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Design and *In vitro* Analysis of Polyethylene Glycol based multidrug delivery systems for combination therapy in the treatment of Breast Cancer

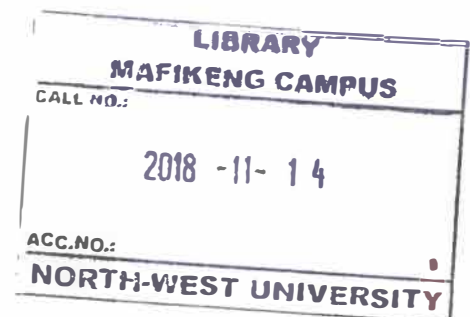
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Master of Science in Biology at the
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DECLARATION BY CANDIDATE

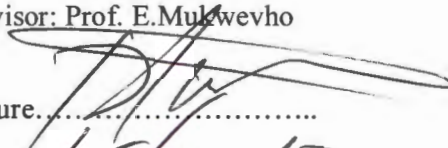
I hereby declare that this dissertation submitted for the degree of MSc in Biology, at the North-West University, is my own original work, and has not previously been submitted to any other institution of higher education. I further declare that all sources cited or quoted are indicated and appropriately acknowledged by means of a comprehensive list of references.

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DEDICATION

This study is dedicated to my loving parents, Professor E.R Sadiku and Mrs. V.A Fasiku, my siblings; Ranti, Kemi, Tola, Morayo, and finally my friends for their unreserved love, prayers and support.

My profound gratitude also goes to Shesan Owonubi for his encouragement, love and support all through this study.

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ABSTRACT

Polymer hydrogels are known to be excellent drug delivery biomaterials for unconventional cancer therapy. The polymer hydrogel was prepared via the free-radical polymerization of acrylamide (AAm) in the presence of poly ethylene glycol (PEG), gum acacia and N-isopropylamide.

Swelling analysis at different pHs (1.2, 5.8 and 7.4) were performed on the hydrogels in order to determine the swelling capacity of the polymer hydrogel and characterization was done by using the Fourier transform infrared (FTIR) spectroscopy, thermogravimetric analysis (TGA), x-ray diffraction (XRD) and the scanning electron microscopy (SEM).

Hydrogels were loaded with doxorubicin (DOX) and curcumin (CUR) drugs which have been identified as anti-proliferation agents and this was done individually and in combination. The successful incorporation of these drugs onto the polymeric network of the gel was confirmed and drug-polymer interaction observed with various characterization techniques employed as exemplified in the spectra data and images obtained. Results from the drug release studies which were intended to mimic the gastrointestinal tract, tumor cells and blood reveal a successful release of drugs from the gel at pH of 1.2, 5.8 and pH 7.4, respectively. These conforms with diffusion models such as the Korsmeyer–Peppas model, suggesting that the hydrogels are potential target materials for drug delivery systems to cancerous cells. Furthermore, cytotoxicity studies and cell viability tests were performed in order to determine the anti-cancer effects of the drugs bound to the hydrogel by treating against MCF-7 adenocarcinoma breast cancer cell lines. Results obtained showed that the growth of cells was inhibited and the hydrogel loaded with the drugs proved to be an excellent targeted drug delivery system.

Keywords: Hydrogel, doxorubicin, curcumin, drug delivery

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ACRONYMS AND ABBREVIATIONS

2D/3D	Two and three dimensional
AAm	Acrylamide
AIP	Apoptosis-inducing protein
AFM	Atomic force microscopy
AML	Acute myelogenous leukemia
ATP	Adenosine triphosphate
Bax	B-cell lymphoma 2 associated X protein
Bcl-2	B-cell lymphoma 2
BCG	Bacillus Calmette-Guerin
BCNU	Bis-chloroethylnitrosourea
BMI	Body mass index
BRCA	Breast Cancer-Associated Gene 1 and 2
CAT	Catalase
CCD	Charged coupled device
CDD	Controlled drug delivery
CPT	Camptothecin
CUR	Curcumin

DCIS	Ductal carcinoma in situ
DDS	Drug delivery system
DDDS	Dual drug delivery system
DES	Diethylstilbestrol
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOX	Doxorubicin
D-C	Doxorubicin-curcumin
DSC	Differential scanning calorimetry
EDTA	Ethylenediaminetetraacetic acid
EGCG	Epigallocatechin-3-gallate
EGFR	Epidermal growth factor receptor
EMA	European medicines agency
ERCC2	Excision repair cross-complementing rodent repair deficiency
ER/PR	Estrogen and progesterone receptors
ERT	Extracellular signal regulated kinase

ERT	Estrogen replacement therapy
EZH2	Enhancer of zeste homolog 2
FDA	Food and drug administration
FTIR	Fourier transform infrared
G-phase	Growth phase
GI	Gastrointestinal
GnRH	Gonadotropin releasing hormone
GPX1	Glutathione peroxidase 1
H-C	Hydrogel-curcumin
H-D	Hydrogel-doxorubicin
H-DC	Hydrogel-doxorubicin/curcumin
HER	Human epidermal growth factor receptor
HPMC	Hydroxypropyl methyl cellulose
HRT	Hormone replacement therapy
IAR	Independent annual review
IBC	Inflammatory breast cancer
IDC	Invasive ductal carcinoma

IL-2	Interleukin-2
ILC	Invasive Lobular Carcinoma
IPN	Interpenetrating network
IR	Infrared radiation
JAMA	Journal of the American medical association
JNK	Jun N-terminal kinase
KPS	Potassium per sulfate
LHRH	Luteinizing hormone releasing hormone
LCST	Lower critical solution temperature
M-phase	Mitosis phase
Maspin	Mammary serine protease inhibitor
mRNA	messenger Ribonucleic acid
MAPK	Mitogen-activated protein kinases
MBA	Methylene bisacrylamide
MCF	Michigan cancer foundation
MDR	Multi drug resistance
MLH1	DNA mismatch repair protein

MMPs Matrix metallo proteinases

MNT Max network transcriptional repressor

MSH2 Mut S homolog 2

NADPH Nicotinamide adenine dinucleotide phosphate reduced

NCI National cancer institute

NF- κ B1 Nuclear factor kappa B subunit 1

NMR Nuclear magnetic resonance

NOS3 Nitric oxide synthase 3 (endothelial cell)

NQO1 NAD(P)H dehydrogenase quinone 1

P-27 Protein 27

P-38 Protein 38

P-53 Protein 53

PARP1 Poly (ADP-ribose) polymerase 1

PBS Phosphate buffered saline

PCBs Polychlorinated biphenyls

PCNA Proliferating cell nuclear antigen

PEG Polyethylene glycol

PK Pharmacokinetics

PkB Protein kinase B

PRL-3 Phosphatase of regenerating liver 3

RNA Ribonucleic acid

ROS Reactive oxygen species

RON Receptor d'origine

RPM Revolution per minute

S-phase Synthesis phase

s-IPN Semi- interpenetrating network

SEM Scanning electron microscopy

SERMs Selective estrogen receptor modulators

SKP 2 S-phase kinase –associated protein 2

SLC22A16 Solute carrier family 22

SM Sphingomylin

SMases Sphingomylinases

SOD1 Superoxide dismutase1

Src Sarcoma

Stat 3 Signal transducer and activator of transcription 3

TcF T-cell factor

TEM Transmission electron microscopy

TGA Thermogravimetric analysis

TNF Tumor necrosis factor

TMEDA Tetra-methylenethyldiamine

TOP 2 Topoisomerase II

TP 53 Tumor protein 53

US United States

UV Ultraviolet

VEGF Vascular Endothelial Growth Factor-A

VEGFR1 Vascular endothelial growth factor receptor-1

WHI Women's health initiative

WHO World health organization

XDH Xanthine dehydrogenase

XRD X-ray diffraction

CHAPTER ONE

INTRODUCTION AND BACKGROUND OF THE STUDY

1.1 BACKGROUND

The incorporation of drugs into carrier materials (a drug delivery system – DDS) has become a powerful methodology for treating various pathologies. These delivery systems are usually targeted drug delivery systems, since they seek to concentrate the therapeutic agents to a particular location of interest in the body and at the same time, reduce its relative concentration in other tissues or parts of the body. In this type of drug delivery system, there is usually an enhanced therapeutic index of the drug because the drug's specificity, due to targeting a particular tissue, cell or intracellular compartment, is increased. In addition, the control over the release kinetics and the protection of the active agent or a combination thereof, of both, is made possible via this form of drug delivery system. All drug delivery systems aim to deliver the incorporated drug at the right place (targeted location), at the right concentration for the right period of time and also to have the ability of administering the drug *in vivo* with little or no toxic effect. It is equally important for any developed drug delivery system to be capable of complete self-degradation of the drug in order to prevent any need for surgical removal of the drug mediated carrier device after serving its desired purpose in the body.

As a result of these necessities for any drug delivery system, discovery and development of better biomaterials with highly enhanced biodegradable, biocompatible and non-toxic properties, have become of great importance to researchers in their quest for an improved targeted drug delivery system. One of such drug carrier biomaterials that has been developed, is the polymer hydrogel. Hydrogels have so many advantages for several drug delivery applications because of their unique characteristics which make them biocompatible, resulting from their similarities to natural living

tissue. Some of these characteristics and similarities includes: softness, flexibility, high water content and their ability to absorb drugs into their webbed network structure.

The focus of this research study was to explore the use of polymer hydrogel as a carrier for multiple drugs delivery for cancer therapy, thus broadening their use in the medical field. This was done by loading hydrogel with two drugs that possess anti-cancer properties, independently and in combination thereof and subsequently *in vitro* analysis on MCF-7 human adenocarcinoma breast cancer cell lines.

In recent times, a lot of improvements have been made by researchers and the pharmaceutical industries in the area of enhanced drug delivery system, and this is greatly due to the considerations made on the diverse factors necessary for achieving a successful drug delivery system. Some of the factors are; the properties of the drug, such as solubility, prolonged duration in the body, reduced side effects (retention of drug bioactivity for the required active period in the body, toxicity etc); and the properties of the drug carrier device, which are non-toxicity, biocompatibility, biodegradability, responsiveness to stimuli and external factors (pH, temperature and its mechanical strength). The aim of developing biocompatible and biodegradable biomaterials by researchers is to enhance the exact dosage release to time ratio of drugs used in the treatment of several diseases. A number of natural materials such as gelatin, chitosan and synthetic materials such as carbon nanotube and silica nanostructure, have been used in recent times and these materials have grown in significant relevance due to the potential advantage in several cell-based therapies, cancer therapy, diabetic therapy and drug delivery (Bertholon et al., 2006, Ethirajan et al., 2008, Li et al., 2003, Ali-Boucetta et al., 2008, Ramachandran et al., 2002, Wang and Levin, 2009, Semmler-Behnke et al., 2008, Yang et al., 2014, Zhang et al., 2001)

1.2 PROBLEM STATEMENT

Breast cancer has become a major problem in the public health sector with about 4.4 million women living with the disease. Over 1 million new cases resulting with over 400,000 deaths are reported annually worldwide (Meade and Dowling, 2012, Parkin et al., 2005). According to Alteri et al. (2011), amongst all diseases, breast cancer is the most frequently diagnosed all over the world, and the scourge accounts for the main cause of death in women who have cancer. In addition, it is responsible for about 14 percent of cancer deaths. Sex, age, race, genetic predisposition, and exposure to environmental carcinogens are multiple factors responsible for the prevalence, geographic distribution and behaviour of particular cancer types (Dumitrescu and Cotarla, 2005). Researches on drug delivery systems and therapeutic agents used in breast cancer treatment have been ongoing for over two decades as an endeavour to improve the overall curative effect of drugs (Fundueanu et al., 2008, Huynh et al., 2008, Wang et al., 2008, Nakamura et al., 1999).

Lately, novel drug delivery systems and combination therapy, which is basically the use of two or more drugs with different curative capacities and abilities have proven to be a productive method in the treatment of diseases. Due to the challenges and limitations faced when targeting originative substances that cause the occurrence of the disease by using one drug, a combination of individual drugs is now dispensed maximally and at different or the same stages of the treatment for optimum overall effects of drugs (Sharan et al., 1997, Wei et al., 2009b). Nevertheless, some challenges have also been associated with combination therapy. The major challenges include drug loading and drug ratio, characterization, development of suitable carriers, inability to control the entire process and the dependent release behaviour of each drug (Wei et al., 2009b, Greco and Vicent, 2009). Due to these and other challenges, dual drug delivery systems that are capable of

overcoming these challenges, are being explored. In recent times, positive outcomes have emerged from limited research that were carried out on dual drug delivery systems (Manna and Patil, 2009, Ma et al., 2010, Qiu and Bae, 2007, Song et al., 2008, Xia et al., 2008, Lee et al., 2008b, Baumann et al., 2009).

The purpose of this project is, therefore, to design a polymer (hydrogel) whose stimuli response can be tapped into and utilized as a drug delivery carrier device in order to provide potential cure to breast cancer. In addition, the project aims to compare the effects of the anticancer drugs individually and in combination when loaded onto the polymer (hydrogel) substrate. This is done in an attempt to proffer a solution to the persistent challenge of combination therapy faced by earlier researchers, especially the inability of the drug carrier device to degrade before the depletion of the administered drug.

Drugs such as doxorubicin (DOX) which belong to a class of drugs known as anthracycline which possess anti-tumor, antibiotic properties, hence its medical application is seen in cancer treatment. DOX, amongst other antitumor drugs that have ever been developed, stands out as the most effective and widely used because of its high antineoplastic activity to breast cancer, soft tissue sarcomas, destructive lymphomas and childhood solid tumours. However, it is noteworthy that the drug has certain drawbacks in its use as an anticancer drug. The major limitation it possesses is its diverse toxic effects, in addition to the fact that breast cancer cells have become resistant to the drug, hence it has become of great necessity to have further studies into the drug in order to develop ways to overcome this challenge and make the drug more effective (Minotti et al., 2004, Quiles et al., 2006, Volkova and Russell, 2011). A few other drugs with anticancer activity, such as curcumin, have also been well researched independently and in combination with other drugs with the aims and hopes to have better effects on breast cancer cells.

1.3. RESEARCH QUESTIONS

The following are the crucial research questions this project aims to answer:

- i. What are the effects of loading curcumin and doxorubicin (individually) and both drugs onto a PEG based hydrogel on the release mechanisms of the drugs from the hydrogel?
- ii. What are the effects of PEG-based hydrogel loaded with both drugs on breast cancer cell lines?
- iii. Does the presence of these drugs in the PEG based hydrogel enhance their targeted release profiles?

1.4. AIM OF RESEARCH

To design a PEG-based hydrogel for dual delivery of anti-cancer drugs with enhanced therapeutic effect.

1.5 OBJECTIVES

The major objectives are:

- ❖ To design and develop a PEG based drug delivery carrier with remarkable physicochemical properties.
 - i To analyse the pH-dependent swelling properties of the hydrogel; and
 - ii To characterize the PEG based hydrogel by using Fourier transform infrared spectroscopy (FTIR), X-ray diffraction (XRD), Thermogravimetric analysis (TGA) and scanning electron microscope (SEM).
- ❖ To load therapeutic agents: curcumin and doxorubicin (independently and in combination thereof) onto the PEG based hydrogel.

i Characterization of the PEG based hydrogel loaded with the therapeutic agents

- ❖ To perform drug release kinetics of the hydrogel containing curcumin, doxorubicin and the combination of both drugs.
- ❖ To perform *In vitro* analysis of the polymer hydrogel loaded with bioactive agents on MCF-7 human adenocarcinoma breast cancer cell lines.

1.6 HYPOTHESIS

It is hypothesized that the hydrogel, loaded with the drugs will exhibit targeted drug release mechanisms with enhanced cytotoxicological effect on the cancer cell lines when compared to the free drugs.

1.7 SCOPE AND LIMITATIONS

The study observed the similarities and dissimilarities between the effects of both drugs loaded onto the hydrogel, with the free drugs in breast cancer treatment. In this study, the free drugs doxorubicin and curcumin were used as the positive control, with the aim to compare the effect of combinational therapy of the free drugs with hydrogel loaded with the drugs. This investigation involved cytotoxicity evaluation on breast cancer cell lines in order to affirm a successful anti-cancer effect in terms of the delivery device used.

1.8. SIGNIFICANCE OF THE STUDY

The study aims to develop a drug delivery device that is capable of proffering a potential solution to breast cancer patients by using combination therapy. It is expected that this research will provide an up to date knowledge and insight about combination therapy and dual drug delivery by using polymeric hydrogel as a substrate (carrier) in treatment of breast cancer. The study hopes to add to the available knowledge in this field, thus leading to possible further research. Also, the outcome

of this study is expected to enlighten the public and society at large on the efficacies of these drugs when bound to the polymer (hydrogel) developed and used in combination therapy as a comparative study on the free drugs individually and in combination. Finally, this study is expected to reduce the challenges faced by breast cancer patients, most especially the need to have oral drugs intake at intervals.

CHAPTER TWO

LITERATURE REVIEW

2.1. INTRODUCTION

The focus of this chapter is on the review of relevant literature on cancer, introduction to cancer, types of cancer, risk factors related to cancer, world statistics on cancer and treatment, however emphasis will be on breast cancer, since breast cancer is the case study of this research. Drug delivery systems (DDS): various types of drug delivery systems, importance and advantages of drug delivery systems, challenges and drawbacks of the systems will also be reviewed. Furthermore, hydrogels and hydrogel design as well as its role and significance in drug delivery system and combination therapy will also be reviewed and discussed in details.

2.2 NUCLEUS OF THE HUMAN CELL- CRITICAL SITE FOR CANCER DEVELOPMENT

The nucleus of the human cell is a conspicuous structure and it is of crucial significance because the genetic material (Deoxyribonucleic acid, DNA), which is responsible for governing the characteristics of the cell and its metabolic functioning, is stored in the nucleus of the cell. Although a glance at the nucleus even with the use of an electron micrograph will not reveal the DNA molecule, and all that is seen is a thread-like material known as the chromatin which consists of the DNA molecule. It is this chromatin that coils to form the rod-like structures before cell division takes place (Loewy and Siekevitz, 1969). Inherited traits and characteristics, such as the eye colour and skin colour are encrypted in the DNA, thus making it a vital part of all cells and for life (Bruce, 2002).

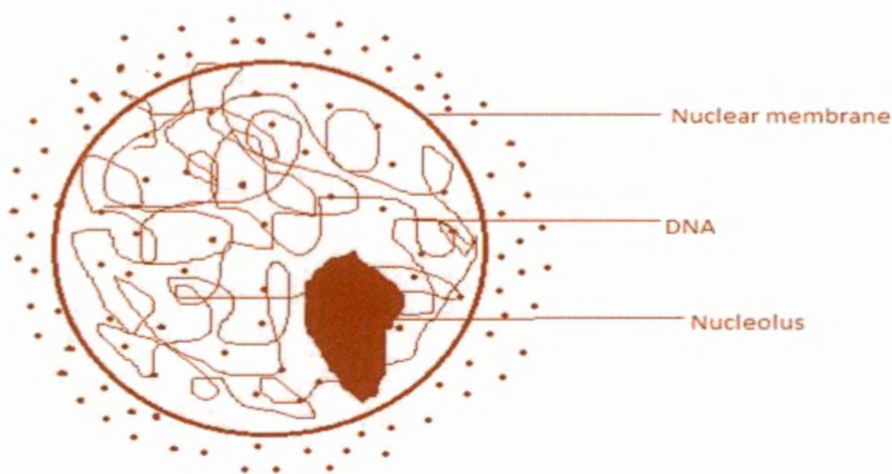


Figure 2.1: A figure showing the nucleus of the human cell

The nucleus serves as the control centre of every living cell, since it maintains the integrity of genes and it controls the cell activities, such as: cell growth, cell division, protein synthesis and it is also involved in regulating gene expression, (Lamond and Earnshaw, 1998). Any alterations in the DNA stored in the nucleus can lead to cells producing the wrong amount of certain protein or make a misshapen protein that does not function properly and because many proteins controls cell behaviour, these changes could cause health problems. In cancer, these changes causes survival of bad cells and uncontrolled growth of the cells thereby causing damage to the surrounding tissues (Bahcall, 2013).

Normal cells become cancerous as a result of gene mutations and most times, several mutations must take place for this to occur. Mutation is known to affect tumour suppressor genes that are responsible for the control of cell growth and division and also genes of normal cells, causing them to become oncogenes (cancer causing genes) (Fearon and Bommer, 2008). An inherited abnormal copy of a gene, makes the buildup of mutations more rapid and with ease; and hence, causing a cell to become cancerous at a faster rate when compared to an abnormal copy of gene that was not

inherited. This accounts for the tendency of inherited cancer to happen earlier in life than cancer that was not inherited (Berger and Pandolfi, 2011). Although persons without inherited cancer gene may still acquire cancer as a result of gene mutation during their life time and in most cases, these acquired mutations may be caused by exposure to cigarette smoke, radiation, hormones and certain diets amongst other environmental factors. Some other causes of mutations have been associated to obscure reasons which seem to be a random occurrence during cell division. There is always a chance for mutation to occur every time cell division takes place; therefore, there is a higher risk of developing cancer as people grow older in life (Ringer and Schnipper, 2001).

2.2.1 CELL DIVISION AND CELL CYCLE

Cell division refers to a series of activities that give rise to 'daughter' cells from a parent cell; and it is a process that occurs as part of the cell cycle. All cell divisions come before a single round of DNA replication, with the aim to maintain the original genome of the cell (Martin and Hine, 2015). For every cell in the human body to divide and multiply, it passes through various stages of cell division. This entire process is often referred to as the cell cycle and it consists of the following phases:

- Interphase (Intermitosis)
 - ✓ G1 phase (Growth): At this stage, nothing conspicuous is happening in the cell's nucleus, however the cells are very active. Growth, development and preparation for cell division take place.
 - ✓ S phase (DNA replication): S represents synthesis and at this stage DNA copying and replication occur.
 - ✓ G2 phase (Growth): Just as in the case of G1, although there is no obvious activity in the cell's nucleus, the cells are active.

- Mitotic phase (Chromosome separation): In here, cell division occurs. Cells are divided into two 'daughter' cells at this point in the cell cycle (Vajpayee, 2014, Cooper, 2000, Wang and Levin, 2009).

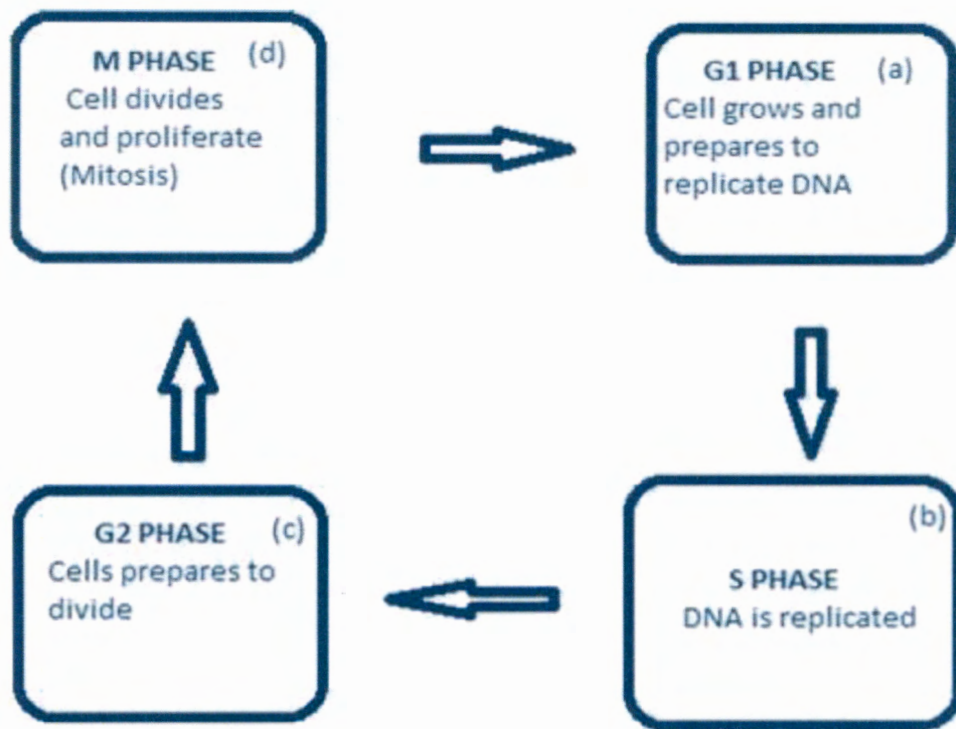


Figure 2.2: A figure showing the various phases of the cell cycle

2.2.2 CELL DIVISION IN NORMAL CELL AND CANCER CELL

- **Cell Division Control:** In normal cells, cell division occurs normally and under favourable environmental conditions and with the existence of appropriate mechanisms for proper DNA replication. Cell division termination, which is a control mechanisms, sets in when there is an unfavourable condition or genetic damage. However, in the case of cancer cells, cell division continues amid inappropriate and unfavourable conditions. In addition, because cancer cells do not stop dividing when genetic damage occurs, the resultant

‘daughter’ cell will therefore contain abnormal number of chromosomes and DNA which are more abnormal than the ‘parent’ cells and continuous division will hence, lead to tumour formation. Furthermore, in normal cells errors, encountered in the course of cell replication are correctable, but not so in cancer cell (Weinberg, 2013, Tsaniras et al., 2014).

- **Cell Division Signaling:** It has been observed in normal cells that although active cell division is not on going, cells still perform their normal functions and these cells divide in response to external signals in the form of proteins or steroid growth factor, however, cancer cells act contrary to all of these. Although, at times, normal cells may stop dividing for some reasons, such as: the absence of external signals, pre-programmed limit to number of times for cell division (Urry et al., 2016). For example, breast cancer cells grow without the need for estrogen which is the normal growth factor. This happens when the ability to respond to estrogen is lost as a result of the turning-off of the estrogen receptor within the cell. In addition, inhibition contact, which is the ability for cells to detect when there is ‘crowding’ by nearby cell, is not exhibited by cancer cells. They do not respond to stop the signal anymore and hence, there is the continual growth leading to cells heaping-up and eventually, tumour mass is formed (Cooper, 2000, Wang and Levin, 2009).

2.3 OVERVIEW OF CANCER

Cancer is a disease that arises when there is an alteration in the processes and mechanisms that guide or control proliferation and differentiation of cells. This alteration leads to growth, excessive proliferation and transformation of abnormal cells into tumours that are capable of invading and compressing adjacent normal structures, such as tissues (Seyfried and Shelton, 2010). The growth of these abnormal cells is a characteristic of the disease condition. These cells express different cell surface antigens, which may either show signs of apparent immaturity, qualitative or

quantitative chromosomal anomalies. In addition, various translocations and appearances of amplified gene sequences may be displayed. A little portion of these cells are the tumour stem cells which are capable of proliferating repeatedly and then travel through a metastatic process to distant parts in the body. Such part of the body (organs) are colonized by them (tumour stem cells) and subsequently, a colony is formed. This neoplastic progression is usually followed by quantitative abnormalities in various metabolic pathways and cellular components. Death is often the result of this invasive and metastatic process, if untreated or poorly managed (Katzung et al., 2011).

Cancer is a disease known to be a principal cause of death globally, and it is responsible for ~7.4 million deaths, annually. In low and middle-income countries, it is recorded that more than 70% of all death that occurs is caused by cancer. In 2009, cancer death was predicted, by World Health Organization to rise continuously with a projected deaths of 11.5 million by the year 2030. (Ta et al., 2008). Of all the risk factors associated with cancer, environmental exposure and chemical carcinogens (particularly those in tobacco smoke), dyes, aflatoxins, asbestos, and benzene, have been clearly discovered to induce cancer in humans and animals (Katzung et al., 2011).

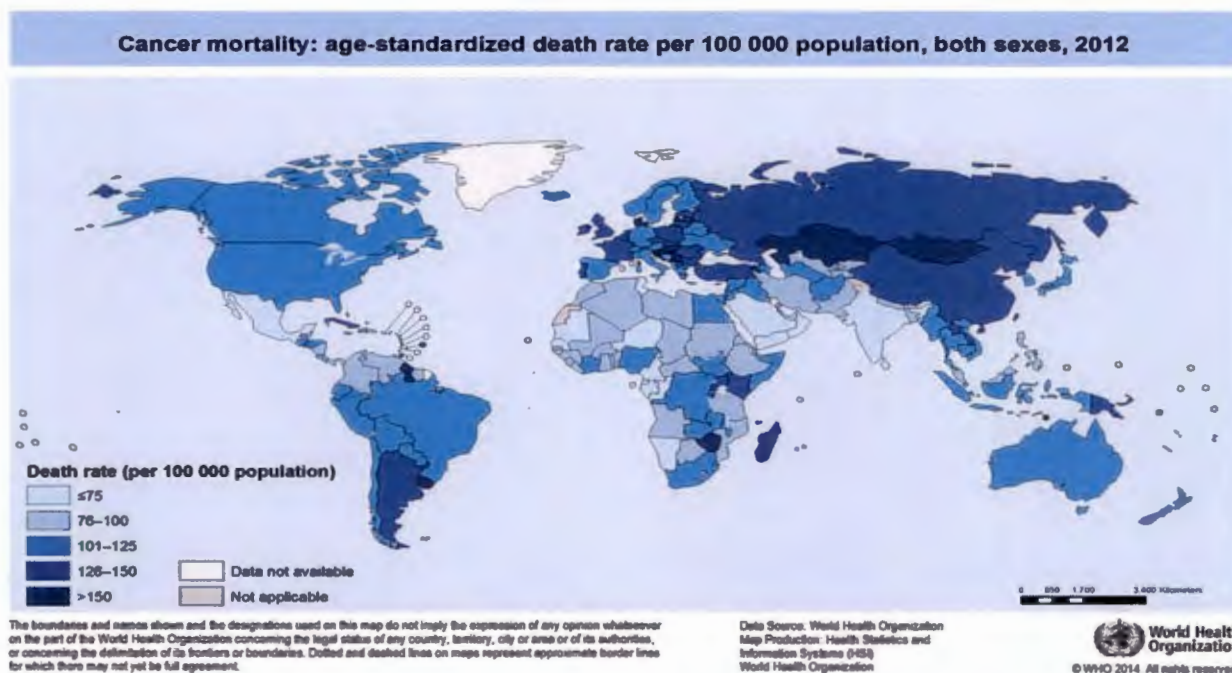


Figure 2.3: A figure showing the statistical indication of the number of individuals living with cancer in the world (World Health Organization, 2014)

2.3.1 TYPES OF CANCER

Various forms of cancers exist and they arise from tumours (lumps), which can either be benign or malignant. Benign tumours are cells that grow at a slow rate and do not spread to other body parts; they are like normal healthy cells in the body, not cancerous and are rarely life threatening. They only turn out to be a problem if they become very large, thus causing discomfort and press on other organs – a typical example is the brain tumour inside the skull. In naming benign tumours, a suffix *-oma* and the name of the affected organ are usually used. An example is leiomyoma, a benign tumour of smooth muscle cell, having its common name as fibroid and it occurs in the uterus (Santen and Mansel, 2005, Schnitt and Connolly, 2004).

On the other hand, malignant tumours grow very fast when compared to benign tumours and they are capable of spreading to tissues close-by and causing destruction to such tissues. Through a process known as metastasis, malignant tumours break away from the main tumour and spread to healthy tissues in other body parts where they undergo continuous cell division and growth. The new secondary site where the metastasis occurs is referred to as metastases, and the disease condition is called metastatic cancer (Burstein et al., 2008).

Usually, the body part where a cancerous cell develops is often used to describe the type of cancer, but because of the presence of multiple types of tissues in different parts of the body, a greater precision is required for the classification. For these reasons, cancers are further classified, based on the type of cell that the tumour cells originate from. They are classified according to the following:

- **Carcinoma:** It is a cancer type that has its origin from the epithelial cells that is, the lining of cells responsible for protecting organs. They are capable of invading the nearby tissues and organs and metastasize to the lymph nodes and other areas of the body. Common examples of this type of cancer includes breast, prostate, lung and colon cancer and they occur particularly in adults (Berman, 2004, Kuriakose et al., 2001, Chang et al., 2007).
- **Sarcoma:** This type of cancer originates from the connective tissues (i.e. muscle, blood vessels bone, cartilage, fat, nerve) and each arises from cells originating in the mesenchymal cells outside the bone marrow. Leiomyosarcoma, liposarcoma and osteosarcoma are common forms of this kind of cancer (Tran et al., 2005, Longhi et al., 2006, Fletcher et al., 2002).
- **Lymphoma:** This type of cancer can occur anywhere in the body because it is a cancer of the lymphatic system, which runs all through the entire body. Hodgkin's lymphoma and

non-Hodgkin's are the two main forms of this cancer type. Non-Hodgkin's is initiated by the unrestrained growth of the white blood cells lymphocytes of the immune system and Hodgkin's lymphoma occurs when the cells of the lymph nodes become cancerous (Bernstein and Burack, 2009, Solimini et al., 2016, Tran et al., 2005).

- **Leukemia:** Leukemia is a cancer associated with the white blood cells and bone marrow, the tissues that forms blood cells. Lymphocytic leukemia and chronic lymphocytic leukemia are common subtypes of this type of cancer amongst several others. Both subtypes of this cancer originate from blood making cells. It is the most common type of cancer in children accounting for about 30% of cancer cases in children (Varricchio, 2004). However, far more adults develop lymphoma and leukemia (Colvin and Elfenbein, 2003, Patlak, 2002, Dearden et al., 2001).
- **Germ cell tumour:** This cancer is known to arise from pluripotent cells. It is most often present in the testicle (seminoma) or the ovary (dysgerminoma) (Ulbright, 2005, Verville and Bozzone, 2009).
- **Blastoma:** It is a cancer that is more common in children when compared to adult and it is derived from immature "precursor" cells or embryonic tissue (Alberts et al., 2008).

