THE ANTIOXIDATIVE AND CYTOTOXIC EFFECTS OF *HIBISCUS SABDARIFFA* ON MCF7 AND MCF12A BREAST CELL LINES

By

MANDISA PAMELA SOBANTU

Thesis submitted in fulfillment of the requirements for the degree Master of Technology: Biomedical Technology

In the

Faculty of Health and Wellness Sciences

At the

CAPE PENINSULA UNIVERSITY OF TECHNOLOGY

Supervisor: **Dr YG Aboua**

Co-supervisor: **Prof M Meyer**

Bellville

October 2015

DECLARATION

I, Mandisa Pamela Sobantu, declare that the contents of this dissertation/thesis represent my own unaided work, and that the dissertation/thesis has not previously been submitted for academic examination towards any qualification. Furthermore, it represents my own opinions and not necessarily those of the Cape Peninsula University of Technology.

Signed **Date**

ABSTRACT

Cancer is the leading cause of death in both developed and developing countries. In particular, breast cancer is regarded as the most common neoplastic disease in females and accounts for the high mortality rates in women. Increased mortality rates could be attributed to ineffective current cancer treatment modalities that have been implicated to cause multidrug resistance, high toxicity and induction of several side effects. In addition, oxidative stress appears to play a role in the development of breast cancer. Therefore, current cancer research aims to search for plant based anticancer compounds with less side effects and toxicity towards the human body. An example of such a plant is *Hibiscus sabdariffa* also known as roselle and is reported to have bioactive compounds that exhibit anticancer and antioxidant effects. However, the effects of *Hibiscus sabdariffa* on breast cancer in relation to oxidative stress and apoptosis have not been investigated.

In this research study, the aim was to evaluate the cytotoxic and antioxidant effects of water and methanolic extracts of *Hibiscus sabdariffa* (HS) on cancerous MCF7 and non-cancerous MCF12A breast cell lines with special reference to oxidative stress and apoptosis. This was done based on the fact that HS has been documented for its traditional use against cancer and other ailments. In the first part of the study, the antioxidant contents and capacities of the HS extracts were determined at a concentrations range of 0.2mg/ml to 1mg/ml. The findings stipulated that HS methanolic extract demonstrated a higher yield of polyphenols than water extract while, HS water extract demonstrated a higher yield of anthocyanins than methanolic extract. The ORAC assay showed that methanolic extract demonstrated a higher total antioxidant capacity than water extract. However FRAP assay, produced no difference on the reducing power between the two HS extracts. These findings confirm the concept that no single assay can fully assess the antioxidant capacity of plant extracts but instead variety of assays should be employed.

In the second part of the study, HS plant extracts were screened for cytotoxicity and pro-apoptotic activity against MCF7 and MCF12A cells. Cell viability was assessed using the WST-1 proliferation assay. Apoptosis was determined using the Apo*Percentage*™ assay, while mitochondrial transmembrane potential was determined using Tetra Methyl Rhodamine Ethyl ester (TMRE) assay. Cell viability results indicated that HS extracts were more cytotoxic on the tumorigenic MCF7 cell line compared to the non-cancerous MCF12A cells and methanolic extract was more potent than water extract. The Apo*Percentage*™ assay indicated that both water and methanolic extracts demonstrated a dose and time dependent

increase in apoptotic cancerous MCF7 cells. However, HS treated non-cancerous MCF12A cell line exhibited low number of apoptotic cells in both dose and time dependent experiments. Molecular data showed that both water and methanolic extracts demonstrated a dose and time dependent increase in MCF7 cells with depolarized mitochondria. However, both water and methanolic extracts exhibited low numbers of treated MCF12A cells with depolarized mitochondria in both dose and time dependent experiments.

The third part of the study examined the oxidative status of the cancerous MCF7 and non-cancerous MCF12A breast cell lines treated with HS extracts using reactive oxygen species (ROS) assay, antioxidant enzyme assay and lipid peroxidation assay. Findings indicated that HS water extract altered intracellular redox status of MCF7 breast cancer cells by depleting intracellular antioxidant enzymes (superoxide dismutase, catalase and total glutathione) and increasing lipid peroxidation and ROS levels. Treatment with hydrogen peroxide and pretreatment with HS extract and then hydrogen peroxide significantly in MCF7 cells decreased antioxidant enzymes (superoxide dismutase, catalase and total glutathione) and increased lipid peroxidation and ROS levels. This suggests HS extract did not offer any antioxidant activity in MCF7 cells upon hydrogen peroxide induced oxidative stress. However, in non-cancerous MCF12A cells treated with HS extract and pretreated with HS extract and then hydrogen peroxide showed a significant increase in intracellular superoxide dismutase and catalase activity and low levels of lipid peroxidation and ROS were observed. This suggests HS extract offered antioxidant activity in MCF12A cells upon hydrogen peroxide induced oxidative stress.

Based on the findings of the study, HS extracts selectively induced apoptosis via ROS generation and the mitochondrial dysfunctional pathway in MCF7 cells. In addition, this study concludes that HS extracts contain compounds that selectively confer pro-oxidant and cytotoxic effects on MCF7 cancer cells.

Keywords: Apoptosis, oxidative stress, antioxidants, breast cancer, cytotoxic, *Hibiscus sabdariffa*, flow cytometry, anticancer, reactive oxygen species.

ACKNOWLEDGEMENTS

I wish to thank:

- The God Almighty for giving me the knowledge, wisdom, understanding, courage and hope throughout this testing time.
- My supervisor Dr YG Aboua and co-supervisor Prof M Meyer, for their support, willingness to share knowledge and guidance. I appreciate your dedication in making my research a success.
- CPUT Oxidative Stress Research centre staff: Mr Fanie Rautenbach, for technical support and your willingness to help at all times.
- CPUT University research funding (URF) for recognizing and financial support for my research project.
- UWC Biotechnology department staff: Nicole and Lauren, for your assistance with some assay and use of equipments.
- My parents Mr and Mrs Sobantu, for their support, encouragement, prayers and understanding. My accomplishment will be meaningless without you. Thank you.
- My family: Judith, Joyce, Judy, Sindie Sobantu, Charmaine, Bomi, Chance, Mirriam, Nkosiyapha. Thank you for the unique support.
- My friends and colleagues: Dumisani Sibanda, Phumuzile Dube, Mediline Goboza, Venessa, Larsen, Rosemary, Audrey, Zenzele. Thank you for keeping me going.
- The financial assistance from the National Research Foundation towards this research, it is acknowledged. Opinions expressed in this thesis and the conclusions arrived at, are those of the author.

DEDICATION

This thesis is dedicated to the Almighty God.

LIST OF OPERATIONAL TERMS AND CONCEPTS

Antioxidants	Substances that reduce damage caused by oxidation and scavenge reactive oxygen species
Apoptosis	Genetically controlled active cell death process
Cell viability assay	An assay to estimate the number of viable cells present in multiwell plates.
Chemoprevention	The use of naturally occurring natural or synthetic substances to prevent or slow down the development of a disease
Cytotoxicity assay	An assay to determine the relative number of live and dead cells in cell populations.
Endogenous	Originating within an organism, tissue or cell
Exogenous	Originating outside an organism, tissue or cell
Free radical	A free radical has a missing electron in the outer shell and it reaches its stable state by gaining electrons from other healthy cells or genes.
Lipid peroxidation	It is the process whereby free radicals gain electrons from the lipids in cell membrane, resulting in cell damage and increased production of free radicals.
Phytochemical	A chemical compound that occurs naturally in plants
ORAC assay	An assay to determine the antioxidant capacity of cells in multiwell plates.

LIST OF ABBREVIATIONS

A	
ATP	Adenosine triphosphate
ANOVA	Analysis of variation
B	
Bcl-2	B cell leukaemia -2
C	
^{o}C	Degrees celsius
CAT	Catalase
D	
DMEM	Dulbecco's modified medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
DETAPAC	Diethylenetriamine penta acetic acid
E	
EDTA	Ethylene diamine tetra acetic acid
F	
FASC	Fluorescence activated cell sorter
FITC	Fluorescein isothiocyanate
FRAP	Ferric reducing antioxidant power assay
G	
GAE	Gallic equivalent
GSH	Reduced glutathionine
H	
HER2	Human epidermal growth factor receptor 2
HS	*Hibiscus sabdariffa*
H_2O_2	Hydrogen peroxide
I	
IC50	Inhibitory concentration at 50
L	
LPO	Lipid peroxidation

M

MCF7	Michigan cancer foundation 7
MCF12A	Michigan cancer foundation 12A
MDA	Malondialdehyde
Mg	Milligram
Ml	Milliliter

N

NADPH	Nicotine adenine dinucleotide phosphate

O

ORAC	Oxygen radical absorbance capacity
OS	Oxidative stress

P

PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PS	Phosphatidylserine
P value	Probability value

R

ROS	Reactive oxygen species
RNS	Reactive nitrogen species

S

SOD	Superoxide dismutase

T

TBARS	Thiobarbituric reacting substance
TMRE	Tetramethylrhodamineethylester
TE	Trolox equivalent

U

μmole	Micromole

CONTENTS

LIST OF FIGURES

Chapter 2: Literature

Chapter 4: Results

LIST OF TABLES

CHAPTER ONE

INTRODUCTION

Cancer is a broad term used for a class of diseases characterized by uncontrolled growth of abnormal cells. Breast cancer starts in the cells of the breast and can metastasizes to other areas of the body. It is one of leading causes of morbidity and mortality worldwide. Scientific evidence reveals a possible link between oxidative stress (OS) and cancer as it is associated with the presence of increased reactive oxygen species (ROS). OS inflicts oxidative damage to DNA, proteins and lipids and initiates the process of carcinogenesis leading to the development of cancer. In recent years, focus on finding chemotherapeutic agents has shifted to natural products. Studies on *Hibiscus sabdariffa* (HS) show that it exhibit antioxidant and apoptotic activities on cancer cells. However, the effects of HS on breast cancer related to oxidative stress and apoptosis have not been assessed. Therefore, this study will focus on the effects of HS on cancerous and non cancerous breast cell lines.

1.1 Background of research problem

Cancer is the leading cause of death and globally it is the most crucial health problem of the current era. It can be defined as unregulated cell division leading to tumour formation. Its constitutive features include uncontrolled growth, ability to invade and metastasize. In its advancement, tumour mass continues to grow invading surrounding tissue and finally gets access to the lymphatic and vascular system from where it spreads to distant organs. Breast cancer presents as a lump in the breast or underarm. It usually starts as a painless swelling in the armpit, which can also be associated with breast pain or tenderness, changes in breast size, nipple retraction, nipple discharge that maybe clear or bloody and dimpling of breast skin (Mann *et al.,* 2012). Breast cancer is diagnosed using mammograms, breast physical examination, ultrasonography and magnetic resonance imaging (MRI) (Benson *et al*., 2004).

The six hallmarks of cancer include sustained proliferative signalling, evading growth suppressors, enabling replicative immortality, induced angiogenesis, activating invasion and metastasis and resisting cell death (Hanahan and Weinberg, 2000; Lazebnik, 2010; Hanahan and Coussens, 2012). Since cancer is characterised by uncontrolled proliferation and evasion of apoptosis, there is a pursuit for anticancer compounds which will induce apoptosis in cancer cells as a way to treat cancer (Wong, 2011). Apoptosis is an active form of

programmed cell death that consists of a set of biochemical pathways leading to non inflammatory cell suicide (Kerr *et al*., 1972). Up-regulation and down-regulation of apoptosis results in neurodegenerative conditions and cancer, respectively. Therefore, apoptosis is vital in the maintenance of homeostasis in multicellular organisms. Apoptosis displays phenotypic characteristics such as cell shrinkage, plasma membrane blebbing, chromatin condensation and DNA fragmentation (Zhao *et al*., 2001).

A relationship has been established between OS and cancer (Hileman *et al*., 2004; Visconti and Grieco, 2009). OS is due to a disturbance in the balance between the production of free radicals and the body's antioxidant defence mechanisms (Durackova, 2010). Free radicals are highly reactive compounds with an unpaired electron and are products of cellular reactions in the cell that breakdown oxygen which is mostly generated by the mitochondrial respiratory chain (Poyton, *et al.,* 2009). Major groups of free radicals are reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Franco and Panayiotidis, 2009). Elevated reactive oxygen species cellular ROS are known to be involved in the initiation and progression of cancer as they can target cellular components such as DNA, lipids and proteins (Powis *et al.,* 1997). Acting to protect the body from these ROS is a system of enzymatic antioxidants that are distributed in the human body such as superoxide dismutase (SOD) and catalase (CAT) and non enzymatic antioxidants such as Vitamin C and E (Florence, 1995; Rahman, 2007). However, under OS these cellular antioxidants are depleted (Conklin, 2000) and accumulation of ROS may result in DNA damage, genome instability and mutations in tumour suppressor genes that initiate the process of carcinogenesis (Kang, 2002).

Different treatments for breast cancer have been proposed and they include chemotherapy (Colleoni *et al.,* 2000), surgery (Petit *et al*., 2003), radiation therapy (Poortman *et al*., 2004), hormonal therapy (Goldhirsch *et al*., 2001) and psychological treatment. Unfortunately most of these treatments are associated with multidrug resistance, toxicities and side effects such as hair loss and vomiting (Bergkvist and Wengstrom, 2006). In addition, the currently and commonly used cancer therapies are ineffective in prolonging life and thus, there is need to search for novel cancer therapeutics such as plant extracts or plant derived compounds since they are natural, non-toxic, easily assessable and affordable (Roja and Rao, 2000). A wide variety of human disorders that include cancer are being treated with the use of plant materials due to their decreased toxicity levels, cost-effectiveness as well as minimal side effects in order to avoid drug resistance caused by pharmacological agents (Pari *et al.,* 2000; Ravi *et al.,* 2012). Administration of antioxidant rich compounds could be a promising chemo preventive and chemotherapeutic approach in the management of breast cancer.

1.2 Study Aim and Objectives

Hibiscus sabdariffa (HS) is an important medicinal plant because it is rich in bioactive compounds such as polyphenolic acids, anthocyanins, flavonoids, amino acids and minerals. Studies indicate that HS exhibit antioxidant activity to combat OS and possess anticancer activity. However, there are no reports on the effects of HS on breast cell lines. Therefore, this study will focus on evaluating the cytotoxic and antioxidant effects of HS on MCF7 and MCF12A breast cell lines with special focus on OS and apoptosis.

This will be achieved by the following objectives:

- To determine antioxidant content of methanolic and water extracts of HS using the total polyphenols and anthocyanins assays;
- To determine antioxidant capacity of water and methanolic extracts of HS using the assays: Oxygen radical absorbance capacity (ORAC) and Ferric reducing antioxidant power assay (FRAP);
- To evaluate the effect of HS extracts on cell morphology, cell viability and cytotoxicity of *in vitro* cell cultures;
- To assess if HS extracts affects mitochondrial transmembrane potential of *in vitro* cell cultures;
- To determine the antioxidant status of *in vitro* cell cultures treated with HS extract using the superoxide dismutase, catalase and total glutathione assays;
- To determine the antioxidant status of *in vitro* cell cultures treated with HS extract using the thiobarbituric reacting substance assay and reactive oxygen species assay.

CHAPTER TWO

LITERATURE REVIEW

The development of human breast tissue occurs in early foetal life. It initially starts as a depression along the lines of the armpit which forms a mammary pit on the mammary gland. Both males and females have breasts but the mammary gland is more developed in females. At puberty female breasts begin to grow to form glandular tissue and fat deposits due to the production of oestrogen and progesterone hormones in preparation for reproduction.

2.1. Breast Anatomy

The breast is a mass of fibrous, glandular and fatty tissue that is located on the superior ventral surface of the body. The glandular tissue consists of 15 to 20 lobes which are the milk producing glands and give shape to the breasts. Each lobe is composed of lobules which are connected together with blood vessels, areolar tissue and ducts. Lactiferous ducts transport milk from lobules to the nipple and each duct has a dilated section known as a lactiferous sinus for accumulating milk. The nipple is a small cuboidal projection surrounded by an area of pigmented skin called the areola as shown in Figure 2.1. The Cooper's ligament gives support to the breast tissue and the lobes (Monifar, 2007; Moore *et al*., 2013).

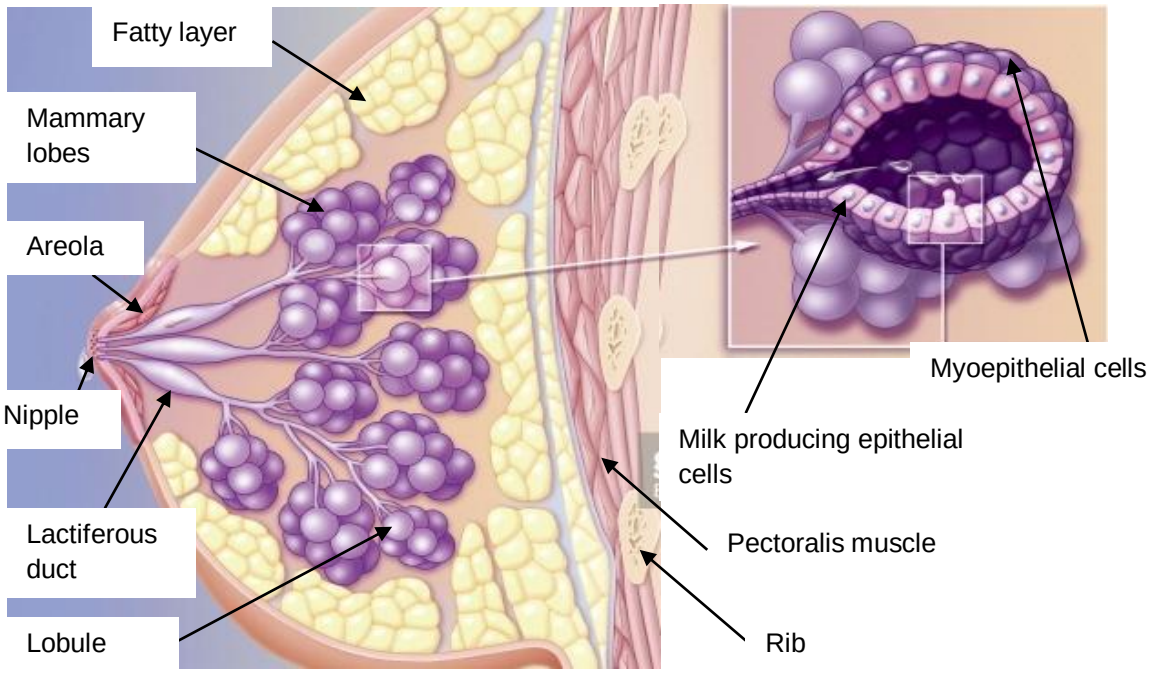

Figure 2.1: Breast anatomy (Adapted from Moore *et al*., 2013)

Pregnancy results in breast tissue changes that include darker, hyper pigmented and larger areola, due to hormonal influence (Gray, 2000). Hormones such as oestrogen stimulate the development of lobules, deposition of fat and growth of mammary gland. Progesterone and prolactin are responsible for the function of these structures (Moore *et al.,* 2013). The breast tissue can develop various disorders which include fibrocystic changes, fibro adenomas, mastitis, breast abscess, gynecomastia and breast cancer. Fibro adenomas are non-cancerous breast masses which are discrete, mobile, solid and have well demarcated borders. They are common in adolescents and are characterised by increased proliferation or neoplasm of stromal cells and reactive glands (Dixon, 2009). Fibrocystic changes are also knows as mammary dysplasia, fibrocystic disease or fibroadenosis. This disorder is characterised by benign or non-cancerous neoplasm of stroma and glandular tissues and is common in women between the ages of 20 to 50 years. It is associated to the classic changes in hormone levels during menstruation and its symptoms include increased density, tenderness of breasts and changes in the size of cysts (Malik *et al*., 2010). Breast abscess are a rare inflammatory condition of the breast which is characterised by collection of pus especially in lactating women (Jatoi and Kaufmann, 2010). Ultimately, breast cancer is a benign or malignant neoplasm that results from rapid cellular proliferation of abnormal cells which lack structural organisation or functional coordination (Gupta and Massague, 2006).

2.2 Epidemiology of Breast cancer

Cancer forms an enormous burden on the society in both developed and developing countries. Increased risk factors such as smoking, obesity, changing reproduction patterns, growth and aging of the population are expected to be the cause of the increase in the burden of cancer. In 2012, the prevalence of cancer worldwide was estimated at about 14.1 million and death cases at 8.2 million (Torre *et al*., 2015). The number of new cases is expected to increase by about 70% over the next decade. Over the years the burden has shifted to developing countries which accounts for 57% of new cases and 65% of death caused by cancer (Torre *et al*., 2015). This is due to worsening economic and social challenges and lack of equipment for early detection and treatment leading to poor prognosis (Anderson *et al*., 2003).

Breast cancer is the most common cancer in women worldwide and it remains the leading cause of cancer death in females (Ferlay *et al*., 2010). In 2012, prevalence of breast cancer was 1.7 million new cases and 521,900 deaths. It account for 25% of all cancers cases and 15% of all cancer deaths among females (Torre *et al*., 2015). Breast cancer is the most common cancer in South Africa and it is increasing in incidence. According to the (2005) National Cancer Registry, 1 in 29 women will develop breast cancer and there are about

5674 new cases per 100 000 people per year. It is the most common and accounts for 20% of all female cancers (South-African-cancer-statistics). These high figures demostrate that breast cancer is an important health issue for women and the society at large. Interventions and strategies to decrease the prevalence and mortality rates include identification and modification of the risks factors, early diagnosis, improved and safer treatment modalities.

2.3 Classification of Breast cancer

Breast cancers are a multifaceted group of tumours that exhibit extensive differences in their morphology, response and clinical presentation. Their classification is paramount as it gives an insight on the biology of breast cancers, prognosis and management strategies. Breast cancers can be divided into histopathological classification which organises according to unique cytology and growth patterns (Tavassoli and Devilee, 2003), molecular classification which employs Immuno-histochemistry and gene expression profiling which identifies unique transcriptomic features (Perou *et al*., 2000). The histological classification has been the key diagnostic tool but it does not demonstrate effectively the biological complexity and clinical course of this disease. Therefore, there is a need to confirm these distinct entities and determine prognosis in the breast cancer subtypes by the use of molecular techniques such as identifying proteins and gene expression (Weigelt *et al*., 2008).

2.3.1 Histopathologic types of breast cancer

Breast cancer can be classified as invasive (infiltrating) or non invasive (*in situ). In situ* refers to tumours that are confined to their site of origin and have not spread to other parts of the body. In contrast, invasive refers to tumours that have invaded the basement membrane and spread through the lymphatic and vascular system to distant organs. The most common types of breast cancer are lobular and ductal carcinoma which can either be invasive or *in situ* type (Jatoi and Kaufmann, 2010).

Ductal carcinoma in situ (DCIS) is characterised by proliferation of malignant epithelial cells that line the breast milk ducts. It comprises of pathologic lesions that include papillary, comedo, cribiform, solid and micro papillary forms that differ in their growth patterns. It's a premalignant lesion that may progress to invade the surrounding tissue and form lesions that are single and unilateral resulting in invasive ductal carcinoma (IDC). IDC is the most common type of breast cancer consisting up to 50 to 80 % of breast cancers (Page, 2003; Weigelt *et al*., 2008). Lobular carcinoma in situ originates from the lobes of the breast and is common in women. It is a premalignant lesion that varies in its growth patterns and progresses to invasive lobular carcinoma (IIC) upon invasion of surrounding tissue. IIC consists of 5% to10% of all breast cancers. Its pathologic lesions are bilateral, multifocal and

multi central and is more common in younger women (Page, 2003; Dixon, 2006; Weigelt *et al*., 2008).

Other types of breast cancer that are rare include medullary carcinoma, mucinous carcinoma, inflammatory breast carcinoma and Paget's disease (World Health Organisation, 1982; Weigelt *et al*., 2008; Dixon, 2009). Invasive carcinomas include tubular, medullary, mucinous, ductal and lobular are further subclassified as well differentiated, moderately differentiated or poorly differentiated based on levels of mitotic index and nuclear pleomorphism (Lester *et al*, 2009).

2.3.2 Molecular classification of breast cancer

Molecular classification of breast cancer has prognostic value and permits the prediction of responses to new targeted therapies (Gatza *et al*., 2010; Malhotra *et al*., 2010). Molecular methods include gene expression profiling, unbiased hierarchical clustering, Immuno-histochemistry, nucleic acid in situ hybridization and Polymerase Chain Reaction (PCR). Previous studies have discovered a number of molecular subtypes of breast cancer that are classified as luminal epithelial subtype A and B, Basal epithelial-like, normal breast-like and Human epidermal growth factor receptor 2 (HER2) subtype (Perou *et al*., 2000; Sorlie *et al*., 2001; Malhotra *et al*., 2010). A previous study indicated that a new subtype has been discovered and is known as claudin-low (Prat *et al*., 2010).

The basal like subtype accounts for 15% of all molecular types and is also known as triple negative meaning it is negative for HER2, oestrogen and progesterone receptors (Badve *et al*. 2011). It is characterised by cytokeratins 5/17, has the worst diagnosis and is common in young women. Luminal A accounts for 40% of all subtypes and is oestrogen receptor positive, HER2 negative. It is low grade type with better prognosis and is common in post menopausal women. Luminal B accounts for 20% of all subtypes, is positive for oestrogen receptor, progesterone and HER 2 hence also known as triple positive. It is a high grade cancer with poor prognosis. Both luminal A and B subtypes are characterised by cytokeratins 8/18 (Hu *et al*., 2006; Kornegoor *et al*., 2012). HER2 accounts for 10% to 15% of all subtypes and is oestrogen receptor negative and HER2 positive. It is high grade with poor prognosis. Claudin-low accounts for 12% to 14% of all subtypes. It is low for claudin 3/4/7, oestrogen receptor negative and E-cadherin positive (Perou *et al*., 2000; Sorlie *et al*., 2001; Malhotra *et al*., 2010). Variation in the subtypes is due to different transcriptional programs in each tumour. Bergamaschi *et al* (2006), showed gene expression in the subtypes display distinct frequencies of genetic DNA copy number alterations with high amplification in luminal B tumours. These findings suggested a different mechanism of genomic instability for the different subtypes.

2.3.3 Functional classification of breast cancer

There has been profound research on breast cancer stem cells (CSCs) with several studies identifying CSCs involvement as tumour initiating cells in various malignancies. The theory for CSCs affirms that, a group of cells within a tumour accounts for the initiation and progression of the tumour while the majority of the tumour cells have a low tumorigenic capacity. Currently there are two proposed theories, the first being the variation seen in breast cancer arises from normal mammary stem or progenitor cells at various levels in the stem cell hierarchy. The second being that they arise from normal mammary stem or progenitor cells that are transformed to oncogenes or tumour suppressor genes which give rise to various breast cancer subtypes. Studies have shown that CSCs are identified with poor prognosis and future studies will encompass CSCs in therapeutic strategies (Stingl and Caldas, 2007).

2.4 Diagnosis of Breast cancer

Innovation in the diagnostic techniques has transformed the detection and diagnostic strategies of breast cancer over the past decade. Implementation of educational programmes with breast self examinations, organised and improved screening procedures have a significant effect in early detection and treatment of breast cancer. Detection of breast cancer includes procedures such as mammography, Magnetic Resonance Imaging (MRI), ultrasound, Positron Emission Tomography (PET), ultrasonography, breast biopsy and molecular tests (Veronesi *et al*., 2005; Lee *et al*., 2010). Diagnostic mammograms are used to confirm malignancy in a patient presenting with symptoms of breast cancer upon clinical or breast self examination (Benson *et al*., 2009). However mammograms are less likely to confirm breast tumours in young women as they have denser breast tissue compared to postmenopausal women. Malignancy presents as micro calcifications in a mammogram (Kneeshaw *et al*., 2003). Breast ultrasonography is an imaging technique that is effective in the diagnosis of women with dense breast tissue and it can differentiate between a cyst and a solid lesion. MRI is a highly sensitive technique that gives images on non palpable lesions and is used in the screening of BRCA positive patients (Veronesi *et al*., 2005; Mann *et al*., 2013). When imaging techniques give positive results for breast cancer, cytological tests are done which include a breast biopsy (Gordon *et al*., 1996).

Molecular techniques that are more reliable and highly sensitive such as proteomic and gene expression profiling and PCR are being employed as diagnostic tools (Malhotra *et al*., 2010). Multiple proteins have been found to be over expressed in breast cancer and they include oestrogen, progesterone and HER2 receptors. The molecular detection of these markers could aid in early diagnosis and provide an insight into the treatment and prognosis of the

diseased protein. Immuno-histochemistry is a key technique that uses specialised stains to determine the presence of hormone receptor oestrogen and progesterone. A tissue biopsy sample is used to evaluate the presence of these hormones; however this technique is associated with technical variability and false negative results (Perou *et al*., 2000). HER-2 protein is an oncogene encoded growth factor receptor that is over expressed in breast cancer due to amplification of HER-2 proto oncogene. Its presence has been associated with poor prognosis of breast cancer (Crystal *et al*., 2003). It is quantified using the fluorescence In Situ Hybridization technique which is also associated with technical variability (Bast *et al*., 2001). Reverse transcriptase- Polymerase chain reaction (RT-PCR) is current diagnostic test that is highly sensitive, simple and rapid for detecting breast cancer cells in blood and lymph nodes and in amplifying oestrogen receptor Ribonucleic acid (RNA) (Raj *et al*., 1998). Molecular genetic testing is a comprehensive screening test which is done on symptomatic individuals and their relatives at risk of BRCA1 and BRCA2 gene mutations (Frank *et al*., 1998; Petrucelli *et al*., 2010).

2.5 Pathophysiology of breast cancer

Cancer involves gene alterations in cellular processes which results in tumour formation. For the past decades, the focus on cancer research has been mainly on the malignant cell. New information has enhanced knowledge on the genetic and non genetic causes that relate to the transformation of normal cells to cancer cells, resulting in malignancy (Franco *et al.,* 2008). Risk of developing breast cancer is increased when an individual has a family history of the disease (Pharoah *et al.,* 1997). Potential modifiable factors associated with increased risk for breast cancer are physical inactivity, obesity (Adebamowo *et al.,* 2003) and the use of oral contraception (Chlebowski *et al*., 2013). Breast cancer is not common among women younger than 30 years but the incidence of breast cancer increases with age (Peto *et al.,* 2000). Reproductive factors that change hormone status affect the risk of breast cancer and they include women who menstruate at a younger age and reach menopause later (Kelsey *et al*., 1993). In addition, risk is also increased by inherited genetic risks in the case of the *BRCA2* gene mutation (Ashworth, 1995), exposure to radiation (Doody *et al.,* 1998; Nguyen *et al*., 2011), previous breast cancer diagnosis and hormone replacement therapy (Beral, 2003). Increase in age could be a determining factor for the acquisition of telomere dysfunction, mutations and high epigenetic silencing that leads to tumour progression (Widschwendter & Jones 2002).

Six hallmarks of cancer were proposed and they give an insight on the alterations in cell physiology that result in the tumorigenic and ultimately malignant cell. The hallmarks are peculiar and integral capabilities that enhance tumour growth and metastasis and provide a

solid framework to comprehend the biology of cancer. They include sustained proliferative signalling, evading growth suppressors, enabling replicative immortality, induced angiogenesis, activating invasion and metastasis and resisting cell death as illustrated in Figure 2.2 (Hanahan and Weinberg, 2000; Lazebnik, 2010; Hanahan and Coussens, 2012). Underlying these capabilities are genomic instabilities and mutations which induces heterogeneity and inflammation. In addition, two emerging capabilities have been added: evading immune response (Cavallo *et al*., 2011) and re programming of energy metabolism (Kroemer and Pouyssegur, 2008; Ward and Thompson, 2012). It has also been noted that tumour cells recruit normal cells to participate in tumorigenesis creating a tumour micro environment (Hanahan and Weinburg, 2011). These capabilities could be employed in the development of treatment strategies. For the purpose of this study, the focus is on sustained proliferative signalling, evading growth suppressors, enabling replicative immortality and resisting cell death.

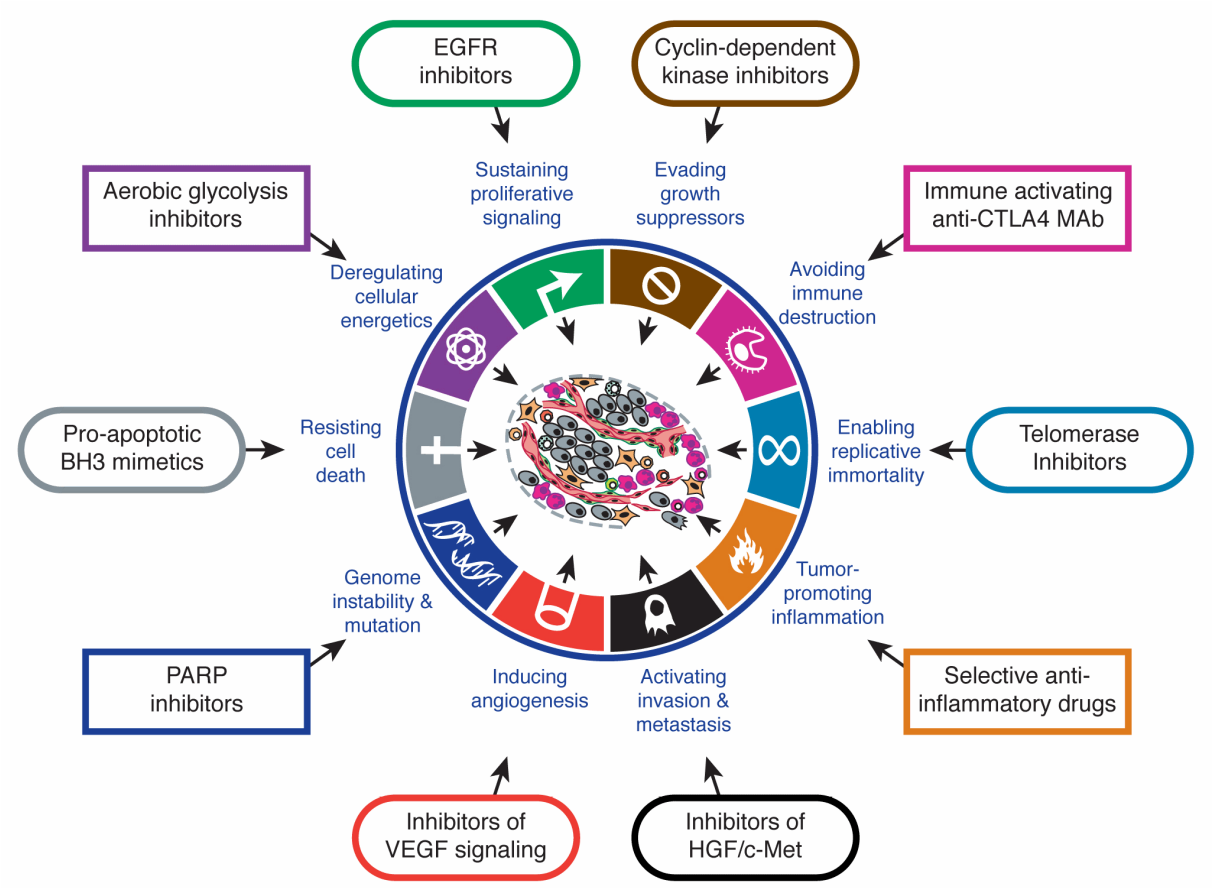

Figure 2.2: Emerging and enabling hallmarks of cancer. (Adapted from Hanahan and Weinburg, 2011)

2.5.1 Sustained Proliferative Signalling

Growth factors are proteins that stimulate cell proliferation through glucose metabolism (Heiden *et al.,* 2001). Normal cells ensure homeostasis by controlling production and release of growth signals for cell proliferation and division (Hanahan and Weinburg, 2011). Cell division precisely occurs when normal cells are supplied with suitable mitogenic factors. (Hanahan and Weinburg, 2000). In contrast, cancer cells produce their own growth factor ligands or send signals to stimulate normal cells within the supporting stroma to provide cancer cells with growth factors (Cheng *et al.,* 2008). Growth independence in cancer cells could be due to deregulation of receptor signalling pathways derived from elevated receptor proteins, downstream to these receptors or mutations on oncogenes or tumour suppressor genes (Kroemer and Pouyssegur, 2008; Hanahan and Weinburg, 2011). They activate receptor tyrosine kinases, which in turn activate the two pathways: the RAS/RAF/MAP kinase (ERK) pathway and the phosphatidyl-inositol 3 kinases (PI3K) pathway which further activate the mammalian target of rapamycin (mTOR) for enhancing cell proliferation (Kroemer and Pouyssegur, 2008). Activation of the PI3K pathway in human cancers has been reported to be caused by mutations on the oncogenic PIKC3A gene and alterations upstream of the kinases (Brachmann *et al.,* 2009). In breast cancer, Human epidermal growth receptor (HER2) oncogene produces HER3, which recruits and activates PI3K pathway, AKT pathway and mTOR kinases at translational level which leads to increased cell proliferation (Lee-Hoeflich *et al.,* 2008). Most human cancers have mutations in Kirsten (K-RAS), Harvey (H-RAS) and Neuroblastoma (N-RAS) or otherwise on downstream effector genes that include AKT kinases (Shaw and Cantley, 2006).

2.5.2 Evading Growth Suppressors

Cancer can result from the deregulation of normal functioning of the cell cycle which underlies increased proliferation (Meeran and Katiyar, 2008). The cell cycle is a process that consists of recurring events that leads to cell division. In eukaryotic cells, the cell cycle is divided into four phases namely, G1 phase which prepares the cell for synthesis, S phase which is the DNA synthesis phase, G2 phase which prepares the cell for division and the M phase which is the period of chromosome separation and cell division (Murray and Hunt, 1993; William and Stoeber, 2012). As the cell cycle proceeds from one phase to the next, it is regulated by certain gene products known as check points which are activated or inactivated depending on the status of the cell (Hartwell and Weinert, 1989; William and Stoeber, 2012). Aberrations due to replication errors, DNA damage from chemicals and radiation or incomplete cell cycle events are detected by checkpoint that halt cell cycle progression or propagates apoptosis (Bartek *et al*., 2004). In some cases checkpoints activates the induction of repair genes (Bartek *et al*., 2004; Musacchio and Salmon, 2007). One of the

checkpoints is the Cyclin-dependent kinases (CDK's) which are protein kinases that are activated upon binding to cyclins (Malumbres and Barbacid, 2006). Failure in the functioning of the checkpoints mechanism results in the development of cancer (Meeran and Katiyar, 2008). Events implicated in the insensitivity of cancer cells to antigrowth signals include mutations in the tumour suppressor genes and inappropriate activation of signalling pathways. Inactivation of p53 or Rb cell cycle checkpoints in cancer cells results in persistent cell proliferation (Harbour and Dean, 2000). Mutations in ligands and receptors such as the HER2 receptor mutation or deregulation of downstream signalling pathways such as the PI3K or the Ras-Raf-MAPK are a common occurrence in cancer cells (Huang *et al*., 2002; Zhang *et al*., 2009). Deregulated signalling results in activation of CDK's and cyclins leading to defective Rb and altered capacity to suppress transcription (Malumbres and Barbacid, 2006). Other studies revealed mutations on the cell cycle genes such as CDK's in different cancer cells (Freier *et al*., 2003). Ultimately, mutations results in continued cell cycle progression, disruption in the cell cycle mechanism, genomic instability and tumour propagation (Malumbres and Barbacid, 2009). Molecular targets for cancer therapy on the cell cycle regulation includes the different stages of the cell cycle, cyclins, CDK's and apoptosis (Meeran and Katiyar, 2008).

2.5.3 Enabling Replicative Immortality

Normal cells undergo a limited number of successful cell divisions before they halt their replication. The cells either enter into a crisis state, known as cell death or apoptosis, which is an irreversible, non proliferative state (Artandi and DePinho, 2010). However, telomerase is the contributing factor in the maintenance of telomeres resulting in immortalized cancer cells (De Vivo *et al*., 2009). Previous studies have revealed that tumours consist of a subpopulation of cells known as cancer stem cells (CSC) (Joseph *et al*., 2010). The CSC's characteristics include tumorigenic potential and self renewal (Balic *et al*., 2006) and have been identified in breast, brain, colon, prostate and ovarian tumours (Visvader and Lindeman, 2008).

2.5.4 Resisting Cell Death

Programmed cell death (PCD) is an essential process for the maintenance of homeostasis and development of biological processes in multi cellular organisms (Hanahan and Weinberg, 2011). Currently, three types of PCD exist, that can be differentiated by morphology, which are apoptosis, necrosis and autophagy (Bialik *et al*., 2010). Apoptosis is an ancient Greek word which denotes falling leaves and was first introduced by Kerr *et al*. (1972) as active form of programmed cell death that is crucial in biological processes and consists of a set of pathways leading to morphogenesis and elimination of unwanted cells

(Adam, 2003; Ola *et al*., 2011). Abnormal up-regulation or down-regulation of apoptosis results in pathological disorders. Excessive down regulation of apoptosis leads to disorders like cancer, auto immune diseases and inflammation, while up regulation leads to neurodegenerative conditions such as Alzheimer's disease (Bidere *et al*., 2006; Ola *et al*., 2011). Apoptosis can be initiated by several stimuli that include cytotoxic drugs (Solary *et al*., 2000), oxidative stress (Meng and Yu, 2010), removal of growth factors, radiation, tumour necrosis factor (Ashkenazi, 2002), ischemia and bacteria (Lancellotti *et al*., 2009; Ola *et al*., 2011).

2.5.4.1 Characteristics of apoptosis

Morphological changes of apoptosis are dense chromatin condensation and margination, nuclear fragmentation, cell shrinkage also known as pyknosis (Kroemer *et al*., 2005; Wong 2011). Chromatin condenses and eventually breaks off with an intact membrane also known karyorrhexis to form apoptotic bodies and usually the plasma membrane is intact. Other features include late mitochondrial disintegration, membrane blebbing, individual cells affected and eventually phagocytosis (Gulbins *et al*., 2000; Ouyang *et al*., 2012). Biochemical changes in apoptosis are DNA fragmentation, caspase activation, poly (ADP- ribose) polymerase proteolysis (Giansanti and Scovassi, 2008; Giansanti *et al*., 2011), phosphatidylserine (PS) externalisation, cleavage of intracellular substrates by enzymes and recognition by phagocytic cells (Kumar *et al*., 2010; Ouyang *et al*., 2012). Apoptosis starts with the externalisation of PS on the outer membrane which results in recognition by phagocytic cells and DNA fragmentation (Vaux and Silke, 2003; Wong, 2011). Caspases are activated to cleave cellular proteins and to activate DNAases which further break down the nuclear DNA (Lavrik *et al*., 2005). However, some studies indicate apoptosis can occur without caspase activation (Galluzi *et al*., 2007).

2.5.4.2 Apoptosis and carcinogenesis

Studies have indicated that apoptosis plays a role in the removal of cancer cells and reduction of tumour progression (Kerr *et al*., 1972; Wong, 2011). Hanahan and Weinberg (2011) reported that most cancer cells acquired resistance to apoptosis and mutations in certain genes that aids cancer cells to evade apoptosis. This is accomplished by several ways that include oncogenes and mutated tumour suppressor genes. An example is the TP53 tumour suppressor gene which encodes the p53 protein and its defect contribute to tumour progression and decreased apoptosis (Bai and Zhu, 2006; Ouyang *et al*., 2012). Studies show that silencing of p53 mutant gene results in decreased cell proliferation due to increased apoptosis (Vikhanskaya *et al*., 2007). Cancer cells also evade apoptosis due to an imbalance between pro-apoptotic and anti-apoptotic proteins of the Bcl family (Ola *et al*.,

2011). Bcl-2 protein family is crucial in modulating apoptosis in cancer cells and consist of anti-apoptotic and pro-apoptotic proteins (Petros *et al*., 2004; Llambi and Green, 2011). Studies indicate that over expression of Blc-2 anti-apoptotic protein leads to resistance in apoptosis in prostate cancer (Raffo *et al*., 1995; Wong, 2011) and breast cancer cells (Fulda *et al*., 2000; Wong, 2011). In addition, an anti-apoptotic protein Bcl-xl contributes to multidrug resistance and resistance to apoptosis in cancer (Minn *et al*., 1995; Wong, 2011). Another contributing factor is reduced or dysfunctional caspase proteins which causes tumourigenesis and has been observed in colorectal (Shen *et al*., 2010), breast, and cervical cancer (Devarajan *et al*., 2002; Wong, 2011). In addition, impaired death receptor signalling pathway in cancer cells has been linked to resistance in apoptosis. Defects or loss of the death receptors such as FAS or Tumour necrosis factor leads to evasion of apoptosis and tumour progression (Reesink-Peters *et al*., 2005). Certain cell proliferation oncogenes such as MYC enhance evasion of apoptosis via increased signalling (Lowe *et al*., 2004; Hanahan and Weinburg, 2011).

