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HOLARRHENA FLORIBUNDA LEAVES AS A POTENTIAL SOURCE OF BIOACTIVE ANTICANCER COMPOUNDS

by

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DECLARATION

I, **Badmus Jelili Abiodun**, hereby declare that the dissertation "***Holarrhena floribunda* leaves as a potential source of bioactive anticancer compounds**" hereby submitted by me for the PhD degree in Medical BioSciences at the University of the Western Cape has not been submitted previously at this or any other university, and that it is my own work in design and in execution, and that all reference materials contained herein have been duly acknowledged.

Badmus J. A. : .....

Date Signed : .....

DEDICATION

This dissertation is dedicated to my parents, Mr and Mrs Badmus, for their efforts towards my education. To my wife, Haleema Oyekemi, for her support and steadfastness towards my academic success and for keeping my home for 3 years while outside the country in pursuance of my doctoral degree. To my children: Oyindamola, Omorinola, Olaonipekun and Olamiposi, for constantly reminding me to come home.

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ABSTRACT

Cancer is one of the leading causes of morbidity and mortality in developed and developing nations. It is estimated that 86% of new cases and 64% of death due to cancer are from Africa and 13.1 million deaths are estimated to occur worldwide by the year 2030. Cancer death rates have not subsided despite recent advances in cancer drug development and treatment. Present cancer drug regimens are limited due to unpredictable efficiency, severe side effects, resistance and high cost. Plants provide a vast array of natural compounds such as terpenoids, phenolics and alkaloids with antiproliferative pro-apoptotic and antioxidant effects. Plants are principal sources of compounds for drug discovery and development of several clinically proven useful anticancer drugs. The present study focused on the isolation of compounds from the *Holarrhena floribunda* (*H. floribunda*) leaves for their potential anticancer activities.

Standard methods were employed to assess the antiproliferative potential, apoptosis, cell cycle analysis and reactive oxygen species of the methanolic leaf extract (MLE) of *H. floribunda*. The standard methods of isolation such as column chromatography, thin layer chromatography, high performance liquid chromatography (HPLC) and nuclear magnetic resonance (NMR) were used to isolate and purify bioactive compounds from the leaves. To elucidate the mechanism of cytotoxicity of the isolated compounds, apoptosis effect was studied by flow cytometry analysis using the Apopercentage^{TM} dye, Annexin-V/PI stain, induction of caspase-3 using the Caspase-3/7 Glo assay kit and PARP-1 deactivation using Western blot analysis. The mode of action was further assessed by evaluating reactive oxygen species (ROS), mitochondrial toxicity, light and fluorescent microscopic morphological evaluations of F-actin and topoisomerase-I relaxation assay. In addition, potential cancer prevention of the plant was also evaluated by assessing the antioxidant activity of the flavonoids compounds isolated from the MLE.

The results of the present study show that the MLE of *H. floribunda* inhibited the proliferation of the cancer cell lines (HeLa, HT-29 and MCF-7) in a dose- and time-dependent manner. The anti-proliferative activity of the methanolic extract is selective towards cancer cells more than normal KMST-6 fibroblast cells used in the study. The extract showed cytotoxicity, cell cycle arrest, induced generation of ROS and apoptosis. The methanolic extract of the leaves led to the isolation of two pure steroidal alkaloid and four flavonoid compounds. The two steroidal alkaloids - holamine and funtumine were found to be selectively cytotoxic to human cancer cells more than the normal fibroblasts. The cytotoxicity of the two steroidal alkaloids was mediated through the induction of apoptosis. The apoptosis induction was found to be triggered by the activation of caspase-3, deactivation of PARP-1, increased ROS, cell cycle arrest at G0/G1 and G2/GM phases, mitochondria toxicity, F-actin disorganization and topoisomerase-I inhibition.

However, four flavonoids (kaempferol-3-O-rutinoside, quercetin-3-galactoside/glucoside, quercetin-3-O-glucoside and kaempferol-3-O-glucoside) isolated were subjected to antioxidant activity assay using oxygen radical absorbance capacity (ORAC), ferric reducing/antioxidant power (FRAP), trolox equivalent antioxidant capacity (TEAC) and lipid peroxidation inhibition. Two flavonoids with a quercetin nucleus were found to be active in the entire antioxidant spectrum, except pro-oxidant activity while the remaining two compounds with a kaempferol nucleus were selective in their activity. Structure-activity relationship analysis shows that the activity of the flavonoids depends on the *ortho* di-hydroxyl group on the B-ring of the flavonoids. The present study on *H. floribunda* has shown that the leaves contain bioactive compounds with potential as anticancer agents and cancer protective and preventive activity. More work is still needed to elucidate other possible anticancer mechanisms that might be relevant to the cytotoxic effects of the two isolated alkaloids.

Key words: alkaloids, antiproliferative acivity, apoptosis, cancer, caspase, cell cycle, chromatography, cytotoxicity, drugs, F-actin, flavonoids, flow cytometry, *Holarrhena floribunda*, lipid peroxidation, mitochondria, mortality, plants and ROS

ABBREVIATIONS

7-AAD	7-Amino actinomycin
ABAP	2'2'-Azobis (2-amidinopropane) dihydrochloride
ABTS	2, 2'azinobis (-3-ethylbenzothiazoline-6-sulphonate)
ANT	Adenine nucleotide translocator
AO	Acridine orange
ATP	Adenosine triphosphate
Annexin-V-PE	Annexin-V-phycoerythrin
Anti-HER	Anti-human epidermal growth factor receptor
Apaf-1	Apoptosis activating factor-1
AIF	Apoptosis inducing factor
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia related
Bax	Bcl-2 associated X-protein
Bcl-2	B cell leukaemia-2
BH	Bcl-2 homology domains
Bid and **tBid**	(Truncated) BH3-interacting domain
BIR	Baculoviral repeat
BrdU	Bromodeoxyuridine or 5-bromo-2'-deoxyuridine
CAD	Caspase-activated deoxyribonulease

CARD	Caspase recruitment domain
Caspase	Cysteine aspartic acid-specific proteases
CDK	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitor(s)
CD95	Cluster of differentiation
CED-3	Cell death abnormality-3
CHK2	Check point kinase-2
Cisplatin	Cis-dichlorodiammineplatinum (II) complex
CLM	Confocal laser microscopy
CM-H_2DCFDA	5-(and-6)-Chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate
CPT	Camptothecin
DAPI	4', 6-diamidino-2-phenylindole
DCM	Dichloromethane
DD	Death domain
DED	Death effector domain
DIABLO	Direct IAP binding protein with low pI
DISC	Death-inducing signalling complex
DMEM	Dulbeco's modified medium
DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
$DiOC_6$(3)	3,3'-diehexyloxocarbocyanine iodide
DcR	Decoy receptor

DR	Death receptor
DR-3	Death receptor-3
E2F	E2 Transcription factor
EBV	Epstein-Barr virus
EDAR	Ectodysplasin A receptor
EDTA	Ethylenediaminetetracetic acid
F-actin	Filamentous actin
FRAP	Ferric reducing antioxidants power
FACS	Florescence activated cell sorter
FADD	Fas-associated death domain
Fas	Fibroblast-associated
FasL	Fas ligand
FCS	Foetal calf serum
FITC	Fluorescein isothiocyanate
FLICE	FADD-like IL-β-converting enzyme
GADD45	Growth arrest DNA damage-inducible 45
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HTLV-1	Human T-cell lymphotropic virus
IAP	Inhibitor of apoptosis
IC_{50}	Concentration at which 50% population of cells die
ICAD	Inhibitor of caspase activated DNase

ICE	Interleukin -1-β-converting caspase enzyme (caspase-1)
IM	Inner membrane
KSHV	Kaposi's sarcoma-associated herpersvirus
MAPK	Mitogen-activated protein kinase
MCPyV	Merkel cell polyomavirus
Mdm2	Murine double-minute 2
MLE	Methanolic Leaf Extract
Myc	Mylocytoma
NFκB	Nuclear factor kappa B
NGFR	Nerve growth factor receptor
NHL	Non-Hodgkin lymphoma
NSCLC	Non-small-cell lung cancer
OM	Outer membrane
OPG	Osteoprotegerin
ORAC	Oxygen radical absorbance capacity
p53	Phosphoprotein 53 (tumour protein/Tp53)
E2F	Family of transcription factors involved in cell cycle
PARP-1	Poly (ADP) ribose polymerase 1
PCD	Programmed cell death
P13K/Akt	Phosphatidylinositol 3-kinase (Serine/threonine-protein kinase)
PKC	Protein kinase c
PS	Phosphotidylserine

Rb	Retinoblastoma
RNS	Reactive nitrogen species
ROCKI	Rho-associated coiled-coil forming kinase
ROS	Reactive oxygen species
RNA	Ribonucleic acid
RPMI	Roswell park memorial institute
SARs	Structural activity relationships
SMs	Secondary metabolites
Smac	Second mitochondrial-derived
TE	Tris-EDTA (ethylenediamine tetraacetic acid)
TEAC	Trolox equivalent antioxidant capacity
TRITC	Tetramethylrhodamine
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labeling
TNF	Tumour necrosis factor
TRADD	Tumour necrosis factor receptor-associated death domain
TRAIL	TNF-related apoptosis-inducing ligand
TSG	Tumour suppressor gene
VDAC	Voltage-dependent anion channel
XRCCI	X-ray repair complementing defective repair in Chinese hamster cells 1.

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CHAPTER 1

INTRODUCTION

1.1 Introduction and Background to the Study

Cancer is a disease at the cellular level involving heritable disorders in cellular control mechanisms. The disease is a manifestation of aetiological and pathogenic disturbances of mechanisms that control cell division, differentiation and homeostasis. It presents more than 100 distinct clinical pathologies (Ji-Hwan et al., 2007). It is the largest single cause of death in both men and women, claiming over 7 million lives each year worldwide. More than 11 million people are diagnosed with cancer every year. It is estimated that globally, there will be 16 million new cases every year by 2020 (Anand et al., 2008). Despite intensive research and advances in scientific knowledge relating to cancer and associated disease conditions, the death rate from some common forms of cancer such as lung, breast and colon continues to rise (Sporn, 1996). In addition, improvements in the detection and treatment of cancer have not translated into decline in the overall rate of mortality for most cancers of epithelial origin in the last 30 years (Sporn, 1997). This is mainly because they are limited by tumour drug resistance and side effects of anticancer drugs on normal tissues and cells (Lee, 2001).

Colorectal cancer accounts for over 90% of malignant tumours of the large bowel (Greenstein et al., 1989). Death due to colon cancer alone is about 639,000 worldwide per year and it is the third leading cause of cancer-related deaths worldwide (WHO, 2009). This malignancy is caused by environmental factors and chemicals in diets capable of promoting tumour growth (Pisani et al., 2002) as well as some drugs and cosmetics (Rojanapo et al., 1996). Skin cancer is also one of the most common of all human cancers, and its incidence is increasing rapidly worldwide. It

contributes approximately 30% of all newly diagnosed cancers in the world, and solar ultraviolet radiation is an established cause of approximately 90% of skin cancer (Armstrong and Kricker, 2001).

Animal investigations supported by epidemiological studies have suggested an inverse relationship between the consumption of fruits and vegetables and the incidence of cancers in multiple organs, including lungs, larynx, oral pharynx, gastrointestinal tract and pancreas (Block et al., 1992). These studies support the view that more than 70% of all types of cancer do not depend on the individual's genetic background and can be prevented by changes in lifestyle, such as a "correct" diet (Wong et al., 2005). Many naturally occurring substances present in the human diet, including micronutrients, phytochemicals, vitamins, vitamin precursors and minerals, have been found to possess both complementary and overlapping mechanisms of chemopreventive activity in multistage carcinogenesis (Wang et al., 2002). Chemoprevention is recognized as an important therapeutic strategy for the management of cancer. The enormous levels of clinical attrition for current chemical-based synthetic anticancer drugs underscore the dire need to develop novel anticancer drugs.

The key to effective chemoprevention is the identification of a chemopreventive agent that can effectively inhibit cancer development without toxic side effects. Herbs and natural products lack much of the toxicity that is present in synthetic chemicals, thus enhancing their appeal for long-term prevention strategies (Zou et al., 2005). Medicinal plants rich in antioxidant phytochemicals are increasingly being explored for chemopreventive potential (Manikandan et al., 2007). Approximately 5-15% of the more than 250,000 species of higher plants have chemically and pharmacologically been investigated for various ailments that afflict humans (Baladrin et al., 1993). Sixty percent of approved drugs for cancer treatment between the 1981-2002 originated from natural plant sources (Newman et al., 2003). Data obtained from scientific research

emphasize the superior role of natural products as both valuable lead compounds and potentially new drugs (Müller, 1998). Moreover, a significant number of the world's flora still need to be explored for the potential bioactive compounds for clinical evaluations.

The plant used in this study is *Holarrhena floribunda*, commonly found in undergrowth close to forests and woodland savannah, where it normally grows on sandy, humid or lateritic soils. Each part of the plant is reputed to have different medicinal uses in folk medicine. The leaves are used for sterility, vaginitis, abortion and hypertension, and the root decoctions are indicated in blennorrhoea, gonorrhoea and sterility, while decoctions of roots and stems are used for abortion, diabetes, dysentery, infectious diseases and local anaesthesia (Kerharo and Adam, 1974; Tamboura et al., 1998; Adjanohoun et al., 1989; Neuwinger, 1996). Conessine, a compound derived from the stem of the plant, is used for destruction of amoeba without emetic effect (Berhaut, 1971). Bioactive compounds isolated from the stem have been shown to possess inhibitory activity against resistant strains of *Plasmodium falciparum*, the malaria pathogen. Also, conessine isolated from the stem is reported to have significant effects against various bacteria (Bogne et al., 2007). Recently, the leaf extract of this plant has been found to have *in vitro* antioxidant activities against reactive oxygen species OH^-, NO^- , DPPH and lipid peroxidation (Badmus et al., 2010).

1.2 Objectives of the Study

The objectives of this study were:

1. To evaluate the anti-tumour activity of crude methanolic extract of the leaves of *H. floribunda* in different cancer cell lines and a normal cell line.

2. To evaluate the mechanism(s) of action of the plant extracts.

3. To perform bioassay-guided isolation, purification and identification of bioactive compounds from the leaves of *H. floribunda.*

4. To test the anti-tumour activity of the isolated compounds in different cancer cell lines and a normal fibroblast cell line.

5. To evaluate the antioxidant activity of the isolated compounds.

6. To evaluate the mechanism(s) of action of isolated compounds.

To achieve these goals, the following steps were taken:

- Collection of the plant material (leaves).

- Evaluation of anticancer activity of the methanolic extract using different bioassay systems.

- Bioassay-guided fractionation of the methanolic extract using chromatographic methods like column chromatography, thin layer chromatography and nuclear magnetic resonance spectroscopy (NMR).

- Cytotoxicity profiling using the 3-(4,5 dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) reduction assay.

- Determination of the best performing fraction(s) in the cancer cell lines by calculating IC_{50} using non-linear regression analysis of dose-response curves.

- Further exploration of fraction(s) that show(s) better inhibition of proliferation of the cancer cell lines to elucidate mechanisms of action via cell cycle analysis, apoptosis induction using Annexin-V FITC-PI staining and Apopercentage™ assays, and reactive oxygen species (ROS) determination.

- Determination of the mechanisms of cell death type I in treated cells by evaluating involvement of PARP using Western blot techniques.

- Evaluation of the effects of the compounds on the activity of topoisomerase-I, an enzyme that cleaves one strand of double-stranded DNA, relaxes the strand, and reanneals the strands.

- Determination of the effects of the compounds on actin filaments, using fluorescence microscopy.

- Estimation of ROS induction using fluorescence microscopy in the presence of 5-(and-6)-chloromethyl-2', 7' dichlorodihydrofluorescein diacetate (CM-H_2DCFDA).

- Assessment the effects of the compounds on the mitochondria using mitotracker red dye

- Microscopic observation of cellular morphology and nuclear morphology.

- Determination of antioxidant activity of the isolated flavonoids compounds.

- Characterization and elucidation of the isolated compounds.

CHAPTER 2

LITERATURE REVIEW

2.1 Cancer Initiation

Cancer is a Greek word indicating growth of epithelial lining surfaces - either external (skin or mucous membranes) or internal (glandular acini or tubules). The growth remains unspecialized or poorly differentiated in appearance (Oertel, 1930). Cancer growth or tumour expansion is the consequence of cells free of physiological controls and maintenance. Cancer was described by Horst Oertel, Professor of Pathology at McGill University, Montreal, as far back as 1930 to be an expression of atypical cell regeneration, a result of disturbances in the normal relations between tissue cells, their vascular supply and innervations which lead to modifications in cell differentiation, metabolic and formative functions. He further stated that cancer cells are independent of physiological environments where they are derived from. They grow as a new entity with their own blood and nerve supply which are adapted only to their growth (Oertel, 1930). Carcinogenesis is defined as a stepwise accumulation of genetic changes that unshackles neoplastic cells from the homeostatic mechanisms that govern normal cell proliferation (Hahn and Weinberg, 2002). The slight and noticeable departure from the description by former and latter definitions of cancer is that the recent description shows that the advancement in cancer biology has delineated the involvement of genetic events in the progression of a normal cell to neoplastic development.

