancer genes. Treatment plans may include surgery, radiation therapy, chemotherapy, hormonal
therapy, targeted therapy, clinical trials, and palliative care (American Society of Clinical Oncology, 2018).

2.5 CANCER EPIDEMIOLOGY

Due to geographical variations in cancer incidence worldwide, there is the need for population-based cancer registration in order to ensure reliable estimation of the number of new cases. Compilation of worldwide epidemiological data on cancer allows for identification of countries and geographic regions where cancer types are more common than others. By deduction, the differences observed would reflect exposure to specific causative environmental factors. This would inform decisions on preventative measures, planning and dissemination of resources (Stewart & Kleihues, 2003).

In the year 2000, about 10.1 million new cases, 6.2 million deaths and 22.4 million persons living with cancer were estimated. This represented a 19% and 18% increase in incidence and mortality since 1990, respectively (United Nations, 1998). In the year 2000, cancer was the cause of 6.2 million deaths, which represented about 12% of all deaths globally behind only deaths caused by cardiovascular disease, which accounted for 30% of all deaths. Low- and middle-income countries were home to over 70% of all cancer deaths, and even though the risk of getting or dying from cancer is higher in the developed countries, inadequate control measures of infectious diseases as well as the ageing population in developing countries give rise to the increase in cancer burden throughout the globe (WHO, 2001). Research has shown that cancers are avoidable. A study as early as in the year 1970 showed that about 75% of cancer cases in most parts of the USA could have been avoided (Doll & Peto, 1981). Parkin et al. (1999) also estimated the possibility of 22.5% fewer cancer cases in developing countries in the year 1990, if hepatitis B and C viruses, human
papillomaviruses, EBV, HTLV-I, HIV, helicobacter pylori, schistosoma, and liver fluke infections had been avoided.

In 2003, the type of cancers that had the highest incidence worldwide were lung cancer (12.3% of all cancers), breast cancer (10.4%) and colorectal cancer (9.4%). This excluded all non-melanoma skin cancers. Lung cancer was reported to be the highest single cause of cancer death worldwide with 1.1 million deaths annually, mainly due to poor prognosis. Breast cancer, however, proved to be less fatal due to more effective interventions. As a result, breast cancer, although it has been ranked second in incidence, was not among the top three causes of cancer deaths. The top three were lung cancer (17.8%), stomach cancer (10.4%) and liver cancer (8.8%).

It has also been observed that generally, the relationship between cancer incidence and mortality is not affected by sex. Also, the major differences in cancer susceptibility between developed and developing countries are that developing countries are more prone to cancers that arise due to infectious diseases (Stewart & Kleihues, 2003). On the other hand, developed countries are more associated with cancers that are attributed to peculiar lifestyle and dietary habits (Stewart & Kleihues, 2003).

Cancer is responsible for 1 in every 8 deaths worldwide, leading AIDS, tuberculosis, and malaria combined (American Cancer Society, 2011). In terms of economic development, cancer is the leading cause of death in developed countries and the second leader in developing nations just after heart disease. Estimates from the International Agency for Research on Cancer (IARC) showed that there were 12.7 million new cancer cases in 2008 worldwide, 5.6 million occurring in economically developed countries and 7.1 million in economically developing countries. Total cancer deaths were 7.6 million, 2.8 million in economically developed countries and 4.8 million in economically developing countries (American Cancer Society, 2011). Worldwide cancer burden
was expected to grow to 21.4 million new cancer cases and 13.2 million cancer deaths due to numerical increase and aging of the population, as well as deaths from infectious diseases in developing countries. Incidence in developing countries was also expected to increase due to the adoption of western lifestyles, such as smoking, poor diet, physical inactivity, and reproductive factors (American Cancer Society, 2011).

In 2011, the World Economic Forum estimated that the total financial burden of Non-Communicable Diseases (NCDs) worldwide could amount to USD 47 trillion in the following 20 years, nearly half of the burden (USD 21 trillion) falling to low- and middle-income countries. By the year 2015, 71% of countries had a functional NCD plan which included cancer, representing a 21% increase in participating countries since the year 2010. Africa and the Americas have shown the most significant progress, however, there remains considerable amount of work needed to translate plans into effective national programmes (UICC, 2016).

There are about 16,600 new cases of cancer in Ghana every year; out of every 100,000 people, there are 109.5 cases (Ministry of Health, 2011). The types of cancers which result in the highest mortalities are cervical, liver, prostate, breast, stomach and colorectal cancer, and Non-Hodgkin’s lymphoma. Data from the Oncology Directorate of Komfo Anokye Teaching Hospital (KATH) and Korle Bu Teaching Hospital (KBTH) show that the main treatment plans for tumors involved Radiotherapy or chemotherapy usually after Surgery (Ministry of Health, 2011). From 2004 to 2006, between 400 and 500 new cancer cases were seen yearly. Cervical cancer accounted for 34.7% of all female cancers reporting to KATH; breast cancer accounted for 20.9% of all cancer cases. The commonest in males were prostate cancer (23.6%), cancers of the accessory sinuses (5.2%), bones (4.3%), larynx (3.7%) and bladder (3.4%). There were 135 deaths during the period. KBTH recorded between 800 and 1100 new cases from the year 2006 to 2009.
2.6 CANCER TREATMENT

There are a number of treatment options for cancer. The treatment used in each case depends on the type of cancer, the stage and to what extent the cancer has spread. Sometimes, more than one treatment may be used, but the three major ones are surgery, chemotherapy and radiotherapy. Other treatment options include hormone therapy, immunotherapy, adjuvant therapy and gene therapy.

2.6.1 SURGERY

Surgery, which is a method of cancer treatment that involves manual and instrumental means of cutting off tumors, has become a specialist discipline. Surgeons have become highly specialized in performing organ-based procedures as opposed to general treatment of all types of cancers. Instrumentation for surgery has evolved over the years. Fibreoptic endoscopy, along with other new technologies, has played a major role in the development of modern surgery (International Agency for Research on Cancer, 2003). Laparoscopy which is endoscopic examination of the interior of the abdomen has also become a very useful procedure in surgical cancer treatment. New procedures are still being explored in the treatment of cancer.

2.6.2 RADIOThERAPY

Radiotherapy involves using radiation, usually X-Rays, to treat disease. It was previously referred to as radium treatment or deep X-Ray therapy (DXT), but due to technical improvements, these names have ceased to be used. Radiation is known to be dangerous and pose certain health risks but recently, some experience and a better understanding has encouraged its use in medicine. It could be performed either from outside the body (external radiotherapy) using X-Rays, electrons
and quite rarely, particles like protons to destroy cancer cells or from inside, (internal radiotherapy / brachytherapy) by placing radioactive material in or close to the tumor (APBN, 2007). The purpose of radiotherapy is to destroy cancer cells in a given area of the body, and though this may affect normal cells, they are better able to repair themselves, allowing relatively lesser damage.

### 2.6.3 IMMUNOTHERAPY

The principle of immunotherapy works with the use of vaccines aimed at stimulating the host’s immune system to reject cancer cells. This has been a major objective of onco-immunologists for much of the 20th century (International Agency for Research on Cancer, 2003). Immunotherapy also involves using biological agents that mimic some of the body’s signals for controlling tumor growth. These natural biological agents are now being synthesized in the lab, and they include interferons, interleukins, cytokines, endogenous angio-inhibitors and antigens. Scientists have been able to design monoclonal antibodies that work against lymphoma and breast cancer (Sudhakar, 2009). Presently, cancer immunotherapy is aimed at developing vaccines that boost the body’s immune response against cancer.

### 2.6.4 HORMONE THERAPY

An estimated 40% of malignant tumours in women and 25% in men are known to be dependent on hormones (Chan & Yeung, 2006). Hormone therapy may cause some dramatic response devoid of the toxicity that comes with chemotherapy. Hormone therapy has been observed to have cytostatic effects on cancer cells, thus inhibiting their growth, however, the exact mechanism by which this happens is not fully understood. It is considered to limit the growth of tumours through
deprivation of hormonal growth stimulus. Ways in which it may act include downregulation of hypothalamic-pituitary-gonadal axis, blocking hormone receptors, inhibition of adrenal steroidogenesis and inhibition of peripheral conversion of sex hormones (Chan & Yeung, 2006).

2.6.5 GENE THERAPY

There have been major advancements in cancer research and genomics over the past 20 years. Due to the wealth of knowledge that now exists on cancer, there has been considerable development of novel therapeutic methods in the management of cancer, particularly with gene therapy. In gene therapy, the procedures are aimed at treating a disease by genetic modification of body cells. Materials to be transferred into the patient’s cells may be genes, gene segments or oligonucleotides (Amer, 2014). Gene therapy involves both in vivo and ex vivo approaches. For the in vivo approach, the method involves direct targeting of cells, such as intradermal injection of a metastatic nodule and for the ex vivo approach, targeted cells from a tumor are selected, collected and cultured in the laboratory with culture media in a controlled microenvironment (Amer, 2014). A new gene is then inserted into the cell genome, and the cells are then introduced back into the patient. Tumors with high level of intratumor heterogenicity and genomic instability are less susceptible to targeted therapies like gene therapy, making it somewhat difficult to achieve with limited success (Amer, 2014).

2.6.6 CHEMOTHERAPY

Chemotherapy employs the use of a number of cytotoxic, drugs. About 60 different drugs are available for use, and new ones are being developed. Chemotherapy drugs are designed to damage
cancer cells even though they may be toxic to normal cells. They do this by targeting cells that divide more rapidly and so have a higher affinity for dividing cells, making them more suitable for destroying cancerous cells, whose hallmark is a high rate of proliferation. Cells that are at rest are less susceptible to damage by chemotherapy. This explains why chemotherapy results in certain side effects since it affects normal cells and tissues that grow continually, for instance, skin, hair and cells of the digestive system. Despite some of the deleterious side effects of this form of treatment, chemotherapy is a major form of treatment for cancer because it thrives on the principle that normal cells should be able to repair themselves more rapidly than cancer cells therefore, damage to healthy cells should not be as severe as that to cancer cells (APBN, 2007). Adjuvant therapy is the use of chemotherapy after surgery to destroy residual cancer cells in the body. It has been used in treating colon and testis cancers (Sudhakar, 2009).