2.5.4.3 Apoptosis pathways

2.5.4.3.1 Intrinsic pathway

The intrinsic pathway is initiated internally by stimuli such as stress, hypoxia, oxidative stress and DNA damage (Karp, 2008; Wong, 2011). This leads to mitochondrial disruption, increased mitochondrial permeability and the release of apoptotic proteins (Danial and Korsmeyer, 2004; Wong, 2011). The Apoptosome is formed when cytochrome c is released into the cytoplasm and binds to Apoptotic Protein Activating Factor 1 (APAF-1) and results in caspase cascade activation (Wen *et al*., 2012). When caspase 8 is triggered, it cleaves pro caspase 3 to form caspase 3, which in turn cleaves other caspases (Ghavami *et al*., 2009). Additionally, the mitochondria also release substances known as Inhibitors of Apoptotic Proteases (IAP's) to block apoptosis that inhibit caspases (Altieri, 2010). Mitondondria also releases nucleases that degrade DNA and the cellular fragments are engulfed by phagocytes to complete apoptosis (Savill and Fadok, 2000; Giansanti *et al*., 2012). The pathway is shown in Figure 2.3 (Wong *et al*., 2011).

2.5.4.3.2 Extrinsic pathway

The extrinsic pathway is initiated by cell death signals which results from death ligands binding to specific death receptors. Common death receptors include TNF-α and FAS and their ligands TNF-α L and FAS-L (Wajant 2002; Ola *et al*., 2011). Death receptors have death domains that recruit adapter proteins such FADD (Fas Associated Death Domain Protein) (Ashkenazi, 2002). DISC (death inducing signalling complex) is formed by trimerization of death ligands, induction of death receptor oligomerazation, which provides a binding site for

adaptor protein (Yan and Shin, 2005). DISC also initiates the activation of caspase 8 which initiates apoptosis (Karp, 2008). The pathway is shown in Figure 2.3 (Wong *et al*., 2011).

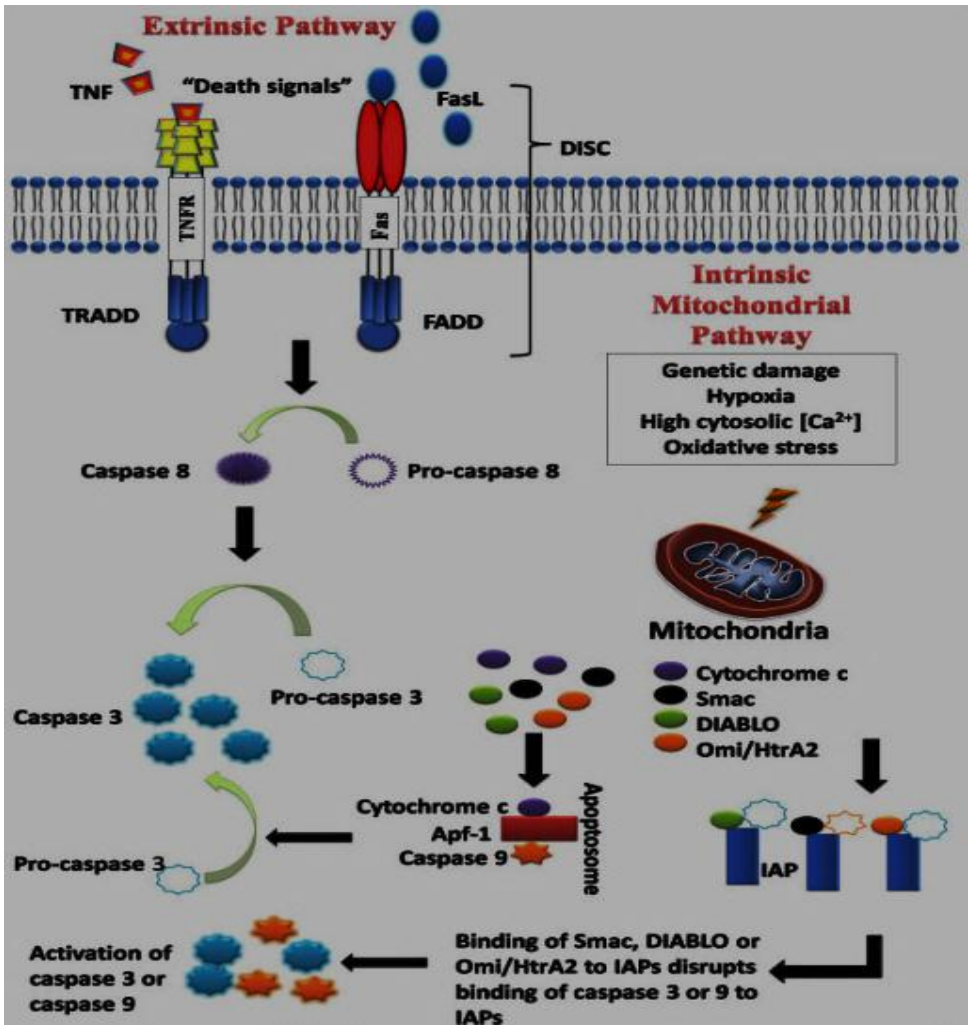

Figure 2.3: Diagram for two major apoptosis pathways (Adapted from Wong *et al*., 2011)

2.5.4.4 Regulatory components of apoptosis

The Bcl-2 protein family is composed of four homology domains named, BH1, BH2, BH3 and BH4 which are responsible for mitochondrial membrane permeability (Danial and Korsmeyer 2004; Wong, 2011). Anti-apoptotic proteins are multi domain and include Bcl-2, Bcl-w, B00, Bcl-xl and A1 (Engel and Henshall, 2009; Ouyang *et al*., 2012). The pro-apoptotic proteins comprise of two groups, the multi-domain members of Bax and Bak and those with the BH3 domain only, which include the Bad, Bim, Bid, Bik and Bmf (Dewson and Kluc, 2010). Bcl-2 family controls apoptosis by forming heterodimers between anti apoptotic and pro apoptotic proteins. Up-regulation of anti-apoptotic proteins prevents cell death, while up regulation of pro-apoptotic proteins results in cell death (Wong, 2011). Anti-apoptotic proteins prevent cell

death by counteracting the activity of pro-apoptotic proteins through interfering with oligomerization (Youle and Strasser, 2008).

The Caspases are cysteine proteases that are responsible for cleaving target proteins, activate latent enzymes crucial in apoptosis (Wang and Lernardo, 2000; Ola *et al*., 2011). Caspases exist as pro enzymes and are activated by a series of events of proteolysis that cause apoptosis (Shi, 2002; Ola *et al*., 2011). Caspases are divided into 2 subfamilies, namely those involved in inflammation and those involved in apoptosis. Apoptotic caspases are further divided into 2 groups, namely the initiators (upstream) and effectors (downstream) caspases (Shi, 2002; Ola *et al*., 2011). Initiator caspases (caspase 1, 2, 4, 5, 9, 11, 12) have long prodomains for protein recruitment (Thomberry and Lazebnik, 1998; Ola *et al*., 2011). Effector caspases (3, 6, and 7) play a role in apoptotic cell death (Sprick and Walczak, 2004). Apoptosis can either be caspase independent or dependent, with the later occurring via apoptosome formation or DISC (death inducing signal complex) formation (Ola *et al*., 2011). Other studies indicate p53 mediated apoptosis, occurs upon DNA damage and is caspase 2 dependent (O'Reilly *et al*., 2002; Ola *et al*., 2011). Caspase independent apoptosis (Lartigue *et al*., 2009) involves the AIF and endonucleases G which upon receiving a death signal, they move to the mitochondria and cause DNA fragmentation independent of caspase activation (Comelli *et al*., 2009). Other components that are responsible for caspase independent apoptosis include heat shock proteins (Laudanski and Wyczechowska, 2006), high temperature requirement protein A (Omi/HItrA2) (Balakrishnan *et al*., 2009) and ste20-like protein kinase 3 (Lin *et al*., 2010).

Other regulatory components in apoptosis include IAP's and micro RNA's. IAP's are a group of proteins that control apoptosis via inhibition of caspases by binging to active sites or promoting degradation of caspases (Wei *et al*., 2008). Micro RNA's are crucial in controlling gene expression and are single stranded non protein coding RNA's (Lima *et al*., 2011; Ouyang *et al*., 2012). Studies indicate some micro RNA's act as oncogenes and enhance tumour progression (Linnstaedt *et al*., 2010), while some are linked to the gene c-Myc which increases cell proliferation and inhibits apoptosis (Croce, 2009).

Potential treatment strategies that target the regulation of apoptosis can be used to eradicate cancer (Wong, 2011). Some drugs have been discovered that target the Bcl2 anti apoptotic proteins in leukaemia (Abou-Nassar and Brown, 2010) and silence anti apoptotic proteins in breast cancer cells (Wu *et al*., 2011). Additionally, some treatment strategies target p53 gene (Chene, 2001) and caspase proteins (Rohn and Noteborn, 2004).

2.6 Multi step carcinogenesis model

Evidence indicates that cancer development is a multi stage and multi step process which results in molecular and cellular events that transform normal cells to malignant cells (Klaunig *et al*., 2010). Carcinogenesis consists of three steps; initiation, promotion and progression as illustrated in Figure 2.4. Initiation is an irreversible phase of interaction between physical or chemical carcinogenic agents and the tissue consequently experiencing in DNA damage (Singh *et al*., 2011). The promotion stage is a reversible stage, where promoters enhance clonal expansion of abnormal initiated cells (Klaunig *et al*., 2010). The final stage is the progression step, which is the irreversible conversion of premalignant cells to neoplastic cells that have the hallmarks of increased proliferation, invasiveness, angiogenesis and metastasis (Srivastava *et al*., 2010; Singh *et al*., 2011).

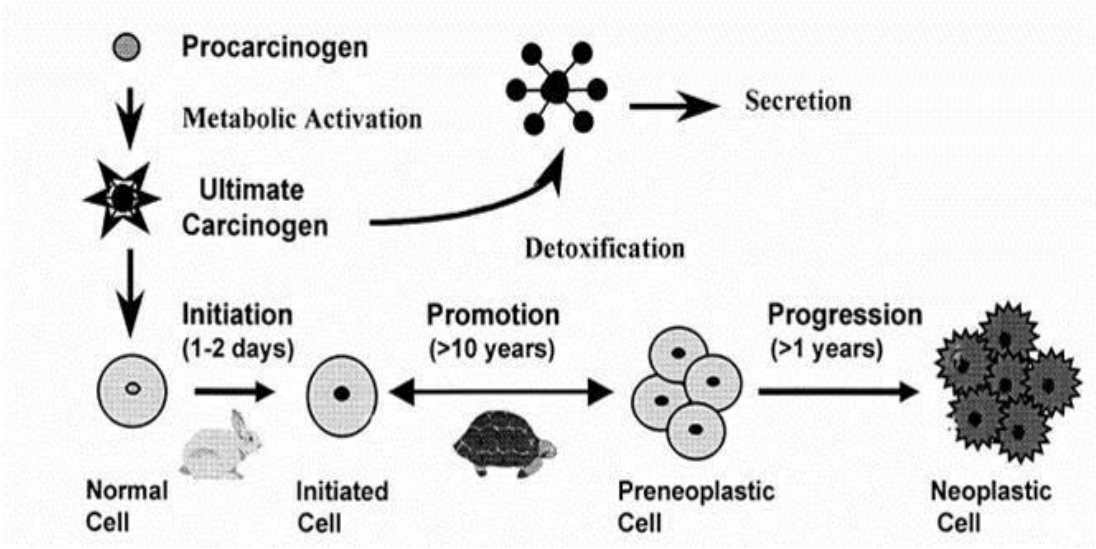

Figure 2.4: Multi step carcinogenesis (Adapted from Barcellos-Hoff *et al*., 2013)

2.6.1 Potential carcinogens

Tumour carcinogenesis is activated by environmental carcinogens which are physical or chemical exogenous factors (Dabre and Charles, 2010). They include ionising radiation (Preston *et al*., 2009), alcohol consumption (Boffetta and Hashibe, 2006), smoking (Reynolds, 2013), use of oral contraceptives (Casey *et al*., 2008; Chlebowski *et al*., 2013) and oestrogen exposure (Yager and Davison, 2006). There is evidence that environmental oestrogen reaches the breast tissue via application of cosmetic chemicals with oestrogenic activity such as deodorants, consumption of organocompounds such as Polychlorinated Biphenyls (PCB's) and use of detergents (Kalantzi *et al* 2004; Donovan *et al*., 2007). Their mode of action is to bind to oestrogen receptors and enabling cell proliferation (Soto *et al*.,

2006). Additionally, animal studies showed that bisphenol and phthalates from plastic increases cell proliferation in breast tissue and acts as an endocrine disruptor (Durando *et al*., 2007). Endogenous sources of carcinogenesis include oxidative stress which has gained popularity among researchers (Klaunig *et al*., 2010). Studies have shown that tumour development and progression is also induced by oxidative stress (Kumar *et al*., 2008). Most of the exogenous carcinogens in the host are able to generate free radicals upon activation. When these free radicals exceed antioxidants, it results in oxidative stress (Panayiotidis 2008; France *et al*., 2009).

2.7 Free radicals

Over the years research has focused on the field of free radicals, which resulted in increase in knowledge on disease progression and therapeutic strategies (Auroma, 2003). Oxygen is a critical component for respiration and its metabolism results in free radical formation (Mohammed and Ibrahim, 2004). Free radicals can either be beneficial or detrimental to multicellular organisms (Bagchi and Puri, 1998; Lobo *et al*., 2010). Free radicals are known as chemical species that comprise of an unpaired electron on the outer orbit and are highly reactive and unstable (Lobo *et al*., 2010; Halliwell, 2012). Therefore, they can target macromolecules such as DNA, RNA, lipids and proteins and prompting cell injury and deregulation of homeostasis (Young and Woodside, 2001; Anglada *et al*., 2015). Free radicals are either classified as reactive oxygen species (ROS) and reactive nitrogen species (RNS) and they both deplete antioxidants and produce reactive species. Most common RNS include nitric acid (NO), nitric oxide and nitroxyl anion. ROS are further divided into radical and non radical species. Non radical species include hypochlorous acid, hydrogen peroxide and ozone. Radical species include hydroxyl radical $^{\bullet}OH$ and superoxide $O_2^{-\bullet}$ (Ray *et al*., 2012; Sie, 2013).

2.8 Sources of ROS

ROS has both endogenous and exogenous sources. Exogenous sources are due to environmental exposure and include radiation, pollutants, smoke, alcohol, hypoxia and strenuous exercise (Schroder and Krutmann, 2005). Most ROS is generated endogenously and the sources are due to incomplete oxygen metabolism during aerobic respiration. They include mitochondrial electron transport chain, NADPH oxidase, xanthine oxidase, nitric oxide synthase and inflammation (Ebadi, 2001; Lobo *et al*., 2010). ROS is generated by reduction of oxygen (O_2) to water (H_2O) which is converted to superoxide anion ($O_2^{-\bullet}$) (Klaunig and Kamendulis, 2004). Other sources of $O_2\bullet^-$ are NADPH oxidase and xanthine oxidase (He *et al*., 2006; Klaunig, 2010).

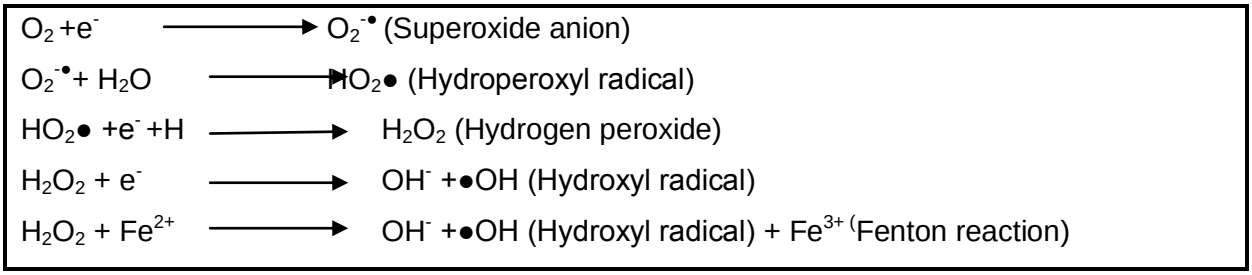

Figure 2.5: Pathways of ROS generation (Klaunig, 2010)

2.9 Physiological roles of ROS

ROS has been found to participate in the physiological processes of normal cells. They act as signalling molecules in the regulation of development processes (Halliwell, 2012). ROS regulates cellular response in hypoxic inducible factor 1 α in fluctuating oxygen levels (Bell *et al*., 2007). Increased uptake of oxygen by neutrophils and macrophages activated upon an immune response causes release of H_2O_2 and superoxide radical which exhibits antimicrobial and antitumour activities (Droge, 2002; Vera-Ramirez *et al*., 2011). Once lymphocytes are activated, they release ROS that evoke the immune response leading to the removal of the offender (Kim *et al*., 2008). Additionally, cells that control regulatory pathways for hormone synthesis, cardiovascular system produce ROS (Wolin, 2009). RNS also play a role in controlling processes such the immune response and nervous system (Klaunig and Kamendulis, 2004).

2.10 Oxidative stress

Oxidative stress is a physiological situation characterised by an imbalance between production of free radicals and the antioxidant defence system resulting in oxidative damage (Ziech *et al*., 2011; Vera-Ramirez *et al*., 2011; Anglada *et al*., 2015). Oxidative stress has been linked to the pathogenesis of several disorders including cancer (Halliwell, 2012). Overproduction of unstable and reactive free radicals in the absence of antioxidants consequently leads to interaction with RNA, DNA, lipids and proteins causing oxidative damage (Sies, 2013). On the contrary, oxidative stress leads to activation of antioxidant defence system to absorb free radicals and prevent cell damage (Halliwell, 2012) as illustrated in Figure 2.5.

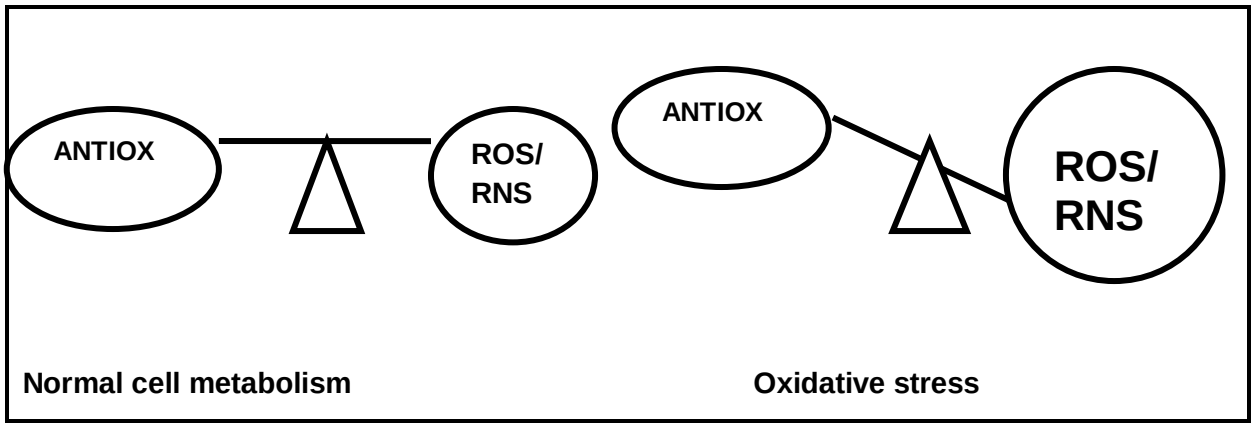

Figure 2.6: Schematic diagram for normal cell metabolism and oxidative stress
Abbreviations: Antiox (Antioxidants), ROS/RNS (reactive oxygen/ nitrogen species)

2.10.1 Lipid peroxidation

Lipid peroxidation is a process by which polyunsaturated fatty acids in the cell membrane are degraded by free radicals. It occurs in 3 phases; initiation, propagation and termination (Valko *et al*., 2006). Hydroxyl radical initiates by oxidising the fatty acids to form lipid radicals that reacts with oxygen to form lipid hydroperoxide and a new radical. Lipid peroxidation is propagated by a new radical that further oxidises fatty acids. Termination is characterised by the formation of conjugated dienes and malano aldehydes, alkanes (Vera-Ramirez *et al*., 2011). These are used as markers for lipid peroxidation in the Thiobarbituric acid reactive substances assay (Gutteridge, 1995; Cejas *et al*., 2004).

2.10.2 Protein damage

Proteins are prone to oxidative damage since they are abundant in the human body. Proteins are oxidised through modifications of amino acids, reactions with lipid peroxidation residues and attack on the peptide bond by free radicals (Lobo *et al*., 2010). Oxidation of proteins is initiated by superoxide radical which reacts with oxygen to form peroxyl radical. Irreversible attack on proteins results in protein fragmentation and aggregation which affects functional activity of enzymes, receptors and transport proteins (Halliwell, 2010). Oxidative attack on proteins results in formation of protein carbonyls and methionine sulfoxide. Protein damage can be quantified using markers such as protein carbonyls (Coskun *et al*., 2007).

2.10.3 DNA damage

Evidence indicates DNA and RNA are prone to oxidative damage and has been indicated in cancer and aging (Woo *et al*., 2008; Lobo *et al*., 2010). Superoxide radical attacks the DNA through intercalating on the double bonds and causing damage that includes DNA breaks,

lesions, modified bases, abberent DNA and protein interaction (Halliwell, 2010). Free radical attack on DNA can result in the formation of 8-hydroxy-2-deoxyguanosine (8-OH-dG) which is potentially mutagenic and is used as an oxidative stress marker (Hattori *et al*., 1997; Lobo *et al*., 2010). Superoxide radical is converted to hydrogen peroxide which is stable and can reach the nucleus causing both nucleus and mitochondrial damage resulting in malfunction of the respiratory chain. Oxidative damage to DNA by ROS disrupts the activity of DNA polymerase and ligase enzymes commonly found in oncogenes and tumour suppressor genes (Nakabeppu *et al*., 2006).

2.11 Implications of oxidative stress on breast cancer

Scientific evidence reveals that most cancer cells have increased ROS production which leads to oxidative stress (Szatrowski and Nathan, 1991; Trachootham *et al*., 2006). Documentation also states that cancer cells have high levels of ROS (Pervaiz *et al* 2004) and several *in vivo* and *in vitro* studies have proved that oxidative stress is higher in patients with cancer as compared to normal subjects (Kumaragumparan *et al.,* 2002*;* Hussien *et al*., 2005; Sener *et al*., 2007). Free radicals have been shown to cause DNA damage which results in mutations that lead to tumourigenesis (Kang, 2002; Kryston *et al*., 2011). Scientific studies stipulate that ROS is involved in multi step carcinogenesis (Kong *et al.,* 2000). In the initiation phase oxidative stress assists in cell transformation through DNA damage (Valko *et al*., 2006). In the promotion stage, OS interferes with cell signalling pathways and inhibits apoptosis and in the progression stage it inflicts additional DNA damage (Klaunig *et al*., 2010; Reuter *et al*., 2010).

Increased ROS production in tumor cells is due to uncontrolled proliferation and deregulated metabolism resulting in increased requirements for ATP. This energy demand causes stress on the mitochondrial respiratory chain resulting in increased ROS generation (Pelicano *et al.,* 2004). Malfunction of the mitochondrial respiratory chain, anticancer agents and oncogenic transformation are other factors leading to increased ROS production (Breimer, 1990). Mild ROS stress results in cells adapting by up regulating antioxidant enzymes. Further increase in ROS causes oxidative stress and disrupts redox state leading to mutations, DNA damage, decreases mitochondrial depolarization and ultimately cell death (Perera *et al.,* 1995; Pelicano *et al*., 2004). ROS plays a crucial role in initiating apoptosis or nercosis in tissues, indicating its anticancer effects. It is therefore crucial to obtain a balance between ROS and antioxidants in tumour cells to make sure cancer progression is inhibited while maintaining apoptosis (Kroemer *et al.,* 1998; Kong *et al.,* 2000).

Recently studies have indicated that free radicals are involved in oestrogen metabolism (Vera-Ramirez *et al*., 2011). Both endogenous and exogenous oestrogen are metabolised to catechol oestrogen which generates ROS and leads to DNA, RNA and protein damage and lipid peroxidation (Sarabia *et al*., 1997; Vera-Ramirez *et al*., 2011). High levels for catechol oestrogen have been discovered in oestrogen positive breast tumours compared to healthy breast tissue (Markides *et al*., 1998). Additionally, MCF7 breast cell lines treated with oestrogen where found to be high in ROS (Parkash *et al*., 2004). In addition to ROS initiating tumour formation and genomic instability, it activates signalling pathways by acting second messenger and ultimately causes tumour development through tumour cell survival, proliferation, metastasis and angiogenesis (Storz, 2005; Reuter *et al*., 2010).

2.11.1 Tumour cell survival

Studies indicate that OS contributes to cell death via apoptosis (Slater *et al*., 1995; Vera-Ramirez *et al*., 2011). However, some studies indicate that oxidative stress inhibits apoptosis in cancer cells from pancreas, retina and colon (Vaquero *et al*., 2004; Groeger *et al*., 2009). Mechanism by which free radicals inhibit apoptosis in tumours is through deactivation of caspases and Fas receptors by binding to cysteine residues or by increasing pH (Akram *et al*., 2006). Another mechanism is through inhibition of transduction pathways that lead to apoptosis such as phosphtase and tensin homology (PTEN) and up regulation of AKT pathway that increases cell proliferation (Manning and Cantley, 2007). AKT inhibits apoptosis by inactivating pro apoptotic and disrupting cell metabolism (Plas and Thompson, 2005).

2.11.2 Tumour cell proliferation

Oxidative stress contributes to increased proliferation by up regulating MAPK/AP-1 and MF-kB signalling pathways (Muller *et al*., 1997; Reuter *et al*., 2010). Hydrogen peroxide induces AP-1 via MAPK cascade and leads to activation of transcription factors and increasing cell proliferation (Chang and Karin, 2000). Nuclear factor kappa B (NF-kB) expression enhances cell proliferation, and has been reported to be expressed in breast and colon cancer (Baldwin 1996; Rath and Aggarwal, 2001; Reuter *et al*., 2010). The link between ROS and NF-kB is debatable, with low oxidative stress causing its activation and high oxidative stress causing inhibition (Gloire *et al*., 2006). ROS up-regulates cell proliferation in most cells and decreasing ROS by use of antioxidants prevents cell proliferation but high ROS results in cell death by apoptosis (Chang *et al*., 2007; Halliwell, 2007).

2.12 Antioxidants

Antioxidants are substances that prevent oxidative damage by acting as radical scavengers, peroxide decomposer, single oxygen quenchers and metal chelators (Lobo *et al*., 2010; Halliwell, 2012). Under normal circumstances antioxidants prevent the formation of free radicals and scavenge free radicals (Kancheva, 2009). Antioxidant system is grouped into enzymatic and non enzymatic antioxidants (Caracho and Ferreira, 2013a). Endogenous enzymatic antioxidants include superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidise (Gpx). Non-enzymatic endogenous antioxidants include enzyme cofactors and glutathione. Endogenous antioxidants are not enough; therefore, human beings depend on exogenous antioxidants from their diet (Lobo *et al*., 2010). Several plant extracts and their secondary metabolites were shown to have strong antioxidant activity and protect against oxidant-induced damage (Wong *et al*., 2006). As a result, much attention has been directed to the research of naturally occurring protective antioxidants and their mechanisms of action.

2.12.1 Endogenous antioxidants

Superoxide dismutase is the first enzyme in ROS detoxification and converts superoxide radical to hydrogen peroxide and oxygen. It mainly resides in the mitochondria and cytosol (Zelko *et al*., 2002). Three major forms include zinc and copper SOD, iron or manganese SOD and nickel SOD (Rahman, 2007; Wuerges *et al*., 2004). Catalase resides in the mitochondria and peroxisomes. Its primary role is catalysing the decomposition of hydrogen peroxide to water and oxygen (Chelikani *et al*., 2004). Glutathione peroxide is an enzyme that contains selenium cofactors and catalyses the breakdown of hydrogen peroxide to water and removes hydro peroxides. It is found in the mitochondria and cytosol (Brigelius-Flone, 1999; Caracho and Ferreira, 2013). Glutathione is non enzymatic and contains cysteine peptides and its function is to recycle vitamin E and contributes in the removal of free radicals. Glutathione reductase converts glutathione disulfide (GSSH) to reduced glutathione (GSH) and in cells is predominantly present as GSH in normal physiological conditions (Steenroorden and Henegouwen, 1997). Coenzyme is another non enzymatic endogenous antioxidant that is found in all cells and it prevents formation of lipid peroxyl radicals and it regenerates vitamin E (Turunen *et al*., 2004).

2.12.2 Exogenous antioxidants

Due to insufficient source of endogenous antioxidants, nutritionists recommends on the intake of exogenous antioxidants. They can either be natural or synthetic, with natural antioxidants being favoured as they are safer and healthier (Pham-Huy *et al*., 2008). Synthetic antioxidants include butylated hydroxytoulene (BHT) and butylated hydroxyanisole

(BHA), while, natural antioxidants include vitamin C (ascorbic acid), vitamin E (tocopherols), flavanoids and polyphenols (Caracho and Ferreira, 2013). Vitamin E is a lipophilic vitamin with antioxidant properties and there are eight tocopherols and tocotrienols and among these, α tocopherol is active in humans (Lobo *et al*., 2010). Vitamin E prevents lipid peroxidation, thereby protecting cell membranes from oxidative damage and has been shown to prevent breast cancer due to its antioxidant activity (Traber and Atkinson, 2007). Vitamin A (Retinol) is a type of carotenoid that prevents lipid peroxidation by binding to peroxyl radical (Jee *et al*., 2006). Vitamin C also known as ascorbic acid is a hydrophilic vitamin that scavenges superoxide radical and hydrogen peroxide (Barros *et al*., 2011). It acts as a pro-oxidant in treatment and prevention of breast cancer (Chen *et al*., 2005).

2.13 Medicinal plants as sources of antioxidants

For generations, medicinal plants have been used as a primary source for the treatment of diseases globally (Fallah-Hosein *et al*., 2006). Despite the increase usage of modern medicine, some countries still rely on herbal medicine due to high cost of modern drugs. According to the WHO, 70% of the world is using herbal medicine as alternative or mainstay therapeutic treatment for a number of diseases (Gurinder and Galjit, 2009). The increased interest in scientific research on herbal medicine is due to their documented antioxidant, antimicrobial, anticancer and anti diabetic effects (Rafieian-Kopaei, 2011). Up to 50% of modern drugs are derived from plants and their derivatives (Preethi *et al*., 2010).

Medicinal plants constist of phytochemicals such as carotenoids, polyphenols, alkaloids, saponins, tannins and vitamins (Madhuri and Pandey, 2009). Most of the phytochemicals have antioxidant activity can decrease the risk of developing diseases (Anderson *et al*., 2001). In addition, they exhibit anticancer, anti inflammatory and anti mutagenic properties (Yen *et al*., 1993; Mothana *et al*., 2009). Polyphenols are a class of chemicals that include flavanoids, phenolic acids, tannins and stilbenes (Ramos, 2007). Recently, dietary polyphenols such as flavanoids and phenolic acids have been of interest due to their health benefits such as antioxidants, anticancer, anti inflammatory and chemo-preventive properties (Ramos, 2007).

2.13.1 Flavanoids and phenolic acids

Flavanoids are classified into catechins, anthocyanins, flavones, isoflavones, flavanones and flavanols (Ramos, 2008). Their antioxidant properties are from the phenolic hydroxyl group that is attached to the benzene ring structure. Their function is to scavenge radicals, activate other antioxidant enzymes and quench singlet oxygen (Halliwell, 2012). Flavanoids have been identified in fruits and vegetables and are associated with decreased risk of cancer

(Ramos, 2008). Phenolic acids consist of one or more rings and hydroxyl groups and are found in plants. They constitute almost a third of polyphenols and its sources include lettuce, spinach, tea and strawberries. They exhibit antioxidant activity by scavenging peroxyl and hydroxyl radicals (Krimmel *et al*., 2010). Anthocyanins are natural colourants that belong to the flavanoid family. They are water soluble and are commonly found in grapes, apples and berries (Castaneda-Ovando *et al*., 2009). Anthocyanidins, an aglycon form of anthocyanins comprises of an aromatic ring attached to a heterocyclic ring with an oxygen molecule (Konczak and Zhang, 2004). Studies indicate that anthocyanins reduce the risk of developing chronic diseases such as cancer due their antioxidant activity (Lule and Xia, 2005). The anticancer properties of anthocyanins includes antioxidant effect by radical scavenging activity, stimulation of phase II detoxifying enzymes, reduced cell proliferation, induction of apoptosis, anti-inflammatory effects, anti-angiogenesis and-anti carcinogenesis (Wang *et al.,* 2008). The phenolic structure of anthocyanins is responsible for their antioxidant activity that is their ability to scavenge ROS. The antioxidant effects of anthocyanins have been demonstrated *in vitro* using several cell lines such as cells from the colon (Renis *et al.,* 2007) and endothelial (Bagchi *et al.,* 2004). In these studies anthocyanins demonstrated anti-carcinogenic and anti-toxic effects by consuming ROS, increasing the oxygen radical absorbing capacity of cells, decrease lipid peroxidation; induce Phase ll antioxidant enzymes and reducing the formation of oxidative adducts in DNA (Feng *et al.,* 2007).

Antioxidants can also behave like pro-oxidants which form free radicals (Singh *et al*., 2010). Flavanoids and polyphenols have been discovered to exhibit pro-oxidant activity (Halliwell, 2012). When polyphenols are oxidised free radicals are formed which can causes cell damage. However, some studies indicated that the pro-oxidant activity is beneficial to the cells as it triggers the release of antioxidant enzymes (Halliwell, 2012). The anticancer properties of polyphenols are attributed to their pro-oxidant activity as they cause ROS production (Ramos, 2008; Caracho and Ferreira, 2013b). Flavanoids increase superoxide radical production in colon cancer cells, resulting in apoptosis (Wenzel *et al*., 2005). Additionally, epigalocatechin (EGC) in green tea cause hydrogen peroxide production in lung cancer cells (Yang *et al*., 2000).

2.14 Medicinal plants as anticancer agents

Epidemiological studies reveal differences in the risk for breast cancer vary according to location, culture and lifestyle (Aggarwal and Shishodia, 2006; Locatelli *et al*., 2006). Suggesting lifestyle factors such as diet can influence chronic diseases such as cancer (Manson, 2003; Wong *et al*., 2005). Plant based diet rich in fruits, vegetables, spices, teas and seeds have been linked to decreased risk of developing cancers due to the polyphenols that they contain (Ramos, 2007). Studies have indicated medicinal plants that are rich in

antioxidants can be used for breast cancer chemoprevention and chemotherapy (Donaldson, 2004). Studies indicate the anticancer mechanism of medicinal plants encompasses targeting anti apoptotic proteins, protein kinases, apoptotic proteins, transcription factors, cell cycle proteins, growth factor pathways and metastasis (Aggarwal and Shishodia, 2006) as illustrated in Figure 2.6. Medicinal plants and other bioactive compounds derived from plants that have anticancer properties are curcumin, resveratrol, ginger and garlic. Curcumin is derived from turmeric and its mechanism of action is induction of apoptosis (by increasing Bax and decreasing Bcl_2) and by cell cycle arrest (by decreasing cyclin D1) (Chiu and Su, 2009, Prasad *et al*., 2009; Maswelli *et al*., 2012). The source of resveratrol is grapes and studies indicate it exhibit anti cancer effects by inducing apoptosis (increasing caspase, decreasing $Bcl\text{-}_{xl}$ and Bcl_2) and inhibiting metastasis (decreasing MMP-2) (Shie *et al*., 2011; Tang *et al*., 2008). Derivatives of ginger (6-gingerol and 6-paradol) exhibit anticancer by inhibiting metastasis (by decreasing MMP-2 and MMP-9), inducing apoptosis (by increasing caspase) and suppressing transcription factor NF-kB (Lee *et al*., 2008; Hsu *et al*., 2010; Hsu *et al*., 2012).

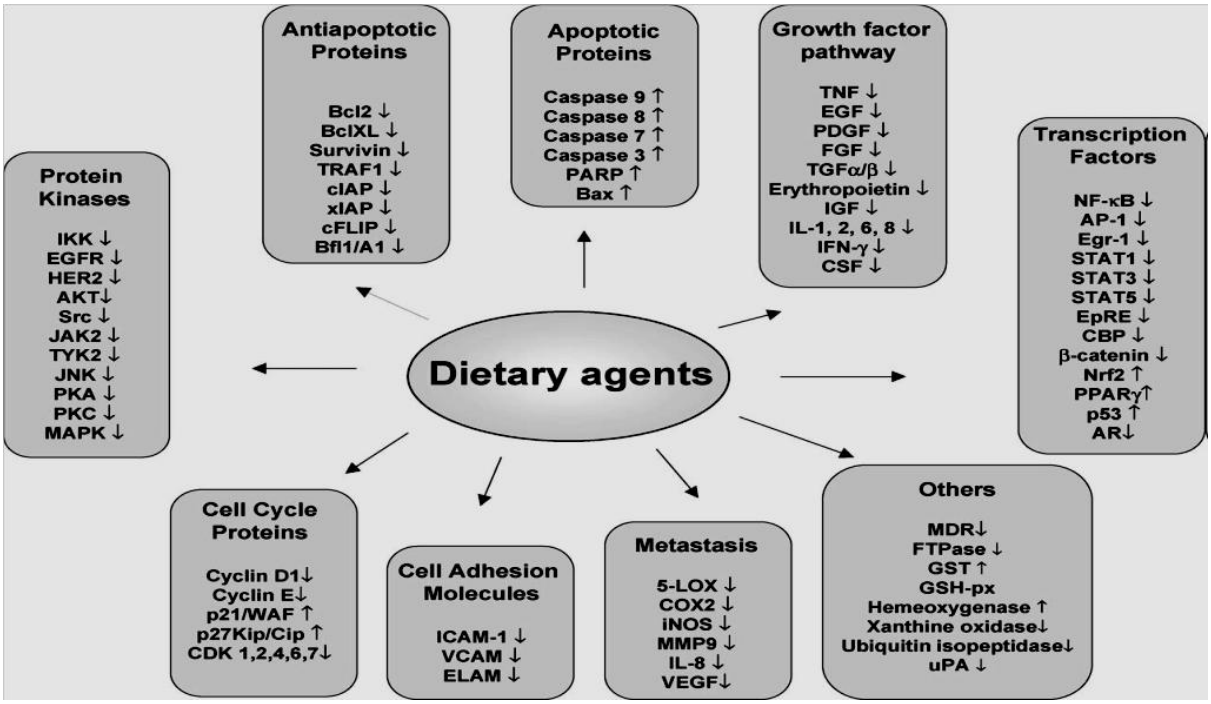

Figure 2.7: Molecular targets of medicinal plants. Adapted from (Aggarwal and Shishodia, 2006)

Numerous modern anticancer drugs were derived from natural products and these compounds include *Vinca* alkaloids, *Camptotheca* alkaloids, *Taxus* diterpenes and *Podophyllum* lignans (Gurib-Fakim, 2006; Itokawa *et al.,* 2008). Cancer drugs derived from Vinca alkaloids include Vincristine and Vinblastine and their derivatives vindesine and vinblastine. Their mechanism of action is inhibition of mitosis by preventing the assembly of mitotic spindle microtubules (Ngan *et al*., 2001). Vinca alkaloids are used in the treatment of

metastatic breast cancer, Hodgkin's lymphoma and malignant melanoma (Itokawa *et al.,* 2008). Taxol a diterpene alkaloid was first identified from the bark of a tree known as Pacific or European yew. Taxol acts by inhibiting mitosis through blocking the cell cycle. It has been used for the treatment of metastatic breast and ovarian cancer (Gragg and Stuffness, 1988; Shigemori and Kobayashi, 2004). Camptothecin is an antitumor alkaloid that is derived from Camptotheca tree from China (Covey *et al*., 1989). Its mechanism of action is to inhibit DHA topoisomerase I, thereby, preventing nucleic acid synthesis (Wang *et al*., 1994). It has been used to treat advanced ovarian and colorectal cancers (Vanhoefer *et al*., 2001). Therefore, natural products and their derivatives from plants can be developed into useful therapeutic drugs.

2.15 Hibiscus sabdariffa

2.15.1 Description, botany and history

Hibiscus sabdariffa (also known as roselle, sour tea or karkade) is a tropical wild plant which is a member of the Malvaceae family and this species belongs to the genus Hibiscus represented by 250 species (Campaore *et al*., 2013; Sindi *et al*., 2014). It is native to West and Central Africa but can be found growing in many tropical areas and subtropical areas of the hemisphere but is widely used in Thailand, Mexico, China, Egypt and Western countries. This annual, bushy, herbaceous plant with a height of up to 2.5metres and characterised by red flowers, green leaves and cylindrical stems (Mohamed *et al*., 2007; Vasudeva *et al.,* 2008) as illustrated in Figure 2.7. The calyces of HS are used to give colour and flavour to beverages, jam, flavouring agents and jellies and it is also popular for making herbal tea mixtures (Wang *et al.,* 2000; Ali *et al*., 2005; Da-Costa-Rocha *et al*., 2014). The hibiscus flower is rich in anthocyanins that confer the red colour (Sindi *et al*., 2014). Traditionally, HS is used as folk medicine for many conditions such as hypertension, liver disease, nerve disorder, cancer, to alleviate constipation and promote circulation (Wang *et al.,* 2000; Da-Costa-Rocha *et al*., 2014).

Figure 2.8: *Hibiscus Sabdariffa* flower. Adapted from (Da-Costa-Rocha *et al*., 2014)

2.15.2 Phytochemical composition

HS has a rich phytochemical profile that enhances its beneficial biological effects (Patel, 2014). It comprises of proteins, carbohydrates, β carotenes, fibre, vitamins, phosphorus, calcium and iron (Ismail *et al.,* 2008; Ajiboye *et al*., 2011). The nutritional composition of HS differs due to environmental and ecological conditions of the plant. The bioactive components of *Hibiscus* flowers that constitutes its pharmacological effects includes polyphenolic acids, anthocyanins, flavonoids and organic acids (Ali *et al.,* 2005; Da-Costa-Rocha *et al*., 2014). Extracts of HS contain organic acids as such hibiscus acid, citric acid, malic acid, tartaric acid and ascorbic acid (Da-Costa-Rocha *et al*., 2014). Common flavanoids in HS extracts include chlorogenic acid, quercetin, protocatechuic acid, luteolin and ergosterol (Ali-Bradeldin *et al*., 2005; Salem *et al*., 2014). Anthocyanins are a very large group of red-blue plant pigments and occur in all higher plants, mostly in flowers and fruits. They carry a positive charge in acidic solution and are water-soluble and, depending upon pH and the presence of chelating metal ions are intensely coloured in blue, purple, or red (Mazza, 1995).The major components of HS are delphinidin-3-sambubioside and cyanidin-3-sambubioside and the minor components are delphinidin-3-glucoside and cyanidin-3-glucoside (Ali *et al.,* 2005; Maganha *et al*., 2010). HS calyces are rich in anthocyanins which confer its antioxidant activity (Ajiboye *et al*., 2011).