Cancer may also be formed from clonal multiplication of cells, which escaped apoptosis and accumulate genetic/epigenetic alterations (Trosko, 2001). Accumulation of aberrant cells with

genetic lesions is a fulcrum of carcinogenesis (Nguyen-Ba and Vasseur, 1999). Neoplasia can be classified as benign or malignant, depending on their cellular characteristics. Malignant neoplasia proliferate uncontrollably, differentiate themselves, invade other tissues and frequently metastasize to a region of the body that are not related to their origin (Hanahan and Weinberg, 2000). Benign neoplasia, on the other hand, grow more slowly with no adverse effects on immediate and surrounding tissue functions, except if they compress vital structures (Player et al., 2004).

The causes of cancer development are due to complex multivariate factors (Clapp et al., 2008). Diet, lifestyle, viral agents, genetics, environment and occupational exposures all can contribute to various stages in the initiation or progression of a tumour (Clapp et al., 2008). Epidemiology studies established the causes of the majority of cancers, including environmental factors such as chemicals, radiation and viruses (Wogan et al., 2004; Weinstein, 1988). Also, an individual genetic disposition to this factor is an important contributor to the emergence of cancer incidence. These environmental agents are termed carcinogens that are categorized as either endogenous or external factors. A carcinogen is a substance that is genotoxic and which initiate carcinogenesis by causing mutations in DNA (mutagen) (Clapp et al., 2008). Carcinogens include ionizing radiation, ultraviolet light, chemicals and viruses (Weinberg, 1989; Doll and Peto, 1981; Borek, 1993). Cells interact with ionizing radiation within a few seconds, reaction with chemicals take a prolonged time while, in contrast, viruses induce new genetic information into the genome of target cells. These reactions modify the normal function of cellular genes (Borek, 1993). The agents that initiate carcinogenesis have the capacity to interact with the DNA base sequence and introduce faulty genetic information. A single exposure to these agents is capable of activating a carcinogenic process (Straub and Burlingame, 1981). Examples of these agents are PAH (polycyclic aromatic hydrocarbons), nitrosamines, halogenated hydrocarbons, benzene, cadmium, formaldehyde.

A co-carcinogen refers to a substance that enhances the potency of genotoxic substances, but lack ability to cause a tumour on its own. A tumour promoter does not cause a tumour, but enhances tumour formation upon repeated exposure to it. Multiple exposures of promoters affect gene expression by enhancing the rate of replication of faulty DNA base sequences, which lead to expression of faulty genetic information (Trosko and Upham, 2005; Warren et al., 1982; Carpenter, 2006).

However, in addition to chemicals, viruses also cause cancer and these are known as tumour viruses. They are either DNA viruses which include EBV, KSHV, HBV and MCPyV or RNA viruses such as HCV and HTLV-1 (Saha et al., 2010). Tumour viruses may directly transform the cell by integrating oncogenes into a cell or activating cellular oncogenes. These mechanisms can lead to tumorigenesis if a dysfunctional immune surveillance system is unable to prevent or modulate tumour development (Carrillo-Infante et al., 2007).

2.2 Cancer Development

Carcinogenesis is a process that involves abnormal accelerated cellular changes in the genes controlling proliferation, differentiation and apoptosis (Sun et al., 2004; Hong and Sporn, 1997). Malignant development of normal cells requires acquisition of six fundamental events: self-sufficient proliferation, insensitivity to anti-proliferative signals, evasion of apoptosis, unlimited replicative potential, the maintenance of vascularization and for malignancy, tissue invasion and metastasis (Hanahan and Weinberg, 2000).

There is no difference in the carcinogenesis process in human and experimental animals. The rates and periods of exposure to different environmental carcinogens determine the speed and frequency of mutation, rates of cell proliferation and phenotypic expression of mutated genes. In addition, individual genetic compositions to the susceptibility of the environmental agent and

their defense mechanism play roles in neoplasic stages (Oliveira et al., 2007). Cancer development is functionally grouped into three phases: initiation, promotion and progression (Kinzler and Vogelstein, 1996; Bertram, 2000; Berenblum and Armuth, 1981; Heidelberger et al., 1983).

Initiation is a series of heritable genomic changes within cancer cells, such as point mutations, gene deletions and amplification, and chromosomal rearrangements leading to irreversible cellular changes. Initiation can also occur through spontaneous mutations of DNA by depurination, deamination and errors in DNA replication (Gomes-Carneiro et al., 1997; Trosko, 2001). Occurrence of cell division after the cells had been initiated before the DNA repair system clears the insult makes the damage to be permanent and irreversible (Farber, 1984; Trosko, 2001). The initiation phase is an additive process because neoplastic development depends on the dose of carcinogen, an increase in the dose expands the multiplicity of neoplasia and reduces the latent period of its manifestation. Mutated cells can be initiated only if the genes that regulate the terminal differentiation are mutated (Trosko, 2001; Farber, 1984).

Initiated cells can remain latent for years and grow into an autonomous tumour mass in a clonal fashion. In this phase, initiated cells ensure symmetrical cell division. The expansion of initiated cells occurs by mitogenic processes caused by an increase in the number of new cells and apoptosis inhibition. Apoptosis promotes the natural death of damaged cells (Trosko, 2001; Trosko, 2003). Survival and clonal expansion of initiated cells by tumour promoters lead to cancer cell development. Irreversible genetic changes prepare normal cells to enter immortality (Trosko, 2001; Shacter and Weitzman, 2002). Initiated cells are similar phenotypically when compared with normal cells, although mutations have occurred, but not differentiation (Trosko, 2003).

The promotion phase is a reversible stage of carcinogenesis that requires several years or decades for its manifestation. It is an expansion of mutated cells to form actively dividing multicellular premalignant lesions (Sun et al., 2004). Progression is substantial growth in tumour size and either growth related or mutually exclusive metastasis. Accumulation of genetic lesions is important to cancer development. The lesion is essential for initiation which also involves promotion or progression of tumour development (Kinzler and Vogelstein, 1996). The sustained genetic lesions induce the activation of cellular proto-oncogenes or inactivation of tumour suppressor genes.

The acquired intrinsic autonomous properties of cancer cells are not mutually exclusive to the ultimate development of cancer. The process of tumorigenesis also involves non-cancer cell participation, many of which consist of heterocellular tumour cell populations. For example, the contribution of vascular endothelial cells to the development of neo-angiogenesis (new blood vessel formation) for the survival of immortal cells (Rakoff-Nahoum, 2006; Kinzler and Vogelstein, 1996).

2.3 Cancer Epidemiology

According to the World Health Organization (WHO) and its cancer research agency, the International Agency for Research on Cancer (IARC), about 12.7 million new cancer cases were diagnosed in 2008 worldwide and cancer caused more than 7.6 million deaths in the same year (http://globocan.iarc.fr/factsheets/populations/factsheet.asp?uno=900; accessed 14 October 2013). Cancers which exceeded 40% of all cases most frequently diagnosed include lung, female breast, colorectal and stomach cancers. Table 2.1 summarizes estimated age-standardized cancer incidence and mortality rate per 100,000 by world area in 2008 (Ferlay *et al*., 2010). Figure 2.1 provides estimates of the 10 most commonly diagnosed cancers in 2012 (Ferlay *et al*., 2010).

2.4 The Cell Cycle

2.4.1 The Normal Cell Cycle

The cell cycle (Figure 2.2) is a series of cell divisions which are basic requirements for the development of a fertile zygote to an adult individual (van den Heuvel, 2005). Each set of divisions requires complete orderly events that are known as the cell cycle. The cell cycle involves a network of interacting proteins that receive signals from outside and inside the cells. The signals are processed and integrated which then evokes the fate of the cell whether to proliferate or to enter the quiescent state (Weinberg, 2007).

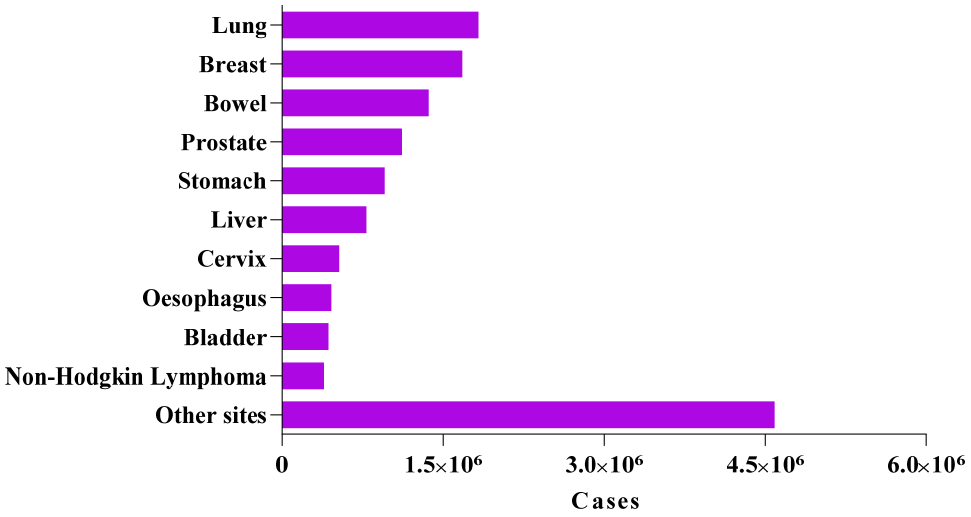
Table 2.1: Estimated age-standardized cancer incidence and mortality rate per 100,000 by world area, 2008.

	Incidence			**Mortality**		
	Male	Female	Overall	Male	Female	Overall
Eastern Africa	121.2	125.3	122.8	105.4	95.9	99.9
Middle Africa	88.1	96.7	91.8	78.5	75.6	76.4
Northern Africa	109.2	98.9	103.2	89.5	68.2	78.0
Southern Africa	235.9	161.0	189.6	172.1	108.1	133.2
Western Africa	92.0	123.5	107.6	80.1	91.2	85.4
Eastern Asia	222.1	158.1	188.4	155.5	87.3	120.1
South-Central Asia	99.7	110.8	104.6	78.0	71.7	74.5
South-Eastern Asia	143.9	141.7	141.5	112.3	89.4	99.5
Western Asia	152.8	119.5	133.8	113.9	74.3	92.2
Caribbean	196.3	153.5	172.6	116.6	86.2	99.9
Central America	136.2	134.4	134.4	84.7	80.6	82.0
Northern America	334.0	274.4	299.9	122.4	91.5	105.1
South America	186.7	162.9	171.9	116.6	88.2	100.3
Central and Eastern Europe	259.2	184.2	210.6	181.5	94.0	128.1

Northern Europe	292.3	249.5	266.1	134.6	99.7	114.5
Southern Europe	289.9	212.2	245.0	149.9	81.2	111.7
Western Europe	337.4	250.9	287.7	138.4	84.3	108.0
Australia/ New Zealand	356.8	276.4	313.3	126.6	86.0	104.1
Melanesia	146.0	133.4	138.5	119.8	95.9	106.8
Micronesia	153.8	164.4	157.5	104.7	70.3	86.1
Polynesia	225.0	201.5	209.8	133.6	87.9	109.1

Table excludes non-melanoma skin cancer (Ferlay *et al*., 2010).

It is an organized and monitored high energy demanding set of events required for proper cell division into two daughter cells (Norbury and Nurse, 1992). The cell cycle is a process alternating between phases: S phase (doubling process during which DNA is synthesized) and M phase (when the cell contents are divided into two equal daughter cells). The periods between these phases are known as gap periods (G phase) (Sandal, 2002). Cells are stimulated by extracellular mitogens and growth factors during the G1 phase. In responding to stimulation, the cell passes through G1 and proceeds with DNA synthesis in the S phase. G2 is the interval between the completion of DNA synthesis and mitosis. M phase is marked by the generation of bipolar mitotic spindles, segregation of sister chromatids and cell division (Israels and Israels, 2000).



Source: (Ferlay et al., 2012).

Figure 2.1: Estimates of the 10 most commonly diagnosed cancers in 2012

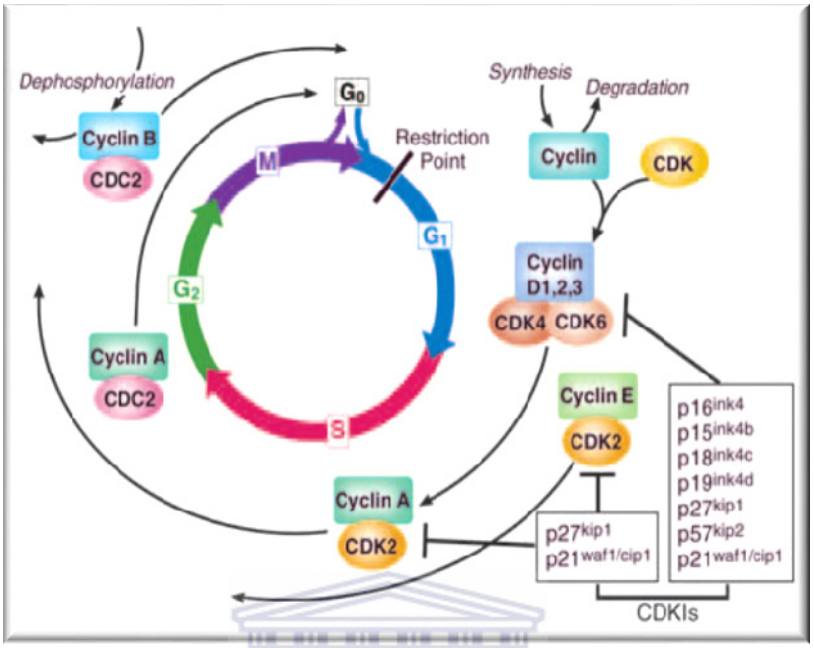
Stages of the M phase include prophase, metaphase, anaphase and telophase. DNA replication occurs in the S phase, which is preceded by a gap called G1 during which the cell prepares for DNA synthesis followed by another gap known as G2 when the cell gets ready for mitosis. Cells in G1 can enter G0 (a resting state) before it commits to DNA replication. The G0 phase accounts for the non-proliferatiing cells (Norbury and Nurse, 1992). Proliferating cells enter G1 phase from G0, after completing cytokinesis. G1 phase is mitogenic dependent up to the restriction point after which cells can proliferate independently of mitogenic stimuli and are committed to S phase, when DNA replication occurs. Progression through the mammalian cell cycle requires the accurate orchestration of this sequence of events (Diaz-Moralli et al., 2013).

The cell is imparted with quality control points known as checkpoints. Checkpoints sense damaged DNA before the cell enters the S phase (G1 checkpoint) or M phase (G2 checkpoint) (Weinberg, 2007). The culmination of any of these checkpoints (Figure 2.2) is the ability to turn off the previous state and promote the future state of the cell cycle (irreversible progression).

Cell cycle checkpoints inhibit DNA replication when DNA is damaged, inhibition of mitosis when DNA has not been replicated or is damaged and inhibition of cytokinesis when mitosis has not been properly executed (Weinberg, 2007). Loss of checkpoint control results in genomic instability, accumulation of damaged DNA and uncontrolled cell proliferation which are implicated in many human cancers (Sherr, 2000; Weinberg, 2007).

Intrinsic and extrinsic pathways are two main classes of regulatory mechanisms driving the cell cycle. The intrinsic mechanism appears in every cycle while the extrinsic mechanism acts only when a defect is detected. The mechanism of regulation is divided into phosphorylation; dephosphorylation and proteolytic degradation (Ekholm and Reed, 2000). The transmission of phases in the cell cycle is propelled by a complex of protein kinases with their respective cyclins with subsequent phosphorylation by an activating kinase (Figure 2.2). Cyclin is the regulatory unit and CDK is its catalytic partner (Israels and Israels, 2000). Cyclin/CDK complexes phosphorylate specific protein substrates to move the cell through the cycle with activation of DNA synthesis (in late G1 and S) and formation of the structural components associated with mitosis (in late G2 and M) (Israels and Israels, 2000).

During the G1 phase, there is increased expression of D cyclins (D1, D2, D3) - these cyclins associate with CDK4 and CDK6 to form complexes which result in phosphorylation and activation of the CDKs. The activated CDKs phosphorylate the retinoblastoma (Rb) tumour suppressor. The Rb plays a critical role in regulating G1 progression through the restriction point (Israels and Israels, 2000). Rb acts as a gatekeeper of the cycle and thus guards the restriction point, preventing cell cycle progression in the hypophosphorylated state while hyperphosphorylated Rb is associated with the release of elongation factor E2F and passage through the cell cycle. Rb is maintained in its hyperphosphorylated state throughout the remainder of the cycle (Israels and Israels, 2000).