Plant products have been used in the treatment of different kinds of diseases for many years. Out of the 92 drugs used for treatment of cancer in the USA along with all other universally recognized chemotherapy drugs, 60% are from natural sources (Shoeb, 2006). Many medicinal plants have shown anti-cancer properties including *Allium sativum*, which is known to contain over 100 biologically active secondary metabolites (Rashed, 2014). Another plant with anticancer properties is *Annona* spp.; it is known to contain acetogenins, which possess anti-leukaemic properties and are useful in the treatment of nasopharyngeal carcinoma (Rashed, 2014). Other such important plants include *Arctium lappa, Luffa aegytiaca, Solenostemma arghe, Cassia italica, Ocimum basilicum, Gossypium barbadense, Gyrophora esculenta, Beta vulgaris, Colocasia antiquorum* and *Capsicum frutescens* fruit. In Ghana, herbalists use different plants as natural remedies for different types of cancers. However, the scientific basis for many of these plants as anticancer agents is yet to be proven. A study by Bayor and Wright (2006) supports the use of the bark of *Z. xanthoxyloides* and the root of *C. membranaceus* as promising anticancer agents. Many other
plants are known to contain phytochemicals which may be useful as agents in combating a number of diseases including cancer. The family of citrus fruits is an example (Silalahi, 2002).

2.7 FREE RADICALS

Oxygen is a necessity of life for aerobic organisms but may become toxic at higher concentrations (Kumar, 2014). Oxygen possesses certain properties that play an important role in a number of biological processes. Aside being essential for life, it can also lead to significant damage within cells (Mahantesh et al., 2012). When cells use oxygen to generate energy, free radicals are formed as a result of ATP production by the mitochondria. These products are also called reactive oxygen species (ROS) (Kabel, 2014). A free radical can be defined as a molecule or fragments of a molecule that possess one or more unpaired electrons in its outermost shell and are capable of existing on their own. Free radicals may be generated by the homolytic cleavage of a chemical bond and also through redox reactions. Examples of ROS include superoxide (O$_2^-$), hydroxyl (OH$^-$), peroxyl (RO$_2^-$), hydroperoxyl (HO$_2^-$), alkoxy (RO$^-$), peroxy (ROO$^-$), nitric oxide (NO$^-$), nitrogen dioxide (NO$_2^-$) and lipid peroxyl (LOO$^-$) (Mahantesh, Gangawane, & Patil, 2012).

Free radicals play a dual role as both destructive and beneficial. At low concentration, they possess beneficial effects on cellular responses and immune function whereas at high levels they result in oxidative stress, a deleterious process that can damage all cellular structures (Kabel, 2014). Free radicals and their destructive effects were discovered in the last decade. They are formed in the body during normal metabolic processes. Bodily energy is produced by the oxidation of carbohydrates, fats and proteins through both aerobic and anaerobic processes, and these result in the production of free radicals as a by-product. Overproduction of free radicals can result in tissue injury. Cell membranes which are made of unsaturated lipids are particularly susceptible to free
radical oxidative damage that can lead to a breakdown or hardening of these lipids which constitute the cell walls. Breakdown or hardening of the lipids is due to lipid peroxidation which leads to death of the cell by limiting its ability for nutrient uptake (Mahantesh et al., 2012). DNA, RNA and protein enzymes are also susceptible to oxidative damage (Mahantesh et al., 2012). Some environmental agents are able to initiate free radical generation. The toxic effects of lead, pesticides, cadmium, ionizing radiation, alcohol, cigarette smoke, UV light and pollution are all because of their ability to initiate free radical production in the body (Mahantesh et al., 2012). Free radicals are responsible for a host of health conditions including cancer, heart disease, gastric problems and even disease conditions associated with aging. Increased generation of ROS and weak cellular antioxidant response can result in a number of health conditions including both type 1 and type 2 diabetes (Kehrer & Klotz, 2015). Increased production of superoxide/hydrogen peroxide through enhanced mitochondrial metabolism has been linked to high blood glucose and saturated fatty acid levels. Also, due to their role in endothelial dysfunction where the regulation of the vascular wall is disrupted, free radicals have been implicated in the pathogenesis of cardiovascular disease, which has endothelial dysfunction as its common trait (Bhattacharya, Ahmed, & Chakraborty, 2011).

2.8 ANTIOXIDANTS

Antioxidants are substances that inhibit or are able to slow down oxidative damage to a target molecule (Mahantesh et al., 2012). Antioxidants cause this effect by neutralizing free radicals. The human body naturally produces antioxidants but the production may sometimes be inadequate to match the free radical load in the body. Production of antioxidants in the body also declines with age. Increasing the antioxidant intake can prevent diseases and lower the risk of health problems.
Research shows that antioxidant-rich foods possess many health benefits. Fruits, vegetables and medicinal herbs are among the richest sources of antioxidant compounds and are loaded with key antioxidants such as vitamin A, C, E and beta-carotene, as well as important minerals, including selenium and zinc (Mahantesh et al., 2012). Many plants are known to have antioxidant components that are able to convert free radicals to less reactive species. Different free radical scavengers can be found in dietary sources like fruits, vegetables and beverages. Regular intake of vegetables and fruits have been linked to the reduction in risk of chronic diseases (Dembinska-Kiec et al., 2008). Studies show that citrus fruits have a high content of natural antioxidants like vitamin C. Blueberries, strawberries, grapes, plums, prunes, red beans, spinach, kale, broccoli flowers and alfalfa sprouts have also been shown to possess significantly high amounts of antioxidants and as such, have been added to many diets (Cao et al., 1998; Grossman et al., 1994). Antioxidants may be grouped into natural and synthetic antioxidants, or dietary and endogenous antioxidants (Yadav et al., 2016).

Antioxidants act in three ways in the body; they may prevent oxidant formation, mop up free radicals or repair oxidized molecules (Noori, 2012). They may also be enzymatic or non-enzymatic (Noori, 2012). The enzymatic system of antioxidants, comprising catalase, superoxide dismutase, glutathione peroxidase, glutathione reductase and thioredoxin defends the body against the ROS while the non-enzymatic antioxidants scavenge for ROS and RNS. They include glutathione, vitamin E and C, uric acid and melatonin.

2.8.1 NATURAL ANTIOXIDANTS

Natural antioxidants are antioxidants found in natural sources such as fruits and vegetables. Natural antioxidants found in foods include Vitamin C (ascorbic acid), Vitamin E (tocopherols),
Vitamin A (carotenoids), various polyphenols including flavonoids, and Anthocyanins which are a type of flavonoid, Lycopene, which is a type of carotenoid, and Coenzyme Q 10 (Ubiquitin), which is a type of protein (Yadav et al., 2016). Natural antioxidants are also generally found in many living plants. This is so because plants are under constant oxidative stress from free radicals, both exogenous (heat and light) and endogenous ($H_2O_2$ and transition metals). Plants have therefore adapted to develop a system of antioxidants in order to counter free radical burden, lipid oxidation, oxidation intermediates, and secondary breakdown products (Yadav et al., 2016). Some of these compounds with antioxidant properties include flavonoids, phenolic acids, carotenoids and tocopherols.

2.8.2 SYNTHETIC ANTIOXIDANTS

Synthetic antioxidants do not occur naturally, and so are chemically produced. They are used as food preservatives in order to keep processed food for long periods of time by preventing lipid oxidation (Shahidi et al., 1992). Depending on their mode of action, synthetic antioxidants may be classified into either primary or secondary antioxidants. Some synthetic antioxidants include butylated hydroxytoluene (BHT), butylated hydroxyanisol (BHA), tertiary butyl hydroquinone (TBHQ) and propyl gallate (PG).

2.8.3 DIETARY ANTIOXIDANTS

Dietary antioxidants are found mainly in food, and include ascorbates, tocopherols and carotenoids, and their roles in health have been well documented (Boskou et al., 2005). Vitamin C, vitamin E, and beta carotene as well as other carotenoids and oxycarotenoids like lycopene are
some of the most studied. Vitamin C for instance, has been described as the most important water-soluble antioxidant because of its ability to annihilate ROS in the aqueous state before lipid peroxidation can occur (Yadav et al., 2016). Vitamin E, which is fat-soluble, is able to break chains within the cell membrane, protecting it from lipid peroxidation. Antioxidants may also have some beneficial effects on other antioxidants. Vitamin C, for instance, is able to regenerate vitamin E in the body and beta carotene may possess synergistic effects on vitamin E (Yadav et al., 2016).

2.8.4 ENDOGENOUS ANTIOXIDANTS

Endogenous antioxidants are antioxidants that are synthesized in the body. These antioxidant enzymes (glutathione peroxidase, catalase, and superoxide dismutase [SOD]) are able to metabolize certain oxidative species. They work in conjunction with micronutrients like selenium, iron, zinc, copper and manganese in order to optimize catalytic activity (Duthie, 1994). Research reveals that this endogenous defense mechanism is largely dependent on the intake of these micronutrients in adequate proportions, and as such, nutritional deficiencies can compromise the system significantly (Duthie, 1994). Absorption of these trace elements may, however, decrease with age.

Glutathione is a water-soluble antioxidant that is a product of amino acid precursors glycine, glutamate, and cysteine. It is able to mop up ROS such as lipid peroxides, and it is an important agent in xenobiotic metabolism (Yadav et al., 2016). Exposure of an individual to high levels of xenobiotics results in the depletion of glutathione making it less available to perform antioxidant activities in the body. Glutathione and vitamin C have been reported to work together in the neutralizing of free radicals and may each have a sparing effect on themselves (Jacob, 1995). Lipoic acid is also an important endogenous antioxidant that contains sulfur and is important in
speeding up the oxidative decarboxylation of alpha-keto acids, such as pyruvate and alphaketoglutarate, in the Krebs cycle (Yadav et al., 2016). Lipoic acid is able to mop up free radicals in both lipid and aqueous phases in the body and as a result, has been termed a universal antioxidant. Lipoic acid may also possess a sparing effect on other antioxidants. Animal studies have suggested a protective effect that lipoic acid produces in subjects with vitamin E and C deficiency (Packer 1995).

2.8.5 PHYTONUTRIENTS

Some antioxidant substances may be found in plants and plant foods that fall outside the scope of traditional vitamin antioxidants. They are called phytochemicals or phytonutrients and have become well-known due to their antioxidant activity. Phenolic compounds such as flavonoids are found commonly within the plant kingdom. In plants, flavonoids are observed to protect against environmental stress while modifying biological response in humans (Percival, 1998). Some flavonoids have been shown to possess anti-inflammatory, antiallergenic, anti-viral, anti-aging, and anti-carcinogenic activity due mainly to their antioxidant effect. They are also able to offer some protection against heart-related diseases by inhibiting the activities of cyclooxygenase and lipoxygenase in platelets and macrophages (Percival, 1998).