2.15.3 Biological activities

HS has been used in the treatment of numerous diseases (Ali *et al.,* 2005). Recently, studies indicated that HS prevent diseases such as hypertension (Maganha *et al*., 2010), hepatic diseases, diabetes and cardiovascular diseases (Chen *et al.*, 2004). Extracts of HS have been shown to act as anticancer, antimutagenic (Liu *et al*., 2010), antimicrobial agents

(Maganha *et al*., 2010; Ashami and Alharbi, 2014) and antioxidant agents (Yang *et al*., 2012; Mense and Golomeke, 2015). HS has been found to have antihypertensive, antioxidant and hypocholesterolemic properties (Ali *et al.,* 2005; Patel, 2014) as illustrated Figure 2.8. Additionally it acts as a diuretic that lowers urinary concentration of potassium, calcium and phosphates (Alarcon and Alonsoa *et al.,* 2012).

2.15.3.1 Antioxidant activity

Assays used to quantify antioxidant properties include ferric reducing ability of plasma (FRAP), catalase, superoxide and oxygen radical absorbing capacity (ORAC). Numerous studies have indicated HS has antioxidant activity due to its ability to scavenge ROS (Tseng *et al*., 1997; Farombi and Fakoya, 2005; Olalye and Rocha, 2007; Mohd-Esa *et al*., 2010). In human studies, a water extract of HS reduced oxidative stress using FRAP assay (Frank *et al*., 2012). Ajiboye *et al*. (2011) determined that HS anthocyanins up regulated antioxidant enzymes and scavenged free radicals in induced oxidative stress on rats. Additionally, HS has the ability to prevent lipid peroxidation and generation of Thiobarbituric acid reactive substances (Farombi and Fakoya, 2005; Olalye and Roche, 2007; Da-Costa-Rocha *et al*., 2014).

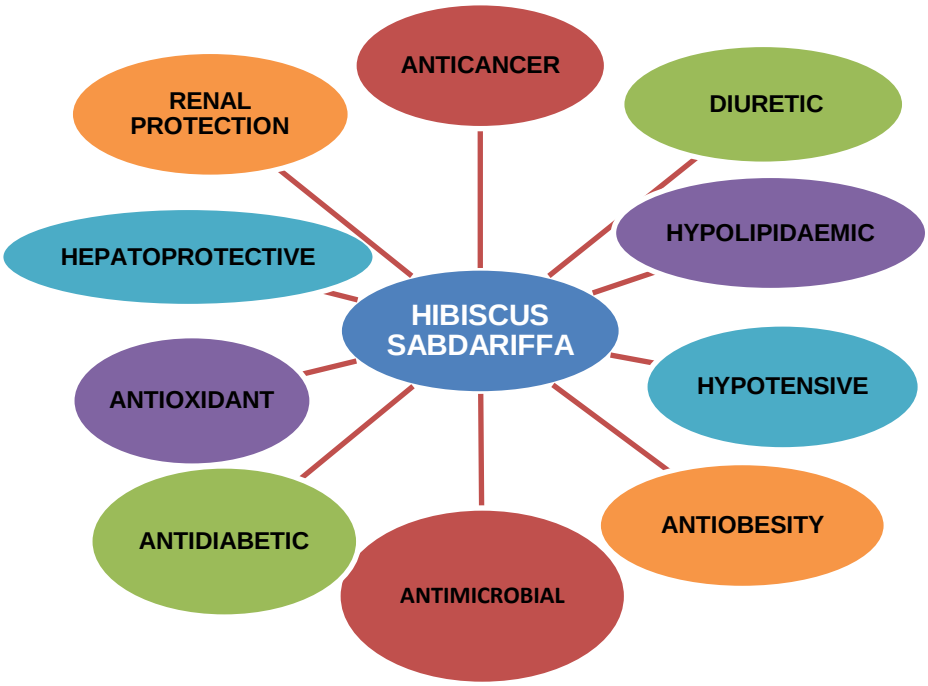

Figure 2.9: *Hibiscus Sabdariffa* and its biological effects, (Adapted from Patel 2014)

2.15.3.2 Antiobesity and hypolipidaemic activity

HS has been found shown to have an anti obesity effect. A study showed a decreased body weight on animals fed on HS extract (Carvajal-Zarrabal *et al*., 2013). Extracts of HS were

shown to reduce weight gain in obese mice (Alarcono-Aguilar *et al*., 2007), blocking sugar and starch absorption (Stone *et al*., 2007) and aiding the absorption and excretion of fats (Carvajal-Zarrabal *et al*., 2007). HS has lipid lowering activity and reduces risk of atherosclerosis and cardiovascular diseases (Gosain *et al*., 2010; Yang *et al*., 2010). HS prevents atherosclerosis by inducing apoptosis in smooth muscle cells (Lo *et al*., 2010) , lowers levels of lipids inside the cells (Chen *et al*., 2013) and in hyperlipidemic animals it reduces cholesterol and triglyceride levels (Hopkins *et al*., 2013). Some studies have demonstrated the extracts cause a reduction in low density lipoproteins and an increase in high density lipoproteins (Ochani and D'Mello, 2007; Yang *et al*., 2010). Human studies indicated a decrease in cholesterol and tryglcerides in patients with metabolic syndrome upon consumption of HS extracts for 6 weeks (Gurrola-Diaz *et al.,* 2010).

2.15.3.3 Hypotensive and antidiabetic activity

Numerous *in vitro* and *in vivo* studies on HS have found to decrease systolic and diastolic blood pressure (Adegunloyo *et al.,* 1996; Inuwa *et al*., 2012). The possible method by which HS extracts lower blood pressure is by relaxing the vascular system or by suppressing angiotension converting enzymes (Adegunloyo *et al.,* 1996; Ojeda *et al*., 2010). Clinical studies indicated a reduction in systolic blood pressure in diabetic patients with moderate hypertension upon consumption of HS tea for twice a day in 4 weeks (Mozaffari-Khosravi *et al*., 2013). Another study showed a reduction in salt induced high blood pressure on rats upon consumption of HS water extract for 6 weeks (Mojiminiyi *et al.*, 2012). Additionally, HS extracts from the calyces lowered systolic blood pressure and reduced ventricle mass in hypertensive rats (Inuwa *et al*., 2012). A polyphenol rich extract of HS has been found to lower hyperglycaemia and hyperinsulinemia in rats with type 2 diabetes mellitus upon treatment with 200mg/kg (Peng *et al*., 2011). Upon feeding diabetic rats with a dose of 1g/kg for 6 weeks on a daily basis, HS ethanolic extract lowered blood glucose levels (Wisetmuen *et al*., 2013).

2.15.3.4 Hepatoprotective actvity

HS extract have been found to protect the liver against toxins such as acetaminophen (Lee *et al*., 2012), azothioprine (Amin and Hamza, 2005), lipopolysaccharides (Lin *et al.,* 2003), carbon tetrachloride (Liu *et al.*, 2006) and radiation (Adaramoye *et al.,* 2008). Upon induced liver damage, extracts of HS have been found to up regulate levels of antioxidant enzymes such as catalase and superoxide dismutase and phase II detoxifying enzymes while reducing lipid peroxidation (Olaleye and Rocha, 2008) and a reduction in levels of liver enzymes such as alkaline phosphatise and alanine transaminase in high blood levels of ammonia was observed (Essa *et al.,* 2006).

2.15.3.5 Renal protection and diuretic actvity

Several studies have indicated that extracts of HS have nephroprotective effects. One study revealed a reduction in creatinine and urea levels on rats after feeding with 50mg/g of water extract of HS for 4 weeks (Olatunji *et al*., 2012). A polyphenol extract of HS reduced kidney mass and improved renal function in rats with diabetic nephropathy possibly through reduction in oxidative stress (Wang *et al*., 2011). A rat model study indicated a reduction in the advancement of kidney disease after being fed with an aqueous HS extract (Seujange *et al*., 2013). Aqueous extract of HS restored diuretic effects in rats that lack the adrenal gland (Jimenez-Ferrer *et al*., 2012). An increased kidney filtration due to vasorelaxation was observed on *in vivo* studies using quercetin from HS (Alarcano-Alonso *et al*., 2012). In male albino rats, HS extract prevented the onset and progression of urolithiasis (Laikangbam and Damayanti-Devi, 2012).

2.15.3.6 Antimicrobial actvity

Methanolic extract of HS exerted an inhibitory effect on numerous bacteria such as *Escherichia coli, Klebsiella pneumonia, Staphylococci aureus* and *Bacillus cereus* (Olaleye, 2007). Inhibition of *E.coli* O157:H7 strain was observed after treatment with 10% methanolic extract (Fullerton *et al*., 2011). An ethanolic extract had an inhibitory effect on *Staphylococci aureus* and *Bacillus subtilis* (Jung *et al*., 2013). Protocatechuic acid and HS water extract showed inhibitory effect on *Pseudomonas aeruginosa* and methicillin resistant *Staphylococci aureus* (Liu *et al*., 2005).

2.15.3.7 Anticancer actvity

A study done on HS demonstrated that it induces smooth muscle cell apoptosis via p38 and p53, thereby slowing down the rate of atherosclerosis (Lo *et al.,* 2007). Anthocyanins rich extracts have also exhibited apoptotic effects on cancerous cells and they induce apoptosis through both intrinsic (mitochondrial) and extrinsic (FAS) pathways (Chang *et al.,* 2005). In the intrinsic pathway, anthocyanin treatment of cancer cells results in a depolarised mitochondrial membrane, cytochrome c release and modulation of caspase-dependent anti and pro-apoptotic proteins. In the extrinsic pathway, anthocyanins modulate the expression of FAS and FASL (FAS ligand) in cancer cells resulting in apoptosis (Chang *et al.,* 2005). Extracts of HS induced apoptosis on human stomach adenocarcinoma cells via the p38 signalling cascade and prevented proliferation of Hela cancer cells (Olvera-Garcia *et al*., 2008). *In vitro* and *in vivo* studies show that leaf extract of HS induced apoptosis via both intrinsic and extrinsic pathways in prostate cancer cells (Lin *et al*., 2012).

In vitro and *in vivo* experimental animal studies have shown that HS extract has anti-inflammatory effects with previous studies showing that this plant exhibit antioxidant properties and could be used to treat various diseases such as cancer where it induces apoptosis (Lo *et al.,* 2007). One study showed that HS induced anticancer activity in a N-Nitroso methyl urea induced rat leukaemia (Tsai *et al.,* 2013). Phenolic rich HS extract reduced DNA damage on *tert*-butyl hydro peroxide induced cytotoxicity in primary hepatocyes extracted from rats and hepatotoxicity in rats (Tseng *et al*., 1996). It demonstrated that HS caused a reduced leakage in lactate dehydrogenase while *in vivo* investigation revealed lowered serum levels of hepatic enzymes and reduced oxidative damage (Wang *et al.,* 2000). HS exhibited the anticancer effect in human gastric adenocarcinoma and in promyelocytic cells *in vitro*, reflecting its chemo-preventive functions (Lin, *et al.,* 2007). Therefore, natural products and their derivatives such as HS extracts can be developed into useful therapeutic drugs.

CHAPTER THREE

RESEARCH DESIGN AND METHODOLOGY

3.1 Study Design

This study was divided into three parts as follows:

Part 1: *In vitro* antioxidant content and capacity determination of *HS* extract.

Part 2: Antioxidant status of cells treated with HS extracts.

Part 3: Apoptotic and cytotoxic study on HS treated cells.

3.2 General stock solutions

3.2.1 Chemicals, assay kits and suppliers

2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH)	Sigma-Aldrich
2,4,6-tri[2-pryridyl]-s-triazine (TPTZ)	Sigma-Aldrich
5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA)	Life technologies
5,5'-Ditho-bis-(2-nitrobenzoic acid) reagent (DNTB)	Sigma-Aldrich
6-hydroxy-2,5,7,8-tetramethylchroman-2carboxylic acid or trolox	Sigma-Aldrich
6-hydroxydopamine (6-HD)	Sigma-Aldrich
APO P*ercentage* ™ apoptosis assay	Bicolor Ltd
Bicinchoninic acid (BCA) Protein Assay	Thermo Scientific
Diethylenetriaminepentaacetic acid (DETAPEC)	Sigma-Aldrich
Dimethyl sulphoxide (DMSO)	Sigma Aldrich
Doxorubicin	Sigma Aldrich
Ethanol	Sigma Aldrich
Ethylenediaminetetraacetic acid (EDTA)	Sigma Aldrich
Folin Ciocalteu's phenol reagent	Sigma Aldrich
Gallic acid	Sigma-Aldrich

Hydrochloric acid	Merck
Hydrogen peroxide	Sigma-Aldrich
Iron chloride hexahydrate	Sigma-Aldrich
L-ascorbic acid	Sigma-Aldrich
Malondialdehyde (MDA) standard	Sigma-Aldrich
Orthophosphoric acid	Sigma-Aldrich
Perchloric acid	Sigma-Aldrich
Potassium chloride (KCl)	Sigma-Aldrich
Potassium phosphate	Sigma-Aldrich
reduced glutathione (GSH)	Sigma-Aldrich
reduced β-nicotinamide adenine dinucleotide phosphate (NADPH)	Sigma-Aldrich
Sodium acetate	Merck
Sodium carbonate	Sigma-Aldrich
Sodium phosphate ($NaPO_4$)	Sigma-Aldrich
Tetramethylrhodamine ethyl ester (TMRE)	Life technologies
Thiobarbituric acid (TBA)	Sigma-Aldrich
Triton X-100 (iso-octylphenoxypolyethoxyethanol)	Sigma Aldrich
Trypan Blue dye	Life technologies
Trypsin	Life technologies
WST-1 cell proliferation assay	Roche

3.2.2 Stock solutions and buffers

ORAC assay buffer: 75mM Sodium phosphate ($NaH_2PO_4H_2O$) buffer, pH 7.4

Potassium chloride buffer: 0.025M Potassium chloride, pH 1.0

Sodium acetate buffer: 0.4M Sodium acetate buffer, pH 4.5

Catalase assay buffer: 50mM Potassium phosphate (KPO_4) buffer, pH 7.0

Superoxide dismutase assay buffer: 50mM Sodium phosphate ($NaPO_4$) buffer, pH 7.4

Glutathione assay buffer: 500mM Sodium phosphate ($NaPO_4$) buffer, 1mM EDTA, pH 7.4

Cell lysis buffer: 50mM Sodium phosphate ($NaPO_4$) buffer; 0.5% (v/v) Triton X-100, pH 7.5

3.2.3 Cell culture media and cell lines

Dulbecco's Modified Eagle Medium (DMEM)	Lonza
Insulin	Lonza
Foetal Bovine Serum (FBS)	Lonza
Phosphate Buffered Saline (PBS) without $CaCl^{2+}$ and $MgCl_2$	Lonza
Penicillin Streptomycin	Lonza
Epidermal growth factor (EGF)	Lonza
Hydrocortisone	Lonza
Dulbecco's Modified Eagle Medium with F12 (DMEM F 12)	Lonza
MCF-7	ATCC
MCF12A	ATCC

Complete DMEM was prepared by combining 450ml of serum free DMEM, 5ml 0.2% Penicillin/Streptomycin and 50ml of 10% FBS. Complete DMEM-F12 was prepared by combining 450ml of serum free DMEM-F12, 5ml of 0.2% Penicillin/Streptomycin, 50ml of 10% FBS and supplemented with 10µg/ml insulin, 20ng/ml EGFand 500ng/ml Hydrocortisone.

3.3 Plant extraction

Hibiscus Sabdariffa plant was collected locally in South Africa in summer. The sample was washed with distilled water and air dried at room temperature in dark room. Dried sample was crushed into powder form and subjected to water extraction by maceration method with distilled water (10% w/v) at 25ºC in a dark room for 48 hours. The extract was filtered through a Whatman filter paper and evaporated using a freeze dry evaporator at -40ºC overnight. Methanolic extract was prepared by maceration method with 1% acidified methanolic (10% w/v) at 25ºC in a dark room for 48 hours. The extract was filtered through a Whatman filter paper and concentrated using a rotary evaporator at 50ºC for 30 minutes. Both extracted powders were weighed and stored at -20ºC in foil paper. Water and methanolic extracts were reconstituted in buffer and DMSO respectively to a stock solution of 1mg/ml and stored at -20ºC.

3.4 Determining the antioxidant content and capacity in extracts

Since different extraction methods were done, antioxidant content and capacity were conducted using the different dilutions of both plant extracts that ranged from 0.1mg/ml to 1mg/ml.

3.4.1 Quantification of Total Polyphenols

A colometric assay was used to quantify the total concentration of phenolic hydroxyl compounds in plant extracts (Singleton and Rossi, 1965). In this assay polyphenols react with redox reagents to form a blue complex that can be quantified by a multiplate reader. The total polyphenol content was determined using the Foli-Ciocalteu method (Singleton and Rossi, 1965). The gallic acid standards were prepared in distilled water at the following concentrations: 0, 20, 50, 100, 250 and 500 mg/ml. The Folin working reagent was prepared by mixing 1ml of the Folin reagent with 9ml of distilled water. A volume (25µl) of each standard or sample was added to a well in a 96 well plate. All these reactions were done in triplicates. This was followed by the addition of 125µl of Folin reagent. After 5minutes, 100µl of 7.5% Sodium carbonate (Na_2CO_3) was added into each well and the plate was incubated for 2hours at room temperature. Thereafter, the absorbance reading was read at 765 nm using a Multiskan Spectrum plate reader (Thermo Scientific, USA). Results were expressed in mg Gallic equivalents (GAE)/L.

3.4.2 Quantification of Total Anthocyanins

The total anthocyanin content in extracts containing other phenolic materials has been determined by the differential method (Dussi *et al.,* 1995). This method measures the absorbance at two different pH values, and relies on the structural transformations of the anthocyanin chromophore. Accurate measurement of the total monomeric anthocyanin pigment content can be obtained, along with indices for polymeric colour, colour density, browning, and degradation. To determine total anthocyanin content, the absorbance at pH 1.0 and 4.5 is measured at the $l_{vis\text{-}max}$ and at 700 nm, which allows for haze correction.

The total anthocyanins content was determined as previously described by Dussi *et al* (1995). A volume (25µl) of each standard or sample was added to a well in a 96 well plate. All these reactions were done in triplicates. This was followed by the addition of 275µl of the prepared Potassium chloride buffer or Sodium acetate buffer at their designed wells. Absorbance was read at $\lambda_{vis\text{-}max}$ and at 700 nm against a blank with distilled water using a Multiskan Spectrum plate reader (Thermo Scientific, USA). Results in absorbances (A) were calculated using formula below:

$A = (A_{\lambda vis\text{-}max} - A_{700})_{pH\ 1.0} - (A_{\lambda vis\text{-}max} - A_{700})_{pH\ 4.5}$

3.4.3 Oxygen radical absorbance capacity (ORAC) assay

Oxygen radical absorbance capacity (ORAC) is a reliable and sensitive method that is used to measure the antioxidant capacity of a substance to scavenge peroxyl radicals (Rautenbach *et al.,* 2010). This assay measures the loss of fluorescence intensity due to free radicals damaging the fluorescent probe and antioxidant ability to scavenge radicals over time. The method is based on the reaction between an antioxidant which is the sample, the fluorescent probe which is the oxidizable substrate and 2,2'-azobis (2-methylpropionamidine) dihydrochloride (AAPH) which is the source of peroxyl radicals upon thermal decomposition. A delay in fluorescence decay is observed when antioxidants bind to AAPH. Trolox, a water soluble Vitamin E analogue which acts as an antioxidant was used as a standard. The antioxidant capacity of the sample is measured by comparing the areas under the fluorescent decay curve to that of the standards.

The assay was done using a modified method by Rautenbach *et al* (2010). AAPH, Trolox and the fluorescent probe were prepared in ORAC assay buffer and standards were prepared at the following concentrations: 0, 83, 167, 250, 333 and 417 µM. The reaction mixture consisted of 138µl of fluorescein (14µM), which was the target for radical attack and 12µl of the sample (diluted 1:10 using ORAC assay buffer prepared). All reactions were done in triplicates in a black 96 well plate. The plate was incubated for 30 minutes at 37ºC in the micro plate reader. The reaction was initiated by the addition of 50µl AAPH (500µM) and the fluorescence (excitation 485 and emission 538nm) was read using a fluoroskan ascent plate reader (Thermor Fisher Scientific, Waltham, Mass, USA). Readings were taken every minute for 2 hours after the plate was shaken. Results were expressed in µM Trolox equivalents (TE)/g tissue. The final ORAC value was determined using a regression equation ($y=a+bx+cx_2$) between Trolox concentration and area under the curve (AUC). The wells on the edge of the plate on column 1, 2 and 12 of the 96 well plate were not used.

3.4.4 Ferric reducing antioxidant power (FRAP) assay

The ferric reducing antioxidant power (FRAP) is a colorimetric assay used to evaluate antioxidant capacity of a substance. Antioxidants in a sample donate electrons and acts as a reductant to convert ferric ion (Fe^{3+}) to ferrous iron (Fe^{2+}) in low pH conditions. In acidic conditions ferric chloride hexahydrate- 2, 4, 6-tri [2-pryridyl]-s-triazine [$Fe_3(TPTZ)_2\,Cl_3\,6H_2O$] used as an oxidant is reduced by biological antioxidants to give an intense blue colour. The FRAP assay was done as described by Benzie and Strain (1996). FRAP reagent was prepared by mixing 30ml of acetate buffer (300mM) at pH 3.6, 3ml of TPTZ (10mM), 3ml of

Iron (lll) chloride hexahydrate ($FeCl_3$) (20mM) and 6.6ml of distilled water. L-ascorbic acid was used as a standard. All reagents were prepared in distilled water except TPTZ which was prepared in 0.1M hydrochloric acid. The plant extract (10µl) or standards (10µl) were added in triplicates into a 96 well plate. This was followed by the addition of 300µl of the FRAP reagent and incubation for 30minute at room temperature. The plate was read at 593nm using a Multiskan Spectrum plate reader (Thermo Scientific, USA). Results were expressed as µmole AAE/L and were obtained by comparison to the calibration curve using the regression equation (y=a+bx).

3.5 Culturing of cells

3.5.1 Thawing of cells

The vials of frozen MCF7 and MCF12A cells were removed from the -150^{o}C freezer and immediately thawed in a water bath heated to 37^{o}C. The contents of the vial were transferred into a 15ml tube containing 4ml pre-warmed media and centrifuged at 300xg for 3minutes. The supernatant was discarded and the cell pellet was resupended in 5ml pre-warmed media and transferred into a 25cm^3 cell culture flasks. The flask was then incubated at 37^{o}C in a humidified incubator containing 5% $CO_{2.}$ The media was changed every 3 days and cell growth observed under a microscope.

3.5.2 Trypsinization of cells

If the cells reach 90% confluency they were sub cultured. The spent media was removed and the flask was rinsed with 4ml phosphate buffered saline (PBS) and 2ml trypsin was added. The flask was incubated for 2 minutes until all cells were detached. Complete media was added to stop trysinization and the cells were transferred to a 15ml tube and centrifuged at 300xg for 3minutes. After centrifugation and decanting of supernatant, the cell pellet was re-suspended and the cells were split into three 25cm^3 cell culture flasks.

3.5.3 Freezing of cells

To freeze down the cells, they were trypsinized and recovered by centrifugation and the cell pellet was re-dissolved in media containing 10% DMSO. The cell suspension was aliquoted into 2ml cryo-vials and placed in a -150^{o}C freezer for long term storage.

3.5.4 Seeding of cells

When the cells have reached 90% confluency, they were trypsinized and centrifuged to obtain the cell pellet. The pellet was re-suspended in 2ml of media and counted using a Countess^{TM} automated cell counter. The cells were seeded at a concentration of 1x 10^5 cells

per well on a 96 well plate and 1x 10^6 cells per well on a 12 well plate. Cells were incubated at 37^{o}C for 24hour prior to treatment.

3.5.5 Cell Count

Cell counts were performed as per the manufacturer's instructions using the Countess™ automated cell counter (Invitrogen). Trypan blue dye was used to distinguish between live and dead cells as described by Stober (2001). It is based on the principle that live cells possess an intact cell membrane that exclude the dye and remain clear while dead cells take up the dye and stain blue. 10µl of cell suspension was mixed with 10µl of trypan blue dye and placed on a Countess™ automated cell counter slide and viewed using the cell counter. Live cells were countered and an automated calculator was used to determine the volume of cells required.

3.6 Apoptotic and cytotoxic study on HS treated cells

3.6.1 Preparation of HS extracts

Stock solutions of 10mg/ml HS water and methanolic extracts were prepared using PBS and DMSO to dissolve the extracts respectively. Working concentrations of 0.1, 0.2, 0.4, 0.6 and 0.8mg/ml were prepared from the stock solution using cell culture media. The final DMSO concentration of the highest concentration of 0.8mg/ml was less than 0.01%.

3.6.2 Morphological evaluation of HS treated cells

MCF7 and MCF12A cells were cultured in a 6 well cell culture plate to 90% confluency. Media was removed and the cells were treated with increasing concentrations of both water and methanolic extracts ranging from 0.1mg/ml to 0.8mg/ml. The negative control was left untreated. The cells were incubated for 24hours at 37^{o}C in a humidified incubator containing 5% CO_2. Following incubation the cells were studied and pictures taken using a 40x magnification inverted Leica EC3 digital camera.

3.6.3 Cell viability assay

WST-1 is a colorimetric assay used to quantify cell viability and cytotoxicity. The stable tetrazolium salt (red) in WST-1 is cleaved to a soluble formazan (yellow) by succinate-tetrazolium reductase system in metabolically active cells (Berridge *et al*., 1996). The amount of formazan formed relates to the metabolically active cells in culture. Formazan dye is quantified using a plate reader.

Cells were seeded at a concentration of 1x 10^5 cells per well into a 96 well plate and incubated for 24 hours, there after the cells were treated according to 3.6.2. Doxorubicin was used as a positive control at the following concentrations: 0.05, 0.07, 0.1, 0.2mg/ml. Effect of the solvent DMSO was determined on cells at the following concentrations 0.01, 0.05, 0.1, 0.5%. Triplicates were done for each concentration. The treated cells were incubated for 24 hours at 37^{o}C in a humidified CO_2 incubator. After 24hours, 10µl of WST-1 reagent was added into each well and the cells were incubated for 2hours. The plate was placed on a shaker for 1 minute. Absorbance values of formazan readings were measured at a wavelength of 420nm and reference wavelength of 620nm using a LabSystem Multiscan Plus microplate reader. Cell viability was calculated using the following formulae:

$$\% \text{ cell viability} = \frac{(\text{Absorbance of sample} - \text{Absorbance of Blank})}{(\text{Absorbance of negative control} - \text{Absorbance of Blank})} \times 100$$

IC_{50} values were tabulated as the concentration that reduced cell viability by 50%.

3.6.4. Apo*Percentage*™ apoptosis assay

The principle behind the Apo*Percentage*™ apoptosis assay is based on the transfer and exposure of phosphatidyl serine on the cell surface membrane upon apoptosis induction. This movement result in apoptotic committed cells taking up the dye. Dye uptake continues and the dye that is accumulated in the cell is not released. Cells that have taken up the dye can be measured using a flow cytometry.

This assay was done as described by Meyer *et al* (2008). The cells were plated at a concentration of 1x 10^6cells per well on a 12 well plate and incubated for 24 hours at 37^{o}C in a humidified CO_2 incubator. The cells were treated cells using the HS extracts at a concentration range of 0.2 to 0.4mg/ml for 24hours. Cells were treated with the IC_{50} value for the positive control doxorubicin and DMSO. A negative control which is the untreated cells was also included. For the time response, the cells were treated with the IC_{50} value for HS extract for 12 or 24hours. After the specified time, floating cells were transferred into a 15ml tube. The adherent cells were washed using PBS, trypsinized and combined with floating cells. The cells were centrifuged at 300xg for 3 minutes and washed with PBS. This was followed by staining the cells using 300µl of Apo*Percentage*™ dye (1:160 dilutions) for 30 minutes at 37^{o}C. The cells were then washed with PBS and analyzed by flow cytometry. Cell staining was measured at 488nm at FL2 on a Becton Dickinson Accuri™ C6. A minimum of 10 000 cells per sample were acquired and analyzed using a BD CSampler™ Software.

3.6.5 Measurement of mitochondrial membrane potential using Tetra Methyl Rhodamine Ethyl ester (TMRE)

TMRE is a cell-permeable, positively charged, red orange dye that detects mitochondrial membrane potential. Loss of mitochondrial membrane potential or inactive mitochondria is an indicator of apoptosis and can be detected using TMRE (Ricci *et al*., 2003). It clumps in the mitochondria of non-apoptotic cells and fluoresce bright orange or red, while in apoptotic cells it diffuses throughout the cell.

This assay was done as described by Ricci *et al* (2003) with some modifications. The cells were plated at a density of 1x 10^5cells per well on a 12 well plate and incubated for 24 hours at 37^{o}C in a humidified CO_2 incubator. The cells were treated with HS extracts at a concentration range of 0.1 to 0.3mg/ml for 24hours. Cells were also treated with the IC_{50} value for the positive control doxorubicin and DMSO. A negative control which is the untreated cells was included. For the time response, the cells were treated with the IC_{50} value for HS extract for 12 or 24hours. After the specified time, floating cells were transferred into a 15ml tube. The adherent cells were washed using PBS, trypsinized and combined with floating cells. The cells were centrifuged at 300xg for 3 minutes and washed with PBS. This was followed by staining the cells using 300µl TMRE dye (1µM) for 30 minutes at 37^{o}C. The cells were then washed with PBS to remove the dye and analyzed by flow cytometry. Cell staining was measured at 488nm at FL3 on a Becton Dickinson Accuri^{TM} C6. A minimum of 10 000 cells per sample were acquired and analyzed using a BD CSampler^{TM} Software.

3.7 Assessing the antioxidant status and capacity of treated cells

3.7.1 Determination of protein concentration

Protein determination was done using the Bicinchoninic Acid (BCA) method. The principle of this method relies on the formation of Cu^{2+} protein complex under alkaline conditions, followed by a reduction of Cu^{2+} to Cu^{1+} (Smith, 1985). BCA forms a water soluble, purple-blue complex in alkaline conditions which can be monitored at an absorbamce reading of 562nm. Protein determination was measured as described by Smith (1985). Treated and untreated cells were subjected to an ice cold cell lysis buffer which was three times the cell suspension volume. The cells were centrifugation at 1600xg for 10minutes at 4^{o}C and the supernatant was collected for immediate analysis or stored in ice. In a 96 well plate, 25µl of sample or standard (bovine serum albumin) and 200µl of BCA working reagent were added into each well. All these reactions were done in triplicates. The 96 well plates was covered and incubated at 37ºC for 30minutes. The absorbance was taken at 562nm using a Multiskan plate reader and protein concentrations were calculated using a standard curve.

3.7.2 Superoxide dismutase assay (SOD)

SOD assay quantifies the auto-oxidation of 6-hydroxydopamine (6-HD). A unit of SOD is the amount of enzyme needed to exhibit 50% dismutation of superoxide radical. This assay is simple, fast and reproducible for measuring SOD activity in cell lysates. The SOD activity was measured as described by Ellerby and Bredesen (2000). Treated and untreated cells were subjected to an ice cold cell lysis buffer and protein determination was done as described in section 3.7.1. In a 96 well plate, 170µl of Diethylenetriaminepentaacetic acid (DETAPEC) solution prepared by dissolving 2mg of DETAPEC in 50ml of superoxide dismutase assay buffer was added to 12µl of the sample or standard in triplicates.18µl of superoxide dismutase assay buffer was added to the mixture. This was followed by the addition of 15µl of 6-hydroxydopamine (6HD) [4mg of 6HD powder dissolved in 10ml of (50µl perchloric acid + 10ml of MilliQ water)]. The absorbance was read immediately at 490nm for 4minutes at 1minute intervals. The activity of SOD was calculated using an equation obtained from linear calibration curve of the standard. One unit is defined as the amount of enzyme needed to exhibit 50% dismutation for the superoxide radical.

3.7.3 Catalase assay (CAT)

Catalase is an antioxidant enzyme that catalyses the decomposition of hydrogen peroxide to water and oxygen. This assay was used to measure catalase in cell lysates. The catalase activity was measured as described by Ellerby and Bredesen (2000). Treated and untreated cells were subjected to an ice cold cell lysis buffer and protein determination was done as described in section 3.7.1. A volume (10µl) of each standard or sample and 170µl of catalase assay buffer were added to a well in a 96 well plate. All these reactions were done in triplicates. To initiate the reaction, 75µl of hydrogen peroxide prepared by mixing 10ml of catalase assay buffer and 34µl of 30% v/v of hydrogen peroxide was added. The absorbance was read at 240nm for 1 minute at 15 seconds interval. The catalase activity was calculated using the equation obtained from the linear regression of the standard curve.

3.7.4 Glutathione assay (GSH)

The Glutathione levels were measured as described by Ellerby and Bredesen (2000). Treated and untreated cells were subjected to an ice cold cell lysis buffer and protein determination was done as described in section 3.7.1. GSH standard solutions were prepared in the following concentrations: 0 (blank), 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0µM. A volume (50µl) of each standard or sample and 50µl of a chromogenic compound 0.3mM

Ditho-bis-(2-nitrobenzoic acid) reagent (DNTB) prepared by mixing 60mg of DNTB in 500ml of Glutathione assay buffer were added to a well in a 96 well plate. Another 50µl of the assay enzyme 0.02U/µl glutathione reductase (GR) prepared by mixing 80µl of GR and 4920µl of Glutathione assay buffer was added to each well. All these reactions were done in triplicates. This was followed by 5minutes incubation at 25^{o} C in a preheated microplate reader. To initiate the reaction, 50µl of 1mM reduced β-nicotinamide adenine dinucleotide phosphate (NADPH) was added. The absorbance values were read at 340nm for 2 minute at 30 seconds interval. The glutathione levels were calculated using the equation obtained from the linear regression of the standard curve.

3.7.5 Thiobarbituric reacting substance assay (TBARS)

The TBARS assay will be used to screen and monitor lipid peroxidation, a major indicator of oxidative stress. The assay provides important information regarding free radical activity and has been used for measurement of antioxidant activity of several compounds. Malondialdehyde (MDA) forms a 1:2 adduct with thiobarbituric acid. Biological specimens contain a mixture of thiobarbituric acid reactive substances, including lipid hydroperoxides and aldehydes, which increase as a result of oxidative stress. TBARS are expressed in terms of malondialdehyde (MDA) equivalents. In this assay, an MDA standard is used to construct a standard curve against which unknown samples can be plotted (Catala, 2009). Treated and untreated cells were subjected to an ice cold cell lysis buffer and protein determination was done as described in section 3.7.1. 12.6µl of 100% ethanol was added to 100µl of the supernatant and 100µl of ortho-phosphoric acid in a tube. The mixture was vortexed for 10 seconds and 12.5µl of Thiobarbituric acid (TBA) reagent (0.11M in 0.1M sodium hydroxide) was added. This was followed by mixing for 10 seconds and heating in a 90^{o} C water bath for 45 minutes. The mixture was then transferred to ice for 2 minutes in order to stop the reaction. The tubes were transferred to room temperature for 5 minutes, followed by addition of 1000µl of n-butanol and 100µl of NaCl. After vortexing for 10 seconds, the samples were centrifuged at 12 000xg for 2 minutes at 4^{o} C. 250µl of the top butanol phase was added into each well in a 96 well plate. All these reactions were done in triplicates. The absorbance values were read at 532 and 572nm on a Multiskan plate reader. TBARS results were expressed in nmoles/mg protein.

3.7.6 Reactive oxygen species assay (ROSA)

5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) is a chloromethyl derivative of 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) that is used to evaluate intracellular ROS. Its advantages over other molecular probes of H_2DCFDA, is that it exhibits better retention in live cells and facilitates long term

studies (Koopman *et al*., 2006). CM-H_2DCFDA passively diffuses into the cells, where it is converted into non fluorescent CM-H_2DCF by esterification of acetate groups by intracellular esterases. Its thiol-reactive chloromethyl group also reacts with intracellular glutathione. Subsequent oxidation by intracellular oxidants such as the hydroxyl radical, superoxide and hydroperoxides converts CM-H_2DCF to a highly fluorescent CM-DCF which is measured using a flow cytometry (Koopman *et al*., 2006).

Evaluation of ROSA was done as described by Ju *et al*., (2007) using CM-H_2DCFDA molecular probe with some modifications. The cells were plated at a density of 1x 10^5cells per well on a 12 well plate and incubated for 24 hours at 37^{o}C in a humidified CO_2 incubator. The cells were treated for 24hours using HS extracts at a concentration range of 0.1mg/ml to 0.5mg/ml and the IC_{50} value of hydrogen peroxide was used as a positive control. A negative control which is the untreated cells was included. After 24hours, spent media with floating cells was removed and cells were spun and the pellet collected and returned to the wells. The cells were stained with 250µl of 7.5µM CM-H_2DCFDA dye for 30 minutes at 37^{o}C. Thereafter, the dye was removed and cells were washed using PBS, trypsinized and transferred to a 15ml tube. The cells were centrifuged at 300xg for 3 minutes and washed with PBS to remove the dye and analyzed using a flow cytometry. Cell staining was measured at 488nm at FL3 on a BD Accuri^{TM} C6. A minimum of 10 000 cells per sample were acquired and analyzed using a BD CSampler^{TM} Software.

3.8 Statistical analysis

All samples were were assessed in triplicates and were presented as (means± SEM). For statistical analysis using Graph pad prism 5, one way analysis of variance (ANOVA) test was used to test for significance between the groups. Bonferoni Multiple test was used to compare differences between the groups. Student T test was used to compare difference between the same concentration. Differences were regarded statistically significant if $P< 0.05$ and highly significant if $P < 0.001$.

CHAPTER FOUR

RESULTS

4.1 Antioxidant characterization of *Hibiscus Sabdariffa* (HS) extracts

4.1.1 Antioxidant contents of HS water and methanolic extracts: total polyphenols and anthocyanins

The procedures for determining total polyphenol content and anthocyanins content were described in section 3.4.1 and 3.4.2 respectively. Figure 4.1 shows total polyphenol content of HS extracts was significantly higher in both water and methanolic extracts respectively at concentrations 0.4mg/ml (8.11±0.29mg/g, $p<0.001$ for water; 9.38±0.48mg/g, $p<0.001$ for methanolic), 0.6mg/ml (10.59±0.11mg/g, $p<0.001$ for water; 12.04±0.33mg/g, $p<0.001$ for methanolic), 0.8mg/ml (14.39±0.51mg/g, $p<0.001$ for water; 16.43±0.125mg/g $p<0.001$ for methanolic) 1mg/ml (17.71±0.39mg/g, $p<0.001$ for water; 21.44±0.66mg/g, $p<0.001$ for methanolic) when compared to 0.2mg/ml (3.17±0.22mg/g for water; 3.65±0.035mg/g for methanolic) respectively. In addition, at concentrations 0.8mg/ml and 1mg/ml, the polyphenols extracted with methanolic were higher than with water (16.43±0.125mg/g for methanolic versus 14.39±0.51mg/g for water at 0.8mg/ml, $p<0.05$) and (21.44±0.66mg/g for methanolic versus 17.71±0.39mg/g for water at 1mg/ml, $p<0.001$. However, there was no significant difference between polyphenols extracted with water and methanolic at 0.2mg/ml (3.17±0.22mg/g versus 3.65±0.03mg/g, $p>0.05$), 0.4mg/ml (8.11±0.29mg/g versus 9.38±0.485mg/g, $p>0.05$) and 0.6mg/ml (10.59±0.11mg/g versus 12.04±0.335mg/g, $p>0.05$).

Figure 4.2 shows that anthocyanins content of HS extracts was significantly higher in both water and methanolic extracts respectively at concentrations 0.4mg/ml (7.65±0.45mg/g, $p<0.001$ for water; 6.556±0.156mg/g, $p<0.001$ for methanolic), 0.6mg/ml (12.67±0.53mg/g, $p<0.001$ for water; 8.8±0.7mg/g, $p<0.001$ for methanolic), 0.8mg/ml (20.67±0.435mg/g, $p<0.001$ for water; 17±0.8mg/g $p<0.001$ for methanolic) 1mg/ml (24.81±0.51mg/g, $p<0.001$ for water; 19.4±0.1mg/g, $p<0.001$ for methanolic) when compared to 0.2mg/ml (4.56±0.46mg/g for water; 5.21±0.01mg/g for methanolic) respectively. In addition, at concentrations 0.6mg/ml, 0.8mg/ml and 1mg/ml, the anthocyanins extracted with water were higher than with methanolic (12.67±0.53mg/g for water versus 8.8±0.7mg/g for methanolic, $p<0.01$), (20.67±0.435mg/g for water versus 17±0.8mg/g for methanolic, $p<0.01$) and (24.81±0.51mg/g for water versus 19.4±0.1mg/g for methanolic, $p<0.001$). However, there were no significant difference between anthocyanins extracted with water and methanolic at concentration 0.2mg/ml (4.56±0.46mg/g versus 5.21±0.01mg/g, $p>0.05$) and 0.4mg/ml (7.65±0.45mg/g versus 6.556±0.156mg/g $p>0.05$).

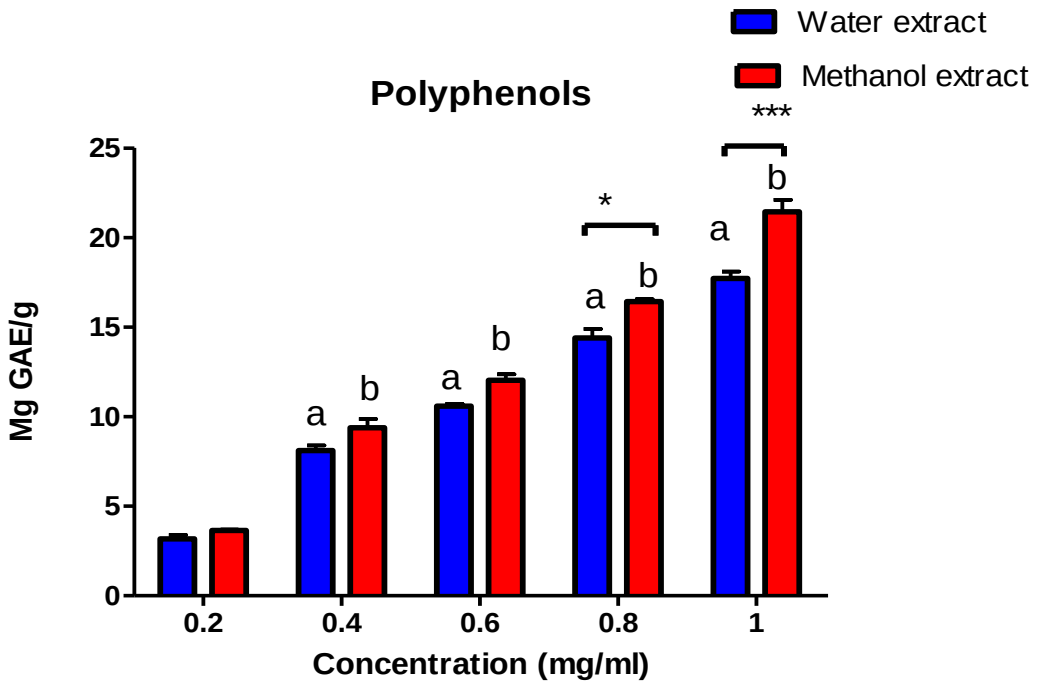

Figure 4.1: Total Polyphenols on water and methanolic extracts of HS. Results are presented as mean ± SEM in triplicates. (a) and (b) indicates significant differences at p<0.001 respectively compared to 0.2mg/ml water and methanolic extracts. (*) indicates p<0.05 and (***) indicates p<0.001 when comparing water to methanolic extract. ***Abbreviations***: GAE (gallic acid equivalent).