G1, S, G2 and M are promoted by cyclin-dependent kinases regulated positively by cyclins and negatively by CDK inhibitors (CDKIs) (Schwartz and Shah, 2005).

Figure 2.2: The four phases of the cell cycle (G1, S, G2 and M)

The integrity of the cell's genome is monitored by the transcription factor p53 which halts progression of the cell cycle to allow time for DNA repair. This is accomplished by p53 inhibition of Rb phosphorylation. The level of p53 is maintained low in normal proliferating cells. In the presence of DNA damage, p53 binds to its sequence-specific DNA site - gene induction results in increased p53 protein synthesis (Israels and Israels, 2000). Thus, p53 controls cell cycle through upregulation of p21 (CKI) an active inhibitor of CDKs 2, 4 and 6. The inhibition of kinase activity prevents phosphorylation of Rb and the cell is arrested at G1 to allow time for DNA repair. The p53 induced un-redeemable damaged cell enters into apoptosis by inducing the expression of the pro-apoptotic protein Bax (Israels and Israels, 2000).

2.4.2 The Cancer Cell Cycle

The delicate balance between the rate of cell cycle progression (cell division) and cell growth (increased cell mass) on the one hand and programmed cell death (apoptosis) on the other is important to cancer development (Reed, 1999). Tumour heterogeneity and diversity do not stand as barriers to share some prominent properties such as unrestricted proliferation. Aberrations in proteins that are key to cell proliferation control and survival are important to all types of tumour formation (Evan and Vousden, 2001). The connection between altered cell cycle regulatory proteins and malignant transformation had been described by several scientific reports which has led to the notion of cancer as a disease of the cell cycle (Diaz-Moralli et al., 2013).

Overexpression of CDKs and cyclins and loss of CKI and pRb expression are altered modifications linked to tumorigenesis that result from chromosome alterations such as amplifications and translocations of oncogenes and deletions of tumour suppressors or epigenetic inactivation such methylation of tumour suppressor promoters (Shapiro, 2006; Malumbres and Barbacid, 2001). Misregulated CDKs is known to induce constitutive mitogenic signalling and defective responses to anti-mitogenic signals cause aberrant proliferation, genomic and chromosomal instability (Malumbres and Barbacid, 2009; Massague, 2004). CDKs (CDK4, 6 and 2) involved in G1 to S transition control and regulations have been shown to be altered in 80-90% of tumours (Tetsu and McCormick, 2003; Malumbres, 2011).

ATM (ataxia-telangiectasia-mutated)-CHK2-p53, a DNA damage checkpoint pathway, reportedly leads to CDK hyperactivity, unregulated cell cycle progression, genomic instability, and cancer when they are dysregulated (Malumbres and Barbacid, 2009). Such genes involved in cancer transformation are termed oncogenes (growth promoting) and tumour suppressor genes (growth inhibiting) (Butel, 2000). More than 100 oncogenes and 30 tumour suppressor genes (TSG) are known and are the major classes of cancer-related genes that are in focus in addition

to tumour viruses some of which are of no cognates (Surekha, 2001). Oncogenes in their *normal* proto-oncogene state drive the cell cycle forward, allowing cells to proceed from one cell cycle stage to the next (Chow, 2010). This highly regulated process becomes dysregulated due to activating genetic alterations that lead to cellular transformation. Tumour suppressor genes, on the other hand, restrict cell cycle progression. Their control over cell division is lost with genetic alterations leading to their inactivation (Chow, 2010).

Epigenetic changes in these genes are also contributing factors to the unrestricted proliferative potential of cancer cells. These changes show the negative impact of mutagens (exogenous and endogenous), germline mutations and various types of genomic instabilities acquired during tumour development (Surekha, 2001). Both the oncogenes and TSG are known to code for diverse functions such as growth factors (cytokines), growth factor receptors, adapter molecules, protein kinases, G-proteins, nuclear transcription factors, molecules that repair DNA, apoptosis, metastasis and invasion (Surekha, 2001). Six essential manifestations of the alterations in these genes have been aptly described by Hanahan and Weinberg to lead to self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of programmed cell death, limitless replicative potential, sustained angiogenesis, tissue invasion and metastasis (Hanahan and Weinberg, 2000).

2.5 Apoptosis

2.5.1 Overview of the Process

The term apoptosis was originally described by John Kerr, an Australian pathologist in 1965. The word was coined in 1972 by John Kerr, Alasrtair Currie and Andrew Wyllie for what is now known as programmed cell death (Kerr et al., 1972). Apoptosis describes a morphological distinct form of biologically programmed cell demise (Elmore, 2007). Programmed cell death

(PCD) is endowed like the cell cycle with a complex network of cell death pro- and anti proteins working in tandem in a delicate balance to achieve tissue homeostasis (Reed, 1999).

PCD occurs naturally as a homeostatic mechanism during development and aging to maintain appropriate cell populations in tissues and as a defence mechanism when cells are damaged by disease or noxious agents (Norbury and Hickson, 2001). It is a genetically regulated active process of removing cells in both physiological and pathological processes. It is a normal physiological response to specific suicide signals or lack of survival signals (Kerr et al., 1994). It is an energy dependent coordinated cascade of events and its response to death signals leads to the activation of a group of cysteine proteases called caspases (Elmore, 2007).

Apoptosis plays a significant role in shaping tissues during morphogenetic development, endocrine dependent atrophy and as an important component of processes of normal cell turnover in many tissues. It also plays a pivotal role in organism survival by limiting the accumulation of harmful cells, such as self-reactive lymphocytes, virus-infected cells and tumour cells, proper development of immune system function, normal embryonic development and maintenance of cell homeostasis (Reed, 1995; Wyllie, 1997; Reed and Tomaselli, 2000).

Morphological changes during apoptosis could be assessed using light and electron microscope (Hacker, 2000). Cell morphological changes are a hallmark that can be used to describe apoptosis, including chromatin condensation, nuclear fragmentation accompanied by cell rounding up, cellular volume reduction (pyknosis) and retraction of pseudopodes (Kroemer et al., 2005). A cell undergoing apoptosis loses microvilli and junctional complexes making it to shrink and to be released (detach) from neighboring cells with enlarged nucleolus and abnormal granular appearance (Wyllie, 1997). Cell shrinkage and pyknosis are the earlier processes of apoptosis, which is characterized by smaller size, dense cytoplasm and tightly packed organelles,

while pkynosis is a result of chromatin condensation (Elmore, 2007) which form a cap like densely heterochromatic regions in the nucleus (Wyllie, 1998). Cell surface molecules changes occur to facilitate recognition of neighbouring cells or phagocytes (Wyllie, 1998). Plasma membrane blebbing, karyorrhexis and separation of cell fragments into apoptotic bodies (apoptosomes) occur during a process called budding.

Apoptotic bodies consist of cytoplasm with tightly packed organelles with or without nuclear fragments. This does not affect the integrity of the organelle as they are enclosed within an intact plasma membrane (Elmore, 2007). Apoptotic bodies are phagocytosed by macrophages, parenchymal cells or neoplastic cells and degraded within phagolysosomes (Elmore, 2007). The phagocytosis process prevents apoptotic cells to release their constituents into the surrounding interstitial tissue, thereby preventing secondary necrosis and also the engulfing cells do not produce inflammatory cytokines (Savill and Fadok, 2000; Kurosaka et al., 2003).

Apoptosis is triggered by a series of stimuli such as DNA lesions as a result of ultraviolet (UV) radiation, ionizing radiation, oxidative stress, errors in replication or recombination and as well as environmental and therapeutic toxins (Kumari et al., 2008). A number of physiological and pathological stimuli that initiate apoptosis have been described and are grouped into four main categories (Pritchard and Watson, 1996; Savitz and Rosenbaum, 1998; Arends and Wyllie, 1991). The first category includes alkylating anticancer drugs and ionizing radiation that cause DNA damage. The second category induces apoptosis by receptor activation through glucocorticoids acting on the thymus, tumour necrosis factor-α or by withdrawal of growth factors such as nerve growth factor and interleukin (IL)-3. The third category encompasses agents that induce apoptotic downstream components which include kinase inhibitors and phosphatases. The agents that cause direct cell membrane damage includes heat, UV light and oxidizing agents (superoxide anion, hydrogen peroxide) fall into the fourth category (Wyllie,

1997; Savitz and Rosenbaum, 1998; Rudin and Thompson, 1997; MacLellan and Schneider, 1997).

The DECISION PHASE of apoptosis is a genetically controlled first stage after induction by an appropriate stimulus. This is followed by an execution phase, which is the stage for the manifestation of morphological changes in apoptosis. The third stage is the engulfment of the dying cell and eventually is followed by degradation of the engulfed cell DNA (Goran, 2005). The two main genes that control apoptosis in the decision stage are known as Bcl-2 and p53 genes. Bcl-2 family of mammalian proteins either inhibits or promotes apoptosis. Bcl-2 and Bcl-xL inhibit apoptosis while Bax, Bad, Bak and Bcl-xs which are Bcl-2 associated proteins promote apoptosis (Hockenbery et al., 1993; Yang et al., 1997; Savitz and Rosenbaum, 1998; Haunstetter and Izumo, 1998). The p53 nuclear phosphoprotein functions by binding to DNA as a transcription factor and controls cell proliferation and repair. C-myc gene also induces apoptosis in the presence of p53 gene (Miyashita and Reed, 1995).

The EXECUTION PHASE involves proteolytic cleavage of enzymes belonging to the cysteine protease family referred to as caspases leading to their activation. Activation of caspases, which occurs when the enzymes cleave after aspartic acid residues is a fundamental event in the apoptotic process (Wyllie, 1997). Caspases are biosynthesized in the zymogen forms that are activated by upstream signals into mature proteases. Initiator caspases (caspase-1.-2,-4, -5, -8, -9, -10 and -14) are activated by oligomerization-induced autoprocesssing while effector caspases (caspase-3, -6 and -7) are activated by other proteases, including initiator caspases and granzyme B (Butt et al., 1998; Li et al., 1997; Martin et al., 1998; Yang et al., 1998).

There are two mains molecular pathways of apoptosis. An additional pathway involves T-cell mediated cytotoxicity and perforin-granzyme-dependent killing of the cell. The evidence that the

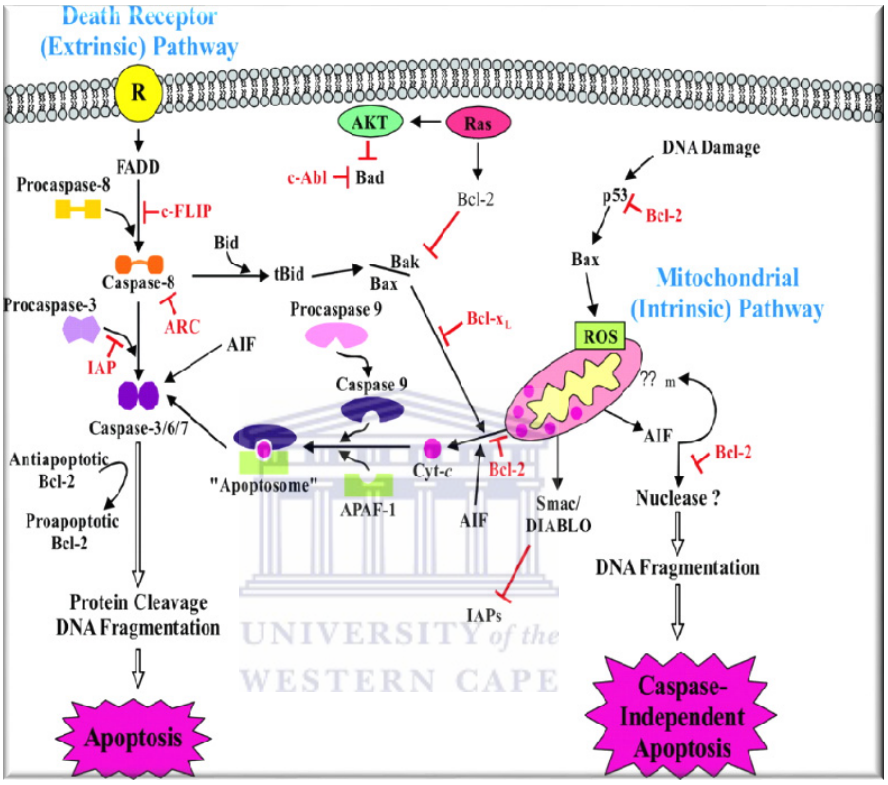
two main pathways interact and one pathway influences the other had been provided by Igney and Krammer (2002). The main two pathways of apoptosis are known as extrinsic and intrinsic while the additional pathway is called the perforin/granzyme pathway and all these mechanisms are all linked to the same terminal or execution pathway (Igney and Krammer, 2002).

The extrinsic mechanism involves transmembrane receptor mediated interactions of death receptors that are members of the tumour necrosis factor (TNF) receptor superfamily. They transmit the death signals from the cell surface to the intracellular signalling pathways. FasL/FasR, TNF-/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 are death ligands and their corresponding receptors had been well characterized (Suliman et al., 2001; Rubio-Moscardo et al., 2005). The aggregation of these receptore and their respective ligand complexes initiate activation of procaspase-8 into its active form which subsequently activates caspase-3 (Goran, 2005).

The intrinsic pathway is a result of damage to the mitochondria inner membrane by various stimuli such as oxidative stress. Damage to the inner mitochondrial membrane leads to mitochondrial membrane transition pore opening which subsequently releases cytochrome c, apoptosis-inducing factor (AIF) and endo G. The complexes of cytochrome c with Apaf-1 activate procaspase-9 in cytosol (Figure 2.3). The activated procaspase-9 (caspase-9) activates downstream effector caspases such as caspase-3, -6 and -7. The endo G released in turn cleaves chromosomal DNA independent of caspase activation (Nakagawa et al., 2000).

T-cell mediated cytotoxicity is a perforin/granzyme pathway of inducing cells to undergo apoptotic cell death. Cytotoxic T lymphocytes (CTLs) kill cells through the extrinsic pathway with the application of FasL/FasR interaction (Brunner et al., 2003). The killing of tumour cells and virus-infected ones occurs through secretion of the transmembrane pore-forming molecule, perforin, with the release of serine proteases granzyme A and granzyme B as cytoplasmic

granules through the pore into target cells (Trapani and Smyth, 2002). Granzyme B cleaves proteins at aspartate residues and therefore activates caspase-10 and cleaves factors like ICAD (inhibitor of caspase activated DNAse).



The major apoptotic pathways involve either membrane death receptor stimulation (extrinsic pathway) and/or mitochondrial disruption (intrinsic pathway)(Remillard and Yuan, 2004).

Figure 2.3: The two major apoptotic pathways

Activation of procaspase-8 leads to cytochrome c release from mitochondria, thereby linking the extrinsic and intrinsic pathways (Goran, 2005). Granzyme A on the other hand induces apoptosis through caspase activation independent pathway (Pardo et al., 2004). Once in the cell, granzyme A activates DNA nicking via DNAse NM23-H1. This DNAse is important in immune surveillance to prevent cancer progression through induction of tumour cell apoptosis (Sakahira et al., 1998).

2.5.2 Apoptosis and Cancer

The human body produces about 10 billion cells per day to balance homeostasis of cells and counteract cells dying through apoptosis. The rate of apoptosis determines the number of cells generated, especially in the disease state, during normal development and aging (Renehan et al., 2001). Deregulated apoptosis is implicated in pathological conditions. Decreased apoptosis is linked to cancer, autoimmune disorders and viral infections while excessive apoptosis is related to ischaemic injury (myocardial infarction, stroke, AIDS, neurodegenerative disease, sepsis and multiple organ dysfunction syndromes) (Zornig et al., 2001; Saikumar et al., 1998).

Cancer is an example of either an overproliferation of cells or suppressed apoptosis. Evasion of apoptosis is one of the hallmarks of cancer development and progression. Escape of cell death due to host inability to eliminate potential malignant cells caused by elevated and ablated expression of tumour suppressor genes and oncogenes as well as interaction with other growth promoting lesions play a vital role in carcinogenesis (Lyons and Clarke, 1997). Mechanisms that lead to evasion of apoptosis has generally been divided into three categories: (1) Imbalance between pro-apoptotic and anti-apoptotic proteins (2) reduced caspase function and (3) disruption of death receptor signalling (Hanahan and Weinberg, 2000).