Most commonly, suffixes such as *-carcinoma*, *-sarcoma* or *-blastoma* are usually used to name cancers in combination with the root word of the organ or tissue which is often in Latin or Greek. Examples includes: hepatocarcinoma (cancer of the liver parenchyma), hepatoblastoma (arises from primitive liver precursor), and liposarcoma (cancer arising from fat cells). However, in some cases the root word is English, a typical example is the breast cancer, which is called ductal (originates from milk duct) carcinoma of the breast. Also, the shapes and sizes of certain cells

under the microscope are used to name certain cancer types. For example, giant cell carcinoma, spindle cell carcinoma, and small-cell carcinoma (Rethinavelsubramanian and Suganya, 2014).

2.3.2 DRUGS USED IN CANCER TREATMENT

From previous discussions, it is clear that the pathogenesis of cancer is closely related to impaired pathway of cell division. The mechanism of action of most anticancer drugs is such that it blocks one or more stages of cell cycle, thus blocking cell division in rapidly growing cells (Urry et al., 2016). A lot of chemotherapeutic drugs used in the treatment of cancer are specifically designed to inhibit the fusion of precursor molecules that are needed for DNA replication; and prevent the cell from completing the 'S' phase of the cell cycle, and in some cases cause extensive DNA damage, which halts replication process. During the mitosis phase in the cell cycle, it is often required that the chromosome separates and for this to occur, spindle fibers made of microtubules are needed; however, a class of drugs referred to as spindle inhibitors stop microtubules from being synthesized (Beckers and Mahboobi, 2003, Sarabia et al., 2006). Cancer cells are very sensitive to these drugs than normal cells because cell division does not occur often in adult cells. However, notable side effects such as gastrointestinal discomfort, loss of hair and low white blood cell count are experienced with the use of these chemotherapy drugs. This is because they tend to kill some adult cells, such as those that line the gastrointestinal tract, bone marrow cells, and hair follicles undergoing rapid cell division.

2.3.3 TYPES OF CHEMOTHERAPY DRUGS

A number of drugs are used for chemotherapy. They are divided into different groups, based on a couple of factors, such as their mechanism of action, their chemical structure, and their relationship to another drug. Some drugs have more than one mechanism of action, thus belonging to more than a group. A good knowledge of the mechanism of action of these drugs improves the

therapeutic effect(s), since this helps in making decisions about how to combine a drug with another, when each drug should be administered (order and frequency) and also predicting the side effects of the drug individually and in combination, in cases where more than a drug would be used.

Alkylating agents: These drugs act by directly damaging the DNA (the genetic material in each cell) in order to prevent cell reproduction. This takes place in all phases of the cell cycle and they are used in treating various types of cancers. Examples of the cancer types that can be treated with alkylating agents, includes: leukemia, lymphoma, breast and ovary, lung, Hodgkin disease, multiple myeloma and sarcoma (Słoczyńska et al., 2014). As a result of the damage on the DNA, a long-term damage to the bone marrow may occur, which can in turn, result to acute leukemia in rare cases because it is “dose-dependent”. This means that the lower the doses, the smaller the risk but an increase in the dose leads to increased risk. After 5 to 10 years of treating cancer with these alkylating agents, the risk of developing leukemia often increases. These alkylating agents are classified into the following different classes: nitrogen mustards (e.g chlorambucil, and melphalan), nitrosoureas (e.g streptozocin, and lomustine) and alkyl sulfonates (e.g busulfan), triazines (e.g dacarbazine and temozolomide), ethylenimines (e.g thiotepa and altretamine) (Ralhan and Kaur, 2007, Colvin, 2003).

Due to the similar manner in which platinum drugs (such as: cisplatin, carboplatin, and oxaloplatin) kill cells, they are grouped with alkylating agents, at times. Although, the likelihood of these platinum drugs to cause leukemia later in life is less than that of the alkylating agents (Tew et al., 1996).

Differentiating agents: These drugs focus on cancer cells by causing them to grow into normal cells. Examples include: the retinoids, tretinoin, with their trade names known as ATRA or Atralin®, bexarotene, with its trade name known as Targretin®, as well as arsenic trioxide, with its trade name known as Arsenox® (Sell, 2004, Nowak et al., 2009, Leszczyniecka et al., 2001).

Antimetabolites: Antimetabolites are therapeutic agents that cause cell damage during the S phase of the cell cycle, during the process of cell copying. The destruction to the cell is achieved by replacing the normal building blocks of RNA and DNA, thereby interfering with the growth of RNA and DNA. Some of the cancer types which these antimetabolites are used to treat, include: leukemias, cancers of the breast, ovary, and the intestinal tract (Peters et al., 2000, Takimoto and Calvo, 2008). Examples of these antimetabolites are 5-fluorouracil, 6-mercaptopurine, capecitabine, cytarabine, floxuridine, fludarabine, gemcitabine, hydroxyurea and methotrexate.

Anti-tumor antibiotics: The mode of action of these drugs is not the same with the regular antibiotics used to treat infections. Anti-tumor antibiotics work by altering the DNA found in cancer cells so as to prevent them from replicating. A representative of this type of drug is the Anthracyclines that obstructs enzymes involved in DNA replication and these drugs work in all phases of the cell cycle. They are used widely for treating different cancers. Examples of this group of drugs are: daunorubicin, doxorubicin, epirubicin and idarubicin.

Permanent heart damage has been discovered to be a major side effect of these drugs, if administered in high doses. Hence, it is important to limit drug dose. Examples of other drugs that are not anthracyclines, but also are anti-tumor antibiotics include: actinomycin-D, bleomycin, mitomycin-C and mitoxantrone (also acts as a topoisomerase II inhibitor) (Salmon and Sartorelli, 2001, Cortés-Funes and Coronado, 2007, Sparreboom et al., 2002).

Topoisomerase inhibitors: As the name implies, these drugs inhibit enzymes (proteins that brings about chemical reaction in living cells) called topoisomerases which are involved in helping DNA strand separation and thus stopping the DNA from being copied during the S phase. Examples of the cancers that can be treated with topoisomerase inhibitors are: some leukemias, also cancer of the lung, ovary and gastrointestinal cancers. The grouping of these drugs is based on the enzyme type that they affect. Examples of the drugs that inhibits topoisomerase-I include: topotecan, irinotecan while those that inhibit topoisomerase-II include: etoposide, teniposide and mitoxantrone (also acts as an anti-tumor antibiotic). However, the risk of developing a second cancer; acute myelogenous leukemia (AML) after 2 or 3 years is increased with the use of topoisomerase-II inhibitors (Pommier, 2006, Pommier et al., 2010, Staker et al., 2005, Teicher, 2008).

Mitotic inhibitors: Mitotic inhibitors are compounds obtained from natural products and plant alkaloids. They act by preventing mitosis in the M phase of the cell cycle, but in the process, they can cause harm to normal cells in all phases, by inhibiting enzymes that are necessary for the synthesis of proteins needed for cell reproduction. Cancer of the breast, lung and myelomas are a few of the different cancers that these drugs have being used to treat and their side effect, is nerve damage, therefore, dose given is always limited. Examples of the drugs that fall in this category include: taxanes (e.g paclitaxel and docetaxel), epothilones (e.g ixabepilone), vinca alkaloids (e.g vinblastine, vincristine and vinorelbine) and estramustine (Lyseng-Williamson and Fenton, 2005, Clarke and Rivory, 1999, Jiang et al., 2006)

Corticosteroids: Corticosteroids are drugs considered as chemotherapeutics when used in combination with other therapeutics for cancer treatment and they are often referred to as steroids. They are natural hormones and drugs like hormones that are found to be useful in the treatment of

various types of cancer and also other illnesses. They can be used before treatment to avoid severe allergic reactions and after treatment to prevent vomiting and nausea after chemotherapy. Examples are: prednisone, methylprednisolone and dexamethasone (Rhen and Cidlowski, 2005, Banuelos et al., 2016, Nelson et al., 2003).

Hormone therapy: This category of chemotherapy drugs consist of sex hormones or hormone-like drugs that are capable of altering the mechanism of action of the male and female hormones. They act by slowing the growth of breast, prostate, and uterus cancers, which often grow in response to the body's natural sex hormones. In addition, they prevent cancerous cells from utilizing the hormone they need for growth, or by stopping the body from making the hormone and this mode of action is different from that of standard chemotherapy drugs. In some cases, the cancerous cell loses its normal responses to growth factors such as hormones (Salmon and Sartorelli, 2001, Brunton et al., 2011, DeVita et al., 2010). Hormone therapy is usually achieved with the use of drugs that disrupts the activity or synthesis of the hormone required for cell growth. Examples of such drugs include: anti-estrogens called selective estrogen receptor modulators (SERMs) (fulvestrant, tamoxifen, and toremifene), aromatase inhibitors (anastrozole and letrozole), progestins (megestrol acetate, estrogens), anti-androgens (bicalutamide, flutamide and nilutamide), gonadotropin-releasing hormone (GnRH), also known as luteinizing hormone releasing hormone (LHRH) agonists or analogs, such as leuprolide (Lupron®) and goserelin (Zoladex®). These drugs have gone through a ten-year clinical trial with about 20,000 women with the aim to investigate their effectiveness in breast cancer prevention (Bahls and Fogarty, 2002, Peng et al., 2009). In recent times, chemotherapeutic drugs that targets specific active proteins or processes in cancer cell signal transduction pathways, such as receptors, growth factors, or kinases, have also been developed (Davies et al., 2002, Veggeberg, 2002).

Immunotherapy: Recently, there has been an emergence of drugs with unique method of treatment for people with cancer. These drugs act by helping their immune systems identify and attack cancerous cells and this uniqueness in the mode of action has made them separate from chemotherapy. Immunotherapy can either be active, that is, stimulate the body's own immune system to fight the disease or passive that is; it does not depend on the body to attack the disease (Adams and Weiner, 2005, Palucka and Banchereau, 2013, Egawa, 2004, Scott et al., 2012). Examples of active immunotherapies, include: monoclonal antibody therapy such as rituximab and alemtuzumab, non-specific immunotherapies and adjuvants (other substances or cells that boost the immune response), e.g. BCG, interleukin-2 (IL-2), and interferon-alfa and immunomodulating drugs, such as thalidomide and lenalidomide.

In 2010, the first vaccine (the Provenge® vaccine for advanced prostate cancer) used to treat cancer was approved by FDA and this proves that cancer vaccines are a type of active specific immunotherapy. Presently, there are ongoing studies for the development of other vaccines that can be used for many different types of cancer (Itano et al., 2015, Yarbro et al., 2005, Hosoya and Miyagawa, 2014, Gaddis, 2004, Fuchs-Tarlovsky, 2013, Cheung-Ong et al., 2013, Armstrong et al., 2006, Baldo and Pham, 2013).

Targeted therapy: Targeted therapy is currently a major focus for many researchers. As a result of more discoveries about the inner workings of cancerous cells, researchers have developed new drugs that are now in use in the treatment of cancer. These drugs function more specifically than traditional chemotherapy drugs. This is accomplished by attacking cells that have altered the version or mutated the versions of some genes and also cells expressing excessively, a lot of copies of a certain gene. These drugs can either be used as part of the main treatment or for post-treatment

in order to prevent the reoccurrence of the disease. Examples of such drugs include: imatinib, gefitinib, sunitinib, bortezomib (Zhukov and Tjulandin, 2008, Katzel et al., 2009)

Other chemotherapy drugs: The mechanism of action of some chemotherapy drugs are somewhat different making it difficult to group them into one of the various categories of chemotherapy drugs. Good examples of such drugs include drugs like L-asparaginase, which is an enzyme and the proteasome inhibitor, bortezomib. On the other hand, some other drugs are involved in cancer treatment but they are not often referred to as chemotherapy drugs because they do not act in similar way with chemotherapy drugs. These drugs act by focusing on other properties that differentiate cancer cells from normal cells and this often make them have reduced side effects because they are targeted to affect cancer cells and not healthy cells. Many are used along with chemotherapy.

There are certain factors to be considered before any drug is chosen for cancer treatment, some of such factors includes:

- Cancer type;
- Present stage of the cancer (spread rate);
- Age of the patient;
- Overall health of the patient;
- Other serious health problems (such as heart, liver, or kidney diseases); and
- Types of past cancer treatments given.

2.4 BREAST CANCER

2.4.1 STATISTICS

According to world statistics, of all cancer cases breast cancer accounts for ~26%. It is the leading cancer that is diagnosed in women and it occurs about 100 times more in women than men, but the outcomes in men are often poorer because of late diagnosis. Of all cancer deaths recorded worldwide in 2008, about 458,503 deaths occurred due to breast cancer and ~13.7% were women (Fentiman et al., 2006, Petrocca et al., 2005, Weiss et al., 2005). At the moment, breast cancer incidences are increasing worldwide, especially in developing countries e.g. India. According to the American Society of Cancer, as at 1940, the life time risk of women developing breast cancer was ~5% or 1 in 20, but recently, it has increased to ~13% or 1 in 8 (Greenlee et al., 2001). According to the American Cancer Society in 2012, in many cases, it is unclear why women develop breast cancer because ~75% of all women with breast cancer have no risk factors, however different women have different risk factors of developing breast cancer during their life time (Alteri et al., 2011).

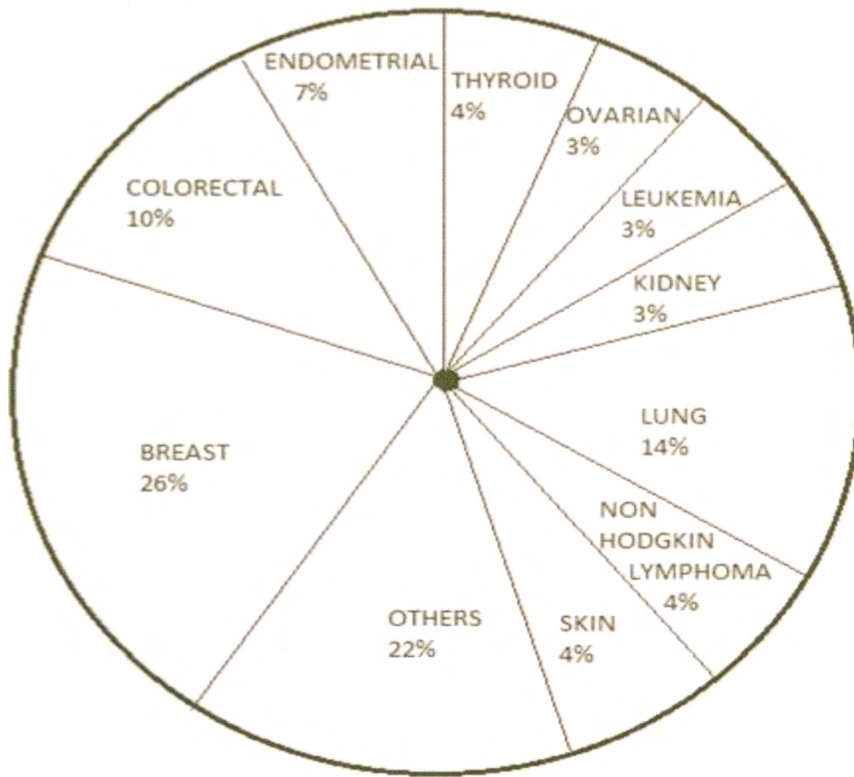


Figure 2.4: A figure showing the statistical occurrence of the various cancer types

2.4.2 BREAST CANCER RISK FACTORS

A risk factor is said to be whatever increases the possibility of developing breast cancer. Some of the most important risk factors for breast cancer are age, family history and medical history which are beyond control (non-preventable); however, some other risk factors are controllable (preventable) and they include weight, physical activities and alcohol consumption. Studies have shown that these risk factors increase the likelihood that a woman will develop breast cancer. However, the absence of breast cancer risk factors does not mean people are not susceptible to developing breast cancer (Lakshmi et al., 2012). Even though a lot of these risk factors cannot be reversed, some can however be modified.

- **PREVENTABLE RISK FACTORS**

- **OVERWEIGHT:** Overweight is generally defined as a condition where the BMI (Body Mass Index) is over 25 in the waist. At this point, there is increased risk of developing breast cancer, especially for women who have reached menopause. Before menopause, a lot of the hormones are produced by the ovaries but after menopause, estrogen is gotten from fat tissues which contain aromatase that converts androgen to estrogen. This implies that the more the fat tissues in the body, the higher the estrogen levels and this increases breast cancer risk. Being overweight also increases the risk of breast cancer reoccurrence. From studies, large tumours, great lymph node involvement and poor breast cancer prognosis with 30% higher risk of mortality are more common in obese women but then, other factors affect breast cancer and there is a complicated link between the disease condition and extra weight. For instance, an extra weight around the belly may increase the risk of developing breast cancer more when compared to extra weight of same amount around the hip or thigh (Protani et al., 2011). On the other hand, women at premenopausal stages who are overweight tend to have a lower risk of developing breast cancer, but the risk of developing breast cancer after menopause is increased by between 30-60% in overweight women when compared to normal weight women. This may be related to the manner in which estrogen is produced before and after menopause (Reeves et al., 2007, Huang et al., 1997, Nelson et al., 2012, Van Den Brandt et al., 2000).
- **DIETS:** In most cancer cases including breast cancer, diet is suspected to be a risk factor. Nevertheless, there is no known evidence that shows a relationship between breast cancer risk and diet. However, since it is suspected that diets rich in fat especially saturated fat, raises the risk, it is therefore important to avoid food that are rich in saturated fats in order

to the promote good overall healthy living (Tsubura et al., 2005). Such foods, include: red meat, food with too much cholesterol, diary fat in cheese, milk and ice cream, which may contain certain hormones, other growth factors and antibiotics and pesticides (Eliassen et al., 2012, Aune et al., 2012, Jung et al., 2013). Low fat diets-rich in fruits and vegetables, are generally recommended as a recent study suggests that risk and reoccurrence of breast cancer may be reduced significantly by eating these low fat diets (Chandran et al., 2012).

- ✓ **LACK OF EXERCISE:** It is becoming evident (from research) that physical activity reduces breast cancer risk and there is a chance of breast cancer risk reduction when exercising regularly at a moderate or intense level of between 4-7 hours per week. However, there are still arguments by various bodies about how much exercise is needed. The Women's Health Initiative recommends as little as between 1¼ to 2½ hours per week of brisk walking as this can reduce a woman's risk by ~18%, while the American Cancer Society recommends engaging in 45-60 minutes of physical exercise per day, 5 or more days a week (Eliassen et al., 2010, Wu et al., 2013, Hildebrand et al., 2013). By exercising, blood sugar limits are reduced and blood levels of insulin growth factor are controlled and a hormone that can affect how the breast cells grow and behaves. Regular exercise prevents excessive fat accumulation and this tends to reduce the risk in such persons and more likely to maintain a healthy weight when compared to people who do not exercise. Since the more the fat cells the more estrogen is produced, people who do not engage in any form of physical exercise become exposed to extra estrogen overtime and then the risk of developing breast cancer becomes higher than when they do not exercise (Caan et al., 2006).

- ✓ **ALCOHOL CONSUMPTION:** Alcohol intake is obviously related to breast cancer and several other cancers. Breast cancer risk tends to increase with the amount of alcohol consumed by women (Dumitrescu and Shields, 2005, Hamajima et al., 2002). Alcohol causes breast cancer primarily by increasing estrogen levels and limiting the ability to control the hormone in the body. In addition, alcohol can damage the cell's DNA thereby increasing breast cancer risk when compared to women who do not drink at all. For women who consume about three alcoholic drinks per week, they are at risk of breast cancer by 15% and from estimation, they are liable to an additional 10% risk increase for each additional drink each day. According to studies conducted on over one million middle aged British women, it was established that there is an increase of breast cancer cases by 11 cases per 1000 women for every additional daily alcohol intake (Room et al., 2005).
- ✓ **SMOKING:** In recent studies, it has been discovered that there is higher risk of breast cancer in women who have indulged in heavy smoking, especially those who started smoking before they had their first child; the reason being that breast tissues appear most sensitive to chemical carcinogen in this phase and that breast cells are not fully differentiated until lactation (Xue et al., 2011). This conclusion is "suggestive but not sufficient" evidence according to the 2014 US Surgeon General's report of breast cancer. Another active focus of research is whether secondhand smoke increases the risk of breast cancer, since both mainstream and second-hand smoke contain chemicals in high concentrations. In a study on rodents, it was observed that both mainstream and second-hand smoke caused breast cancer. However, this study remains unclear in a way because the link between breast cancer and smoke is unclear in second-hand smoke, but then it is said that chemicals in tobacco smoke reach breast tissue and are found in breast milk. Going

by the conclusion made by the California Environmental Protection Agency in 2005 about second-hand smoke and breast cancer, there seems to be a “consistency with a causal association” in younger, mainly pre-menopausal women hence a “suggestive but also not sufficient” evidence of a link at this point. In any case, this implies that second-hand smoke should be avoided because there is a 75% breast cancer risk increase by breathing second-hand smoke, especially in premenopausal women and young women (Sasco et al., 2003, Oze et al., 2011, Ha et al., 2007, Marcus et al., 2000).

- ✓ **ENVIRONMENTAL FACTORS:** Even though there are several uncertainties as to which environmental factors are involved in breast cancer, there is an increased evidence to support a convincing theory that states that exposure to certain man-made chemicals that can mimic hormones is the cause of breast cancer. Yet, few of the manmade chemicals in use today have gone through thorough safety and toxicity assessments (Brody and Rudel, 2003), and from findings it is now obvious that a few of these chemicals have undesirable properties. Usually, these environmental compounds act by mimicking hormones, such as estrogen or they affect the susceptibility to carcinogenesis (Gray et al., 2009). Particular focus is on those chemicals which are known to cause cancer in the mammary (breast) tissue (“mammary carcinogens”) and chemicals that can mimic estrogen. These chemicals are called hormone disruptors or “endocrine disrupting” chemicals and examples are: diethylstilbestrol (the synthetic estrogen DES), dioxin and dioxin-like compounds, polychlorinated biphenyls (PCBs), and some other pesticides. (Weyandt et al., 2008).
- ✓ **ORAL CONTRACEPTIVES/PILLS:** Drugs, such as diethylstilbestrol (DES), contraceptive pill and estrogen therapy, are artificial estrogens that have been shown to increase a woman’s risk of breast cancer. In the 1950s and 60s, diethylstilbestrol (DES)

was given to women in order to prevent miscarriage, but it was ineffective and research has shown that there was a doubled breast cancer risk for the daughters of the women who took the drug (Palmer et al., 2006). Also, the use of oral contraceptives is linked to a slight increase in the risk of a woman developing breast cancer, but only for a short period of time. Suggestions from studies are that there is no increased breast cancer risk in women who stopped the use of contraceptives for over 10 years and then greater risk of breast cancer exists for women who started using hormonal contraceptives before the age of 20 or before their first full-term pregnancy (Parkin, 2011).

- ✓ **HORMONE REPLACEMENT THERAPY(HRT):** According to the results of Women's Health Initiative (WHI), when the effect of estrogen and the combination of estrogen and progestin with placebo on chronic disease risk was observed, users of combination therapy for hormone replacement have 53% increased invasive breast cancer risk while users of estrogen alone have 34% increased breast cancer risk, especially if used for over 5 years (Rossouw et al., 2007). Prior this study being carried out, there were suggestions by earlier studies that cancers developed as a result of HRT, were of more benign pathology and had a more favourable prognosis (Smith et al., 1996). However, a re-assessment of the WHI data proved these suggestions are invalid, since invasive breast cancers, associated with estrogen plus progestin usage were larger (1.7 cm vs 1.5 cm, $p = 0.04$), were more likely to be node positive (26% vs 16%, $p = 0.03$), and were diagnosed at a significantly more advanced stage (regional/metastatic 25.4% vs 16%, $p = 0.04$). There were similarities in the estrogen and progestin group when compared to the placebo group in terms of the percentages and distribution of invasive ductal, invasive lobular, mixed ductal, and lobular as well as tubular carcinomas (Chlebowski et al., 2003). There was a

statistically significant increase in breast density in the estrogen plus progestin group, resulting in increased incidence of abnormal mammograms (9.4% vs 5.4%, $p=0.001$) over a one year observation period (Chlebowski et al., 2003). Gann and Morrow in a JAMA editorial noted that in combined hormone therapy, the agent increases the risk of developing a disease, yet at the same time delays its detection and this ability possessed by combined hormone therapy creates an almost unique situation (Gann and Morrow, 2003). In addition, reports by Li et al. (2003) reveal that women who used unopposed estrogen replacement therapy (ERT) had no significant increase in breast cancer risk. However, women who used combined estrogen and progestin hormone replacement therapy, had an overall 1.7-fold (95% CI 1.3– 2.2) increased risk of breast cancer, including: (i) a 2.7-fold (95% CI 1.7–4.3) increased risk of invasive lobular carcinoma, (ii) a 1.5-fold (95% CI, 1.1–2.0) increased risk of invasive ductal carcinoma, and (iii) a 2-fold (95% CI 1.5–2.7) increased risk of ER1/PR1 breast cancers (Li et al., 2003). Hormonal replacement therapy was also associated with a substantial increase in abnormal mammograms and a higher breast cancer risk is generally observed in women who are currently using hormonal replacement therapy or have used it in the past, whereas, the use of hormones for treatment of menopausal symptoms for a short period of time appears to have little or no breast cancer risk (Colditz, 1999).

▪ **NON-PREVENTABLE RISK FACTORS**

- ✓ **AGE:** Age has been a risk factor largely associated to breast cancer. This is because from studies, it has been observed that the longer one lives, there are more opportunities for genetic damage (mutation) in the body and the body becomes less able to repair genetic damages. Statistically, at age 20, one's risk is 1 in 1,681, at around age 40 (1 in 69) and as

the age increases to 70 or older (1 in 27) and if all women lived to age 95 (1 in 8), hence more risk increase, but most women tend to die before age 95 due to other diseases aside breast cancer. Also, about 1 out of 8 invasive breast cancers are found in women younger than 45, while about 2 of 3 invasive breast cancers are found in women aged 55 or older. The probability of breast cancer rises with age but breast cancer tends to be more aggressive in younger people (Nelson, 2006).

- **SEX:** When compared to any other risk factors, gender is the greatest factor, since it is obvious that most breast cancer cases occur 100 times more in women than in men. This is largely because the breast tissues in men is less than in women and also that they have less female hormone (estrogen and progesterone), which promotes the growth of breast cancer (Fentiman et al., 2006, Petrocca et al., 2005, Weiss et al., 2005). For a woman, breast cells are constantly changing and growing mainly due to the activity of the female hormones estrogen and progesterone, hence they are more susceptible to breast cancer. Studies show that <1% of all breast cancer cases in the United States and ~0.1% of cancer mortality, occurs in men. However, in Africa this situation may be different, since between 5-15% of breast cancer in Uganda and Zambia, may occur in males (Fentiman et al., 2006, Okobia and Osime, 2001, Sano et al., 1997, Kidmas et al., 2005, Loeffler, 1997, Smith et al., 1996). Although men still develop the diseases, in most cases, they are more likely to be diagnosed with hormone receptor positive tumours, with about six out of seven cases being estrogen-receptor positive and it occurs often in men who are older than women, however, the overall outcome remains worse for women than men (Giordano et al., 2004).
- **FAMILY HISTORY:** Often, breast cancer tends to be higher in women who have a family history of the disease. When a parent, sibling or child develops breast cancer, it is

considered a first-degree risk for other family members, especially if it occurs in conjunction with ovarian cancer or affects both breasts or before menopause or age 50. The risk is increased by 3-4 times if more than one first degree family member develop breast cancer when compared with women who do not have any relative that is affected by the disease (Hoskins et al., 1995). Cases where the father or brother has a family history of breast cancer, the woman has an increased risk of breast cancer, although the exact risk is unknown. From statistics, less than 15% of women with breast cancer have a family member with this disease and this implies that most women (~85%) who develop breast cancer, do not have a family history of this disease (Greene, 1997). Risk ratios for women who have one, two, three affected family members are 1:8, 2:9 and 3:9, respectively (Dumitrescu and Cotarla, 2005). It is advised that breast cancer screening should be conducted for such women at an early age. In 5% of breast cancer cases, there is a strong inherited familial risk (Lacroix and Leclercq, 2005). A father or brother diagnosed with breast cancer, also increases the risk (Valeri et al., 2000, Anderson and Badzioch, 1993, Beebe Dimmer et al., 2015).

- **GENETICS:** The role of genetics as a risk factor for breast cancer is limited because only between 5-6% breast cancer cases are hereditary (Malone et al., 1998). Two tumour suppressor genes; BRCA-1 and BRCA-2 account for an estimated 80% of hereditary breast cancer. They are important in DNA damage processing, genomic integrity preservation and they are inherited in an autosomal dominant fashion with varying penetrance. Women who are positive to these genes have a 50 to 85% lifetime risk of developing breast cancer and between 15 to 65% risk of developing other cancer at age 25 (Haber, 2002, Buchholz et al., 2002). There is a relatively high prevalence in European Ashkenazi Jewish population

and their descendants when compared to Europe and North America, BRCA1 is found in 0.1% of the general population when compared with 20% in the Ashkenazi Jewish population and it is found in 70% of women with inherited early-onset breast cancer (Bland, 2005). It is suggestive that there may be significant differences in the genetics of hereditary breast cancer in Africa because it has been discovered that there is uniqueness in the BRCA-1 and BRCA-2 mutation and sequence (McKean-Cowdin et al., 2005, Nanda et al., 2005). Apart from the fact that between 50-87% of women carrying a mutated BRCA-1 gene develop breast cancer during their lifetime, there is also the risk for such women to develop other cancer types, such ovarian and prostate cancers. On the other hand, the percentage risk of women who have BRCA-2 mutations to develop breast and ovarian cancer is between 10-20%, but generally, between 25- 30% is the estimated lifetime risk of developing breast cancer. In men who are carriers of BRCA-2, the lifetime risk of developing breast cancer is 6%. Other cancer types associated with BRCA-2 are: prostate, pancreatic, fallopian tube, bladder, non-Hodgkin lymphoma, and basal cell carcinoma. Below are the strategies for BRCA-1 and BRCA-2 carriers' management:

- Prophylactic mastectomy and reconstruction
- Prophylactic oophorectomy and hormone replacement therapy
- Intensive surveillance for breast and ovarian cancer
- Chemoprevention by using Tamoxifen or raloxifene (post-menopausal women)

(Nanda et al., 2005, McKean-Cowdin et al., 2005, Polite and Olopade, 2005).

- **RACE AND ETHNICITY:** Various risk factors are responsible for the decrease or increase in breast cancer risk factors observed among different races and ethnic groups. The Caucasian race has more risk, while Asian Americans or Pacific Islanders have decreased risk of breast cancer. Age at menarche, age at menopause, age at first birth, number of birth, weight of the body, patterns of breastfeeding are risk factors connected with these differences.

Overall, comparing the risk of different races and ethnic groups, Caucasian women are slightly more likely to come down with cancer of the breast than African American women, but African-American women are liable to die more of this cancer due to changeable or improvable factors, such as limited access to mammography and poor quality of medical care and the pattern of their lifestyle (eating habits, weight issues, etc.) while Asian, Hispanic and native American women have lower risk of developing and dying from breast cancer (Siegel et al., 2015). However, African American women who are below 40 years of age, tend to develop more destructive and more advanced stage of breast cancer than Caucasian women below the age of 40. Triple- negative breast cancer which is estrogen receptor negative, progesterone-receptor negative and HER-2 negative is more aggressive than other types is more common in African American women (Hirschman et al., 2007).

- **PERSONAL HISTORY OF BREAST CANCER:** A woman that has been previously diagnosed of breast cancer is at risk of developing a new cancer in the other breast or another part of the previously affected breast and this risk is higher if previous diagnosis was made at a tender or young age; although this is different from a recurrence or return of the initial cancer (Gao et al., 2003, Trentham-Dietz et al., 2007, Ricceri et al., 2015).

Research has shown that women who had prior history of invasive carcinoma or ductal carcinoma in situ have between 0.5-1% per year risk of developing a new invasive breast carcinoma, while those with prior cases of atypia ductal or lobular hyperplasia have between four to five times higher risk of developing breast cancer. Those with proliferative lesions without atypia, such as moderate hyperplasia and sclerosing adenosis, are associated with a slightly increased risk of between 1.5-2%. According to (Santen and Mansel, 2005), palpable cysts, fibroadenomas and duct papillomas which are the other common non proliferative changes, are not associated with a significantly increased risk. Furthermore, people with previous cancer cases of other types, are at a higher risk of future reoccurrence of breast cancer. Also, mothers of children with soft-tissue sarcoma and men with prostate cancer may have an elevated risk of breast cancer, although, the absolute risk remains low in both cases (Levi et al., 2006).

2.4.3 TYPES OF BREAST CANCER

According to Sinn and Kreipe (2013), breast cancers are known to be very heterogeneous since they can either be invasive or non-invasive.

NON-INVASIVE BREAST CANCER

Non-invasive cancer called in situ (“in the same place”) or pre-cancer is that which remains in a place usually within the milk ducts or lobules in the breast. Their growth does not spread into or invade normal tissues that are within or beyond the breast.

INVASIVE BREAST CANCER: On the other hand, invasive cancer is that which spreads or invades normal tissues that are within or beyond the breast and it is of different types. The

following are the various classification of invasive breast cancers, based on the region of the breast where they originate.

(a) Invasive ductal carcinoma (IDC): This cancer type originates in the milk ducts of the breast, capable of spreading outside the membrane of the duct or lobule and it accounts for between 70-80% of all invasive breast cancers (Dushkin and Cristofanilli, 2011).

(b) Invasive lobular carcinoma (ILC): This is the second most common cancer type which originates in lobules that produce milk in the breast and accounts for ~10% of all invasive breast cancers. Due to the formation of a thick line rather than a lump by the cells, it is often less sensitive to mammogram; that is, it is harder to detect (Eheman et al., 2009).