2.8.5.1 POLYPHENOLS

Polyphenols, are widely distributed in plants and contribute to the protection of the body against ultraviolet radiation or dangerous pathogens. They are also responsible for the distinguishing colours of plants. They are mainly found in plant foods such as fruits, vegetables, legumes and
cereals, and beverages such as wine, tea and coffee. They are responsible for many of the organoleptic properties of plants (Fejzić & Ćavar, 2014). The health benefits of phenolic compounds have become evident in recent years being largely as a result of their antioxidant potential, their abundance in many plant foods, and their ability to reduce oxidative stress and its associated diseases (Fejzić & Ćavar, 2014). There are over 8000 phenolic structures currently known. All plant phenolics have a common intermediate amino acid from which they stem, called phenylalanine, or a close precursor, shikimic acid. They mainly occur in different forms, usually having one or more sugar residues linked to hydroxyl groups. Phenolic compounds may form associations with other compounds such as carboxylic and organic acids, amines, lipids, as well as with other phenols. Polyphenols may be categorized into different groups depending on the number of phenol rings that they have and other constituents of their structure which link the rings together (Pandey & Rizvi, 2009). The most common phenolic compounds in foods are phenolic acids, flavonoids and tannins. Phenolic compounds have at least one aromatic ring with one or more hydroxyl groups. Phenolics can also be classified as either flavonoids or non-flavonoids. Polyphenols are involved in the elimination of free radicals and may also have some synergistic effects on other antioxidants like vitamin E (Lima et al., 2014). They may also be involved in immune modulatory, anti-cancer and antibacterial activities, and may have significant wound healing potential (Lima et al., 2014).

2.8.5.2 FLAVONOIDS

Flavonoids are the most studied group of polyphenols. More than 4,000 varieties of flavonoids have been described, and many of these cause the colours of some flowers, fruits and leaves (Pandey & Rizvi, 2009). The structure of flavonoids is distinguished by a di-phenyl-propane
moiety (C6-C3-C6) which occurs naturally in plants and can be grouped into six. These are flavones, flavonols, anthocyanidins, flavanones, isoflavones and flavan-3-ols (Lima et al., 2014). Individual differences within each group arise from the variation in number and arrangement of the hydroxyl groups and their extent of alkylation and/or glycosylation. Quercetin, myricetin, catechins etc., are some most common flavonoids (Pandey & Rizvi, 2009). Flavonoids possess other beneficial properties apart from free radical scavenging including antimutagenic activity, anti-proliferative potential for tumors, protection against heart-related conditions like atherosclerosis, radio-protective action, and antimicrobial properties (Lima et al., 2014).

2.9 CITRUS FRUITS

Historically, the pharmacological properties of citrus fruits have been well documented dating as far back as the 4th to 5th century B.C. A paper by Arias and Ramon-Laca (2005), presented detailed ethnobotanical information acquired from medieval and ancient documents and manuscripts which depicted chemical and pharmacological uses and knowledge of citrus fruits. According to Theophrastus, (1968) if the fruit of the citrus tree was ingested, it was useful when one had been poisoned by a toxic substance. Also, when administered in wine, it acted as an emetic (causing an individual to vomit), expelling ingested toxins.

A number of studies have shown that citrus fruit consumption is protective in different human cancers (Ajaiyeoba et al., 2003; Alshatwi et al., 2011; Mohanapriya, Ramaswamy, & Rajendran, 2013a; Silalahi, 2002b; Umashanker & Shruti, 2011). Presumably, most of this protective effect is attributed to vitamin C. However, a study by Miller et al. (1994) has suggested that the consumption frequency of citrus fruit is more closely related to the reduction of risk as compared to the intake of vitamin C. This may suggest that citrus fruits contain multiple cancer preventive
agents (Diab, 2016). Citrus fruits, such as lemons, oranges, grape fruits and limes are a major source of dietary fiber, folate, vitamin C, and other bioactive components. These components include limonoids, carotenoids (especially β-carotene), and flavonoids, which have been shown to prevent a large number of different cancers as well as cardiovascular diseases (Silalahi, 2002b).

The peel of citrus fruits has been identified as a rich source of flavanones, which are not commonly found in other plants (Dhanavade et al., 2011). Flavonoids in citrus fruits have a large spectrum of biological activity including antibacterial, anticancer, antidiabetic and antiviral activities (Burt, 2004; Ortuno et al., 2006). Flavonoid is a strong inhibitor of lump progression in cancerous tissue (Garg, 2015). Flavonoids can function as direct antioxidants and free radical scavengers and have the capacity to modulate enzymatic activities and inhibit cell proliferation (Duthie and Crozier, 2000). In plants, they appear to play a defensive role against invading pathogens, including bacteria, fungi and viruses (Sohn et al., 2004).

Phytochemicals are biologically active, naturally occurring chemical compounds found in plants, which protect plant cells from environmental hazards such as pollution, stress, drought, UV exposure and pathogenic attack (Nyamai et al., 2016). Citrus fruits are particularly high in a class of phytochemicals known as limonoids which is responsible for the bitter taste in citrus fruits (Lam, Zhang, & Hasegawa, 1994). It has also been determined by animal studies that citrus limonoids have certain biological activities that may be used as chemo-preventive agents for cancer (Garg et al., 2015). Limonoids have been shown to inhibit the growth of oestrogen positive and negative receptor human breast cancer cells in culture by significantly slowing down the proliferation of MCF7 cells which are responsible for breast cancer in vivo (Alshatwi et al., 2011; Tian et al., 2001). This inhibitory action depends on the dose used and the duration of exposure. Glutathione S-transferase (GST) is one of the major detoxifying enzyme systems that catalyzes the
conjugation of glutathione with electrophiles which cause induction of activated carcinogens (Silalahi, 2002). A number of GST enhancers have been found to inhibit chemically induced carcinogenesis (Silalahi, 2002). The inhibitory effects of limonin are of a similar trend as their ability to induce GST activity.

2.9.1 LEMONS

*Citrus limon* (lemon) is a flowering plant of the family Rutaceae, and the main distinguishing features of the plant are thorny branches, white flowers with purple edges. The edible fruit is oval-shaped, with a rind that is aromatic and may be yellow or green depending on ripeness and environmental conditions (Chaturvedi & Shrivastava Suhane, 2016).

The specific origin of lemons is quite unclear but it is believed to have originated in the east Himalayan region of India. Due to their close relation to the citron, lemons are thought to be a hybrid between citron and lime. They were well established in Iraq by 900AD and by the 12th Century, had reached China and the near East (Hardy, 2004). They were introduced to Europe and America by the 15th century and by mid-seventeenth Century, South Africa by the Dutch. Lemons are grown in the semi-arid and arid subtropical regions of the world. They produce many crops annually and are in season all year round. They have a relatively high acidity (5-8%) and are mostly used for their juice, peel and oil. The oil can be found in the peel, juice sacs and seeds and can be used as flavour in food and drinks (Hardy, 2004).

The main *Cirrus limon* varieties include Eureka, Fino, Lisbon, Meyer, Verna and Yen Ben (Hardy, 2004). Eureka originated in California in the year 1858 from seeds imported from Sicily. Eureka is the most widely grown lemon variety in Australia, California, South Africa, Israel and Argentina. For Fino, the origin is quite unknown but it could have come from an older Spanish
variety. The Fino variety can also be called Mesero or Primofiori in Spain. The Lisbon variety is of Portuguese origin but is popular in California, Arizona, Uruguay and Argentina. Meyer is thought to be a hybrid between *Citrus limon* and either orange or mandarin, as it is not considered a true lemon. It was first introduced into the USA from China in 1908 by F. N. Meyer (Hardy, 2004). The Verna variety of *Citrus limon* is a Spanish variety, the origin of which is not known, but it accounts for about 60% of the annual *Citrus limon* produce of Spain. It is also grown in Algeria and Morocco. The Yen Ben variety originated from New Zealand in 1978 and since then, it has been grown on a larger scale because of its good fruit quality characteristics (Hardy, 2004).

The length of the leaves of *Citrus limon* range between 66 to 100 mm in length and are jointed to the petiole (Chaturvedi & Shrivastava Suhane, 2016). They possess narrow wings with leaf blades that are elliptic to ovate, an apex that is usually mucronate and a margin that is crenulate. The lemon fruit is ovoid and some varieties may be yellow when ripe. The shape of the fruit may vary as the plant gets older and may also be a subject of variety. The size is also subject to variety, crop load, rootstock and irrigation practices. Mature *Citrus limon* fruits may be green to yellow, weigh between 50 and 80 g and may be 5 to 8 cm in diameter. The seeds are found aggregated in the pulp close to the center of the fruit. Their sizes vary according to variety but most are white, hard and oval shaped, measuring about 3/8 of an inch in length. *C. limon* rind contains fibre of about 15.18%, crude fat of 4.8%, proteins of 9.42% and an ash content of 6.46% (Chaturvedi & Shrivastava Suhane, 2016). The juice of *C. limon* is made up of about 5% acid, and the pH lies between 2 and 3, accounting for its sour taste. *C. limon* juice contains acids, mainly citric acid (8%) as well as sugars. The rind consists of the outer layer, which is the pericarp or zest, which contains essential oil (6%) and limonene (90%) and the mesocarp which contains coumarin derivatives, bitter flavone glycosides, a little potash, sugar and gum (Chaturvedi & Shrivastava Suhane, 2016).
*C. limon* has been shown to possess many health benefits ranging from uses in skin care, treatment of scurvy (vitamin C deficiency), aiding in digestion, prevent constipation, peptic ulcer, respiratory disorders, gout, piles, urinary disorders and help in weight loss and improve vision (Mohanapriya, Ramaswamy, & Rajendran, 2013). Citrus fruits like *C. limon* contain limonoids which may be responsible for antimicrobial activity against some significant, isolated strains of bacteria. Limonoids obtained from *C. limon* have shown good antibacterial and antifungal activity (Kadhim Hindi & Ghani Chabuck, 2013).