2.5.2.1 Imbalance Between Pro- and Anti-Apoptotic Bcl-2 Proteins

The Bcl-2 family of proteins is known to have more than 30 members with either pro- or anti-apoptotic functions, indicating that they might be involved in different roles in carcinogenesis (Cory et al., 2003). Alteration in the expression of anti-apoptosis and pro-apoptosis members of the Bcl-2 family in human cancer was the first to be described (Reed, 1997b; Reed, 1997a). The mechanism involved in abnormal levels of Bcl-2 family proteins in most instances imply changes in the transcriptional and posttranscriptional regulatory networks that control the eventual products of their genes (Reed, 1999). It involves structural alterations to the genes such

as t(14;18) chromosomal translocations that activate Bcl-2 in most non-Hodgkin's lymphomas (Tsujimoto et al., 1985). B cell lymphoma overexpresses Bcl-2 which serves as evidence for suppressed apoptosis for cancer incidence. Single nucleotide substitution and frame shift mutations that inactivate Bax in malignancies of the colon, stomach and haematopoietic system and retrovirus gene insertions that activate the *bcl-XL* gene in murine leukaemias (Thomas et al., 1998).

The expression of either Bcl-2 an anti-apoptotic protein and downregulation of Bax pro-poptotic protein is what tumour cells need to acquire resistance to apoptosis (Elmore, 2007). The expression of both proteins is controlled by the p53 tumour suppressor gene (Miyashita et al., 1994). The condition that favours anti-apoptotic over pro-apoptotic signatures in Bcl-2 family members renders tumour cells resistant to a wide range of cell death stimuli, including essentially all chemotherapeutic drugs, radiation, hypoxia, cell detachment from extracellular matrix, growth factor withdrawal, glucose deprivation, elevated cytosolic Ca^{2+}, oxidants, tumour suppressors such as p53 that induce apoptosis, and oncogenes such as *myc* and cyclin D1/Bcl-l that drive cell division, but also promote death (Reed, 1994). The resistance to cell death that occurs due to overexpressed Bcl-2 in cancer has relevance to cancer cell behaviour and newer cancer therapies which include angiogenesis inhibitors (hypoxia, hypoglycaemia), tumour invasion and metastasis (cell adhesion), multistep carcinogenesis (myc, cyclin D1), and antibodies designed to block growth factor receptors (anti-HER2) (Reed, 1999).

Bcl-2 transgenic mice have been shown to develop malignancy at old age, which implies that overexpression of *bcl-2* is not sufficient for malignant transformation. In addition, *bcl-2* transgene expression has been reported to significantly extend the lifespan of B lymphoid tumours, indicating that *bcl-2* overexpression provides a predisposition for the development of B-cell lymphomas (Strasser et al., 1990). The assumption that Bcl-2 prolong lifespan of normally

short lived cells to accumulate additional oncogenic mutation was corroborated by observations that leukaemias develop very rapidly in mice with the combination of a *bcl-2* and *c-myc* transgene than in mice bearing either of the two alone (Zhivotovsky and Orrenius, 2006).

2.5.2.2 Reduced Caspase Function

Human caspases are classified according to their function, size of their pro-domain or cleavage specificity. Group I caspases are proteases that are basically associated with innate immunity against pathogens (inflammatory) which includes caspase-1, -4 and -5. Group II caspases are involved in the regulation of apoptosis (Olsson and Zhivotovsky, 2011). Group II caspases are divided into two classes: initiator caspases, which include caspase-2, -8, -9 and -10 and effector caspases also known as executioner caspases which include caspase-3, -6 and -7. Initiator caspases are monomeric zymogens while the effector is constitutively produced in cells as dimers. Dimerization triggered by the formation of multi-component complexes activates initiator caspases while the proteolytic activity of initiator caspases is required to trigger the activity of effector caspases (Boatright et al., 2003). The vital role played by the caspases in both the upstream and downstream process of apoptosis conceivably implies that dysregulation of their functions might influence cell proliferation (Zhivotovsky and Orrenius, 2006). It is not, however, out of a place to hold that low levels of caspases or impairment in caspase function may result in a decrease in apoptosis or carcinogenesis.

Caspase-9 is not mutated or silenced in the majority of cancers (Kim et al., 2010). Decreased levels were reported in 46% of colon cancers while its expression was found to be increased in gastric cancer (Yoo et al., 2002; Yoo et al., 2004; Palmerini et al., 2001). Somatic mutation without altering amino acid sequence occurs at very low frequency in colorectal, gastric and lung carcinomas (Soung et al., 2006). It has been observed that 55% of caspase-9 was phosphorylated

at Thr125 in gastric carcinomas, presumably preventing the activation of the enzyme (Allan and Clarke, 2009; Yoo et al., 2007).

Initiator caspases are known to allow one specific caspase to respond to one death stimulus. This sole obligatory role makes them an important candidates for putative tumour suppressors (Olsson and Zhivotovsky, 2011). Caspase-8 and -10 are found in a genomic locus 2q33-34 frequently known to be affected in cancer (Grenet et al., 1999). Caspase-10 was not mutated in breast cancer, lung cancer and hepatocellular carcinoma, but in 4.3% of colon cancer samples analyzed (Oh et al., 2010). Increased levels of expression of caspase-10 are observed in gastric cancers compared to normal mucosa. It is therefore implied that caspase-10 function is of no relevance to gastric cancer (Park et al., 2002).

Paediatric tumours and their cell lines contain silenced caspase-8 owing either to gene deletion or promoter methylation (Teitz et al., 2000a). Loss of caspase-8 expression is known to often correlate with *mycn* oncogene amplification and increased levels of the corresponding protein (Harada et al., 2002). Cells with low apoptosis capacity is linked to all the described alterations in caspase-8 compared to cells harbouring normal caspase-8 which strongly suggests tumour suppressor functions (Olsson and Zhivotovsky, 2011). Caspase-8 is involved in the suppression of oncogenic transformation independent of its role in cell death induced by death receptors (Krelin et al., 2008). Neuroblastoma, medulloblastoma and small-cell lung carcinoma (SCLC) are reported to lack caspase-8 due to gene silencing (Shivapurkar et al., 2002; Takita et al., 2000; Zuzak et al., 2002). It has therefore been suggested that caspase-8 acts as a tumour suppressor in the neuroectodermal or neuroendocrine cell lineage (Mazumder and Almasan, 2002; Teitz et al., 2000b). It is likely that DNA methylation of caspase-8 occurs via DNA methylation in 52% cases (Hopkins-Donaldson et al., 2003).

Detailed investigations of caspases in execution phase produced conflicting results. The expression of caspase-3 in some cancer cells is induced, but suppression occurs in non-cancerous cells (Nakagawara et al., 1997; Fujikawa et al., 2000). The expression of this protein and malignant grade has been reported to be either positively or negatively correlated (Nakagawara et al., 1997; Satoh et al., 2000). Localization of caspase-3 in both nuclear fractions of pancreatic and neuroblastoma tumours was found to be correlated with low malignancy. Low frequencies of mutated caspase-3 are detected in some human cancers such as colon and stomach, non-Hodgkin lymphoma (NHL) and hepatocellular carcinoma (Soung et al., 2004). Breast and prostate cancers were observed to express non-mutated caspase-3 and the expression increases with tumour progression (Kim et al., 2010). Caspase-6 and -7 are rarely found in mutated form in human cancers (Anwar et al., 2004; Jager and Zwacka, 2010).

High levels of caspase-3 expression while low levels of caspase-6 and -7 are reported in gastric carcinoma more than in normal mucosa (Yoo et al., 2002; Yoo et al., 2004). In the majority of cases of invasive carcinoma of the breast caspase-6 was found to be expressed (Vakkala et al., 1999). However, only small fractions of human cancer are related to inactivating mutations or silencing of caspase genes (Jager and Zwacka, 2010). It is not yet certain whether mutation in these caspases play role in tumorigenesis. Some of prevalent cancer type lack mutant caspase genes and show normal expression of caspases (Jager and Zwacka, 2010).

2.5.2.3 Disruption of Death Receptor Signalling

Death receptors are part of the TNF receptor gene family with over 20 proteins of different biological activities which include the regulation of cell death, cell survival, differentiation or immune regulation (Ashkenazi and Dixit, 1998). They have similar cysteine-rich extracellular domains and are identified by their cytoplasmic domain of about 80 amino acids known as the death domain (Walczak and Krammer, 2000). Death domain functions essentially as an apoptotic

inducer by transmitting death signals from the cell's surface to intracellular targets and play major roles in the extrinsic pathway of apoptosis (Debatin and Krammer, 2004).

Examples of death receptors are TNFR1 (DR1), Fas (DR2, CD95 or APO-1), DR3 (APO-3), DR4 [or TNF-related apoptosis inducing ligand receptor 1 (TRAIL-1)], DR5 (TRAIL-2), DR6, ectodysplasin A receptor (EDAR) and nerve growth factor receptor (NGFR) (Wong, 2011). The best characterized death receptors are CD95 (APO-1/Fas), TNF receptor 1 (TNFR1), TRAIL-R1 and TRAIL-R2 (Debatin and Krammer, 2004). Death receptors are activated in response to binding of ligands upon oligomerization. The corresponding ligands of the TNF superfamily of death receptors include CD95 ligand, TNF alpha, lymphotoxin-alpha, TRAIL and TWEAK (Walczak and Krammer, 2000).

Several abnormalities in death signalling pathways such as downregulation or defects in receptor functions, mutations within the genes encoding receptors and apoptotic signalling pathway defects are the reported mechanisms by which cells evade apoptosis (Wong, 2011; Landowski et al., 1997; Hitoshi et al., 1998). Heterozygous mutations of CD95 gene on chromosome 10q23 are found in a number of human tumours (Takayama et al., 2002; Muschen et al., 2002). Also, loss of expression of TRAIL receptors I and II localized on chromosome 8p due to loss of heterozygosity or hypermethylation are reported in several tumours (Pai et al., 1998; van Noesel et al., 2002). Downregulation of FAS surface expression levels are found to be associated with melanoma, oesophageal cancer and pulmonary adenocarcinomas. FAS mutations are also reported in nasal NK/T cell mutation and testicular germ cell tumours (Takakuwa et al., 2002; Takayama et al., 2002). Substitution of guanine with adenine at position 422 which changes an arginine to histidine and also cytosine to guanine at position 626 replacing threonine with arginine in the extra domain of DR4 are associated with risk of lung and head and neck cancer

(Fisher et al., 2001). Missense mutations in DR5 are reported in 7% of gastric cancer samples. A mutated protein inhibits TRAIL-induced apoptosis when transfected into cells (Park et al., 2001).

Some mutations induced loss of apoptotic activity when overexpressed while some are neutral. Mutant DR5 corresponding to the Ipr locus was found to be 50% less in inducing apoptosis while guanine to adenosine substitution at nucleotide base 1063 led to a change of glutamic acid to lysine, resulted in total loss of apoptotic function (McDonald et al., 2001; Lee et al., 1999). Reduced expression of CD95 is reported to be involved in treatment-resistance in leukemia or neuroblastoma cells (Friesen et al., 1997; Fulda et al., 1998). Reduced membrane expression of death receptor and abnormal expression of decoy receptors play roles in the evasion of the death signalling pathways in cancers (Fulda, 2010a).

Accumulating scientific evidence suggests that down-regulation of pro-apoptotic proteins, in combination with the expression of other structural and regulatory proteins are essential for metastatic progression. The overall status of the cell death machinery, however, might explain how it affects tumorigenesis rather than an expression of the individual proteins (Zhivotovsky and Orrenius, 2006).

2.5.3 Orchestration of Apoptosis by p53

The p53 protein is also called tumour protein 53. It is one of the best characterized tumour suppressor proteins encoded by the tumour suppressor gene located on the short arm of chromosome 17 (17p13.1) (Wong, 2011). The p53 protein regulates the transcription of genes responsible for the cyclin-dependent kinase inhibitor p21 waf1/cip1, the protein GADD45, and apoptosis proteins Bax and Bcl-2, and thereby controls cell cycle arrest and apoptosis and its own function by regulating Mdm2 transcription (van Oijen and Slootweg, 2000). In addition to its role in induction of apoptosis and cell cycle regulation, some other key functions such as cell

development and differentiation, gene amplification, DNA recombination, chromosomal segregation and cellular senescence have been adduced for it (Lane, 1992).

A mouse model carrying mutant p53 lacking proline-rich domain that confers inability to induce cell cycle arrest, but retain ability to induce apoptosis was found to be protected from tumour development, suggesting the importance of the tumour suppressing ability of p53 (Toledo et al., 2006). In addition, the second mouse model with functional cell cycle arrest, but inactive apoptosis induction ability in mutant p53 (p53-R172P) suggests that cell cycle arrest and chromosome stability are also critical to tumour development (Liu et al., 2004). These models show that p53 activities contribute to its tumour suppressor potential in different cellular contexts (Freed-Pastor and Prives, 2012).

The main mutations found in p53 are point mutations, although small gene deletion or insertions have also been detected (van Oijen and Slootweg, 2000). The p53 tumour suppressor gene defects had been shown to be associated with more than 50% of human cancers (Elmore, 2007). Oncogenic property due to p53 mutation is referred to as a gain of oncogenic function.

It has been reported that some target genes of p53 involved in apoptosis and cell regulation are aberrantly expressed in melanoma cells, leading to abnormal activity of p53 and contributing to the disproportionate proliferation of these cells. It has been demonstrated that mice with N-terminal mutant deletion of p53 (122p53) that corresponds to 133p53 had decreased survival, a different and more aggressive tumour spectrum, conferred a marked proliferative advantage on cells, reduced apoptosis and profound proinflammatory phenotype (Lane, 1992).

2.5.4 Inhibitors of Apoptosis Proteins (IAPs)

IAPs are highly conserved structurally and functionally related family of proteins that regulate apoptosis, cytokinesis and signal transduction (LaCasse et al., 2008). Examples of IAPs in humans are XIAP, cIAP1, CIAP2, survivin and livin. Their functions are not limited to regulation of apoptosis, but also mitosis as found in survivin (Altieri, 2001; Holcik and Korneluk, 2001; Debatin and Krammer, 2004).

They directly inhibit caspase-3 and caspase-7 and block caspase-9 activation, but caspases -1, -6, -8 and -10 are not under its regulation. IAPs regulate caspases in the distal portions of the apoptotic proteolytic cascades when ectopically expressed in cells with some functioning as the ultimate effectors as found in caspase-3 and -7 (Reed, 1999). Its inhibitory effect is through the presence of one or more baculoviral repeat (BIR) motifs in their molecules. They inhibit caspase activity by binding their conserved BIR domains to the active sites of caspases or by keeping the caspases away from their substrates (Wei et al., 2008).

It has been suggested that the expression of IAPs as downstream inhibitors of apoptosis might be essential to both tumour formation and chemoresistance and also that IAPs can function as oncogenes (Zhivotovsky and Orrenius, 2006). The increased levels of IAPs have been reportedly found in a wide variety of cancer cells and primary tumour biopsies. (Zhivotovsky and Orrenius, 2006). The chromosomal amplification of the 11q21-q23 region, which contains both c-IAP1 and c-IAP2 was observed in a variety of malignancies and oesophageal squamous cell carcinomas frequently display amplification of IAPs (Uren et al., 2000).

Abnormal IAP expression has been reported in pancreatic cancer cells and is found to be also responsible for resistance to chemotherapy (Wong, 2011). In addition, Livin was observed to be

expressed in melanoma and lymphoma and Apollon was upregulated in gliomas and was responsible for cisplatin and camptothecin resistance (Vucic et al., 2000; Ashhab et al., 2001).

Survivin was noted to be overexpressed in haematological malignancies and also in small-cell lung carcinomas (Chen et al., 1999). It is suggested that overexpression of survivin in the majority of NSCLCs together with abundant or upregulated expression of XIAP indicate that these tumours are endowed with resistance against a variety of apoptosis-inducing conditions (Small et al., 2010; Krepela et al., 2009).

Accumulating evidences suggest that survivin expression can identify the lesions at highest risk for malignant transformation and invasion. Its presence in the body fluids, however, might be an important biological marker and predictive sign of treatment outcome (Wang et al., 2004). Abnormal expression of the survivin gene in different tumours and its role in tumourigenesis are related to function during mitosis, rather than inhibition of apoptosis (Earnshaw, 2005). IAPs are the most important regulators of apoptosis because they synchronize both the intrinsic and extrinsic pathways of apoptosis. Eight human IAP proteins are known to date with survivin and XIAP as the most characterized.

2.6 Free Radicals and Reactive Oxygen Species (ROS)

2.6.1 ROS Signalling in Normal and Cancer Cells

Free radicals are commonly known as reactive oxygen species (ROS) while some are referred to as reactive nitrogen species (RNS) (Valko et al., 2006). ROS is defined as intermediate oxygen carrying metabolites with or without an unpaired electron with oxidizing capacity. They are persistently generated molecules that are transformed and consumed in all aerobic organisms (Dickinson and Chang, 2011).