(c) Inflammatory breast cancer: This cancer type is usually very aggressive and always, it can be advanced locally, if not metastatic. Its diagnosis is basically clinical with diffuse erythema and edema, often without a mass on the majority of the breast. It probably accounts for between 1-5% of invasive breast cancers (Sinn and Kreipe, 2013).

(d) Paget's disease: This cancer type originates in the nipple then further spread to the areola causing redness and itchiness of the nipple and areola too. It accounts for less than 5% of invasive breast cancer cases and it has been observed that most patients with this type of cancer already have cancer in another region(s) of the breast (Dushkin and Cristofanilli, 2011).

(e) Phyllodes tumors: These tumours rarely metastasize and their growth pattern is like a leaf and it accounts for <1% of breast cancer (Eheman et al., 2009).

2.4.4 SIGNS AND SYMPTOMS OF BREAST CANCER

With the introduction of mammograms for breast cancer screening, the number of detected breast cancer cases has increased even before any symptom is identified. However, some breast cancers are still not detected because of reasons, such as non-performance of test or mammogram's inability to detect the mass or lump, which is often the most common symptom of breast cancer. The different characteristics exhibited by this mass can hinder self-diagnosis because a mass can be painless, hard, and have uneven edges, it also can be tender, soft, or rounded and painful. Therefore, it is necessary to have any new breast mass, lump, or change be examined by some medical personnel that are experienced in diagnosing breast diseases. Other symptoms related to cancer of the breast are: swelling of all or part of a breast even in the absence of a distinct lump, irritation or dimpling of the skin, pain in the breast or nipple, nipple retraction (turning inward), redness, scaliness, or thickening of the nipple or breast skin and discharge from the nipple other than breast milk. Because other things may be responsible for any of these symptoms, it is therefore, advisable to contact a health provider for proper diagnosis (Girish et al., 2014).

2.4.5 BREAST CANCER TREATMENT

Most pharmaceutical drugs that are generally used in the treatment of cancer, as earlier discussed, have been employed and used over the years to cure breast cancer. However, these anticancer drugs, used for clinical treatments, are faced with encumbrances, such as: toxicity, de novo refracton, and acquired multidrug resistance (MDR), which limit their efficacy, hence the need to explore and discover combined therapeutic procedures, by combining two or more drugs for better and more effective treatment of the disease. Paclitaxel (Taxol®), which belongs to the taxane class of anticancer drugs, is one of such common drugs used for treating different types of cancer. It acts by stabilizing microtubule that is responsible for selective arrests of cells in the G2/M phase of the

cell cycle, and it also induces cytotoxicity in a time and it is a concentration-dependent form of therapy (Peltier et al., 2006, Brito et al., 2008, Jordan and Wilson, 2004, Ganguly et al., 2010, Bharadwaj and Yu, 2004).

2.4.6 COMBINATION THERAPY IN BREAST CANCER TREATMENT

This excellent therapeutic approach was first formulated by Skipper and Schabel and described as the log-kill hypothesis (Katzung et al., 2011). The major basis for introducing the use of combination therapy in breast cancer treatment is to apply different methodologies that exhibit various mechanisms of action. This combined treatment (use of two or more approaches) aims to target different key signal transduction pathways, thereby having a method with better efficient cancer cells destruction and eluding the cellular resistance mechanisms, thereby enhancing the overall therapeutic efficacy of drugs. Also, an additional advantage and even synergistic effects, such as a reduction of the dosage of the most toxic component, is sometimes possible, with decreased or total elimination of noxious side effects (Cavo et al., 2011).

Over the last few decades, the basis for combination chemotherapy development has remained constant. Currently, the fast growing interest of combining drugs for breast cancer treatment is hinged on the following principles:

- i) To use drugs that do not have overlapping toxicities, such that the administration of each drug can be at near-maximal dose;
- ii) To combine agents with different mechanisms of action and minimal cross-resistance in order to inhibit the emergence of broad spectrum of drug resistance;
- iii) To preferentially use drugs with proven activity as single drugs;

- iv) To overcome the limited log kill time of individual anticancer drugs; and
- v) To administer the combination at early stage of the disease and at a schedule with a minimal treatment-free period between cycles but still allows the recovery of sensitive target tissues (Mayer and Janoff, 2007, Harasym et al., 2006, Ramsay et al., 2005, Zoli et al., 2001).

Furthermore, sub clones resistant to only one of the agents can potentially be eradicated (Kang and Park, 2012). Some of the notable advantages of combination chemotherapy, include: (a) improved patient compliance due to the reduced number of administrations of drugs, (b) the emergence of additive or synergistic interaction effects, (c) the ability to overcome or delay multi drug resistance and (d) the reduction of drug dose with consequent diminishing of toxicity to healthy tissues (Chou, 2006, Chou, 2010, Ramsay et al., 2005). Quite a number of methods of quantitative evaluation of drug-combined interaction effects have been employed and this has revealed the various effects of these drugs when combined. Their effect at different concentrations can be synergistic (superior), antagonistic or additive (equal or inferior respectively) to the expected sum of individual effects (Zoli et al., 2001, Merlin, 1993, Pinto et al., 2011). Additive means that each constituent contributes to the effect in accordance with its own potency (Chou, 2006, Pinto et al., 2011). These effects can be identified by carrying out systematic screening analysis of drug combinations (Mayer and Janoff, 2007). For a maximal therapeutic efficacy *in vivo*, it is important to schedule drug dosage so that the tumour cell will be exposed to a well defined concentration of the drug. This is because a synergistic activity, depends largely, on drug to drug ratio and that the dependence has profound implications on clinical application, since *in vivo* activity relies on the maintenance of those therapeutic ratios at the disease site (Mayer and Janoff, 2007, Harasym et al., 2006, Mayer et al., 2006, Zhao et al., 2008).

2.5 CURCUMIN

Curcumin is the active component in the traditional herbal remedy and dietary spice turmeric. It is a yellow spice obtained from the roots (rhizomes) of the plant, *Curcuma longa*. *Curcuma longa* is a perennial plant with short stems, curved leaves, oblong rhizomes that grows to about 100cm in height and it is naturally found throughout the Indian subcontinent and in tropical countries, particularly the South East Asia. As far back as over 5,000 years, curcumin has been used in "Ayurvedic medicine" and also as an ancient Indian healing system as an "herbal aspirin" and "herbal cortisone", used to relieve discomfort and inflammation associated with an extraordinary spectrum of infectious and autoimmune diseases (Sharma et al., 2005, Pal et al., 2001).



(a)



(b)

Figure 2.5: A figure showing Curcumin in different forms: (a) crystalized curcumin powder and (b) the root of turmeric (Yang et al., 2014)

The chemical formula of curcumin is diferuloylmethane (shown in Figure 2.6 below) and has its structural formula as $(C_{21}H_{20}O_6)$. Its antioxidant, anti-toxic, anti-inflammatory, cancer chemopreventive and potentially chemotherapeutic properties have been widely studied and acknowledged by many researchers (Sharma et al., 2005, Pal et al., 2001, Pal et al., 2005, Oyama et al., 1998, Sugimoto et al., 2002, Campbell and Collett, 2005). Even though these pharmacological and supposed anticancer properties of curcumin have been the subject of

investigations and paper publications, the superiority of the phytochemicals present in curcumin over other chemotherapeutic agents that also induce apoptosis successfully in cancer cells has not been proven.

From studies, cancer cell apoptosis induction by most chemotherapeutic agents, including those isolated from plants, e.g. taxol and vincristin also cause serious damage to the normal cells of the host, with the immune system being affected more severely (Vial and Descotes, 2003). On the contrary, curcumin is used as part of our daily food and even in large quantities from ancient time, without any known side effects, thereby proving its safety (Sharma et al., 2005). It has been observed that death is much more pronounced in tumour cells than normal cells, since highly proliferating cells are the major targets during apoptosis induction by curcumin (Choudhuri et al., 2002). Furthermore, laboratory report revealed that curcumin did not induce apoptosis in non-malignant (benign, i.e not cancerous) cells in the G0 phase of the life cycle of cells, rather it only arrests the cells in a reversible manner (Choudhuri et al., 2005). More studies on the mechanism of action of this phytochemicals and chemotherapeutic agents show that they protect the T cells of the cancer bearer from cancer (Choudhuri et al., 2002, Bhattacharyya et al., 2007).

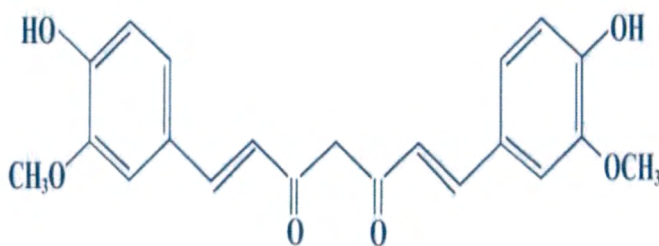


Figure 2.6: A figure showing the chemical structure of curcumin (Maheshwari et al., 2006)

2.5.1 MECHANISIM OF ACTION OF CURCUMIN IN BREAST CANCER TREATMENT

One of the mechanisms of action of curcumin is seen in its effect on the oncogene proteins of proliferation and apoptosis. This mechanism of action is exhibited by inhibiting the proliferation of tumour cells and inhibiting oncogene proteins of proliferation and apoptosis. This inhibitory mechanism is seen when curcumin is used alone or in combination with other therapeutics (Kunnumakkara et al., 2008). According to (Banerjee et al., 2010), curcumin also acts by inducing G2/M arrest and apoptosis, inhibiting cell proliferation by inhibiting the assembly dynamics of microtubules and then activating the mitotic checkpoint in the MCF-7 cells. In addition, curcumin suppresses the expression of zeste homolog 2 (EZH2) genes in cells that are accumulated in the G1 phase of the cell cycle through the stimulation of three major members of the mitogen-activated protein kinase (MAPK) pathway: c-JunNH2-terminal kinase (JNK), extracellular signal-regulated kinase (ERK) and p38 kinase (Hua et al., 2010). Other routes by which tumor cell growth is prevented or stopped by curcumin are inhibiting the expression of Ki-67, proliferating cell nuclear antigen (PCNA) and p53 mRNAs in breast cancer cells. It also induces Bax mRNA expression with the down-regulation of p21 mRNA in the human mammary epithelial cell line (Ramachandran and You, 1999). Furthermore, since growth of breast cancer is associated with the abnormal activation of the Wnt/ β -catenin signaling pathway, curcumin therefore acts by inhibiting the expression of β -catenin, cyclin D1 and slug in both MCF-7 and MDA-MB-231 cells (Prasad et al., 2009). Further studies have shown that curcumin also acts by increasing the gene expression of maspin, a serine protease inhibitor, which suppresses tumour growth and metastasis *in vivo*, tumour cell motility and invasion *in vitro* in MCF-7 cells with the up-regulation of p53 protein and down regulation of Bcl-2. From another report, curcumin acts by reducing the expression of Bcl-2 by up-regulating the expression of miR-15a and miR-16 in MCF-7 cells (Yang et al., 2010). Data

obtained from studies carried out by Chiu and Su (2009) revealed that curcumin can also inhibit the proliferation of MDA-MB-231 cells either through up-regulating p21 expression or up-regulating the Bax to Bcl-2 ratio. In malignant breast cancer cells, AIP-1/Alix protein, which is an inhibitor protein of paraptosis, is progressively down regulated by treating with curcumin (Yoon et al., 2010). Furthermore, curcumin directly inhibits the function of $\alpha 6\beta 4$ integrin, thereby inhibiting ultimately, breast cancer cell motility and invasion (Im Kim et al., 2008).

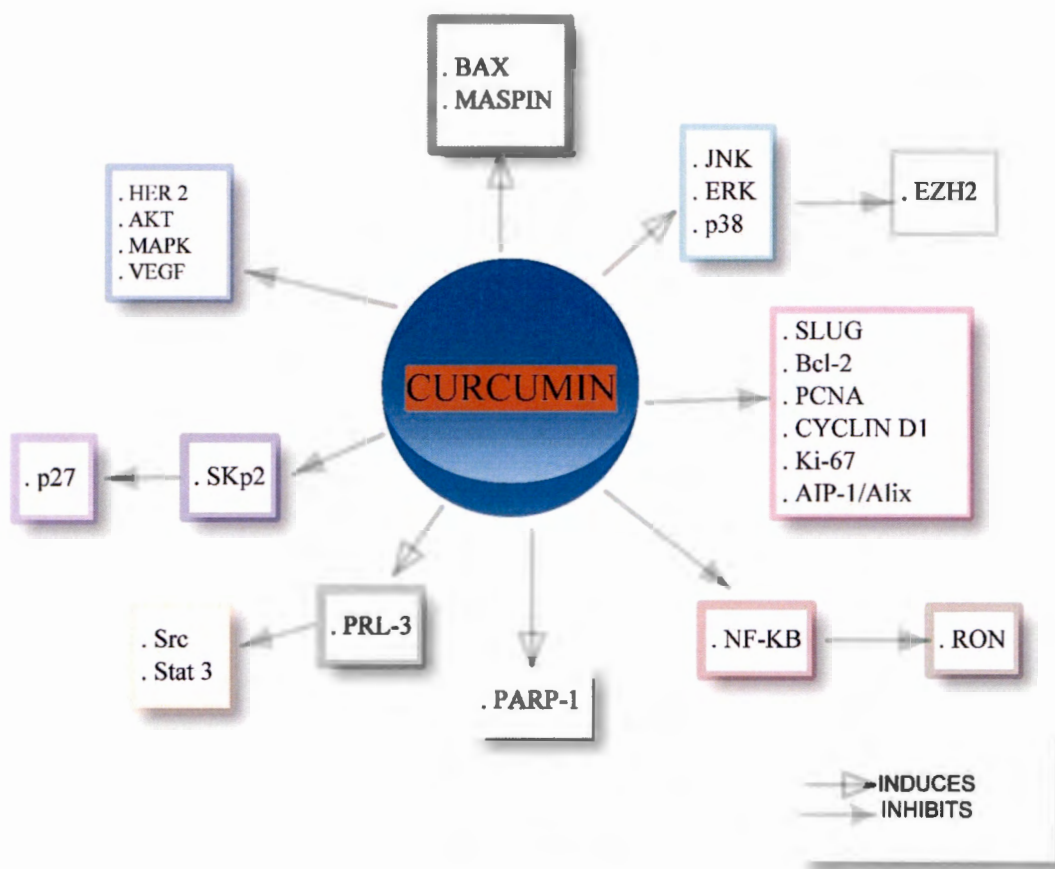


Figure 2.7: A figure showing the mechanism of action of curcumin

Another anti-cancer effect of curcumin is also seen on nuclear factor- κ B (NF- κ B) which is responsible for mediating cell survival, proliferation and metastasis. Most therapeutic agents with anticancer activities act by activating nuclear factor- κ B; therefore, curcumin plays its anticancer role by inhibiting the migratory activity of breast cancer cells, proliferative rate, adhesion, and invasion by down-regulating the expression of NF- κ Bp65 (Chiu and Su, 2009, Kang et al., 2009, Zong et al., 2012). Curcumin has also been found to decrease the human epidermal growth factor 2 (HER2), which is a major oncoprotein, often overexpressed in 15 to 25% of breast cancers HER2. Also, a decrease in phosphorylation of Akt, MAPK, and the expression of NF- κ B in both BT-474 and SK-BR-3-hr cells, have been associated with the activity of curcumin (Lai et al., 2012).

Furthermore, the efficacy of chemotherapy is enhanced by curcumin and this was achieved by modifying p65NF- κ B-p300 cross-talk in favor of p53-p300 for breast cancer (Sen et al., 2011). In addition, as a result of the effect of curcumin on p65, protein expression and transcriptional activity via NF- κ B in blocking recepteur d'origine nantais (RON) tyrosine kinase-mediated invasion of carcinoma cells a potential therapeutic role is further exhibited (Narasimhan and Ammanamanchi, 2008).

Beside all these effects exhibited by curcumin, it has also been discovered that curcumin affects the vascular endothelial growth factor (VEGF). From clinical observations, curcumin may be beneficial in suppressing the elaboration of vascular endothelial growth factor in tumour cells because it has been revealed that curcumin suppressed breast tumour angiogenesis by abrogating osteopontin or medroxyprogesterone acetate induced by VEGF expression (Chakraborty et al., 2008, Carroll et al., 2008). An alteration in the intracellular localization of α 6 β 4 by curcumin is known to prevent the association of α 6 β 4 with signalling receptors such as the epidermal growth factor receptor (EGFR) and Akt, thus it can be concluded that curcumin inhibits α 6 β 4 signaling and functions (Soung and Chung, 2011, Somers-Edgar et al., 2008). Furthermore, a better efficacy has been observed by combining epigallocatechin gallate (EGCG) and curcumin *in vitro* and *in vivo* models of ER α -breast cancer. It is important to know that the regulation of VEGFR-1 can play a key role in the antitumor activities in all these processes (Somers-Edgar et al., 2008).

2.6 DOXORUBICIN

Doxorubicin (DOX), alongside daunorubicin, idarubicin and epirubicin, which are its analogues belongs to anthracyclines family. Before the discovery of the doxorubicin from *Streptomyces peucetius* var. *caesius*, a mutagenic *Streptomyces peucetius* in 1969, the first anthracycline antibiotics that was isolated in the early 1957 from the actinobacterium *Streptomyces peucetius* is

daunorubicin (Arcamone et al., 2000, Alsaad et al., 2012, Di Marco et al., 1980). Ever since the discovery of DOX, it has become one of the most effective and widely used drugs in the treatment of several ailments, especially cancer, due to its high antineoplastic activity to breast cancer, aggressive lymphomas, childhood solid tumours and soft tissue sarcomas (Volkova and Russell, 2011, Minotti et al., 2004, Quiles et al., 2006). It has been a successful therapeutic agent for diverse forms of malignant tumours, such as lymphomas and various solid tumours such as breast cancer, small cell lung cancer and ovarian cancer (Ogura, 2001, Zhong et al., 2013). However, there has been limitations in the use of DOX in chemotherapy because of its diverse side effects such as toxicities, hematopoietic suppression, nausea, vomiting and alopecia and cardiac toxicity; this is the most dreaded side effect amongst them all, since it is cumulative and dose dependent. Doxorubicin has a complex structure that is closely associated with its main mechanisms of action and metabolism.

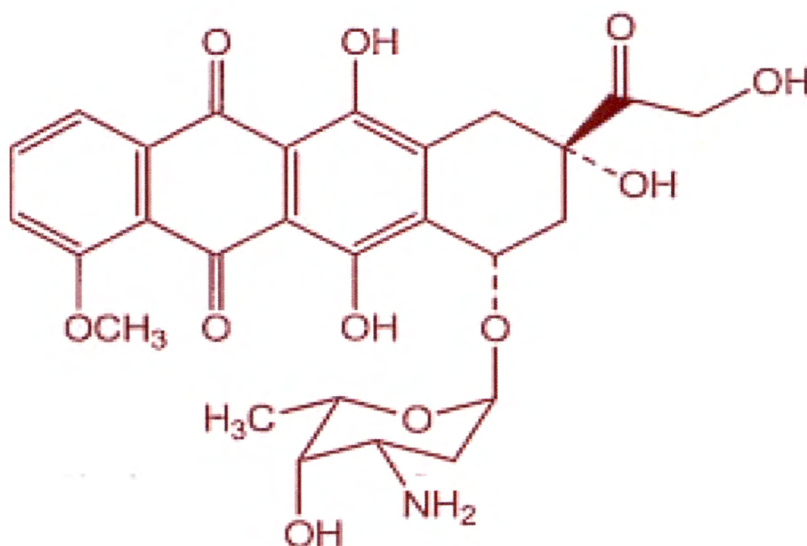


Figure 2.8: A figure showing the chemical structure of doxorubicin (Minotti et al., 2004)

Doxorubicin has a tetracyclic aglycone structure that is linked to an amino sugar by a glycosidic bond at the C-7 in ring A, called daunosamine. It is this amino sugar that provides a hydrophilic center for doxorubicin. It consists of a short chain with a carbonyl group and a hydroxyl group at the C-13 and C-14 respectively. The hydroxyl group is attached at C-9 to ring A and in addition, a quinine group and a hydroquinone group are present in rings C and B respectively. In addition, a methoxyl substituent is seen to be linked to ring D at C-4. (Minotti et al., 2004, Arcamone et al., 2000). The hydroxyl group at C-14 in doxorubicin, and the methyl group at C-14 in daunorubicin are responsible for the only structural difference between both drugs.

2.6.1 MECHANISM OF ACTION OF DOXORUBICIN IN BREAST CANCER

TREATMENT

A lot of different mechanisms of action of doxorubicin-induced anticancer cytotoxicity have been studied but certain issues are still obscure and debatable thus the need for further intensive studies of the mechanisms of action. Despite the lack of clarity surrounding the mechanism of action of DOX, Minotti and his colleagues recently appraised the discovery already made in line with the mechanism of action of anticancer cytotoxic activities of doxorubicin and then summarized them as follows (Minotti et al., 2004):

- **Inhibition of topoisomerase II (topo II) triggered by DNA intercalation:** This is the first mechanism of action that was characterized (Tewey et al., 1984, Cortés-Funes and Coronado, 2007). It has been discovered that during cell replication, topoisomerase II, a nuclear enzyme is responsible for the alteration of the topological state of double-stranded DNA, which leads to the breakage of transitory double-stranded DNA and rejoining the DNA after altering the twisted status of the double helix. This activity and function of topoisomerase II is however inhibited whenever cells are treated with doxorubicin.

Doxorubicin directly intercalates into the DNA double-stranded helix in the nucleus when rings B and C of doxorubicin overlap with the base pairs and its ring D passes through the intercalation site. In addition, there is an interaction between the amino sugar located in the DNA minor groove and the flanking base pairs that are immediately adjacent to the intercalation site (Minotti et al., 2004). Stabilization of topoisomerase II-DNA complex is experienced when there is an intercalation of doxorubicin with DNA. This prevents the DNA double-stranded helix to re-join after the breakage of the transient double-stranded DNA by topoisomerase II, thus cell replication is disrupted. This mechanism is referred to as the main cytotoxicity mechanism, based on the observation made when cancer cells were exposed to low concentration of doxorubicin of between 1-2 μM (equivalent to 0.544-1.087 $\mu\text{g/mL}$) *in vitro* (Gewirtz, 1999, Chabner and Longo, 2011). The peak plasma concentrations (C_{max}) and the tumour tissue concentrations of doxorubicin after a large dose of doxorubicin administered to patients can achieve the *in vitro* concentration in clinical settings (Lee et al., 1980), since it has been observed that the concentrations of doxorubicin in the cells of patients with lymphoma and multiple myeloma is above the concentration for a few days (La-Beck et al., 2012, Speth et al., 1988, Andersen et al., 1999).

- **Oxidative damage mediated by generation of free radicals:** Cell death can be linked to oxidative damage mediated by the generation of free radicals. For this free radicals to be generated, quinone group in doxorubicin ring C is catalyzed by a number of NAD(P)H (nicotinamide adenine dinucleotide phosphate in the reduced form) oxidoreductases, including cytochrome P450 or b5 reductases, mitochondrial NADH dehydrogenase, xanthine dehydrogenase and endothelial nitric oxide synthase (reductase domain) to accept

a free electron to form semiquinone doxorubicin and this free radical semiquinone further reacts with molecular oxygen to produce the superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radicals (OH). These free radicals are often found in the cells when doxorubicin concentrations *in vitro* are far greater than $2\mu M$. This validates the report that free radicals contribute to the anticancer toxicity of doxorubicin through direct DNA cleavage, the production of lipid peroxidation to damage cell membrane and the increase of ceramide generation to activate proapoptotic cycle (Speth et al., 1988, Minotti et al., 2004).

- **Apoptosis induced by tumor protein p53-dependent and/or tumour protein p53-independent pathway:** Another route by which doxorubicin exerts its effect as an extracellular signal in inducing apoptosis has been observed in the activation of tumour protein p53-dependent pathway and/or tumour protein p53-independent pathway. Doxorubicin has the ability to increase the level of the tumour protein p53, which is a DNA binding protein. An increase subsequently results in the up-regulation of the cyclin-dependent kinase inhibition of protein p21 waf1/cip1, thus inhibiting DNA synthesis and causing G1 arrest (Minotti et al., 2004). There are also other several tumour protein p53-independent pathways through which doxorubicin has been observed to induce apoptosis. One of such pathways is the direct stimulation of sphingomyelinases (SMases) by doxorubicin to hydrolyze sphingomyelin (SM) to generate ceramide. Further activation of JNK (c-Jun N-terminal kinase) pathway and blockage of serine-threonine Akt/PKB (protein kinase B) survival pathway, is displayed by the generated ceramide, a second messenger and thus, inducing apoptosis (Minotti et al., 2004, Kolesnick, 2002). The alternate pathway is observed when doxorubicin causes damage to the mitochondria

directly, leading to cytochrome-C release and subsequently causing apoptosis in cancer cells (Minotti et al., 2004).

- **Interactions with proteasome to mediate-transport of doxorubicin into the nucleus:**

Accumulation of doxorubicin in the nucleus is of great importance, chiefly because the inhibitory anticancer cytotoxicity mechanism of doxorubicin on topoisomerase II occurs in the nucleus. From recent research, it has been shown that the entrance of doxorubicin into the nucleus does not happen passively, but rather by the aid of a specific carrier known as 26S proteasomes, a normal degradation protein. Doxorubicin-proteasome complex is usually formed in the cytoplasm when there is a high affinity binding between doxorubicin and 26S proteasome. After the formation of this complex, translocation through the nuclear pores takes place, allowing the complex arrive at the nucleus. On arrival, doxorubicin dissociates from the proteasome and then binds with DNA with higher affinity (Kiyomiya et al., 2001, Kiyomiya et al., 1998, Minotti et al., 2004). Preclinical and clinical studies have adopted this mechanism for combinational chemotherapy of doxorubicin. An example is bortezomib, a novel proteasome inhibition anticancer drug. When bortezomib inhibits proteasome, degradation of inhibitory protein I κ B is prevented, thus inhibiting NF- κ B antiapoptotic activity. Synergic effects were observed when the two drugs were used together during preclinical studies due to the distinct binding sites of proteasome between bortezomib and doxorubicin (Minotti et al., 2004). The combinational use of the two drugs in the treatment of patients with advanced cancer has been successfully proven in few clinical studies done recently (LoConte et al., 2008, Lee et al., 2008a). The figure below shows the intercalation into DNA, leading to: (a) the inhibition of the DNA synthesis or (ii) the poisoning of topoisomerase II (TOPII), generation of free radicals that leads to

DNA and cell membrane damage, in addition to (iii) how doxorubicin affects the PK path way.

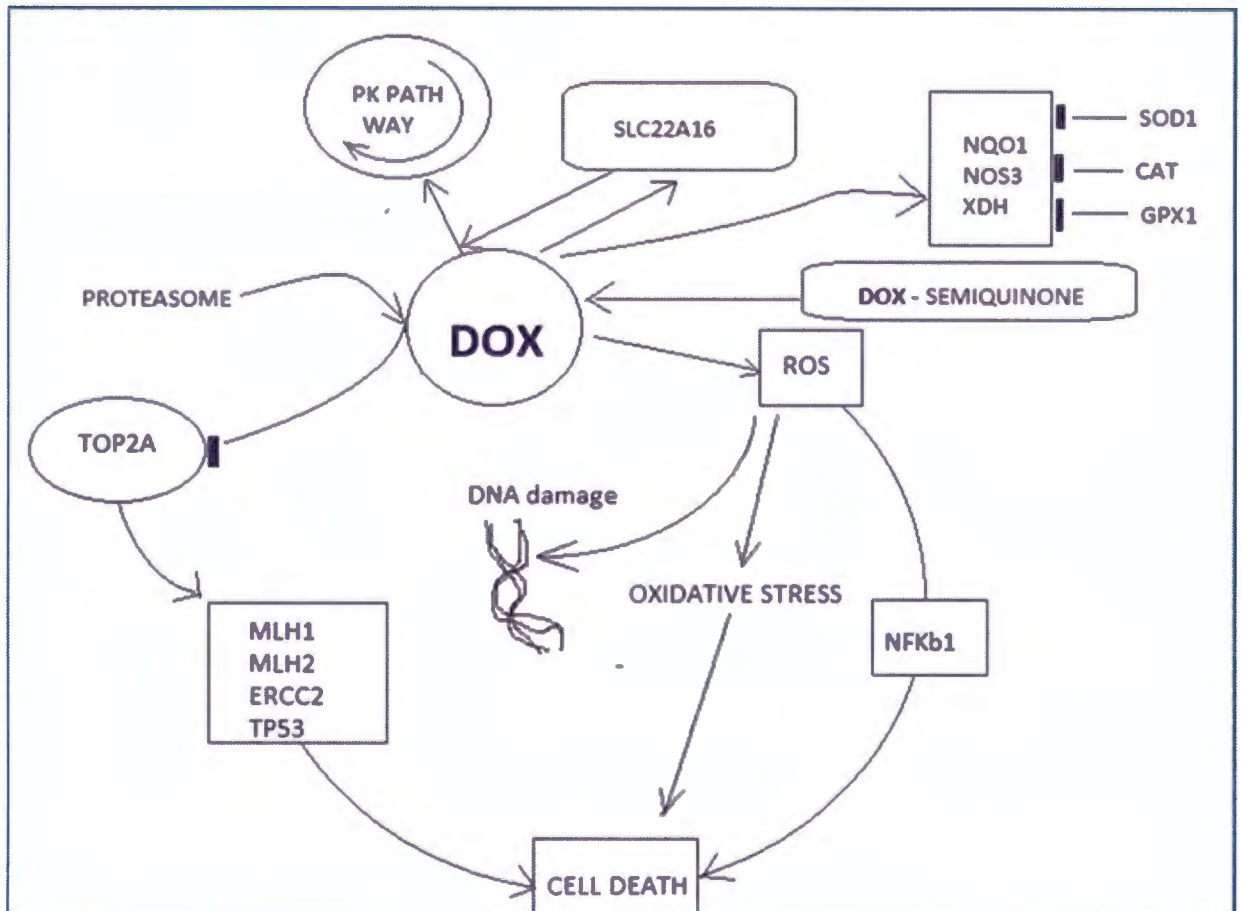


Figure 2.9: A figure showing the mechanism of action of doxorubicin.

2.7 DRUG DELIVERY SYSTEM (DDS)

For over two decades, researchers have embarked on studies into drug delivery systems with the aim to improve the therapeutic and curative effects of drugs when administered to humans. Drug delivery systems take cognizance of the: (a) methods, (b) approaches or processes employed for administering and (c) transporting one or more pharmaceutical compound as required in the body systems or parts of humans in order to achieve a therapeutic effect. These systems work by

targeting specific sites in the body or enhancing systemic pharmacokinetics, but whichever way it works, the quantity and duration of the drug's presence in the system are factored into consideration. These delivery systems are fast becoming promising alternate routes for drug delivery, especially peptides and protein therapeutics (Shantha Kumar et al., 2006). As a result of the successes attained by these alternate drug delivery systems, several drug delivery systems have been formulated, developed and studied. Examples are: prodrugs, liposomes, microspheres, gels among others. Usually, drug delivery is achieved by using a drug's chemical formulation, but sometimes it may require medical devices or combination of both. A variety of systems, such as: particulate carriers, polymer gels, and lipids among others, have been used as drug delivery systems (Bae and Kataoka, 2006, Kim et al., 2008, Nguyen et al., 2008, Tamilvanan et al., 2008, Tang and Singh, 2008, Watanabe et al., 2008).

Any drug delivery technology aims to adjust drug release profile, absorption, distribution and elimination for better effect and safety, also for convenience and compliance by patients. Diffusion, degradation, swelling, and affinity-based mechanisms are basic forms by which drugs are released (Fundueanu et al., 2008, Huynh et al., 2008, Nakamura et al., 1999, Wang and Von Recum, 2011, Wang et al., 2008, Kumar et al., 2008). For any device to be considered appropriate for drug delivery or used as a biomedical system, it must possess some properties (Jain, 2008). These properties include:

- Structural control over size and shape of drug or imaging-agent cargo-space;
- Biocompatible, nontoxic polymer/pendant functionality;
- Precise, nanoscale-container and/or scaffolding properties with high drug or imaging-agent capacity features;

- Well-defined scaffolding and/or surface modifiable functionality for cell specific targeting moieties;
- Lack of immunogenicity;
- Appropriate cellular adhesion, endocytosis and intracellular trafficking in order to permit therapeutic delivery or imaging in the cytoplasm or nucleus;
- Acceptable bio-elimination or biodegradation;
- Controlled or trigger-able drug release possibility;
- Molecular level isolation and protection of the drug against inactivation during transit to target cells;
- Minimal non-specific cellular and blood-protein binding properties; and
- Ease of consistent, reproducible and clinical-grade synthesis.

2.7.1 TYPES OF DELIVERY SYSTEMS

Thus far, different types of drug delivery systems have been discovered and have gained wide range of application in various medical fields. All the different types of drug delivery systems in use have been classified into two, viz: conventional drug delivery system and the novel drug delivery system

2.7.1.1 Conventional Drug Delivery System

Conventional drug delivery system often known as traditional system is the one that combines the usual and common methods for the delivery of pharmaceutical compounds into the body. It tends to have a lot of benefits (Singh et al., 2011). Examples of this type of delivery systems include (a) oral delivery (tablets, capsules and suspensions), (b) buccal / sublingual delivery (tablets and chewing gums), (c) rectal delivery, (d) intravenous delivery, (e) subcutaneous delivery and (f)

intramuscular delivery amongst others. These individual delivery systems have different advantages over one another and also disadvantages.