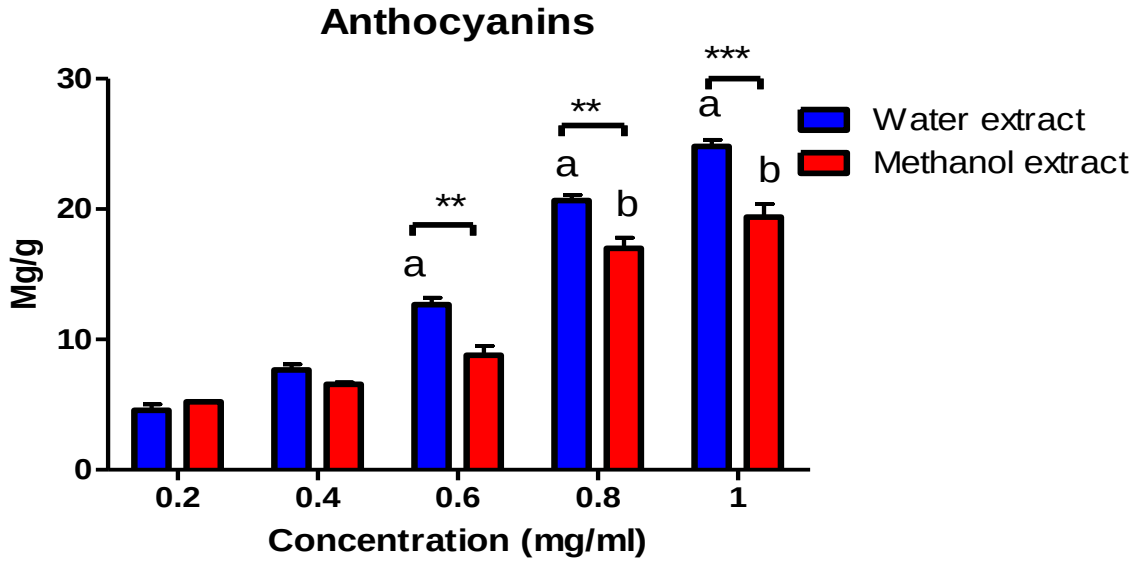

Figure 4.2: Anthocyanins concentration on water and methanolic extracts of HS. Results are presented as mean ± SEM in triplicates. a and b represent significant differences p<0.001 respectively compared to 0.2mg/ml water and methanolic extracts. ** indicates p<0.01 and *** indicates P<0.001 when comparing water to methanolic extract. ***Abbreviations***: Mg/g (milligrams per gram).

4.1.2 Antioxidant capacity of HS water and methanolic extracts: FRAP and ORAC

The procedures for determining FRAP and ORAC assays were described in section 3.4.3 and 3.4.4 respectively. Figure 4.3 shows that water and methanolic extract antioxidant capacity using FRAP assay was significantly higher in both water and methanolic extracts

respectively at concentrations 0.4mg/ml (151.7±7.59μmole/g, $p<0.05$ for water; 151.2±7.59μmole/g, $p<0.05$ for methanolic), 0.6mg/ml (267.3±17.96μmole/g, $p<0.001$ for water; 263.5±11.07 μmole/g, $p<0.001$ for methanolic), 0.8mg/ml (327.2±4.425μmole/g, $p<0.001$ for water; 326.7±8.73μmole/g, $p<0.001$ for methanolic), 1mg/ml (428.9±3.79μmole/g, $p<0.001$ for water; 438.1±2.375μmole/g, $p<0.001$ for methanolic) when compared to 0.2mg/ml (81.04±4.64 μmole/g for water; 77.91±10.01μmole/g for methanolic) respectively. However, there were no significant difference between antioxidant capacity using FRAP on water and methanolic at concentration 0.2mg/ml (81.04±4.64 μmole/g versus 77.91±10.01μmole/g, $p>0.05$), 0.4mg/ml (151.7±7.59μmole/g versus 151.2±7.59μmole/g $p>0.0.5$), 6mg/ml (267.3±17.96μmole/g versus 263.5±11.07 μmole/g, $p>0.0.5$), 0.8mg/ml (327.2±4.425μmole/g versus 326.7±8.73μmole/g, $p>0.05$) and 1mg/ml (428.9±3.79μmole/g versus 438.1±2.375μmole/g, $p>0.0.5$).

Figure 4.4 shows that water and methanolic extract antioxidant capacity using ORAC assay was significantly higher in both water and methanolic extracts respectively at concentrations of 0.6mg/ml (769.2±16.2μmole/g, $p<0.001$ for water; 1345±46.65 μmole/g, $p<0.001$ for methanolic), 0.8mg/ml (1001±15.35μmole/g, $p<0.001$ for water; 1465±20.15μmole/g, $p<0.001$ for methanolic), 1mg/ml (1506±30.05μmole/g, $p<0.001$ for water; 2057±79.30μmole/g, $p<0.001$ for methanolic) when compared to 0.2mg/ml (237.9±13 μmole/g for water; 718.7±20.65μmole/g for methanolic) respectively. In addition, at concentrations of 0.2mg/ml to 1mg/ml, the antioxidant capacity using ORAC was higher in methanolic than water (237.9±13 μmole/g for water versus 718.7±20.65μmole/g for methanolic, $p<0.001$ at 0.2mg/ml), (470.8±22.8μmole/g for water versus 1021±52.86μmole/g for methanolic at 0.4mg/ml, $p<0.001$ at 0.4mg/ml), (769.2±16.2μmole/g versus 1345±46.65 μmole/g for methanolic, $p<0.001$ at 6mg/ml), (1001±15.35μmole/g for water versus 1465±20.15μmole/g for methanolic, $p<0.001$ at 0.8mg/ml) and (1506±30.05μmole/g for water versus 2057±79.30μmole/g for methanolic, $p<0.001$ at 1mg/ml).

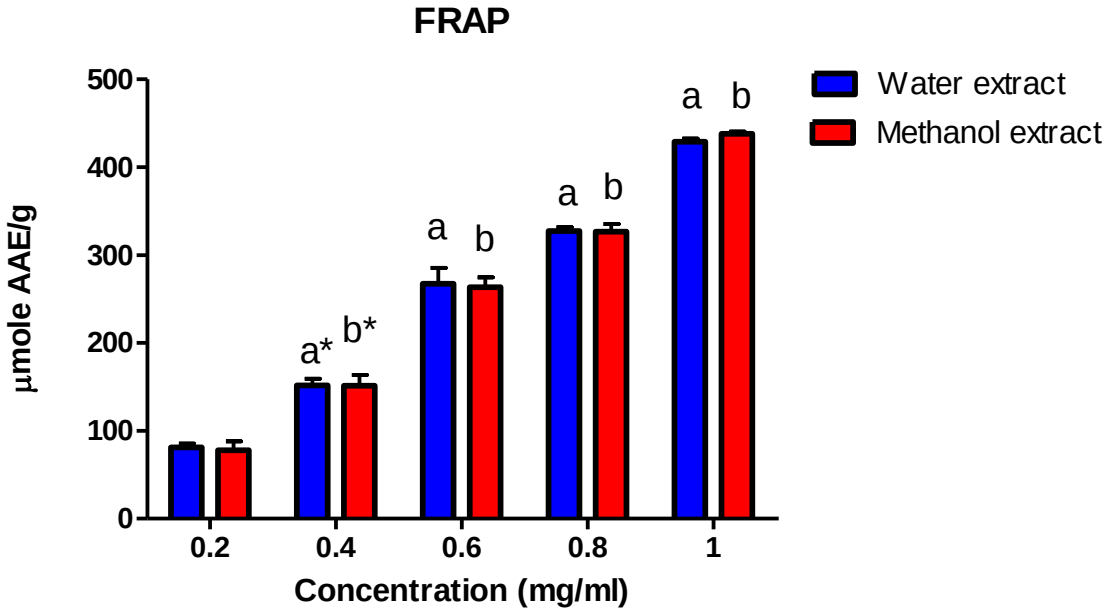

Figure 4.3: Ferric reducing antioxidant power of water and methanolic extracts of HS
Results are presented as mean ± SEM in triplicates. (a) and (b) represent significant differences P< 0.001 compared to 0.2mg/ml water and methanolic extracts , (a*) and (b*) indicates P< 0.05 respectively compared to 0.2mg/ml. ***Abbreviations***: AAE (ascorbic acid equivalent).

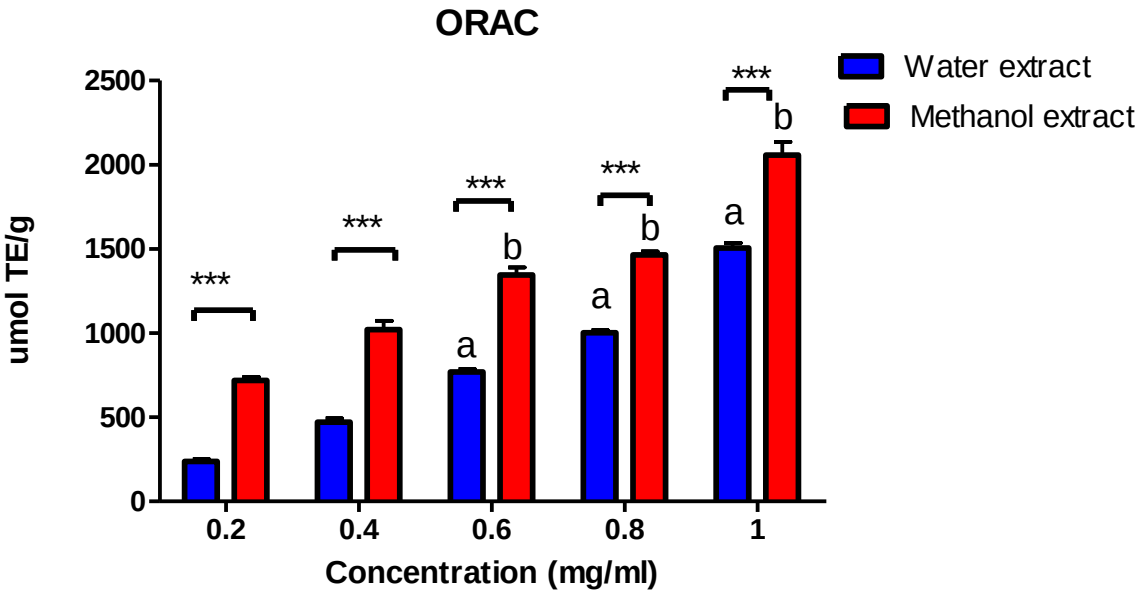

Figure 4.4: Oxygen radical absorbance capacity of water and methanolic extracts of HS
Results are presented as mean ± SEM in triplicates. (a) and (b) represent significant differences (p< 0.001) respectively compared to 0.2mg/ml water and methanolic extracts. (***) indicates p<0.001 when comparing water to methanolic extract. ***Abbreviations***: TE (trolox equivalent).

4.2 Apoptotic and cytotoxic study on HS treated cells.

4.2.1 Evaluating the effects of HS extracts on cell morphology

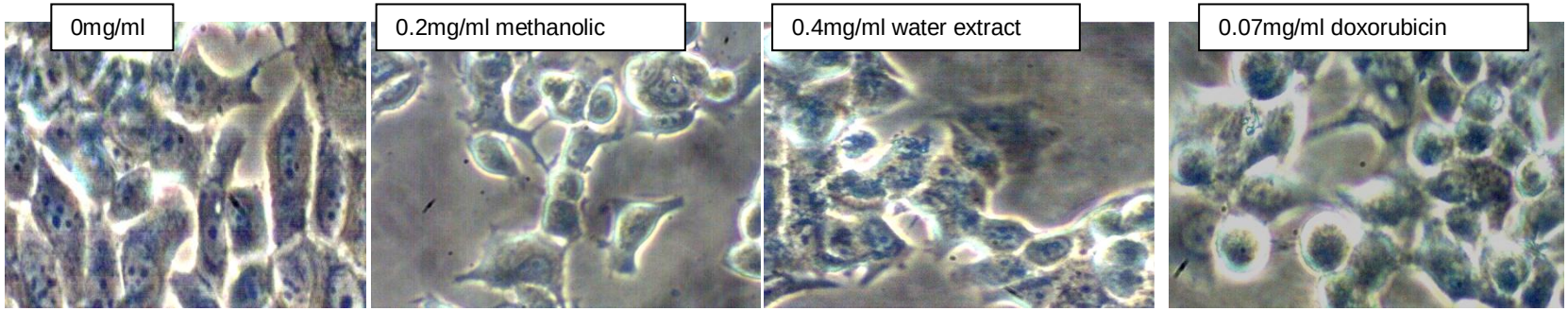

Figure 4.5: Morphological changes on MCF 7 cells

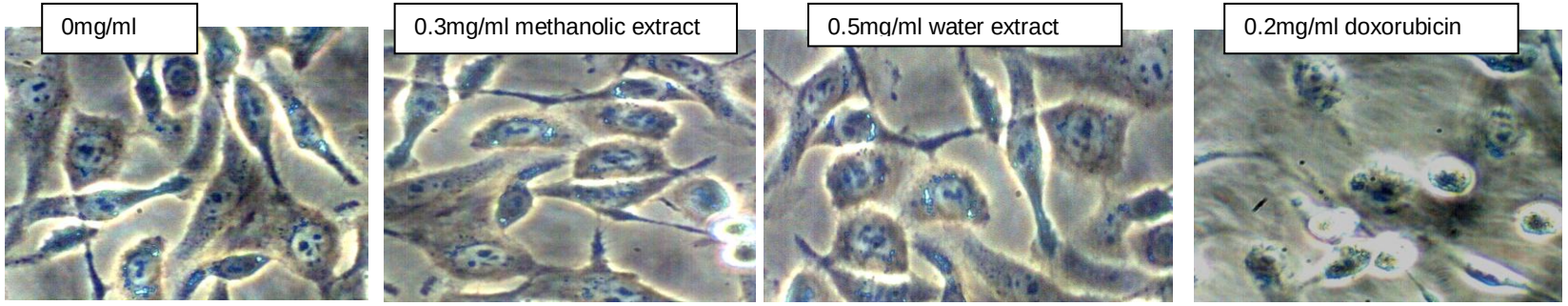

Figure 4.6: Morphological changes on MCF 12A cells

The morphological effects of water and methanolic extracts of HS on the two cell lines were observed using a 40x inverted light microscope following treatment for 24hours. Figure 4.5 shows treated and untreated cancerous MCF7 cells. Untreated cells were polygonal and adhered to culture dishes. Cells treated with water and methanolic extracts IC_{50} showed morphological changes with some cells that appeared to be detached from the plate and spherical in shape. In addition, cell density was low as some cells had detached from the flask. Doxorubicin is an anticancer agent and its use on MCF7 cells reveals morphological changes similar to those of cells treated with HS. Figure 4.6 shows treated and untreated non-cancerous MCF12A cells. Untreated cells were spindle shaped and adhered to culture dishes. Treatment with HS extracts shows some morphological changes. Treatment with doxorubicin reveals morphological changes as cells are spherical in shape.

4.2.2 Measurement of cell viability using WST-1

WST-1 is a cell viability assay. HS water and methanolic extracts were used to screen for possible anti-proliferative effects against MCF7 and MCF12A cells in a dose dependent manner. The procedure was done as described in section 3.6.3. Results obtained in Figure 4.7 shows that HS water extract significantly induced growth inhibition on MCF7 and MCF12A cells respectively at concentration 0.1mg/ml (72.86±3.68%, $p<0.001$ for MCF7 cells; 84.96±1.97%, $p<0.001$ for MCF12A cells), 0.2mg/ml (59.01±1.17%, $p<0.001$ for MCF7 cells; 69.56±2.70%, $p<0.001$ for MCF12A cells), 0.4mg/ml (49.42±2.73%, $p<0.001$ for MCF7 cells; 55.23±1.49%, $p<0.001$ for MCF12A cells), 0.6mg/ml (36.95±2.99%, $p<0.001$ for MCF7 cells ;43.8±1.34%, $p<0.001$ for MCF12A cells), 0.8mg/ml (25.4±3.89%, $p<0.001$ for MCF7 cells; 39.67±0.89%, $p<0.001$ for MCF12A cells) and 1mg/ml (23.69±3.35%, $p<0.001$ for MCF7 cells; 36.24±1.92%, $p<0.001$ for MCF12A cells) compared to untreated cells (100% for MCF7 cells and MCF12A cells). In addition, decreased cell viability of MCF7 cells compared to MCF12A cells was observed at a concentration of 0.2mg/ml (59.01±1.17% for MCF7 cells versus 69.56±2.70% for MCF12A cells, $p<0.05$), 0.8mg/ml (25.4±3.89% for MCF7 cells versus 39.67±0.89% for MCF12A cells, $p<0.01$) and 1mg/ml (23.69±3.35% for MCF7 cells versus 36.24±1.92% for MCF12A cells, $p<0.001$). However, no significant difference on treated MCF7 versus treated MCF12A cells was noted at a concentration of 0.1mg/ml (72.86±3.68%, versus 84.96±1.97%, $p>0.05$), 0.4mg/ml (49.42±2.73% versus 55.23±1.49%, $p>0.05$) and 0.6mg/ml (36.95±2.99% versus 43.8±1.34%, $p>0.05$).

Figure 4.8 shows that HS methanolic extract significantly induced growth inhibition on MCF7 and MCF12A cells respectively at concentration 0.1mg/ml (66.11±1.08%, $p<0.001$ for MCF7 cells; 78.58±4.46%, $p<0.001$ for MCF12A cells), 0.2mg/ml (49.71±1.29%, $p<0.001$ for MCF7 cells; 61.6±1.97%, $p<0.001$ for MCF12A cells), 0.4mg/ml (38.48±0.78%, $p<0.001$ for MCF7 cells; 47.26±3.10%, $p<0.001$ for MCF12A cells), 0.6mg/ml (32.72±2.82%, $p<0.001$ for MCF7

cells ;39.86±1.39%, p<0.001 for MCF12A cells), 0.8mg/ml (25.25±1.70%, p<0.001 for MCF7 cells; 37.36±2.94%, p<0.001 for MCF12A cells) and 1mg/ml (15.89±4.06%, p<0.001 for MCF7 cells; 35.68±2.05%, p<0.001 for MCF12A cells) compared to untreated cells MCF7 and MCF12A cells. In addition, decreased cell viability of MCF7 cells compared to MCF12A cells was observed at a concentration of 0.1mg/ml (66.11±1.08%, versus 78.58±4.46%, p<0.001), 0.2mg/ml (49.71±1.29% for MCF7 cells versus 61.6±1.97% for MCF12A cells, p<0.001), 0.4mg/ml (38.48±0.78% versus 47.26±3.10%, p<0.01), 0.6mg/ml (32.72±2.82% versus 39.86±1.39%, p<0.05), 0.8mg/ml (25.25±1.70% for MCF7 cells versus 37.36±2.94% for MCF12A cells, p<0.001) and 1mg/ml (15.89±4.06% for MCF7 cells versus 35.68±2.05% for MCF12A cells, p<0.001).

Decreased cell viability on MCF12A cells treated with methanolic compared to water was observed at concentration 0.2mg/ml (69.56±2.70% for water versus 61.6±1.97% for methanolic, p<0.01) and 0.4mg/ml (55.23±1.49% for water versus 47.26±3.10% for methanolic, p<0.05). On MCF7 cells decreased cell viability was observed upon treatment with methanolic compared to water at concentration 0.4mg/ml (49.42±2.73% for water versus 38.48±0.77% for methanolic, p<0.05). Results were not indicated on the graph.

Anti-proliferative activity of plant extract on breast cell lines was determined using IC_{50} value which is based on the concentration of the plant that causes 50% cell death. The lower the IC_{50} value the more potent a plant extract is (Griffiths and Sundaram, 2011). IC50 values are summarized in Table 4.1. Both HS extracts had a low IC_{50} value on MCF7 cancerous cells (0.2mg/ml for methanolic extract and 0.4mg/ml for water extract) compared to non cancerous MCF12A cells (0.4mg/ml for methanolic extract and 0.5mg/ml for water extract). The IC_{50} for positive controls was low in MCF7 cells (0.05% for DMSO; 0.075% hydrogen peroxide and 0.07mg/ml for doxorubicin) compared to non-cancerous MCF12A cells (0.1% for DMSO, 0.2% hydrogen peroxide and 0.2mg/ml doxorubicin).

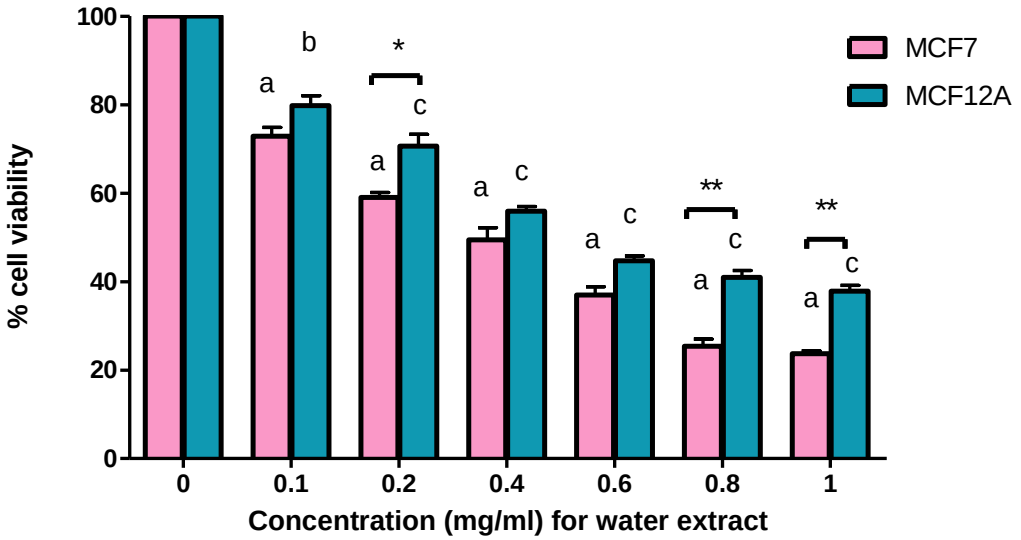

Figure 4.7: The cell viability of MCF7 and MCF12A cells treated for 24 hours with HS water extract at a concentration range of 0.1 to 1mg. Results are presented as mean ± SEM, (n=3). (a) indicates significant differences p< 0.001 compared to untreated MCF7 cells. (b) and (c) indicates significant differences p< 0.01 and p< 0.001 respectively, compared to untreated MCF12A cells. (*) indicates p<0.05 and (**) indicates p<0.01 when comparing MCF7 cells to MCF12A cells.

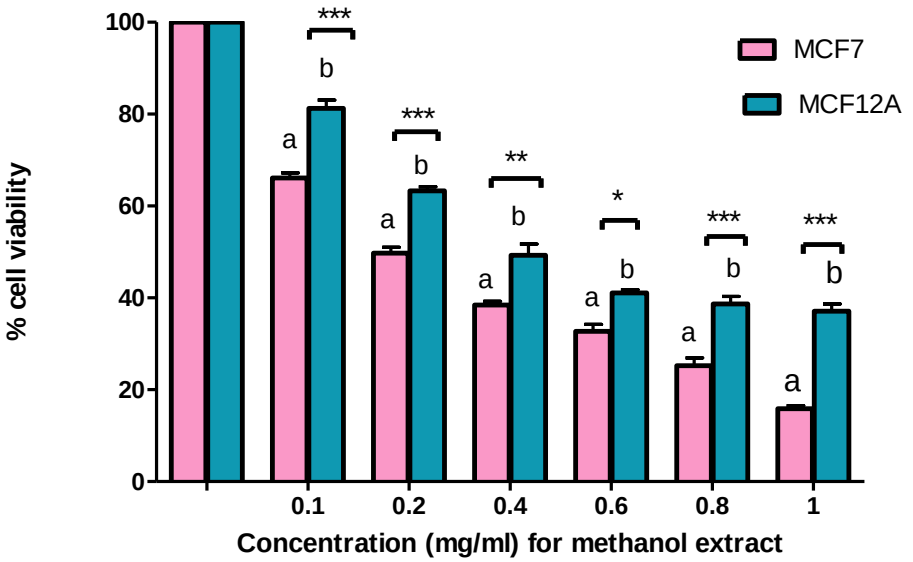

Figure 4.8: The cell viability of MCF7 and MCF12A cells treated for 24 hours with HS methanolic extract at a concentration range of 0.1 to 1mg/ml. Results are expressed as mean ± SEM with (n=3). (a) represents significant differences p< 0.001 compared to untreated MCF7 cells. (b) represents significant differences p< 0.001 compared to untreated MCF12A cells. (*) indicates P<0.05, (**) indicates p<0.01 and (***) indicates p<0.001 when comparing MCF7 cells to MCF12A cells.

Table 4.1: Summary for IC50 values for treated MCF7 and MCF12A cells

Treatments	MCF 7 cells (IC50)	MCF 12A cells (IC50)
HS water extract	0.4mg/ml	0.5mg/ml
HS methanolic extract	0.2mg/ml	0.4mg/ml
DMSO	0.05%	0.1%
Doxorubicin	0.07mg/ml	0.2mg/ml
Hydrogen peroxide	0.075%	0.2%

4.2.3 Evaluating the activation of apoptosis using the Apo*Percentage*™ assay

Many compounds can inhibit the growth of tumor cells but not all of them trigger apoptosis. Therefore, in order to ascertain if the cytotoxicity of HS extracts is due to apoptosis, the Apo*Percentage*™ assay was done as described in section 3.6.4. Table 4.2 and Figure 4.12 indicated that MCF7 cells exhibited sensitivity to both water and methanolic extracts respectively at concentrations of 0.2mg/ml (54.57±0.89% for HS water exract, 54.57±0.89% for HS methanolic extract $p<0.001$), 0.3mg/ml (58.90±1.686% for water, 58.77±3.67% for methanolic $p<0.001$) and 0.4mg/ml (70.40±2.44% for water, 69.50±1.55% for methanolic $p<0.001$) in comparison to untreated MCF7 cells (19.10±0.665%). In addition, both positive controls induced apoptosis with DMSO (62.50±2.639%, $p<0.001$) and doxorubicin (69.37±2.669%, $p<0.001$) in comparison to untreated MCF7 cells (19.10±0.665%). MCF12A cells treated with HS showed non significant apoptotic cells at the concentration of 0.2mg/ml (11.33±1.453% for water, 9.00±1.026% for methanolic, $p>0.05$), 0.3mg/ml (12.30±1.350% for water, 12.37±1.32 for methanolic $p>0.05$) and 0.4mg/ml (15.33±1.419% for water, 14.43±1.946 for methanolic $p>0.05$) in comparison to untreated MCF12A cells. However, both positive controls DMSO and doxorubicin induced apoptosis with 46.7±2.359% and 58.83±0.99% respectively cells positive for apoptosis in comparison to untreated MCF12A cells with significant difference ($p<0.001$). Significantly high number of apoptotic MCF7 cells were noted in comparison to MCF12A cells at 0.2mg/ml, 0.3mg/ml and 0.4mg/ml concentration of HS extracts at $p<0.001$. Figure 4.9 shows examples of flow cytometry data in the form of histograms.

Table 4.3 illustrates time response for apoptosis at times 12hours and 24hours. Both extracts demonstrated a significant increase in apoptotic cells ($p<0.001$) at both 12hours (54.5±2.598% for water, 51.07±1.084% for methanolic, $p<0.001$) and 24 hours 74.6±2.330% for water, 73.17±1.855% for methanolic, $p<0.001$) on treated MCF7 cells compared to untreated MCF7 cells (19.1±0.665). In non-cancerous MCF12A cells, no significant difference ($p>0.05$) was seen between the specified times compared to untreated MCF12A cells (10.53 ±1.178%). A significant increase in apoptotic cells on MCF7 cells at times 12 and

24 hours using both extracts was observed compared to treated MCF12A cells ($p<0.001$). These findings suggest that induction of apoptosis is dose and time dependent on MCF7 cells.

Table 4.2: Evaluating apoptosis on MCF7 and MCF12A cells treated with HS water and methanolic extract for 24hours

Treatments	Concentrations	Apoptotic cells (%) MCF12A	Apoptotic cells (%) MCF7
Negative control	Media only	10.53 ±1.178	$19.10\pm0.665^{*}$
Water extract	0.2 mg/ml	11.33±1.453	54.57 ± 0.89^{bc}
	0.3 mg/ml	12.30±1.350	58.90 ± 1.686^{bc}
	0.4 mg/ml	15.33±1.419	70.40 ± 2.44^{bc}
Methanolic extract	0.2 mg/ml	9.00±1.026	54.57 ± 0.89^{bc}
	0.3 mg/ml	12.37±1.32	58.77 ± 3.67^{bc}
	0.4 mg/ml	14.43±1.946	69.50 ± 1.55^{bc}
Doxorubicin (Positive control)	IC_{50}(mg/ml)	46.7 ± 2.359^{a}	62.50 ± 2.639^{bc}
DMSO (Positive control)	IC_{50} (%)	58.83 ± 0.99^{a}	69.37 ± 2.669^{bc}

Values are expressed as mean ± SEM (n=6) with (a) and (b) representing significant differences ($p< 0.001$), compared to untreated MCF7 and MCF12A cells respectively. (*) indicates ($p<0.05$) and (c) indicates ($p<0.001$) when comparing MCF7 cells to MCF12A cells.

Table 4.3: Evaluating apoptosis on MCF7 and MCF12A cells treated with HS water and methanolic extract for 12 and 24 hour

Treatments	Time (hours)	Apoptotic MCF12A cells (%)	Apoptotic MCF7 cells (%)
Negative control (media only)	0	$10.53\pm1.178^{*}$	19.1±0.665
Water extract	12	$12.30\pm1.253^{***}$	54.5 ± 2.598^{b}
IC_{50}	24	$12.00\pm1.050^{***}$	74.6 ± 2.330^{bd}
Methanolic extract	12	$12.67\pm0.600^{***}$	51.07 ± 1.084^{b}
IC_{50}	24	$11.87\pm0.968^{***}$	73.17 ± 1.855^{bd}
Doxorubicin IC_{50}(mg/ml)	24	$46.7\pm2.359^{a***}$	62.50 ± 2.639^{b}
DMSO IC_{50} (%)	24	$58.83\pm0.99^{a***}$	69.37 ± 2.669^{b}

Values are expressed as mean ± SEM (n=6). a and b represents significant differences ($p< 0.001$), compared to untreated MCF12A and MCF7 cells respectively. d indicates ($p<0.001$) compared to MCF7 12hour. * indicates $P<0.05$ and *** indicates $P<0.001$ when comparing MCF7 to MCF12A cells.

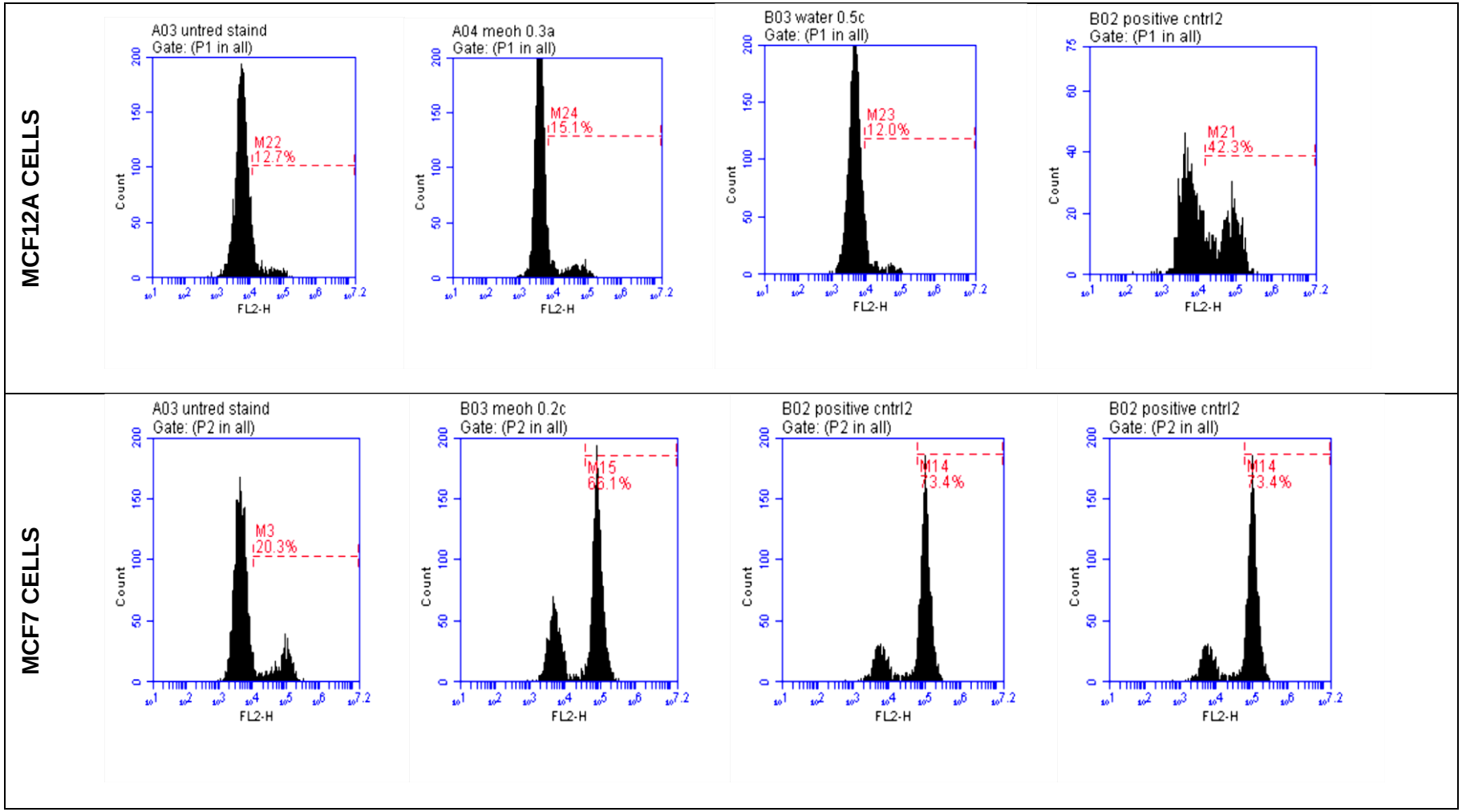

Figure 4.9: Histograms represent apoptotic cells harvested from untreated cells, cells treated with HS water and methanolic extract and a positive control DMSO and stained with apo percentage dye. The region marker represents the % of apoptotic cells while FL2-H represents fluorescent intensity (apoptosis).

4.2.4 Evaluating mitochondrial depolarization using Tetra Methyl Rhodamine Ethyl ester (TMRE)

Mitochondrial depolarization is a common feature in cells undergoing apoptosis. Mitochondrial dysfunction is associated with the loss of mitochondrial transmembrane potential which is linked to apoptosis and loss of cell viability. MCF7 and MCF12A cells were treated HS extracts and evaluated for mitochondrial depolarization. Table 4.4 and Figure 4.10 indicates that MCF7 cells treated with HS extracts showed an increase in number of cells with depolarized mitochondrial with significant difference at concentrations 0.1mg/ml (24.75±0.7% for water, $p< 0.001$), 0.2mg/ml (38.30±0.30% for water, 58.77±3.67% for methanolic $p< 0.001$) and 0.3mg/ml (42.50±0.50% for water, 38.05±0.45% for methanolic $p<0.001$) except for 0.1mg/ml (19.50±0.50% methanolic $p>0.05$) when compared to untreated MCF7 cells (15.50±2.20%). In addition, both positive controls induced mitochondrial depolarization with DMSO (52.60±0.8%, $p<0.001$) and doxorubicin (62.65±0.65%, $p<0.001$) in comparison to untreated MCF7 cells (15.50±2.20%). No significant difference was observed on MCF12A cells treated with both extracts at concentration 0.1mg/ml to 0.3mg/ml, $p>0.05$ compared to untreated MCF12A cells (4.75±0.55%). In addition, both positive controls induced mitochondrial depolarization with DMSO (47.5±9.50%, $p<0.001$) and doxorubicin (36.5±1.5%, $p<0.001$) in comparison to untreated MCF12A cells (4.75±0.55%). Significantly high numbers of treated MCF7 cells with depolarized mitochondria ($p<0.001$) were noted in comparison to treated MCF12A cells at all concentrations and at 0.1mg/ml methanolic treated cells ($p<0.01$). This suggests both HS methanolic and water extracts induced mitochondrial depolarization on MCF7 cells. Figure 4.10 shows examples of flow cytometry data in the form of histograms.

Table 4.5 shows a time response for mitochondrial depolarization at times 12hours and 24hours. High numbers of treated MCF7 cells with depolarized mitochondria was noted, while low numbers were noted in MCF12A cells. Both extracts demonstrated a significant increase in mitochondrial depolarization at 24 hours (42.00±1.0% for water, 38.00±1.0% for methanolic, $p<0.001$) and no significant difference at 12hours (28.40±0.60% for water, 19.75±0.75% for methanolic, $p>0.05$) on treated MCF7 cells compared to untreated MCF7 cells (15.50±2.20%). On the non cancerous MCF12A cells no significant difference was seen between all specified times compared to untreated MCF12A cells (4.75±0.55%, $p>0.05$). A significant increase in mitochondrial depolarization ($p<0.001$) on MCF7 cells at times 12 and 24 hours using water extract and ($p<0.01$) at time 24hours using methanolic extract was observed compared to treated MCF12A cells. However, no significant difference was noted between untreated MCF 7 and MCF12A, $p>0.05$. These findings suggest mitochondrial depolarization is time dependent on MCF7 cells.

Table 4.4: **Evaluating mitochondrial depolarization of MCF7 and MCF12A cells on a dose response experiment in treated with HS water and methanolic extract for 24hours**

Treatments	Concentrations	Mitochondrial depolarization (%) MCF12A cells	Mitochondrial depolarization (%) MCF7 cells
Negative control	Media only	4.75±0.55	15.50±2.20*
Water extract	0.1 mg/ml	4.80±0.30	24.75±0.75^{a***}
	0.2 mg/ml	4.55±0.35	38.30±0.30^{a***}
	0.3 mg/ml	4.55±0.25	42.50±0.50^{a***}
Methanolic extract	0.1 mg/ml	4.10±0.40	19.50±0.50**
	0.2 mg/ml	4.00±0.50	27.25±0.75^{a***}
	0.3 mg/ml	4.10±0.20	38.05±0.45^{a***}
Doxorubicin (Positive control)	IC_{50} (mg/ml)	47.5±9.50^{b}	52.60±0.80^{a***}
DMSO (Positive control)	IC_{50} (%)	36.5±1.50^{b}	62.65±0.65^{a}

Values are expressed as mean ± SEM (n=3) with a and b representing ($p<0.001$) compared to untreated MCF7 and MCF12A cells respectively. * for indicates ($p<0.05$), ** indicates ($p< 0.01$) and *** indicates ($p<0.001$) when comparing MCF7 cells to MCF12A cells.

Table 4.5: Evaluating mitochondrial depolarization of MCF7 and MCF12A cells on a time response experiment on treated with HS water and methanolic extract for 12 and 24 hours

Treatments	Treatment time (hours)	Mitochondrial depolarization (%) MCF12A cells	Mitochondrial depolarization (%) MCF7 cells
Negative control	0	4.75±0.55	15.50±2.20
Water extract	12	5.10±0.30	28.40±0.60***
	24	5.95±0.15	42.00±1.00^{a***}
Methanolic extract	12	4.55±0.25	19.75±0.75**
	24	4.80±0.30	38.00±1.00^{a***}
Doxorubicin (Positive control)	IC_{50} (mg/ml)	47.5±9.50^{b}	52.60±0.80^{a***}
Doxorubicin (Positive control)	IC_{50} (%)	36.5±1.50^{b}	62.65±0.65^{a}

Values are expressed as mean ± SEM (n=3). a and b representing $p<0.001$ compared to untreated MCF7 and MCF12A cells respectively. ** indicates ($p< 0.01$) and *** indicates ($p< 0.001$) when comparing MCF7 cells to MCF12A cells.

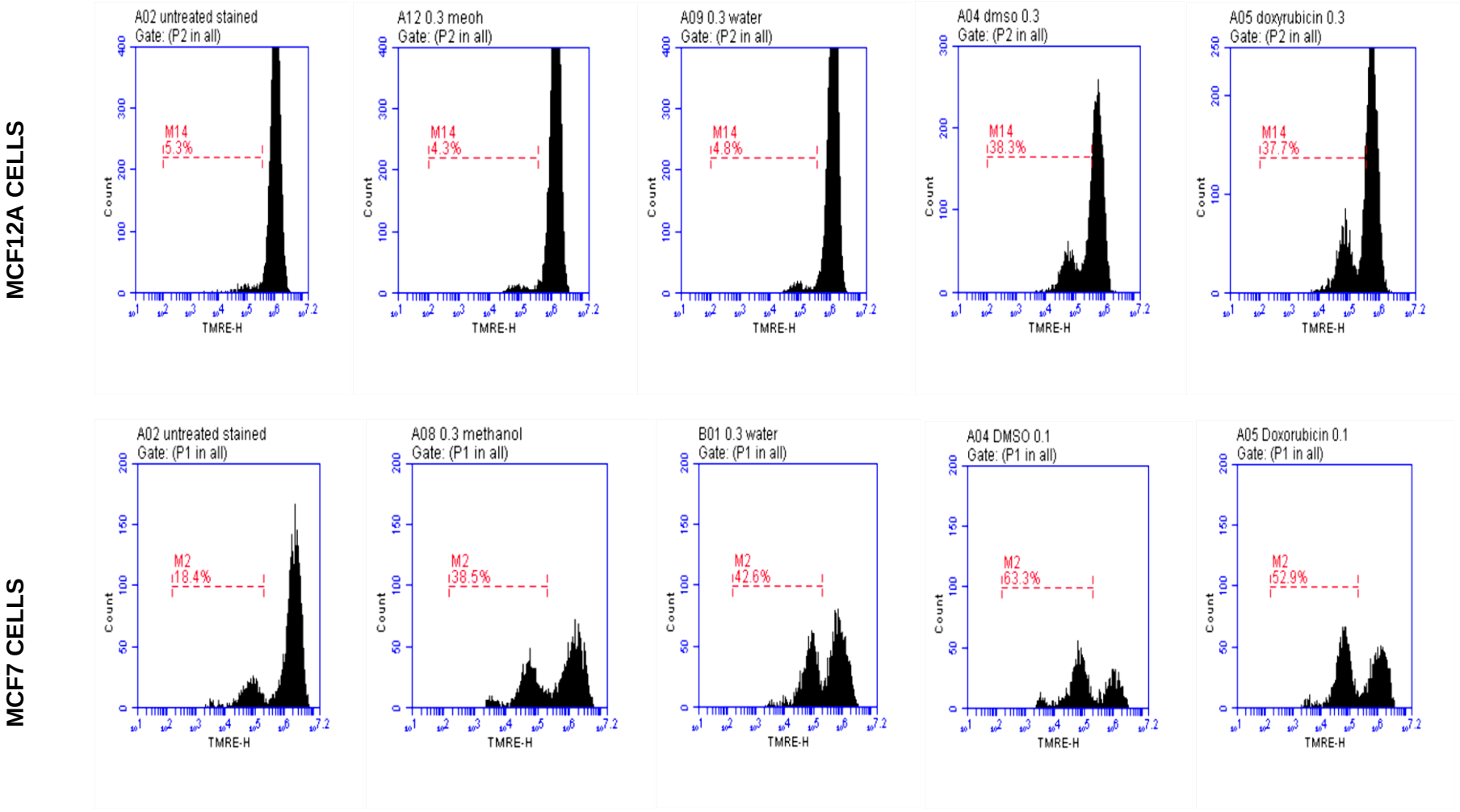

Figure 4.10: Histograms represent cells with depolarized mitochondria that are harvested from untreated cells, cells treated with HS water and methanolic extract and a positive control DMSO and stained with TMRE dye. The region marker represents the % of cells with depolarized mitochondria and TMRE-H represents fluorescent intensity.

4.3 Antioxidant status of cells treated with HS extracts

4.3.1 Determining Superoxide dismutase (SOD) activity

SOD activity in treated and untreated cell was determined as shown in Figure 4.11.