ROS describes oxygen derived radicals such as superoxide anions ($O_2^{\cdot-}$), hydroxyl radicals ($OH^{\cdot}$), peroxyl ($ROO^{\cdot}$), alkoxyl ($RO^{\cdot}$) as well as oxygen derived non-radical species such as hydrogen peroxide (H_2O_2) while RNS include nitric oxide ($NO^{\cdot}$), peroxynitrite (ONOO-), nitrogen dioxide and nitrite (NO_2^-) (Halliwell and Cross, 1994; Mahelkova et al., 2008). They are chemically active prooxidant products of incomplete oxygen reduction (Sreevalsan and Safe, 2013; Valko et al., 2006). They convert other components in a chain reaction into free radicals through oxidation (Rammal et al., 2010; Valko et al., 2007).

ROS are produced either to serve essential biological functions or represent byproducts of metabolic processes (Shen et al., 1996). Oxygen free radicals either as reactive oxygen or reactive nitrogen species are products of normal biological systems which play paradoxical roles in living systems (Nyska and Kohen, 2002; Uttara et al., 2009). ROS are generated either from both endogenous and exogenous sources (Valko et al., 2006). Endogenous source of ROS includes mitochondria, cytochrome P450 metabolism, peroxisome and inflammatory cell activation (Inoue et al., 2003).

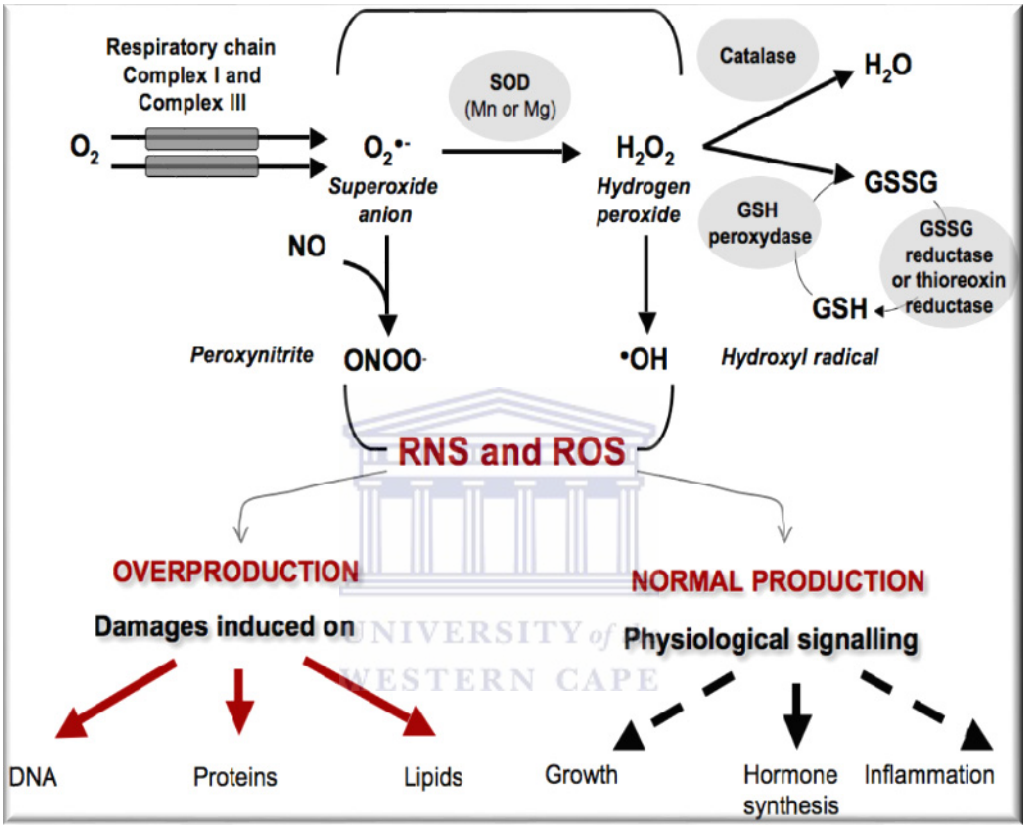
Electron transport chains of mitochondria and endoplasmic reticulum are the major sources of superoxide anion in aerobic cells. A significant increase is observed in the presence of uncouplers of oxidative phosphorylation, hyperbaric oxygen treatment, pathologic conditions and alterations of mitochondrial lipids (Mates and Sanchez-Jimenez, 2000). Superoxide can also be generated from leaking of electrons onto oxygen in nuclear membranes in the presence of NAD(P)H, decomposition of oxyhaemoglobin, photo-irradiation of tryptophan, eumelanin, pheomelanin by UV-light, endothelium autoxidation of catecholamines, thiols, a reduced form of riboflavin and its derivatives, enzymes such as xanthine oxidase, dehydrogenases and oxidases (Mates and Sanchez-Jimenez, 2000). Other endogenous sources of ROS are neutrophils, eosinophils and macrophages. Activated macrophages induce an increase in oxygen uptake and

give rise to ROS, which include superoxide anion, nitric oxide and hydrogen peroxide (Conner and Grisham, 1996). Microsomes and peroxisomes are also responsible for ROS production. Microsomes is associated with 80% of hydrogen peroxide produced *in vivo* at hyperoxia site while peroxisomes produce hydrogen peroxide under physiological conditions (Gupta et al., 1997).

An exogenous source of ROS includes xenobiotics, chlorinated compounds, metal ions, radiation, barbiturates and a host of other environmental agents. These are known to directly or indirectly generate reactive species in cells (Klaunig and Kamendulis, 2004). ROS/RNS are produced during irradiation by UV light; X-ray and gamma-rays; products of metal-catalyzed reactions; are present as pollutants in the atmosphere; produced by neutrophils and macrophages during inflammation; by-products of mitochondria-catalyzed electron transport reactions (Cadenas, 1989). They play paradoxical roles in biological systems such as defence against infectious agents which involve cellular signalling systems and also at low concentrations they induce a mitogenic response. In contrast to their beneficial roles they are mediators of damage to cell structures, including lipids and membranes, proteins and nucleic acid (Valko et al., 2004). Figure 2.4 shows the integrated ROS signalling network in cell mitochondria and its downstream physiologic and pathologic effects.

It is estimated that 10,000 to 20,000 free radicals attack each body cell per day (Valko et al., 2006). The overwhelming generation of ROS/RNS either through an endogenous or exogenous source in excess of the capability threshold of the cell antioxidant defense system is termed oxidative stress (Valko et al., 2006). Oxidative stress leads to oxidative degeneration of biomolecules such as proteins, lipids and DNA. The accumulation of oxidative damage is known to alter physiological functions of the cell as found in alterations in signal transduction and gene expression to mitogenesis, transformation, mutagenesis and cell death (Hunt et al., 1998; Mills et

al., 1998). Pathological conditions attributed to oxidative lesion include myocardial infection, atherosclerosis, neurodegenerative disorders, rheumatoid arthritis and cancer (Devasagayam et al., 2004).



Mitochondrial respiratory chain is recognize as a major site of ROS production in the form of superoxide, hydrogen peroxide and hydroxyl free radical (Bellance et al., 2009)

Figure 2.4: ROS signalling and its downstream physiologic and pathologic effects

2.6.2 Reactive Oxygen Species (ROS) and Carcinogenesis

Mammalian cells are naturally endowed with an antioxidant defense system that buffers the effects of oxidants produced in the cells (Lokanatha et al., 2014). The overwhelming production of radicals over the body's antioxidant capability or due to the inability of the body system to mop the excess radicals can lead to the pathway of carcinogenesis. Oxidative stress is

established, however, when the there is a disruption in the antioxidant/oxidant delicate balance (Lokanatha et al., 2014). Building up of the oxidants in excess of normal body antioxidant threshold limit leads to deleterious effects on macromolecules. Oxidative DNA mutation can be single or double stranded DNA breaks, purine, pyrimidine or deoxyribose modifications and DNA cross-links (Funda and Hakan, 2008). The damage can lead to either arrest or induction of transcription, induction of signal transduction pathways, replication errors, genomic instability (Valko et al., 2006), gene amplification and rearrangements that result in the activation of proto-oncogenes or inactivation of tumour suppressor genes, all of which are associated with carcinogenesis (Tanaka et al., 1998).

Free radicals such as hydroxy radicals modify DNA by different mechanisms which include sugar and base modifications, strand breaks and DNA protein cross-links (Wallace, 1998). Guanine (G)-cytosine (C) to thymine (T)-adenine (A) DNA base transversion modification is reported to have mutagenesis potential (Grollman and Moriya, 1993). G-C to T-A transversion is reported in either inactivation of tumour suppressor genes or activation of oncogenes, suggesting that ROS-related mutations play pivotal roles in the initiation step in the development of cancer (Lokanatha et al., 2014). Free radical generation with high rates of oxidative DNA damage give some tumour potential to mutate, inhibit anti-proteases and damage surrounding tissues.

Oxidative modification of DNA is also a factor that contributes to the genetic instability and metastatic potential of tumour cells in fully developed cancers (Malins et al., 1996). The exact mechanism of action of ROS in carcinogenesis has not been fully deciphered. However, two mechanisms have been proposed to play a role in oxidative damage and in the development of carcinogenesis (Valko et al., 2004). The first mechanism involves modulation of gene expression while the second is radicals induce genetic alterations (Valko et al., 2004). The first mechanism is through epigenetic effects on gene expression which lead to the stimulation of growth signals

and proliferation (Crawford et al., 1995). Strand breakage misrepair, alterations in gene expression and loss of heterozygosity contribute to gene expression modulation (Bohr et al., 1995). Stimulation of protein kinase and poly (ADP-ribosylation) shows that signal transduction pathways are also involved in ROS modulation of essential genes (Cerutti and Trump, 1991). The second mechanism by which ROS induce carcinogenesis implicates gene mutations and chromosomal rearrangements which play roles in the initiation of carcinogenesis (Guyton and Kensler, 1993). Oxidative DNA damage leads to chromosomal abnormalities, blockage of DNA replication and cytotoxicity (Bohr et al., 1995). A mutation can occur through misrepair and incorrect replication while chromosomal rearrangement can be the result of strand breakage misrepair (Halliwell and Aruoma, 1991).

Therefore, it has been shown that oxidants contribute to carcinogenesis by their abilities to induce DNA base changes in certain oncogenes and tumour suppressor genes (Jackson, 1994). For example, K-ras and C-Raf-1 have been shown to be activated by hydroxyl radicals due to induction of DNA point mutations in G-C base pairs and N-terminal deletions in these genes (Jackson, 1994). CpG base point mutations induced by ROS are also found to be present in some tumour suppressor genes such as p53 and retinoblastoma (Rb) leading to their inactivation (Nigro et al., 1989).

In addition, the tumorigenesis inducing role of ROS is not only limited to DNA oxidation, but also by activating signalling pathways that regulate cellular proliferation, angiogenesis and metastasis (Storz, 2005). The effect of ROS as stated above is dependent largely on the level of expression of ROS. The low levels are known to activate proliferative signals while high levels reduce cell proliferation by activating damage or cell death signals (Rosa et al., 2012). It has been observed that ROS activate kinases, protein serine/threonine kinases, small G-proteins or transcription factor signaling (including nuclear factor kB, activator protein 1 and hypoxia

inducible factor 1 alpha) and inactivate phosphatase, all of which lead to loss of proliferation control (Rosa et al., 2012).

2.7 Antioxidants

An antioxidant is any substance that delays, prevents or removes oxidative damage to a target molecule (Halliwell and Gutteridge, 2007). Evidences have shown that oxidative stress is important for cancer development (Halliwell, 2007) and paradoxically apart from the oxidative damage of DNA, proteins and lipid attributed to ROS, they still play significant beneficial roles in all aerobes at certain conditions (Halliwell, 2006a). The balance between antioxidants and free radicals permits useful biological functions unhindered (Halliwell and Gutteridge, 2007). An antioxidant is, however, expected to allow the beneficial roles performed by the ROS with minimal oxidative damage (Halliwell and Gutteridge, 2007). Increased oxidative damage occurs not only through induced oxidative stress, but also via failure to repair or replace damaged biomolecules and/or decrease antioxidant levels (Halliwell, 2006b). Coping with oxidative damage, therefore, requires repair of oxidized DNA, reassembling of [Fe-S] clusters in enzymes, repair of oxidized Met sulfoxide reductases and destroy oxidized lipids and proteins (Moskovitz and Stadtman, 2003).

Antioxidants act by scavenging radicals, donating hydrogen and electrons, decomposing peroxide, quenching singlet oxygen, inhibiting enzyme and chelating metals (Lobo et al., 2010). They are categorized into two groups of enzymatic antioxidants - superoxide dismutase (SOD), catalase, glutathione peroxidase (GPx) and non-enzymatic (ascorbic acid, apha-tocopherol) found in the intracellular and extracellular environment (Kohen and Nyska, 2002). They function at three different stages of ROS reactions which are prevention (keeping the formation of ROS at bearable levels), interception (scavenging reactive species either by catalytic and/or non-catalytic molecules) and repair (repairing damage tissue) (Kohen and Nyska, 2002). Prevention is known

as the first line of defense, radical scavenging is the second line of defense while repair and *de novo* is the third line of defense (Lobo et al., 2010). The first line defense antioxidants act by quenching superoxide anion, decomposing hydrogen peroxide and sequestration of metal ions. The antioxidants in this category are SOD, catalase, GPx, glutathione reductase, glutathione-S-transferase (GST). Also, antioxidant minerals that act as metalloenzymes such as Se, Mn, Cu and Zn are also part of the first line of defence (Irshad and Chaudhuri, 2002). Glutathione (GSH), vitamin C, uric acid, albumin, bilirubin, vitamin E, carotenoids, flavonoid and ubiquinol are radical scavenging antioxidants that form the second line antioxidant defense(Irshad and Chaudhuri, 2002). Some of these important antioxidants such as β-carotene, vitamin C and E are required in the diets as they cannot be synthesized by most mammals, including human (Irshad and Chaudhuri, 2002). Third line of defense are enzymes that involve in repair damaged biomolecules and reconstitute damaged cell membrane such as DNA repair enzymes (glycosylases and nucleases), transferases, proteases, methionine sulphoxide reductase (Henle and Linn, 1997). They still perform another important function known as adaptation where signals for the production and reactions of free radicals trigger the formation and transportation of the appropriate antioxidant to the right site (Niki, 1993).

It is well established that inability of inherent antioxidant capacity of the body to clear the overwhelming productions of ROS from the body systems lead to degenerative diseases (Saeed et al., 2012). Epidemiological studies and metal analyses have shown that diets rich in plant polyphenols protect against development of cancers, cardiovascular diseases, diabetes, osteoporosis, etc. (Pandey and Rizvi, 2009). This is because ingestion of plant antioxidants have been inversely correlated with morbidity and mortality from degenerative diseases (Gulcin, 2012). Antioxidant compounds from plants prevent or delay oxidative modification of macromolecules by inhibiting the initiation or propagation of oxidative chain reactions (Velioglu et al., 1998). This has led to growing interest in medicinal plants with antioxidant constituents

such as phenolic compounds, terpenoids and vitamins as potential food additives in the prevention of oxidative related diseases (Craig, 1999).

2.8 Medicinal Plants

2.8.1 Plants as Natural Health Remedies

Plants are the basic sources of folklore medicine based on the empirical findings of our forebears for the treatment of illness common in their contemporary period (Gurib-Fakim, 2006). At that time, they did not understand the scientific basis for its use, but they knew from the experience that it is effective when used at the right doses (Gurib-Fakim, 2006). A medicinal plant is any plant with parts (leaves, stem bark, roots, etc.) that can be put to use for the relief of pain, prevention or cure a disease or ability to alter physiological and pathological processes, or any plant used as a source of drugs or their precursor (Rates, 2001).

Medicinal plants contain compounds that either act singly, additively or in synergy to alleviate health challenges. A single plant may contain substances that stimulate digestion, or anti-inflammatory compounds that reduce swelling and pain, phenolic compounds with antioxidant activity, tannins with natural antibiotics, antidiuretic substances that enhance the elimination of waste products and toxins and alkaloids that enhance mood and give a sense of well-being. The presence of secondary metabolites in plants serves as the basis for its medicinal value. Although, the presence of these compounds does not enjoy any biochemical justification, but they provide an invaluable resource for the discovery of novel and potent drugs for diseases that plague humanity (Gurib-Fakim, 2006).