(a) Oral delivery: Advantages of this form of drug delivery system include: (i) convenience of administration, (ii) non-invasive, (iii) accurate and measured dose, (iv) unit dosage form and (v) cheap for the patient, while the disadvantages include: (i) cannot be used by unconscious patients, (ii) low solubility and permeability, (iii) degradation by gastro-intestinal enzymes or flora, (iv) food interactions and (v) irregular absorption (Verma et al., 2010).

(b) Buccal / sublingual delivery: Advantages includes: (i) by-pass first pass metabolism (an event during drug metabolism whereby the drug's concentration is reduced before it gets to the systemic circulation), (ii) rapid absorption and (iii) low enzymatic activity while disadvantages include: (i) discomfort during dissolution, (ii) probability of swallowing- loss of effect and (iii) small doses (Rawas-Qalaji et al., 2006).

(c) Rectal delivery: (i) By-pass first pass metabolism and (ii) the fact that it is useful to administer drugs to children are the advantages of this form of drug delivery system while its disadvantages are: (i) absorption depends on disease state, (ii) degradation by bacterial flora and (iii) discomfort (Chang et al., 1981, Verma et al., 2010).

(d) Intravenous delivery: The major advantages of this form of drug delivery include: (i) drug's 100% bioavailable, (ii) rapid response, (iii) total control of blood concentration, (iv) maximize incorporation of degradable drugs and (v) by-pass first pass metabolism while its disadvantages are: (i) that it is invasive, (ii) trained personnel is required to administer the drug and (iii) possible toxicity due to incorrect dosing and sterility.

(e) Subcutaneous delivery: Advantages include: (i) patient self-administration, (ii) slow but complete absorption, (iii) by-pass first pass metabolism while the disadvantages include: (i) irritation, (ii) inflammation and (iii) it has a maximum dose volume (Verma et al., 2010).

(f) Intramuscular delivery: Advantages include: (i) patients can administer the drug themselves, (ii) larger volume than subcutaneous can be administered and (iii) by-pass first pass metabolism while disadvantages include: (i) invasiveness (patient discomfort), (ii) irritation, (iii) inflammation and (iv) may require some training to administer (Verma et al., 2010).

2.7.1.2 Novel Drug Delivery Systems

Novel drug delivery systems basically comprise of three individual delivery systems. These are:

(a) targeted, (b) controlled and (c) sustained/modified release drug delivery system.

(a) Targeted Drug Delivery System

Smart drug delivery, as it sometimes called, is a method that delivers medication to patients in such a way that the drug is made available for a prolonged period of time and the concentration of the drug is relatively increased in the targeted site when compared to other parts of the body. In contrast to the conventional drug delivery system, drugs are absorbed across a biological membrane and targeted drug delivery systems release therapeutic compounds in dosage form. Some of the advantages this method of drug delivery system provides, are: reduction in frequency of doses ingested by patients, reduction in the drug circulation fluctuations, decreased side effects and these lead to a more even effect of the drug and it retains the required drug levels in the plasma and tissue, thereby protecting healthy tissues from damage. However, a few disadvantages have been observed with this method of drug delivery system. These include: the limitation in dosage adjustment and high cost, hence making production more difficult.

Targeted drug delivery system involves and requires a wide range of disciplines for adequate optimization of the delivery system. Chemists, material scientists, biologist and engineers, are required for combined effort to achieve this. Before a targeted drug delivery system is fully implemented, the drug properties, side effects of the drugs, the drug delivery route, the targeted site, and the disease to be treated, are factors to be considered (Mark and Torchilin, 2011, Muller and Keck, 2004, Vyas and Khar, 2004, Allen and Cullis, 2004).

There are two fundamental approaches employed in targeted drug delivery system. This is because it does not only seek to improve the overall therapeutic effects of the drug, but also to decrease the toxicity of the drug by permitting drug dosage reduction. These two approaches are widely in use and are otherwise known as the classifications of targeted drug delivery system.

- **Passive targeting:** Passive targeting in simpler terms, is a drug delivery system that delivers drugs through blood circulation. This is the gathering of drug enveloped in drug carrier systems at the particular site of the disease in the body. In this type of targeting, the action of the drug and drug release are restricted to the sites in the body that is affected. A typical example is cancer, whereby anti-cancer drugs are targeted to the tumour site and not the liver. Hence, a specific control of the size and surface properties of drug delivery carrier is required in order to avoid uptake by other organs (Gref et al., 1994, Rani and Paliwal, 2014, Gupta and Sharma, 2011).
- **Active targeting:** Active targeting occurs mainly after blood circulation and extravasations. It is an action that involves specific type of ligand– receptor interaction for intracellular localization. Further classification of active targeting method based on the different levels of targeting are:

- ✓ First order targeting which is the restriction of the distribution of the drug carrier systems to the capillary bed of a predetermined target site, organ or tissue, e.g. compartmental targeting in lymphatics, peritoneal cavity, plural cavity, cerebral ventricles and eyes and the joints (Rani and Paliwal, 2014).
- ✓ Second order targeting involves selecting the particular cite (specific cell type) for delivery of drugs. For example, the targeting of tumour cells and not the normal cells and also the selective drug delivery to kupffer cells in the liver (Akanksha et al., 2014).
- ✓ Third order targeting is the delivery of drugs precisely to the intracellular site of targeted cells e.g. receptor based ligand mediated entry of a drug complex into a cell by endocytosis (Kannagi et al., 2004, Muller and Keck, 2004, Gref et al., 1994).

(b) Controlled Drug Delivery System: Controlled Drug Delivery (CDD) is usually the incorporation of therapeutic agents in a drug delivery device and subsequently, there is the release of therapeutic agent from the delivery device in a pre-designed form and manner. At the moment, stimuli-sensitive drug delivery systems have been an attractive theme for controlled release because the release behaviours of drugs can be easily controlled and triggered by environmental or external events, such as pH (Lee et al., 2008b, Connal et al., 2008) temperature (Kurkuri et al., 2008, Chen et al., 2007), ionic strength (Shah et al., 2006) and electric field (Nolkrantz et al., 2001). This release may happen constantly over a long period or cyclic over a long period of time. However, as the release takes place, the ultimate priority is to achieve a more efficient therapy at the same time avoiding under dosing and overdosing. A controlled release drug delivery system is one which therapeutic drugs are administered at a predetermined rate and time, taking into consideration the required concentration of the drug and the drug's pharmacokinetic characteristics. This type of drug delivery system makes use of devices capable of encapsulating

the therapeutic agent which can be released at a controlled rate, days, weeks or months. This system has numerous advantages and these include: tailored drug release rates, protection of fragile drugs, patient's compliance and comfort, among others.

The difference between controlled release and sustained release is that: controlled release is a perfectly zero order release; that is, the drug releases over time, irrespective of concentration. Sustained release implies slow release of the drug over a time period. It may or may not be controlled release (Bhowmik et al., 2012, Lachman et al., 1986). Although, Controlled and Sustained Release drug delivery systems are separate delivery processes, they are often used in inconsistent and confusing manner, due to their similarities in process. However, there is a difference between them and it is majorly that a controlled release system releases drug over time regardless of the drug's concentration (zero order release), whereas a sustained release system releases drug at a slow rate over a period of time and not time dependent. It can either be controlled or uncontrolled (Li et al., 1987, Wani, 2008, Brahmanekar and Jaiswal, 2009).

(c) Sustained/ Modified Release Drug Delivery System: Sustained release system is a form of drug delivery system that is modified and can serve as a substitute to conventional drug delivery system, but the administration of drugs to patients is usually in a more efficient manner. This is accomplished by sustaining the release of drug and maintaining the therapeutic window concentration of the drug in the plasma. The mode of action of a sustained drug delivery system is seen in the prevention of peak and trough in dosing and there is a constant plasma drug concentration in therapeutic window. The design of sustained delivery systems offers a lot of benefits which includes; reduction in the frequency of dose, increment in the effectiveness of the drug by localization at the site of action, reduction in the required dose and providing uniform drug delivery. Furthermore, the advantages of this delivery system are: patient compliance, evasion of

multiple dosing, increased plasma drug concentration, little or no side effects and also the challenges encountered in conventional drug delivery systems are greatly overcome by this drug delivery system(Kumar et al., 2012, Kutmalge et al., 2014, Patnaik et al., 2013).

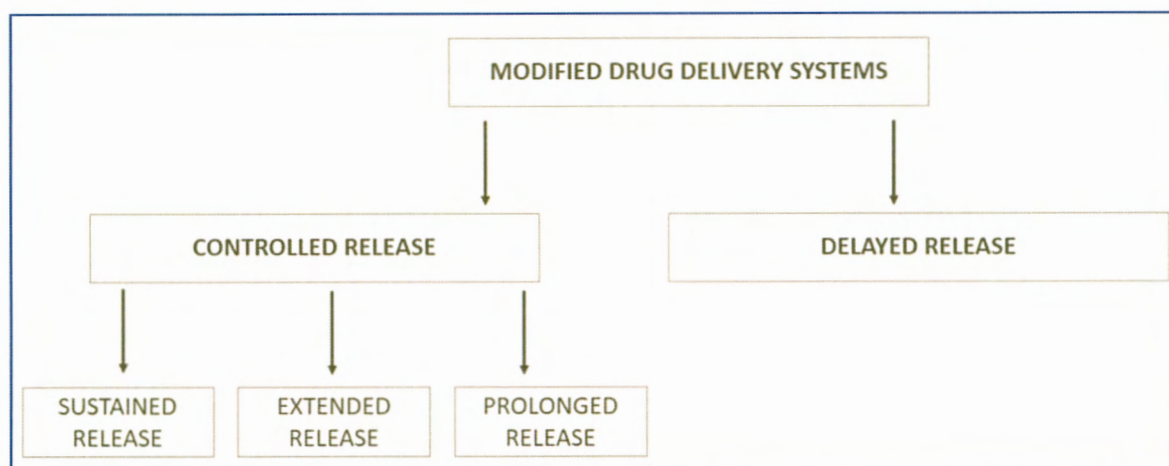


Figure 2.10: A figure showing the classification of sustained/modified drug delivery system

Several physiochemical and biological factors have been associated to the way by which drugs are administered through a sustained drug delivery system. These includes:

- Dose size: Generally, in a conventional dosage a single dose of drug which contains between 500 mg-1000 mg of the drug is considered the maximum for any drug that is administered to patients and same is considered as the maximum dose in sustained drug delivery system.
- Ionization, pKa and aqueous solubility: Some drugs which are usually in unchanged form permeate across lipid membranes while most drugs are either weak acids or bases. It is therefore important to consider the relationship of the pKa of the compound and the absorptive environment. In addition, delivery system that depends on diffusion or dissolution will likewise be dependent on the solubility of the drug in aqueous media. Due

to the fact that the stomach is acidic and the small intestine is relatively more neutral, the dosage forms of any drug must be designed in such a way that it can function in a pH changing environment.

- **Partition Coefficient:** Administered drug compulsorily passes through a number of biological membranes, which are commonly lipid-based before its therapeutic effect in the targeted area of the body is felt, hence the need to consider the partition coefficient of oil-soluble drugs in order to determine the effectiveness of membrane barrier penetration. Lipophilic compounds with high partition coefficients are poorly aqueous soluble, hence they remain in the lipophilic tissue for a longer time, while the membrane of compounds with very low partition coefficient are very difficult to penetrate, resulting in poor bioavailability.
- **Stability:** The stability of drugs is a factor which is greatly put into consideration in any drug delivery system before drug is administered. This is because it has been observed that drugs that are orally administered are subjected to both acid base hydrolysis and enzymatic degradation. Usually, a continuous reduced degradation rate is noticed for drugs in solid state thus making it the preferred composition of delivery for problem cases. In cases of drugs with unstable dosage form in the stomach, systems that prolong delivery over the entire course of transit in the gastrointestinal (GI) tract are of huge advantage. This is also true for systems that delay release until the dosage form reaches the small intestine because compounds which tend to be unstable in the small intestine may show decreased bioavailability when administered from a sustained dosage form (Li et al., 1987, Deshpande et al., 1996).

- **Half-life:** The half-life of a drug is the time it stays in the body system (residence time).
The preferred half-life of any drug should, ordinarily be between 3-4 hours for many formulations of drug delivery systems. The dosage form of a drug with short half-life (< 2 hours) may contain excessive large quantity of the drug. On the other hand, a drug with longer half-life (≥ 8 hours), however it is administered, i.e., either through conventional dosage or sustained release, are sufficiently controlled in the body.
- **Therapeutic index:** A drug that has a high conventional dosage form is a less appropriate drug for sustained release drug delivery system because it will be impossible to administer such drugs without difficulty, due to the size of a unit dose of oral formulation.
- **Absorption window:** Some drugs when orally administered can only be absorbed from a particular part of the gastrointestinal tract and this is known as 'absorption window'. Such drugs are equally not suitable for sustained releases drug delivery system.
- **Plasma concentration response relationship:** The pharmacological activity of a drug is largely dependent on plasma drug concentration rather than the dosage. Hence, a drug that its pharmacological activity, is independent of plasma concentrations and are not ideal for oral sustained release drug delivery system.
- **Metabolism:** Orally administered drugs undergo significant metabolism before they are absorbed into the lumen or tissues of the intestine and the drug is released slowly thus resulting in decreased bioavailability of the drug due to enzymatic degradation and thereby making available low active drug ingredients. Therefore, it is necessary to give keen attention when formulating drug dosage forms in order to prevent them from being affected by such enzymatic actions or the need of the delivery system capable of withstanding the

level of degradation by the enzymes, in order for it to have enough bioactive drugs in the formulation for efficient and sustained release.

- Concentration dependency on transfer of drug: For a drug to be transferred from one compartment to other, it either follows the zero-order kinetics or second order kinetics. A drug therefore is considered a poor choice for oral sustained release delivery system if it follows zero order kinetic process rather than the first order kinetics (Jantzen and Robinson, 1996, Bhatt, 2010, Popli and Sharma, 1989).

2.8 DUAL DRUG DELIVERY SYSTEM (DDDS)

In order to improve the curative effect of drugs, DDSs have been developed and studied in great depth for the past two decades and it has been discovered that drug delivery system can amend the problems of conventional administration by enhancing drug solubility, prolonging the duration or time of release, reducing side effect, and retaining drug bioactivity among others (He et al., 2008). One of such systems developed is the dual drug delivery system. Dual drug delivery systems are systems which have the ability to control the release behaviour of more than one drug (multiple drugs). In recent times, the use of combination therapy has increased and it has become quite attractive in the treatment of various diseases because it has been discovered that the combination of drugs has better therapeutic effect and also brings about tissue regeneration. For the optimization of their effects, it is required that the different drugs be used at their optimal dose and at different periods in the treatment. A major challenge of this combination therapy, however, is the inability to control the release behaviour of each drug independently, hence the development of DDDS, which can control the release behaviour of each drug as needed, since single drug delivery systems are not sufficient to overcome the challenge. (Lee et al., 1999, Ma et al., 2010, Lee et al., 2008b, Mok et al., 2008, Manna and Patil, 2009, Fundueanu et al., 2008, Huynh et al., 2008, Baumann et

al., 2009, Nakamura et al., 1999, Wang et al., 2008, Qiu and Bae, 2007, Song et al., 2008, Xia et al., 2008). However, thus far there has been limited research on dual drug delivery systems.

2.9 DRUG DELIVERY DEVICES

As a result of the development of alternate drug delivery systems, it became important and hence highly imperative for delivery devices with compatible properties to be designed. The major biomaterials that are generally and commonly used as drug delivery devices are polymeric drug carriers. Polymeric delivery systems targeted at specific sites in the body, can either be a reservoir type, or a matrix type. In the case of the reservoir type, the polymeric envelope where the drug is enveloped, serves as a diffusional rate-controlled membrane while a matrix type is that which the drug is evenly dispersed in a polymer matrix (Fulzele et al., 2003, Sharma et al., 2011). However, most biodegradable systems make use of the latter type, where drug release is by a combination of diffusion, erosion and dissolution factors. Currently, researchers are focusing on the use of two or more polymer types (hybrids of polymers) thereby, taking advantage of the collective properties of the individual polymers to enhance the capacity of the drug delivery systems (Omanathanu and Panchagnula, 2001). These polymeric substrates are widely used because they are degradable, nontoxic and biocompatible, and they can be eliminated from the body, without the need of surgery, after the encapsulated drug has been released. Such drug delivery systems that make use of polymeric carrier to deliver drugs via targeting are known as smart drug delivery systems (Tatsuya and Satoru, 2012). These biomaterials, made of polymers are therefore classified thus:

- ✓ Based on interaction with water
 - Non- biodegradable hydrophobic Polymers: - E.g. Polyvinyl chloride,
 - Soluble Polymers: - E.g. HPMC, PEG
 - Hydro gels: - E.g. Polyvinyl pyrrolidine

- ✓ Based on polymerization method
 - Addition Polymers: - E.g. Alkane Polymers
 - Condensation polymers: - E.g. Polyesterene and Polyamide
- ✓ Based on polymerization mechanisms
 - Chain Polymerization
 - Step growth Polymerization
- ✓ Based on chemical structure
 - Activated C- C Polymer
 - Inorganic polymers
 - Natural polymers
- ✓ Based on occurrence
 - Natural polymers: - E.g. Proteins- collagen, keratin, albumin, cellulose
 - Synthetic polymers: - E.g. Polyesters, polyamides
- ✓ Based on bio- stability
 - Bio- degradable
 - Non-Bio- degradable

(Gandhi et al., 2012, Omanathanu and Panchagnula, 2001, Mody, 2010, Fulzele et al., 2003, Sharma et al., 2011, Raizada et al., 2010).

2.9.1 SMART POLYMERIC DRUG DELIVERY DEVICES

A lot of high molecular weight materials have their generic names as polymers. These materials contain a very large number of atoms and different types of atoms, thereby making them to exist in countless forms with different chemical structures, physical properties, mechanical behaviour, and thermal characteristics among others, hence their different classifications and diverse

applications in different fields, such as: controlled drug delivery, biomedical applications, and tissue engineering. Certain synthetic polymers (hydrogels) are called ‘smart’ polymers because of their ability to mimic the non-linear response of biopolymers (DNA, proteins etc.), caused by cooperative interactions between monomers and also due to their ability to excellently absorb water and undergo changes in their structure in response to different stimuli and reverse to its initial state. In addition, they resemble natural living tissues, they are permeable and possess swelling characteristics and hence, they are termed ‘intelligent’ or ‘stimuli responsive drug delivery systems’ (Galaev and Mattiasson, 1999, Lowman and Peppas, 1999, Kost and Langer, 2012). Apart from these characteristics, other properties that make them excellent for drug delivery, include: ease of manufacturing, biocompatibility, strength, resilience, flexibility, ability to keep the drug stable, excellent carriers of nutrient to the cells, easily charged by using adhesion ligands and they can be injected *in vitro* as liquid to create a gel with the body temperature (Mahajan and Aggarwal, 2011, Stuart et al., 2010).

2.10 HYDROGELS

Hydrogels are cross-linked, three-dimensional hydrophilic networks, which when in contact with water, can swell but cannot dissolve. It has been established that the water-loving nature of the hydrogel network which leads to its swelling is basically because of the hydroxylic, carboxylic, primary amidic and the sulphonic chemical residues found within the polymer backbone or its lateral chains. This ability to swell enables them to serve as ideal materials for biomedical applications (Gao et al., 2007). It can be prepared in different physical forms, such as: slabs, micro-particles, nanoparticles, coatings, and films thus making them widely used in clinical practice and experimental medicine for a wide range of applications, such as: biosensors, biomolecules or cells

separation, barrier materials to regulate biological adhesions, tissue engineering and regenerative medicine (Fujiwara et al., 2006, Prasad et al., 2008, Entezami and Massoumi, 2006, Baroli, 2007).

Hydrogels can be classified on different basis; they can be classified as neutral or ionic, as affine or phantom, as homopolymers or copolymers based. They are classified based on the nature of the side groups, mechanical and structural characteristics, methods of preparation, physical properties of the network such as amorphous, semi-crystalline, hydrogen bonded structures, super-molecular structures and hydrocolloidal aggregates (Peppas et al., 2006). In neutral hydrogels, swelling occurs as a result of the elastic polymer contribution and water-polymer thermodynamic mixing contribution, to the overall free energy (Peppas et al., 2006). Stimuli-responsive gels are of great importance when compared to other classes of hydrogels. This is because their swelling behaviour is dependent on their physical environment, such as: pH, ionic strength, temperature, light, enzyme and magnetic field variation (Kato et al., 1997, Liu et al., 2007, Gu et al., 2009, Straley and Heilshorn, 2009, Zhao and Stoddart, 2009), hence they permit a wide range of applications (Lin and Metters, 2006). Therefore, these stimuli sensitive gels can act as 'smart' drug delivery systems (Kuckling et al., 2002, Wu et al., 2006, Feil et al., 1992).

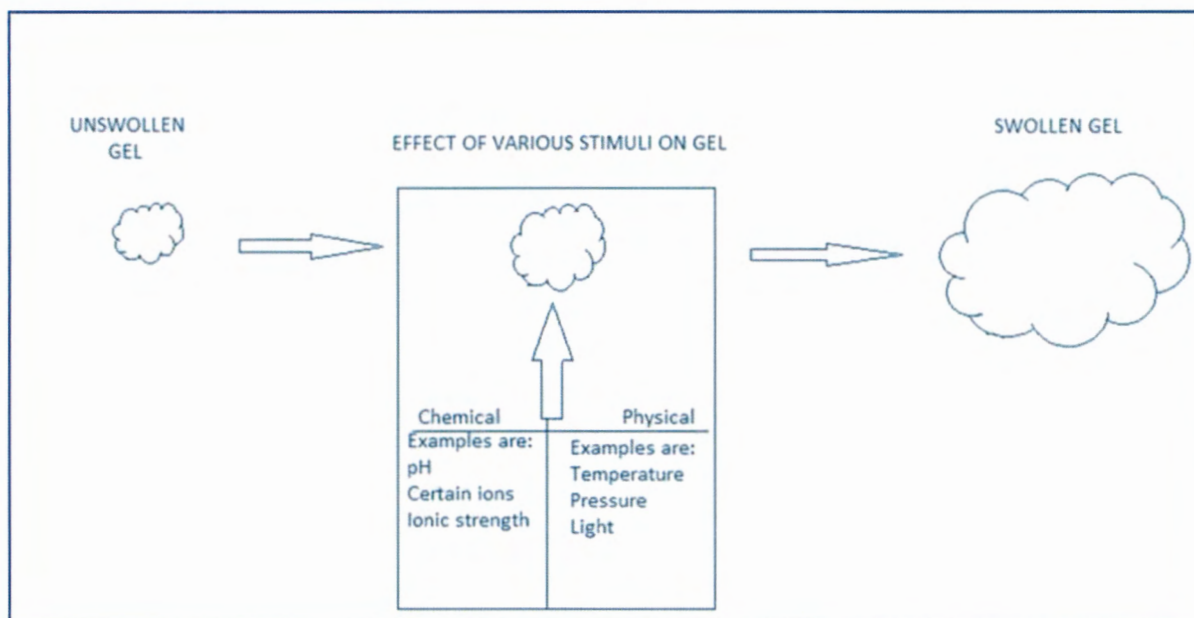


Figure 2.11: A figure showing the effect of stimuli on hydrogel

Furthermore, due to their unique biocompatibility, flexible methods of synthesis, range of constituents and desirable physical characteristics, hydrogels are extensively used in different biomedical fields. Polymers used for fabricating hydrogel scaffolds, provide: structural integrity to tissue constructs, control drug and protein delivery to tissues and serve as adhesives or barriers between tissue and material surfaces. The properties of hydrogels are important for tissue engineering and other areas of biomedical field. The increasing attractiveness of hydrogels is largely because they can hold drugs in the mesh of their polymeric network and control the release of such drugs to the body by means of diffusion, swelling or degradation (Lin and Metters, 2006), thereby avoiding the need for surgery in order to eliminate the carrier vehicle (hydrogel) from the body (Colombo et al., 2012). A lot of simple and complex models of hydrogels have been developed based on the rate-limiting step for controlled drug release carriers by using water-

soluble, biodegradable polymeric materials, either synthetic or natural polymers (Gudeman and Peppas, 1995, Park et al., 1998b, Wang et al., 2004, Wen and Stevenson, 1993).

In medicine, stimuli-responsive properties of hydrogels are noted for their response within the setting of biological conditions (Schmaljohann, 2006). There is usually a response from the hydrogel when there is a sudden change in the pH range or temperature, resulting in the interactions between the repeating units of the polymers and the solvent in which the polymer is put and this is translated to a chain transition between extended and compacted coil states (da Silva et al., 2008, Lupitskyy et al., 2005, Uhlmann et al., 2005, Yamato et al., 2002). In the structures of pH-sensitive polymers, which are polyelectrolytes, there exist weak acidic or basic groups attached to their hydrophobic backbone capable of either accepting or releasing protons in response to changes in the environmental pH. When they are ionized, a dramatic extension of the coiled chains is experienced in response to the electrostatic repulsions of the generated anions or cations (Aguilar et al., 2007). For the purpose of this research, emphasis is on a few selected polymer-hydrogels from different sources, based on their occurrence. These are:

- **Polyethylene glycol (PEG):** It is an example of a synthetic polymer-hydrogel, which is injectable. It is a very popular polymer with outstanding positive properties, since this assertion can be easily confirmed in the literature, thus allowing its usage to be very broad. It is used in everyday products, industrial applications, as well as in many biomedical drug delivery systems. PEG as a polymer has indeed played a vital role in drug delivery and its success in the field of biomedical drug delivery system is seen in several pharmaceutical products that have been approved in the last 20 years by the Food and Drug Administration (FDA) and European Medicines Agency (EMA). However, some structural parameters which influence the biological characteristics of PEG and even out its effect have to be

wisely considered before it is used (Torchilin and Trubetskoy, 1995, Kumari et al., 2010). The monomer unit of PEG is $\text{O}(\text{CH}_2)_2$ and each monomer of PEG has both a polar oxygen atom (O), and a non-polar $(\text{CH}_2)_2$ group thus making PEG to be soluble in a wide variety of both polar and nonpolar solvents (Schmaljohann, 2006) and has also been broadly used as a carrier for hydrophobic drug molecules in order to enhance their aqueous solubility or dissolution characteristics (Chiou and Riegelman, 1971, Vasconcelos et al., 2007, Leuner and Dressman, 2000). Polyethylene glycols are non-toxic, water soluble polymers capable of shielding drugs encapsulated into the polymer matrix due to their hydrodynamic volume, thereby guarding the drug from renal clearance, enzymatic degradation and recognition cells on the immune system (Kavitha and BhalaMurugan, 2013). The water loving nature of PEG has contributed to its success, especially because not every non-ionic hydrophilic polymer can provide stealth behaviour; this hydrophilic nature is based on its decreased interaction with blood components and its high biocompatibility (Knop et al., 2010).

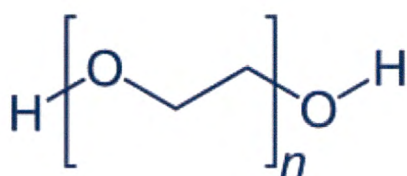


Figure 2.12: A figure showing the chemical structure of PEG (Kahovec et al., 2002)

- **N-Isopropylacrylamide:** It is a synthetic, biodegradable polymer that is temperature-sensitive and shows a sharp phase transition at a low critical solution temperature (LCST) of about 32°C in pure water. This polymer, when injected into the human body, it turns into a gel at body temperature and forms a small deposit, which makes controlled drug delivery

of any incorporated drug possible. This makes this polymer a widely studied material in drug delivery systems (Nakayama et al., 2006, Wei et al., 2009a).

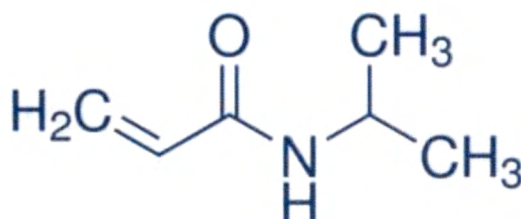


Figure 2.13: A figure showing the chemical structure of N-Isopropylacrylamide (Li et al., 2014)

- **Gum acacia:** Acacia gum is an edible and natural biopolymer, basically grown in the African region of the Sahel, Sudan. It is a non-viscous liquid that is rich in soluble fiber and it is obtained from the branches and stems of mature trees of Acacia Senegal or/and Acacia Seyel whenever there is drought, poor soil fertility, injury or any other harsh condition. This polymer is known to hydrate and form gel when they are in contact with water. It has been in use as adhesive and inks by the Egyptians as far back as the second millennium BC. Then, it was gradually exported to other parts of the world, especially Europe where it was named “gum acacia” because of the place it was imported from (Arabia)(Williams and Phillips, 2009). It is chemically composed of macromolecules of carbohydrate (D-galactose and L-arabinose) and proteins of different sizes, thereby making it a complex biomaterial (Islam et al., 1997). Factors, such as: origin, harvest season, age of the tree and processing condition (e.g. spray drying) influence the chemical composition of the gum, thereby causing slight variations in the chemical composition of the gum obtained from Acacia Senegal and Acacia Seyel. Although, the sugar residues are same however, lower contents of rhamnose and glucuronic acid while higher contents of arabinose and 4-O methyl glucuronic acid are found in Acacia Seyel gum when compared

to acacia Senegal (Flindt et al., 2005, Hassan et al., 2005, Siddig et al., 2005, Islam et al., 1997).

The use of this gum has been broadly explored and its application is seen in the industrial sectors such as: textiles, ceramics, lithography, cosmetics and also in the food industry as stabilizer, thickener or/and emulsifying agent (Verbeken et al., 2003). In addition, the pharmaceutical sector has also employed the gum as suspensions, in tablets and also in sustained drug release. Drugs released from the polymer are usually sustained over a long period because it is usually via diffusion, hence making it a good drug delivery device (Jain et al., 2008, Maitiet al., 2010, Tiwari et al., 2014). It can therefore be concluded, based on its physiological harmlessness and evidences, that the positive health related role of gum acacia cannot be over emphasized, hence its use as a drug delivery system.

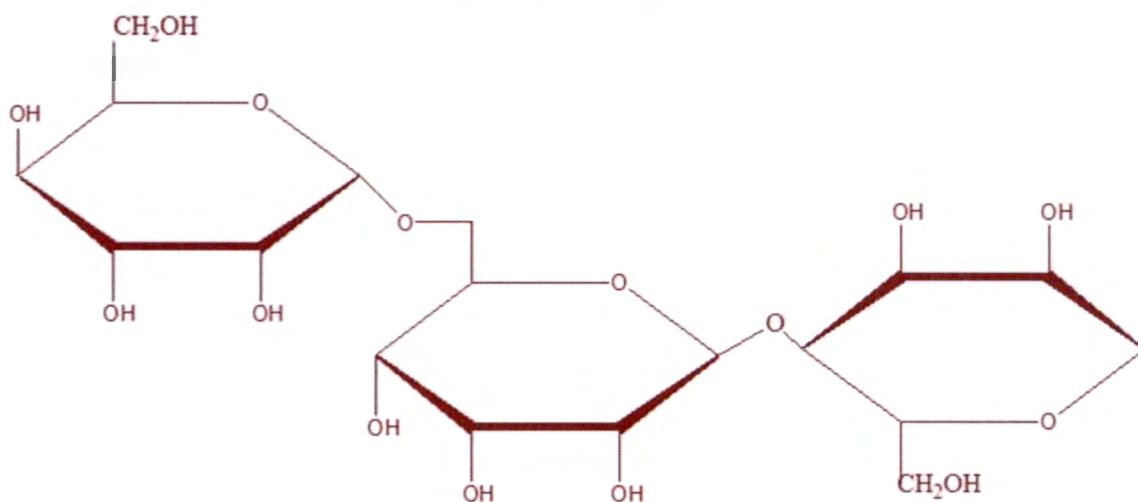


Figure 2.14: A figure showing the chemical structure of gum acacia (Azzaoui et al., 2015)

2.10.1 HYDROGEL DESIGN

Hydrogels are widely used in many foods and bioprocessing applications, aside the pharmaceutical field, because of their biocompatible and biodegradable properties, therefore any method of

hydrogel design or preparation must ensure that these properties are retained (Gunasekaran et al., 2007). Several methods have been employed over the years for hydrogel preparation, one of such is crosslinking and it is one of the majorly employed methods. Crosslinking can either be thermal crosslinking, physical crosslinking, ionic crosslinking or covalent crosslinking. Thermal crosslinking is known to be relatively easy, physical and ionic crosslinking mechanism are difficult to control in a lot of natural hydrogels and this affects the final network and structure. However, the crosslinking density and structural properties of cross-linked hydrogels are controllable and this makes them advantageous method because they permit the design of a wide range of polymeric networks for cell encapsulation and tissue growth (Durmaz and Okay, 2000).

Simultaneous copolymerization and crosslinking is also a method of hydrogel preparation. It involves one or more mono-functional and one multi-functional monomer or by crosslinking of a homopolymer or copolymer in solution (Rosiak and Yoshii, 1999). Quite a number of researches on the properties and swelling behaviour of hydrogels prepared by this method in the presence of an initiator and a crosslinking agents have been investigated (Tanaka, 1981, Okay, 2000, Liu et al., 1999, Durmaz and Okay, 2000).

Furthermore, free radical polymerization, in the presence of aqueous solutions, a monomer, crosslinker and an initiator, is another method that has been employed in hydrogel preparation. The component for the reaction is made up of various materials. An example is the use of a monomer–acrylamide (AAm), a crosslinker–methylene bisacrylamide (MBA), an initiator for the polymerization reaction process–potassium per sulfate (KPS) and an activator tetramethylenethyldiamine (TMEDA). Polymerization, reaction occurs first between KPS and TMEDA leaving TMEDA molecule with an unpaired valance electron. Activated TMEDA molecule then combines with acrylamide and the crosslinker molecules; in the process the

monomeric units receive unpaired electron, and thus become reactive. If the active center is continually shifted to the free end of the chain, it gives room for continuous indefinite growth of the polymer. Also simultaneously, the crosslinkers molecules are incorporated into the chains, thereby forming a permanent link between one another (Karadag and Saraydin 2002, Tanaka, 1981). Further attachment and activation of another monomer or polymer is made possible in the same manner (Park et al., 1998a, Park et al., 1998b, Yao et al., 1993, Wang et al., 2004).