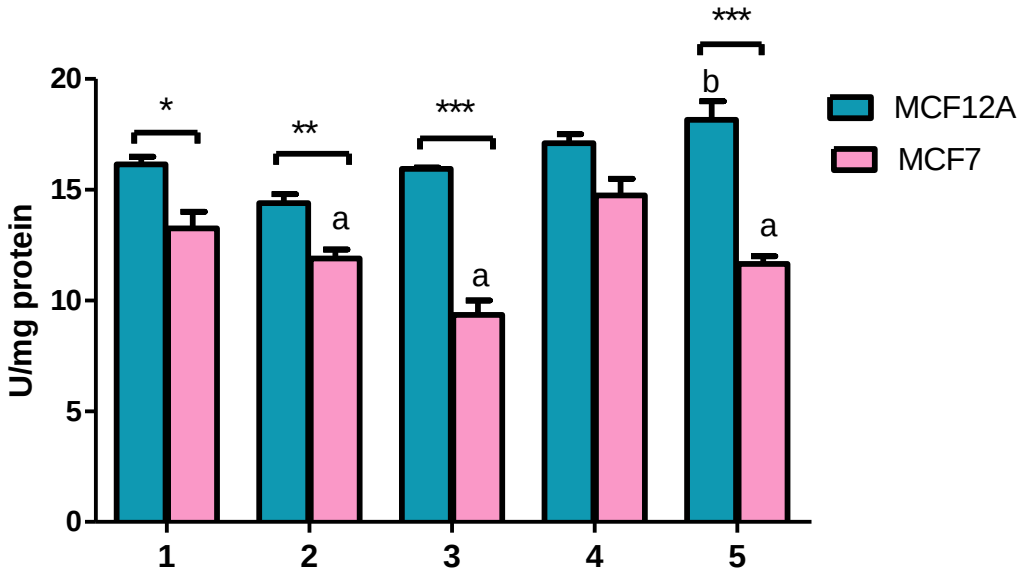

Figure 4.11: SOD activity in cells treated with HS water extracts for 24hours
Values are expressed as mean values ± SEM in triplicates. a indicates ($p<0.01$), b indicates ($p<0.05$) compared to untreated cells. * indicates ($p<0.05$), ** indicates ($p<0.01$) and *** indicates ($p<0.001$) when comparing MCF7 cells to MCF12A cells. ***Experimental groups-*** 1: untreated cells (control group), 2: cells treated with 0.2% hydrogen peroxide, 3: cells pretreated with HS extract 0.2mg/ml and then treated with hydrogen peroxide $IC_{50,}$ 4: cells treated with 0.2mg/ml HS and 5: cells treated with 0.4mg/ml HS.

In MCF12A cells a significant higher SOD activity was noted upon treatment with HS extract 0.4mg/ml (18.65±0.35U/mg, $p<0.05$) compared to untreated MCF12A cells (16.5±0.35U/mg). No significant difference was noted on MCF12A cells treated respectively with (IC_{50}) 0.2% hydrogen peroxide (14.4±0.4U/mg, $p>0.05$), 0.2mg/ml HS extract (17.1±0.4U/mg, $p>0.05$) and pretreatment with HS extract 0.2mg/ml and then treated with hydrogen peroxide 0.2% (15.95±0.05U/mg, $p>0.05$) compared to untreated MCF12A cells (16.5±0.35U/mg). In MCF7 cells, a significant lower SOD activity was observed upon treatment with hydrogen peroxide (IC_{50}) 0.075% (11.9±1.1U/mg, $p<0.01$), 0.4mg/ml HS (14.65±0.65U/mg, $p<0.01$) and pretreatment with HS extract 0.2mg/ml and then treated with hydrogen peroxide 0.075% (9.35±0.65U/mg, $p<0.01$) when compared to untreated MCF7 cells (13.25±0.75U/mg). However, no significant difference was seen at 0.2mg/ml treatment (14.75±1.25U/mg, $p>0.05$) when compared to untreated MCF7 cells (13.25±0.75U/mg). Higher catalase activity was observed in MCF12A in comparison to MCF7 cells respectively on untreated cells (16.5±0.35U/mg versus 13.25±0.75U/mg, $p<0.05$), hydrogen peroxide, (14.4±0.4U/mg versus 11.9±1.1U/mg, $p<0.01$), pretreatment with HS extract 0.2mg/ml and then treated with

hydrogen peroxide (15.95±0.05U/mg versus 9.35±0.65U/mg, $p<0.001$), and 0.4mg/ml (18.65±0.35U/mg versus 11.65±0.65U/mg). No significant difference was observed in MCF12A in comparison to MCF7 cells with 0.2mg/ml treatment (17.1±0.4U/mg versus 14.75±1.25U/mg, $p>0.05$).

4.3.2 Assessing catalase activity

Catalase activity in treated and untreated cell was determined as shown in Figure 4.12. In MCF12A cells a significant lower catalase levels were noted upon treatment with HS extract 0.4mg/ml (4.24±0.04U/mg, $p<0.05$) and a significant decrease with 0.2% hydrogen peroxide (IC_{50}) treatment (3.385±0.1385U/mg, $p<0.05$) compared to untreated MCF12A cells (3.8±0.1U/mg). No significant difference was noted on MCF12A cells treated respectively with 0.2mg/ml HS extract (3.825±0.075U/mg, $p>0.05$) and pretreatment with HS extract 0.2mg/ml and then treated with hydrogen peroxide 0.2% (3.725±0.125U/mg, $p>0.05$) compared to untreated MCF12A cells (3.8±0.1U/mg). In MCF7 cells, a significant lower catalase activity were indicated upon treatment with hydrogen peroxide (IC_{50}) 0.075% (2.3±0.1U/mg, $p<0.01$), 0.4mg/ml HS (2.35±0.05U/mg, $p<0.01$) and pretreatment with HS extract 0.2mg/ml and then treated with hydrogen peroxide 0.075% (2.075±0.175U/mg, $p<0.001$) when compared to untreated MCF7 cells (2.90±0.1U/mg). However, no significant difference was seen at 0.2mg/ml treatment (3.07±0.08U/mg, $p>0.05$) when compared to untreated MCF7 cells (2.90±0.1U/mg). Higher catalase activity was observed in MCF12A in comparison to MCF7 cells respectively on untreated cells (3.8±0.1U/mg versus 2.90±0.1U/mg, $p<0.001$), hydrogen peroxide, (3.385±0.1385U/mg versus 2.3±0.1U/mg, $p<0.001$), pretreatment with HS extract 0.2mg/ml and then treated with hydrogen peroxide (3.725±0.125U/mg versus 2.075±0.175U/mg, $p<0.001$), 0.2mg/ml (3.825±0.075U/mg versus 3.07±0.08U/mg, $p<0.001$) and 0.4mg/ml (4.24±0.04U/mg versus 2.35±0.05U/mg, $p<0.001$).

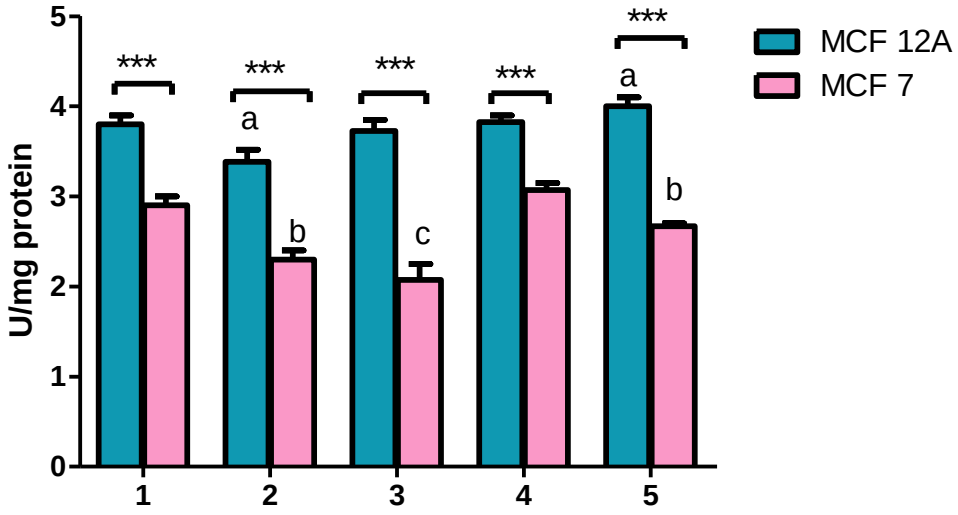

Figure 4.12: Catalase activity in cells treated with HS water extracts for 24hours
Values are mean values ± SEM in triplicates. a indicates ($p<0.05$), b indicates ($p<0.01$) and c indicates ($p<0.001$) compared to untreated cells. *** indicates ($p<0.001$) when comparing MCF7 cells to MCF12A cells. ***Experimental groups-*** 1: untreated cells (control group), 2: cells treated with 0.2% hydrogen peroxide, 3: cells pretreated with HS extract 0.2mg/ml and then treated with hydrogen peroxide $IC_{50,}$ 4: cells treated with 0.2mg/ml HS and 5: cells treated with 0.4mg/ml HS.

4.3.3 Determining glutathione levels

Glutathione (GSH) concentration in HS treated cells was determined as shown in Figure 4.13. In MCF12A cells a significant lower GSH levels were noted upon treatment with 0.2% hydrogen peroxide (IC_{50}) (44.05±0.7nmole/mg, $p<0.01$) compared to untreated MCF12A cells (48.8±0.9nmole/mg). No significant difference was noted on MCF12A cells treated respectively with 0.2mg/ml HS extract (48.75±0.45nmole/mg, $p>0.05$), 0.4mg/ml HS extract (50.05±0.95nmole/mg, $p>0.05$) and pretreatment with HS extract 0.2mg/ml and then treated with hydrogen peroxide 0.2% (47.05±1.15nmole/mg, $p>0.05$) compared to untreated MCF12A cells (48.8±0.9nmole/mg). In MCF7 cells, a significant reduction in GSH levels was indicated upon treatment with hydrogen peroxide (IC_{50}) 0.075% (34.00±1.0nmole/mg, $p<0.05$), 0.4mg/ml HS (34.25±0.95nmole/mg, $p<0.05$) and pretreatment with HS extract 0.2mg/ml and then treated with hydrogen peroxide 0.075% (31.55±0.45nmole/mg, $p<0.01$) when compared to untreated MCF7 cells (37.9±1.2nmole/mg). However, no significant difference was seen at 0.2mg/ml treatment (36.7±1.3nmole/mg, $p>0.05$) when compared to untreated MCF7 cells (37.9±1.2nmole/mg). Higher GSH levels was observed in MCF12A in comparison to MCF7 cells respectively on untreated cells (48.8±0.9nmole/mg versus 37.9±1.2nmole/mg, $p<0.001$), hydrogen peroxide, (44.05±0.7nmole/mg versus 34.00±1.0nmole/mg, $p<0.001$), pretreatment with HS extract 0.2mg/ml and then treated with hydrogen peroxide (47.05±1.15nmole/mg versus 31.55±0.45nmole/mg, $p<0.001$), 0.2mg/ml

(48.75±0.45nmole/mg versus 36.7±1.3nmole/mg, p<0.001) and 0.4mg/ml (50.05±0.95nmole/mg versus 34.25±0.95nmole/mg /mg, p<0.001).

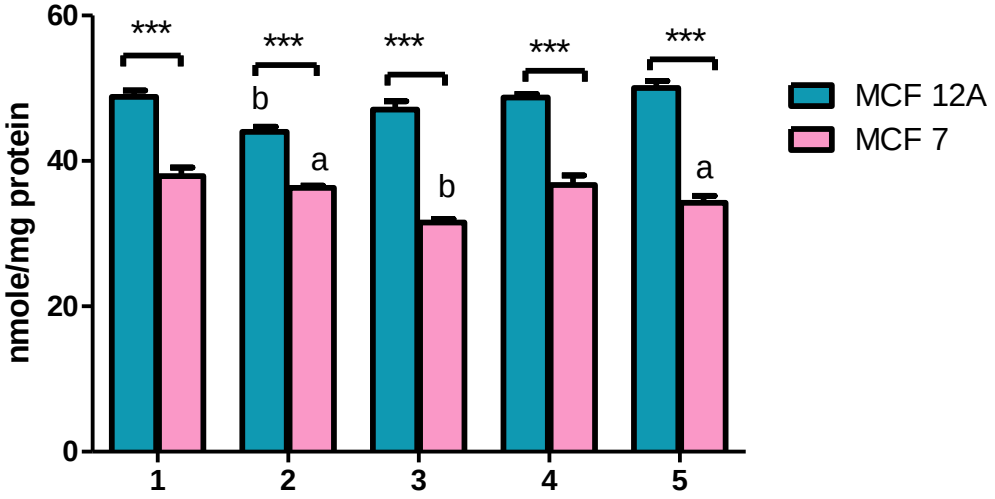

Figure 4.13: Glutathione levels in cells treated with HS water extracts for 24hours
Values are mean values ± SEM in triplicates. a indicates (p<0.05), b indicates (p<0.01) compared to untreated cells. *** indicates (p<0.001) when comparing MCF7 cells to MCF12A cells. ***Experimental groups-*** 1: untreated cells (control group), 2: cells treated with 0.2% hydrogen peroxide, 3: cells pretreated with HS extract 0.2mg/ml and then treated with hydrogen peroxide $IC_{50,}$ 4: cells treated with 0.2mg/ml HS and 5: cells treated with 0.4mg/ml HS.

4.3.4 Assessing lipid peroxidation using TBARS assay

Thiobarbituric reacting substance assay (TBARS) was used to determine levels of lipid peroxidation in treated and untreated cell as shown in Figure 4.14. In MCF12A cells, no significant difference in levels of lipid peroxidation was noted with 0.2% (IC_{50}) hydrogen peroxide (0.62±0.04nmole/mg, p>0.05), 0.2mg/ml HS extract (0.49±0.05nmole/mg, p>0.05), 0.4mg/ml HS extract (0.37±0.03nmole/mg, p>0.05) and pretreatment with HS extract 0.2mg/ml and then treated with hydrogen peroxide 0.2% (0.53±0.017nmole/mg, p>0.05) compared to untreated MCF12A cells (0.5±0.057nmole/mg). In MCF7 cells, a significant higher levels of lipid peroxidation were indicated upon treatment with (IC_{50}) 0.075% hydrogen peroxide (2.03±0.08nmole/mg, p<0.001), 0.4mg/ml HS (1.82±0.04nmole/mg, p<0.001) and pretreatment with HS extract 0.2mg/ml and then treated with hydrogen peroxide 0.075% (2.2±0.10nmole/mg, p<0.001) when compared to untreated MCF7 cells (1.42±0.014nmole/mg). However, no significant difference was seen at 0.2mg/ml (1.49±0.052nmole/mg, p>0.05) when compared to untreated MCF7 cells (1.42±0.014nmole/mg). Higher levels of lipid peroxidation were observed in MCF7 cells in comparison to MCF12A respectively on untreated cells (1.42±0.014nmole/mg versus 0.5±0.057nmole/mg, p<0.001), hydrogen peroxide (2.03±0.08nmole/mg versus

0.62±0.04nmole/mg, p<0.001), pretreatment with HS extract 0.2mg/ml plus hydrogen peroxide (2.2±0.10nmole/mg versus 0.53±0.017nmole/mg, p<0.001), 0.2mg/ml (1.49±0.052nmole/mg versus 0.49±0.05nmole/mg, p<0.001) and 0.4mg/ml (1.82±0.04nmole/mg versus 0.37±0.03nmole/mg /mg, p<0.001).

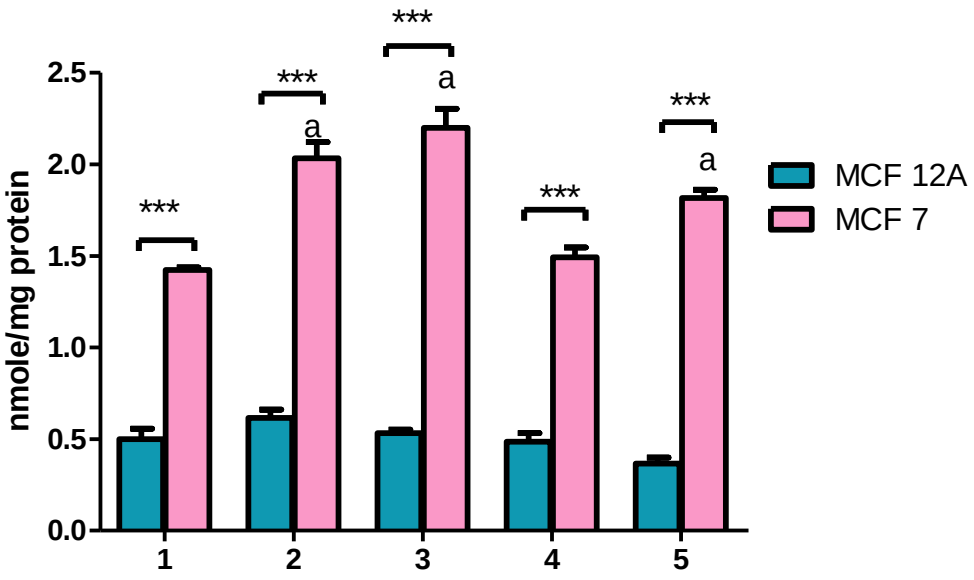

Figure 4.14: Lipid peroxidation levels in cells treated with HS water extracts for 24hours. Values are mean values ± SEM in triplicates. a indicates (p<0.001) compared to untreated cells. *** indicates (p<0.001) when comparing MCF7 cells to MCF12A cells. ***Experimental groups-*** 1: untreated cells (control group), 2: cells treated with 0.2% hydrogen peroxide, 3: cells pretreated with HS extract 0.2mg/ml and then treated with hydrogen peroxide $IC_{50,}$ 4: cells treated with 0.2mg/ml HS and 5: cells treated with 0.4mg/ml HS.

4.3.5 Evaluating the ROS generation

Using CM-H_2DCFDA staining, intracellular ROS was determined in HS treated MCF7 and MCF12A cells. Table 4.6 shows a time response of ROS levels after treatment periods of 1hour, 3hours, 6hours, 9hours, 12hours, 18hours and 24hours with HS water extract on both cell lines. On MCF7 cells a time dependent increase in ROS levels was observed with with significant difference noted at 1hour (76±0.15%, p<0.001), 3hours (67.5±1.5%, p<0.001), 6hours (61±0.5%, p<0.001), 9hours (63.5±0.5%, p<0.001), 12hours (76±0.1%, p<0.001), and 24hours (59.5±0.5%, p<0.01) compared to untreated MCF7 cells (39.5±3.5%). No significant difference was noted at 18hours (45±3%, p>0.05) compared to untreated MCF7 cells (39.5±3.5%). For treated MCF12A, no significant difference was noted at times 1hour (7.35±0.15%, p>0.05), 3hours (6.15±0.05%, p>0.05), 6hours (8.25±1.75%, p>0.05), 9hours (8.6±1.8%, p>0.05), 12hours (3.75±0.15%, p>0.05), 18hours (3.1±0.3%, p>0.05) and 24hours (3.15±0.15%, p>0.05) compared to untreated MCF12A cells (8.45±0.45%). In

addition, when comparing MCF12A and MCF7 cells treated with HS water, significant higher ROS levels were noted in MCF7 cells compared to MCF12A cells at all times ($p<0.001$).

Table 4.6: ROS levels in cells treated with 0.2mg/ml HS water extracts in a time response experiment from 1hour to 24hours

Treatment time (hours)	MCF12A positive for ROSA (%)	MCF7 positive for ROSA (%)
0	8.45±0.45	39.5±3.5
1	7.35±0.15	$76.0\pm1.0^{a***}$
3	6.15±0.05	$67.5\pm1.5^{a***}$
6	8.25±1.75	$61.0\pm5.0^{a***}$
9	8.60±1.80	$63.5\pm0.5^{a***}$
12	3.75±0.15	$76.0\pm1.0^{a***}$
18	3.10±0.30	$45.0\pm3.0^{***}$
24	3.15±0.15	$59.5\pm0.5^{b***}$

Results are mean values ± SEM in triplicates. a represents ($p<0.001$), b indicates ($p<0.01$) compared to untreated MCF7 cells. *** indicates ($p<0.001$) when comparing MCF7 cells to MCF12A cells.

ROS levels were determined in cells treated with HS water extract at a dose dependent manner. Figure 4.15 demonstrated treatment with HS water extract on MCF7 cells significantly increased intracellular ROS at concentration 0.2mg/ml (60±0.5%, $p<0.01$), 0.3mg/ml (73.50±1.5% $p< 0.001$) and 0.4mg/ml (74.5±2.5% $p<0.001$) in comparison to untreated MCF7 cells (42.5±2.5%). In addition, the positive control 0.1% hydrogen peroxide increased intracellular ROS (62.50±0.5%, $p<0.001$) in comparison to untreated MCF7 cells (42.5±2.5%). However, no significant difference was observed on MCF7 cells treated at a concentration 0.1mg/ml (53±1.0% $p>0.05$) compared to untreated MCF7 cells (42.5±2.5%). In MCF12A cells no significant difference was observed on cells treated at concentrations of 0.1mg/ml (4.1±0.3% $p>0.05$), 0.2mg/ml (4.1±0.8% $p>0.05$) 0.3mg/ml (3.75±0.35% $p>0.05$) and 0.4mg/ml (4.2±0.6% $p> 0.05$) in comparison to untreated MCF12A cells (5.65±1.55%). In addition, the positive control 0.3% hydrogen peroxide increased intracellular ROS (34.6±1.4%, $p<0.001$) in comparison to untreated MCF12A cells (5.65±1.55%). In addition, when comparing MCF12A and MCF7 cells treated with HS water extract, significantly higher ROS levels were noted in MCF7 cells compared to MCF12A cells at all concentrations ($p<0.001$). Figure 4.16 shows examples of flow cytometry data in the form of histograms.

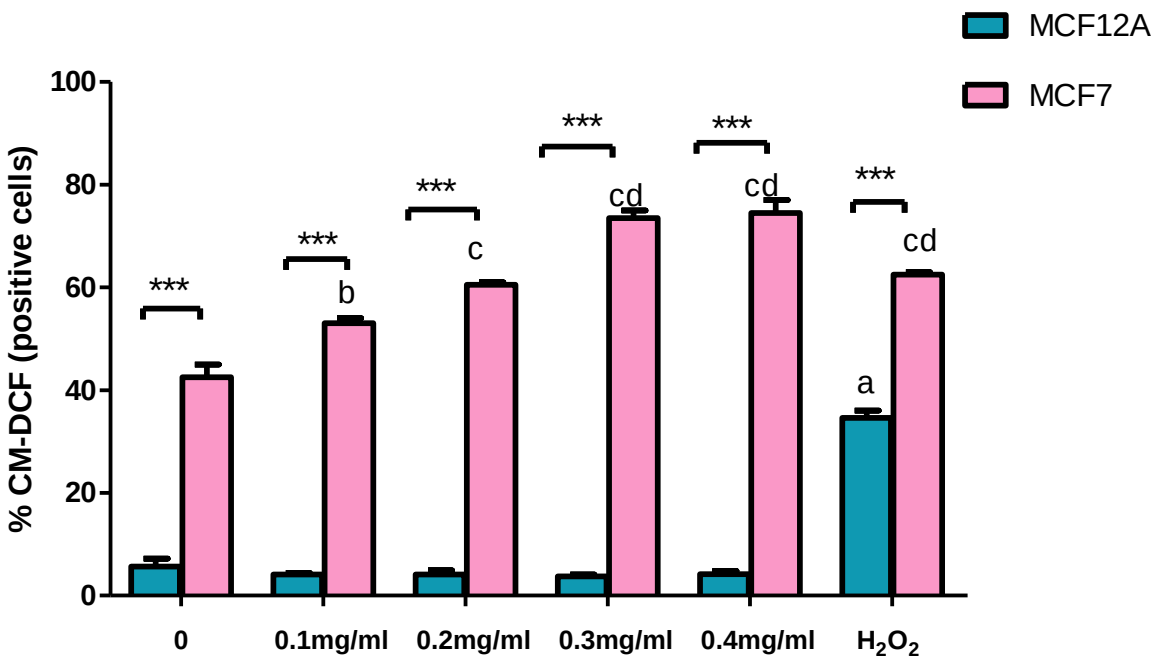

Figure 4.15: ROS levels in cells treated with HS water extracts in a dose response experiment with concentrations from 0.1mg/ml to 0.4mg/ml. Results are mean values ± SEM in triplicates. With a representing ($p<0.001$) compared to untreated MCF12A cells, b indicating ($p<0.01$) and c indicating ($p<0.001$) compared to untreated MCF7 cells. d represent ($p<0.001$) compared to 0.1mg/ml and *** indicates ($p<0.001$) when comparing MCF7 cells to MCF12A cells.

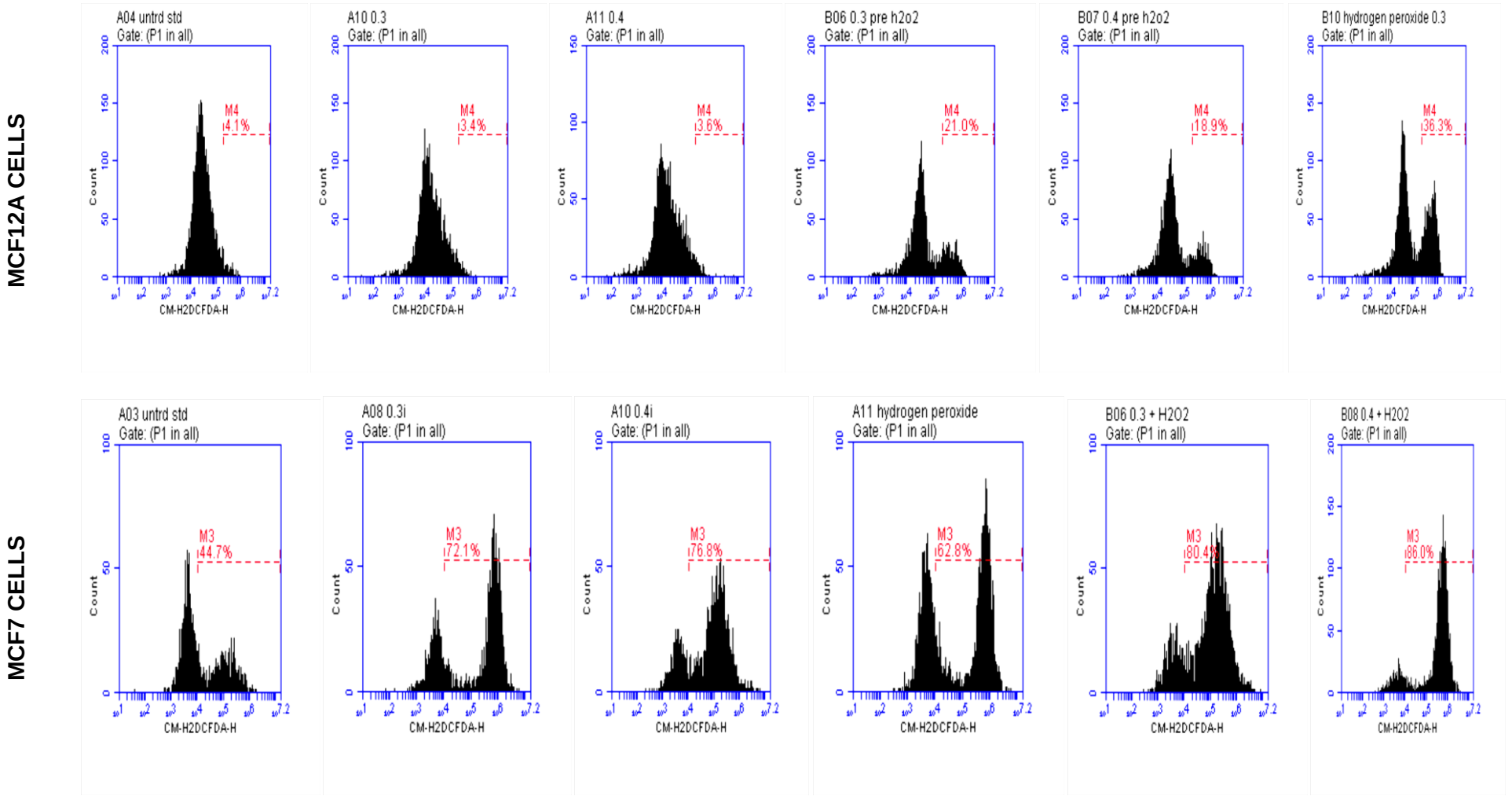

Figure 4.16 Histograms represent cells positive for ROSA harvested from untreated cells, cells treated with HS water and methanolic extract and a positive control hydrogen peroxide and stained with ROSA dye. The region marker represents the % of cells positive for ROSA while CM-H_2DCFDA-H represents fluorescent intensity (ROSA).

The protective effect of HS water extract on hydrogen peroxide induced OS on both cell lines was determined using CM-H_2DCFDA staining. Figure 4.17 demonstrated that treatment with HS water extract and pre-treatment with HS water extract and then treatment with hydrogen peroxide respectively on MCF7 cells significantly increased intracellular ROS at concentration 0.1mg/ml (59.5±1.5% for HS and then treated with hydrogen peroxide, $p<0.01$), 0.2mg/ml (60.5±0.5% for HS water extract, $p<0.01$; 72±3% for HS and then treated with hydrogen peroxide, $p<0.001$), 0.3mg/ml (71.50±3.5% for HS water extract; 80.5±0.5% for HS and then treated with hydrogen peroxide, $p< 0.001$) and 0.4mg/ml (74.50±2.5% for HS water extract; 83±3% for HS plus hydrogen peroxide, $p< 0.001$) in comparison to untreated MCF7 cells (42.5±2.5%). In addition, the positive control 0.1% hydrogen peroxide increased intracellular ROS (62.50±0.5%, $p<0.001$) in comparison to untreated MCF7 cells (42.5±2.5%). However, no significant difference was observed on MCF7 cells treated at concentration 0.1mg/ml (73.50±1.5% for HS water extract, $p>0.05$) compared to untreated MCF7 cells (42.5±2.5%). Furthermore, there was an increase in ROS levels in MCF7 cells treated with HS and hydrogen peroxide versus cells treated with HS water extract with a significant difference at 0.2mg/ml (60.5±0.5% for HS water extract versus 72±3% for HS plus hydrogen peroxide, $p<0.01$) and 0.4mg/ml (74.50±2.5% for HS water extract versus 83±3% for HS plus hydrogen peroxide $p<0.05$). This implies that HS plant increase intracellular ROS levels in MCF7 cells and does not offer protection against hydrogen peroxide induced ROS but instead aggravates ROS levels.

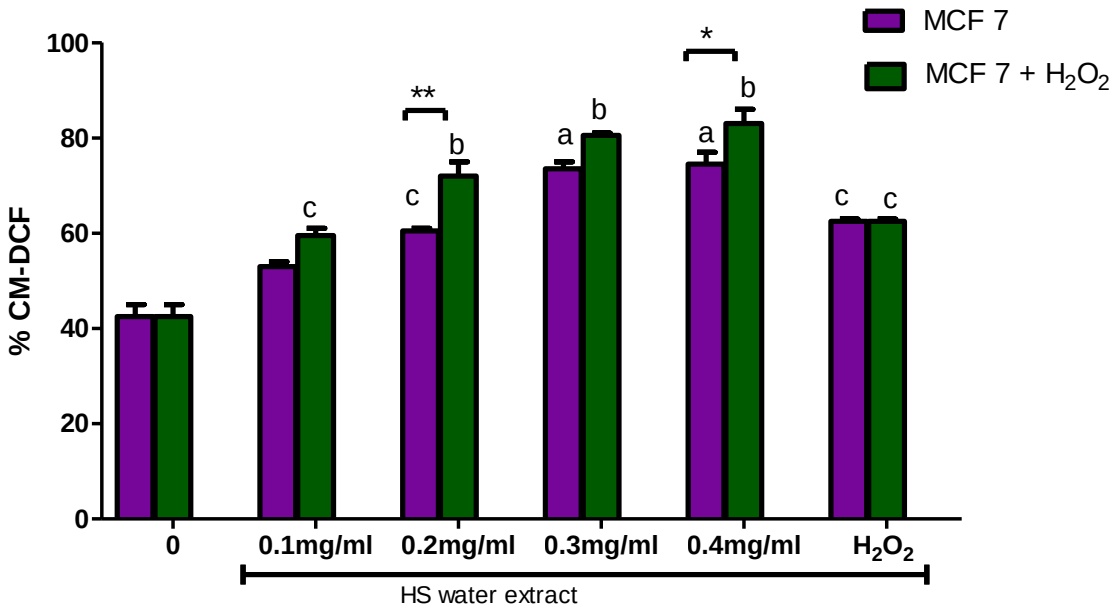

Figure 4.17: The effect on ROS levels in hydrogen peroxide induced OS on MCF7 cells treated with HS water extracts at a concentration range of 0.1mg/ml to 0.4mg/ml for 18 hours and pre-treatment with plant and then treatment with hydrogen peroxide 0.1% for 30minutes. Results are expressed as mean values ± SEM in triplicates. a and b represent ($p<0.001$), c indicates ($p<0.01$) compared to untreated MCF7 cells. * represents ($p<0.05$) and ** represents ($p<0.01$).

Figure 4.18 indicates that in MCF12A cells no significant difference in ROS levels was observed on cells treated at concentration 0.1mg/ml (4.1±0.3% p>0.05), 0.2mg/ml (4.1±0.8% p>0.05) 0.3mg/ml (3.75±0.35% p>0.05) and 0.4mg/ml (4.2±0.6% p> 0.05) in comparison to untreated MCF12A cells (5.65±1.55%). However, a significant increase in ROS was noted on MCF12A pre-treated with HS and then treated with hydrogen peroxide at concentration 0.1mg/ml (25.5±1.5%, p<0.001), 0.2mg/ml (21.5±1.5%, p<0.001), 0.3mg/ml (19.75±1.25%, p<0.001) and 0.4mg/ml (16.0±2% p<0.01) relative to untreated MCF12A cells (5.65±1.55%). Furthermore, they was an increase in ROS levels in MCF12A cells treated with HS and then treated with hydrogen peroxide versus with HS water extract with a significant difference at 0.1mg/ml (4.1±0.3% for HS water extract versus 25.5±1.5% for HS and then treated with hydrogen peroxide, p<0.001), 0.2mg/ml (4.1±0.8% for HS water extract versus 21.5±1.5%for HS and then treated with hydrogen peroxide, p<0.001), 0.3mg/ml (3.75±0.35% for HS water extract versus 19.75±1.25% for HS plus hydrogen peroxide, p<0.001) and 0.4mg/ml (4.2±0.6% for HS water extract versus 16.0±2% for HS plus hydrogen peroxide p<0.001). A decline in ROS levels was observed in MCF12A cells treated with HS plus hydrogen peroxide 0.3% at 0.2 mg/ml, 0.3 mg/ml, 0.4mg/ml (p<0.001) and 0.1mg/ml (p<0.05) compared to hydrogen peroxide 0.3% (34.6±1.4%). These findings imply that HS extract protects MCF12A cells from hydrogen peroxide induced ROS.

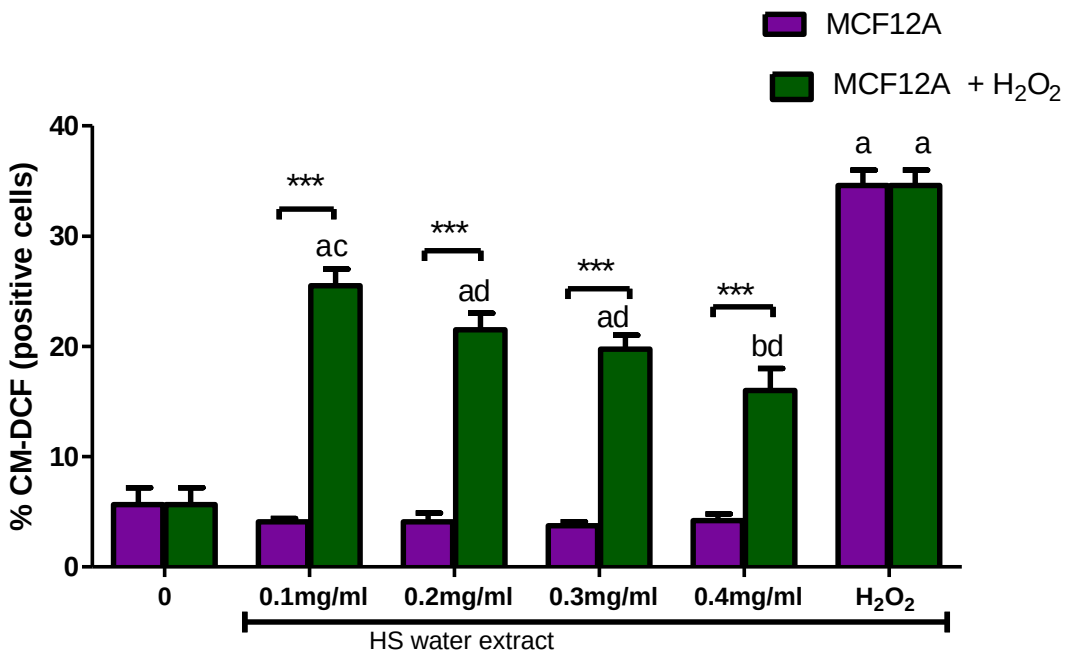

Figure 4.18: **The effect on ROS levels in hydrogen peroxide induced OS on MCF12A cells treated with HS water extracts** at a concentration range of 0.1mg/ml to 0.4mg/ml and pre-treatment with HS for 18hours then hydrogen peroxide 0.3% for 30minutes. Results are expressed as mean values ± SEM in triplicates. a represents significant difference (p<0.001) and b (p<0.01) compared to untreated MCF12A cells. c and d represent significant difference (p<0.05) and (p<0.001) respectively compared to MCF12A treated with hydrogen peroxide. *** indicates (p<0.001).

CHAPTER FIVE

DISCUSSION

Breast cancer is regarded as the most common neoplastic disease in females and accounts for the high mortality rates in women (Ferlay *et al*., 2010). Neoplastic cells are characterised by uncontrolled proliferation and evasion of apoptosis. Investigating the induction of apoptosis will assist in obtaining clues about effective anticancer therapy as many chemotherapeutic agents are reported to confer their anti tumour effects by inducing apoptosis. Therefore, it is crucial to search for and identify anticancer compounds which will induce apoptosis in cancer cells as a way of treating cancer (Wong, 2011). In addition, a link between oxidative stress and cancer has been established (Hileman *et al*., 2004) with scientific evidence revealing that most cancer cells trigger increased ROS production (Trachootham *et al*., 2006; Pervaiz *et al.,* 2004). This suggests that enhancing the cellular antioxidant defence system could be a promising approach to prevent and slow down the progression of oxidative stress-related diseases such cancer. Nowadays, breast cancer treatment includes the use of cytotoxic agents that include doxorubicin and cisplatin (Colleoni *et al.,* 2000). These modalities have been implicated to cause multidrug resistance, high toxicity, high cost and the induction of several side effects such as alopecia (hair loss) and vomiting (Bergkvist and Wengstrom, 2006). In order to overcome these drawbacks, it is crucial to develop alternative therapies that are natural, non-toxic, easily assessable and affordable (Roja and Rao, 2000). Medicinal plants have been considered as suitable candidates in the development of alternative and complementary therapeutic strategies against cancer and other oxidative stress-related conditions.

Hibiscus Sabdariffa (HS) is a medicinal plant known for its high content in bioactive compounds such as polyphenolic acids, anthocyanins, flavonoids, amino acids and minerals (Patel, 2014). HS is used traditionally for a variety of ailments such as hypertension, liver disease, nerve disorders, alleviating constipation and poor circulation (Wang *et al.,* 2000). Research shows that HS is a potential medicinal plant for the development of cancer novel therapies (Liu *et al*., 2010) and exhibits excellent antioxidant activities (Ali *et al.,* 2005). However, the effects of HS on breast cancer in relation to oxidative stress and apoptosis have not been assessed. Therefore, in this study the potential antioxidant and anticancer effects of HS water and methanolic extracts on cancerous and non-cancerous breast cell lines were investigated.

5.1 Antioxidant characterization of Hibiscus Sabdariffa (HS) extracts

Naturally, plants are rich in antioxidants that enable them to survive in various environmental conditions by counteracting free radicals and enhancing internal growth and metabolism (Al-Hashmini, 2012). Numerous studies have been done to determine antioxidant content and capacity on HS extracts. The focus of this study was to quantify total polyphenols and anthocyanins content on HS extracts, as well as the antioxidant capacity, measured by the FRAP and ORAC assays on HS extracts. It has been demonstrated that the choice of solvent used and the extraction method influence the yield of components being investigated (Mohammedi and Atik, 2001). Polyphenolic compounds have been shown to be highly bioactive and commonly attributed to their antioxidant activities (Ramos, 2007; Mohd-Esa *et al.,* 2010). In this study both methanolic and water extracts were used inorder to explore solvents with different polarities. Findings of the present study, as shown in section 4.1.1, stipulates that HS methanolic extract demonstrated a higher yield of polyphenols than the HS water extract. In addition, the polyphenol content of all samples increased with increasing concentration of the extracts. Therefore, methanolic is a suitable solvent for polyphenol extraction possibly due to its amphiphilic state (extract both polar and non polar components) and ability to inhibit the action of the enzyme polyphenol oxidase that causes oxidation of polyphenols (Yao *et al*., 2004). This was confirmed by a previous study that also indicated that alcoholic extracts of HS contained higher polyphenols content compared to water extract, suggesting water was found to be less effective for the extraction of polyphenols (Al-Hashmini, 2012). Anokwuru and co-workers (2011) also demonstrated similar results with methanolic extraction showing a higher yield of polyphenols than extraction in water. However, these findings were in disagreement with Sindi and colleagues (2014), who indicated that water was the best solvent for extraction of total polyphenols extracted from HS. The discrepancies are possible due to difference in extraction method, solvent polarities and harvesting procedure (Ali *et al*., 2005).

High antioxidant activity of anthocyanins is a significant feature, which is paramount in the prevention of chronic diseases such as cancer and diabetes (Konczak and Zhang, 2004). Studies have established that anthocyanins in plants have higher antioxidant activity than exogenous antioxidants such as Vitamin C and E (Bagch *et al*., 1998). Anthocyanins are polar molecules that can be extracted from plants using various solvents. The findings of the present study, as shown in chapter 4, indicate that the water extract demonstrated a higher yield of anthocyanins than methanolic extract and that the anthocyanins content of all samples increased with increasing concentration of the extracts. Similar results were obtained that indicated that water was the best solvent for extraction of anthocyanins from HS when compared to alcohols (Sindi *et al*., 2014). These results contradict with other

studies that show effective anthocyanins extraction using an alcohol compared to water. In black currants, anthocyanins extraction using methanolic was more effective than water (Metivier *et al*., 1980; Kapasakalidis *et al*., 2006). These differences are possible due to different extraction time and temperature and different extract polarities. Different extracts have different polarities and a solvent with high polarity results in an increase in extractable compounds and extraction yield (Mohd-Esa *et al*., 2010). Therefore, high anthocyanins extraction using water could be attributed to its hydrophilic nature.

Numerous methods have been employed to determine total antioxidant capacity such as Ferric reducing antioxidant power (FRAP), Oxygen radical absorbance capacity (ORAC), 2,2-diphenyl-1-picrylhydrazyl (DPPH) and trolox equivalent antioxidant capacity (TEAC). The antioxidant capacity assays that were used in the current study were FRAP and ORAC, however they differ in their principles. The FRAP assay measures antioxidant capacity based on the reduction of ferric (III) ions to ferrous (II) ions (Benzie and Strain, 1996). The ORAC assay is a fluorescent technique based on the reaction between an antioxidant rich sample, the fluorescent probe which is the oxidizable substrate and AAPH a source of peroxyl radicals. The antioxidant capacity is determined by calculating the area under the fluorescent decay curve (Rautenbach *et al.,* 2010). The antioxidant capacity of a bioactive compound against free radicals does not certainly match its reducing ability. Frequently, the antioxidant capacity has been implicated to be responsible for suggested protective properties against human diseases (Wong *et al*., 2006). According to Duh and Yen (1997), HS extracts have the capacity to act as electron donors and can react with free radicals to form stable products, therefore, disconnecting radical chain reactions. In addition, the HS extract was found to be high in natural antioxidant such as ascorbic acid (Falade *et al*., 2006).