Plants have been sources of medicine in the form of crude drugs such as tinctures, teas, poultices, powders and other herbal combinations/condiments for various illnesses for thousands of years (Samuelsson, 2004). The type of plant or part of a plant to be used for a particular ailment is passed down through an oral rendition which, are known to have played significant roles in

traditional medicine (Douglas Kinghorn, 2001; Samuelsson, 2004). The WHO estimated that 80% of the population in developing countries rely on the use of plants for curing different ailments while plants also play a significant secondary role in the health care sector of the developed countries (Jabeena et al., 2014). The development and deployment of technology since the 19th century, however, have led to the isolation of particular bioactive agents responsible for the observed effects in complex mixtures of compounds present in crude formulations or condiments (Newman et al., 2000; Samuelsson, 2004). The isolated bioactive compounds are presently being used as drugs or in the various stages of preclinical and clinical trials. The isolated compounds in use as drugs represent a very good platform for the exploitation of medicinal plants (Balunas and Kinghorn, 2005).

Drugs of plant origin contribute about 25% of the world's total drug prescriptions with 121 active compounds that are currently being used. The WHO considered 252 drugs as the basic essential need out of which 11% are of plant origin and a number are precursors of synthetic drugs, e.g., digoxin from *Digitalis* spp., quinine and quinidine from *Chinchona* spp., vincristine and vinblastine from *Catharanthus roseus*. Reliance on the structural base diversity of natural products which give it an edge to be a lead compound and essential template for the design and rational planning of new drugs, biomimetic synthesis development and discovery of new therapeutic properties not yet attributed to known compounds (Hamburger and Hostettmann, 1991). The importance of natural product, notwithstanding, exploration of potential of plants for the discovery of novel drugs is still very poor because out of about 250,000-500,000 plant species only about 5000 species have been studied for medicinal use (Payne et al., 1991).

2.8.2 Plants as Sources of Anticancer Drugs

The importance of natural plant products for the treatment of disease has been recognized since ancient times. Natural products are small-molecule secondary metabolites from terrestrial and

marine plants, microorganisms and animals with diverse structural quality for novel drug discovery (Pan et al., 2010). The advancement in technology and progress in combinatorial chemistry, notwithstanding, plant agents still make a tremendous contribution to the drug discovery process most of which are currently in clinical trials (Cragg et al., 1997; Farnsworth et al., 1985). More than 60% of drugs currently used as anticancer have their origin from the natural sources (Newman et al., 2003) (Figure 2.5). A review of anticancer drugs in North America, Europe and Japan between the period of 1981-2006 show that 47.1% of 155 clinically approved anticancer drugs are of natural origin (Newman and Cragg, 2007).

The discovery of anticancer agents from plant sources started in the 1950s with the development of the vinca alkaloids, vinblastine and podophyllotoxins. It is remarkable to note that most of the new cancer drugs from natural sources in the last 50 years have been from soil microorganisms, terrestrial fungi and higher plants (Kinghorn, 2008). The search has also led to the discovery of more anticancer agents with different cytotoxic mechanisms of action, including the taxanes and camptothecins (Cragg and Newman, 2005). The upsurge in the number of naturally based anticancer drugs might not be unconnected to the adaptation of new technologies, such as combinatorial synthesis and automated high throughput screening, which enhances bioactive screening, purification and structural analysis from natural products (Nobili et al., 2009).

The development of drugs that are selective to cancer cells with no effects on normal cells are desperately needed to solve the challenges of the burgeoning increase in deaths due to various forms of cancer, which stands as another reason for the increased exploration for anticancer drugs from the natural sources (Sporn and Liby, 2005). There are four major structural classes of plant derived compounds presently in use in Western medicine as a single chemical entity: vinca alkaloids (vinblastine, vincristine, vinorelbine), epipodophyllotoxin lignans (etoposide,

teniposide, etoposide phosphate), taxane diterpenoids (paclitaxel, docetaxel) and camptothecin quinoline alkaloid derivatives (topotecan, irinotecan) (Brunton et al., 2005; DeVita et al., 2008).

2.8.1.1 Vinca Alkaloids

Vinblastine and vincristine are alkaloids isolated from the Madagascar periwinkle, *Catharantus roseus* G. Don (*Apocynaceae*) (Figure 2.5). The mechanisms of action of the two alkaloids involves blocking the polymerization of tubulin molecules into microtubules, preventing the formation of the mitotic spindle which results in metaphase arrest and apoptosis (Verma and Singh, 2010). Vindesine is semisynthetic analogue of the vinca alkaloid vinblastine which acetyl group was changed to amido. It is used mainly to treat acute lymphocytic leukaemia, but less prescribed for used in breast cancer, colorectal cancer, non-small-cell lung carcinoma (NSCLC) and renal cancer. Some countries such as Britain, South Africa and certain European countries have approved its use, but the USA Food and Drug Administration (FDA or USFDA) is yet to give approval (Jordan and Wilson, 2004). Vinorelbine, trading under the name Navelbine, is a semisynthetic analogue of vinblastine. It is differs from vinblastine because of loss of water from the piperidine ring and also the bridge linking the indole ring to the piperidine nitrogen is one carbon shorter. It was approved for treatment of NSCLC in France in 1989 and metastatic breast cancer (MBC) in 1991. It was, however, approved for use by FDA in December 1994 (Smith, 1995; Jabeena et al., 2014).

Dihydro-fluoro derivatives of vinorelbine known as vinflunine was approved by the European Medical Agency (EMEA) in 2009 as a second line chemotherapy in metastatic urothelial cancer. The mechanism of this derivative is similar to the other members of vinca alkaloids by interacting with tubulin molecules, inhibiting microtubule polymerization and the formation of tubulin paracrystals. This leads to G2/M phase arrest and induction of apoptosis. It is in Phase II/III clinical trial for the treatment of MBC and NSCLC. Phase I/II trials to evaluate its potential

in other advanced solid tumours have been alluded to (Bachner and De Santis, 2008; Mamtani and Vaughn, 2011). Vincristine sulphate, a nanoparticle-encapsulated molecule designed specifically to reduce potential toxicity associated with cancer therapeutic agents was approved for use as treatment of adult patients with Philadelphia chromosome-negative (Ph^-) acute lymphoblastic leukaemia (ALL) in second or greater relapse whose disease has progressed after two or more anti-leukaemia therapies (Jabeena et al., 2014).

2.8.1.2 Taxane Diterpenoids

Paclitaxel was discovered from the bark of the Pacific Yew, *Taxus brevifolia* Nutt (*Taxaceae*) provides further evidence for the success of natural product drug discovery (Figure 2.5). It promotes microtubule formation and it has been used in the treatment of ovarian cancer, breast cancer and also NSCLC (Kinghorn and Seo, 1996). Docetaxel is the first derivative of paclitaxel to reach clinical trials. It shows a wide range of clinical activity against tumours from different origin with the attendant toxicity pattern than the parent compound. Different structural modifications have been made to improve the efficacy of this compound with enhanced cytotoxicity in resistant tumours, decreased toxicity in normal tissue and improved solubility. The inability of paclitaxel and docetaxel to cross the blood brain barrier (BBB) is the major obstacle for these compounds (Bissery et al., 1995; Gelmon, 1994). Cabazitaxel is a semi-synthetic taxane that binds with tubulin leading to microtubule depolymerization and cell division inhibitions, G2/M cell cycle arrest and the inhibition of tumour cell proliferation (Paller and Antonarakis, 2011).

Cabazitaxel is a poor substrate for the membrane bound multidrug resistance efflux pump P-glycoprotein (Pgp), it has ability to penetrate the BBB unlike other taxanes and may be useful for treating MDR tumours. Its combination with prednisone significantly extends overall survival in men with hormone-refractory prostate cancer previously treated with a docetaxel-containing

regimen and also improves disease control (Jabeena et al., 2014). Abraxane is an albumin bound nanoparticle formulation of paclitaxel approved in the US in 2005 for use as a breast cancer treatment and in 2012 approved as first line of treatment of metastatic NSCLC among patients who could not receive radiation therapy or curative therapy (Jabeena et al., 2014). Paclitaxel poliglumex is a semi synthetic innovative macromolecular taxane designed to improve the therapeutic index of paclitaxel. It is undergoing Phase III clinical evaluation of NSCLC and also Phase III trial in combination with carboplatin for the treatment of performance status (PS 2) patients with chemotherapy naive advanced NSCLC. Although it does not prove to be better in respect to the superior survival in the first line treatment of PS 2 patients with NSCLC when compared with paclitaxel/carboplatin regimen, it was more convenient with respect to progression-free survival and overall survival (Bao and Pokman, 2011). Taxaprexin (7-DHA-Taxol) is made by linking paclitaxel to docosahexaaenoic acid (DHA). It is a fatty acid that is easily taken up by tumour cells. Its limitation is the low activity in patients with advanced NSCLC as a single therapy. Despite this limitation of low objective response rate, treatment with this agent is associated with survival comparable to platinum based combination therapy. Presently, however, the agent is undergoing different phases of clinical trials in advanced lung cancers, eye melanoma and advanced skin melanoma (Payne et al., 2006).

2.8.1.3 Epipodophyllotoxin Lignans

Podophyllotoxin is a compound obtained from *Podophyllum peltatum*. Its mechanism of action as an anticancer agent is the ability to bind reversibly to tubulin (Wilson, 1975). Etoposide and teniposide are analogues of podophyllotoxin which have different mechanisms of action compared to the parent compound (Srivastava et al., 2005). They act as inhibitors of the enzyme topoisomerase-II and can be used as treatment of various cancers. Many semi-synthetic analogues of podophyllotoxin have been synthesized to overcome shortcomings of the lead compound such as poor water solubility, acquired drug resistance and metabolic inactivation,

e.g., NK-611, GL-331, azatoxin, Top-53, etoposide phosphate and tafluposide are produced as either clinical drugs or novel clinical trial candidates for various cancers (Sargent et al., 2003; Liu et al., 2002; Huang et al., 2000).

2.8.1.4 Camptothecin Quinoline Alkaloid Derivatives

Camptothecin (CPT) is a quinoline alkaloid isolated from *Camptotheca acuminate* (Figure 2.5). It is a known topoisomerase-I inhibitor that induces cell death by DNA damage (Hsiang et al., 1985). A number of analogues have been synthesized and approved for clinical use such as topotecan, irinotecan and belatecan which effectively inhibit DNA topoisomerase-I and overcome low aqueous solubility and severe toxicity associated with the lead compound (Oberlies and Kroll, 2004). 9-Aminocamptothecin (9-AC) is another analogue of CPT with potent preclinical studies, but a phase II trial was not as successful as the phase I as it was found not to have activity against lung and colon cancer despite modest activity against ovarian and malignant lymphoma (Pazdur et al., 1997; Vey et al., 1999). However, in combination with other drugs, Phase I/II trials are determined to evaluate its safety, efficacy and tolerability (Farray et al., 2006). Diflomotecan is the first homo E-ring modified CPT analogue to be developed (Jabeena et al., 2014). In addition to its topoisomerase inhibitory capacity, it possesses better lactone stability than the others. Ipsen pharmaceutical has completed a Phase II open label study on the drug at a fixed dose of 7 mg as a 20 min intravenous infusion once every 3 weeks in patients with sensitive small cell lung cancer who have had failed platinum based regimens (Scott et al., 2007).

Gimatecan is an oral topoisomerase-I inhibitor, which is undergoing a Phase I trial for the treatment of advanced solid tumour and treatment of recurrent epithelial ovarian and fallopian cancer in a Phase II trial in a patient that has previously undergone platinum and taxane regimens (Zhu et al., 2009; Pecorelli et al., 2010). In addition, different conjugated CPT has been produced

by conjugating small molecules at the C-20 hydroxyl for stabilization of the lactone ring. This stabilization is to delay opening and reducing affinity to serum albumin - ultimately to slow release of the active drug and alter therapeutic index (Jabeena et al., 2014). Examples of this category of CPT are TP-300 and EZN-2208 which are currently undergoing a Phase I trial against solid tumour and advanced malignancies, respectively (Anthoney et al., 2012; Kurzrock et al., 2012).

Combretastatin is a drug isolated from the bark of *Combretum caffrum* Kuntze (*Combretaceae*) from South Africa known to act by inhibiting tubulin assembly. Combrestastatin A4 has been found to be active against cancers of colon, lung and leukaemia (Jabeena et al., 2014). It is known to act by binding to the colchicine site of tubulin leading to the disruption of microtubule formation (Sackett, 1993). CA-4P and CA-1P prodrugs developed from combretastatins bind with the colchicine site of beta-tubulin and are known to be most active prodrugs from plant origin (Cirla and Mann, 2003). Clinical trials in Phase I/II of CA-4P focus on the safety, tolerability of its combination with other treatment regimens like chemotherapy, radiotherapy and antiangiogenic therapies (Nathan et al., 2012). Combination of conventional therapies and CA-4P has led to Phase II studies in the treatment of NSCLC and anaplastic thyroid cancer (Jabeena et al., 2014).

Other anticancer agents from natural plants are omacetaxine mepesuccinate, an alkaloid from *Cephalotaxus harringtonia*, is a known protein translation inhibitor (Wetzler and Segal, 2011). Ingenol mebutate is a substance found from the sap of *Euphorbia peplus* (Fallen and Gooderham, 2012). Pentacyclic triterpenoid known as Betulinic acid was isolated from *Betula* species (*Betulaceae*) and also from *Mauritiana rugosa* and *Mauritiana oenoplia* (*Zizyphus* species) (Pisha et al., 1995; Shoeb et al., 2006). Betulinic acid expresses different biological

activities such as anti-HIV, anti-inflammatory, antioxidant, antiretroviral and antibacterial (Tzakos et al., 2012; Nguemfo et al., 2009).

2.8.3 The *Apocynaceae* Family

The *Apocynaceae* family referred commonly to as oleander or dogbane, has about 250 genera and over 2,000 species of tropical trees, shrubs and vines found mostly in tropical and subtropical regions (Wiart, 2006). The inclusion of *Asclepiadaceae* species has enlarged the family from two to five subfamilies (Endress and Bruyns, 2000). The subdivision of *Apocynaceae* into the *Plumerioideae*, *Cerberoideae* and *Apocynoideae* significantly corresponds with the bearing of different types of alkaloids in the plants. Therefore, alkaloids can stand as another taxonomical tool for the *Apocynaceae* family. Indole alkaloids are synonymous only to representatives of *Plumerioideae* and it has never been found in the other two sub divisions while skytanthus alkaloids are only found in *Cerberoideae*. Steroidal alkaloids are the exclusive preserve of *Apocynoideae*, a subdivision that *Holarrhena* belongs to (Endress et al., 1989).

The family is characterized with almost all species producing milky sap, simple opposite or whorled leaves, large colorful and slightly fragrant flowers with five contorted lobes with paired fruits (Wiart, 2006). The family is known for its poisonous plants and sources of bioactive substances like alkaloids, terpenoids and iridoids used extensively for medicinal purposes (Connor, 1977).