2.11 CHARACTERIZATION OF HYDROGEL

2.11.1 FOURIER TRANSFORM INFRARED SPECTROSCOPY (FTIR)

Fourier Transform Infra-Red spectroscopy (FT-IR) is widely used for both qualitative and quantitative analyses in diverse science fields and it is widely applied in elucidating chemically synthesized structures or those of natural origin. Fourier Transform Infra-Red (FT-IR), when compared to dispersive infra-red technology, has a lot of advantages and applications (Baravkar et al., 2011). It is an absorption spectroscopy technique that utilizes the infrared portion of the electromagnetic spectrum to examine samples chemical structure. This is achieved when the spectrophotometer produces a beam of infrared light that is split into two (Brain and Smith, 1996). One of the two beams is at a fixed length and the other at a variable length (mirror A and mirror B respectively) (Silverstein et al., 2014). Destructive and constructive interferences are produced due to the varying distances between the two path lengths, thereby causing a variation in the intensities of the interferogram. The interferograms are converted by the Fourier transformation into one spectral point on the more familiar form of the frequency domain (Silverstein et al., 2014). From the variation of the length between mirrors A (fixed length) and mirror B (variable length), a complete IR spectrum is produced. For identification purposes, sample analysis is then carried out

by placing sample in the path of the generated beam and a frequency at which the sample absorbs the light, is determined (Griffiths and De Haseth, 2007, Brault, 1996).

2.11.2 SCANNING ELECTRON MICROSCOPY (SEM)

SEM is one of the most flexible and most heavily used analytical instruments with higher magnification, greater resolution, compositional and crystallographic information and a larger depth of field (than the ordinary light microscope) that permits a large amount of sample to be focused on, at the same time. It is used to examine and characterize microstructure morphology and chemical compositions of matter (Goldstein et al., 2003). Micro-view images of materials that is a good representation of the three dimensional sample, are usually formed, however, the naked eye and light microscope cannot view the materials because of their size. These images formed, depend on the acquisition of signals produced from the electron beam and specimen interactions (McMullan, 1995). These interactions can either be elastic or inelastic. The three major components that make up SEM are: electron-optical column, vacuum system and 52 electron and display system (Lawes, 1987). An electron beam, which is produced by an electron gun accelerated at high voltage and passed through an electron-optical column that is held under high vacuum is used to illuminate the image. A series of deflection coils move the electron beam across the surface of the specimen and signals are produced. Afterwards, an image is formed when these signals are collected, compiled and interpreted by a detector (Chapman, 1986).

2.11.3 THERMOGRAVIMETRIC ANALYSIS (TGA)

TGA is one of the thermal analytical techniques used to characterize and study the thermal properties of materials. It helps to give complimentary and supplementary information to other commonly used techniques such as DSC. It is an instrument composed of a thermo-balance, which

measures the rate and mass change (weight of the sample either reduces or increases) of a sample as a function of temperature or time, under a defined and controlled environment with respect to the heating rate, gas atmosphere, flow rate, crucible type (Coats and Redfern, 1963, Price et al., 2000). The aim is to measure the thermal and/or oxidative stabilities of materials, estimate product lifetimes, determine decomposition kinetics, determine the effects of the reactive atmospheres on materials, identify material purity, measure the filler content of materials, determine moisture and volatile contents as well as analyse the multi-components of materials. It is a technique that is very useful for the study of polymeric materials, including: thermoplastics, thermosets, elastomers, composites, films, fibers, coatings and paints (Liu and Yu, 2006). The TGA operates at temperatures ranging from 37°C to 1000°C. The point at which weight loss is most apparent is determined by a derivative weight loss curve. This curve provides information about changes in sample composition, thermal stability and the kinetic parameters for chemical reactions in the sample (Prime et al., 2009, Settle, 1997). A lot of factors are associated with the mass change during this thermal analysis, some of which include: physical phenomena, such as: gas adsorption, gas desorption, phase transitions, vaporization, sublimation and chemical phenomena such as: decomposition, break down reactions, gas reactions and chemisorption (adsorption by means of chemical instead of physical forces).

2.11.4 X-RAY DIFFRACTION (XRD)

Since the discovery of X-rays in 1895 (Rullhusen et al., 1998), the crystallinity of samples at the atomic level, has been examined and it has become a major tool for characterization in solid state chemistry and material science. It determines the structure of polymers by comparing the X-ray wavelengths to the interatomic distances in crystals. The working principle of XRD is such that samples are placed in the path of a monochromatized X-ray beam of low divergence, where the

scattered X-rays from the regularly placed atoms interfere with each other, thereby producing strong diffraction signals in particular directions, which are related to the slope and dimensions of the unit cell of the crystalline lattice, and the diffraction intensity depend on the disposition of the atoms within the unit cell. It is important to note that the intensity of the X-ray scattering is dependent on the number of electrons in the atoms (Atkins, 1989). Structure determination and fingerprint characterization of crystalline materials are the two major areas where this characterization technique has been greatly explored (Ealick, 2000). There is usually a unique characteristic X-ray powder pattern for every crystalline solid, and this may be used as a "fingerprint" for its identification. X-ray crystallography may then be used to determine its structure that is, how the atoms pack together in the crystalline state and the interatomic distance and angle, after the identification of the material (Scapin, 2006). The main information obtained from the result of the diffraction is the structure of the semi crystalline polymers with a range of interatomic distances of between 1 – 50 Å. In addition, the determination of the size and the shape of the unit cell for any compound, is made possible by using X-ray diffraction (Willard Jr, 1974, Alexander, 1969, Sawyer et al., 2008, Atkins, 1989, Formica, 1997).

CHAPTER THREE

3.0 EXPERIMENTAL METHODOLOGY

3.1. INTRODUCTION

All the experimental methods employed to achieve the set-out objectives stated earlier in chapter 1, are outlined and explained in this chapter. These experiments are: the preparation of the carrier polymer hydrogel by free radical polymerization, pH and swelling analysis, drug loading and release studies, cell culture and treatment techniques. The results obtained from these procedures are discussed in chapter 4, under “Results and Discussions”

3.2. CHEMICALS AND REAGENTS

MCF-7 cell lines were purchased from Cellonex cell line, LOT: 01. All chemicals used in this project were analytical grade and they were purchased from Sigma Aldrich Company and Separations Technology, South Africa. Acrylamide (AAM), Curcumin and Doxorubicin were supplied by Sigma Aldrich (Darmstadt, Germany) and N,N-methylenebisacrylamide (MBA), gum acacia, acrylic acid, acrylamide, N-isopropylacrylamide (NIPAM), N,N,N,N-Tetramethylethylenediamine (TEMED), potassium persulfate (KPS), Polyethylene glycol (PEG), were supplied by Sigma Aldrich Chemical Co. (South Africa). Cell culture reagents were obtained from Separations Technology, South Africa.

3.3 SAMPLES PREPARATIONS AND CHARACTERIZATION

3.3.1 INSTRUMENTATION

Fourier Transform Infrared (FTIR) spectroscopy, X-Ray Diffraction (XRD), Thermogravimetric Analysis (TGA) and Scanning Electron Microscope (SEM) were employed for the different characterizations.

3.3.2. PREPARATION OF SAMPLES FOR CHARACTERIZATION

3.3.2.1 FOURIER TRANSFORM INFRARED (FTIR)

About 70 mg of the hydrogel sample (drug loaded and unloaded) was analysed by placing the sample on the diamond sample surface of a Perkin Elmer Spectrum 100 FTIR spectrometer in the wavelength range of between 4000-500 cm^{-1} . The number of scans used was set at 16 at a resolution of 4 cm^{-1} . The background was scanned to act as a control before the sample was analysed.

3.3.2.2 X-RAY DIFFRACTION (XRD)

Identification of the interaction of the drugs within the polymer hydrogel was achieved by using X-ray diffraction. This experiment was carried out on dried samples on a PANalytical X'Pert PRO diffractometer (CuK_α radiation, with a wavelength, $\lambda = 0.1546 \text{ nm}$) running at a voltage of 40 kV and current of 40 mA. The sample was packed into a hole of between 1–2 mm in diameter, in a piece of metal of $\sim 1.5 \text{ mm}$ thick. Afterwards, the metal piece was placed on a specimen holder in order to allow the X-ray beam pass through. The XRD technique enables a clear understanding of the changes in the morphological structure and the changes in the crystalline nature of the sample by resolving its phase structure.

3.3.2.3 SCANNING ELECTRON MICROSCOPE (SEM)

Before the sample was analysed, the SEM machine was filled with liquid nitrogen. Having fixed a carbon tape on a slide sample holder, the sample was mounted onto a stub with a carbon tape for analysis. The sample was coated with gold in order to make it electrically-conductive and then loaded into a Jeol JSM-5600 SEM. Images were formed at different magnifications after appropriate focusing was done.

3.3.2.4 THERMOGRAVIMETRIC ANALYSIS (TGA)

Samples of about 7 mg were measured in the sample holder and loaded onto a TA TGA Q500 machine, an inert gas (nitrogen) was used to purge the samples in order to avoid oxidative decomposition. It was ensured that there was no moisture on the plate so that the results are not interfered with. The instrument was set at a heating rate of 10°C per minute, under air and ramped up to 900°C. From this analysis, the plot of the weight loss is obtained and further analysis was done on the data in order to obtain the points of inflection.

3.3.2.5 UV-VISIBLE SPECTROPHOTOMETER

The amount of drug released from the hydrogel was determined by using a Cary 100 UV-Visible spectrophotometer. Prior the analysis, the spectrophotometer was switched on for 20 minutes in order to allow sufficient heating up time. The wavelength used for the analysis was dependent on the wavelength of the drug loaded onto the hydrogel. Before recording the absorbance of the samples, auto zero and standards were run in order to ensure that accurate results are obtained.

3.4. EXPERIMENTAL PROCEDURE

The following subheads consist of all the experimental procedures employed in this investigation:

3.4.1. THE SYNTHESIS OF THE HYDROGEL

Table 3.1: A table showing the composition of hydrogel

Material	Quantity
Gum acacia	0.05 g
Acrylic acid	0.5 g
Acrylamide	1 g
PEG (4000)	0.5 g

MBA (6.49mM)	2 mL
N-isopropylacrylamide (1% solution)	2 mL
KPS (36.7mM)	3 mL
TEMED(86mM)	3 mL

The PEG-based hydrogel was prepared by free radical polymerization technique. 0.05 g of gum acacia was weighed into a beaker, 2 mL MBA (cross linker) was added and stirred until gum acacia had dissolved. 1 g of acrylamide and 0.5 g of PEG was added to the solution with continuous stirring, followed by the addition of 0.5 g of acrylic acid, 2 mL of N-isopropylacrylamide, 3 mL of KPS (initiator) and 3 mL of TEMED (accelerator), respectively. The resultant solution was placed on a hot plate at a temperature of 40-50°C, resulting in the formation of a gel. The formed gel was soaked in distilled water for 24 hours so as to get rid of unreacted amines and dried for 3-4 days.

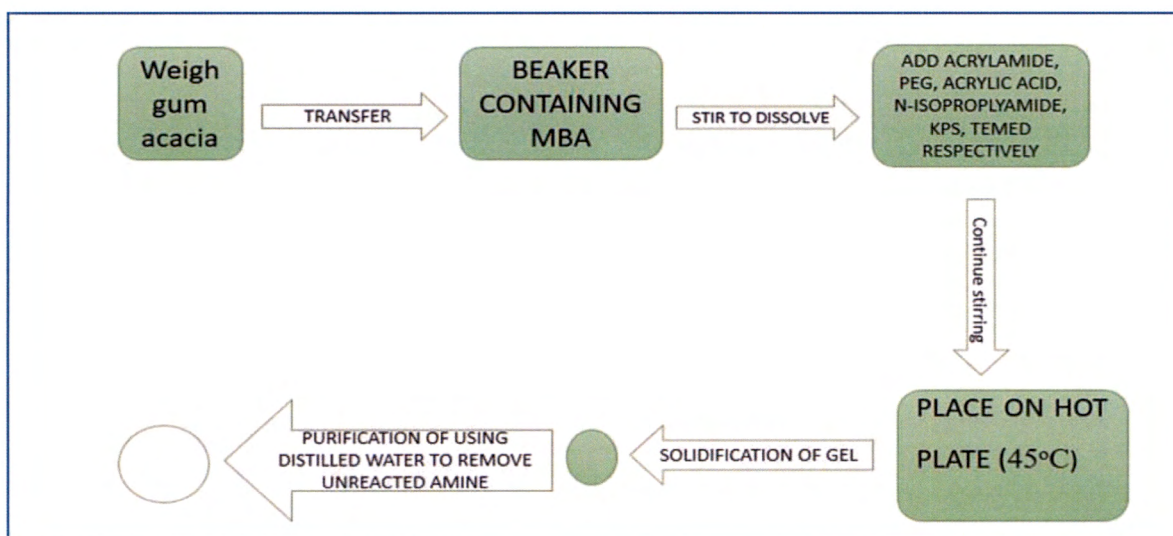


Fig 3.1: A figure showing the preparation of hydrogel by free radical polymerization

3.4.2. SWELLING STUDIES OF HYDROGEL

pH swelling study was performed after the hydrogel was prepared. Swelling ability is a characteristic of smart DDS, enabling them to hold drugs in the mesh of the polymeric network and control the release of such drugs to the body by means of diffusion, swelling or degradation (Lin and Metters, 2006). As shown in Figure 3.2, gravimetric method was employed to measure the equilibrium swelling behaviour of the hydrogel (Varaprasad et al., 2010). At different pHs, the swelling capacity of the hydrogel was tested by allowing it to swell in varying pH solutions. About 0.3 g, which is the initial weight of the gel, was weighed and put in beakers containing buffer solutions (pHs: 1.2, 5.8 and 7.4) of about 25 mL at ambient temperature. Samples were weighed after blotting with filter paper, at various time intervals in order to obtain varied weights (W1, W2, W3, W4, W5, W6, W7, W8, W9, W10, W12, W13, W14, W15 and at equilibrium W16) of the water absorbed by the hydrogel. This was achieved by using an electronic weighing balance. The readings were taken at intervals of 30 minutes for 6 hours, then 12, 24 and 48 hours respectively and finally the equilibrium weight was determined at 72 hours.

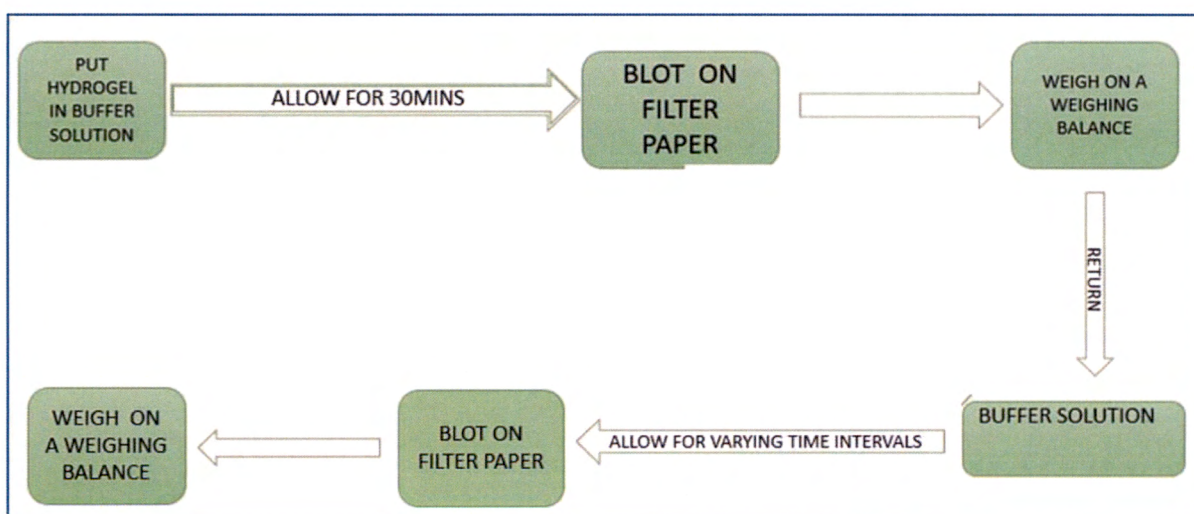


Fig 3.2: A figure showing the pH swelling analysis of the synthesized hydrogel

3.4.3 SWELLING CAPACITY DETERMINATION PROCEDURE FOR THE HYDROGEL

The swelling capacity of the hydrogel was determined by performing the experiment by using the procedure of (Varaprasad et al., 2010). The hydrogel was put in selected buffer solutions at ambient temperature and allowed to swell until the swelling equilibrium was reached (72 hours). It was then weighed after getting rid of excess water on the surface of the hydrogel by blotting with a filter paper. The hydrogel was weighed every 30 minutes for 6 hours and subsequently, 24, 48 and 72 hours, respectively. The swelling ratio and equilibrium swelling ratio of the hydrogel in the buffer solution was calculated gravimetrically and from the following relation.

Equilibrium swelling ratio:

$$Sr = \left(\frac{W_t - W_o}{W_o} \right) \dots\dots\dots \text{equation 3.1}$$

$$Seq = \left(\frac{W_s - W_t}{W_s} \right) \dots\dots\dots \text{equation 3.2}$$

$$S \% = \left(\frac{W_t - W_o}{W_o} \right) \times 100 \dots\dots\dots \text{equation 3.3}$$

$$\frac{W_t}{W_{\infty}} = Kt^n \dots\dots\dots \text{equation 3.4}$$

where:

Sr = Swelling Ratio

Wt = Weight at time t

W₀ = Initial hydrogel weight

W_s = Swollen hydrogel weight

S% = Swelling percentage

S_{eq} = Equilibrium swelling ratio

The solvent diffusion and polymer matrix relaxation effect was calculated and analysed by examining the exponent n from equation 3.4.

$$\frac{W_t}{W_\infty} = Kt^n \dots\dots\dots \text{equation 3.4}$$

where:

W_t = Weight of hydrogel at time, t

W_∞ =Weight of hydrogel at equilibrium

K = Diffusion constant

n = Diffusion exponent.

When n = 0.5, it indicates a case I diffusion (i.e. a perfect Fickian diffusion), which implies that the rate of the network relaxation is faster than the rate of diffusion, but when n = 1.0, it indicates a non-Fickian diffusion, where water transport is controlled and the rate of diffusion is faster than the network relaxation. However, in a case where 0.5<n< 1.0, it indicates that the rate of penetration, mobility and segmental relaxation are compatible (Ritger and Peppas, 1987).

The diffusion exponent was determined from the slope of the graph of Ln (M_t/M_∞) versus Ln (t) for swelling ratio.

The diffusion coefficient was calculated by using the equation:

$$S = 4 \left[\frac{D}{\pi r^2} \right]^{\frac{1}{2}} \cdot t^{\frac{1}{2}} \dots\dots\dots \text{equation 3.5}$$

where D = diffusion coefficient of the hydrogel

r = radius of the hydrogel

S = fractional swelling

t = time

The diffusion coefficients were evaluated from the plot of S versus $t^{\frac{1}{2}}$ and these were obtained from the slopes.

3.4.4. DRUG LOAD PROCEDURE AND DETERMINATION OF CONCENTRATION OF DRUGS RELEASED FROM THE HYDROGEL

Five mg of the drugs were loaded onto ~100 mg of hydrogel, following the procedure of Varaprasad et al. (2011), but for the hydrogel containing both drugs, 2.5 mg each of the drug were loaded. In Table 3.2, the various drug dissolution formulation used for the experiment is clearly shown, where PCur, PDox and PDoxCur represent PEG-Curcumin, PEG-Doxorubicin and PEG-Doxorubicin-Curcumin, respectively. The hydrogel was soaked in the drug solutions at ambient temperature, until they reached equilibrium in the drug solutions. Distilled water was then used to rinse the gels in order to get rid of any excess drug present on the surface of the gels and then dried at ambient temperature for 5 days. Curcumin is not soluble in water and hence it was first dissolved

in sodium hydroxide before distilled water was added to make up the volume of solvent required for drug uptake of the hydrogel.

Table 3.2: A table showing the drug(s) loading unto the PEG-based gel

Gels	Cur (mg)	Dox (mg)	Distilled H ₂ O (mL)	NaOH (mL)
A (PCur)	5	-	9.5	0.5
B (PDox)	-	5	10	-
C (PDoxCur)	2.5	2.5	9.5	0.5

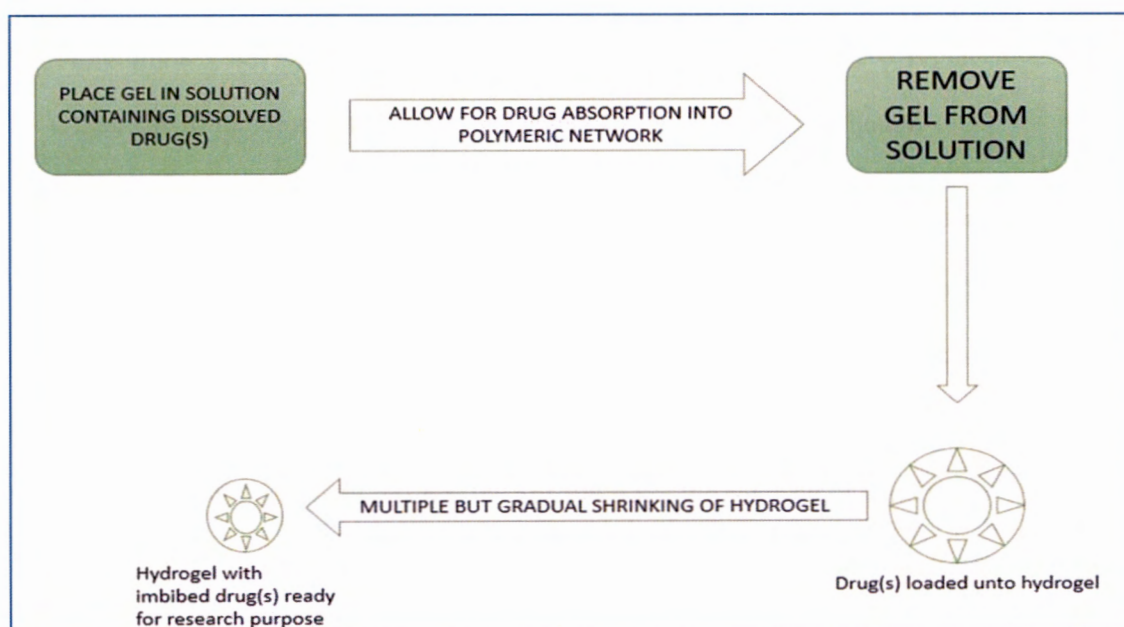


Fig 3.3: A figure showing the drug loading process unto the hydrogel

3.4.5. DRUG RELEASE STUDIES

It was necessary to carry out drug release study on the samples due to specific environmental change/stimuli such as pH. Figure 3.4 shows how this experiment was performed and afterwards, the drug release profile of the loaded hydrogel was calculated.

The various buffer solutions used were prepared and placed in a water bath shaker-BS-06 Lab Companion (set at 37°C) overnight in order to equilibrate. The hydrogel was then placed in 3 mL of the buffer in the water bath, set to 50 rpm at 37°C. At 30 minutes' interval, the entire 3 mL of the buffer solution in the water bath was pipetted and replaced with another 3 mL of the same buffer solution. They were stored in the fridge for further analysis. Buffer replacement was done for 6 hours at 30 minutes' interval and subsequently at: 24, 28 and 72 hours.

A UV spectrophotometer was used to analyse the stored buffer solutions in order to determine the drug release concentrations at the varied time intervals. Three buffers were used (at pHs: 1.2, 5.8 and 7.4) in order to stimulate the environment of the gastric juice in the stomach, the tumour cells and the human blood respectively. The amount of drug released was measured using the UV-Vis spectrophotometer in all three buffers; the absorption of the solution of curcumin was measured at 427 nm, while doxorubicin was at 480 nm. For the determination of the individual drug release from the combined drug loaded gel, the absorbance of the individual drugs were taken relative to the other.

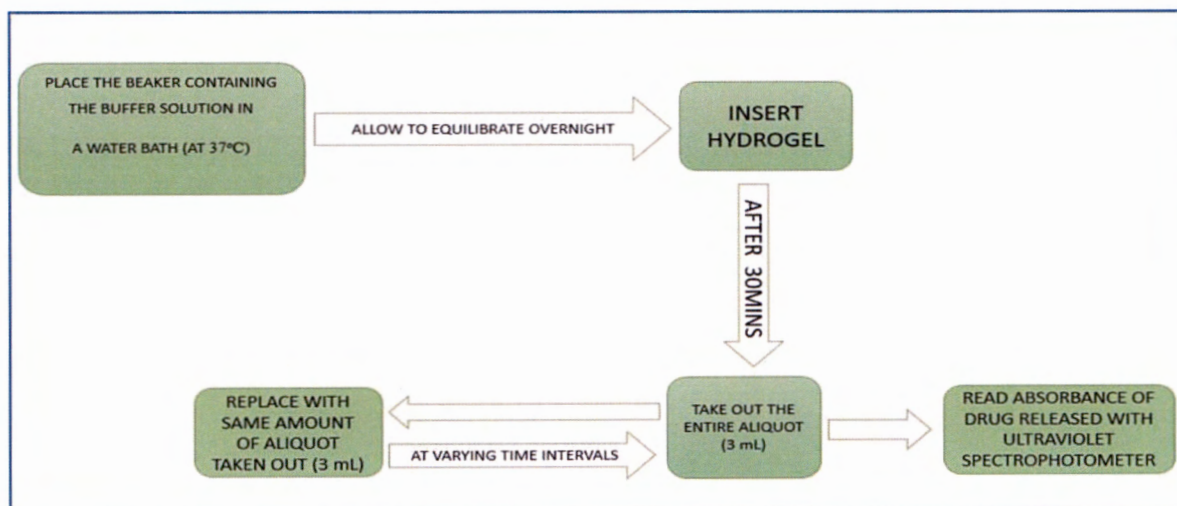


Fig 3.4: A figure showing the drug release from hydrogel

By using the percentage of cumulative release data obtained from equation 3.6, drug release kinetics was done as performed by (Aderibigbe et al., 2015).

$$\text{Percentage cumulative drug release} = \left(\frac{I_o}{I_f} \right) \times 100 \dots\dots\dots \text{equation 3.6}$$

where:

I_o = initial amount of drug released at time t

I_f = Total amount of drug loaded onto the hydrogel

Using the percentage of cumulative release data obtained from equation 3.6, different drug release kinetics models were employed in this studies. They are;

Korsmeyer Peppas:

$$\left(\frac{M_t}{M_\infty} \right) = K t^n \dots\dots\dots \text{equation 3.7}$$

where,

$\frac{M_t}{M_\infty}$ = fraction of drug released.

K = constant incorporating the structural geometric characteristics of the hydrogel.

n = release exponent

t = time of release

Zero order:

$$C = C_0 - K_0 t \dots\dots\dots \text{equation 3.8}$$

where,

C = amount of dissolved or released drug (assuming the release occurred rapidly after the drug dissolved)

C₀ = initial amount of drug in solution (usually zero)

K₀ = zero order rate constant

t = time

Hixson Crowell:

$$C_0^{\frac{1}{3}} - C_t^{\frac{1}{3}} = K_{HC} t \dots\dots\dots \text{equation 3.9}$$

where,

C_t = amount of drug released at time 't'

C_0 = initial amount of drug

K_{HC} = Hixson Crowell's rate constant

Higuchi:

$$C = [D(2qt - Cs)Cst]^{\frac{1}{2}} \dots\dots\dots \text{equation 3.10}$$

where,

C = total amount of released drug per unit area of the matrix (mg/cm^2)

D = diffusion coefficient for the drug in the matrix (cm^2/hr)

qt = total amount of drug in a unit volume of matrix (mg/cm^3)

Cs = dimensional solubility of drug in the polymer matrix (mg/cm^3)

t = time (hr)

3.5 CELL CULTURE STUDIES

The human breast adenocarcinoma, MCF-7 cell line was used to perform this study, consisting of different groups. Negative control (untreated group), Positive control (free drug(s), without the substrate, treated groups) and test group (PEG-based hydrogel loaded with drug(s)).

CELL GROWTH

The cells were grown in a 10% complete growth media, which contains FBS and MEM/EBSS with non-essential amino acids. 2 mM L-glutamine, 1mM sodium pyruvate and 1500 mg/L sodium bicarbonate, 5 mL insulin, 1% Penicillin/streptomycin, 2 mL trypsin EDTA solution for 1 week (media was replaced on alternate days) at 37°C and at a 95% relative humidity with 5% CO₂ following the procedures below, accordingly:

Cell resuscitation

The frozen cells were thawed quickly in a 37°C water bath and 1 mL of the cells was transferred to a centrifuge tube with a growth media (20% complete media, MEM/EBSS + FBS + insulin). Cells were centrifuged at 1500 rpm, 20°C for 5 min and media was removed and then a 20% complete growth media was added. Cells were re-suspended and transferred to a T25 flask and then incubated at 37°C for between 1- 3 days.

Splitting/passaging cells

Incubated cells were taken out of the incubator and checked for 80- 90% confluence. Old media was aspirated with Pasteur pipette and 3- 4 mLs of PBS was added twice in order to get rid of all waste materials which include trypsin inhibitor and serum. 3 mL of trypsin was added and

incubated for 5 minutes at 37°C. 10% complete media was added immediately in order to stop trypsin activity, which could damage the cells and vigorous pipetting was done in order to obtain a single- cell suspension. Cell suspension was spun in a 15 mL centrifuge tube for 5 min and at 4°C at 1000rpm. The liquid was aspirated and fresh media was added and then the cells were re-suspended by pipetting up and down in order to separate cells and eliminate clumps and finally, the cells were divided into 2 flasks. Cell splitting depends on the number of plates required for study.

Cell counting:

A 50 µL of cell suspension was put into a microcentrifuge tube and then 50 µL of trypan blue stock solution (0.5% (w/v)) in 1:1 ratio and thoroughly mixed in order to obtain a homogenous mixture. 15 µL of the mixture was pipetted into each of the two counting chambers of the haemocytometer in order to count the number of cells. The number of cells per mL in the suspension was calculated by counting the number of cells in the middle square and multiplying by 10^{-4} , since each square volume is 10^{-4} mL (Cell number/mL = Average cell number of two chambers $\times$ Dilution factor $\times 10^{-4}$).

Freezing cells

Freezing of cells is often necessary for backup cultures or cell reservation. This was done by freezing cells slowly with ice crystals in order to avoid killing the cells. Cells were trypsinized in order to detach and separate cells, then serum and the media (10% complete growth media) were added. Cells were spun for 5 minutes at 1500 rpm and at 4°C, the media was aspirated and appropriate amount of freezing media (10% DMSO and 90% FBS which is equivalent to the freezing media (10mL), 9 mL FBS and 1 mL DMSO) for the number of vials frozen (about 1 mL

per vial; 1mL/10million cells) was added. Cells were re-suspended in freezing media, transferred into a cryovial and frozen at - 80°C in an alcohol bath for between 1- 2 days before they were transferred to liquid nitrogen.

3.5.1 CELL VIABILITY ASSAY

Cell viability experiments are mostly carried out in order to determine a drug's efficacy. It is an experiment that is often done in the pharmaceutical or academic research and this can involve the synthesis of a new drug or modification of an existing test compound (drug). Usually, the outcomes of the assay can either be cytotoxicity or cell proliferation. In this study, water soluble tetrazolium (WST) proliferation assay was done in order to determine the cell toxicity, however, there are other experiments that can be done to determine the cytotoxicity of cells.

3.5.2 DRUG LOADING ON HYDROGEL

Five mg each of Curcumin (Cur), and Doxorubicin (Dox) were weighed, dissolved in appropriate solvents and filtered into separate vials, respectively. 10 different concentrations, ranging from 0.055-28 µg/mL were prepared and loaded onto the hydrogel.