The findings of the present study, as described in section 4.1.2, indicate that the antioxidant capacity levels for both ORAC and FRAP assay displayed a dose-dependent relationship with increasing concentration for both extracts. When using ORAC assay, methanolic extract demonstrated a higher total antioxidant capacity than water extract possible due to the elevated polyphenol content at high concentration. Previous work indicated a high positive correlation between antioxidant capacity and polyphenol content of HS extracts (Ramakrishna *et al*., 2008; Sindi *et al*., 2014). The differences on the antioxidant capacity of the extracts were due to the different types of phenol compounds extracted by different solvents. Using the FRAP assay, no difference on the reducing power was noted between the two extracts. Similar results were obtained that indicated that no significant results were obtained for FRAP between and methanolic extracts (Sindi *et al*., 2014). These findings contradict Koffi and colleagues (2011), who indicated that alcoholic extracts of HS had higher reducing ability. A similar study indicated different findings with the alcoholic extract of HS

exhibiting high reducing power (Al-Hashimi, 2012). These striking differences could possibly be due to variation in the plants natural growing environments, storage before analysis, processing at analysis and the methodology used that could potentially affect the final structures of polyphenols and other bioactive compounds that contribute to the antioxidant activity.

5.2 Apoptotic and cytotoxic study on cells treated with HS extracts

Cell death is denoted by morphological changes that includes dense chromatin condensation, cell shrinkage and membrane blebbing (Zhao *et al*., 2001). Cell morphological changes were investigated using a light microscope. In this study, as shown in chapter 4, untreated MCF7 breast cancer cells were polygonal and adhered to culture dishes. However, treatment of MCF7 cells with concentrations of HS water and methanolic extracts equivalent to the IC_{50} values resulted in morphological changes such as cell detachment from the plate, inhibition of cell growth and cell shrinkage which are shown to be the result of apoptosis. Doxorubicin, an anticancer agent was used as a positive control and revealed apoptotic features on MCF7 cells. Previous results indicated that doxorubicin induced apoptosis in HL-60 human leukaemia cells (Mizutani *et al*., 2005) and MCF7 breast cells (Syamsuddin *et al*., 2010). On the contrary, non-cancerous MCF12A untreated cells appeared spindle shaped and adhered to culture dishes. Treatment with HS extracts showed morphological changes similar apoptosis. Similar findings were obtained by Lin and colleagues (2007) with HS water extract showing morphological changes characteristic of apoptosis in gastric carcinoma cells. Although HS caused significant death in MCF12A cells it was less cytotoxic to non-cancerous MCF12A compared to MCF7 cells.

Cell viability and cytotoxicity assays measure inhibition of cell growth or cell death and have been widely used to identify potential anticancer drugs (Liu *et al*., 2008). Medicinal plants have anticancer activity and over 50% of drugs in clinical trials are isolated from plants (Gragg and Newman, 2000). Scientific research on the anti-proliferative effects of HS extracts on MCF7 and MCF12A breast cells are lacking. WST-1 proliferation assay was used to assess variations on the inhibitory effects of HS extracts on MCF7 cancer and MCF12A non cancerous breast cells. It was also used to determine the IC_{50}, which is the concentration of the plant extract that inhibits 50% of the cell growth. Untreated cells were included as a negative control and doxorubicin or DMSO as a positive control.

In this study, as described in section 4.2.2, both HS water and methanolic extract significantly induced growth inhibition in MCF7 and MCF12A cells with increasing concentration of the extracts. However, HS was more cytotoxic on the tumorigenic MCF7 cell line compared to the non cancerous MCF12A cells. This suggests that HS plant extracts

induced different levels of cytotoxicity on the two cell lines. The methanolic extract was found to be more potent than water extract possibly due to its high polyphenol content and antioxidant activity. Evidence reveals that antioxidant activity of certain medicinal plants correlates with anticancer effects on MCF7 cancer cells (Li *et al*., 2007; Abrahim *et al*., 2012). Similar findings indicated that roselle juice made from HS exhibited antiproliferative effects on various cell lines such as ovarian (Caov-3), breast (MCF-7) and cervical (Hela) cancer cells (Akim *et al.,* 2011). A phenolic compound, protocatechuic acid (PCA) that was isolated from HS flower inhibited cell growth in human promyelocytic leukaemia (HL-60) cells (Tseng *et al*., 1997). Khaghani and co-workers (2011) indicated that HS aqueous extract exhibited cytotoxic effects on MCF7 breast cancer cells.

Both HS extracts had a low IC_{50} value on MCF7 cancerous cells (0.2mg/ml for methanolic extract and 0.4mg/ml for water extract) compared to non cancerous MCF12A cells (0.4mg/ml for methanolic extract and 0.5mg/ml for water extract). This suggests that HS was more potent on MCF7 cancer cells compared to MCF12A cells. The guidelines of the American National Cancer Institute (NCI) stipulate that the IC50 for a crude extract should be less than 30µg/ml after 72hours exposure (Stuffness and Pezzuto, 1990). IC_{50} concentrations for this study were higher possibly due to the treatment exposure of 24hours compared to 72hours for NCI. Higher IC_{50} concentrations could also raise concerns about its safety and *in vivo* use. However, *in vivo* studies using rats show that consumption of HS was not associated with any toxic effects (Gaya *et al*., 2009; Ndu *et al.,* 2011). A recent study indicated that HS extracts have a high lethal dose and low acute toxicity suggesting its safety when consumed at moderate levels (Hopkins *et al*., 2013). In addition, HS extracts are safe and have a long standing traditional use in the food industry to make fruit colored drinks and in medicine (Fasoyiro *et al*., 2005; Da-Cosat-Rocha *et al*., 2014).

Resistance to apoptosis is a known hallmark for cancer (Hanahan and Weinberg, 2000). Considerable attention has focused on targeting and manipulating apoptosis as a strategy for the development of anticancer drugs. Cells undergoing apoptosis are characterized by exposing phosphatidyl-serine (PS) on their surfaces. PS externalization is observed with dyes such as APOPercerntage. In this study, as shown in section 4.2.3, the ApoPercerntage assay confirmed cell death in MCF7 cells treated with HS plant extracts was due to apoptosis. Both water and methanolic extracts demonstrated a dose and time dependent increase in apoptotic MCF7 cancer cells. However, HS treated MCF12A non-cancerous cell line exhibited low number of apoptotic cells in both dose and time dependent experiments. Significantly high apoptotic MCF7 cells ($p<0.001$) were noted in comparison to MCF12A cells. This suggests HS extracts showed selective induction of apoptosis towards the MCF7 cancer cell line. This biochemical variance could be due to genetic differences between the

different cell lines. A similar study indicated that *Hibiscus* anthocyanins induced apoptosis in a dose dependent manner (Chang *et al*., 2005). A phenolic compound, protocatechuic acid (PCA) that was isolated from HS flower induced apoptosis in human promyelocytic leukaemia (HL-60) cells (Tseng *et al*., 2000). Another previous study indicated HS methanolic extract induced apoptosis in human gastric carcinoma cells (Lin *et al*., 2005). Numerous previous studies have shown that other natural bioactive compounds from plants induce apoptosis in breast cancer cells (Deng *et al*., 2010; Luo *et al*., 2010; Lee *et al*., 2012; Na *et al*., 2012).

Other markers or assays that confirm apoptosis include mitochondrial depolarization, caspase-3 cleavage and DNA fragmentation. Studies indicate MCF7 cells are characterized by caspase-3 gene mutation which results in the expression of a non functional caspase protein (Kurokawa *et al*., 1999), henceforth in this study caspase-3 assay was excluded. This study focused on the bioactivity of HS extract on mitochondrial membrane potential using Tetra Methyl Rhodamine Ethyl ester (TMRE) dye. Findings, as described in section 4.2.4, indicate TMRE assay using a flow cytometer confirmed mitochondrial depolarization in MCF7 cancer cells treated with HS plant extracts. Both water and methanolic extracts demonstrated a dose and time dependent increase in mitochondrial depolarization on MCF7 cancer cells. However, treated non-cancerous MCF12A cell line exhibited low number of cells with mitochondrial depolarization in both dose and time dependent experiments. Significantly high mitochondrial depolarization in MCF7 cells ($p<0.001$) was noted in comparison to MCF12A cells. This suggests HS extracts showed selective induction of mitochondrial depolarization towards the MCF7 cancer cell line. This confirms that mitochondrial depolarization plays a role in the HS induced apoptosis of MCF7 cells. Studies indicated mitochondrial dysfunction which includes mitochondrial depolarization and release of pro apoptotic factors from the mitochondria are associated with apoptosis via the intrinsic pathway (Hou *et al*., 2005). Similar studies have indicated the mechanism of apoptosis is via the mitochondrial pathway in various cancer cells (Cao *et al.*, 2010). Previous studies have shown that bioactive compounds such as diallyl trisulphide (Na *et al*., 2012), arctigenin (Hsieh *et al*., 2014) and dryofrag (Zhang *et al*., 2012) induce apoptosis via the mitochondrial pathway in breast cancer cells. Mitochondrial depolarization could be due to the HS extract increasing ROS generation from the mitochondria. This ROS mediated damage to the mitochondria causes malfunction of the respiratory chain and further increases ROS generation which stimulates mutations. The generated ROS damages the mitochondria and results in the loss of mitochondrial membrane potential or mitochondrial depolarization (Crompton *et al*., 2002; Cao *et al*., 2010). Loss of mitochondrial potential triggers the release of cytochrome c which activates the apoptotic cascade leading to apoptosis (Boya *et al*., 2003; Pelicano *et al*., 2004). Therefore, in this study one might speculate that apoptosis was due to mitochondrial

depolarization and increased intracellular ROS generation. These results give us an insight into new possible molecular mechanism of HS extracts and its potential value as an anticancer agent. This study shows that HS extracts are promising chemotherapeutic agents that induces apoptosis in cancer cells while inducing minimal cytotoxic effects on normal cells.

5.3 Antioxidant status of cells treated with HS extracts.

Oxidative stress is a characterized by an altered intracellular redox homeostasis due to high levels of free radicals that exceed antioxidants (Ziech *et al*., 2011; Vera-Ramirez *et al*., 2011). It plays a role in the pathogenesis of several diseases including cancer (Schiff *et al.,* 2000). It has been proposed that carcinogenesis is also indicated by decreased expression or low activity of antioxidant enzymes (Sharma *et al*., 2009; Khan *et al*., 2010). Numerous studies have indicated HS is naturally rich in anthocyanins and polyphenols, hence, possessing antioxidant potential (Tseng *et al*., 1997; Ajiboye *et al*. 2011; Frank *et al*., 2012). Therefore, HS extract is expected to act as an antioxidant. However, the anti-oxidative effects of HS on the different cancers are controversial. In this study, levels of antioxidant enzyme status were elucidated by measuring activities of SOD and catalase, glutathione and TBARS contents in HS treated MCF7 and MCF12A cells. In section of the study, water extract only was used in the determining antioxidant status as it is of greatest interest when testing bioactivity of plant extracts due to its traditional preparation procedure and its great significance to public health (McKay *et al*., 2010).

It should be noted that this was the first study on the effects of HS treatment on MCF7 and MCF12A cells with reference to antioxidant status. However, similar studies have been done using different plants on various cell lines. In the present study, SOD, catalase and GSH levels were significantly higher in non-cancerous MCF12A cells compared to cancerous MCF7 cells. This is possibly due to the fact that cancer cells are under oxidative stress and antioxidants are constantly detoxifying or scavenging free radicals resulting in low intracellular antioxidant enzyme levels. Similar results were obtained, with higher antioxidant enzymes SOD and catalase in normal cells versus cancer cells (Khan, 2013). SOD is the first enzyme in ROS detoxification and is converted into superoxide radical and hydrogen peroxide. Catalase plays a primary role in scavenging the hydrogen peroxide produced by SOD. In addition, GSH is the principal intracellular antioxidant and is a good indicator of redox status in cells (Khan, 2013). Hydrogen peroxide has been considerably used as an inducer of oxidative stress in numerous *in vitro* models (Hwang *et al*., 2008). Studies postulate an increase in cell proliferation at low concentrations of exogenous hydrogen peroxide but at high concentration it induces oxidative stress and cytotoxic effects on cells (Kim *et al*., 2001).

In non-cancerous MCF12A cells, as shown in section 4.3, no significant change was observed on SOD, catalase and GSH upon treatment with a low concentration of HS alone and pretreated with HS then treated with hydrogen peroxide. However, a significant increase in GSH and catalase activity was observed upon treatment with hydrogen peroxide only as these enzymes are the main hydrogen peroxide scavengers. In addition, a significant increase in SOD and catalase activity was also observed at high concentration of HS treatment. This could be attributed to the natural antioxidants in HS plant that increase endogenous antioxidant activities and enhance antioxidant defence mechanism of the cell by protecting against hydrogen peroxide induced oxidative stress. In tumourigenic MCF7 cells, as shown in chapter 4, a significant decrease in SOD, GSH and catalase activity was observed upon all treatments except treatment with low concentration of HS only. This postulates that low doses of HS slightly triggers the activity of antioxidant enzymes due to MCF7 tumour cells responding to increasing ROS imposed by HS extract. However, treatment with high concentration of HS further increases intracellular ROS resulting in the MCF7 cells being overwhelmed leading to exhaustion of antioxidant enzymes. This stipulates that HS extract is cell selective and acts as a pro-oxidant by increasing ROS intracellular levels in MCF7 cells leading to the depletion of antioxidant enzymes. Previous reports have indicated similar findings that green tea polyphenol at high concentrations acts as a pro-oxidants by increasing ROS levels in oral carcinoma cell lines (Yamamoto *et al*., 2003). Treatment with hydrogen peroxide only and pretreatment with HS then treatment with hydrogen peroxide significantly decreases SOD, GSH and catalase activity in MCF7 cancer cells. This suggests HS does not offer any antioxidant activity in MCF7 cells upon hydrogen peroxide induced oxidative stress. Similar results were obtained with a decrease in SOD and catalase activity on hepatic cancer cell line (HepG2) upon treatment with ginger (Hanif *et al*., 2005). However, these findings contradict other studies that indicate an increase in catalase and SOD activity upon treatment with resveratrol in breast MCF7 cancer cells and prostate PC-3 cancer cells (Khan *et al*., 2013) and treatment with *Piper betle* on breast MCF7 cancer cells (Abrahim *et al.,* 2012). These discrepancies could be due to different bioactive compounds found in various plants.

One of the main indicators of oxidative stress is lipid peroxidation, which is a free radical mediated oxidative damage on lipids. By-products of lipid peroxidation include malondialdehydes (MDA) which are mutagenic, are involved in tumour promotion and have been used as a biomarker for lipid peroxidation (Esterbauer and Cheeseman, 1990; Esme *et al*., 2008; Ayala *et al.,* 2014). A significant increase in MDA is related to an increase in oxidative stress (OS). Under low lipid peroxidation levels due to OS the cell responds by up regulating antioxidant enzymes in an adaptive stress response. However, high lipid peroxidation levels due to high OS causes the cells to be overwhelmed and induce cell death

(Barrera, 2012). Lipid peroxidation has been indicated in different types of cancers such as breast (Tas *et al.,* 2005), lung (Gonenc *et al.,* 2001), cervix (Beevi *et al.,* 2007) and prostate (Ozmen *et al*., 2006).

In this study, as shown in section 4.3.4, lipid peroxidation levels were significantly higher in cancer cells compared to normal cells. This is possibly due to the fact that cancer cells are under oxidative stress and depletion of antioxidant enzymes (Zadi *et al*., 2005). In normal MCF12A cells, as shown in chapter 4, no significant change on was observed on lipid peroxidation levels upon all treatments. This suggests that HS extract protected normal cells against lipid peroxidation induced by hydrogen peroxide due to its antioxidant properties and the ability of the phenolic compounds to bind to lipid peroxides and inhibit lipid peroxidation. In tumourigenic MCF7 cells, a significant increase in lipid peroxidation was observed upon all treatments. The increase in lipid peroxidation could be due to increased intracellular ROS resulting in oxidative damage accompanied by depletion of antioxidant enzymes in MCF7 cells. This suggests that HS plant is cell selective and acts as a pro-oxidant by increasing ROS intracellular levels in MCF7 cells and attacking lipids which can lead to changes in membrane permeability. Previous work in agreement with this study indicated that MCF7 breast cancer cells treated with hydrogen peroxide increased malondialdehyde concentration an indicator for oxidative stress (Chua *et al*., 2009). Similar findings indicated an increase in lipid peroxidation in MCF7 breast cancer cells treated with eugenol (Vidhya and Niranjali Devaraj, 2011). Filomeni and co-workers (2007) indicated an increase in MDA levels on MCF7 cells treated with trans-resveratrol due to a ROS burst. However, these finding contradict a study that indicates ginger does not affect lipid peroxidation levels on the hepatic cancer cell line, HepG2 (Hanif *et al*., 2005). This variation could be due to the use of different cell lines and different bioactive compounds found in various plants.

Evidence indicates that ROS is implicated in the process of carcinogenesis (Kong *et al.,* 2000). Many studies have shown that cancer cells have higher ROS levels than their normal counterpart (Szatrowski and Nathan, 1991). Increased ROS production in tumour cells is due to uncontrolled proliferation and deregulated metabolism resulting in increased requirements for ATP. This high energy demand causes stress on the mitochondrial respiratory chain resulting in increased ROS generation (Pelicano *et al.,* 2004). Increased ROS production is also attributed to the malfunction of the mitochondrial respiratory chain, anticancer agents and oncogenic transformation. In normal cells DNA damage is repaired to remove genetic errors while in tumour cells the repair system is overwhelmed by high ROS mediated damage leading to multiple mutations (Jackson and Loeb, 2001). It is therefore crucial to obtain a balance between ROS and antioxidants in tumour cells to make sure cancer

progression is inhibited while by maintaining apoptosis (Kroemer *et al.,* 1998; Kong *et al.,* 2000).

In this study, as shown in section 4.3.5, dose and time response experiments indicated that ROS levels were significantly higher in cancer cells compared to normal cells. This is possible due to the fact that cancer cells are under oxidative stress and depletion of antioxidant enzymes (Zadi *et al*., 2005). For non-cancerous MCF12A cells, no significant increase in ROS levels was noted at all time points and with increasing concentrations of HS extract compared to untreated MCF12A cells. Similar findings were obtained by Yamamoto and collegues, (2003) which demonstrated that green tea polyphenols failed to induce ROS formation in normal keratinocytes.

Oxidative stress can lead to increased cell proliferation or cell death and this is determined by the type of tissue, concentration of ROS produced and cellular antioxidants (Storz, 2005). Mild ROS stress results in cells adapting by up regulating antioxidant enzymes. Further increase in ROS could exhaust cellular antioxidants and overwhelm the cells due to high ROS leading to apoptosis (Kong *et al*., 2000). Previous studies have indicated anticancer agents simultaneously induce apoptosis and generate ROS (Valko *et al*., 2007). Natural compounds such as curcumin have been found to generate ROS and exhibit pro-oxidant effects (Woo *et al.,* 2003). HS extracts are rich in polyphenols that can behave as pro-oxidants by inducing ROS production (Halliwell, 2012). However, it should be noted that the mechanism in which phytochemicals behave as pro-oxidants is not clearly understood.

In this study, a dramatic ROS burst was observed in MCF7 cells with increasing treatment time and the highest peak was indicated at 1hour and 12hour post treatment and the lowest level at 18hour post treatment. HS plant extract also increased intracellular ROS levels in MCF7 cells with increasing concentration. Moreover, HS plant increased intracellular ROS levels in MCF7 cells but did not offer protection against hydrogen peroxide induced ROS and instead aggravated ROS levels further. HS extract protected MCF12A cells from hydrogen peroxide induced ROS. These findings suggest HS is able to create differential oxidative environment, by acting as a ROS suppressor in normal cells and ROS inducer in cancer cells. MCF7 cancer cells are characterised by increased cell proliferation which leads to stress and increased ATP requirements that strains the mitochondrial electron transport chain. HS extract elevated ROS and did not offer protection from hydrogen peroxide induced ROS in MCF7 cancer cells possibly due to polyphenols in HS that have been found to target the strained mitochondrial electron transport chain and cause mitochondrial dysfunction. Malfunction of the mitochondrial respiratory chain leads to increased ROS formation (Hodnick *et al.,* 1998). However, in non-cancerous MCF12A cells, HS extracts offered

protection from hydrogen peroxide induced ROS possibly by exhibiting antioxidant activity and by preventing mitochondrial damage.

Similar results were obtained that indicated high ROS levels in human leukaemia cells treated with 2-methoxyestradiol (Hileman *et al.,* 2003) and MCF7 cells treated with trans-resveratrol (Filomeni *et al*., 2007). Since high ROS results in apoptosis it is predicted that cancer cells with increased intracellular ROS are sensitive to anti tumour agents that act as ROS inducers. In this study, there was a positive relationship between intracellular ROS and apoptosis in cancer cells suggesting HS causes ROS mediated apoptosis in MCF7 cancer cells. A related study showed that the higher the ROS in chronic lymphocytic leukaemia the more sensitive the cells were to HS (Zhou *et al*., 2003). Studies have reported that polyphenols are structurally unstable and can undergo spontaneous oxidation in the presence of metal ions or cell cultures to form ROS or interfere with antioxidant enzymes in target cells (Halliwell, 2003). An example of this is the polyphenol Epigallocatechin gallate which has been found to produce notable amounts of hydrogen peroxide unser cell culture conditions (Akagawa *et al*., 2003). It should also be noted that the probe may not be able to identify all types of free radicals, since Wardman, 2007 showed that the CM-H_2DCFDA molecular probe has low reactivity for the oxygen radical.

Depletion of antioxidant enzymes renders the cancer cells more susceptible to apoptotic agents (Dai *et al.,* 2010). The products of lipid peroxidation are toxic and have been found to stimulate internucleosomal DNA fragmentation and increased lipid peroxidation which is associated with apoptosis (Briehl and Baker, 1996). These points indicate that intracellular oxidative metabolites influence apoptosis. In this study a link between oxidative stress and apoptosis was indicated by the depleted antioxidant enzymes, increased lipid peroxidation, increased ROS levels, mitochondrial depolarization and induction of apoptosis by HS extracts selectively in MCF7 cancer cells. Therefore, in this study apoptosis on MCF7 cancer cells was due to mitochondrial depolarization and increased intracellular ROS generation. Similar results were obtained on MCF7 cells using various natural bioactive compounds from plants that the mechanism of apoptosis was via ROS generation and mitochondrial dysfunctional pathway (Luo *et al.*, 2010; Na *et al*., 2012; Paul *et al.,* 2012; Shi *et al.,* 2013).

CHAPTER SIX

CONCLUSION

Various medicinal plants exhibit health benefits that are attributed to the synergistic interaction of various phytochemicals that they are comprised of. The biochemical function of medicinal plants in biological systems and their mechanism of actions have gained attention in modern science due to their perception that there are natural and safe. The first part of this study focused on the antioxidant characterization of HS in terms of antioxidant content and capacity. The results provided evidence that phytochemical analysis indicated that both HS extracts were rich in polyphenols and anthocyanins and conferred high antioxidant capacity and reducing power. Furthermore, the differences in antioxidant content between water and HS methanolic extract were attributed to the difference in polarity.

In the second part of the study, water and methanolic extracts of HS were screened for their anti-proliferative activity on cancerous MCF7 and non-cancerous MCF12A cell lines. HS extracts exerted differential cytotoxic effects on MCF7 and MCF12A cells. MCF7 cancer cells were more susceptible to HS extracts than MCF12A cells. Inducers of apoptosis are regarded as suitable agents in anti cancer therapies since cancer cells are characterized by the evasion of apoptosis. Morphological studies and ApoPercentage assay revealed that HS extracts induced apoptosis in MCF7 cancer cells in both dose and time dependent experiments. This renders HS extracts suitable candidates in anticancer drug discoveries. However, in non cancerous MCF12A cell line HS exhibited a low number of apoptotic cells in both dose and time dependent experiments. In addition, HS treated non-cancerous MCF12A cell line exhibited a low number of cells with depolarized mitochondrial in both dose and time dependent experiments. Cancerous MCF7 cells treated with HS extracts exhibited high number of cells with depolarized mitochondrial in both dose and time dependent experiments. This indicates that apoptosis was due to mitochondrial depolarization. The differential induction of apoptosis and mitochondrial depolarization in cancerous MCF7 cells in preference to non-cancerous MCF12A cells makes HS extract a desirable anti cancer agent.

Finally, the effect of HS extracts on the antioxidant status of cancerous MCF7 and non-cancerous MCF12A cells was investigated in the third part of the study. This study indicated that HS water extract altered intracellular redox status of MCF7 breast cancer cells by depleting the antioxidant enzymes (SOD, catalase and GSH) and increasing lipid peroxidation and ROS levels. Treatment with hydrogen peroxide only and pretreatment with HS extract and then treatment with hydrogen peroxide significantly decreased antioxidant

enzymes (SOD, catalase and GSH) and increasing lipid peroxidation and ROS levels. This suggests the HS extract did not offer any antioxidant activity in MCF7 cells upon hydrogen peroxide induced oxidative stress. However, in non-cancerous MCF12A cells a significant increase in SOD and catalase activity and low levels of lipid peroxidation and ROS were observed at high concentration of HS treatment. Pretreatment with HS extract and then treatment with hydrogen peroxide significantly increased levels of GSH and catalase activity in MCF12A as they are the main hydrogen peroxide detoxifiers. This suggests the HS extract offered antioxidant activity in MCF12A cells upon hydrogen peroxide induced oxidative stress. It could be attributed to the natural antioxidants in the HS plant that increased endogenous antioxidant activities and enhance antioxidant defence mechanism of the cell. These findings stipulates that HS water extract was cell selective and acted as a pro-oxidant by increasing ROS intracellular levels in cancerous MCF7 cells leading to the depletion of antioxidant enzymes while in non-cancerous MCF12A cells, HS extract exhibited antioxidant activity.

The results summarize that pathways activated by HS water extract in non-cancerous MCF12A cells versus cancerous MCF7 cells create differential oxidative environments that favour minimal destruction in MCF12A cells and considerable cell destruction in cancerous MCF7 cells. HS extracts initiates cell death by decreasing cell proliferation, antioxidant enzymes, increasing ROS and lipid peroxidation, induction of apoptosis and mitochondrial depolarization in MCF7 cancer cells. These findings demonstrate that apoptosis is induced via ROS generation and the mitochondrial dysfunctional pathway. This study concludes that HS extract selectively conferred pro-oxidant and cytotoxic effects on cancerous MCF7 cells while exhibiting antioxidant effects and minimal cytotoxic effects on non-cancerous MCF12A cells. These results enhance our knowledge on the anticancer function of HS extracts in herbal medicine.

There is great potential to develop HS extracts as chemotherapeutic agents in breast cancer treatment, however, further research need to be done. Future scientific research could include upgrading this study to an *in vivo* experiment using a nude mice model. Since this study speculate that apoptosis was induced via the mitochondrial pathway determining the mechanism of apoptosis induction such as assessing cytochrome c release is crucial. Further work includes also includes determining the anti-metastatic effects of HS extracts and formation of nanoparticles from the HS extracts. It is also paramount to elucidate on the isolation and characterization of biological active components of HS plant and revealing possible biochemical and molecular mechanism of action of the components.

REFERENCES

Abou-Nassar, K. & Brown, J.R. 2010. Novel agents for the treatment of chronic lymphocytic leukemia. *Clinical Advanced Hematology Oncology*, *8*(12): 886-95.

Abrahim, N.N., Kanthimathi, M.S. & Abdul-Aziz, A. 2012. Piper betle shows antioxidant activities, inhibits MCF-7 cell proliferation and increases activities of catalase and superoxide dismutase. *BMC complementary and alternative medicine*, *12*(1): 220.

Adams, J.M. 2003. Ways of dying: multiple pathways to apoptosis. *Genes & Development,* 17: 2481–2495.

Adaramoye, O., Ogungbenro, B., Anyaegbu, O. & Fafunso, M. 2008. Protective effects of extracts of Vernonia amygdalina, Hibiscus sabdariffa and vitamin C against radiation-induced liver damage in rats. *Journal of Radiation Research*, 49(2): 123–131.

Adebamowo, C.A., Ogundiran, T.O., Adenipekun, A.A., Oyesegun, R.A., Campbell, O.B., Akang, E.U., Rotimi, C.N. & Olopade, O.I. 2003. Obesity and height in urban Nigerian women with breast cancer. *Annals of Epidemiology,* 13: 455–461.

Adegunloye, B.J., Omoniyi, J.O., Owolabi, O.A., Ajagbonna, O.P., Sofola, O.A. & Coker, H.A. 1996. Mechanisms of the blood pressure lowering effect of the calyx extract of Hibiscus sabdariffa in rats. *African Journal of Medicine and Medical Sciences*, 25(3): 235–238.

Aggarwal, B.B. & Shishodia, S. 2006. Molecular targets of dietary agents for prevention and therapy of cancer. *Biochemical pharmacology*, *71*(10): 1397-1421.

Ajiboye, T.O., Salawu, N.A., Yakubu, M.Y., Oladiji, A.T., Akanji, M.A., Okogun, J.I. 2011. Antioxidant and drug detoxification potentials of Hibiscus sabdariffa anthocyanin extract. *Drug Chemical Toxicology,* 34:109-115.

Akagawa, M., Shigemitsu, T., & Suyama, K. 2003. Production of hydrogen peroxide by polyphenols and polyphenol-rich beverages under quasi-physiological conditions. *Bioscience, biotechnology, and biochemistry*, *67*(12): 2632-2640.

Akim, A., Ling, L.C., Rahmat, A. & Zakaria, Z.A. 2011. *Antioxidant and anti-proliferative activities of Roselle juice on Caov-3, MCF-7*. MDA-MB-231 and HeLa cancer cell lines. *African Journal of Pharmacy and Pharmacology,* 5 (7): 957-965.

Akram, S., Teong, H.F.C., Fliegel, L., Pervaiz, S. & Clement, M.V. 2006. Reactive oxygen species-mediated regulation of the Na+–H+ exchanger 1 gene expression connects intracellular redox status with cells' sensitivity to death triggers. *Cell Death & Differentiation*, *13*(4): 628-641.

Alarcon-Aguilar, F.J., Zamilpa, A., Perez-Garcia, M.D., Almanza-Perez, J.C., RomeroNunez, E. & Campos-Sepulveda, E.A. 2007. Effect of Hibiscus sabdariffa on obesity in MSG mice. *Journal of Ethnopharmacology*, 114(1): 66–71.

Alarcón-Alonso, J., Zamilpa, A., Aguilar, F.A., Herrera-Ruiz, M., Tortoriello, J., & Jimenez-Ferrer, E. 2012. Pharmacological characterization of the diuretic effect of Hibiscus sabdariffa Linn (Malvaceae) extract. *Journal of Ethnopharmacology*, *139*(3): 751-756.

Al-Hashimi, A.G. 2012. Antioxidant and antibacterial activities of Hibiscus sabdariffa L. extracts. *African Journal of Food Science*, *6*(21): 506-511.

Ali, B.H., Wabel, N.A., & Blunden, G. 2005. Phytochemical, pharmacological and toxicological aspects of Hibiscus sabdariffa L.: a review. *Phytotherapy research*, *19*(5): 369-375.

Ali-Bradeldin, H., Al-Wabel, N. & Gerald, B. 2005. Phytochemical pharmacological and toxicological aspects of Hibiscus sabdariffa: *A review. On Phytotherapy Research*, 19: 369-375.

Alshami, I. & Alharbi, A.E. 2014. Antimicrobial activity of Hibiscus sabdariffa extract against uropathogenic strains isolated from recurrent urinary tract infections. *Asian Pacific Journal of Tropical Disease*, *4*(4): 317-322.

Altieri, D. 2010. Survivin and IAP proteins in cell-death mechanisms. *Biochemical Journal*, *430*: 199-205.

Amin, A. & Hamza, A.A. 2005. Hepatoprotective effects of Hibiscus, Rosmarinus and Salvia on azathioprine-induced toxicity in rats. *Life Sciences*, 77(3): 266–278.

Anderson, B.O., Braun, S., Lim, S., Smith, R.A., Taplin, S. & Thomas, D.B. 2003. Early detection of breast cancer in countries with limited resources. *The breast journal*, *9*(s2): S51-S59.

Anderson, K.J., Teuber, S.S., Gobeille, A., Cremin, P., Waterhouse, A.L. & Steinberg, F.M. 2001. Walnut polyphenolics inhibit *in vitro* human plasma and LDL oxidation. *The Journal of nutrition*, *131*(11): 2837-2842.

Anglada, J.M., Martins-Costa, M., Francisco, J.S. & Ruiz-López, M.F. 2015. Interconnection of Reactive Oxygen Species Chemistry across the Interfaces of Atmospheric, Environmental, and Biological Processes. *Accounts of chemical research*, *48*(3): 575-583.

Anokwuru, C.P., Esiaba, I., Ajibaye, O. & Adesuyi, A.O. 2011. Polyphenolic content and antioxidant activity of Hibiscus sabdariffa calyx. *Research Journal of Medicinal Plant*, *5*(5): 557-66.

Artandi, S.E., & DePinho, R.A. 2010. Telomeres and telomerase in cancer. *Carcinogenesis*, *31*(1): 9-18.

Aruoma, O.I. 2003. Methodological consideration for characterization for potential antioxidant actions of bioactive components in plants foods. *Mutation Research,* 532: 9–20.

Ashkenazi, A. 2002. Targeting death and decoy receptors of the tumour-necrosis factor super family. *Nature Reviews Cancer*, *2*(6): 420-430.

Ashworth, A. 1995. Identification of the breast cancer susceptibility gene BRCA2. *Nature*, *378*: 789-792.

Ayala, A., Muñoz, M.F. & Argüelles, S. 2014. Lipid peroxidation: production, metabolism, and signaling mechanisms of malondialdehyde and 4-hydroxy-2-nonenal. *Oxidative medicine and cellular longevity*, *2014*.

Bagchi, D., Garg, A., Krohn, R.L., Bagchi, M., Bagchi, B.J. & Balmoori, J. 1998. Protective effects of grape seed proanthocyanidins and selected antioxidants against TPA-induced hepatic and brain lipid peroxidation and DNA fragmentation, and peritoneal macrophage activation in mice. *General Pharmacology*, 30(5): 771–776.

Bagchi, K. & Puri, S. 1998. Free radicals and antioxidants in health and disease. *Eastern Mediterranean Health Journal*, *4*(2): 350-360.

Bai, L. & Zhu, W. G. 2006. p53: structure, function and therapeutic applications. *Journal of Cancer Molecules*, *2*(4): 141-153.

Balakrishnan, M.P., Cilenti, L., Mashak, Z., Popat, P., Alnemri, E.S. & Zervos, A.S. 2009. THAP5 is a human cardiac-specific inhibitor of cell cycle that is cleaved by the proapoptotic Omi/HtrA2 protease during cell death. *American Journal of Physiology. Heart Circulatory Physiology*, 297: H643–H653.

Baldwin, Jr., A.S. 1996. The NF-kappa B and I kappa B proteins: new discoveries and Insights. *Annual Review of Immunology*, 14: 649–683.

Balic, M., Lin, H., Young, L., Hawes, D., Giuliano, A., McNamara, G., & Cote, R.J. 2006. Most early disseminated cancer cells detected in bone marrow of breast cancer patients have a putative breast cancer stem cell phenotype. *Clinical Cancer Research*, *12*(19): 5615-5621.

Badve, S., Dabbs, D.J., Schnitt, S.J., Baehner, F.L., Decker, T., Eusebi, V. & Reis-Filho, J.S. 2011. Basal-like and triple-negative breast cancers: a critical review with an emphasis on the implications for pathologists and oncologists. *Modern Pathology*, *24*(2): 157-167.

Barrera, G. 2012. Oxidative stress and lipid peroxidation products in cancer progression and therapy, *ISRN oncology*, *2012*.

Barros, A.I.R.N.A., Nunes, F.M., Gonçalves, B., Bennett, R.N. & Silva, A.P. 2011. Effect of cooking on total vitamin C contents and antioxidant activity of sweet chestnuts (Castanea sativa Mill.). *Food Chemistry*, 128: 165–172.

Bartek, J., Lukas, C. & Lukas, J. 2004. Checking on DNA damage in S phase. *Nature reviews Molecular cell biology*, 5: 792–804.

Bast, R.C. Jr, Ravdin, P., Hayes, D.F., Bates, S., Fritsche, H., Jessup, M.J., Kemeny, N., Locker, G.Y., Mennel, R.G. & Sommerfield, M.R. 2001. 2000 Update of recommendations for the use of tumor markers in breast and colorectal cancer: clinical practice guidelines of the American Society of Clinical Oncology. *Journal of Clinical Oncology*, 19: 1865–1878.

Beevi, S.S., Rasheed, M.H., & Geetha, A. 2007. Evidence of oxidative and nitrosative stress in patients with cervical squamous cell carcinoma. *Clinica chimica acta*, *375*(1): 119-123.

Bell, E.L., Klimova, T.A., Eisenbart, J., Moraes, C.T., Murphy, M.P., Budinger, G.R. & Chandel, N.S. 2007. The Qo site of the mitochondrial complex III is required for the transduction of hypoxic signaling via reactive oxygen species production. *Journal of Cell Biology,* 177: 1029–36.

Benson, J.R., Jatoi, I., Keisch, M., Esteva, F.J., Makris, A. & Jordan, V.C. 2009. Early breast cancer. *The Lancet*, *373*(9673): 1463-1479.

Benson, S.R.C., Blue, J., Judd, K. & Harman, J.E. 2004. Ultrasound is now better than mammography for the detection of invasive breast cancer. *The American journal of surgery*, *188*(4): 381-385.

Benzie, I.F.F. & Strain, J. 1996. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. *Analytical Biochemistry*, 239(1): 70-76.

Beral, V. 2003. Breast cancer and hormone-replacement therapy in the Million: Women Study. *Lancet*, 362: 419-427.

Bergamaschi, A., Kim, Y.H., Wang, P., Sørlie, T., Hernandez-Boussard, T., Lonning, P.E., Tibshirani, R., Dale, A.B & Pollack, J.R. 2006. Distinct patterns of DNA copy number alteration are associated with different clinicopathological features and gene-expression subtypes of breast cancer. *Genes, Chromosomes and Cancer*, *45*(11): 1033-1040.

Bergkvist, K.I., & Wengstrom, Y., 2006. Symptoms experiences during chemotherapy treatment –With focus to nausea and vomiting. *European Journal of Oncology nursing,* 10:21-29.

Berridge, M.V., Tan, A.S., McCoy, K.D. & Wang, R. 1996. The biochemical and cellular basis of cell proliferation assays that use tetrazolium salts. *Biochemica*, *4*(1): 14-19.

Bialik, S., Zalckvar, E., Ber, Y., Rubinstein, A.D. & Kimchi, A. 2010. Systems biology analysis of programmed cell death. *Trends in Biochemical Sciences*, 35: 556–564.

Bidère, N., Su, H.C. & Lenardo, M.J. 2006. Genetic Disorders Of Programmed Cell Death In The Immune System. *Annual Review in Immunology*, *24*: 321-352.

Boffetta, P. & Hashibe, M. 2006. Alcohol and cancer. *The lancet oncology*, *7*(2): 149-156.

Boya, P., Gonzalez-Polo, R.A., Poncet, D., Andreau, K., Vieira, H.L., Roumier, T. & Kroemer, G. 2003. Mitochondrial membrane permeabilization is a critical step of lysosome-initiated apoptosis induced by hydroxychloroquine. *Oncogene*, *22*(25): 3927-3936.

Brachmann, S.M., Hofmann, I., Schnell, C., Fritsch, C., Wee, S., Lane, H., Wang, S., Garcia-Echeverria, C. & Maira, S.M. 2009. Specific apoptosis induction by the dual PI3K/mTor inhibitor NVP-BEZ235 in HER2 amplified and PIK3CA mutant breast cancer cells. *Proceedings of the National Academy of Sciences*, *106*(52): 22299-22304.

Breimer, L.H. 1990. Molecular mechanisms of oxygen radical carcinogenesis and mutagenesis: the role of DNA base damage. *Molecular carcinogenesis*, *3*(4): 188-197.

Briehl, M. M., & Baker, A. F. 1996. Modulation of the antioxidant defence as a factor in apoptosis. *Cell death and Differentiation*, *3*(1): 63-70.

Brigelius-Flohé, R. 1999. Tissue-specific functions of individual glutathione peroxidases. *Free Radical Biology and Medicine*, *27*(9): 951-965.

Cao, X.H., Wang, A.H., Wang, C.L., Mao, D.Z., Lu, M.F., Cui, Y.Q. & Jiao, R.Z. 2010. Surfactin induces apoptosis in human breast cancer MCF-7 cells through a ROS/JNK-mediated mitochondrial/caspase pathway. *Chemico-biological interactions*, *183*(3): 357-362.

Carocho, M., & CFR Ferreira, I. 2013b. The role of phenolic compounds in the fight against cancer–a review. *Anti-Cancer Agents in Medicinal Chemistry (Formerly Current Medicinal Chemistry-Anti-Cancer Agents)*, *13*(8): 1236-1258.

Carocho, M., & Ferreira, I.C. 2013a. A review on antioxidants, prooxidants and related controversy: Natural and synthetic compounds, screening and analysis methodologies and future perspectives. *Food and Chemical Toxicology*, *51*: 15-25.

Carvajal-Zarrabal, O., Waliszewski, S., Barradas-Dermitz, D., Orta-Flores, Z., Hayward-Jones, P., Nolasco-Hipólito, C., Angulo-Guerrero, O., Sanchez-Ricano, R., Infanzon, R.M. & Trujillo, P.R. 2005. The consumption of Hibiscus sabdariffa dried calyx ethanolic extract reduced lipid profile in rats. *Plant foods for Human nutrition*, 60(4): 153-159.

Casey, P.M., Cerhan, J.R. & Pruthi, S. 2008. Oral contraceptive use and the risk of breast cancer. In *Mayo Clinic Proceedings,* 83(1): 86-91). Elsevier.

Castaneda-Ovando, A., de Lourdes Pacheco-Hernández, M., Páez-Hernández, M.E., Rodríguez, J.A. & Galán-Vidal, C.A. 2009. Chemical studies of anthocyanins: A review. *Food chemistry*, *113*(4), 859-871.

Catalá, A. 2009. Lipid peroxidation of membrane phospholipids generates hydroxy-alkenals and oxidized phospholipids active in physiological and/or pathological conditions. *Chemistry and physics of lipids*, *157*(1): 1-11.

Cejas, P., Casado, E., Belda-Iniesta, C., De Castro, J., Espinosa, E., Redondo, A. & González-Barón, M. 2004. Implications of oxidative stress and cell membrane lipid peroxidation in human cancer (Spain). *Cancer Causes & Control*, *15*(7): 707-719.

Chang, M.C., Chan, C.P., Wang, Y.J., Lee, P.H., Chen, L.I., Tsai, Y.L. & Jeng, J.H. 2007. Induction of necrosis and apoptosis to KB cancer cells by sanguinarine is associated with reactive oxygen species production and mitochondrial membrane depolarization. *Toxicology and applied pharmacology*, *218*(2): 143-151.

Chang, L. & Karin, M. 2001.Mammalian MAP kinase signalling cascades. *Nature*, 410: 37–40.

Chang, Y.C., Huang, H.P., Hsu, J.D., Yang, S.F. & Wang, C.J. 2005. Hibiscus anthocyanins rich extract-induced apoptotic cell death in human promyelocytic leukemia cells. *Toxicology and Applied pharmacology*, *205*(3): 201-212.

Chelikani, P., Fita, I. & Loewen, P.C. 2004. Diversity of structures and properties among catalases. *Cellular and Molecular Life Sciences CMLS*, *61*(2): 192-208.

Chen, C.C., Chou, F.P., Ho, Y.C., Lin, W.L., Wang, C.P., Kao, E.S. & Wang, C.J. 2004. Inhibitory effects of Hibiscus sabdariffa L extract on low-density lipoprotein oxidation and anti-hyperlipidemia in fructose-fed and cholesterol-fed rats. *Journal of the science of food and agriculture*, *84*(15): 1989-1996.

Chen, J.H., Wang, C.J., Wang, C.P., Sheu, J.Y., Lin, C.L. & Lin, H.H. 2013. Hibiscus sabdariffa leaf polyphenolic extract inhibits LDL oxidation and foam cell formation involving up-regulation of LXRα/ABCA1 pathway. *Food chemistry*, *141*(1): 397-406.

Chène, P. 2001. p53 as a drug target in cancer therapy. *Expert Opinion on Therapeutic Patents*, *11*(6): 923-935.