### 2.9.2 PHYTOCHEMICAL AND ANTIOXIDANT PROPERTIES OF CITRUS

Citrus fruits include oranges, lemons, limes and grapefruits, and fruits such as these are an important source of certain vital nutrients. They contain vitamin C, folate and dietary fibre (Silalahi, 2002). They also contain many bioactive compounds like carotenoids and flavonoids, which are suggested to offer protective effects against chronic and degenerative diseases (Silalahi, 2002). Citrus fruits are particularly high in a class of phytochemicals known as the limonoids, as well as other components including ascorbic acid, carotenoids (especially β-carotene), folate, flavonoids and dietary fibres.

According to studies by Johann *et al.*, (2007) and Ghasemi *et al.*, (2009) citrus plants have been shown to be a rich source of phytochemicals which are able to produce a broad spectrum of biological activities. Aleksandra (2007) reported high antioxidant activity in the peels of citrus fruits including Sampion cultivar and white grapes, as well as in the seeds of the Idared cultivar and orange. Citrus plants have been shown to contain significant amounts of polyphenols that serve as a natural defense against free radical activity, making them beneficial for human health as they exhibit good antioxidant activity (Ighodaro, 2012).
CHAPTER THREE

3.0 METHODOLOGY

3.1 MATERIALS AND METHODS

3.1.1 CELL LINES AND REAGENTS

Analytical grade solvents such as ethanol, petroleum ether and ethyl acetate used for the extraction of the lemon extracts, Folin-Ciocalteau reagent, sodium carbonate solution, phosphate buffered saline (PBS) were obtained from Sigma-Aldrich Company (Missouri, USA). Rose Park Memorial Institute (RPMI) - 1640 (Nissui Pharmaceutical Co., Ltd., Tokyo, Japan), Fetal Bovine Serum (FBS) (Hyclone Lab. Inc., South Logan, Utah, U.S.A.), penicillin streptomycin L-glutamine (PSG) (Wako Pure Chem. Ind., Tokyo, Japan), Trypsin (Wako Pure Chem. Ind., Tokyo, Japan), Curcumin, 2, 2'-diphenyl-1-picryl hydrazyl free radical reagent (DPPH) (Hamburg, Germany), 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide (MTT) (Wako Pure Chem. Ind., Tokyo, Japan), dimethyl sulphoxide (DMSO), butylated hydroxyl toluene (BHT) (Hamburg, Germany), Gallic Acid (Hamburg, Germany), Trypan Blue (Wako Pure Chem. Ind., Tokyo, Japan). Human hormone-responsive breast carcinoma (MCF-7), human normal prostate (PNT2) and human prostate carcinoma (PC3) cell lines which had been passed not more than seven times were obtained from the Department of Clinical Pathology, Noguchi Memorial Institute for Medical Research (NMIMR), University of Ghana, where the studies were performed.
3.2 STUDY DESIGN

This study employed in vitro culture system to evaluate anticancer property (cytotoxicity) of C. limon extracts. Test and control experiments were set up. Total antioxidant activity of the extracts was also assessed in vitro. For the chemical determination of antioxidant activity as well as cytotoxicity, experiments were done in triplicates in order to produce verifiable results. The control groups were used to validate results obtained from the experimental groups. For the various controls of the experiments, there were positive controls (standards), colour controls and blanks for the chemical analysis of antioxidants. For the cytotoxicity assay, the use of negative controls was employed in addition to the positive controls, colour controls and blanks.

Four sets of extractions were done on the rind, pulp, seeds and juice of C. limon to obtain a total of 16 extracts. For each extract, the total antioxidant activity, total phenolics content and total flavonoids content were evaluated. The cytotoxic activity of each extract was also determined on a breast cancer, prostate cancer and normal prostate cell line to determine the selectivity indices of the extracts.

3.3 SAMPLE COLLECTION AND PROCESSING

Green lemons were purchased from Citiveg (Department of Crop Science, University of Ghana). The fruits were washed thoroughly and manually separated into seed, pulp, juice and rind. The seed, pulp and rind were washed thoroughly and air-dried to remove all moisture. They were then pulverized into fine particles using a dry blender. Sieving was done using double layered muslin cloth to ensure only fine particles were used. The samples were then stored in zip-lock bags for further use. The juice was collected in zip-lock bags, frozen and freeze-dried to obtain a solid powdered form.
3.4 EXTRACTION

Sequential extraction was done for all the samples using Petroleum ether, Ethyl acetate, Ethanol and distilled water in that order. Extraction was done in order of increasing polarity so that non-polar compounds would dissolve first, followed by semi polar, then polar, and finally, the highly polar compounds.

Preparation of the extracts of the various parts was carried out by weighing 50g of each powdered sample on a beam balance, and then suspending it in 500 mL of solvent in a conical flask, each starting with petroleum ether. The ratio of weight of the powdered sample to volume of the solvent was 1:10 (w/v) as is the ratio observed to achieve optimum yield. The extraction was done by cold maceration (Azwanida, 2015). Each mixture was placed on an electric shaker and shaking was done for 3 days at room temperature. The constant shaking ensured agitation of the particles of the solute in order to increase the surface area for contact with the solvent, thus enhancing dissolution. The supernatant was then filtered through Whatman No.1 filter paper and concentrated using a rotary evaporator to remove the solvent so as to obtain the seed, pulp, juice and rind crude petroleum ether extracts. The residues of the samples were then air-dried for a day to remove all solvent and the process was repeated using Ethyl acetate, Ethanol and then distilled water in that order.

When performing aqueous extraction, the mixtures were heated for 1 hour at 80°C with the exception of the juice, for which cold maceration on an electric shaker was done for 3 days. The aqueous extraction was repeated with the pellet after centrifugation and separation of the initial supernatant. The two supernatants were pooled, frozen at -20°C and freeze-dried using a lyotrap freeze-drier (Labconco, England). Extraction on all four samples using the four different solvents
yielded a total of 16 different crude extracts – petroleum ether, ethyl acetate, ethanolic and aqueous fractions of rind, pulp, seed and juice each. The yield of each extract was calculated as (Final weight of crude extract / Initial weight of powder) x 100.

3.5 IN VITRO ANTIOXIDANT ACTIVITY - 2, 2-DIPHENYL-1-PICRYLHYDRAZYL (DPPH)

3.5.1 PRINCIPLE

The 2, 2-diphenyl-1-picrylhydrazyl (DPPH) compound is one of the few stable and commercially available organic nitrogen radicals, and it operates on the theory that a hydrogen donor is an antioxidant (Lewis, 2012). The DPPH assay method is based on the reduction of DPPH, a stable free radical. The free radical DPPH gives a maximum absorption at a wavelength of 517 nm and a purple colour. When antioxidants react with DPPH, it becomes paired off by receiving the hydrogen donated by the antioxidant and is reduced to the DPPH form which has a yellow colour. The extent of decolourization gives an indication of the antioxidant potential of whatever substance is being measured (Shekhar & Anju, 2014).

![Figure 3.1: Reduction reaction of DPPH](image)

(Lewis, 2012)
3.5.2 PROCEDURE

The effects of the crude extracts on the scavenging activity of 2, 2-diphenyl-1-picrylhydrazyl (DPPH) were determined using a method by Acheampong et al. (2015) with slight modifications. A solubility test was done on each extract to determine which solvent would dissolve them. The solvents used were methanol and water. A weight of 20mg of each extract as well as 1mg of Butylated Hydroxytoluene (BHT), which was used as the positive control, were transferred into Eppendorf tubes, and 1 ml of solvent added to obtain stock solutions. The concentration of the stock solutions of the extracts was 20 mg/ml while that of the BHT was 1 mg/ml. Two-fold serial dilutions were done with the extracts in their respective solvents (methanol or water) into eppendorf tubes to obtain a concentration range of 0.3125 - 20 mg/ml and a total of 7 working solutions for each extract. Two-fold serial dilutions of BHT were also done in methanol to obtain a concentration range of 0.015625 - 1 mg/ml and a total of 7 working solutions of BHT. The Eppendorf tubes were labelled appropriately in order to identify each extract and their respective concentrations.

Some 96 well plates were labelled appropriately to identify extracts, their different concentrations, blanks and positive and colour controls. The solvents methanol and distilled water were used as blanks or the negative control. A working concentration of DPPH (0.1972mg/ml) was prepared by first calculating what mass of the dye would be needed to dissolve in a given volume of methanol in order to obtain the volume of DPPH solution sufficient for all the well plates. For each working solution of each extract, 100 µl was pipetted in triplicate and 100 µl for colour control in 96-well microtiter plates. Aliquots of 100 µl of DPPH were added to each well including the blanks, except for colour control wells. The plates were then covered, shaken gently and kept in the dark for 20 minutes after which the absorbance was read on a Tecan-PC infinite M200 Pro Plate reader.
(Austria), at the absorbance wavelength of 517 nm. Percentage DPPH scavenging activity was determined by the equation:

\[
\% \text{ Scavenging (Antioxidant) activity} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100
\]

Where \(A_0\) is the mean absorbance of the wells containing the negative control; \(A_1\) is the mean absorbance of the wells with the test sample or standard compound, (BHT) that scavenged DPPH free radicals. The mean percentage scavenging (antioxidant) activity for the triplicate experiments was then plotted against sample concentrations to obtain effective concentrations at 50% (EC\(_{50}\)) values, the EC\(_{50}\) value being the sample concentration required to scavenge 50% of DPPH radicals.