3.5.3 TREATMENT OF CELLS

When the subcultured cells were 80% confluent, they were seeded in a 96 well plate at a density of 1×10^5 cell/mL in complete media without antibiotics for 48hrs. The medium was then aspirated, washed with fresh media and then treated with 100 µL of the drug(s) at different concentrations and the hydrogel loaded with the drugs. Cells were incubated for 24, 48 and 72 hours and then followed by the assay experiment. The WST assay test was carried out according to the manufacturer's instruction, which is 10 µL of WST, it was added to the wells and the plate was

incubated for 1½ hours. The absorbance was measured by using a microtiter plate reader at a wavelength of 450 nm and individual experiment was carried out three times. Figure 3.5 shows the cell differentiation as viewed under the light microscope at different days. Figure 3.6 shows the visual model for the 96 well plates for the WST assay cell treatment. The minimum inhibitory concentration (IC₅₀) value was determined by using Graph Pad prism software version 6.0

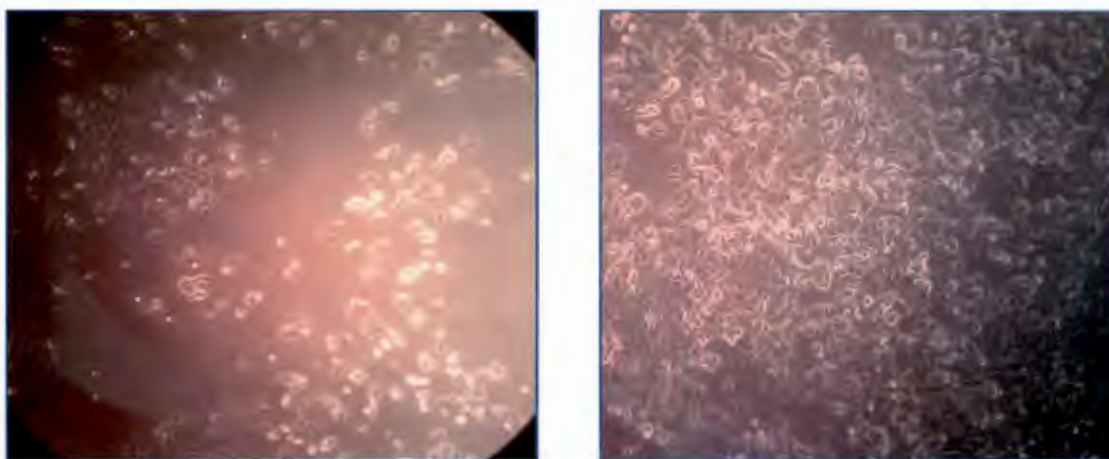


Fig 3.5: A figure showing (a) MCF-7 cell growth at day 3 and (b) MCF-7 cell growth at day 7

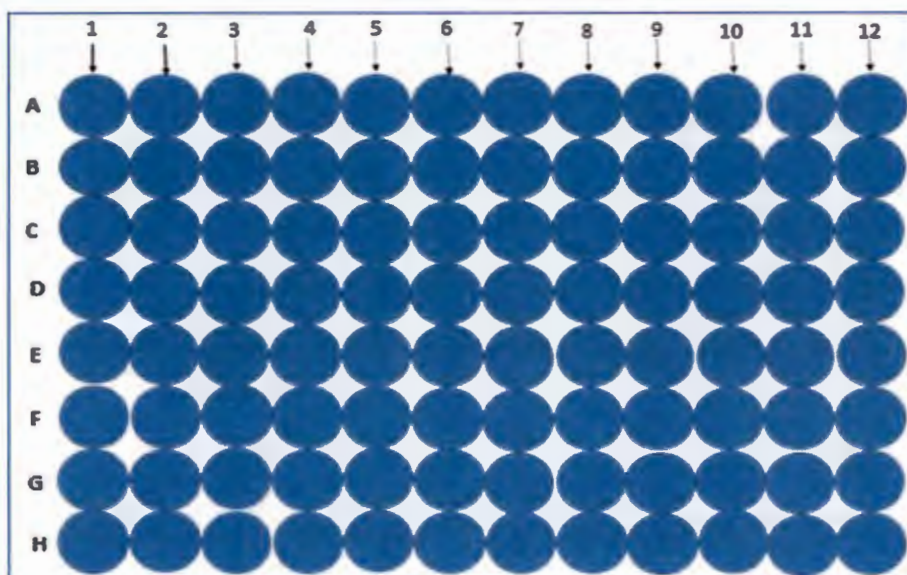


Figure 3.6: A diagram showing the visual model of the 96 well plates for WST assay cell treatment

A1-A12, A1-H1, H1-H12 and A12-H12 = wells without cells in order to remove interferences

B2-B11 = treatment (at different concentrations)

C2-C11 = treatment (duplicate of the different concentrations)

D2-D11 = treatment (triplicates of the different concentrations)

E2-E4 = Control

F2-F4 = Base

G2-G4 = hydrogel (without drugs)

CHAPTER FOUR

4.0 RESULTS AND DISCUSSIONS

4.1. INTRODUCTION

In this chapter, the results obtained from the experimental procedures: swelling at different pHs and drug loading analysis, morphological characterization, drug release and the *in vitro* cell studies of tests performed, are analysed, discussed and the relevance of these results is emphasized in order to adequately contribute to the field of breast cancer treatment.

4.2 RESULTS AND DISCUSSIONS OF DATA OBTAINED FROM STUDIES

4.2.1 HYDROGEL PREPARATION AND SWELLING

According to Shi and Jackowski (1998), the rate of free radical formation from radicals of KPS was accelerated by TEMED and polymerization was in turn catalysed. For polymerization chain reaction to begin, KPS free radicals, converts acrylamide monomers to free radicals which react with inactivated monomers (Shi and Jackowski, 1998). Elongating polymer chains are randomly cross linked by MBA giving rise to a gel with a characteristic porosity that is dependent on the polymerization conditions and monomer concentrations. From literatures, it is observed that the rate at which hydrogel swells is slower than the rate at which drugs are diffused from the polymer matrix of the hydrogel during drug release from the hydrogels and thus it is important to carry out swelling studies on the hydrogel because they are very relevant in the biomedical fields (Colombo et al., 1996). This swelling study was done at room temperature in varying buffer solutions (pHs of: 1.2, 5.8 and 7.4) over a period of 1 week. These buffers of 1.2, 5.8 and 7.4 were selected in order to mimic the gastrointestinal tract, tumour cells and intestinal fluid-blood respectively and also to observe the effect of different pHs on the swelling capacity of the hydrogel, since it is an important factor affecting the swelling capacity of hydrogels.

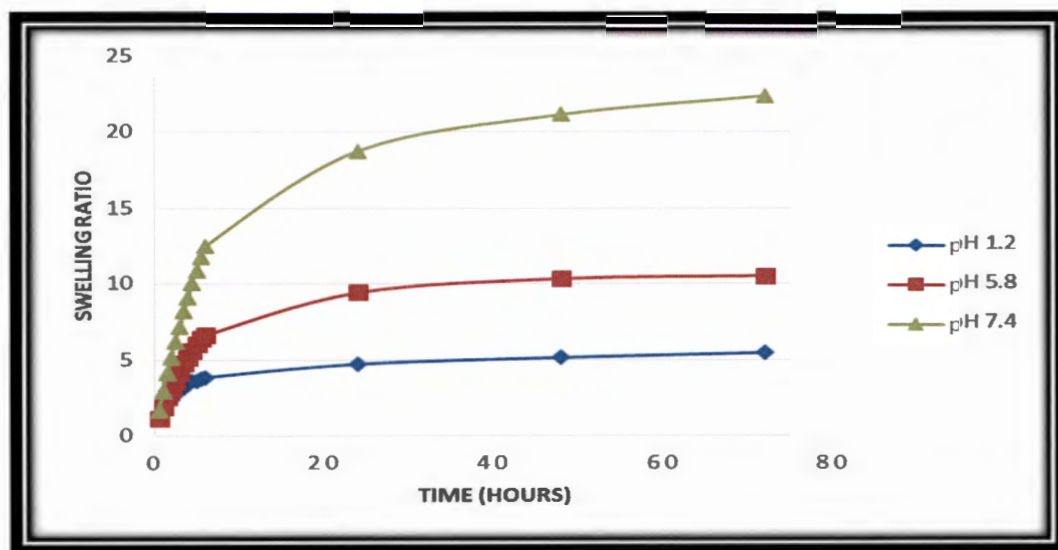


Fig 4.1: A figure showing the swelling of the PEG –based hydrogel in varying buffers at ambient temperature

It is observed from figure 4.1 that all the hydrogels in the different buffer solutions were swollen. This is because a shift in pH generally causes a change on the polymer chains thereby leading to swelling. Although the swelling rate for the hydrogel in the buffer of pH 7.4 was most rapid and had swollen more than half its maximum swelling capacity within 6 hours when compared to the other buffers (pHs: 1.2 and 5.8). Furthermore, at the end of 48 hours of the swelling studies, the same hydrogel (in buffer pH 7.4) attained the highest swelling capacity. Swelling was enhanced in pH 7.4 because the pH is above the pK_a of the ionizable group of the hydrogel and thus, as the degree of ionization increases (increased pH of the system), the number of fixed chains also increases, hence leading to an increase in the electrostatic repulsion between the chains which results in an increased network hydrophilicity and greater swelling (Brannon-Peppas and Peppas, 1991, Skouri et al., 1995, Ohmine and Tanaka, 1982). The results obtained suggest that the hydrogel has a super absorption characteristics and this is due to the presence of hydrophilic components in the hydrogel, as similarly reported by (Varaprasad et al., 2011). The dissolution of

the polymer chains in the hydrogel is prevented because of the cross linking between the chains, however, it makes room for the network of the hydrogel to expand. However, at one week, a slight reduction in weight was observed in the hydrogels in all the buffers when compared to the weight recorded at 72 hours. Similar observation was reported in studies carried out by Cozic et al. (2009) on gum acacia, thus the reduction in weight observed can be attributed to the presence of gum acacia as it is capable of undergoing water desorption.

Table 4.1 shows the analysis of the swelling ratio of the hydrogel in the different buffer solutions. The swelling exponent (n) was determined by the slope of the graph when $\ln (M_t/M_\infty)$ was plotted as a function of $\ln t$ and it was found to be: 0.504, 0.716, and 0.814 and this indicates that the solvent diffusion was Fickian in the buffer of pH 1.2 and non-Fickian (anomalous) in buffers of pHs 5.8 also 7.4 because the values of n are 0.5 and $0.5 < n < 1.00$ respectively while R^2 which is the coefficient of determination was 0.99 indicating an excellent linearity. Furthermore, the slope of the graph of SR as a function of $t^{1/2}$, which is the diffusion coefficient (R^2) of the hydrogel is the same in all the buffer solutions, as seen in Table 4.1. This is because the hydrogels in the different buffer solutions consist of the same preparation materials.

Table 4.1: A table showing the analysis of swelling ratio data obtained

pH	R^2 (Graph of $\ln$ SR vs $\ln$ Time)	n	k	R^2 (Graph of SR vs. $t^{1/2}$)	n	k
1.2	0.99	0.504	1.56	0.99	0.204	1.14
5.8	0.99	0.716	2.29	0.99	0.423	1.39
7.4	0.99	0.814	2.25	0.99	0.824	3.52

4.2.2 DRUG RELEASE

Anti-cancer drugs (curcumin and doxorubicin) were incorporated into the polymer gel, individually and in combination following the procedure of Varaprasad et al. (2011), according

to the drug loading formulation shown in Table 3.2. The ability of a hydrogel to absorb these two drugs revealed that the pore size of the hydrogel is large enough to entrap the drugs into their polymeric network, thus causing the hydrogel to swell. However, the swelling ability and capacity of hydrogel was influenced by the pH.

The plot of the *in-vitro* cumulative drug release of the hydrogels, loaded with the drug(s) against the time of release in the various buffer solutions (pHs of: 1.2, 5.8 and 7.4) is shown in the Figures 4.2 to 4.5 below using different drug release models. The percentage cumulative drug release indicating the percentage of drug that was released over a period of 72 hours in different pH is seen in the Table 4.2 below. The release of the individual drug(s) from the PEG-based hydrogel was faster than the hydrogel containing both drugs in all pHs (1.2, 5.8 and 7.4). The release of curcumin from hydrogel containing both drugs was higher than doxorubicin at pH 7.4 and 5.8.

Table 4.2: Showing the % cumulative drug release indicating the % of drug released over a period of 72 hours in different pHs

DRUGS	pH 1.2	pH 5.8	pH 7.4
Doxorubicin	79	68	62
Curcumin	42	91	72
Doxorubicin from DoxCur	65	57	50
Curcumin from DoxCur	34	83	67

From the Figures 4.2 to 4.5 below, it was observed that the hydrogel was sensitive to pH since the release profiles of the drug(s), individually and in combination, from the polymeric network of the hydrogel was influenced by pH and time. However, the release of doxorubicin was enhanced at a pH of 1.2. This is probably because of the solubility of doxorubicin, which increases in acidic pH environment. Furthermore, it can equally be due to the protonation of the groups present in the

doxorubicin and the hydrogel, thereby causing electrostatic repulsion between the hydrogel and doxorubicin and thus weakening the interaction between the hydrogel and doxorubicin. In the pHs of 5.8 and 7.4, the hydroxyl groups in PEG are not protonated, especially at a pH of 7.4. Thus the higher the pH, the more the number of un-protonated hydroxyl groups, and stronger the hydrogel/drug interaction. Therefore, an increase in the medium pH value leads to the reduction in the doxorubicin dissociation and hence the release rate. This observation is similar to the reports of Manocha and Margaritis (2010) and Dadsetan et al. (2013). On the other hand, in the drug release profile of curcumin, an enhanced release at pH 5.8 was observed. This is as a result of the anti-tumour properties possessed by curcumin, thereby making it more sensitive at a pH characteristic environment for cancer cells. This is in line with reports by Tabatabaei Mirakabad et al. (2016) and Zeighamian et al. (2016). Thus, this suggests that the hydrogel is a suitable device for targeting curcumin to cancerous cells which would bring about an increase in the efficacy of the drug as an effective anti-cancer agent. Generally, it is often desired that the release of drug from a targeted drug delivery system should be slow or minimal at physiological pH in blood circulation. The reason is that this reduces drug loss in its path to the target region of the body, and this was the case in this study, as adjudged from the results obtained. Therefore, this study suggests that the hydrogel is a capable device for targeted drug delivery over a long period of time.

For a clearer understanding of the mechanism by which these drugs were released, some widely employed mathematical kinetics release models for drug release were applied in this study.

Korsmeyer–Peppas model: It is a model which describes the first 60% of drug released from a polymeric system. The graph is plotted by using the logarithm of the cumulative percentage of drug released as a function of logarithm of time. This kinetic release model is represented by the equation below;

$$\left(\frac{Mt}{M_{\infty}} \right) = Kt^n \dots\dots\dots \text{equation 4.1}$$

where,

$\frac{Mt}{M_{\infty}}$ = fraction of drug released, at time t.

K = the constant incorporating the structural geometric characteristics of the hydrogel.

n = release exponent

t = time of release

According to Costa and Lobo (2001), there are different forms of diffusion and each is determined by the value of n when $\text{Log } Mt/M_{\infty}$ is plotted as a function of $\text{Log } t$ (for the first 24 hours of drug release). When $n = 0.5$, it indicates a Fickian diffusion, when $0.5 < n < 1$, it indicates an anomalous or non-Fickian diffusion, which refers to a combination of diffusion and erosion-controlled rate release. When $n = 1$, it indicates a case II transport phenomenon and when $n > 1$, it indicates a super case transport II phenomenon (Costa and Lobo, 2001). The linearity and diffusion exponent (n) were determined from the plot of $\text{Log } Mt/M_{\infty}$ is as a function of $\text{Log } t$ by using the data obtained according to equation 4.1.

A good linearity was observed in all buffer solutions with the R^2 , release exponent (n) and the correlation coefficients obtained, shown in Table 4.3. Result indicates the applicability of Peppas equation to this drug delivery system. At all buffer solutions, the value of n ranged between $0.5 < n < 1$ to $n > 1$, indicating non-Fickian diffusion to super case transport II, as shown in Table 4.4

Table 4.3: A table showing the release exponents, n and correlation coefficients, R^2 of the hydrogel with the loaded drug(s)

DRUGS	pH 1.2			pH 5.8			pH 7.4		
	n	R^2	k	n	R^2	K	n	R^2	k
Doxorubicin	0.823	0.98	1.22	1.000	0.99	1.22	0.903	0.99	1.25
Curcumin	1.000	0.99	1.10	1.335	0.98	1.78	0.950	0.99	1.09
Doxorubicin from DoxCur	0.747	0.98	1.73	0.984	0.99	1.03	1.131	0.99	1.62
Curcumin from DoxCur	1.210	0.98	2.19	1.448	0.98	2.13	1.014	0.99	1.46

At pH 1.2, the release of doxorubicin and doxorubicin from dox-cur was anomalous diffusion while the release of curcumin and curcumin from dox-cur was case II transport and super case II transport diffusion respectively. At pH 5.8, the release of doxorubicin and doxorubicin from dox-cur was case II transport diffusion and anomalous diffusion respectively while the release of curcumin and curcumin from dox-cur was super case II transport diffusion. At pH 7.4, the release of doxorubicin and doxorubicin from dox-cur was anomalous diffusion and super case II transport respectively, while the release of curcumin and curcumin from dox-cur was anomalous diffusion and case II transport diffusion respectively. In addition to these observations, as the n value increased, the k values also increased in all the pHs (1.2, 5.8 and 7.4).

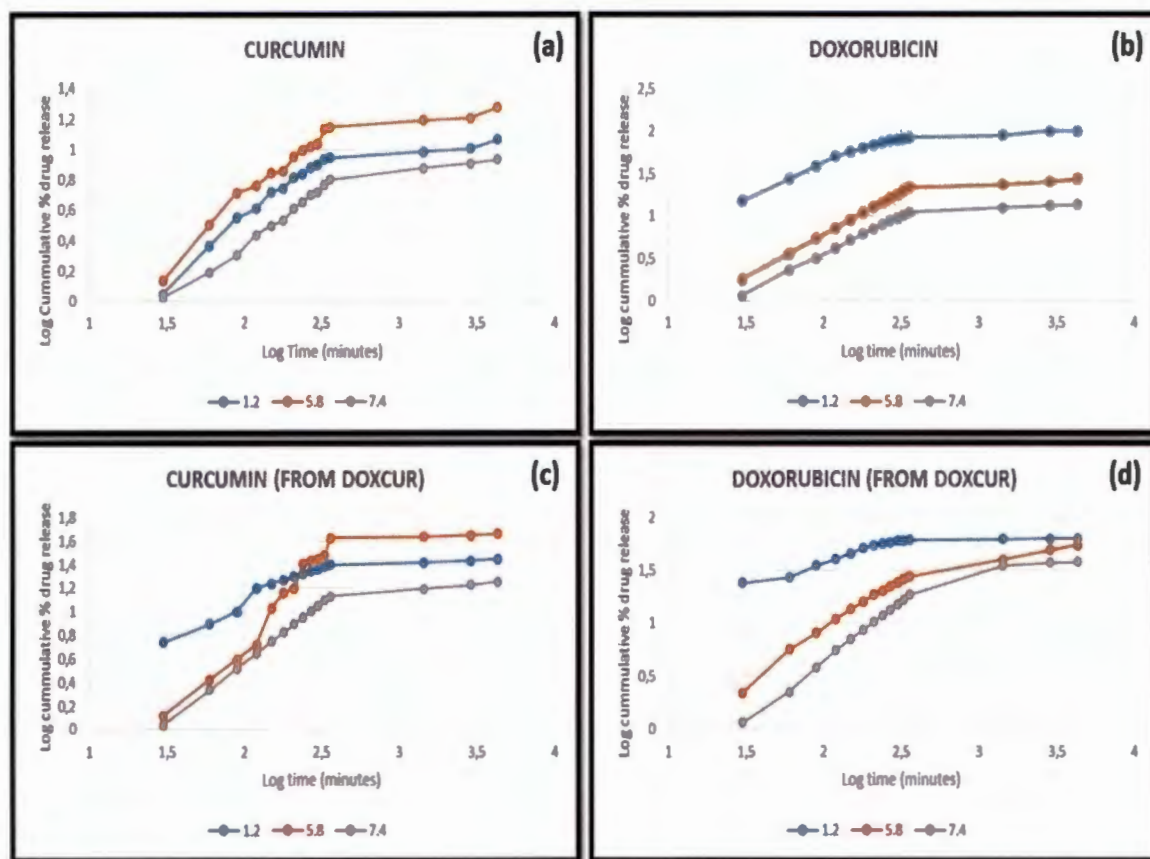


Figure 4.2: A figure showing the plot of Korsmeyer-peppas release kinetics of (a) curcumin from the hydrogel (b) doxorubicin from the hydrogel (c) curcumin from the hydrogel containing both drugs and (d) doxorubicin from the hydrogel containing both drugs.

Hixson crowell model: This model describes the drug dose released from a system where the surface area and diameter of the particle or tablet changes. In using this model, the drug particle dissolution rate is considered to limit the release rate, rather than the diffusion that may take place through the polymeric network. The graph is plotted as the cube root of the percentage of drug remaining in the polymer matrix as a function of time and this can be mathematically expressed by using the equation below:

$$C_0^{\frac{1}{3}} - C_t^{\frac{1}{3}} = K_{HC} t \dots\dots\dots \text{equation 4.2}$$

where,

C_t = amount of drug released at time 't'

C_0 = initial amount of drug

K_{HC} = Hixson-Crowell's rate constant

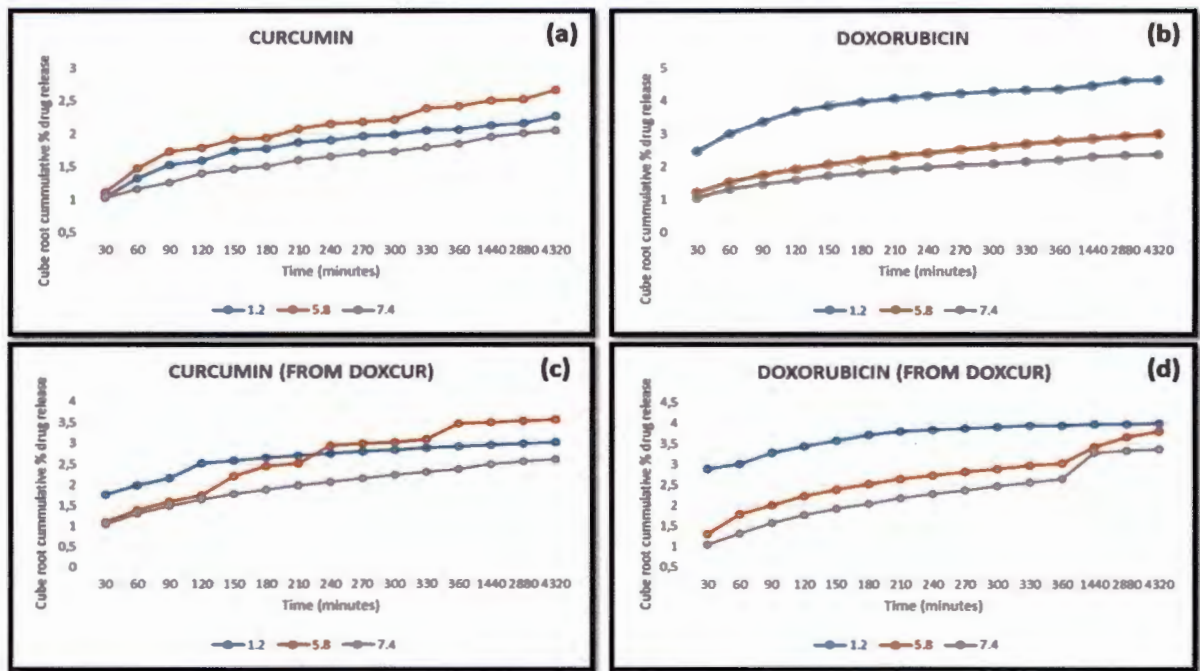


Figure 4.3: A figure showing the plot of Hixson Crowell's release kinetics of (a) curcumin from the hydrogel (b) doxorubicin from the hydrogel (c) curcumin from the hydrogel containing both drugs and (d) doxorubicin from the hydrogel containing both drugs.

Table 4.4: A table showing the constant, k and correlation coefficient, R^2 of drug release from hydrogel

DRUGS	pH 1.2		pH 5.8		pH 7.4	
	R^2	k	R^2	k	R^2	K
Doxorubicin	0.98	2.12	0.98	1.07	0.98	0.85
Curcumin	0.98	0.90	0.99	0.81	0.99	0.92
Doxorubicin from DoxCur	0.98	2.75	0.98	0.99	0.98	0.91
Curcumin from DoxCur	0.99	1.58	0.98	0.86	0.98	0.89

Zero order model: It is a drug release model that describes a system whereby the drug release rate is independent of its concentration. This relationship can be used to describe the dissolution of drugs from a lot of modified release pharmaceutical dosage forms, such as a matrix tablet with low soluble drugs, in a coated form. The graph is plotted as the cumulative amount of drug released as a function of time and the equation is as below;

$$C = C_0 - K_0 t \dots\dots\dots \text{equation 4.3}$$

where,

C = amount of dissolved or released drug (assuming the release occurred rapidly after the drug is dissolved)

C_0 = initial amount of drug in solution (usually zero)

K_0 = zero order rate constant

t = time

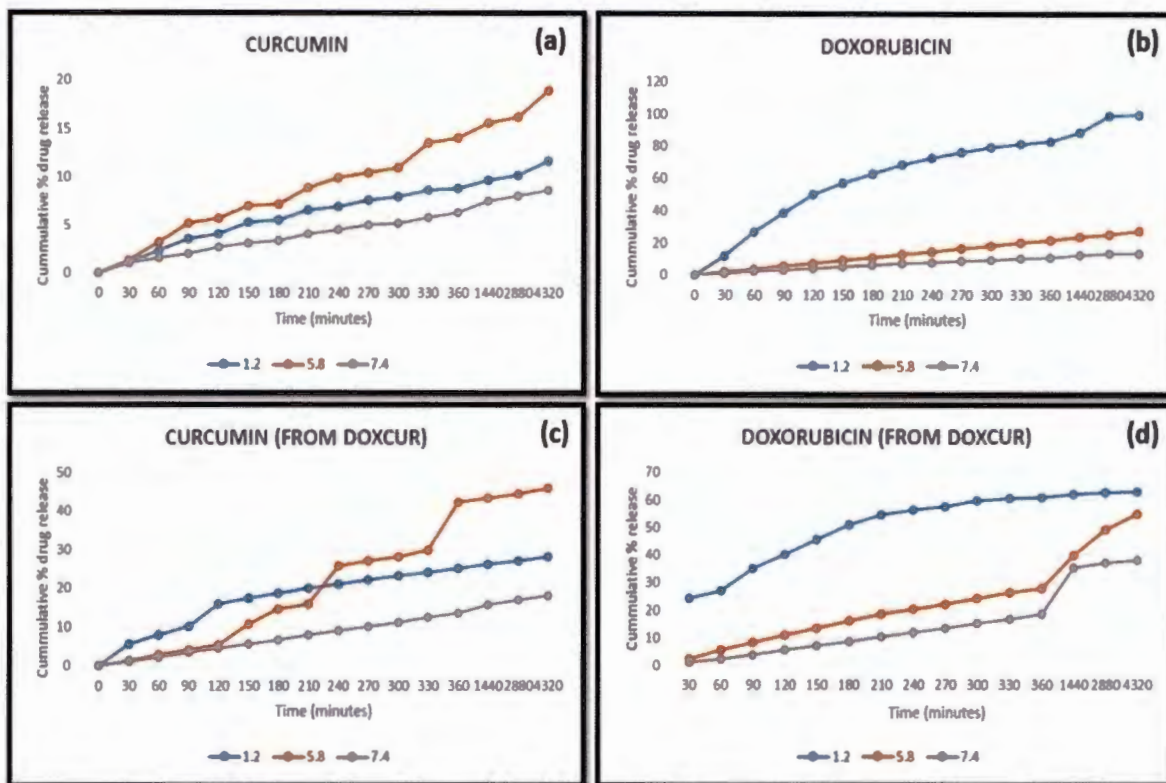


Figure 4.4: A figure showing the plot of zero order release kinetics of (a) curcumin from the hydrogel (b) doxorubicin from the hydrogel (c) curcumin from the hydrogel containing both drugs and (d) doxorubicin from the hydrogel containing both drugs.

Table 4.5: A table showing the constant, k and correlation coefficient, R^2 of drug release from hydrogel

DRUGS	pH 1.2		pH 5.8		pH 7.4	
	R^2	k	R^2	k	R^2	K
Doxorubicin	0.97	3.01	0.97	0.001	0.96	0.58
Curcumin	0.97	1.09	0.96	1.139	0.95	0.66
Doxorubicin from DoxCur	0.95	19.47	0.97	1.375	0.96	0.80
Curcumin from DoxCur	0.96	3.34	0.95	0.026	0.97	0.09

Higuchi model: It is a mathematical model that is probably the most famous and widely used to describe the release of drug from the matrix of a system. It is applicable to various geometric and porous systems. The graph is plotted as the cumulative percentage drug released versus the square root of time. The simple equation that represents this model is;

$$C = [D(2qt - Cs)Cst]^{\frac{1}{2}} \dots\dots\dots \text{equation 4.4}$$

where,

C = total amount of released drug per unit area of the matrix (mg/cm²)

D = diffusion coefficient for the drug in the matrix (cm²/hr)

qt = total amount of drug in a unit volume of matrix (mg/cm³)

Cs = dimensional solubility of drug in the polymer matrix (mg/cm³)

t = time (hr)

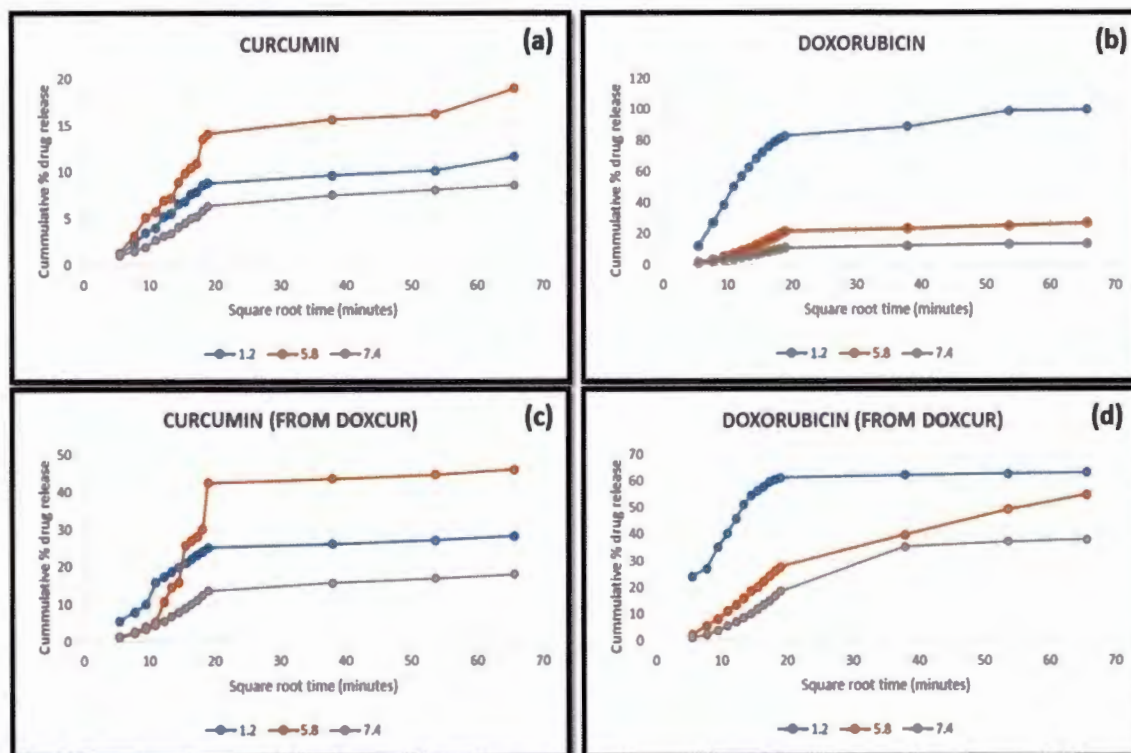
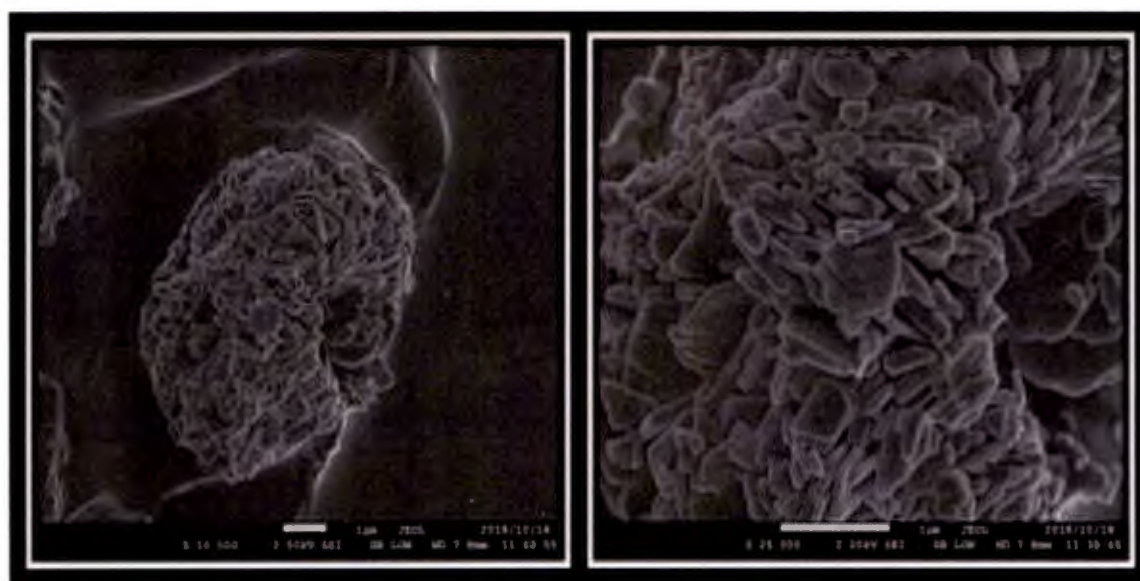


Figure 4.5: A figure showing the plot of Higuchi release kinetics of (a) curcumin from the hydrogel (b) doxorubicin from the hydrogel (c) curcumin from the hydrogel containing both drugs and (d) doxorubicin from the hydrogel containing both drugs.