Cheng, N., Chytil, A., Shyr, Y., Joly, A., & Moses, H.L. 2008. Transforming growth factor-beta signaling-deficient fibroblasts enhance hepatocyte growth factor signaling in mammary carcinoma cells to promote scattering and invasion. Molecular Cancer Research, 6: 1521–1533.

Chiu, T. L. & Su, C.C. 2009. Curcumin inhibits proliferation and migration by increasing the Bax to Bcl-2 ratio and decreasing NF-κBp65 expression in breast cancer MDA-MB-231 cells. *International journal of molecular medicine*, *23*(4): 469-475.

Chlebowski, R.T., Manson, J.E., Anderson, G.L., Cauley, J.A., Aragaki, A.K., Stefanick, M.L., Lane, D., Johnson, K., Wactawski-Wende, J., Chen, C., Qi, L., Yasmeen, S., Newcomb, P. & Prentice, R.L. 2013. Estrogen plus progestin and breast cancer incidence and mortality in the Women's Health Initiative Observational Study. *Journal of the National Cancer Institute*, *105*(8): 526-535.

Chua, P.J., Yip, G.W.C. & Bay, B.H. 2009. Cell cycle arrest induced by hydrogen peroxide is associated with modulation of oxidative stress-related genes in breast cancer cells. *Experimental Biology and Medicine*, *234*(9): 1086-1094.

Colleoni, M., Minchella, I., Mazzarol, G., Nole, F., Peruzzotti, G., Rocca, A., Viale, G., Orlando, L., Ferretti, G., Gurigliano, G., Veronesi, P., Intra, M. & Goldhirsch, A., 2000. Response to primary chemotherapy in breast cancer patients with tumors not expressing estrogen and progesterone receptors. *Annals of oncology*, *11*(8): 1057-1059.

Comelli, M., Genero, N. & Mavelli, I. 2009. Caspase-independent apoptosis in Friend's erythroleukemia cells: role of mitochondrial ATP synthesis impairment in relocation of apoptosis-inducing factor and endonuclease G. *Journal of Bioenergetics & Biomembranes*, 41:49–59.

Compaoré, C.S., Nielsen, D.S., Ouoba, L.I., Berner, T.S., Nielsen, K.F., Sawadogo-Lingani, H. & Thorsen, L. 2013. Co-production of surfactin and a novel bacteriocin by Bacillus subtilis subsp. subtilis H4 isolated from Bikalga, an African alkaline Hibiscus sabdariffa seed fermented condiment. *International journal of food microbiology*, *162*(3): 297-307.

Conklin, K.A., 2000. Dietary antioxidants during cancer chemotherapy: impact on chemotherapeutic effectiveness and development of side effects. *Nutrition and cancer*, *37*(1): 1-18.

Coşkun, C., Kural, A., Döventaş, Y., Koldaş, M., Oezturk, H., Inal, B.B. & Gümüş, A. 2007. Hemodialysis and protein oxidation products. *Annals of the New York Academy of Sciences*, *1100*(1): 404-408.

Covey, J.M., Jaxel, C., Konn, K.W. & Pommer, Y. 1989. Protein-linked DNA strand breaks induced in mammalian cells by camptothecin, an inhibitor of topoisomerase I. *Cancer Research,* 49: 5016– 5522.

Cragg, G.M. & Suffness, M., 1988. Metabolism of plant-derived anticancer agents. *Pharmacol Therapy,* 37: 425–461.

Croce, C.M. 2009. Causes and consequences of microRNA dysregulation in cancer. *Nature reviews genetics*, *10*(10): 704-714.

Crompton, M., Barksby, E., Johnson, N. & Capano, M. 2002. Mitochondrial intermembrane junctional complexes and their involvement in cell death. *Biochimie*, *84*(2): 143-152.

Crystal, P., Strano, S. D., Shcharynski, S., & Koretz, M. J. 2003. Using sonography to screen women with mammographically dense breasts. *American Journal of Roentgenology*, *181*(1): 177-182.

Da-Costa-Rocha, I., Bonnlaender, B., Sievers, H., Pischel, I. & Heinrich, M. 2014. Hibiscus sabdariffa L.–A phytochemical and pharmacological review. *Food chemistry*, *165*: 424-443.

Dai, J. & Mumper R. J. 2010. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. *Molecules*, *15*(10): 7313-7352.

Danial, N.N. & Korsmeyer, S.J. 2004. Cell death: critical control points. *Cell*, *116*(2): 205-219.

Darbre, P.D. 2006. Environmental oestrogens, cosmetics and breast cancer. *Best practice & research clinical endocrinology & metabolism*, *20*(1): 121-143.

Darbre, P.D. & Charles, A.K. 2010. Environmental oestrogens and breast cancer: evidence for combined involvement of dietary, household and cosmetic xenoestrogens. *Anticancer research*, *30*(3): 815-827.

De Vivo, I., Prescott, J., Wong, J.Y., Kraft, P., Hankinson, S.E., & Hunter, D. J. 2009. A prospective study of relative telomere length and postmenopausal breast cancer risk. *Cancer Epidemiology Biomarkers & Prevention*, *18*(4): 1152-1156.

Deng, Y.T., Huang, H.C. & Lin, J.K. 2010. Rotenone induces apoptosis in MCF-7 human breast cancer cell-mediated ROS through JNK and p38 signaling. *Molecular carcinogenesis*, *49*(2): 141-151.

Devarajan, E., Sahin, A.A., Chen, J.S., Krishnamurthy, R.R., Aggarwal, N., Brun, A.M. & Mehta, K. 2002. Down-regulation of caspase 3 in breast cancer: a possible mechanism for chemoresistance. *Oncogene*, *21*(57): 8843-8851.

Dewson, G.K.R.M. & Kluck, R.M. 2010. Bcl-2 family-regulated apoptosis in health and disease. *Cell Health and Cytoskeleton*, *2*: 9-22.

Dirat, B., Bochet, L., Dabek, M., Daviaud, D., Dauvillier, S., Majed, B., & Muller, C. 2011. Cancer-associated adipocytes exhibit an activated phenotype and contribute to breast cancer invasion. *Cancer research*, *71*(7): 2455-2465.

Dixon, J.M. (Ed.). 2009. *ABC of breast diseases* (Vol. 69).John Wiley & Sons.

Donaldson, M.S. 2004. Nutrition and cancer: a review of the evidence for an anti-cancer diet. *Nutrition journal*, *3*(1): 19.

Donovan, M., Tiwary, C.M., Axelrod, D., Sasco, A. J., Jones, L., Hajek, R., & Davis, D.L. 2007. Personal care products that contain estrogens or xenoestrogens may increase breast cancer risk. *Medical hypotheses*, *68*(4), 756-766.

Doody, M.M., Mandel, J.S., Lubin, J.H., & Boice Jr, J.D. 1998. Mortality among United States radiologic technologists, 1926-90. *Cancer Causes & Control*, *9*(1): 67-75.

Dröge, W. 2002. The plasma redox state and ageing. *Ageing research reviews*, *1*(2): 257-278.

Duh, P.D., & Yen, G.C. 1997. Antioxidative activity of three herbal water extracts. *Food Chemistry*, *60*(4): 639-645.

Durackova, Z., 2010. Some current insights into oxidative stress. *Physiology Research*, *59*: 459-469.

Durando, M., Kass, L., Piva, J., Sonnenschein, C., Soto, A.M., Luque, E.H., & Muñoz-de-Toro, M. 2007. Prenatal bisphenol A exposure induces preneoplastic lesions in the mammary gland in Wistar rats. *Environmental Health Perspectives*, 80-86.

Dussi, M.C., Sugar, D., & Wrolstad, R.E. 1995. Characterizing and quantifying anthocyanins in red pears and the effect of light quality on fruit color. *Journal of the American Society for Horticultural Science*, *120*(5): 785-789.

Ebadi, M. 2001. Antioxidants and free radicals in health and disease: An introduction to reactive oxygen species, oxidative injury, neuronal cell death and therapy in neurodegenerative diseases.

Ellerby, LM, and Bredesen, D. 2000. Catalase. *Methods in Enzymology*, 322: 413-421.

Ellerby, LM, and Bredesen, D. 2000. Superoxide Dismutase. *Methods in enzymology*, 322: 413-421.

Engel, T., & Henshall, D.C. 2009. Apoptosis, Bcl-2 family proteins and caspases: the ABCs of seizure-damage and epileptogenesis? *International journal of physiology, pathophysiology and pharmacology*, *1*(2): 97.

Erten Şener, D., Gönenç, A., Akıncı, M., & Torun, M. 2007. Lipid peroxidation and total antioxidant status in patients with breast cancer. *Cell biochemistry and function*, *25*(4), 377-382.

Esme, H., Cemek, M., Sezer, M., Saglam, H., Demir, A., Melek, H. & Unlu, M. 2008. High levels of oxidative stress in patients with advanced lung cancer. *Respirology*, *13*(1): 112-116.

Essa, M.M., Subramanian, P., Suthakar, G., Manivasagam, T., Dakshayani, K.B., Sivaperumal, R., 2006.Influence of Hibiscus sabdariffa (Gongura) on the levels of circulatory lipid peroxidation products and liver marker enzymes in experimental hyperammonemia. *Journal of Applied Biomedicine*, 4(1), 53–58.

Falade, O.S., Otemuyiwa, I.O., Oladipo, A., Oyedapo, O.O., Akinpelu, B.A. & Adewusi, S.R.A. 2005. The chemical composition and membrane stability activity of some herbs used in local therapy for anemia. *Journal of ethnopharmacology*, *102*(1): 15-22.

Fallah-Hoseini, H., Fakhrzadeh, H., Larijani, B. & Shikhsaman, A. 2006. Review of anti-diabetic medicinal plant used in traditional medicine. *Jornal of Medicinal Plant,* 5: 1–8.

Farombi, E.O., & Fakoya, A. 2005. Free radical scavenging and antigenotoxic activities of natural phenolic compounds in dried flowers of Hibiscus sabdariffa L. *Molecular Nutrition & Food Research*, 49(12): 1120–1128.

Fasoyiro, S.B., Ashaye, O.A., Adeola, A., & Samuel, F.O. 2005. Chemical and storability of fruit-flavoured (Hibiscus sabdariffa) drinks. *World Journal of Agricultural Sciences*, *1*(2): 165-168.

Feng, R., Ni, H.M., Wang, S.Y., Tourkova, I.L., Shurin, M.R., Harada, H. & Yin, X.M. 2007. Cyanidin-3-rutinoside, a natural polyphenol antioxidant, selectively kills leukemic cells by induction of oxidative stress. *Journal of Biological Chemistry*, *282*(18): 13468-13476.

Ferlay, J., Shin, H.R., Bray, F., Forman, D., Mathers, C. & Parkin, D.M. 2010. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *International journal of cancer*, *127*(12): 2893-2917.

Filomeni, G., Graziani, I., Rotilio, G., & Ciriolo, M.R. 2007. trans-Resveratrol induces apoptosis in human breast cancer cells MCF-7 by the activation of MAP kinases pathways. *Genes & nutrition*, *2*(3), 295-305.

Florence, T.M., 1995.The role of free radicals in disease. *Australian and New Zealand journal of ophthalmology*, *23*(1): 3-7.

Franco, R. & Panayiotidis, M.I. 2009. Environmental toxicity, oxidative stress, human disease and the" black box" of their synergism: how much have we revealed? *Mutation Research*, 674: 1-2.

Franco, R., Sánchez-Olea, R., Reyes-Reyes, E.M. & Panayiotidis, M.I. 2009. Environmental toxicity, oxidative stress and apoptosis: menage a trois. *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, *674*(1): 3-22.

Franco, R., Schoneveld, O., Georgakilas, A.G. & Panayiotidis, M.I. 2008. Oxidative stress, DNA methylation and carcinogenesis. *Cancer letters*, *266*(1): 6-11.

Frank, T.S., Manley, S.A., Olopade, O.I., Cummings, S., Garber, J.E., Bernhardt, B., Antman, K., Russo, D., Wood, M.E., Mullineau, L., Isaacs, L., Peshkin, B., Buys, S., Venne, V., Rowky, P.T., Loader, S., Offit, K., Robson, M., Hampel, H., Brener, D., Winer, E.P., Clark, S., Weber, E.P., Strong, L.C.,. & Thomas, A. 1998. Sequence analysis of BRCA1 and BRCA2: correlation of mutations with family history and ovarian cancer risk. *Journal of Clinical Oncology*, *16*(7): 2417-2425.

Frank, T., Netzel, G., Kammerer, D.R., Carle, R., Kler, A., Kriesl, E. & Netzel, M. 2012. Consumption of Hibiscus sabdariffa L. aqueous extract and its impact on systemic antioxidant potential in healthy subjects. *Journal of the Science of Food and Agriculture*, *92*(10), 2207-2218.

Freier, K., Joos, S., Flechtenmacher, C., Devens, F., Benner, A., Bosch, F.X. & Hofele, C. 2003. Tissue microarray analysis reveals site-specific prevalence of oncogene amplifications in head and neck squamous cell carcinoma. *Cancer research*, *63*(6): 1179-1182.

Fulda, S., Meyer, E., & Debatin, K.M. 2000. Metabolic inhibitors sensitize for CD95 (APO-1/Fas)-induced apoptosis by down-regulating Fas-associated death domain-like interleukin 1-converting enzyme inhibitory protein expression. *Cancer research*, *60*(14): 3947-3956.

Fullerton, M., Khatiwada, J., Johnson, J.U., Davis, S. & Williams, L.L. 2011. Determination of antimicrobial activity of sorrel (Hibiscus sabdariffa) on Escherichia coli O157: H7 isolated from food, veterinary, and clinical samples. *Journal of medicinal food*, *14*(9): 950-956.

Galluzzi, L., Morselli, E., Kepp, O., Vitale, I., Rigoni, A., Vacchelli, E. & Kroemer, G. 2010. Mitochondrial gateways to cancer. *Molecular aspects of medicine*, *31*(1): 1-20.

Gatza, M. L., Lucas, J. E., Barry, W. T., Kim, J. W., Wang, Q., Crawford, M. D., Datto, M.B., Kelley, M., Mathey-Prevot, B., Potti, A., & Nevins, J. R. 2010. A pathway-based classification of human breast cancer. *Proceedings of the National Academy of Sciences*, *107*(15): 6994-6999.

Gaya, I.B., Mohammad, O.M.A., Suleiman, A.M., Maje, M.I. & Adekunle, A.B. 2009. Toxicological and lactogenic studies on the seeds of Hibiscus sabdariffa linn (Malvaceae) extract on serum prolactin levels of albino wistar rats. *Internet journal of Endocrinology*.

Ghavami, S., Hashemi, M, Ande, S.R., Yeganeh, B., Xiao, W., Eshraghi, M., Bus, C.J., Kadkhoda K, Wiechec, E, Halayko, AJ.., Los, M. 2009. Apoptosis and cancer: mutations within caspase genes. *Journal of Medical Genetetics,* 46: 497–510

Giansanti, V. & Scovassi, A.I. 2008. Cell death: a one-way journey to the graveyard. *Open Biological Journal*, *1*: 27-34.

Giansanti, V., Torriglia, A., & Scovassi, A.I. 2011. Conversation between apoptosis and autophagy “Is it your turn or mine?” *Apoptosis*, *16*(4): 321-333.

Gloire, G., Legrand-Poels, S., Piette, J. 2006. NF-kappaB activation by reactive oxygen species: fifteen years later. *Biochemical Pharmacology*, 72: 1493–1505.

Goldhirsch, A., Gelber, R.D., Yothers, G., Gray, R.J., Green, S., Bryant, J., Gelber, S., Castiglione, M. & Coates, A.S. 2001. Adjuvant therapy for very young women with breast cancer: need for tailored treatments. *MONOGRAPHS-NATIONAL CANCER INSTITUTE*, *30*: 44-51.

Gönenç, A., Özkan, Y., Torun, M., & Şimşek, B. 2001. Plasma malondialdehyde (MDA) levels in breast and lung cancer patients. *Journal of clinical pharmacy and therapeutics*, *26*(2): 141-144.

Gordon, P.B., Goldenberg, S.L. & Chan, N.H., 1993. Solid breast lesions: diagnosis with US-guided fine-needle aspiration biopsy. *Radiology*, *189*(2): 573-580.

Gray, H. 2000. Anatomy of the Human Body.New York: Bartleby.

Griffiths, M. & Sundaram, H. 2011. Drug design and testing: profiling of antiproliferative agents for cancer therapy using a cell-based methyl-[3H]-thymidine incorporation assay. In *Cancer Cell Culture,* (pp. 451-465). Humana Press.

Groeger, G., Mackey, A.M., Pettigrew, C.A., Bhatt, L. & Cotter, T.G. 2009. Stress-induced activation of Nox contributes to cell survival signalling via production of hydrogen peroxide. *Journal of neurochemistry*, *109*(5), 1544-1554.

Gulbins, E., Jekle, A., Ferlinz, K., Grassme, H., Lang, F. 2000. Physiology of apoptosis. *American Journal of Physiology.Renal Physioliology,* 279: 605–615.

Gupta, G.P. & Massagué, J. 2006. Cancer metastasis: building a framework. *Cell*, *127*(4): 679-695.

Gurib-Fakim, A. 2006. Medicinal plants: traditions of yesterday and drugs of tomorrow. *Molecular aspects of Medicine*, *27*(1): 1-93.

Gurinder, J.K., & Daljit, S.A., 2009. Antibacterial and phytochemical screening of *Anethum graveolens***,** *Foeniculum vulgare* and *Trachyspermum ammi*. *BMC Complementary and Alternative Medicine,* 9: 30.

Gurrola-Diaz, C.M., Garcia-Lopez, P.M., Sanchez-Enriquez, S., Troyo-Sanroman, R., Andrade-Gonzalez, I. & Gomez-Leyva, J.F. 2010. Effects of Hibiscus sabdariffa extract powder and preventive treatment (diet) on the lipid profiles of patients with metabolic syndrome (MeSy). *Phytomedicine*, 17(7): 500–505.

Gutteridge, J.M. 1995. Lipid peroxidation and antioxidants as biomarkers of tissue damage. *Clinical chemistry*, *41*(12): 1819-1828.

Halliwell, B. 2003. Oxidative stress in cell culture: an under-appreciated problem?. *FEBS letters*, *540*(1): 3-6.

Halliwell, B. 2007. Oxidative stress and cancer: have we moved forward? *Biochemical Journal,* 401: 1–11.

Halliwell, B. 2012. Free radicals and antioxidants: updating a personal view. *Nutrition reviews*, *70*(5): 257-265.

Hanahan, D. & Coussens, L. M. 2012. Accessories to the crime: functions of cells recruited to the tumor microenvironment. *Cancer cell*, *21*(3): 309-322.

Hanahan, D., & Folkman, J. 1996. Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis. *Cell*, *86*(3): 353-364.

Hanahan, D. & Weinberg, R.A. 2000. The hallmarks of cancer. *Cell*, *100*(1): 57-70.

Hanahan, D., & Weinberg, R.A., 2011. Hallmarks of cancer: the next generation."*cell*,"*144*(5): 646-674.

Hanif, H.A., Murad, N.A., Ngah, W.Z.W. & Yusof, Y.A.M. 2008. Effects of Zingiber officinale on superoxide dismutase, glutathione peroxidase, catalase, glutathione and malondialdehyde content in HepG2 cell line. *Malaysian Journal of Biochemistry and Molecular Biology*, *12*(1).

Harbour, J.W. & Dean, D.C. 2000. The Rb/E2F pathway: expanding roles and emerging paradigms. *Genes & development*, *14*(19): 2393-2409.

Hartwell, L.H. & Weinert, T.A 1989. Checkpoints: controls that ensure the order of cell cycle events. *Science*, 246: 629–634.

Hattori, Y., Nishigori, C., Tanaka, T., Ushida, K., Nikaido, O. & Osawa, T. 1997. 8 Hydroxy-2-deoxyguanosine is increased in epidermal cells of hairless mice after chronic ultraviolet B exposure. *Journal of Investigative Dermatology*, 89: 10405–9.

He, L., Dinger, B., Gonzalez, C., Obeso, A., & Fidone, S. 2006. Function of NADPH oxidase and signaling by reactive oxygen species in rat carotid body type I cells. In *The Arterial Chemoreceptors,* 155-160. Springer US.

Herrera-Arellano, A., Miranda-Sanchez, J., Avila-Castro, P., Herrera-Alvarez, S., Jimenez-Ferrer, J.E. & Zamilpa, A. 2007 .Clinical effects produced by a standardized herbal medicinal product of Hibiscus sabdariffa on patients with hypertension. A randomized, double-blind, lisinopril-controlled clinical trial. *Planta Medica*, 73(1): 6–12.

Hileman, E.O., Liu, J., Albitar, M., Keating, M.J., & Huang, P., 2004. Intrinsic oxidative stress in cancer cells: a biochemical basis for therapeutic selectivity. *Cancer chemotherapy and pharmacology*, *53*(3): 209-219.

Hodnick, W.F., Ahmad, S. & Pardini, R.S. 1998. Induction of oxidative stress by redox active flavonoids. *In Flavonoids in the Living System*, 131-150. Springer US.

Hopkins, A.L., Lamm, M.G., Funk, J.L. & Ritenbaugh, C. 2013. Hibiscus sabdariffa L. in the treatment of hypertension and hyperlipidemia: a comprehensive review of animal and human studies. *Fitoterapia*, *85*: 84-94.

Hou, D.X., Tong, X., Terahara, N., Luo, D., & Fujii, M. 2005. Delphinidin 3-sambubioside, a Hibiscus anthocyanin, induces apoptosis in human leukemia cells through reactive oxygen species-mediated mitochondrial pathway. *Archives of biochemistry and biophysics*, *440*(1): 101-109.

Hsieh, C.J., Kuo, P.L., Hsu, Y.C., Huang, Y.F., Tsai, E.M. & Hsu, Y.L. 2014. Arctigenin, a dietary phytoestrogen, induces apoptosis of estrogen receptor-negative breast cancer cells through the ROS/p38 MAPK pathway and epigenetic regulation. *Free Radical Biology and Medicine*, *67*: 159-170.

Hsu, Y.L., Chen, C.Y., Hou, M.F., Tsai, E.M., Jong, Y.J., Hung, C.H. & Kuo, P.L. 2010. 6-Dehydrogingerdione, an active constituent of dietary ginger, induces cell cycle arrest and apoptosis through reactive oxygen species/c-Jun N-terminal kinase pathways in human breast cancer cells. *Molecular nutrition & food research*, *54*(9): 1307-1317.

Hsu, Y.L., Chen, C.Y., Lin, I.P., Tsai, E.M., Kuo, P.L. & Hou, M.F. 2012. 4-Shogaol, an active constituent of dietary ginger, inhibits metastasis of MDA-MB-231 human breast adenocarcinoma cells by decreasing the repression of NF-κB/Snail on RKIP. *Journal of agricultural and food chemistry*, *60*(3): 852-861.

Huang, Z.Y., Baldwin, R.L., Hedrick, N.M. & Gutmann, D.H. 2002. Astrocyte-specific expression of CDK4 is not sufficient for tumor formation, but cooperates with p53

heterozygosity to provide a growth advantage for astrocytes *in vivo*. *Oncogene*, *21*(9): 1325-1334.

Hussien, M.M.I., McNulty, H., Armstrong, N., Johnston, P.G., Spence, R.A.J. & Barnett, Y. 2005. Investigation of systemic folate status, impact of alcohol intake and levels of DNA damage in mononuclear cells of breast cancer patients. *British journal of cancer*, *92*(8): 1524-1530.

Hwang, S.L. & Yen, G.C. 2008. Neuroprotective effects of the citrus flavanones against H2O2-induced cytotoxicity in PC12 cells. *Journal of agricultural and food chemistry*, *56*(3): 859-864.

Inuwa, I., Ali, B.H., Al-Lawati, I., Beegam, S., Ziada, A., & Blunden, G. 2012. Long-term ingestion of Hibiscus sabdariffa calyx extract enhances myocardial capillarization in the spontaneously hypertensive rat. *Experimental Biology and Medicine*, *237*(5): 563-569.

Ismail, A., Ikram, E.H.K., & Nazri, H.S.M. 2008. Roselle (Hibiscus sabdariffa L.) Seeds-nutritional composition, protein quality and health benefits. *Food*, *2*(1): 1-16.

Itokawa, H., Morris-Natschke, S.L., Akiyama, T. & Lee, K.H., 2008. Plant-derived natural product research aimed at new drug discovery. *Journal of natural medicines*, *62*(3): 263-280.

Jackson, A.L. & Loeb, L.A. 2001. The contribution of endogenous sources of DNA damage to the multiple mutations in cancer. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *477*(1): 7-21.

Jatoi, I. & Kaufmann, M. 2010. *Management of breast diseases*. Berlin Heidelberg New York: Springer.

Jee, J.P., Lim, S.J., Park, J.S. & Kim, C.K. 2006. Stabilization of all-trans retinol by loading lipophilic antioxidants in solid lipid nanoparticles. *European journal of pharmaceutics and biopharmaceutics*, *63*(2): 134-139.

Jiménez-Ferrer, E., Alarcón-Alonso, J., Aguilar-Rojas, A., Zamilpa, A., Jiménez-Ferrer, C.I., Tortoriello, J. & Herrera-Ruiz, M. 2012. Diuretic effect of compounds from Hibiscus sabdariffa by modulation of the aldosterone activity. *Planta medica*, *78*(18): 1893-1898.

Joseph, I., Tressler, R., Bassett, E., Harley, C., Buseman, C.M., Pattamatta, P., Wright, W.E., Shay, J.W. & Go, N.F., 2010. The telomerase inhibitor imetelstat depletes cancer stem cells in breast and pancreatic cancer cell lines. *Cancer research*, *70*(22): 9494-9504.

Ju, W., Wang, X., Shi, H., Chen, W., Belinsky, S.A. & Lin, Y. 2007. A critical role of luteolin-induced reactive oxygen species in blockage of tumor necrosis factor-activated nuclear factor-κB pathway and sensitization of apoptosis in lung cancer cells. *Molecular pharmacology*, *71*(5): 1381-1388.

Jung, E., Kim, Y. & Joo, N. 2013. Physicochemical properties and antimicrobial activity of Roselle (Hibiscus sabdariffa L.). *Journal of the Science of Food and Agriculture*, *93*(15): 3769-3776.

Kalantzi, O.I., Martin, F.L., Thomas, G.O., Alcock, R.E., Tang, H.R., Drury, S.C., & Jones, K.C. 2004. Different levels of polybrominated diphenyl ethers (PBDEs) and chlorinated compounds in breast milk from two UK regions. *Environmental Health Perspectives*, *112*(10): 1085.

Kancheva, V.D. 2009. Phenolic antioxidants – radical-scavenging and chainbreaking activity: a comparative study. *European Journal of Lipid Science & Technology*. 111, 1072– 1089.

Kang, D.H. 2002. Oxidative stress, DNA damage and breast cancer. *AACN Advanced Critical Care*, *13*(4): 540-549.

Kapasakalidis, P.G., Rastall, R.A. & Gordon, M.H. 2006. Extraction of polyphenols from processed black currant (Ribes nigrum L.) residues. *Journal of agricultural and food chemistry*, *54*(11): 4016-4021.

Karin, M. 1995. The regulation of AP-1 activity by mitogen-activated protein kinases. *Journal of Biological Chemistry*, 270: 16483–16486.

Karp G: 2008. Cell and molecular biology: Concepts and experiments. 5 edition. John New Jersey: Wiley and Sons; 653-657.

Kelsey, J.L., Gammon, M.D. & John, E.M. 1993. Reproductive factors and breast cancer. *Epidemiologic reviews*, *15*(1): 36.

Kerr, J.F., Wyllie, A.H. & Currie, A.R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British journal of cancer, 26*: 239-257.

Khaghani, S., Yajloo, M.M., Paknejad, M., Shariftabrizi, A., Pasalar, P. & Razi, F. 2011. Selective cytotoxicity and apoptogenic activity of Hibiscus sabdariffa aqueous extract against MCF-7 human breast cancer cell line. *Journal of Cancer Therapy*, *2*(03): 394.

Khan, M.A., Chen, H.C., Wan, X.X., Tania, M., Xu, A.H., Chen, F.Z. & Zhang, D.Z. 2013. Regulatory effects of resveratrol on antioxidant enzymes: a mechanism of growth inhibition and apoptosis induction in cancer cells. *Molecules and cells*, *35*(3): 219-225.

Khan, M.A., Tania, M., Zhang, D.Z. & Chen, H.C. 2010. Antioxidant enzymes and cancer. *Chinese Journal of Cancer Research*. 22, 87-92.

Kim, B.Y., Han, M.J. & Chung, A.S. 2001. Effects of reactive oxygen species on proliferation of Chinese hamster lung fibroblast (V79) cells. *Free Radical Biology and Medicine*, *30*(6): 686-698.

Kim, C.M., & Kim, J.H. 2008. BMB reports: Mini Review; Cytosolic phospholipase A2, Lipoxygenase metabolites, and reactive oxygen species. *Biochemistry and Molecular Biology reports*, *41*(8), 555-559.

Klaunig, J.E., Kamendulis, L.M. & Hocevar, B.A. 2010. Oxidative stress and oxidative damage in carcinogenesis. *Toxicologic pathology*, *38*(1): 96-109.

Klauning, J.E. & Kamendulis, L.M. 2004. The role of oxidative stress in carcinogenesis. *Annual Review Pharmacology Toxicology*, 44: 239–67.

Kneeshaw, P.J., Turnbull, L.W. & Drew, P.J. 2003. Current applications and future direction of MR mammography. *British journal of cancer*, *88*(1): 4-10.

Koffi, E., Sea, T., Dodehe, Y. & Soro, S. 2010. Effect of solvent type on extraction of polyphenols from twenty three Ivorian plants. *Journal of Animal and Plant Sciences (JAPS)*, *5*(3): 550-558.

Konczak, I. & Zhang, W. 2004. Anthocyanins-more than nature´ s colours (2004). *Journal of Biomedicine and Biotechnology*, (5): 239–240.

Kong, Q., Beel, J.A. & Lillehei, K.O. 2000. A threshold concept for cancer therapy. *Medical hypotheses*, *55*(1): 29-35.

Koopman, W.J., Verkaart, S., van Emst-de Vries, S.E., Grefte, S., Smeitink, J.A. & Willems, P.H. 2006. Simultaneous quantification of oxidative stress and cell spreading using 5-(and-6)-chloromethyl-2′, 7′-dichlorofluorescein. *Cytometry Part A*, *69*(12): 1184-1192.

Korkola, J. & Gray, J.W. 2010. Breast cancer genomes—form and function. *Current opinion in genetics & development*, *20*(1): 4-14.

Kornegoor, R., Verschuur-Maes, A.H., Buerger, H., Hogenes, M.C., de Bruin, P.C., Oudejans, J.J. & van Diest, P.J. 2012. Molecular subtyping of male breast cancer by immunohistochemistry. *Modern Pathology*, *25*(3): 398-404.

Krimmel, B., Swoboda, F., Solar, S. & Reznicek, G. 2010. OH-radical induced degradation of hydroxybenzoic- and hydroxycinnamic acids and formation of aromatic products – a gamma radiolysis study. *Radiation Physics & Chemistry*, 79: 1247– 1254.

Kroemer, G. & Pouyssegur, J. 2008. Tumor cell metabolism: cancer's Achilles' heel. *Cancer cell*, *13*(6): 472-482.

Kroemer, G., Dallaporta, B. & Resche-Rigon, M. 1998. The mitochondrial death/life regulator in apoptosis and necrosis, *Annual review of physiology*, *60*(1): 619-642.

Kroemer, G., Galluzzi, L., Vandenabeele, P., Abrams, J., Alnemri, E.S., Baehrecke, E.H. & Melino, G. 2009. Classification of cell death: recommendations of the Nomenclature Committee on Cell Death 2009. *Cell Death & Differentiation*, *16*(1): 3-11.

Kryston, T.B., Georgiev, A.B., Pissis, P. & Georgakilas, A.G. 2011. Role of oxidative stress and DNA damage in human carcinogenesis. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *711*(1), 193-201.

Kumar, V., Abbas, A.K., Fausto, N. & Aster, J.C. 2010. Cellular responses to stress and toxic insults: adaptation, injury, and death. *Robbins and Cotran pathologic basis of disease, 8th edn. Saunders Elsevier, Philadelphia*, 38-39.

Kumar, B., Koul, S., Khandrika, L., Meacham, R. B., and Koul, H.K. 2008. Oxidative stress is inherent in prostate cancer cells and is required for aggressive phenotype. *Cancer Research,* 68: 1777–85.

Kumaraguruparan, R., Subapriya, R., Kabalimoorthy, J. & Nagini, S. 2002. Antioxidant profile in the circulation of patients with fibroadenoma and adenocarcinoma of the breast. *Clinical Biochemistry*, *35*(4): 275-279.

Kurokawa, H.I.R.O.K.A.Z.U., Nishio, K.A.Z.U.T.O., Fukumoto, H.I.S.A.O., Tomonari, A.K.I.R.A., Suzuki, T.O.S.H.I.H.I.R.O. & Saijo, N.A.G.A.H.I.R.O. 1999. Alteration of caspase-3 (CPP32/Yama/apopain) in wild-type MCF-7, breast cancer cells. *Oncology reports*, *6*(1): 33-40.

Kwon, J., Lee, S.R., Yang, K.S., Ahn, Y., Kim, Y.J., Stadtman, E.R. & Rhee, S.G. 2004. Reversible oxidation and inactivation of the tumor suppressor PTEN in cells stimulated with peptide growth factors. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(47), 16419-16424.

Laikangbam, R. & Damayanti Devi, M. 2012. Inhibition of calcium oxalate crystal deposition on kidneys of urolithiatic rats by Hibiscus sabdariffa L. extract. *Urological Research*, 40(3): 211–218.

Lancellotti, M., Pereira, R.F.C., Cury, G.G. & Hollanda, L.M.D. 2009. Pathogenic and opportunistic respiratory bacteria-induced apoptosis. *Brazilian Journal of Infectious Diseases*, *13*(3): 226-231.

Lartigue, L., Kushnareva, Y., Seong, Y., Lin, H., Faustin, B. & Newmeyer, D.D. 2009. Caspase-independent mitochondrial cell death results from loss of respiration, not cytotoxic protein release. *Molecular Biology of the Cell,* 20: 4871–4884.

Laudanski, K. & Wyczechowska, D., 2006. The distinctive role of small heat shock proteins in oncogenesis. *Archivum Immunologiae et Therapiae Experimentalis,* (Warsz) 54:103–111.

Lavrik, I.N., Golks, A., & Krammer, P.H. 2005. Caspases: pharmacological manipulation of cell death. *Journal of Clinical Investigation*, *115*(10): 2665.

Lazebnik, Y., 2010.What are the hallmarks of cancer?. *Nature Reviews Cancer*, *10*(4): 232-233.

Lee, C.H., Dershaw, D.D., Kopans, D., Evans, P., Monsees, B., Monticciolo, D. & Burhenne, L.W. 2010. Breast cancer screening with imaging: recommendations from the Society of Breast Imaging and the ACR on the use of mammography, breast MRI, breast ultrasound, and other technologies for the detection of clinically occult breast cancer. *Journal of the American college of radiology*, *7*(1): 18-27.

Lee, H.S., Ha, A.W. & Kim, W.K. 2012. Effect of resveratrol on the metastasis of 4T1 mouse breast cancer cells *in vitro* and *in vivo*. *Nutrition research and practice*, *6*(4): 294-300.

Lee, H.S., Seo, E.Y., Kang, N.E. & Kim, W.K. 2008. [6]-Gingerol inhibits metastasis of MDA-MB-231 human breast cancer cells. *The Journal of nutritional biochemistry*, *19*(5): 313-319.

Lee, J.H., Yeon, J.H., Kim, H., Roh, W., Chae, J., Park, H.O. & Kim, D.M. 2012. The natural anticancer agent plumbagin induces potent cytotoxicity in MCF-7 human breast cancer cells by inhibiting a PI-5 kinase for ROS generation.

Lee-Hoeflich, S.T., Crocker, L., Yao, E., Pham, T., Munroe, X., Hoeflich, K.P., Sliwkowski, M.X. & Stern, H.M., 2008. A central role for HER3 in HER2-amplified breast cancer: implications for targeted therapy. *Cancer research*, *68*(14): 5878-5887.

Lester, S.C., Bose, S., Chen, Y.Y., Connolly, J.L., de Baca, M.E., Fitzgibbons, P.L., Hayes, D.F., Hill, K.A, Kleer, C., O'Malley, F.P., Page, D.L, Smith, B.L., Tan, L.K., Weaver, D.L., Winer, E. & Simpson, J.F., 2009. Protocol for the examination of specimens from patients with invasive carcinoma of the breast. *Archives of Pathology & Laboratory Medicine*, *133*(10): 1515-1538.

Li, W.Y., Chan, S.W., Guo, D.J., & Yu, P.H.F. 2007. Correlation between antioxidative power and anticancer activity in herbs from traditional Chinese medicine formulae with anticancer therapeutic effect. *Pharmaceutical Biology*, *45*(7): 541-546.

Lima, R.T., Busacca, S., Almeida, G.M., Gaudino, G., Fennell, D.A. & Vasconcelos, M.H. 2011. MicroRNA regulation of core apoptosis pathways in cancer. *European Journal of Cancer* 47: 163–174.

Lin, C.Y., Wu, H.Y., Wang, P.L. & Yuan, C.J., 2010. Mammalian Ste20- like protein kinase 3 induces a caspase-independent apoptotic pathway. *International Journal of Biochemistry & Cell Biology* 42:98–105.

Lin, H.H., Chan, K.C., Sheu, J.Y., Hsuan, S.W., Wang, C.J. & Chen, J.H. 2012. Hibiscus sabdariffa leaf induces apoptosis of human prostate cancer cells *in vitro* and *in vivo*. *Food Chemistry*, *132*(2): 880-891.

Lin, H.H., Chen, J.H., Kuo, W.H. & Wang, C.J. 2007. Chemopreventive properties of Hibiscus sabdariffa L. on human gastric carcinoma cells through apoptosis induction and JNK/p38 MAPK signaling activation. *Chemico-biological interactions*, *165*(1): 59-75.

Lin, H.H., Huang, H.P., Huang, C.C., Chen, J.H. & Wang, C.J. 2005. Hibiscus polyphenol-rich extract induces apoptosis in human gastric carcinoma cells via p53 phosphorylation and p38 MAPK/FasL cascade pathway. *Molecular Carcinogenesis*, *43*(2), 86-99.

Lin, W.L., Hsieh, Y.J., Chou, F.P., Wang, C.J., Cheng, M.T. & Tseng, T.H. 2003. Hibiscus protocatechuic acid inhibits lipopolysaccharide-induced rat hepatic damage. *Archives of Toxicology*, 77(1): 42–47.

Linnstaedt, S.D., Gottwein, E., Skalsky, R.L., Luftig, M.A. & Cullen, B.R. 2010. Virally induced cellular microRNA miR-155 plays a key role in B-cell immortalization by Epstein-Barr virus. *Journal of Virology*. 84: 11670–11678.

Liu, J.Y., Chen, C.C., Wang, W.H., Hsu, J.D., Yang, M.Y. & Wang, C.J. 2006. The protective effects of Hibiscus sabdariffa extract on CCl4-induced liver fibrosis in rats. *Food and Chemical Toxicology*, 44(3): 336–343.

Liu, T.Z., Cheng, J.T., Yiin, S.J., Chen, C.Y., Chen, C.H., Wu, M.J. & Chern, C.L. 2008. Isoobtusilactone A induces both caspase-dependent and-independent apoptosis in Hep G2 cells. *Food and chemical toxicology*, *46*(1), 321-327.

Liu, K.S., Tsao, S.M. & Yin, M.C. 2005. *In vitro* antibacterial activity of roselle calyx and protocatechuic acid. *Phytotherapy Research*, 19(11): 942–945.

Liu, L.C., Wang, C.J., Lee, C.C., Su, S.C., Chen, H.L., Hsu, J.D. & Lee, H.J. 2010. Aqueous extract of Hibiscus sabdariffa L. decelerates acetaminophen-induced acute liver damage by reducing cell death and oxidative stress in mouse experimental models. *Journal of the Science of Food and Agriculture*, *90*(2): 329-337.

Liu, S., Ginestier, C., Ou, S. J., Clouthier, S.G., Patel, S.H., Monville, F. & Wicha, M.S. 2011. Breast cancer stem cells are regulated by mesenchymal stem cells through cytokine networks. *Cancer research*, *71*(2): 614-624.

Llambi, F., & Green, D.R. 2011. Apoptosis and oncogenesis: give and take in the BCL-2 family. *Current opinion in genetics & development*, *21*(1): 12-20.

Lo, C.W., Huang, H.P., Chan, K.C., Wu, C.H. & Wang, C.J. 2010. Hibiscus Sabdariffa extract induced apoptosis of proliferating smooth muscle cell. *Journal of food biochemistry*, *34*(3), 549-563.

Lo, C.W., Huang, H.P., Lin, H.M., Chien, C.T., & Wang, C.J., 2007. Effect of Hibiscus anthocyanins-rich extract induces apoptosis of proliferating smooth muscle cell via activation of P38 MAPK and p53 pathway. *Molecular nutrition & food research*, *51*(12): 1452-1460.

Lobo, V., Patil, A., Phatak, A. & Chandra, N. 2010. Free radicals, antioxidants and functional foods: Impact on human health. *Pharmacognosy reviews*, *4*(8): 118.

Locatelli, I., Lichtenstein, P. & Yashin, A.I. 2004. The heritability of breast cancer: a Bayesian correlated frailty model applied to Swedish twins data. *Twin Research*, *7*(02): 182-191.

Longatto Filho, A., Lopes, J.M., & Schmitt, F.C. 2010. Angiogenesis and breast cancer. *Journal of oncology*, *2010*.

Lowe, S.W., Cepero, E., & Evan, G. 2004. Intrinsic tumour suppression. *Nature,* 432: 307–315.

Lule, S.U. & Xia, W. 2005. Food phenolics, pros and cons: A review. *Food Reviews International*, 21(4): 367–388.

Luo, M., Liu, X., Zu, Y., Fu, Y., Zhang, S., Yao, L., & Efferth, T. 2010. Cajanol, a novel anticancer agent from Pigeonpea [Cajanus cajan (L.) Millsp.] roots, induces apoptosis in human breast cancer cells through a ROS-mediated mitochondrial pathway. *Chemico-biological interactions*, *188*(1): 151-160.

Madhuri, S. & Pandey, G. 2009. Some anticancer medicinal plants of foreign origin. *Current science*, *96*(6), 779-783.

Malhotra, G. K., Zhao, X., Band, H. & Band, V. 2010. Histological, molecular and functional subtypes of breast cancers. *Cancer biology & therapy*, *10*(10): 955-960.

Malik, J. 2011. Plants for erectile dysfunction. *International Journal of Natural Product Science,* 1: 9-14.

Malumbres, M. & Barbacid, M. 2006. Is Cyclin D1-CDK4 kinase a bonafide cancer target? *Cancer Cell*, 9: 2–4.

Malumbres, M. & Barbacid, M. 2009. Cell cycle, CDKs and cancer: a changing paradigm. *Nature Reviews Cancer*, *9*(3): 153-166.

Mann, R.M., Bult, P., van Laarhoven, H.W.M., Span, P.N., Schlooz, M., Veltman, J. & Hoogerbrugge, N. 2013. Breast cancer size estimation with MRI in BRCA mutation carriers and other high risk patients. *European journal of radiology*, *82*(9): 1416-1422.

Mann, E., Smith, M.J., Hellier, J., Balabanovic, J.A., Hamed, H., Grunfield, E.A. & Hunter, M.S. 2012. Cognitive behavioural treatment for women who have menopausal symptoms after breast cancer treatment (menos1): a randomised trial*. Lancet oncolology*, 13: 309-18.

Manning, B.D. & Cantley, L.C. 2007. AKT/PKB signaling: navigating downstream. *Cell*, *129*(7): 1261-1274.

Manson, M.M. 2003. Cancer prevention–the potential for diet to modulate molecular signalling. *Trends in molecular medicine*, *9*(1): 11-18.

Markides, C.S., Roy, D. & Liehr, J.G. 1998. Concentration dependence of prooxidant and antioxidant properties of catecholestrogens. *Archieves of Biochemistry Biophysics,* 360: 105–12.