### 3.6 DETERMINATION OF TOTAL PHENOLIC CONTENT

#### 3.6.1 PRINCIPLE

Total Phenolic Content can be determined with the use of the Folin Ciocalteau method. It is a method developed from the Folin Denis reagent used in the early 19th century for the determination of tyrosine in proteins. The basic mechanism involved in this assay is an oxidation-reduction reaction with the phenolic group being oxidized and the metal ion reduced (Agbor, Vinson, & Donnelly, 2014). The Folin Ciocalteau reagent contains phosphomolybdic/phosphotungstic acid complexes, and the method relies on the transfer of electrons in alkaline medium from the phenolic compounds to the Folin Ciocalteau reagent (yellow) (Wollgast & Anklam, 2007) to form a blue phosphotungstic-phosphomolybdenum complex, which gives an indication of the extent of the reaction by spectrophotometry. Maximum absorption would be dependent on the alkaline solution and the concentration of phenolic compounds (Blainski, Lopes, & De Mello, 2013).
3.6.2 PROCEDURE

Two-fold serial dilutions were carried out on the standard compound, gallic acid, and on each extract to obtain a concentration range of 0.03125-1 mg/ml for gallic acid and 10 - 20 mg/mL for the extracts (Larbie et al., 2015). Absolute ethanol was used as a blank for the gallic acid samples. Volumes of 10μL of each dilution were aliquoted into Eppendorf tubes followed by the addition of 790 μL of distilled water and 50 μL of Folin-Ciocalteau reagent. The tubes were incubated in the dark at room temperature (26°C) for 8 minutes, and 150 μL of 7% sodium carbonate solution was subsequently added to each tube, after which incubation was continued for 2 hours in the dark at room temperature. A volume of 200 μL of each reaction mixture was aliquoted into wells on a 24-well plate in triplicates and absorbance read at a wavelength of 750 nm using a microplate spectrophotometer. The total phenolic content of each crude extract was calculated from the regression equation of the gallic acid calibration curve (y = 0.3633x - 0.0089, R2 = 0.9976) and expressed as gallic acid equivalents (GAE).

3.7 TOTAL FLAVONOIDS

3.7.1 PRINCIPLE

The aluminum chloride method is a colorimetric method which works on the principle that aluminium chloride forms acid stable complexes with the C-4 keto group and either the C-3 or C-5 hydroxyl group of flavones and flavonols. Aluminum chloride also forms complexes with the orthohydroxyl groups in the A- or B-ring of flavonoids which are subject to change in acidic environments (Hassan, Aqil, & Attimarad, 2013). The reaction produces a subsequent yellow colour from the complex (Pękal & Pyrzynska, 2014).
3.7.2 PROCEDURE

The total flavonoid contents (TFC) of the extracts were determined as described by Ordónez et al., (2006). One hundred microlitres of samples (extracts/standard) was pipetted into 96 well plates. One hundred microlitres of 2% Aluminium Chloride (AlCl₃) was pipetted and added to each of the extracts in the wells. The reaction mixtures were allowed to incubate at room temperature for 20 minutes. The absorbance of the mixtures was then read at a wavelength of 415nm using the Tecan Infinite M200 Pro, microplate reader (Austria). A standard curve for the standard, quercetin was plotted and the concentrations of quercetin equivalence in each of the extracts extrapolated from the curve.

3.8 CELL CULTURE

Cells were recovered from the -80°C freezer and allowed to thaw. A volume of 1ml of cells was pipetted into a 25cm³ culture flask and 5 ml of culture media was added. PC3 and PNT2 cells were cultured in RPMI-1640 medium, and MCF-7 cells were cultured in DMEM medium. All the cells were adhesive cell lines. The culture media were supplemented with 10% fetal bovine serum and 1% Penicillin-Streptomycin-L-Glutamine (PSG) making them complete media. Cultured cells were kept in a humidified incubator at 37°C with regular changing of spent media. After attaining about 90% confluence, cells were sub-cultured not less than twice before use for the cytotoxicity (MTT) assay.
3.9 IN VITRO CELL VIABILITY ASSAY

3.9.1 CELL PASSAGE

Spent media from each culture flask was pipetted off from the flask. Cells were washed with Phosphate Buffered Saline (PBS) to stabilize the pH and also get rid of dead cells. The cells were subsequently treated with 0.5 ml of trypsin and incubated in a humidified incubator for 3 min to detach the cells from the walls of the flasks. A volume of 8.5 ml of media was then added to each flask and the mixture pipetted into 15 ml centrifuge tubes and spun at 1000 rpm using (Tomy LC200 centrifuge, Japan) for 5 minutes. The supernatant was then discarded and the pellets (cells) carefully dislodged and resuspended in fresh 1 ml complete media (culture media supplemented with fetal bovine serum and 1% penicillin-streptomycin). To know the number of cells, the trypan blue method of counting cells was employed. A volume of 40 μl of the trypan blue dye was pipetted into an Eppendorf tube and 10μl of the cells were mixed thoroughly. A volume of 10 ml of the mixture was loaded onto a haemocytometer for counting using a light microscope. The cells were then further passed for future use.

3.9.2 3-(4, 5-DIMETHYLTHIAZOL-2-YL)-2, 5-DIPHENYLTTETRAZOLIUM BROMIDE (MTT) ASSAY

3.9.2.1 PRINCIPLE

The purpose of the MTT assay is to measure viability of cells, usually in high numbers, without the tedious task of counting cells singly. It is commonly used to ascertain the cytotoxicity of drugs...
in varying concentrations. Mitochondrial activity is a hallmark of living cells and therefore, increase or decrease in mitochondrial activity is indicative of corresponding increase or decrease in cell viability (Meerloo & Cloos, 2011). The yellow tetrazolium MTT is reduced by the action of reductases or dehydrogenase enzymes, which are products of metabolic activity, to form reducing equivalents like NADH and NADPH. The result is the formation of formazan, a purple crystalline compound which can be solubilized and quantified by spectrophotometric means (American Type Culture Collection, 2011). Thus, any increase or decrease in viable cell number can be detected by measuring formazan concentration reflected in optical density (OD) using a plate reader at 570 nm (Meerloo & Cloos, 2011).

3.9.2.2 PROCEDURE

The cytotoxic effects of crude lemon extracts on cancer and normal cell lines were determined using the MTT assay. The in vitro cytotoxicity assay was performed on PC3, PNT2 and MCF-7 cell lines.

Cells were seeded at a concentration of $1 \times 10^5$ cells/mL into 96-well plates and incubated overnight in humidified chamber at 37°C in the presence of 5% CO$_2$ for 24 hours. The cells were then treated with various concentrations of crude lemon extracts at a concentration range of 62.5-1000 µg/ml in triplicates using DMSO as the solvent for the extracts. A negative control (untreated cells) was included and the cells were incubated for 72 h. After the incubation, the cells were treated with 20 µL of 2.5 mg/mL MTT solution and re-incubated for 4 h. The samples were subsequently treated with 150 µL of acidified isopropanol (1.7% (v/v) aqueous HCl in isopropanol) containing 1% Triton-X and the plates were incubated in the dark overnight at room temperature to dissolve any formazan crystals formed. The optical density was read using a spectrophotometer (Tecan Infinite
M200 Pro plate reader, Austria) at a wavelength of 570nm. Curcumin was used as a positive control at a concentration range of 23.00 – 368.04 µg/ml. The negative control consisted of 1% of DMSO.

In order to ensure equal concentrations of DMSO in each well, 10 µL from the stock solution of each extract and curcumin was added to 90 µL of media and mixed properly. Serial dilution was done by adding 50 µL of the mixture to 50 µL of media containing 10% DMSO. This step was repeated three more times to obtain five different concentrations of the extracts, each containing 10% DMSO. The concentrations of DMSO were further stepped down by adding 10 µL of each concentration of extract to each well containing the cells. This gave a final concentration of 1% DMSO in each well. The percentage cell viability was calculated using the formula:

% Cell viability = \[\frac{ODT_0 - ODT_1}{ODU_0 - ODU_1}\] X 100%

Where ODT₀ is the absorbance of wells treated with test extracts or standard drug (Curcumin) for all the cell lines, ODT₁ is the absorbance of test wells minus the colour control for test extracts or curcumin, ODU₀ is the absorbance of wells with negative control (untreated cells) for all cell lines, ODU₁ is the absorbance of wells containing blank (culture media only). A graph of a dose response curve of the % cell viability against concentration of the extracts was plotted to determine the inhibition concentration at which 50% cell viability was attained (IC₅₀). According to the National Cancer Institute (USA), crude extracts with IC₅₀ values < 30 µg/ml can be considered as having strong cytotoxic potential as anticancer agents. Those with IC₅₀ values between 30 and 200 µg/ml have moderate potential, whereas those with IC₅₀ > 200 µg/ml are unlikely to have any activity (Geran et al., 1972). The selectivity indices (SI) are evaluated in order to determine the selectivity of the extracts for cancer cells. This is the ratio of the IC₅₀ values of each crude extract or curcumin in the normal prostate cell line (PNT2) to the IC₅₀ values in the cancer cell lines MCF-7 and PC3.
SI values ≥ 2 were considered significant or an indication of a promising therapeutic agent (Badisa et al, 2009).

3.9.3 STATISTICAL ANALYSIS

Microsoft Excel 2016 for Windows 10 was used to plot graphs and calculate the mean and standard deviation values of triplicate experiments. The experimental results were expressed as the mean ± standard error of the mean. Significant differences between control and test experiments were determined using the Student’s t-test. Values for which p<0.05 were considered statistically significant.
CHAPTER FOUR

4.0 RESULTS

4.1 YIELD OF EXTRACTS

Table 4.1 yield of extracts

Table 4.1 shows results on determination of the yield of the Citrus limon extracts.

<table>
<thead>
<tr>
<th>Extracts</th>
<th>Weight of Crude Extract / g</th>
<th>Percentage Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Petroleum Ether Rind (PER)</td>
<td>0.16</td>
<td>0.32</td>
</tr>
<tr>
<td>Petroleum Ether Pulp (PEP)</td>
<td>0.28</td>
<td>0.56</td>
</tr>
<tr>
<td>Petroleum Ether Seed (PES)</td>
<td>1.56</td>
<td>3.12</td>
</tr>
<tr>
<td>Petroleum Ether Juice (PEJ)</td>
<td>0.03</td>
<td>0.06*</td>
</tr>
<tr>
<td>Ethyl Acetate Rind (EAR)</td>
<td>0.79</td>
<td>1.58</td>
</tr>
<tr>
<td>Ethyl Acetate Pulp (EAP)</td>
<td>0.55</td>
<td>1.1</td>
</tr>
<tr>
<td>Ethyl Acetate Seed (EAS)</td>
<td>0.56</td>
<td>1.12</td>
</tr>
<tr>
<td>Ethyl Acetate Juice (EAJ)</td>
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</tr>
<tr>
<td>Ethanolic Rind (EoR)</td>
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</tr>
<tr>
<td>Ethanolic Pulp (EoP)</td>
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</tr>
<tr>
<td>Ethanolic Seed (EoS)</td>
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<td>0.86</td>
</tr>
<tr>
<td>Ethanolic Juice (EoJ)</td>
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<td>25.58</td>
</tr>
<tr>
<td>Aqueous Rind (AqR)</td>
<td>3.46</td>
<td>6.92</td>
</tr>
<tr>
<td>Aqueous Pulp (AqP)</td>
<td>3.96</td>
<td>7.92</td>
</tr>
<tr>
<td>Aqueous Seed (AqS)</td>
<td>3.73</td>
<td>7.46</td>
</tr>
<tr>
<td>Aqueous Juice (AqJ)</td>
<td>5.29</td>
<td>10.59</td>
</tr>
</tbody>
</table>