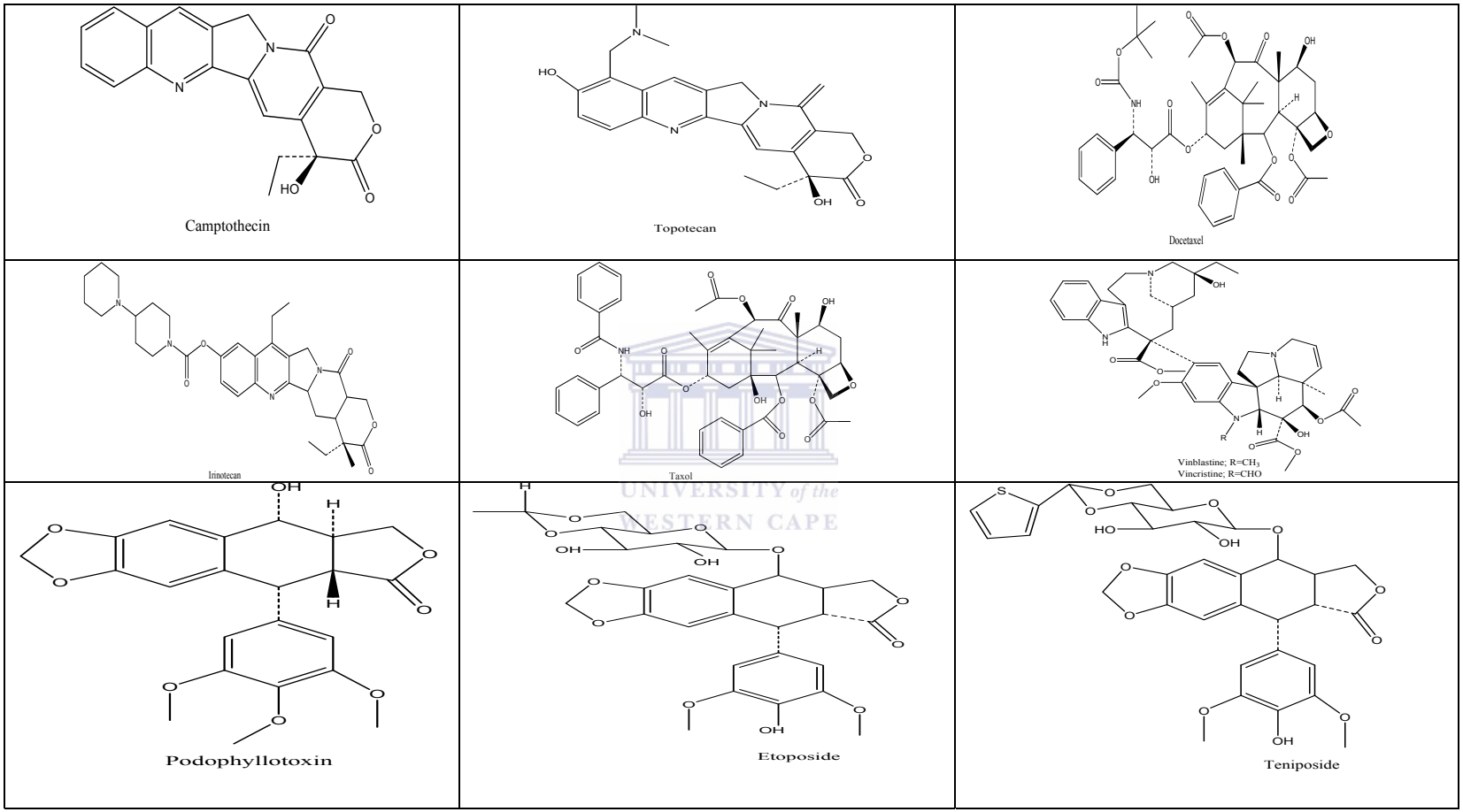


Figure 2.5: Structures of plant-derived anticancer agents in clinical use

Numerous medicinal functions have been attributed to this family in folklore medicine. Treatment ranges from malaria, cancer, headache, leprosy, diarrhoea, diabetes, ulcers, toothache and also the reputed hypoglycemic, purgative, emetic and antiseptic effects (Lin, 2005; Rajakaruna et al., 2002; Nalwaya et al., 2009; De Padua et al., 1999). In addition to traditional importance attributed to this family, several bioactive compounds with broad biological activities had been isolated from the family and published in scientific journals which lend credence to folklore claims.

2.8.4 Bioactive Compounds Isolated from *Apocynaceae*

Nerium oleander is a plant commonly found in Mediterranean regions and sub-tropical Asia and is reputed to have various medicinal properties (Chopra et al., 1956). (Siddiqui et al., 1995) reported the isolation of two novel pentacyclic triterpenoids (*cis*-karenin and *trans*-karenin) with cytotoxic activities against a KB carcinoma cell line from *Nerium oleander*. Another study on *Nerium oleander* also led to the isolation of odoroside A, odoroside B and oleandrin which were active against human breast cancer cell line (Siddiqui et al., 2012). *Carissa lanceolata* is used traditionally by Aboriginals of western Australia for the treatment of medical conditions like toothache and respiratory infections (Lassak and McCarthy, 1983). Carissone, dehydrocarissone and carindone were isolated from the dichloromethane extract of the wood of *C. lanceolata* (Lindsay et al., 2000).

The isolated compounds from *C. lanceolata* were found to be active against *Staphylococcus aureus*, *Escherichia coli* and *Pseudomonas aeruginosa* (Lindsay et al., 2000). The work of Girardot and co-workers led to the isolation of four vobasinyl-iboga bisindole and one 2-acyl monomeric indole alkaloids from the stem of *Muntafara sessilifolia* (Girardot et al., 2012). Some of the indole alkaloids isolated from *Muntafara sessilifolia* were found to be active against chloroquine resistance strain FcB1 of *Plasmodium falciparum* and cytotoxic against the human lung cell line MRC-5 and skeletal muscle cell line L-6 (Girardot et al., 2012). Eight novel pregnane-type steroidal glycosides were isolated from methanolic extract of

Gymnema griffithii fruits pericarp (Srisurichan et al., 2014). Some of these compounds are reported to either have cytotoxicity ability or α-glucosidase inhibitory activity (Srisurichan et al., 2014). Several studies have also reported bioactivity of the pregnane glycosides as antiproliferative, cytotoxic, antidepressant and antifugal (Cioffi et al., 2006; Plaza et al., 2005; De Leo et al., 2005; Waheed et al., 2011; Yoon et al., 2011).

Vincristine and vinblastine are well known antineoplastic drugs derived from *Catharanthus roseus* plant. Vincristine is used for the treatment of Hodgkin's lymphoma and vinblastine for childhood leukemia (Johnson et al., 1963). It has been suggested that the plant still holds potential for the isolation of more chemotherapeutic compounds (El-Sayed et al., 1983). Wang and his co-workers reported the isolation of pregnanone named calotropone and cardiac glycoside gofruside from the ethanol extract of *Calotropis gigantea* L. The two compounds showed remarkable cytotoxicity against K562 and SGC-7901 cell lines (Wang et al., 2008).

Perakine N_4-oxide, raucaffrinoline N_4-oxide and vinorine N_4-oxide isolated from *Alstonia yunnanensis* showed significant cytotoxicity and selective inhibition of Cox-2 (Cao et al., 2012). Triterpenoids isolated from the leaves of *Pleicocarpa pycnantha* are also found to possess antineoplastic activities against panel of cancer cell lines (Omoyeni et al., 2014). The activities of isolated compounds from the Apocynaceae tend more toward the antiproliferative which might not be unconnected to the diverse presence of alkaloids. It is however, not surprising that some of the drugs presently in used and those under clinical evaluations take their roots from the Apocynaceae family. It is on this premises that the bioactive constituents of *Holarrhena floribunda* leaves are being investigated for possible activity against cancer.

2.8.5 *Holarrhena floribunda* (G. Don) Dur. et Schinz

2.8.5.1 Taxonomy

Holarrhena floribunda (G. Don) is a tree that belongs to the *Apocynaceae* family commonly referred to as the false rubber tree (Schmelzer, 2008). *Holarrhena* is a small paleotropical genus of 4 species restricted to tropical west Africa and southern Asia (Endress et al., 1989). It is at variance both morphologically and chemically with other *Apocynaceae* family members (Endress et al., 1989). Considering the aestivation of the corolla lobes, fruits, seed, pollen structure and the alkaloids chemistry *Holarrhena* belongs to the *Apocynoideae* sub division of *Apocynaceae* (Endress et al., 1989). It is a big tree with an approximate height of 17 m by 1 m girth of deciduous forest, savannah wood land in secondary regeneration commonly found from Senegal east to Sudan and South to DR Congo and Cabinda (Angola) (Schmelzer, 2008). It has smooth gray bark with a pale brown slash yielding copious latex (Iwu, 2014). Its leaves are simple, opposite, and entire; stipules are obscure; petiole is up to 1 cm long, shortly hairy and glandular at base; blade is ovate to elliptical, 2-14 (-20) cm x 2.5-8-(-11) cm, base cuneate to rounded, apex acuminate to acute, shortly hairy to glabrous, pinnately veined with 5-14 pairs of lateral veins (Schmelzer, 2008).

Inflorescence is a seemingly axillary cyme, dense and many-flowered; peduncle is between 0.5-1.5 cm long; bract is linear, 1-2 mm long, shortly hairy. Flowers is bisexual, regular, 5 merous, fragrant; pedicel is 0.5-1 mm long; sepals elliptical, 1-4.5 mm long, free or fused at base, shortly hairy; corolla tube cylindrical, 6.5-9 mm x 0.5-1.5 mm, shortly hairy outside and inside form the throat to the insertion of the stamens, lobes elliptical, 7-11.5 mm x 2-3.5 mm, spreading to recurved, shortly hairy to glabrous, white; stamen inserted 2-3 mm from the corolla base, included; ovary superior, ovoid, constisting of 2 carpels connate at extreme base, style cylindrical 2 mm long, pistil head ovoid, stigma 2-lobed. Fruits composed of 2 long and slender follicles 30-60 cm x 5-7 mm, pendulous, dehiscent, pale grey to dark brown, many- seeded. Narrowly oblong 11-16 mm long grooved seeds, glabrous and with dense tuft

of 4-6 cm long hairs at apex. Seedling with epigeal germination; cotyledons are 2.5-3 mm long.

2.8.5.2 Traditional Medicinal Uses

The leaves of *Holarrhena floribunda* are used in folklore medicine as an anti-malarial in Ghana (Bouquet and Debray, 1974). The bark is put to use in Ivory Coast as treatment for the diarrhoea and leaves for amenorrhoea are used either in decoction or macerated in palm wine (Kerharo and Adam, 1974). The bark is also used in Ivory Coast as an enema or in baths to treat skin infection (Schmelzer, 2008). Diuretic activity is obtained with a mixture of the leaves with kolanut while in Sierra Leone it is used as a cure for gonorrhoea and also for the management of diabetes (Kerharo and Adam, 1974). The decoction and concotions with palm kernel oil extraction are given to children with febrile convulsions probably due to cerebral malaria in Nigeria and to both children and adults by Baka pygmies in Cameroon (Fotie et al., 2006). The bark is used as a febrifuge and tonic in the eastern and southern Africa. The root is boiled in milk and used to bathe boys attaining puberty and it is also used as a cure for snakebite and venereal disease (Iwu, 2014). Its fruits are used in Guinea Bissau to prepare a remedy for dropsy. The leaves sap is sprinkled on wounds as a heamostatic. The leaves are marched with those of *Myrianthus arboreus* P. Beauv and fruits of *Capsicum* pepper in water and applied as enema against kidney pain (Schmelzer, 2008).

The tree is known to yield a large number of alkaloids (Kerharo and Adam, 1974). Total alkaloids are greatest in the roots (2%), stem bark (1-1.5%) and least in the leaves (<1.0%). In addition, seasonal variation is known to play a role on the percentage of alkaloids present in the different parts of the plant (Kerharo and Adam, 1974). The plant also contains non-steroidal alkaloids, triacanthine, phenolic acids, flavonoids kaempferol and quercetin (Iwu, 2014). Acid phenols (p-hydroxybenzoic, protocathechuic and p-coumaric acids) are present in the leaves and seeds (Oliver-Bever, 1986).

Major Phytochemicals present in *Holarrhena floribunda* are steroid alkaloids from two main chemical families: counanin and pregnen-5. Conessine, holarrhenine, holadienine, holamine, holaphylline, holaphyllamine, kurchicine, progesterone and some flavones (robinoside) had also been reported (Tamboura et al., 1998). Connesine make up 50% of the total alkaloids and it is responsible for antibiotic activity of the plant (Janot et al., 1950). Most of the main alkaloids are derivatives of pregnane with 0.6% of non-steroidal alkaloids tricanthine (an adenine derivative) (Janot et al., 1950). Many of the steroids alkaloids derived from conamine, holarrhenine are reported to possess hypotensive activity and are also used as a local anaesthetic and spasmolytic (Foussard-Blanpin et al., 1969). Conessine and holarrheine are limited by their cardiotoxic activities despite their hypotensive activities while tricanthine possesses both hypotensive and cardiotonic activity on the heart of rabbit in doses of $1/30^{th}$ of the LD_{50} (Foussard-Blanpin et al., 1969). It causes beneficial long-lasting vasodilation of the coronary arteries in addition to its antispasmodic and respiratory analeptic properties. Its clinical use has been considered against cardiovascular misnormal and it is also found to stimulate erythropoiesis in an experimental anaemia rabbit model (Foussard-Blanpin et al., 1969). Holamine, holaphylline and holaphyllamine have anti-inflammatory properties, cause sodium retention and act as antidiuretic in rats (Schmelzer, 2008). Pregnane derivatives can be an important starting material for the partial synthesis of pharmaceutical steroids such as adrenocortical hormones (corticosteroids) and sex hormones (oestrogens, progestrogens and androgens) (Schmelzer, 2008).

However, the plant is reported to have activity against chloroquine-resistant and chloroquine-sensitive strains of plasmodia. The activity that was found to be more of synergestic effects of the complex mixture contained in the plant rather than the single component (Fotie et al., 2006). *Holarrhena floribunda* exhibited significant cytotoxicity activity out of some plant extracts screened (Abreu et al., 1999). Recently, the phytochemical constituents such alkaloids, saponins cardiac glycoside and tannins have also reported. In addition, some MLEs of *Holarrhena floribunda* fractions are reported to have antioxidant, antimutagenic and lipid

peroxidation inhibitory activities in the liver, brain and egg yolk homogenates (Badmus et al., 2010; Badmus et al., 2013).

Figure 2.6 shows a photomicrograph of *Holarrhena floribunda* and its taxonomic classification. The geographical distribution of the plant on the African continent is depicted in Figure 2.7.

Kingdom	*Plantae*
Phylum	*Tracheophyta*
Class	*Magnoliopsida*
Order	*Gentianales*
Family	*Apocynaceae*
Genus	*Holarrhena*
Species	*Holarrhena floribunda*

Figure 2.6: Photomicrograph of *Holarrhena floribunda* (G. Don) and its taxonomy

(Source: Schmelzer, 2008).

Figure 2.7: Map of Africa showing geographical distribution (part shaded) of *Holarrhena floribunda*

2.8.5.3 Applicability of *Holarrhena floribunda* to this Study

The relevance of *Holarrhena floribunda* used in this study has been covered in detail in Chapter 1, especially with regard to the set objectives of the study and the steps taken to achieve these objectives. Chapter 3 describes the materials and methodology applied in this study.

CHAPTER 3

MATERIALS AND METHODS

3.1 Introduction

This chapter describes the setting, design and methodology used for this study. These encompassed the plant material, maintenance of cancer cells, the chemicals and assay kits, and the types of experiment carried out at each stage of the study.

3.2 Plant Material

Holarrhena floribunda leaves were collected in Igbajo, Osun state, Nigeria, during the rainy season in July, 2011. It was identified and authenticated by the duo of Chukwuma, E.C. and Ugbogu, O.A. at the Federal Research Institute of Nigeria (FRIN). It was deposited at the Institute's herbarium and voucher number FHI 109764 was issued. The plant material was made to undergo different processes of chromatographic separation discussed below to obtain pure compounds used in the study.

3.3 Methods

3.3.1 Maintenance of Cancer Cells

All tissue culture operations were carried out in a model NU-5510E NuAire DHD autoflow automatic CO_2 air-jacketed incubator and an AireGard NU-201-430E horizontal laminar airflow tabletop workstation that provides a HEPA filtered clean work area (NuAire). Cancer cells such as HT-29 (human colon adenocarcinoma), Hela (human cervical cancer) and KMST-6 (normal human fibroblasts) were a kind gift from Dr Mervin Meyer of the Department of Biotechnology, University of The Western Cape, while MCF-7 (Human breast adenocarcinoma) was kindly provided by Professor Maryna De Kock, Department of

Medical Biosciences, University of the Western Cape, South Africa. The cell lines were maintained in complete growth medium containing Dulbecco's Modified Eagle Medium (DMEM) and supplemented with 10% foetal bovine serum (FBS), 1% penicillin/streptomycin (100 U/ml penicillin and 100 µg/ml streptomycin) and grown as monolayer cultures at 37°C in a humidified incubator (Relative Humidity/RH 80%) in an atmosphere of 5% CO_2:95% air.

3.3.2 Cell Growth and Propagation

Cells in cryovials were removed from a -120°C storage freezer and thawed immediately in a 37°C waterbath. The content of the vial was transferred to a T-25 culture flask (surface area (SA) 2500 mm^2) containing 5 ml complete growth medium. The T-25 culture flask was placed on a PrimoVert phase-contrast microscope to visualize the presence of cells, and then placed it in a 5% CO_2incubator at 37°C. The cells were allowed to attach for 24h in the incubator after which the flask was removed and attachment confirmed by microscopy. The flask was incubated until 80-90% confluency was reached.

3.3.3 Subculturing of Cells

Cells were trypsinized at 80-90% confluency. The complete medium was aspirated from the culture flask containing confluent cells. The cells were washed with 2 ml PBS for 1 min. The PBS was replaced with 0.5 ml 0.25% trypsin-EDTA and placed in the incubator for 3-5 min in order to allow for the cells to detach. The flask was then removed from the incubator and placed in a laminar flow cabinet. The trypsin action was deactivated by the addition of 2 ml complete medium. The detached cells were aspirated and transferred to a 15-ml conical centrifuge tube, placed in a centrifuge and spun for 5 min at 2500 rpm to separate the cells from the medium-trypsin solution. After centrifugation, the supernatant was discarded and the cell pellet resuspended in 5 ml complete medium. The cells were mixed to ensure a homogeneous cell suspension, 1 ml of which was transferred to T-75 culture flask (SA 7500 mm^2) containing 12 ml complete growth medium to maintain stock cultures.