Masuelli, L., Benvenuto, M., Fantini, M., Marzocchella, L., Sacchetti, P., Di Stefano, E. & Bei, R. 2012. Curcumin induces apoptosis in breast cancer cell lines and delays the growth of mammary tumors in neu transgenic mice. *Journal of biological regulators and homeostatic agents*, *27*(1): 105-119.

Mense, S.M., Hei, T.K., Ganju, R.K., & Bhat, H.K. 2008. Phytoestrogens and breast cancer prevention: possible mechanisms of action.

Metivier, R.P., Francis, F.J. & Clydesdale, F.M. 1980. Solvent extraction of anthocyanins from wine pomace. *Journal of Food Science*, *45*(4): 1099-1100.

Mohammedi, Z. & Atik, F. 2011. Impact of solvent extraction type on total polyphenols content and biological activity from Tamarix aphylla (L.) karst. *International Journal of* 129:1261–1274.

McKay, D.L., Chen, C.O., Saltzman, E., & Blumberg, J.B. 2010. Hibiscus sabdariffa L. tea (tisane) lowers blood pressure in prehypertensive and mildly hypertensive adults. *The Journal of nutrition*, *140*(2), 298-303.

Meeran, S.M. & Katiyar, S.K. 2008. Cell cycle control as a basis for cancer chemoprevention through dietary agents. *Frontiers in bioscience: a journal and virtual library*, *13*: 2191.

Meng, S.J., & Yu, L.J. 2010. Oxidative stress, molecular inflammation and sarcopenia. *International journal of molecular sciences*, *11*(4): 1509-1526.

Mensah, J.K. & Golomeke, D. 2015. Antioxidant and antimicrobial activities of the extracts of the Calyx of Hibiscus Sabdariffa Linn. *Current Science Perspectives*, *1*(2): 69-76.

Meyer, M., Essack, M., Kanyanda, S. & Rees, J. 2008. A low-cost flow cytometric assay for the detection and quantification of apoptosis using an anionic halogenated fluorescein dye. *Biotechniques*, *45*(3): 317-320.

Minn, A.J., Rudin, C.M., Boise, L.H. & Thompson, C.B. 1995. Expression of bcl-xL can confer a multidrug resistance phenotype. *Blood*, *86*(5), 1903-1910.

Mizutani, H., Tada-Oikawa, S., Hiraku, Y., Kojima, M. & Kawanishi, S. 2005. Mechanism of apoptosis induced by doxorubicin through the generation of hydrogen peroxide. *Life sciences*, *76*(13), 1439-1453.

Mohamed, R., Fernandez, J., Pineda, M. & Aguilar, M. 2007. Roselle (Hibiscus sabdariffa) seed oil is a rich source of gamma-tocopherol. *Journal of Food Science*, 72(3): S207–S21.

Mohammed, A.A. & Ibrahim, A.A. 2004. Pathological roles of reactive oxygen species and their defence mechanism. *Saudi Pharmacology Journal*, 12: 1–18.

Mohd-Esa, N., Hern, F.S., Ismail, A. & Yee, C.L. 2010. Antioxidant activity in different parts of roselle (Hibiscus sabdariffa L.) extracts and potential exploitation of the seeds. *Food Chemistry*, *122*(4): 1055-1060.

Moinfar, F. 2007. *Essentials of diagnostic breast pathology: a practical approach*. Springer Science & Business Media.

Mojiminiyi, F.B.O., Audu, Z., Etuk, E.U. & Ajagbonna, O.P. 2013. Attenuation of salt-induced hypertension by aqueous calyx extract of Hibiscus sabdariffa. *Nigerian Journal of Physiological Sciences*, *27*(2): 195-200.

Moore, K.L., Dalley, A.F. & Agur, A.M., 2013. *Clinically oriented anatomy*.Lippincott Williams & Wilkins.

Mothana, R.A., Lindequist, U., Gruenert, R. & Bednarski, P.J. 2009. Studies of the *in vitro* anticancer, antimicrobial and antioxidant potentials of selected Yemeni medicinal plants from the island Soqotra. *BMC Complementary and Alternative Medicine*, *9*(1): 7.

Mozaffari-Khosravi, H., Ahadi, Z. & Barzegar, K. 2013. The effect of green tea and sour tea on blood pressure of patients with type 2 diabetes: a randomized clinical trial. *Journal of Dietary Supplements,* 10(2): 105–115.

Murray, A. & Hunt, T., 1993. *The Cell Cycle: An Introduction*. Oxford University Press: New York.

Murray, A.W. 2004. Recycling the cell cycle: cyclins revisited. *Cell*, 116:221–234.

Murrell, G.A., Francis, M.J. & Bromley, L. 1990. Modulation of fibroblast proliferation by oxygen free radicals. *Biochemical Journal*, *265*, 659-665.

Musacchio, A. & Salmon, E.D. 2007.The spindle-assembly checkpoint in space and time. *Nature reviews Molecular cell biology*, *8*(5), 379-393.

Na, H.K., Kim, E.H., Choi, M.A., Park, J.M., Kim, D.H. & Surh, Y.J. 2012. Diallyl trisulfide induces apoptosis in human breast cancer cells through ROS-mediated activation of JNK and AP-1. *Biochemical pharmacology*, *84*(10): 1241-1250.

Nakabeppu Y, Sakumi K, Sakamoto K, Tsuchimoto D, Tsuzuki T,Nakatsu Y. 2006. Mutagenesis and carcinogenesis caused by the oxidation of nucleic acids. *Biological Chemistry*, 387: 373–9.

Ndu, O.O., Nworu, C.S., Ehiemere, C.O., Ndukwe, N.C., & Ochiogu, I.S. 2011. Herb–drug interaction between the extract of Hibiscus sabdariffa L. and hydrochlorothiazide in experimental animals. *Journal of medicinal food*, *14*(6): 640-644.

Nelson, K.K. & Melendez, J.A. 2004. Mitochondrial redox control of matrix metalloproteinases. *Free Radical Biology. Medicine*, 37: 768–784.

Newman, D.J., Cragg, G.M. & Snader, K.M. 2000. The influence of natural products upon drug discovery. *Natural product reports*, *17*(3): 215-234.

Ngan, V.K., Bellman, K., Hill, B.T., Wilson, L. & Jordan, M.A. 2001. Mechanism of mitotic block and inhibition of cell proliferation by the semisynthetic Vinca alkaloids vinorelbine and its newer derivative vinflunine. *Molecular pharmacology*, *60*(1): 225-232.

Nguyen, D.H., Oketch-Rabah, H.A., Illa-Bochaca, I., Geyer, F. C., Reis-Filho, J. S., Mao, J.H., Ravani, S.A., Zavadil, J., Borowsky, D., Jerry, J.D., Dunphy, K.A., Seo, J.H, Haslam, S. & Barcellos-Hoff, M.H. 2011. Radiation acts on the microenvironment to affect breast carcinogenesis by distinct mechanisms that decrease cancer latency and affect tumor type. *Cancer cell*, *19*(5): 640-651.

Ochani, P.C. & D'Mello, P. 2009. Antioxidant and antihyperlipidemic activity of Hibiscus sabdariffa Linn. leaves and calyces extracts in rats. *Indian Journal of Experimental Biology*, 47(4): 276–282.

Ojeda, D., Jimenez-Ferrer, E., Zamilpa, A., Herrera-Arellano, A., Tortoriello, J. & Alvarez, L. 2010.Inhibition of angiotensin converting enzyme (ACE) activity by the anthocyanins delphinidin- and cyanidin-3-O-sambubiosides from *Hibiscus sabdariffa*. *Journal of Ethnopharmacology*, 127(1): 7–10.

Ola, M.S., Nawaz, M. & Ahsan, H. 2011. Role of Bcl-2 family proteins and caspases in the regulation of apoptosis. *Molecular and cellular biochemistry*, *351*(1-2): 41-58.

Olaleye, M.T. 2007. Cytotoxicity and antibacterial activity of Methanolicic extract of Hibiscus sabdariffa. *Journal of Medicinal Plants Research*, 1(1): 9–13.

Olaleye, M.T. & Rocha, B.T. 2008. Acetaminophen-induced liver damage in mice. Effects of some medicinal plants on the oxidative defense system. *Experimental and Toxicologic Pathology*, 59(5): 319–327.

Olalye, M.T. & Rocha, J.B. 2007. Commonly used tropical medicinal plants exhibit distinct *in vitro* antioxidant activities against hepatotoxins in rat liver. *Experimental and Toxicologic Pathology*, 58(6): 433–438.

Olatunji, L.A., Usman, T.O., Adebayo, J.O. & Olatunji, V.A. 2012. Effects of aqueous extract of *Hibiscus sabdariffa* on renal Na (+)-K (+)-ATPase and Ca (2+)-Mg (2+)-ATPase activities in Wistar rats. *Zhong xi yi jie he xue bao= Journal of Chinese integrative medicine*, *10*(9): 1049-1055.

Olvera-García, V., Castaño-Tostado, E., Rezendiz-Lopez, R.I., Reynoso-Camacho, R., González de Mejía, E., Elizondo, G. & Loarca-Piña, G. 2008. Hibiscus sabdariffa L. extracts inhibit the mutagenicity in microsuspension assay and the proliferation of HeLa cells. *Journal of food science*, *73*(5): T75-T81.

O'Reilly, L.A., Ekert, P., Harvey, N., Marsden, V., Cullen, L., Vaux, D. L. & Kumar, S. 2002. Caspase-2 is not required for thymocyte or neuronal apoptosis even though cleavage of caspase-2 is dependent on both Apaf1 and caspase-9. *Cell death and differentiation*, *9*(8): 832-841.

Ouyang, L., Shi, Z., Zhao, S., Wang, F.T., Zhou, T.T., Liu, B. & Bao, J.K. 2012. Programmed cell death pathways in cancer: a review of apoptosis, autophagy and programmed necrosis. *Cell proliferation*, *45*(6): 487-498.

Ozmen, H., Erulas, F.A., Karatas, F., Cukurovali, A. & Yalcin, O. 2006. Comparison of the concentration of trace metals (Ni, Zn, Co, Cu and Se), Fe, vitamins A, C and E, and lipid peroxidation in patients with prostate cancer. *Clinical Chemical Laboratory Medicine*, *44*(2): 175-179.

Page, D.L. 2003. Special types of invasive breast cancer, with clinical implications. *The American journal of surgical pathology*, *27*(6): 832-835.

Panayiotidis, M. 2008. Reactive Oxygen Species (ROS) in multistage carcinogenesis, *Cancer Letters*, 266: 3–5.

Pari, L. & Umamaheswari, J. 2000. Antihyperglcaemic activity of Musa sapientum flowers: effect on lipid peroxidation in alloxan diabetic rats. *Phytotherapy research*, 14: 1-3.

Parkash, J., Felty, Q. & Roy, D. 2004. Differential regulation of high and low capacity mitochondrial calcium in breast cancer. In *Proc. Am. Assoc. Cancer Res,* 44:715).

Patel, S. 2014. *Hibiscus sabdariffa*: An ideal yet under-exploited candidate for nutraceutical applications. *Biomedicine & Preventive Nutrition*, *4*(1): 23-27.

Paul, S., Sengupta, S., Bandyopadhyay, T.K. & Bhattacharyya, A. 2012. Stevioside induced ROS-mediated apoptosis through mitochondrial pathway in human breast cancer cell line MCF-7. *Nutrition and cancer*, *64*(7): 1087-1094.

Pelicano, H., Carney, D. & Huang, P. 2004. ROS stress in cancer cells and therapeutic implications. *Drug Resistance Updates*, *7*(2): 97-110.

Peng, C.H., Chyau, C.C., Chan, K.C., Chan, T.H., Wang, C.J. & Huang, C.N. 2011. *Hibiscus sabdariffa* polyphenolic extract inhibits hyperglycemia, hyperlipidemia, and glycation-oxidative stress while improving insulin resistance. *Journal of agricultural and food chemistry*, *59*(18): 9901-9909.

Perera, C.S., Clair, D.K.S. & McClain, C.J. 1995. Differential regulation of manganese superoxide dismutase activity by alcohol and TNF in human hepatoma cells. *Archives of biochemistry and biophysics*, *323*(2): 471-476.

Perou, C.M., Sorlie, T., Eisen, M.B., van de Rijn M, Jeffrey, S.S. & Rees, C.A. 2000. Molecular portraits of human breast tumours. *Nature*, 406: 747–752.

Pervaiz, S. & Clement, M.V. 2004. Tumor intracellular redox status and drug resistance-serendipity or a causal relationship? *Current pharmaceutical design*, *10*(16): 1969-1977.

Petit, J.Y., Veronesi, U., Orecchia, R., Rey, P., Didier, F., Giraldo, A., Luini, A., Delorenzi, F., Rietjens, M., Garusi, C., Intra, M., Yamaguchi, S. & Martella, S. 2002. The nipple-sparing mastectomy: early results of a feasibility study of a new application of perioperative radiotherapy (ELIOT) in the treatment of breast cancer when mastectomy is indicated. *Tumori*, *89*(3): 288-291.

Peto, R., Boreham, J., Clarke, M., Davies, C. & Beral, V. 2000. UK and USA breast cancer deaths down 25% in year 2000 at ages 20-69 years. *Lancet*, *355*(9217): 1822.

Petros, A. M., Olejniczak, E. T., & Fesik, S. W. 2004. Structural biology of the Bcl-2 family of proteins. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1644*(2), 83-94.

Petrucelli, N., Daly, M.B., & Feldman, G.L. 2010. Hereditary breast and ovarian cancer due to mutations in BRCA1 and BRCA2. *Genetics in Medicine*, *12*(5): 245-259.

Pham-Huy, L.A., He, H. & Pham-Huy, C. 2008. Free radicals, antioxidants in disease and health. *International journal of biomedical science: IJBS*, *4*(2): 89.

Pharoah, P D., Day, N.E., Duffy, S., Easton, D.F. & Ponder, B.A. 1997. Family history and the risk of breast cancer: A systematic review and meta-analysis. *International Journal of cancer*, *71*(5): 800-809.

Plas, D.R. & Thompson, C.B. 2005. Akt-dependent transformation: there is more to growth than just surviving. *Oncogene,* 24: 7435–7442.

Poortmans, P., Bartelink, H., Horiot, J. C., Struikmans, H., Van den Bogaert, W., Fourquet, A., Jager, J., Hoogenraad, W., Rodrigus, P., Warlam-Rodenhuis, C., Collette, L. & Pierart, M. 2004. EORTC Radiotherapy and Breast Cancer Groups. The influence of the boost technique on local control in breast conserving treatment in the EORTC 'boost versus no boost'randomised trial. *Radiotherapy Oncology*, *72*(1): 25-33.

Powis, G. & Baker A. 1997. Redox signaling and the control of cell growth and death.*Advanced Pharmacolology,* 38: 329–59.

Poyton, R.O., Ball, K.A. & Castello, P.R. 2009. Mitochondrial generation of free radicals and hypoxic signaling. *Trends in Endocrinology & Metabolism*, *20*(7): 332-340.

Prasad, C.P., Rath, G., Mathur, S., Bhatnagar, D. & Ralhan, R. 2009. Potent growth suppressive activity of curcumin in human breast cancer cells: Modulation of Wnt/β-catenin signaling. *Chemico-biological interactions*, *181*(2): 263-271.

Prat, A., Parker, J. S., Karginova, O., Fan, C., Livasy, C., Herschkowitz, J.I., He, X. & Perou, C.M. 2010. Phenotypic and molecular characterization of the claudin-low intrinsic subtype of breast cancer. *Breast Cancer Res*, *12*(5): R68.

Preethi, R., Devanathan, V.V. & Loganathan, M. 2010. Antimicrobial and antioxidant efficacy of some medicinal plants against food borne pathogens. *Advances in Biological Research*, *4*(2): 122-125.

Preston, D.L., Mattsson, A., Holmberg, E., Shore, R., Hildreth, N.G. & Boice Jr, J.D. 2009. Radiation effects on breast cancer risk: a pooled analysis of eight cohorts.

Raffo, A.J., Perlman, H., Chen, M.W., Day, M.L., Streitman, J.S. & Buttyan, R. 1995. Overexpression of bcl-2 protects prostate cancer cells from apoptosis *in vitro* and confers resistance to androgen depletion *in vivo*. *Cancer Research*, *55*(19): 4438-4445.

Rafieian-Kopaei, M., 2011. Medicinal plants and the human needs. *Journal of Biololgy*, *35*: 635-9.

Rahman, K. 2007. Studies on free radicals, antioxidants, and co-factors. *Clinical Interventions in Aging,* 2: 219-236.

Raj, G.V., Moreno, J.G. & Gomella, L.G. 1998. Utilization of polymerase chain reaction technology in the detection of solid tumors. *Cancer*, *82*(8): 1419-1442.

Ramakrishna, B.V., Jayaprakasha, G., Jena, B. S. & Singh, R.P. 2008. Antioxidant activities of roselle (*Hibiscus sabdariffa*) calyces and fruit extracts. *Journal of Food Science and Technology*, *45*(3): 223-227.

Ramos, S. 2007. Effects of dietary flavonoids on apoptotic pathways related to cancer chemoprevention. *The Journal of nutritional biochemistry*, *18*(7): 427-442.

Ramos, S. 2008. Cancer chemoprevention and chemotherapy: dietary polyphenols and signalling pathways. *Molecular nutrition & food research*, *52*(5): 507-526.

Rath, P.C. & Aggarwal, B.B. 2001. Antiproliferative effects of IFN-alpha correlate with the downregulation of nuclear factor-kappa B in human Burkitt lymphoma Daudi cells. *Journal of Interferon and Cytokine Research,* 21: 523–528.

Rautenbach, F., Faber, M., Laurie, S. & Laurie, R. 2010. Antioxidant capacity and antioxidant content in roots of 4 sweetpotato varieties. *Journal of Food Science*, 75(5): C400-C405.

Ravi, A., Mallika, A., Venkatesh, S., Arunasree, M.K., Vamshi, K.I., & Madhava, R.B., 2012. Anticancer activity of pupalialappacea on chronic myeloid leukaemia K562 cells. DARU *Journal of Pharmarcological Sciences*, 20: 86-90.

Ray, P.D., Huang, B.W., & Tsuji, Y. 2012. Reactive oxygen species (ROS) homeostasis and redox regulation in cellular signaling. *Cellular signalling*, *24*(5): 981-990.

Reesink-Peters, N., Hougardy, B.M.T., Van den Heuvel, F.A.J., Ten Hoor, K.A., Hollema, H., Boezen, H.M. & van der Zee, A.G.J. 2005. Death receptors and ligands in cervical carcinogenesis: an immunohistochemical study. *Gynecologic oncology*, *96*(3): 705-713.

Renis, M., Calandra, L., Scifo, C., Tomasello, B., Cardile, V., Vanella, L., & Galvano, F. 2008. Response of cell cycle/stress-related protein expression and DNA damage upon treatment of CaCo2 cells with anthocyanins. *British journal of nutrition*, *100*(01): 27-35.

Reuter, S., Gupta, S.C., Chaturvedi, M.M. & Aggarwal, B.B. 2010. Oxidative stress, inflammation, and cancer: how are they linked? *Free Radical Biology and Medicine*, *49*(11): 1603-1616.

Reynolds, P., 2013. Smoking and breast cancer. *Journal of mammary gland biology and neoplasia*, *18*(1): 15-23.

Ricci, J.E., Gottlieb, R.A. & Green, D.R. 2003. Caspase-mediated loss of mitochondrial function and generation of reactive oxygen species during apoptosis. *The Journal of cell biology*, *160*(1): 65-75.

Rohn, J.L. & Noteborn, M.H.M. 2004. The viral death effector Apoptin reveals tumor-specific processes. *Apoptosis*, *9*(3), 315-322.

Roja, G. & Rao, P.S. 2000. Anticancer compounds from tissue cultures of medicinal plant. *Journal of Herbs, Spices.and Medicinal Plants*, 7: 71-102.

Russo, M., Spagnuolo, C., Tedesco, I., & Russo, G. L. 2010. Phytochemicals in cancer prevention and therapy: truth or dare? *Toxins*, *2*(4): 517-551.

Rutqvist, L.E., Cedermark, B., Glas, U., Mattsson, A., Skoog, L., Somell, A. & Ringborg, U. 1991. Contralateral primary tumors in breast cancer patients in a randomized trial of adjuvant tamoxifen therapy. *Journal of the National Cancer Institute*, *83*(18): 1299-1306.

Salem, M.Z., Olivares-Pérez, J., & Salem, A.Z.M. 2014. Studies on biological activities and phytochemicals composition of Hibiscus species-A review. *Life Science Journal*, *11*(5).

Sarabia, S.F., Zhu, B.T., Kurosawa, T., Tohma, M. & Liehr, J.G. 1997. Mechanism of cytochrome P450-catalyzed aromatic hydroxylation of estrogens. *Chemical research in toxicology*, *10*(7): 767-771.

Savill, J. & Fadok V. 2000. Corpse clearance defines the meaning of cell death. *Nature* 407:784–788.

Schiff, R., Reddy, P., Ahotupa, M., Coronado-Heinsohn, E., Grim, M., Hilsenbeck, S.G. & Osborne, C.K. 2000. Oxidative stress and AP-1 activity in tamoxifen-resistant breast tumors *in vivo*. *Journal of the National Cancer Institute*, *92*(23): 1926-1934.

Schröder, P. & Krutmann, J. 2000. Environmental oxidative stress–Environmental sources of ROS. In *Reactions, Processes,* (pp. 19-31). Springer Berlin Heidelberg.

Seujange, Y., Leelahavanichkul, A., Yisarakun, W., Khawsuk, W., Meepool, A., Phamonleatmongkol, P. & Eiam-Ong, S. 2013. *Hibiscus sabdariffa* Linnaeus aqueous extracts attenuate the progression of renal injury in 5/6 nephrectomy rats. *Renal failure*, *35*(1), 118-125.

Sharma, A., Tripathi, M., Satyam, A., and Kumar, L. 2009. Study of antioxidant levels in patients with multiple myeloma. *Leukemia & Lymphoma* 50: 809-815.

Shaw, R.J. & Cantley, L.C. 2006. Ras, PI (3)K and mTOR signalling controls tumour cell growth. *Nature* 441: 424–430.

Shen, J., Terry, M.B., Gurvich, I., Liao, Y., Senie, R.T. & Santella, R. M., 2007. Short telomere length and breast cancer risk: a study in sister sets. *Cancer Research*, *67*(11): 5538-5544.

Shen, X.G., Wang, C., Li, Y., Wang, L., Zhou, B., Xu, B. & Sun, X. F. 2010. Downregulation of caspase-9 is a frequent event in patients with stage II colorectal cancer and correlates with poor clinical outcome. *Colorectal Disease*, *12*(12): 1213-1218.

Shi, X., Zhao, Y., Jiao, Y., Shi, T., & Yang, X. 2013. ROS-dependent mitochondria molecular mechanisms underlying antitumor activity of Pleurotus abalonus acidic polysaccharides in human breast cancer MCF-7 cells.

Shi, Y. 2002. Mechanisms of caspase activation and inhibition during apoptosis. *Molecular cell*, *9*(3), 459-470.

Shi, Y., Yang, S., Troup, S., Lu, X., Callaghan, S., Park, D.S., & Yang, X. 2011. Resveratrol induces apoptosis in breast cancer cells by E2F1-mediated up-regulation of ASPP1. *Oncology reports*, *25*(6): 1713-1719.

Shigemori, H. & Kobayashi, J.I. 2004. Biological Activity and Chemistry of Taxoids from the Japanese Yew, Taxus c uspidata⊥. *Journal of natural products*, *67*(2): 245-256.

Sies, H. (Ed.). 2013. *Oxidative stress*. Elsevier.

Sindi, H.A., Marshall, L.J. & Morgan, M.R. 2014. Comparative chemical and biochemical analysis of extracts of *Hibiscus sabdariffa*. *Food chemistry*, *164*: 23-29.

Singh, B.N., Shankar, S. & Srivastava, R.K. 2011. Green tea catechin, epigallocatechin-3-gallate (EGCG): mechanisms, perspectives and clinical applications. *Biochemical pharmacology*, *82*(12), 1807-1821.

Singh, P.P., Chandra, A., Mahdi, F., Ray, A. & Sharma, P., 2010. Reconvene and reconnect the antioxidant hypothesis in Human health and disease. *Indian Journal of Clinical Biochemistry.* 25, 225–243.

Singleton, V.L. & Rossi, J.A. 1965. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. *American journal of Enology and Viticulture*, *16*(3): 144-158.

Slater, A.F., Stefan, C., Nobel, I., van den Dobbelsteen, D.J. & Orrenius, S. 1995. Signalling mechanisms and oxidative stress in apoptosis. *Toxicology Letters*, 82–83: 149–53.

Slatter, T.L., Hung, N., Campbell, H., Rubio, C., Mehta, R., Renshaw, P., & Braithwaite, A.W. 2011. Hyperproliferation, cancer, and inflammation in mice expressing a Δ133p53-like isoform. *Blood*, *117*(19): 5166-5177.

Smith, P.K., Krohn, R.I, Hermanson, G.T, Mallia, A.K, Gartner, F.H, Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J. & Klenk, D.C. 1985. Measurement of protein using bicinchoninic acid. *Analytical Biochemistry*, 150(1): 76–85.

Solary, E., Droin, N., Bettaieb, A., Corcos, L., Dimanche-Boitrel, M.T. & Garrido, C. 2000. Positive and negative regulation of apoptotic pathways by cytotoxic agents in hematological malignancies. *Leukemia*, *14*(10): 1833-1849.

Sørlie, T., Perou, C. M., Tibshirani, R., Aas, T., Geisler, S., Johnsen, H., Hastie, T., Eisein, M.B., Van de Rijin, M., Jeffrey, S.S., Thosern, T., Quist, H., Matese, J.c., Brown, P.O., Botstein, D., Eystein Lonning, P., & Børresen-Dale, A. L. 2001. Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. *Proceedings of the National Academy of Sciences*, *98*(19): 10869-10874.

Soto, A.M., Maffini, M.V., Schaeberle, C.M. & Sonnenschein, C. 2006. Strengths and weaknesses of *in vitro* assays for estrogenic and androgenic activity. *Best Practice & Research Clinical Endocrinology & Metabolism*, *20*(1): 15-33.

Sprick, M.R. & Walczak, H. 2004. The interplay between the Bcl-2 family and death receptor-mediated apoptosis. *Biochimica et Biophysica Acta (BBA)-Molecular Cell Research*, *1644*(2): 125-132.

Srivastava, R. K., Kurzrock, R. & Shankar, S. 2010. MS-275 sensitizes TRAIL-resistant breast cancer cells, inhibits angiogenesis and metastasis, and reverses epithelial-mesenchymal transition *in vivo*. *Molecular cancer therapeutics*, *9*(12), 3254-3266.

Stedman T., 2000. Stedman's Medical Dictionary, 27th Edition Baltimore Lippincott, Williams & Wilkins

Steenvoorden, D.P., & van Henegouwen, G.M.B. 1997. The use of endogenous antioxidants to improve photoprotection. *Journal of Photochemistry and Photobiology B: Biology*, *41*(1): 1-10.

Stingl, J. & Caldas, C., 2007. Molecular heterogeneity of breast carcinomas and the cancer stem cell hypothesis. *Nature Reviews Cancer*, *7*(10): 791-799.

Storz, P. 2005. Reactive oxygen species in tumor progression. *Frontier Bioscience*, *10*(1-3), 1881-1896.

Stover, D.G., Bierie, B. & Moses, H.L. 2007. A delicate balance: TGF-β and the tumor microenvironment. *Journal of cellular biochemistry*, *101*(4), 851-861.

Strober, W. 2001. Trypan blue exclusion test of cell viability. *Current protocols in immunology*, A-3B.

Stuffness, M. & Pezzuto J.M. 1990. Assays related to cancer drug discovery K. Hostettmann (Ed.), *Methods in Plant Biochemistry*: Assays for Bioactivity, Academic Press, London 6:71–133.

Syamsuddin, S.P., Djamil, R. & Heffen, W. L. 2010. Apoptosis of human breast cancer cells induced by ethylacetate extracts of propolis. *American Journal of Biochemical & Biotechnology*, *6*(2): 84-88.

Szatrowski, T.P. & Nathan, C.F. 1991. Production of large amounts of hydrogen peroxide by human tumor cells. *Cancer research*, *51*(3): 794-798.

Tang, F.Y., Su, Y.C., Chen, N.C., Hsieh, H.S., & Chen, K.S. 2008. Resveratrol inhibits migration and invasion of human breast-cancer cells. *Molecular nutrition & food research*, *52*(6): 683-691.

Tarin, D. 2008. Comparisons of metastases in different organs: biological and clinical implications. *Clinical Cancer Research*, *14*(7): 1923-1925.

Tas, F., Hansel, H., Belce, A., Ilvan, S., Argon, A., Camlica, H., & Topuz, E. 2005. Oxidative stress in breast cancer. *Medical Oncology*, *22*(1): 11-15.

Tavassoli, F.A., Devilee, P., 2003. *World Health Organization Classification of Tumours. Pathology and Genetics of Tumours of the Breast and Female Genital Organs*. IARC Press: Lyon.

Thornberry, N.A. & Lazebnik, Y. 1998. Caspases: enemies within. *Science*, *281*(5381): 1312-1316.

Torre, L.A., Bray, F., Siegel, R.L., Ferlay, J., Lortet-Tieulent, J. & Jemal, A. 2015. Global cancer statistics, 2012. *CA. A Cancer Journal for Clinicians*, *65*(2): 87-108.

Traber, M.G., & Atkinson, J. 2007. Vitamin E, antioxidant and nothing more. *Free Radical Biology and Medicine*, *43*(1), 4-15.

Trachootham, D., Zhou, Y., Zhang, H., Demizu, Y., Chen, Z., Pelicano, H., & Huang, P. 2006. Selective killing of oncogenically transformed cells through a ROS-mediated mechanism by β-phenylethyl isothiocyanate. *Cancer cell*, *10*(3): 241-252.

Tsai, T.C., Huang, H.P., Chang, Y.C. & Wang., C.J. 2013. An anthocyanin rich extract from HBA Linnaeus inhibits N-Nitrosomethylurea induced leukaemia in rats. *Journal of agricultural and food chemistry*, 10: 1021.

Tseng, T.H., Kao, T.W., Chu, C.Y., Chou, F.P., Lin, W.L. & Wang, C.J. 2000. Induction of apoptosis by hibiscus protocatechuic acid in human leukemia cells via reduction of retinoblastoma (RB) phosphorylation and Bcl-2 expression. *Biochemical Pharmacology*, *60*(3), 307-315.

Tseng, T.H., Wang, C.J., Kao, E.S. & Chu, H.Y. 1996. Hibiscus protocatechuic acid protects against oxidative damage induced by tert-butylhydroperoxide in rat primary hepatocytes. *Chemico-Biological Interactions*, 101(2): 137–148.

Tseng, T.H., Kao, E.S., Chu, C.Y., Chou, F.P., Lin Wu, H.W. & Wang, C.J. 1997.Protective effects of dried flower extracts of *Hibiscus sabdariffa* L. against oxidative stress in rat primary hepatocytes. *Food and Chemical Toxicology*, 35(12): 1159–1164.

Turunen, M., Olsson, J. & Dallner, G. 2004. Metabolism and function of coenzyme Q. *Biochmica et Biophysica. Acta,* 1660, 171–199.

Valko, M., Leibfritz, D., Moncol, J., Cronin, M. T., Mazur, M. & Telser, J. 2007. Free radicals and antioxidants in normal physiological functions and human disease. *The international journal of biochemistry & cell biology*, *39*(1), 44-84.

Valko, M., Rhodes, C.J., Moncol, J., Izakovic, M. & Mazur, M. 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chemical Biological Interaction,* 160: 1–40.

Vander Heiden, M.G., Plas, D.R., Rathmell, J.C., Fox, C.J., Harris, M.H. & Thompson, C.B. 2001. Growth factors can influence cell growth and survival through effects on glucose metabolism. *Molecular and cellular biology*, *21*(17): 5899-5912.

Vanhoefer, U., Harstrick, A., Achterrath, W., Cao, S., Seeber, S. & Rustum, Y.M. 2001. Irinotecan in the treatment of colorectal cancer: clinical overview. *Journal of Clinical Oncology*, *19*(5): 1501-1518.

Vaquero, E.C., Edderkaoui, M., Pandol, S.J., Gukovsky, I. & Gukovskaya, A.S. 2004. Reactive oxygen species produced by NAD (P) H oxidase inhibit apoptosis in pancreatic cancer cells. *Journal of Biological Chemistry*, *279*(33): 34643-34654.

Vasudeva, N. & Sharma, S.K. 2008. Post-coital antifertility activity of Hibiscus rosa-sinensis Linn.roots: Evidence-Based Complement. *Alternative Medicine*, 5: 91−94.

Vaux, D.L. & Silke, J. 2003. Mammalian mitochondrial IAP binding proteins. *Biochemical and biophysical research communications*, *304*(3): 499-504.

Vera-Ramirez, L., Sanchez-Rovira, P., Ramirez-Tortosa, M.C., Ramirez-Tortosa, C.L., Granados-Principal, S., Lorente, J.A. & Quiles, J.L. 2011.Free radicals in breast carcinogenesis, breast cancer progression and cancer stem cells. Biological bases to develop oxidative-based therapies. *Critical reviews in oncology/hematology*, *80*(3): 347-368.

Veronesi, U., Boyle, P., Goldhirsch, A., Orecchia, R. & Viale, G. 2005. Breast Cancer. *Lancet*, 365: 1727-1741.

Vidhya, N., & Niranjali Devaraj, S. 2011. Induction of apoptosis by eugenol in human breast cancer cells. *Indian journal of experimental biology*, *49*(11), 871.

Vikhanskaya, F., Lee, M. K., Mazzoletti, M., Broggini, M., & Sabapathy, K. 2007. Cancer-derived p53 mutants suppress p53-target gene expression—potential mechanism for gain of function of mutant p53. *Nucleic acids research*, *35*(6): 2093-2104.

Visconti, R. & Grieco, D. 2009. New insights on oxidative stress in cancer.*Current opinion in drug discovery & development*, *12*(2): 240-245.

Visvader, J.E. 2009.Keeping abreast of the mammary epithelial hierarchy and breast tumorigenesis. *Genes & development*, *23*(22): 2563-2577.

Visvader, J.E. & Lindeman, G.J. 2008.Cancer stem cells in solid tumours: accumulating evidence and unresolved questions. *Nature Reviews Cancer*; 8:755–68.

Wajant, H. 2002. The Fas signaling pathway: more than a paradigm. *Science* 296:1635–1636.

Wang, C. J., Wang, J. M., Lin, W. L., Chu, C.Y., Chou, F.P. & Tseng, T.H. 2000. Protective effect of *Hibiscus Sabdariffa* against tert-butyl hydroperoxide-induced hepatic toxicity in rats. *Food Chemical Toxicolology*, 38(5):411-416.

Wang, H.K., Liu, S.Y., Hwang, K.M., Taylor, G. & Lee, K.H. 1994. Synthesis of novel water-soluble 7-(aminoacylhydrazono)-formyl camptothecins with potent inhibition of DNA topoisomerase I. *Bioorganic & medicinal chemistry*, *2*(12): 1397-1402.

Wang, J. & Lenardo, M.J. 2000. Roles of caspases in apoptosis, development, and cytokine maturation revealed by homozygous gene deficiencies. *Journal of Cell Science*, *113*(5), 753-757.

Wang, L.S. & Stoner, G.D. 2008. Anthocyanins and their role in cancer prevention. *Cancer Letters*, 269:281-290.

Wang, S.C., Lee, S.F., Wang, C.J., Lee, C.H., Lee, W.C. & Lee, H.J. 2011. Aqueous extract from *Hibiscus sabdariffa* Linnaeus ameliorate diabetic nephropathy via regulating oxidative status and Akt/Bad/14-3-3γ in an experimental animal model. *Evidence-Based Complementary and Alternative Medicine*, *2011*.

Ward, P.S. & Thompson, C.B., 2012. Metabolic reprogramming: a cancer hallmark even warburg did not anticipate. *Cancer cell*, *21*(3): 297-308.

Wardman, P. 2007. Fluorescent and luminescent probes for measurement of oxidative and nitrosative species in cells and tissues: progress, pitfalls, and prospects. *Free radical biology and medicine*, *43*(7), 995-1022.

Wei, Y., Fan, T. & Yu, M. 2008. Inhibitor of apoptosis proteins and apoptosis. *Acta biochimica et biophysica Sinica*, *40*(4): 278-288.

Weigelt, B., Horlings, H.M., Kreike, B., Hayes, M.M., Hauptmann, M., Wessels, L.F.A., de Jong, D., Van de Vijver, M.J., Van't Vee, L.T., & Peterse, J.L. 2008. Refinement of breast cancer classification by molecular characterization of histological special types. *The Journal of pathology*, *216*(2): 141-150.

Wen, X., Lin, Z. Q., Liu, B., & Wei, Y. Q. 2012. Caspase-mediated programmed cell death pathways as potential therapeutic targets in cancer. *Cell proliferation*, *45*(3), 217-224.

Wenzel, U., Schoberl, K., Lohner, K. & Daniel, H. 2005. Activation of mitochondrial lactate uptake by flavone induces apoptosis in human colon cancer cells. *Journal of cellular physiology*, *202*(2): 379-390.

Widschwendter, M. & Jones, P.A., 2002. DNA methylation and breast carcinogenesis. *Oncogene*, *21*(35): 5462-5482.

Williams, G.H. & Stoeber K. 2012. The cell cycle and cancer. *The Journal of pathology*, *226*(2): 352-364.

Wisetmuen, E., Pannangpetch, P., Kongyingyoes, B., Kukongviriyapan, U., Yutanawiboonchai, W., & Itharat, A. 2013. Insulin secretion enhancing activity of roselle calyx extract in normal and streptozotocin-induced diabetic rats. *Pharmacognosy research*, *5*(2): 65.

Wolin, M.S. 2009. Reactive oxygen species and the control of vascular function. *American Journal of Physiology-Heart and Circulatory Physiology*, *296*(3): H539-H549.

Wong, A.H., Gottesman, I.I., & Petronis, A. 2005. Phenotypic differences in genetically identical organisms: the epigenetic perspective. *Human molecular genetics*, *14*(suppl 1), R11-R18.

Wong, C.C., Li, H.B., Cheng, K.W., & Chen, F., 2006. A systematic survey of antioxidant activity of 30 Chinese medicinal plants using the ferric reducing antioxidant power assay. *Food Chemistry*, *97*(4): 705-711.

Wong, R.S. 2011. Apoptosis in cancer: from pathogenesis to treatment. *Journal of Experimental & Clinical Cancer Research*, *30*(1): 87.

Woo, R.A., Melure, K.G., Lee, P.W. 1998. DNA dependent protein kinase acts upstream of p53 in response to DNA damage. *Nature*., 394: 700–4.

Woo, J.H., Kim, Y.H., Choi, Y.J., Kim, D.G., Lee, K.S., Bae, J.H. & Kwon, T.K. 2003. Molecular mechanisms of curcumin-induced cytotoxicity: induction of apoptosis through generation of reactive oxygen species, down-regulation of Bcl-XL and IAP, the release of cytochrome c and inhibition of Akt. *Carcinogenesis*, *24*(7), 1199-1208.

World Health Organization. 1982. The World Health Organization histological typing of breast tumors. *American Journal of Clinical Pathology*, *78*(6): 806-816.

Wu, X., Liu, X., Sengupta, J., Bu, Y., Yi, F., Wang, C. & Song, F. 2011. Silencing of Bmi-1 gene by RNA interference enhances sensitivity to doxorubicin in breast cancer cells.

Wuerges, J., Lee, J.W., Yim, Y.I., Yim, H.S., Kang, S.O. & Carugo, K.D. 2004. Crystal structure of nickel-containing superoxide dismutase reveals another type of active site. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(23), 8569-8574.

www.cansa.org.za/South-African-cancer-statistics

Yager, J.D. & Davidson, N.E. 2006. Estrogen carcinogenesis in breast cancer. *New England Journal of Medicine*, *354*(3): 270-282.

Yamamoto, T., Hsu, S., Lewis, J., Wataha, J., Dickinson, D., Singh, B. & Schuster, G. 2003. Green tea polyphenol causes differential oxidative environments in tumor versus normal epithelial cells. *Journal of Pharmacology and Experimental therapeutics*, *307*(1), 230-236.

Yan, N. & Shi, Y. 2005. Mechanisms of apoptosis through structural biology.*Annual. Review of. Cell & Developmental. Biology.*, *21*: 35-56.

Yang, G.Y., Liao, J., Li, C., Chung, J., Yurkow, E. J., Ho, C.T. & Yang, C.S. 2000. Effect of black and green tea polyphenols on c-jun phosphorylation and H2O2 production in transformed and non-transformed human bronchial cell lines: possible mechanisms of cell growth inhibition and apoptosis induction. *Carcinogenesis*, *21*(11): 2035-2039.

Yang, L., Gou, Y., Zhao, T., Zhao, J., Li, F., Zhang, B., & Wu, X. 2012. Antioxidant capacity of extracts from calyx fruits of roselle (*Hibiscus sabdariffa* L.). African *Journal of Biotechnology*, 11, 4063–4068

Yang, M.Y., Peng, C.H., Chan, K.C., Yang, Y.S., Huang, C.N. & Wang, C. J. 2010. The hypolipidemic effect of *Hibiscus sabdariffa* polyphenols via inhibiting lipogenesis and promoting hepatic lipid clearance. *Journal of Agricultural and Food Chemistry*, 58(2), 850–859.

Yao, L., Jiang, Y., Datta, N., Singanusong, R., Liu, X., Duan, J., & Xu, Y. 2004. HPLC analyses of flavanols and phenolic acids in the fresh young shoots of tea (Camellia sinensis) grown in Australia. *Food Chemistry*, *84*(2): 253-263.

Yen, G.C., Duh, P.D. & Tsai, C.L., 1993. Relationship between antioxidant activity and maturity of peanut hulls. *Journal of agricultural and food chemistry*, 41**:**67-70.

Youle, R. J., & Strasser, A. 2008. The BCL-2 protein family: opposing activities that mediate cell death. *Nature reviews Molecular cell biology*, *9*(1): 47-59.

Young, I.S. & Woodside, J.V. 2001.Antioxidants in health and disease. *Journal of Clinical Pathology, 54(3): 176-86.*

Zafra-Stone, S., Yasmin, T., Bagchi, M., Chatterjee, A., Vinson, J. A., & Bagchi, D. 2007. Research Article Berry anthocyanins as novel antioxidants in human health and disease prevention. *Molecular Nutrition. Food & Reseach*, *51*, 675-683.

Zaidi, S.K.R., Al-Qirim, T.M. & Banu, N. 2005. Effects of antioxidant vitamins on glutathione depletion and lipid peroxidation induced by restraint stress in the rat liver. *Drugs in R & D*, *6*(3): 157-165.

Zelko, I.N., Mariani, T.J. & Folz, R.J. 2002. Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. *Free Radical Biology and Medicine*, *33*(3): 337-349.

Zhang, J., Yang, P.L. & Gray, N.S., 2009. Targeting cancer with small molecule kinase inhibitors. *Nature reviews Molecular cell biology*, 9: 28–39.

Zhang, Y., Luo, M., Zu, Y., Fu, Y., Gu, C., Wang, W. & Efferth, T. 2012. Dryofragin, a phloroglucinol derivative, induces apoptosis in human breast cancer MCF-7 cells through ROS-mediated mitochondrial pathway. *Chemico-biological interactions*, *199*(2): 129-136.

Zhao, Y., Wu, M., Shen, Y. & Zhai, Z., 2001. Analysis of nuclear apoptotic process in a cell-free system. *Cellular and Molecular Life Sciences, 58*: 298-306.

Zhou, Y., Hileman, E.O., Plunkett, W., Keating, M.J. & Huang, P., 2003. Free radical stress in chronic lymphocytic leukemia cells and its role in cellular sensitivity to ROS-generating anticancer agents. *Blood,* 101: 4098–4104.

Ziech, D., Franco, R., Pappa, A. & Panayiotidis, M.I. 2011. Reactive Oxygen Species (ROS)––Induced genetic and epigenetic alterations in human carcinogenesis. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *711*(1): 167-173.