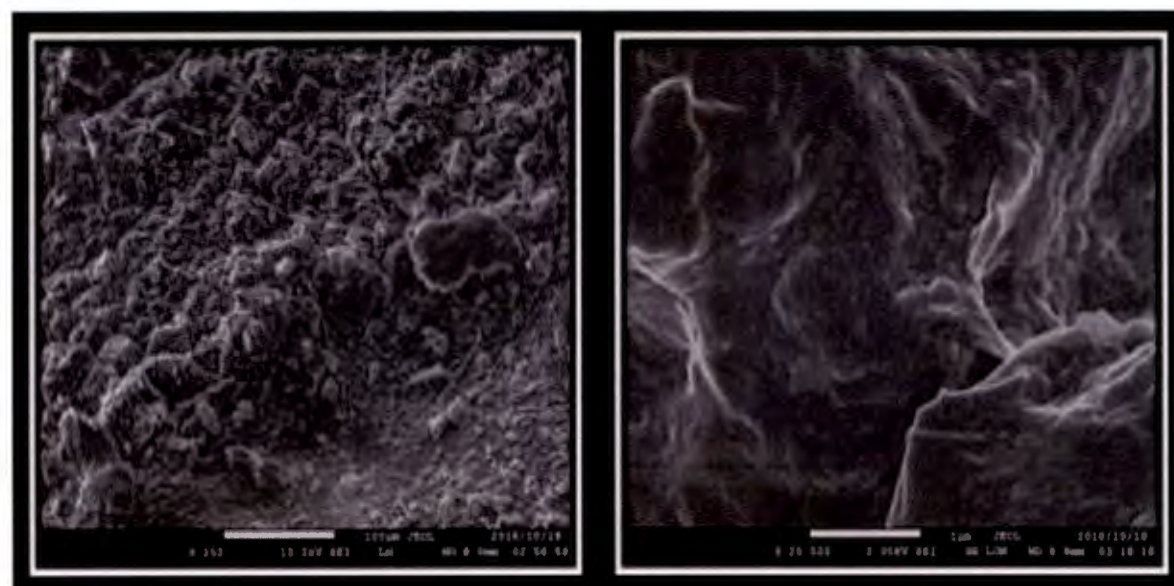
Table 4.6: A table showing the constant, k and correlation coefficient, R^2 of drug release from hydrogel

DRUGS	pH 1.2		pH 5.8		pH 7.4	
	R^2	k	R^2	k	R^2	K
Doxorubicin	0.98	11.84	0.98	8.35	0.98	3.60
Curcumin	0.99	2.12	0.98	3.94	0.98	1.55
Doxorubicin from DoxCur	0.98	5.73	0.99	9.59	0.98	5.51
Curcumin from DoxCur	0.98	2.48	0.99	2.71	0.98	5.32



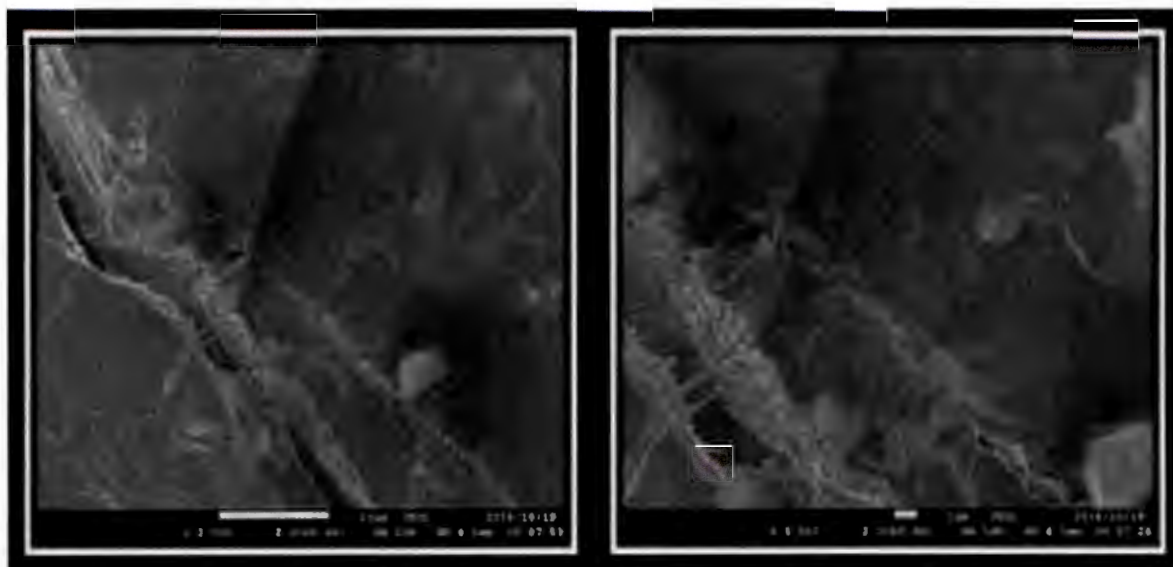
DOX

Figure 4.6b: A figure showing SEM images of doxorubicin at two different magnifications Left $\times 10000$ and Right $\times 25000$



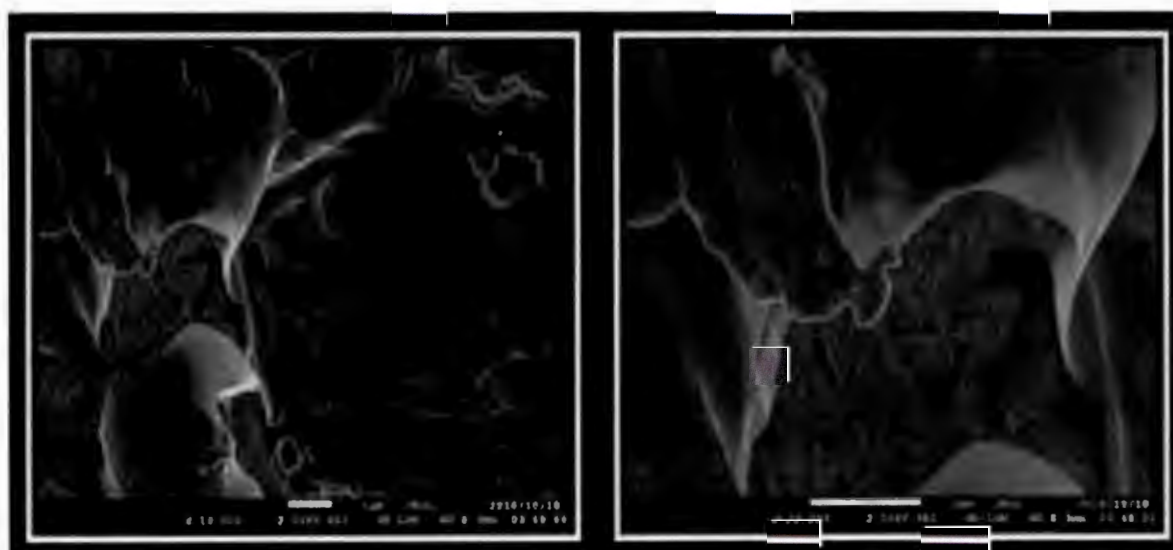
PEG

Figure 4.6c: A figure showing SEM images of PEG-based hydrogel without drug(s) at two different magnifications: Left $\times 250$ and Right $\times 25000$



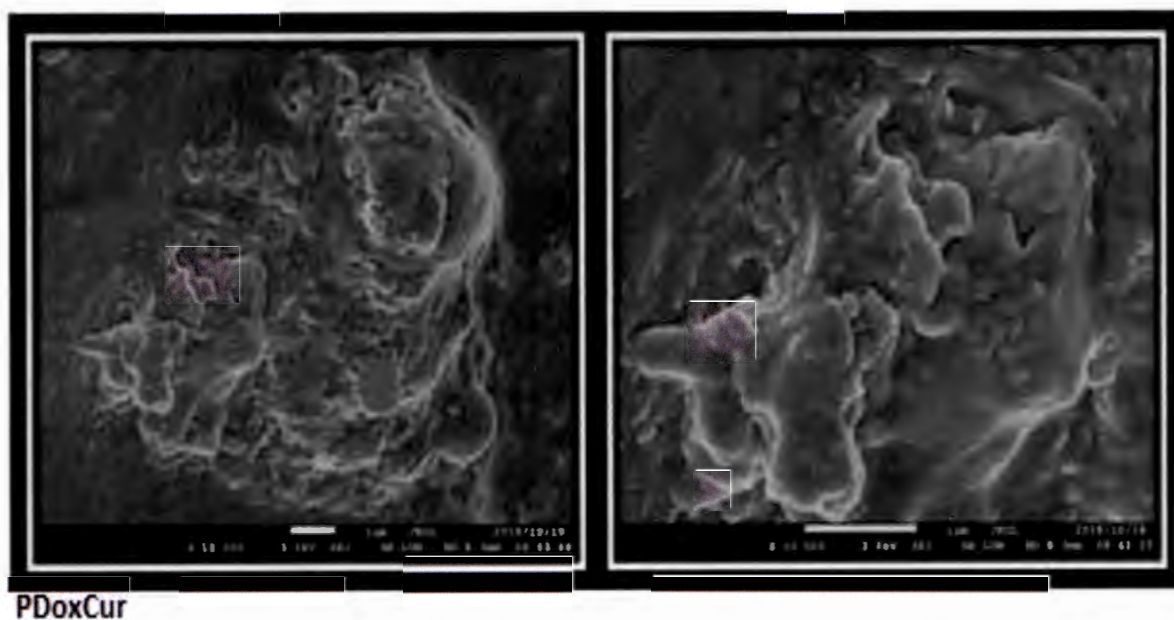
PCUR

Figures 4.6d: A figure showing SEM images of PEG-based hydrogel loaded with curcumin at two different magnifications: Left $\times 2500$ and Right $\times 5000$



PDOX

Figures 4.6e: A figure showing SEM images of PEG based hydrogel loaded with doxorubicin at two different magnifications Left $\times 10000$ and Right $\times 25000$



Figures 4.6f: A figure showing SEM images of PEG loaded with doxorubicin/curcumin at two different magnifications: Left $\times 10000$ and Right $\times 25000$

An irregular topographies of the surfaces of the samples (PEG, curcumin and doxorubicin) are as observed in Figures 4.6 (a-c), and these are similar to the images obtained by (Rachmawati et al., 2013, Jayakumar et al., 2012). The PEG-based hydrogel appears coarse and irregular in shape and the curcumin block-like shaped, while doxorubicin is spherical in shape. In the case of PDox, PCur and PDoxCur, there are changes in the morphology because the original morphology of the individual components had disappeared. These changes in the feature of the hydrogel as seen in Figures 4.6 (e-f) are as a result of the incorporated drugs and this alteration in the pattern noticed confirms that the drugs were absorbed by the polymer matrix of the hydrogel. Furthermore, the loaded drug(s) appear(s) to be more compact and this may be due to the strong aggregation of the hydrogel and drug(s) via noncovalent forces, mediated by the drug(s) and similar to the reports of (She et al., 2013).

4.2.4. X-RAY DIFFRACTION

The changes in the morphological structure and crystallinity of the hydrogel, before and after loading with drug(s) were studied by using XRD. These changes can be seen in Figures 4.7 and 4.8

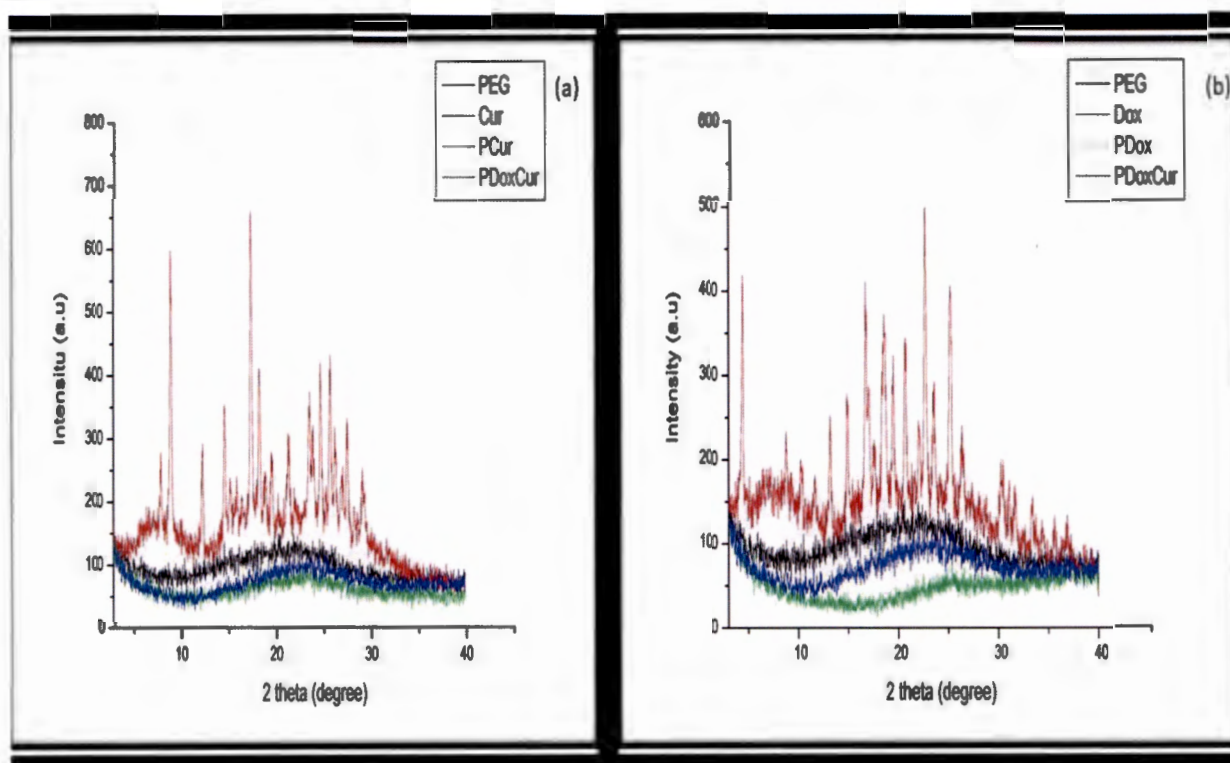
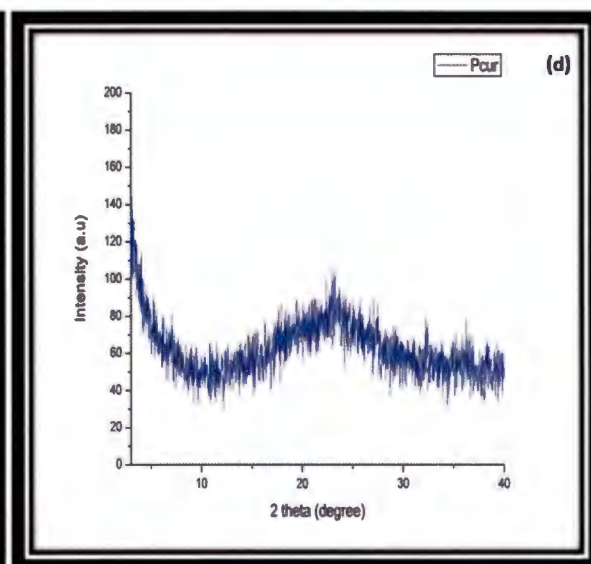
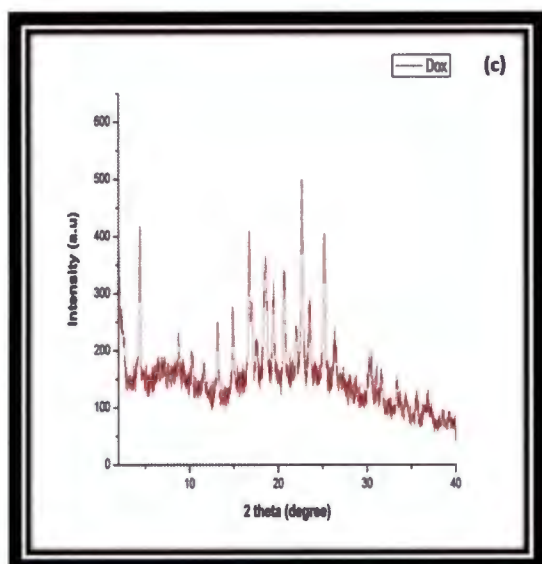
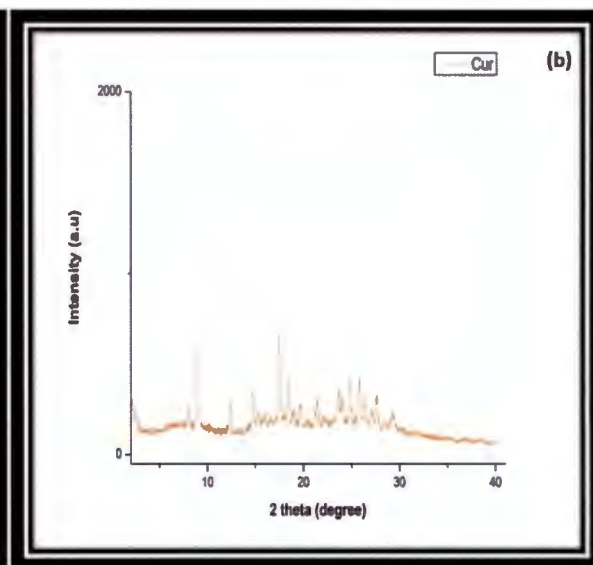
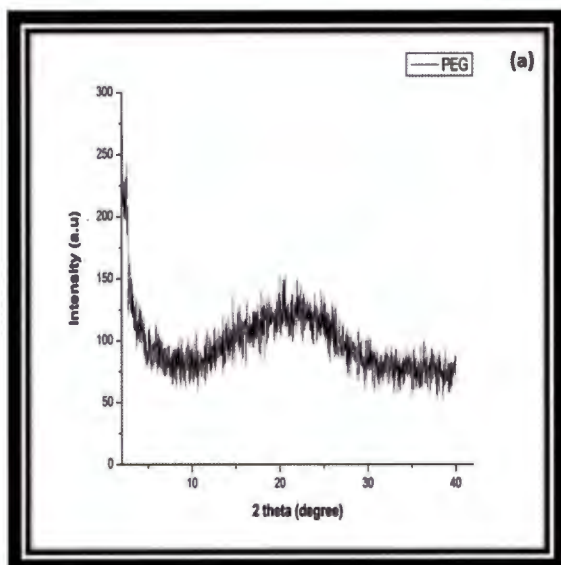


Figure 4.7: A figure showing XRD patterns of: (a) PEG, Cur, PCur and PDoxCur (b) PEG, Dox, PDox and PDoxCur

For a better understanding of the morphological changes that occurred, the individual XRD patterns of the hydrogel (with and without the drugs) and the individual drug(s) are depicted in the Figure 4.8 (a-f) below.



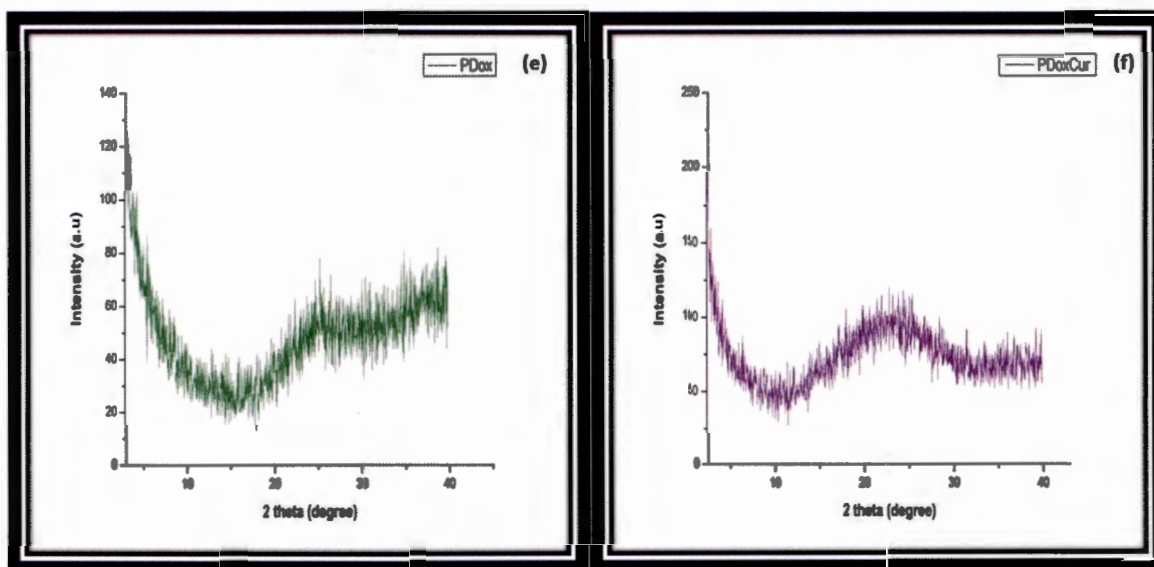


Figure 4.8: A figure showing XRD patterns of: (a) PEG (b) Cur (c) Dox (d) PCur (e)PDox and (f)PDoxCur

The Bragg's angles of the drug(s) show a diffraction curve that reveals a significant change in the diffraction pattern after it has been loaded unto the hydrogel. The drugs show the characteristic crystalline peaks. Major reflections corresponding to the Bragg's angle, 2θ are observed at: 8° , 9° , 12° , 15° - 19.5° , 21° , 24° - 29° , but strongest are observed at 9° and 18° for curcumin and the major reflections observed in doxorubicin are at: 4° , 8° , 10° , 11° , 13.5° , 15° , 17° , 18° , 19.5° , 21° , 24° , 25° , 26° , 30.5° , 31° , 33.5° , 34.5° , 35.5° , 36.5° with the strongest at: 4° , 17° , 18° , 19.5° , 21° , whereas the PEG-based hydrogel reveals amorphous characteristics without any prominent peak. This result is similar to that obtained by Qiao et al. (2016). In the case of PDox, PCur and PDoxCur, the crystalline peaks of the loaded drug(s) disappeared thus confirming the incorporation of the drug(s) and this observation is comparable to reports made by Anitha et al. (2011). However, broad peaks were seen at: 12° - 28° , 12° - 19° , 22° - 25° and 13° - 28° for PDoxCur, PDox and PCur respectively. The absence of crystalline peaks is possibly because of the formation of an amorphous complex,

which can be ascribed to the intermolecular interaction between the PEG based hydrogel and the drug(s) within the matrix (Shaikh et al., 2009, Yallapu et al., 2010).

Furthermore, a decrease in intensity is observed in both drugs when loaded on the hydrogel. This is due to the interaction between the drugs and the polymeric network of the hydrogel, thus leading to a decrease in the intermolecular interaction between the drugs. Also, no crystallinity was observed in the drug-loaded hydrogels and this is also because there is dispersion of the drug at the molecular level of the polymer matrix. This observation is similar to that of (Chavis et al., 2012). For ease of degradation, it is necessary for hydrogel to have less structural stability. This is accomplished in the case of this hydrogel as seen in its low crystallinity since low crystallinity indicates less structural stability.

4.2.5. FOURIER TRANSFORM INFRARED SPECTROSCOPY

In Figure 4.9a and 4.9b, the FTIR spectra reveal the relevant characteristic peaks of the hydrogel formulations. Inferences made, based on the peaks observed are highlighted in the Table 4.7(a-c) below. The presence of the various groups, aside the –enol and –amine groups in curcumin and doxorubicin respectively are shown in Table 4.7(a) and 4.7(b). This result agrees with reports of Pawar et al. (2014) and Shah Sumit (2013), respectively. The supplementary bands seen in the spectrum of the PEG-based gel, is as a result of the other components of the hydrogel.

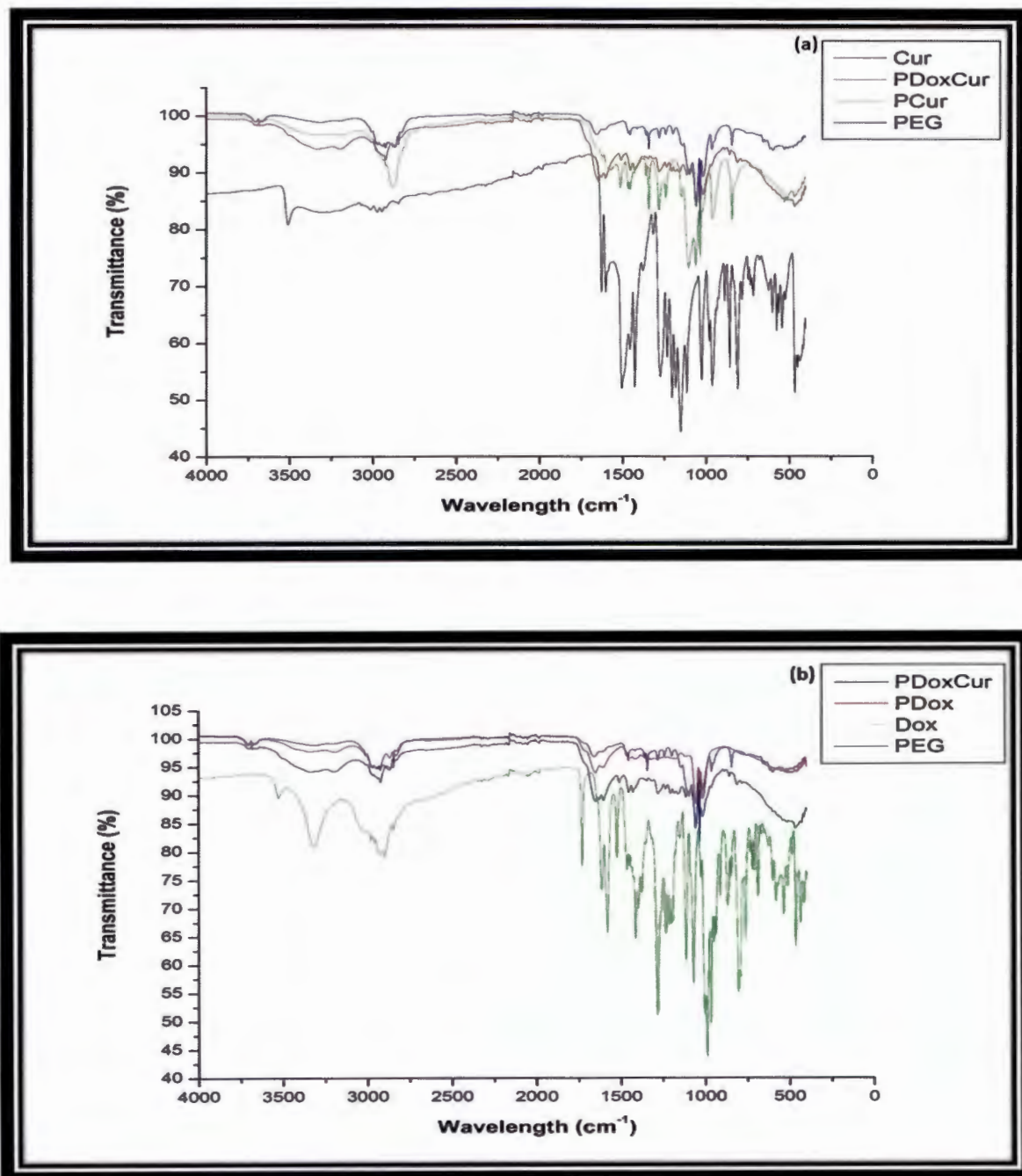


Figure 4.9: A figure showing FTIR spectra of: (a) PEG, Cur, PCur and PDoxCur (b) PEG, Dox, PDox and PDoxCur

Table 4.7 (a): A table showing the peaks and frequencies of Doxorubicin

Frequency (cm ⁻¹)	Indication (type of bond)	Inference
3357-3560	O-H	Hydroxyl stretching
2750-2980	N-H and O-H	Amine and Hydroxyl stretching
1730	C=O	Ketone stretching
1309	C-O-C	Ether stretching
1120	C-O	Tertiary alcohol
1000	C-O	Secondary alcohol
590-899	C-O	Primary alcohol

Table 4.7 (b): A table showing the peaks and frequencies of Curcumin

Frequency (cm ⁻¹)	Indication (type of bond)	Inference
3500	O-H	Hydroxyl stretching
2938	C-H	Alkanes stretching
1590-1615	C=O	Ketone stretching
1500-1400	C=C	Aromatic stretching
1402	C-O	Phenol stretching
1205-1290	C-O	Ethanol stretching
650-960	C-H	Benzoate trans C-H vibration

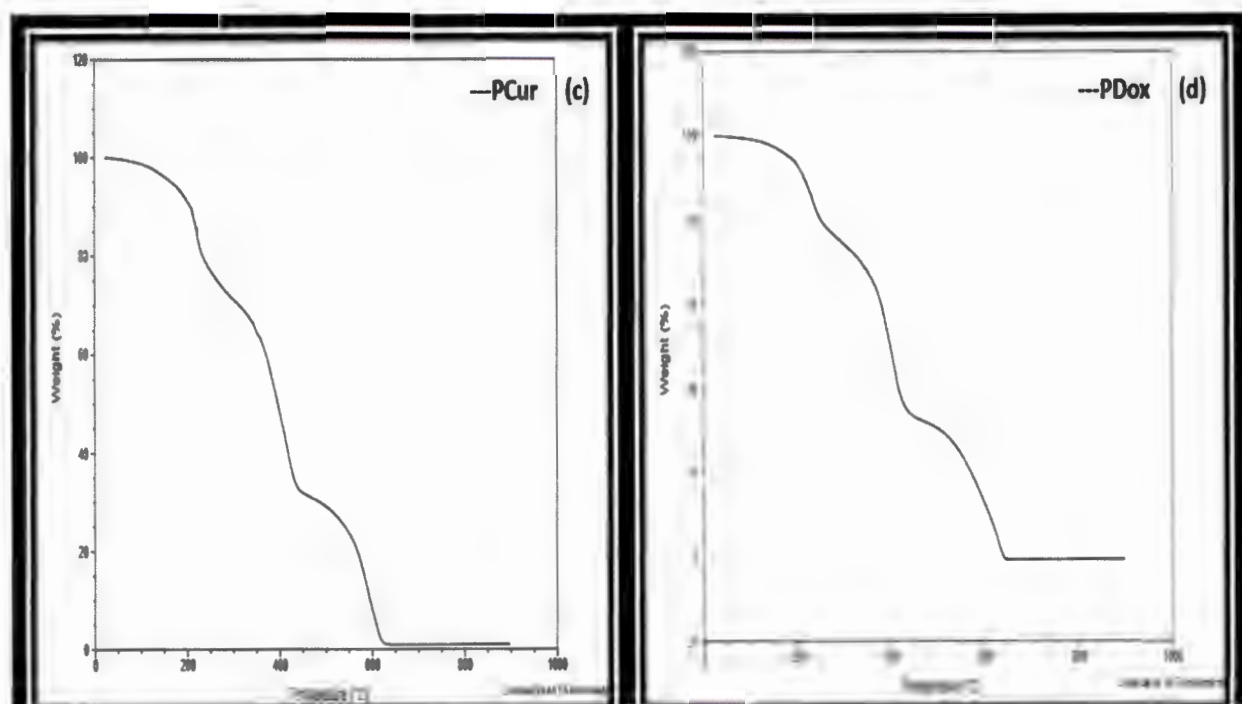
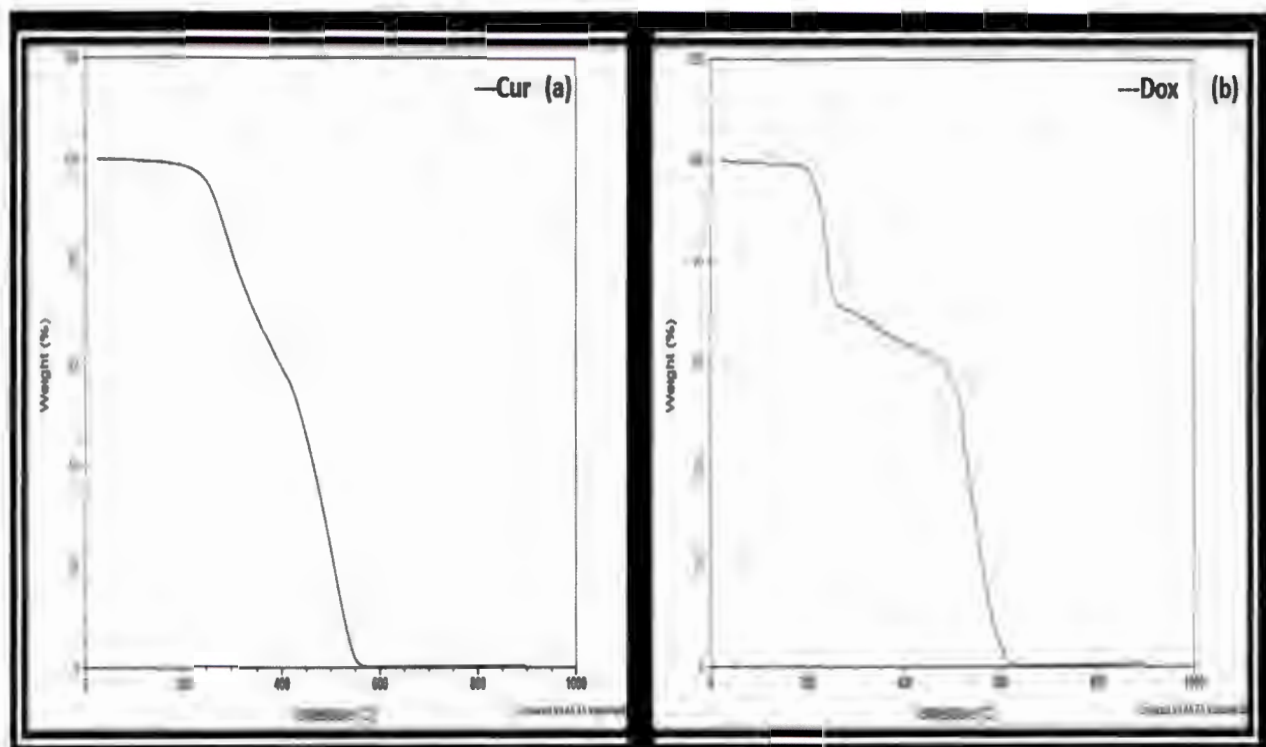
Table 4.7 (c): A table showing the peaks and frequencies of PEG

Frequency (cm ⁻¹)	Indication (type of bond)	Inference
3300-3600	O-H	Hydroxyl stretching
2980-3000	C-H	Alkanes stretching
1700-1600	C=O	Amide stretching
1392-1506	C-H	Alkanes scissoring/bending
972-1050	C-O	Alcohol stretching
435-560	C-C	Vibration

On comparing the FTIR spectra of the drug(s) and the PEG-based hydrogel loaded with the drug(s), the peaks representing the various groups present in each drug still appeared. The curcumin peaks in the region between 1205-1290 cm^{-1} and doxorubicin peaks in the region between 2750-2980 cm^{-1} , respectively, were still visible in the hydrogel loaded with the drug(s), thus confirming the presence of the drug(s) in the hydrogel. In addition, there are notable shifts in the spectra of the drug loaded hydrogels when compared with that of unloaded hydrogel and this confirms the complexation and presence of intermolecular hydrogen bonding between the drug(s) and the hydrogel (Kayal and Ramanujan, 2010).