N/B Petroleum ether juice extract yielded very little hence it was not used for any of the assays.
4.2 TOTAL PHENOLIC CONTENT (TPC)

Figure 4.2a shows the curve plotted for the standard compound Gallic acid, using the absorbance from different concentrations of the compound. The total phenolic contents of the lemon extracts were derived from this standard calibration curve by extrapolation of their absorbance to obtain the curve. Figure 4.2b shows the total phenolic contents of the *Citrus limon* extracts. The ethyl acetate rind extract showed the highest total phenolic content of 6415.268 ± 0.0095mg Gallic Acid Equivalent, followed by the ethanolic rind extract (5885.861 ± 0.0111mg Gallic Acid Equivalent).

\[ Y = 0.3633x + 0.0089 \quad R^2 = 0.9976 \]

**Figure 4.2a: Standard curve for Gallic acid**

Figure 4.2a shows the standard curve for Gallic acid. Each point on the curve represents a mean of three determinations. The graph shows a dose-dependent relationship between concentration of gallic acid and the absorbance. The total phenolic contents of the extracts were extrapolated from this curve; \( R^2 = 0.9976 \)
Figure 4.2b: Total phenolic content of *C. limon* extracts

Figure 4.2b represents the total phenolic content of the *C. limon* extracts. The *C. limon* rind ethyl acetate extract recorded the highest total phenol of 6415.268 ± 0.0095mg Gallic acid equivalent / 100g of Lemon rind extract. TPC represents total phenolic content; PER = Petroleum Ether Rind; PEP = Petroleum Ether Pulp; PES = Petroleum Ether Seed; EAR = Ethyl Acetate Rind; EAP = Ethyl Acetate Pulp; EAS = Ethyl Acetate Seed; EAJ = Ethyl Acetate Juice; E-oIR = Ethanolic Rind; E-oIP = Ethanolic Pulp; E-oIS = Ethanolic Seed; E-oIJ = Ethanolic Juice; AqR = Aqueous Rind; AqP = Aqueous Pulp; AqS = Aqueous Seed; AqJ = Aqueous Juice.

### 4.3 TOTAL FLAVONOID CONTENT (TFC)

Figure 4.3a shows the curve representative of the standard compound quercetin. Experiments were conducted in triplicates and each point plotted is a mean of the triplicate experiment ± SD. The
total flavonoid contents of the *C. limon* extracts were extrapolated from the standard equation. Figure 4.3b represents the total flavonoid contents of the *C. limon* extracts. The petroleum ether rind extract showed the highest total flavonoid content of 2603.025 ± 0.0121 mg/100g Quercetin equivalent followed closely by the ethyl acetate rind extract with 2497.709 ± 0.0113 mg/100g Quercetin equivalent. The ethyl acetate juice extract showed the least total flavonoid content with 67.368 ± 0.0119 mg/100g Quercetin equivalent.

\[ y = 12.078x - 0.0037 \quad R^2 = 0.9993 \]

**Figure 4.3a: Total flavonoid content for standard quercetin in mg/ml**

Figure 4.3a represents the standard curve for quercetin in mg/ml. Each point represents a mean of three experiments. The curve shows a dose-dependent relationship between the concentration of quercetin and the absorbance. The total flavonoid contents of the extracts were extrapolated from this curve. \( R^2 = 0.9993 \)
Figure 4.3b: Total flavonoid content of lemon extracts

Figure 4.3b represents the total flavonoid content of the *Citrus limon* extracts. The *Citrus limon* rind petroleum ether extract showed the highest total flavonoid content of 2603.025 ± 0.0121 mg/100g Quercetin equivalent.

### 4.4 CHEMICAL ANALYSIS OF ANTIOXIDANTS

#### 4.4.1 TOTAL ANTIOXIDANT ACTIVITY

The total antioxidant activity of the various extracts and positive control, BHT, are shown in Figures 4.4.1a – 4.4.1e. The antioxidant activity is a measure of DPPH free radical scavenging ability of each extract. Absorbance readings were taken at a wavelength of 517 nm. The lower the absorbance readings, the higher the total antioxidant activity. The extracts generally demonstrated a dose-dependent increase in antioxidant activity. The rind extracts generally showed the highest antioxidant activity. The ethyl acetate rind extract showed the strongest antioxidant activity with a calculated EC$_{50}$ value of 0.42 ± 0.02 mg/ml, $p = 0.001$. The petroleum ether pulp and seed
extracts as well as the aqueous seed extract showed the weakest antioxidant activities with EC\textsubscript{50} values >10 mg/ml. All other extracts showed significant antioxidant activity.

![Figure 4.4.1a: Total antioxidant activity of the standard, butylated hydroxytoluene (BHT)](image)

Each point is a mean of triplicate experiments. The curve shows a dose-dependent relationship between percent free radical scavenging activity and concentration of BHT. Antioxidant activity increased with increasing concentration of the compound.

**Figure 4.4.1a: Total antioxidant activity of the standard, butylated hydroxytoluene (BHT)**

Each point is a mean of triplicate experiments. The curve shows a dose-dependent relationship between percent free radical scavenging activity and concentration of BHT. Antioxidant activity increased with increasing concentration of the compound.
Figure 4.4.1b: Total antioxidant activity of rind extracts

All rind extracts showed significant antioxidant activity; the petroleum ether rind extract showed strongest antioxidant activity with 100% increase in antioxidant activity at 5mg/ml concentration. Ethyl acetate rind extract however had the highest EC$_{50}$ value of 0.4 ± 0.02 mg/ml.
All the pulp extracts showed a dose-dependent increase in antioxidant activity. Ethyl acetate and ethanolic pulp extracts each showed antioxidant >80% at the concentration of 10mg/ml with the ethanolic pulp extract showing the highest EC$_{50}$ value of 1.4 ± 0.2 mg/ml.

The ethanolic seed extract exhibited the strongest antioxidant activity, exhibiting a 90% increase in antioxidant activity at a concentration of 5 mg/ml. It also showed the highest EC$_{50}$ value of 1.0 ± 0.1 mg/ml.
All juice extracts showed dose-dependent increase in antioxidant activity with the ethanolic juice extract showing relatively strongest activity with a 60% increase in antioxidant activity at a concentration of 5 mg/ml. The aqueous juice extract however had the lowest EC$_{50}$ value of 4.2 ± 0.3mg/ml.
Table 4.4.1: Antioxidant activities of *C. limon* extracts and BHT

Comparison of total antioxidant activities of various extracts and the standard, BHT, was done using EC$_{50}$ values. Each calculated EC$_{50}$ value is the concentration of the extract or BHT at which 50% free radical scavenging activity was exhibited.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Petroleum Ether</th>
<th>Ethyl Acetate</th>
<th>Ethanolic</th>
<th>Aqueous</th>
<th>BHT</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lemon Rind</td>
<td>1.4 ± 0.1*</td>
<td>0.4 ± 0.02*</td>
<td>1.3 ± 0.2*</td>
<td>1.1 ± 0.1*</td>
<td></td>
</tr>
<tr>
<td>Lemon Pulp</td>
<td>&gt;10</td>
<td>2.4 ± 0.1*</td>
<td>1.4 ± 0.2*</td>
<td>4.1 ± 0.3*</td>
<td></td>
</tr>
<tr>
<td>Lemon Seed</td>
<td>&gt;10</td>
<td>6.3 ± 0.1*</td>
<td>1.0 ± 0.1*</td>
<td>&gt;10</td>
<td>0.02 ± 0.00</td>
</tr>
<tr>
<td>Lemon Juice</td>
<td>N/A</td>
<td>7.0 ± 1.0*</td>
<td>6.4 ± 0.5*</td>
<td>4.2 ± 0.3*</td>
<td></td>
</tr>
</tbody>
</table>

Each determination is the mean ± SD of triplicate experiments (n=3). Values with * connote significant difference (p < 0.05) in cytotoxicity in comparison to the positive control BHT.

4.5 *IN VITRO* CELL VIABILITY ASSAY

4.5.1 CYTOTOXIC EFFECT OF EXTRACTS ON MCF-7 CELL LINE

The cytotoxic effects of the extracts on the breast cancer cell line, MCF-7, were evaluated as shown in Figures 4.5.1a – 4.5.1e. Two of the extracts (PER & PES) showed significant cytotoxicity differences comparative to the positive control, curcumin. The petroleum ether rind extract exhibited the highest cytotoxic effect on MCF-7 cells (IC$_{50}$ = 384.6 ± 17.1 µg/ml, p = 0.045). The remaining extracts did not show significant cytotoxic activity on the cells for the concentration range used (0 – 1000 µg/ml).
Figure 4.5.1a: Cytotoxic effect of curcumin on MCF-7 cell line

Each point on the chart is the mean ± SD of three experiments. The curve shows an inverse proportionality between the concentration of curcumin and percent cell viability. It recorded an IC$_{50}$ of 577.1 ± 35.6 µg/ml.
Figure 4.5.1b: Cytotoxic effect of rind extracts on MCF-7 cell line

The petroleum ether extract did not show significant activity within the concentration range of 0 - 250 µg/ml but exhibited a 90% increase in cytotoxic activity between the concentration range of 250 – 500 µg/ml. The ethyl acetate rind extract also exhibited strong cytotoxic activity with a 70% increase in cytotoxic activity within the concentration range of 500 – 1000 µg/ml. Both ethanolic and aqueous rind extracts did not show significant cytotoxic activity on the MCF-7 cells.
Figure 4.5.1c: Cytotoxic effect of pulp extracts on MCF-7 cell line

The petroleum ether pulp extract showed an 80% increase in viability of the cancer cells within the range of 0 – 62.5 µg/ml. The ethyl acetate pulp extract exhibited strong cytotoxic activity on the MCF-7 cells with a 60% decrease in cell viability within the range of 500 – 1000 µg/ml.
Figure 4.5.1d: Cytotoxic effects seed extracts on MCF-7 cell line

The petroleum ether seed extract showed a gradual increase in cell viability above 100% but exhibited strong cytotoxic activity (110% increase) between concentrations 500 - 1000 µg/ml.
Figure 4.5.1e: Cytotoxic effects of juice extracts on MCF-7 cell line

The aqueous juice extract showed weak cytotoxic activity on the MCF-7 cells, exhibiting stronger activity at concentrations >500μg/ml. The ethyl acetate and ethanolic juice extracts exhibited no significant cytotoxic activity on the MCF-7 cells, but rather enhanced their growth.