4.2.6. THERMOGRAVIMETRIC ANALYSIS

The hydrogels (drug free and drug loaded) and drugs were subjected to thermal analysis (TGA) in order to study the thermal behaviour and the possible interaction between the drug(s) and polymer. Figure 4.10 (a-f) reveals the various degradation steps of the hydrogel, drug loaded hydrogel and the drugs.



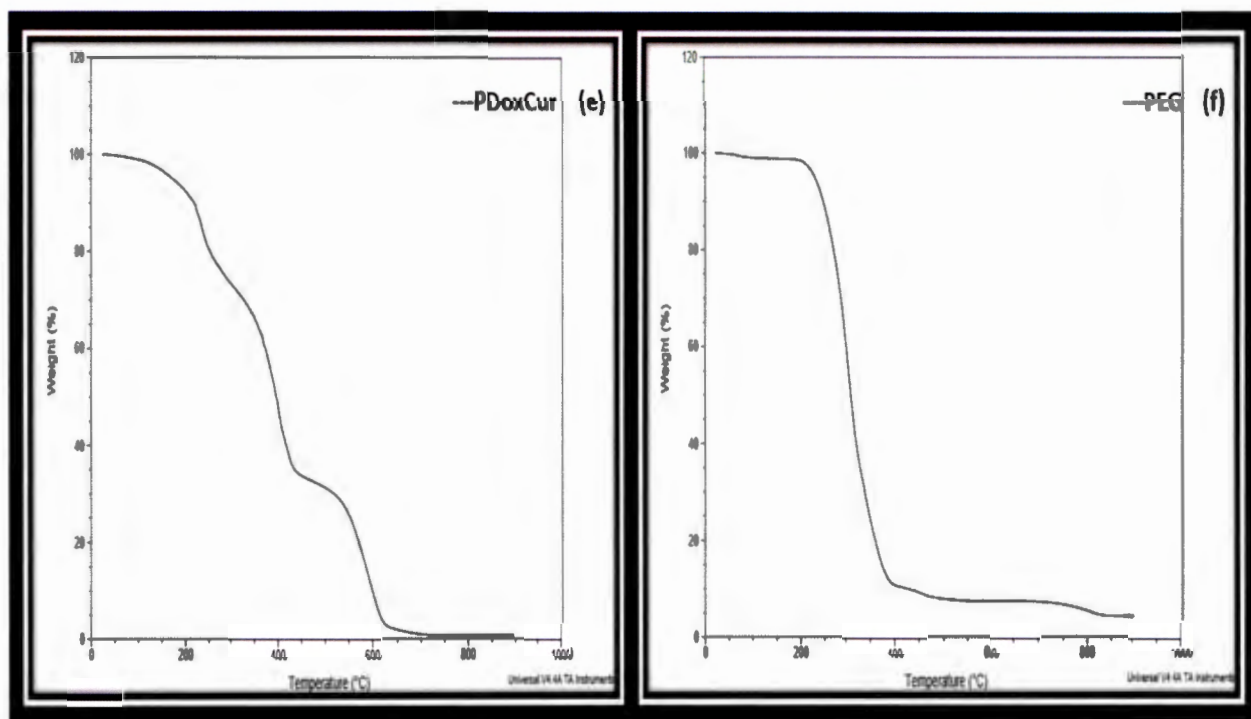


Figure 4.10: A figure showing TGA analysis of (a) Curcumin, (b) Doxorubicin, (c) PCur, (d) PDox, (e) PDoxCur, (f) PEG

Multiple degradation steps were observed in the TGA thermograph of the drugs and the polymer loaded with drugs (Fig. 4.10a-e), whereas the neat polymer has only one degradation step (Fig. 4.10f). An initial weight loss was observed in all samples at different temperatures and this is as a result of the removal of water molecules from the samples; ~4% at 239°C for curcumin, ~3% at 204°C for doxorubicin, ~3% at 212°C for the PEG-based hydrogel, ~9% at 202°C for hydrogel bound to curcumin, ~7% at 200°C for hydrogel-loaded with doxorubicin and ~11% at 222°C for the hydrogel-loaded with both drugs. Subsequent secondary and final weight losses were further observed in all the samples, with the presence of char residue. It was observed that the secondary and final weight losses were, for: curcumin (~54 and ~98%) occurred at 422°C and 547°C, respectively, doxorubicin (~49 and ~99%) occurred at 518°C and 617°C, respectively, hydrogel loaded with curcumin (~37 and ~79%) at 360°C and 564°C, respectively, hydrogel loaded with

doxorubicin (~38 and ~77%) at 372°C and 557°C, respectively, hydrogel loaded with both drugs (~34 and ~73%) at 351°C and 537°C, respectively (Fig. 4.10a-e), while the hydrogel that has just one degradation step had its subsequent weight loss of ~90% at 418°C (Fig. 4.10f). Also, the results for the polymer loaded drugs, showed a combination of the individual behaviours of the two neat drugs, indicating that the two drugs existed separately in the hydrogel with very minimal or no chemical interaction between the drugs.

Furthermore, according to the observed pattern of degradation, it is found that hydrogels loaded with the drug(s) were more stable than the drugs (curcumin and doxorubicin). Rapid degradation was observed in the drugs, whereas a steady degradation was found in the case of the hydrogel, thus confirming the usefulness of hydrogel as a good drug carrier because the thermal stability of the drugs is improved when loaded on the hydrogel substrate. This is in line with reports by Thakur et al. (2012). This improvement is attributed to the hydrogel-drug interaction. In particular, complexing of a drug and a polymer leads to the absence/shifting of endothermic peaks, indicating a change in the crystal lattice, melting, boiling, or sublimation points (Yallapu et al., 2010).

4.2.7 CYTOTOXICITY STUDY ON MCF-7 CELL LINES

The minimum inhibitory concentration (IC_{50}) studies and dose/time-dependent studies that were carried out revealed the effects of free drug(s) and drug(s) loaded unto the hydrogel against breast cancer cells.

Minimum inhibitory concentration of drug(s) IC_{50} and time dependent studies:

The minimum inhibitory concentration represents the concentration of drug required to inhibit 50% growth of the cell. Table 4.5 below shows the IC_{50} value of the drugs used for this study.

Figures 4.12-4.14 shown below reveal the effect of the therapeutic agents and the hydrogel on various test groups over the period of 72 hours treatment when compared with the control groups.

Table 4.8: A table showing the IC_{50} values of the various test groups

INCUBATION TIME	IC ₅₀ VALUES (µg/mL)					
	Cur	PCur	Dox	PDox	DoxCur	PDoxCur
24 hours	0.72	0.51	1.00	0.64	0.59	0.93
48 hours	0.68	0.48	0.80	0.50	0.53	0.42
72 hours	0.60	0.41	0.74	0.47	0.50	0.31

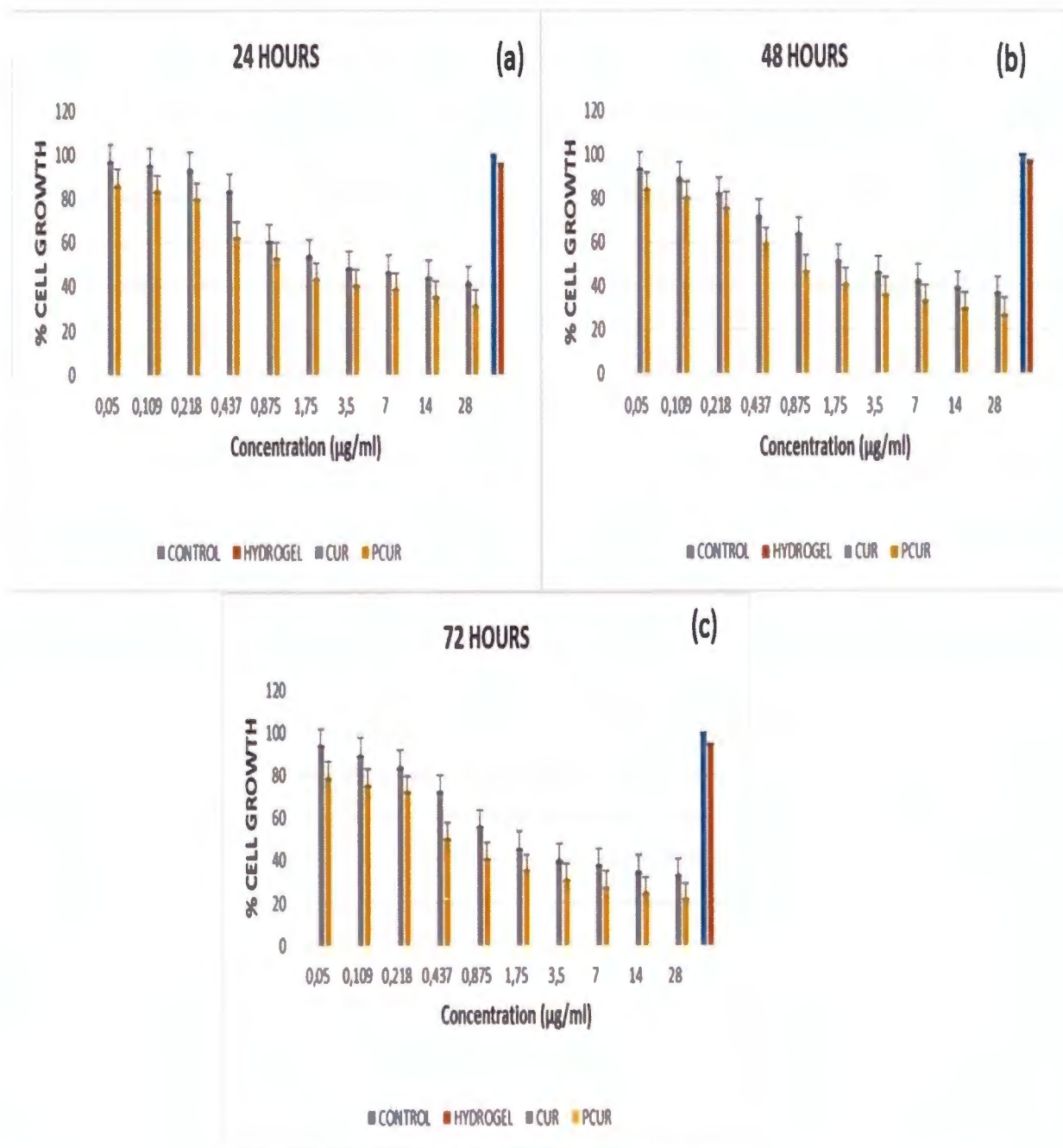


Figure 4.11: A figure showing the percentage cell growth of curcumin and PEG-curcumin at (a) 24 hours (b) 48 hours and (c) 72 hours.

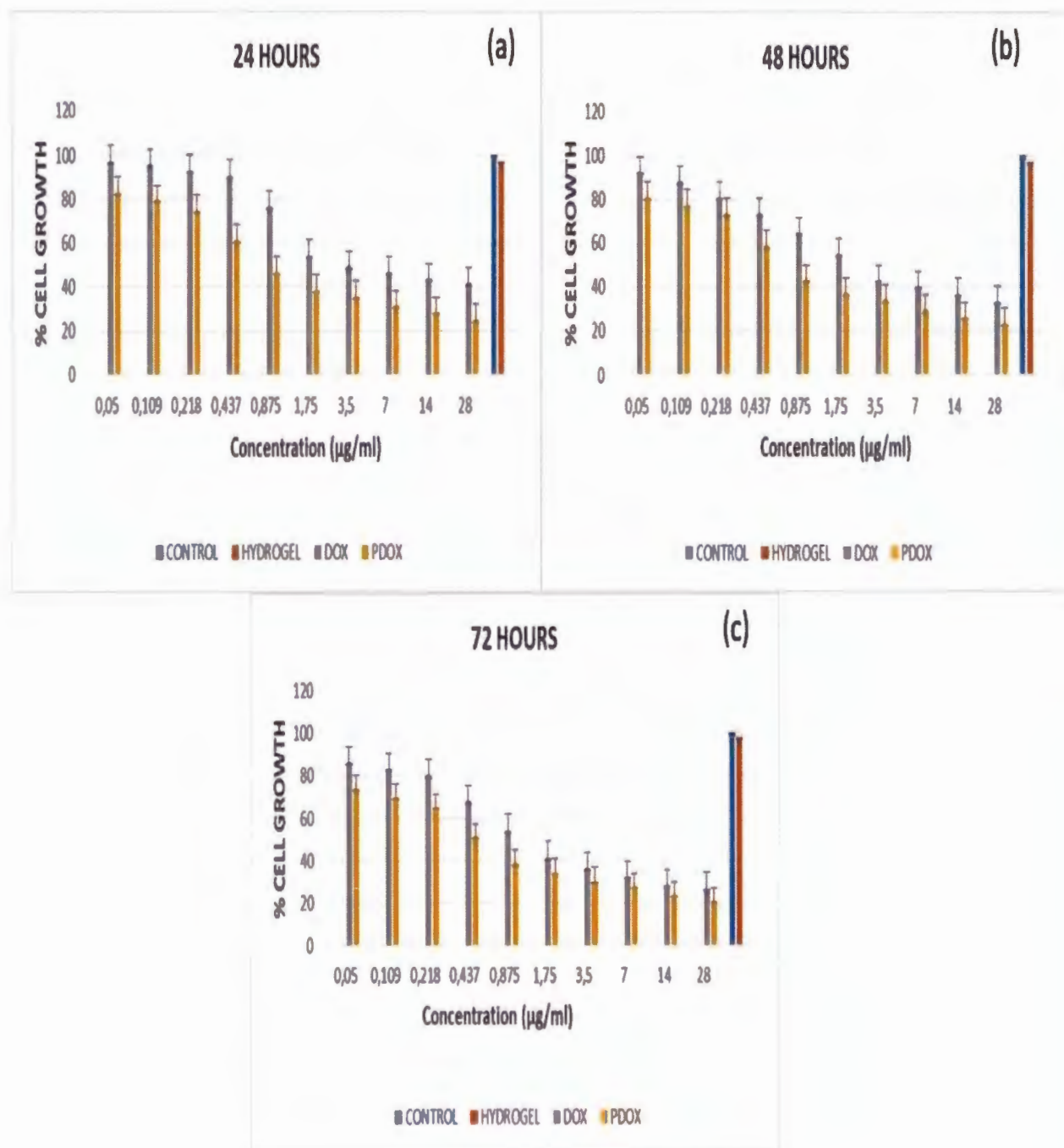


Figure 4.12: A figure showing the percentage cell growth of doxorubicin and PEG-doxorubicin at (a) 24 hours (b) 48 hours and (c) 72 hours.

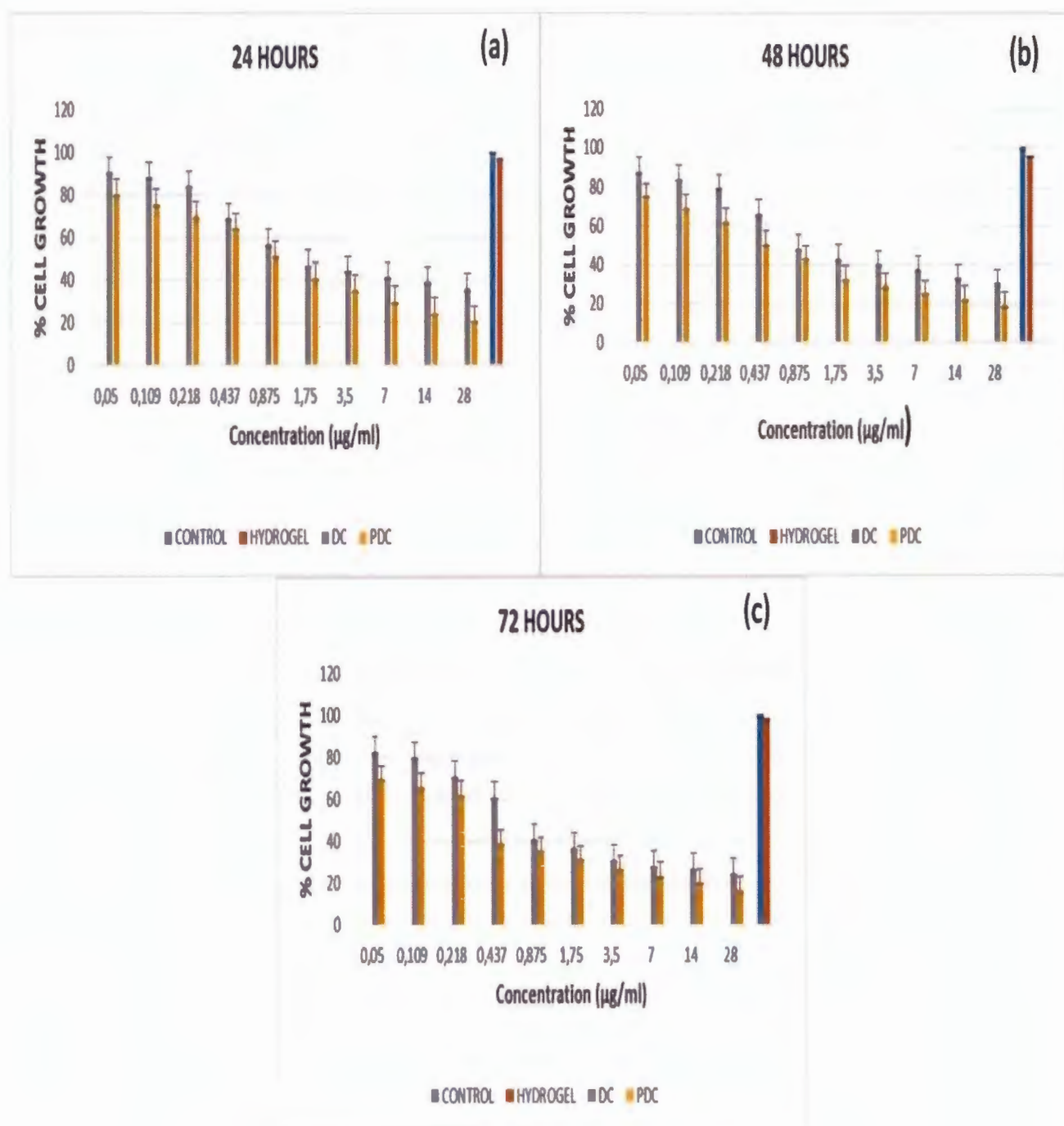


Figure 4.13: A figure showing the percentage cell growth of doxorubicin/curcumin and PEG-doxorubicin/curcumin at (a) 24 hours (b) 48 hours and (c) 72 hours.

The enhanced anti-cancer effect of the PEG-based hydrogel loaded with the two drug(s) was investigated by testing the drug-loaded PEG hydrogels against MCF-7 breast cancer cells. The treatment with free drugs (curcumin, doxorubicin and in combination, i.e. curcumin/doxorubicin) were used as positive control while untreated cells were used as negative control. The results show a significantly suppressed MCF-7 proliferation rate in drug dose and in a time dependent manner when compared to the untreated groups. Cells treated with PEG-doxorubicin, showed a lower effectiveness when compared to the PEG-curcumin. However, the anti-tumour effect of PEG-doxorubicin/curcumin, was clearly greater than the other groups as the dose increased as shown in Figure 4.13. The combination of free curcumin and doxorubicin showed an enhanced anti-tumour effect when compared to the free doxorubicin and curcumin treated groups respectively, as seen in Figure 4.13. Furthermore, it was observed that the effect of PEG-curcumin at 24hours is similar to the free doxorubicin/curcumin at 48hours while PEG-doxorubicin at 48hours is similar to the free doxorubicin/curcumin at 72 hours. This implies that the use of the PEG based hydrogel improves the overall effectiveness of the individual drug(s). Further observations is represented in the higher IC_{50} value of PEG-doxorubicin/curcumin at 24 hours when compared to the free doxorubicin/curcumin, although it greatly lowered over time (after 24hours). Consequently, greater cell death after 24 hours was observed in groups treated with PEG-doxorubicin/curcumin as observed in the reduced IC_{50} values shown in Table 4.8. This initial increase in the IC_{50} value of PEG-doxorubicin/curcumin can be attributed to the interaction of both drugs within the hydrogel and the continuous accumulation of the drugs released from the hydrogel after the disassembling and internalization of the PEG-doxorubicin/curcumin, respectively. Similar observation was reported by Ganta and Amiji (2009). The higher IC_{50} value observed in the free drug(s) treatment group is attributed to the quick removal and internalization of the free drug(s)

doxorubicin/curcumin via passive diffusion by the cells. Similar findings were reported by Zhang and his colleagues (Zhang et al., 2016). Also, the IC_{50} values of doxorubicin and PEG-doxorubicin remained highest at the end of the entire study; this might be because of the cells resistance to doxorubicin. Drug resistance by cancerous cells have been a reported challenge by researchers, e.g. Smith and his co-workers. (Smith et al., 2006). However, with the combination of curcumin, a better therapeutic effect was observed. Furthermore, with the use of the hydrogel, the dose of the drug was reduced and a prolong circulation time for the drug delivery was observed. These observations from the *in-vitro* cytotoxicity studies indicate that there is an enhanced anti-tumour effect by the drugs (doxorubicin/curcumin) released from the PEG-based hydrogel and a synergistic inhibitory effect of doxorubicin and curcumin on the cell growth of breast cancer cells was observed. These observations are similar to that which was reported by Zhang et al. (2016) and Barui et al. (2014).

4.3 DISCUSSION

The micrographs and spectra data obtained from the different characterization techniques, such as SEM and FTIR show a fine integration of the drugs onto the polymer hydrogel substrate and thus the observed significant benefits associated with the use of the PEG-based polymer incorporated with drug(s) in the treatment of MCF-7 breast cancer lines. This benefit is evident in the major positive effects seen in the hydrogel loaded with both drugs (in combination) when compared to the controls, free drugs (unloaded onto the polymer). The hydrogel increased the efficacy of the drug(s), individually and in combination, despite the fact that cancerous cells are known to become drug resistant. The next chapter (chapter 5) focuses on the conclusions and recommendations, based on the results obtained.

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATION

5.1 CONCLUSIONS

The purpose of this study was to develop a PEG-based hydrogel for dual drug delivery. Swelling studies was performed on the hydrogel in order to determine its swelling capacity, followed by drug release studies in order to observe the drug release profile of the drug(s), and *in-vitro* cytotoxicity test on MCF-7 breast cancer cell lines using the hydrogel loaded with the drugs in varying combination ratio for comparative combination therapy study.

Free radical polymerization method was employed in the synthesis of the hydrogel, in the presence of PEG, N-isopropylamide and gum acacia. The characterization of the hydrogel was carried out using the: FTIR, SEM, TGA, and XRD for the confirmation of incorporated anti-cancer drugs (doxorubicin and curcumin).

Results from the swelling ratio analysis revealed that the hydrogel is pH sensitive. The drug release studies of both drugs further confirmed the pH sensitive nature of the hydrogel. This information can be used to specifically load drugs individually or in combination and subsequently trigger the release of the drugs at physiological pH values. Furthermore, it can be established that the PEG-based hydrogel can be used as a potential targeted drug delivery device because from study, the hydrogel in the acidic buffer did not release a large amount of the drug(s) incorporated in it suggesting it can by-pass the gastric acid of the stomach, hence its usefulness as a drug delivery device to the intestine in a controlled manner.

From the results obtained after the minimum inhibitory concentration test and *in-vitro* toxicity, studies were performed on MCF-7 breast cancer cell lines. There was no significant toxicity to the

cells treated with the hydrogel without drug(s), but there was an increased efficacy of the PEG-based hydrogel containing both drugs (combined). At the end of the 72 hours treatment, all IC_{50} values reduced in all the treated groups, however a significant reduction was observed in groups the treated with the hydrogel and drug(s), and thus it can be said that there is a relationship between the duration of treatment and the concentration required to cause 50% inhibition of cell growth.

In summary, combination therapy by using this PEG-based hydrogel was observed to have a significant difference on the effects observed on the cell lines when compared to the control test groups and even the other hydrogel loaded with just one drug. It can, therefore be concluded that the PEG based hydrogel is capable of delivering these drugs over a long time, thereby making it a suitable therapeutic agent for combination breast cancer therapy.

5.2 RECOMMENDATION.

Even though all the proposed objectives of this research were accomplished and the data obtained indicate the inhibitory effect of the drug(s)-loaded unto the PEG-based hydrogel on the proliferation of breast cancer cells, further investigation and research can be done at the molecular level. This can involve the investigation of the effect of the PEG-based hydrogel *in-vivo*, thus allowing a better clarity and understanding on the effect of the PEG-based gel at a deeper level, since more studies are required to elucidate the underlying mechanisms of the drugs on MCF-7 breast cancer cell lines.

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APPENDICES

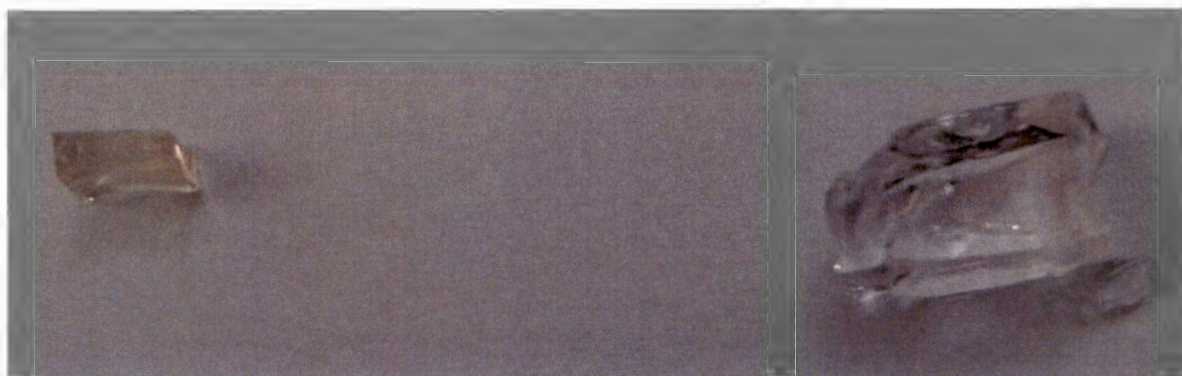


Figure 1: PEG –based hydrogel in buffer solution at ambient temperature: left (before swelling) and right (after swelling).

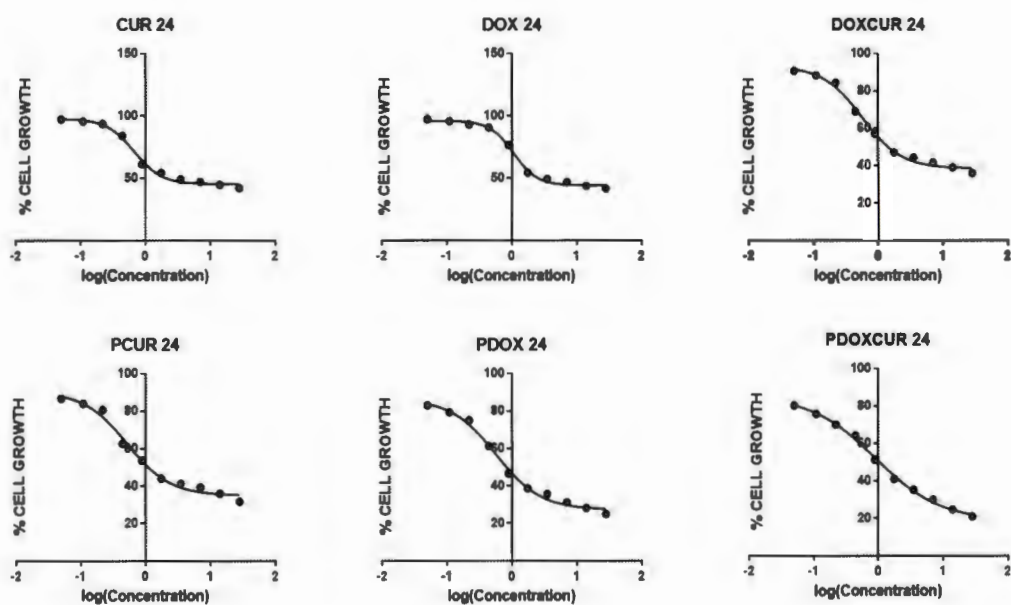


Figure 2: Minimum inhibitory (IC₅₀) curve of various test groups at 24 hours

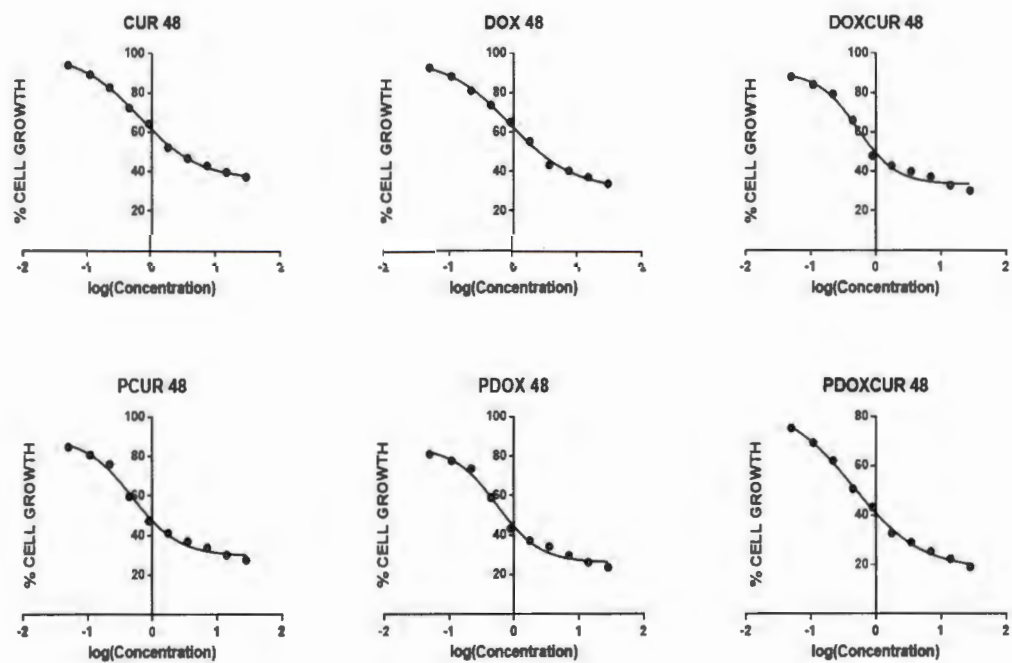


Figure 3: Minimum inhibitory (IC_{50}) curve of various test groups at 48 hours

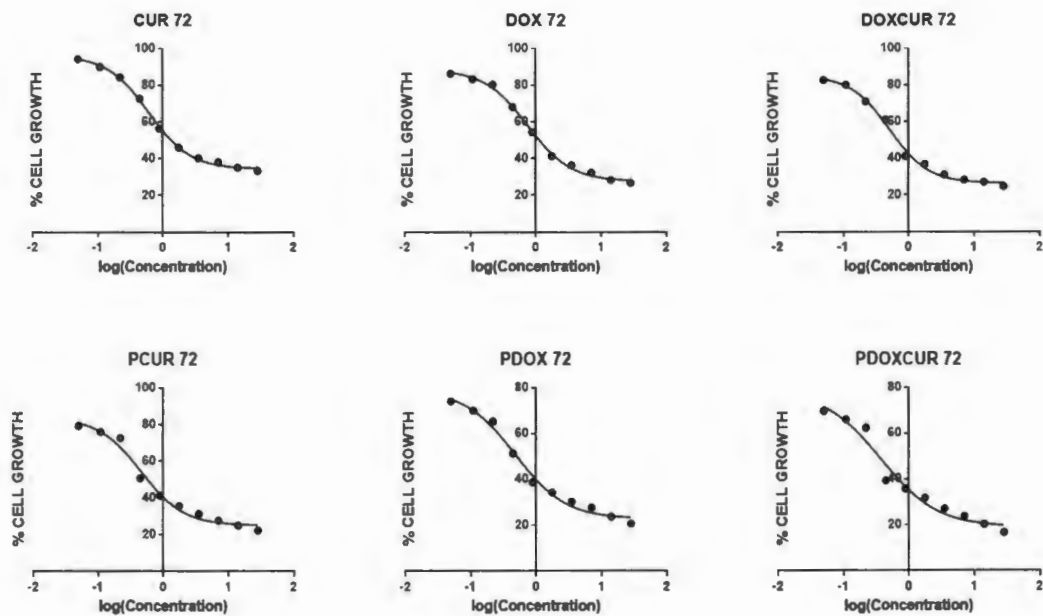


Figure 4: Minimum inhibitory (IC_{50}) curve of various test groups at 72 hours