4.5.2 CYTOTOXIC EFFECT OF EXTRACTS ON PNT2 CELL LINE

The cytotoxic effect of the extracts on the normal prostate cell line, PNT2 was evaluated as shown in Figures 4.5.2a – 4.5.2e. Six of the extracts showed significant cytotoxicity differences comparative to the positive control, curcumin. The petroleum ether seed extract showed the highest cytotoxicity (IC₅₀ = 36.4 ± 3.5 μg/ml, p = 0.0038) followed by the petroleum ether rind extract (IC₅₀ = 363.6 ± 27.0 μg/ml, p = 0.0354), and the ethyl acetate rind extract (IC₅₀ = 366.6 ± 22.5 μg/ml, p = 0.0293). The ethyl acetate pulp, seed and juice extracts also exhibited significant cytotoxic activity on the normal prostate cells, PNT2 cell line. All other extracts did not show significant cytotoxic activity on the cells.
Figure 4.5.2a: Cytotoxic effect of curcumin on PNT2 cell line

Each point on the chart is the mean ± SD of three experiments. The concentration of curcumin is inversely proportional to percent cell viability.
Figure 4.5.2b: Cytotoxic effects of rind extracts on PNT2 cell line

The ethyl acetate rind extract showed >80% increase in cytotoxic activity at a concentration of 250 µg/ml. The petroleum ether rind extract also showed a dose-dependent increase in cytotoxic activity, reaching 90% at a concentration of 500 µg/ml.
Figure 4.5.2c: Cytotoxic effects of pulp extracts on PNT2 cell line

The ethyl acetate pulp extract showed significant cytotoxicity to the PNT2 cells, becoming stronger at concentrations > 500 µg/ml. The petroleum ether, ethanolic and aqueous pulp extracts showed no significant cytotoxicity to the PNT2 cells.
The petroleum ether seed extract showed strongest activity (90% increase) between the range of 0 – 62.5 µg/ml and the ethyl acetate seed extract exhibited a 55% increase in cytotoxicity to the PNT2 cells within the range of 0 – 1000 µg/ml.
Figure 4.5.2e: Cytotoxic effects of juice extracts on PNT2 cell line

The Ethanol and aqueous juice extracts did not exhibit significant cytotoxicity to the PNT2 cells. The ethyl acetate juice extract exhibited weak cytotoxic activity towards the PNT2 cells increasing strongly at concentrations >500 µg/ml.

4.5.3 CYTOTOXIC EFFECTS OF EXTRACTS ON PC3 CELL LINE

The cytotoxic effects of the extracts on the prostate cancer cell line, PC3, were evaluated as shown in Figures 4.5.3a – 4.5.3e. Out of all the extracts, six showed significant differences in cytotoxicity towards PC3 cells compared to the positive control, curcumin. The ethyl acetate rind extract showed the greatest cytotoxicity to the PC3 cells with an IC$_{50}$ value of 99.1 ± 8.4 µg/ml, p = 0.009. The ethanolic rind, petroleum ether and ethyl acetate pulp extracts, and the ethyl acetate and ethanolic juice extracts also exhibited significant cytotoxic activity on the PC3 cells. The other extracts, however, did not have any significant cytotoxic effect on the cells.
**Figure 4.5.3a: Cytotoxic effect of curcumin on PC3 cell line**

Each value is the mean ± SD of three experiments. The concentration of curcumin is inversely proportional to percent cell viability.

**Figure 4.5.3b: Cytotoxic effects of rind extracts on PC3 cell line**

Petroleum ether and ethyl acetate rind extracts showed strong cytotoxicity to the PC3 cells, becoming absolutely cytotoxic to the cells at concentrations of 500 µg/ml and 250 µg/ml respectively. The ethanolic rind extract showed weak cytotoxic activity becoming stronger at concentrations >500 µg/ml.
The ethyl acetate pulp extract shows weak cytotoxic activity becoming stronger at concentrations $>500 \mu g/ml$ from where it shows an 80% increase in cytotoxicity.

None of the extracts showed significant cytotoxic activity on the PC3 cells.
Figure 4.5.3e: Cytotoxic effects of juice extracts on PC3 cell line

The ethanolic and ethyl acetate juice extracts showed significant but weak cytotoxic activity on the PC3 cells with the ethyl acetate juice extract exhibiting stronger activity at concentrations >500 µg/ml. The aqueous juice extract showed no significant cytotoxic activity on the PC3 cells.
Table 4.5.1: Cytotoxic IC$_{50}$ values of *C. limon* extracts and standard, curcumin

<table>
<thead>
<tr>
<th>EXTRACT</th>
<th>MCF-7</th>
<th>PNT2</th>
<th>PC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>577.1 ± 35.6</td>
<td>19.6 ± 2.3</td>
<td>57.8 ± 11.3</td>
</tr>
<tr>
<td>Petroleum ether rind</td>
<td>384.6 ± 17.1*</td>
<td>363.6 ± 27.0*</td>
<td>61.0 ± 0.9</td>
</tr>
<tr>
<td>Ethyl acetate rind</td>
<td>653.6 ± 33.3</td>
<td>366.6 ± 22.5*</td>
<td>99.1 ± 8.4*</td>
</tr>
<tr>
<td>Ethanol rind</td>
<td>&gt;1000</td>
<td>142.7 ± 27.5</td>
<td>729.2 ± 12.9*</td>
</tr>
<tr>
<td>Aqueous rind</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Petroleum ether pulp</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>959.1 ± 19.2*</td>
</tr>
<tr>
<td>Ethanol pulp</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Aqueous pulp</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Petroleum ether seed</td>
<td>853.1 ± 45.4*</td>
<td>36.4 ± 3.5*</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Ethanol seed</td>
<td>&gt;1000</td>
<td>793.8 ± 29.6*</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Aqueous seed</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
<tr>
<td>Ethanol juice</td>
<td>&gt;1000</td>
<td>689.1 ± 10.7*</td>
<td>624.3 ± 25.7*</td>
</tr>
<tr>
<td>Aqueous juice</td>
<td>669.1 ± 20.2</td>
<td>&gt;1000</td>
<td>&gt;1000</td>
</tr>
</tbody>
</table>

Each determination is the mean ± SD of triplicate experiments. Values with * connote significant difference (p < 0.05) in cytotoxicity in comparison to curcumin, the positive control.
4.5.4 SELECTIVITY INDICES OF EXTRACTS

The selectivity index (SI) of each extract was calculated as a ratio of the IC\textsubscript{50} of the crude extracts in the normal prostate cell line (PNT2) to that in the cancer cell lines (MCF-7 and PC3). The petroleum ether rind extract exhibited the greatest SI for PC3 cells (5.96) which was about 17 times greater than the SI for the standard curcumin. This was followed by the ethyl acetate rind extract (3.7). For MCF-7 cells, none of the extracts had SI values greater than 2 even though petroleum ether rind, ethyl acetate rind and ethyl acetate pulp had SI values many folds greater than the SI of the standard curcumin. Extracts with no calculable IC\textsubscript{50} for the concentration range (0 – 1000 µg/ml) tested for any of the cancer cell lines did not have an SI determined for that cancer cell line.
Table 4.5.2: Selectivity indices of *C. limon* extracts and standard, curcumin

<table>
<thead>
<tr>
<th>EXTRACT</th>
<th>MCF-7</th>
<th>PC3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Curcumin</td>
<td>0.03</td>
<td>0.34</td>
</tr>
<tr>
<td>Petroleum ether rind</td>
<td>0.94</td>
<td>5.96</td>
</tr>
<tr>
<td>Ethyl acetate rind</td>
<td>0.56</td>
<td>3.70</td>
</tr>
<tr>
<td>Ethanol rind</td>
<td>N/A</td>
<td>0.20</td>
</tr>
<tr>
<td>Aqueous rind</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Petroleum ether pulp</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Ethyl acetate pulp</td>
<td>0.65</td>
<td>0.85</td>
</tr>
<tr>
<td>Ethanol pulp</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Aqueous pulp</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Petroleum ether seed</td>
<td>0.04</td>
<td>N/A</td>
</tr>
<tr>
<td>Ethyl acetate seed</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Ethanol seed</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Aqueous seed</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Ethyl acetate juice</td>
<td>N/A</td>
<td>1.10</td>
</tr>
<tr>
<td>Ethanol juice</td>
<td>N/A</td>
<td>N/A</td>
</tr>
<tr>
<td>Aqueous juice</td>
<td>N/A</td>
<td>N/A</td>
</tr>
</tbody>
</table>

*N/A means Not Applicable*
CHAPTER FIVE

5.0 DISCUSSION

Cancer is a leading cause of death worldwide, affecting both economically developed and developing countries, with an ever-increasing burden due to the aging and growth of populations (Torre et al., 2015). Due to challenges like difficulty in dosage selection, cytotoxicity to normal cells, rapid drug metabolism, drug resistance and harmful side effects, chemotherapy, which is a major means of cancer treatment has been greatly limited (Khuda-Bukhsh et al., 2014), hence the search for new anticancer agents. Medicinal plants are important for the treatment of cancer because of their multiple bioactive chemical compounds which may have anticancer properties (Kooti et al., 2017). Research shows that phytochemicals are important chemical and biological functional agents which may have a constructive effect on human health particularly because of their ability to neutralize free radicals (Mollakhalili et al., 2017). Citrus limon is known to contain a number of phytochemicals like phenols and flavonoids that exhibit antioxidant properties. In this study, the total phenols, total flavonoids, DPPH free radical scavenging activity and anticancer properties of the extracts of lemons were investigated.

The results from the study revealed that extracts from the Citrus limon rind possessed the highest total phenolic content compared to the other parts. The ethyl acetate rind extract showed the highest phenolic content followed by the ethanolic rind extract, and then the petroleum ether rind extract. These findings of high phenolic content in lemon rind are consistent with findings by Fežič Ćavaro (2014), who reported a higher polyphenol content in Citrus limon rind compared to the juice. Also, in a study involving the extraction of natural antioxidants from Citrus limon peels, they were shown to have higher values for total polyphenols when compared to three other plants Taraxacum oficinale, Urtica dioica & Onopordum acanthium (Diankov, Karsheva, & Hinkov, 2011).
Generally, extracts from the organic solvents showed higher total phenolic content compared to their corresponding aqueous extracts. This is probably due to the fact that many polyphenol compounds are nonpolar and are therefore more soluble in nonpolar solvents (Brglez et al., 2016). Results from the determination of total flavonoid content indicated that the petroleum ether rind extract contained the highest total flavonoids followed by the ethyl acetate rind extract, and then the ethanolic rind extract (figure 4.3b). Generally, the rind extracts contained the highest amounts of flavonoids. This may be attributed to a relatively high total phenolic in the rind extracts. However, the petroleum ether rind extract contained a lower quantity of flavonoids, suggesting that there may be other inherent non-flavonoid polyphenol compounds which also dissolve in petroleum ether. The aqueous extracts had the least flavonoid content, which is also consistent with results from the determination of the total phenolic content. A study involving extraction methods of citrus peel phenolic compounds showed that flavonoids are very characteristic of citrus fruits and are found to be more highly concentrated in the peel compared to other parts of the fruit, naming neoerioicitrin and hesperidin as some of the flavonoids found in Citrus limon peels (M’hiri, Ioannou, Ghoul, & Boudhrioua, 2014).

The ability of the Citrus limon extracts to scavenge free radicals was also determined and the results showed that the scavenging ability of the extracts increased in a concentration-dependent manner. The EC50 refers to concentration required to obtain a 50% antioxidant activity or free radical inhibition (Chen, Bertin, & Froldi, 2013), therefore, lower EC50 values imply higher antioxidant activity. In this study, the Citrus limon rind extracts showed the highest antioxidant activity as their relatively small EC50 values showed. The ethyl acetate rind extract had the least EC50 value (0.4 ± 0.02) followed by the ethanolic seed extract (1.0 ± 0.1), the aqueous rind extract (1.1 ± 0.1), the ethanolic rind extract (1.3 ± 0.2), the petroleum ether rind extract (1.4 ± 0.1) and
the ethanolic pulp extract (1.4 ± 0.2). The reason for the observed high antioxidant activity in the ethyl acetate, petroleum ether and ethanolic rind extracts could be due to their high polyphenol and flavonoid contents. The ethanolic seed, aqueous rind and ethanolic pulp extracts, however, showed relatively little phenolic and flavonoid content, suggesting that their observed antioxidant activity could be as a result of the action of other phytochemicals with antioxidant properties.

In a study to evaluate the phytochemical components of *Citrus limon* pulp and peels, the ethanolic pulp extract was shown to contain a number of phytochemicals including fixed oils, reducing sugar, cardiac glycosides, steroids and phytosterols, and a strong absence of phenols (Mathew, Jatawa, & Tiwari, 2012). This is consistent with findings from this study where the ethanolic pulp extract showed high antioxidant activity but little total phenolic content. In a study to investigate the phytochemical and antioxidant content of citrus seed oils, lemon seeds showed good antioxidant activity, which had a significant correlation to their unsaturated fatty acid and tocopherol content (Malacrida, Kimura, & Jorge, 2012). These results are consistent with the findings of this study, where the ethanolic seed extract showed some significant amounts of phenols and flavonoids as well as a high antioxidant activity suggesting the action of other phytochemicals with antioxidant properties apart from phenols and flavonoids.

The results of this study also show a generally low phenolic, flavonoid and antioxidant content in the *Citrus limon* juice extracts which is consistent with the findings of a study by Hajimahmoodi *et al.* (2012), who reported that *Citrus limon* juice has a low phenolic content. It however contradicts a study which revealed high antioxidant activity in both unripe and ripe *C. limon* juice (Kumari, Sarmah, & Handique, 2014). This difference could possibly be as a result of differences in the solvents that were used for extraction.
Increased mortality associated with cancer, along with high treatment cost calls for continuous research into possible anticancer drugs that are more suitable for treating cancer with reduced side effects. Secondary metabolites such as phenols, flavonoids, alkaloids and tannins have been shown to possess anticancer properties (Kooti et al., 2017). The anticancer properties of the petroleum ether, ethyl acetate, ethanolic and aqueous extracts of lemon against human cancer cell lines MCF-7 (breast) and PC3 (prostate), and normal prostate cell lines (PNT2) were investigated. The cytotoxic effects shown by the extracts on the cell lines were observed to be in concentration-dependent pattern. The results of this study revealed that the petroleum ether rind extract was the most cytotoxic to cancer cells having the lowest IC\textsubscript{50} values in both breast (384.6 ± 17.1) and prostate cancer cells (61.0 ± 0.9). This was followed by the ethyl acetate rind extract which also had relatively low IC\textsubscript{50} values in breast (653.6 ± 33.3) and prostate cancer cells (99.1 ± 8.4). The cytotoxicity of the rind extracts to MCF-7 cells is consistent with the findings of a study by Wang et al. (2014), which showed that the citrus rind polymethoxyflavone, hesperetin was responsible for the inhibition of growth of aromatase-expressing MCF-7 tumors in ovariectomized athymic mice by reducing cyclin D1, CDK4, and Bcl-x(L). In addition, consistent with the findings of this study, is a report by Rawson, Ho and Li (2014), who observed that citrus peel extracts caused inhibition of PC3 xenograft tumor growth in immune-deficient mice even at lower dose. The IC\textsubscript{50} values of the petroleum ether and ethyl acetate rind extracts in prostate and breast cancer cells, respectively, when compared to the standard curcumin, were however not significant. The extracts that showed significant cytotoxicity to breast cancer cells when compared to the standard were petroleum ether seed and rind extracts. Those that showed significant cytotoxicity to prostate cancer cells when compared to the standard were ethyl acetate and ethanolic rind, petroleum ether and ethyl acetate pulp, and ethyl acetate and ethanolic juice extracts. The observed cytotoxicity of
the ethyl acetate and petroleum ether rind extracts could be due to the fact that they showed generally high total phenolic, flavonoid and antioxidant content.

In spite of the observed cytotoxicity to cancer cells, petroleum ether and ethyl acetate rind extracts also exhibited significant cytotoxicity to normal prostate cells with IC$_{50}$ values of 363.6 ± 27.0 and 366.6 ± 22.5, respectively. Petroleum ether seed, ethyl acetate seed, pulp and juice extracts also showed significant cytotoxicity to the normal prostate cells.

Selectivity index demonstrates the differential activity of a compound and so the greater the index value is, the better it is as a potential anticancer drug. An SI value less than 2.0 indicates general toxicity of the compound (Badisa, Ayuk-Takem, Ikediobi, & Walker, 2006). The results of this study showed the petroleum ether rind extract to have the highest selectivity index for the prostate cancer cell line PC3 (5.96) followed by the ethyl acetate rind extract (3.7). All other extracts showed little or no selectivity to the prostate cancer cells. For MCF-7, none of the extracts showed good selectivity, as all the SI values recorded were less than 2. The observed cytotoxicity, yet little selectivity to breast cancer cells due to significant cytotoxicity to normal cells could be as a result of trace amounts of organic solvents in the extracts. Jamalzadeh et al. (2016) reported that though useful in increasing solubility, some organic solvents may have some cytotoxic interference in cellular based assays. Furthermore, lesser selectivity to MCF-7 compared to PC3 cells could be attributed to a generally lower cytotoxicity to the breast cancer cells. This could be as a result of the fact that MCF-7 is a hormone-dependent cell line while PC3 is not. Certain phytohormones have been shown to promote growth of hormone-dependent cancer cells. A study by Kwon (2014) showed that the phytoestrogen Genistein stimulated the growth of estrogen-dependent MCF-7 cells at low doses in vitro with no such effect on hormone-independent breast cancer cells MDA-MB-231. Ganna et al. (2017) reported naringenin, which is found in some citrus fruits and is a precursor
to genistein, to be capable of exhibiting some protumor activity. However, a study by Zhang et al. (2016) suggested that naringenin is able to prevent growth of breast cancer cells by inhibiting the secretion of Transforming Growth Factor TGF-β1, which is a protein responsible for cell growth and proliferation.

Overall, this study has shown that a number of *Citrus limon* components possess anticancer activity. This property however resides mostly in the ethyl acetate and petroleum ether rind extracts.
CHAPTER SIX

6.0 CONCLUSION, LIMITATIONS AND RECOMMENDATIONS

6.1 CONCLUSION

The findings of this study show that the total phenolic contents differ in different parts of *Citrus limon* and the nonpolar extracts contain more phenols and flavonoids than the aqueous extracts. Ethyl acetate and petroleum ether were found to be good solvents for extraction of bioactive components compared to water. The study revealed that antioxidant activity in *Citrus limon* is highest in the rind and is attributable to phenols and flavonoids. The study also demonstrated that the anticancer activity of lemon is more pronounced in the rind, especially in the ethyl acetate and petroleum ether rind extracts. These extracts showed good selectivity towards prostate cancer cells (PC3) making them suitable as potential chemotherapy agents.

6.2 LIMITATIONS

A challenge encountered during the course of this study was the very little yield of the petroleum ether juice extract, making it insufficient for use in the assays performed.

6.3 RECOMMENDATIONS

The bioactive compounds responsible for the antioxidant and anticancer properties in *Citrus limon* parts should be isolated and characterized. The specific anticancer mechanisms of the bioactive compounds in the lemon extracts should also be identified. The apoptotic potential of the bioactive extracts should particularly be determined due to apoptosis being a preferred mechanism of action. This study should be repeated using different solvents to determine whether other bioactive compounds can be extracted, isolated and characterized. Further studies should also involve the
use of different cells lines taking into account hormone-dependent properties. The experiments
could also be repeated and rat liver homogenates could be added to the cultures for metabolism of
components of the extracts since the extracts may be acting in a manner similar to prodrugs which
must be biotransformed *in vivo* or by adding enzymes before significant activity may be observed.
Thus, subsequent studies should involve *in vivo* animal experiments and ultimately clinical trials.
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APPENDIX

Figure A1: Gallic acid standard curve

Figure A2: Quercetin standard curve