3.3.4 Cell Counting and Viability Testing

Cell counts were carried out using TC20^{TM} automated cell counter (Bio-Rad). The automated cell counter uses an optical method to count the cells in fluid samples enclosed in a cell counting slide or chamber. Cell viability was assessed by mixing cell suspension with 0.4% trypan blue stain in a 1:1 ratio (v/v) and 10 µl trypan blue stained cell suspensions was placed in a counting slide chamber and inserted into the TC20TM automated cell counter, with the total number of cells counted together with the number of viable cells displayed on the screen of the cell counter in 30 sec. Thereafter, the required number of cells needed for a particular experiment was calculated in relation to the number of viable cells displayed on the cell counter.

3.3.5 Stock Solutions and Buffers

Details of stock solutions and buffers are summarized in Table 3.1.

Table 3.1: Preparation of stock solutions and buffers

Stock Solution	Preparation
Ammonium Persulfate (APS) (10%)	APS (10%) solution was prepared fresh by dissolving 40 mg of APS in 400 µl distilled water in a microcentrifuge tube and maintained on ice for until used (<5h).
Fluorescein Stock Solution	Fluorescein ($C_{20}H_{10}Na_2O_5$) (22.5 mg) was dissolved in 50 ml of phosphate buffer (pH 7.4), mixed and protected from light. Fluorescein stock solution is stable for a year at 4^0C in the dark.
Glycerol Bromophenol Blue (GBB)	This contains 30% glycerol (v/v), 15 mM ethylenediaminetetracetic acid (EDTA; pH 8.0) and 0.5% bromophenol blue (w/v)
MTT (5 mg/ml)	A 5 mg/ml stock solution of MTT 3-(4, 5-dimethyl-thiazol-2-yl-2, 5-diphenyltetrazolium bromide) was prepared in PBS.
Paraformaldehyde Fixative (4%)	Paraformaldehyde (16 g) was dissolved in distilled water by stirring at 70°C in a fume cupboard. A stepwise drop of 2 M NAOH was added until the mixture remained cleared and the mixture was made up to 80 ml with distilled water. The solution was filtered through a 0.45-µm filter and stored at 4°C.

TBSTween	Tris (2.4 g), NaCl (8.8 g) and 0.1% Tween 20 were made up in 1 L distilled water (pH 7.8).
Peroxyl Radical (25 mg/ml)	AAPH (2, 2'-Azobis (2-methylpropionamidine) dihydrochloride (150 mg) in 6 ml of phosphate buffer (pH 7.4).
Phenylmethanesulfonyl-fluoride (PMSF)	A 10 mM stock solution was prepared in isopropanol.
75 mM Phosphate buffer (pH 7.4)	Sodium dihydrogen monophosphate ($NaH_2PO_4.H_20$) 10.36 g of was dissolved in 800 ml distilled water. The pH was adjusted to 7.4 with 75 mM di-sodium hydrogen monophosphate ($Na_2HPO_4.H_2O$). The buffer volume was made up to 1000 ml with sodium dihydrogen monophosphate. The buffer is stable for a month at 4°C.
Propidium Iodide (PI)	A stock solution of 1 mg/ml was prepared by dissolving 1 mg of PI in 1 ml 3.8 mM sodium citrate (pH 7.0) solution. A working solution consists of 950 µl PBS, 400 µl PI and 100 µl RNase.
RNase (DNase-free)	A 20 mg/ml stock solution was prepared in a 0.1 M sodium citrate and 0.3 mM EDTA (pH 4.8). This solution was boiled for 15 min and cooled quickly by placing it in ice and stored at -20°C.
Trolox Stock Solution	Trolox (6-hydroxy-2, 5, 7, 8-tetra methylchroman-2-carboxylic acid) (0.00625 g) was dissolved in 50 ml phosphate buffer (pH 7.4) and mixed until dissolved. The stock solution was kept in a 2-ml Eppendorf tube and stored at -20°C. Trolox sock solution is stable for 6 months at -20°C.

3.3.6 Preparation of the Methanolic Leaf Extract of *Holarrhena floribunda*

The leaves of *Holarrhena floribunda* were allowed to dry at room temperature. The dried leaves weighing 1.748 kg were soaked in 5.25 L of absolute methanol. The mixture was thoroughly mixed and filtered after 48 hr using a Buchner vacuum filter. The residue was reconstituted in fresh absolute methanol for 24 hr and filtered again using a Buchner vacuum filter. The filtered supernatant was evaporated to dryness with a Rotary evaporator. The weight of dried methaolic extract of the leaves was 380.70 g representing 21.78% yield in relation to the weight of the leaves used.

3.3.7 Fractionation of the Methanolic Leaf Extract of *Holarrhena floribunda*

The fractionation of the methanolic leaf extract (MLE) was carried out by using a silica gel packed column with gradient mixture of ethylacetate and methanol. The ratio of ethylacetate:methanol used were 9:1 (2 l), 8:2 (2 l), 6:4 (2 l) and 4:6 (2 litre), respectively. A total of 51 eluents (500 ml each) was collected. The eluents were monitored by thin layer chromatography (TLC) using the chloroform:methanol mixture (8:2) as mobile phase. The eluents with similar patterns and relative retention factor (RR_f) values after being sprayed with vanillin-sulphuric acid and Dragendorff reagent were combined to yield 18 fractions.

3.3.8 Bioassay-Guided Fractionation

The 18 fractions obtained from the MLE were subjected to bioassay-guided fractionation using the tetrazolium salt 3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay to check for the fractions that can be further purified for anticancer activity.

3.3.9 Isolation and Characterization of Cytotoxic Compounds

The bioassay-guided fractionation of 18 sub-fractions led to the identification of 3 sub-fractions with significant cytotoxic activities. The 3 sub-fractions were found to contain the same active compounds according to their TLC profiles. One of the sub-fractions (0.6 g of sub-fraction 17) was, however, subjected to alumina oxide (Fluka AG, Buch SA) Fluka type 507C neutral packed column chromatography. The gradient mixture of mobile phase started from 0.1% (300 ml), 0.3% (300 ml), 0.5% (300 ml), 0.7% (200 ml), 1% (2.5 L), 3% (200 ml), 5% (1 l) to 10% (100 ml) of dichloromethane (DCM):methanol (MeOH). Eighty four (84) eluents were collected and stratified according to the pattern observed on the alumina coated TLC plate to generate 21 sub-sub-fractions which were subjected to nuclear magnetic resonance (NMR) for possible characterization and identification of pure compounds.

3.3.9.1 Nuclear Magnetic Resonance (NMR)

Identification and characterization of isolated compounds was performed on NMR. The NMR spectra were recorded on 200 MHz Varian Unity Inova spectrometer (Gemini 2000) equipped with an Oxford magnet (14.09 T) and a 5 mm indirect detection PFG probe housed at the Department of Chemistry, University of The Western Cape. The VNMR 6.1C, 1D and 2D software was used for the operation and generation of spectra. The isolated compounds (10 mg) were dissolved in D-chloroform (1 ml). ^{1}H chemicals shifts were referenced to the residual signals of the protons of solvent and were quoted in ppm downfield from tetramethylsilane (TMS).

3.3.9.2 Isolation and Characterization of Flavonoids

Sub-fractions 13 and 14 from the main total extract were selected for further purification based on the TLC profile displayed by the sub-fractions. The sub-fractions had some spots on the TLC profiles that were positive to vanillin-sulphuric acid and also UV visible. The sub-fractions were subjected to silica gel packed column chromatography with 1% DCM:MeOH (1 L), 3% (1 L), 5% (1 L), 7% (1 L) and 10% solvent systems. This was followed by DCM:MeOH:H_2O in ratio 88:12:1 (1 L), 86:14:3 (1 L) and 85:15:3 (2 L), respectively. However, these latter solvent systems were not employed for sub-fraction 14. The sub-fractions obtained were subjected to Sephadex column chromatography using 100% ethanol as mobile phase. The compounds obtained were purified using high performance liquid chromatography (HPLC).

3.3.9.3 High Performance Liquid Chromatography (HPLC)

HPLC separates compounds on the basis of their polarity. It can be used for analyzing, purifying and quantifying compounds (Levin, 2010). HPLC was carried out using an Agilent 1100 HPLC system (Agilent Technologies, Santa Clara, CA, USA) consisting of a quaternary solvent delivery system, an on-line degasser, an auto-sampler, a column temperature controller and ultraviolet (UV) detector coupled with an analytical workstation

and Discovery® C18 column, 5 µm, 250 mm x 10 mm i.d. (Sigma-Aldrich, St. Louis, MO, USA). The experiment was carried out using a reversed phase Phenomenex C-18 silica (250 x 10 mm, 5 micron) column as a stationary phase at a temperature of 30 °C. The optimum resolution was obtained using a linear gradient elution of 30-60% mobile phase A 0.01% acetic acid in methanol and mobile phase B 0.01% acetic acid in water for 30 min, 60-100% at 30-40 min at a constant flow rate of 1.5 ml with a wavelength of 240 nm.

3.3.9.4 Thin Layer Chromatography (TLC)

TLC is a solid-liquid form of chromatography where the stationary phase is normally a polar absorbent and the mobile phase can be a single solvent or combination of solvents. TLC uses thin glass/aluminum plates coated with either aluminum oxide or silica gel as solid phase. TLC distributes compounds between a solid phase applied and a liquid phase (eluting solvent) that is moving over the solid phase (Bele and Khale, 2011). TLC was performed on normal phase Merck Silica gel 60 F_{254} on pre-coated aluminum plates (0.2 mm; Merck, Dermstadt, Germany).

3.3.10 Morphological Evaluation of Cells

Different cell lines were cultured in 6-well plates and treated after 24-hour incubation in 37°C in a humidified CO_2 incubator. The cell morphology was studied using a Zeiss light microscope.

3.3.11 MTT Cytotoxicity Assay

The MTT assay is a measure of viability, which based on the ability of mitochondrial succinate dehydrogenase enzymes in living cells to metabolize yellow 3-(4, 5-dimethyl thiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) into an insoluble coloured purple formazan product which can be measured at wavelengths between 500 and 600 nm by spectrophotometry (van de Loosdrecht et al., 1994; Slater et al., 1963). Viable cells were seeded at a density of 5 x10^4 (100 µl/well) in 96-well plate and incubated in a humidified

atmosphere of 5% CO_2 and 95% air at 37°C for 24 hours to form a cell monolayer. After 24 h, the supernatant on the monolayer was gently discarded and 100 µl of medium and varying concentrations of test compounds in log dose were added and incubated for various times under standard incubation settings.

After each specific time point (24, 48 and 72 h), 20 µl of 5 mg/ml MTT in PBS was added to each well and incubated for 3 hours at 37 ˚C in 5 % CO_2 atmosphere. Supernatant was removed and 150 µl of isopropanol was added and the plates were gently shaken for 15 min to solubilize the formazan and absorbance was measured at 560 nm using a Glomax Multi Detection System (Promega, USA). The percentage inhibition of proliferation was calculated using the formula below and IC_{50} values calculated from log dose-response curves using GraphPad Prism version 6.05 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

$$\% \text{ Inhibition of Proliferation} = 100 - \frac{\text{Test OD}}{\text{Untreated OD}} \text{ X } 100\%$$

3.3.12 Trypan Blue Dye Exclusion Assay

Trypan blue is a ~960 Dalton molecule with two azo chromophore groups. Trypan blue dye exclusion is one of the methods used to measure cell viability, based on the principle that intact membranes are not permeable while compromised cell membranes permit entry of the dye and binds with intracellular proteins.

This allows for the counting of unstained live cells and blue dead cells in a cell population (Denizot and Lang, 1986; Altman et al., 1993). Viable cells were seeded at the cell density of 5 x 10^4 (1 ml/well) in 12-well plates and incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C for 24 hours to form adherent cell monolayers. After 24 h, supernatant on the monolayer was gently discarded and cells treated with 1 ml medium containing varying concentrations of extract (100, 200, 300, 400 and 500 µg/ml) for 24 h. The adherent

cells were detached by trypsinization using 0.25% EDTA trypsin. The cells were centrifuged using Bio-Rad tabletop centrifuge at 730 x g for 5 min and the supernatant discarded. Cell pellets were re-suspended in fresh medium from where 10 µl of cell suspension was added to an equal volume of 0.4% trypan blue dye and loaded into BioRad TC^{20} cell counter. Readings were automatically generated from the machine and recorded based on the formula below;

$$\% \text{ Inhibition} = 100 - \frac{\text{Total Cells - Dead Cells}}{\text{Total Cells}} \text{ X } 100\%$$

3.3.13 Clonogenic Assay

The clonogenic assay evaluates the ability of a cell to form colony of 50 or more cells after treatment with either cytotoxic agent or ionizing radiation. The cells (5 x 10^4/ml) were plated in 6-well plates and allowed to attach for 24 h. After 24 h, the cells were treated with MLE of *Holarrhena floribunda* (100, 200, 300, 400 and 500 µg/ml) and incubated for 24 hours in a humidified CO_2 incubator. The medium containing extract was removed and replenished with fresh neat medium for 5 days in an incubator. After incubation, the medium was removed and the cells washed with PBS. The cells were fixed in 500 µl fixative (75% methanol and 25% acetic acid) for 5 min at room temperature. The fixative was removed and the cell stained with 0.5% crystal violet for 30 min at room temperature. The cells were washed with running tap water to remove the dye. Colonies were counted and the survival rate was calculated as the percentage of cells treated over untreated controls.

3.3.14 Cytotox-Glo™ Cytotoxicity Assay

The MultiTox-Glo multiplex cytotoxicity assay gives ratiometric, inversely correlated measures of cell viability and cytotoxicity. The assay measures live and dead cell protease activities using fluorogenic, cell permeant, peptide substrate (glycyl-phenylalanyl-aminofluorocoumerin, GF-AFC) for live cells while luminogenic cell-impermeant peptide

substrate (alanyl-alanyl-phenylalanyl-aminoluciferin, AAF- Glo™) for dead cells (Promega, 2012). Cells were seeded in 96-well white-walled tissue culture plates and treated using different concentrations of MLE of *Holarrhena floribunda* as reported above. The experiment was carried out according to the instructions of the manufacturer as contained in Promega Technical Bulletin (Promega, 2012).

3.3.15 Apopercentage™ Apoptosis Assay

Viable cells contain an asymmetrical distribution of phospholipids between the inner and outer leaflet of their plasma membranes. Phosphatidylcholine and sphingomyelin are mainly found in the outer membrane while phosphatidylethanolamine and phosphatidylserine (PS) are located in the inner plasma membrane. It is known that during the process of apoptosis, externalization of PS occurs and this can be used as a biomarker for apoptosis (Bretscher, 1972). The APOPercentage™ assay makes use of an anionic halogenated fluorescein dye, the disodium salt of 3, 4, 5, 6,-tetrachloro-2, 4, 5, 7-tetraiodofluorescein (TCTF), which is taken up by apoptotic cells by binding to externalized PS, which gives such cells a purple-red appearance that can be analyzed by flow cytometry (Meyer et al., 2008).

The cells were treated as reported for the trypan blue assay above. Following incubation for 24 h, floating cells in each respective treatment were transferred to a 15-ml centrifuge tube and the adherent cells trypsinized and added to the respective tubes containing floating cells. The cells were washed with 1% PBS and resuspended in residual PBS. APOPecerntage dye (100 µl) in complete culture medium (diluted 1:160, v/v) was added to the tubes and allowed to incubate for 30 min at 37°C in a humidified CO_2 incubator. After the incubation, 500 µl of 1% PBS was added to the tubes containing cells and centrifuged for 5 min at 1,050 x g. After the second washing at the same centrifugation speed, the cell pellet obtained was resuspended in PBS and analyzed on a Becton Dickinson FACScan instrument (BD Biosciences Pharmingen, San Diego, CA, USA) fitted with a 488 nm argon laser. A

minimum of 10,000 cells per sample were acquired and analyzed using the CELLQuest PRO® software (BD Biosciences).

3.3.16 Caspase Glo®-3/7 and -9 Assay

Caspase-3/7 is considered as an executioner caspase and one of the hallmarks of apoptosis induction (Kroemer et al., 2009). Caspase-3/7 was estimated by the Caspase-Glo®-3/7 assay system kit (Promega, USA). Evaluation of the caspase-3/7 was performed according to the manufacturer's instructions. Briefly, 100 µl of 5 x 10^4 cells/ml were seeded in a white walled 96-well microplate for 24 h. The cells were treated with the MLE of *Holarrhena floribunda* (100, 200, 300, 400, 500 µg/ml) for 24 h. After treatment, an equal volume of Caspase-Glo®-3/7 reagent was added, agitated for 30 sec and the luminescence signal recorded using GloMax Multi Detection System (Promega, USA) after incubation for 1 hour. The same procedure was followed for the evaluation of caspase-9.

3.3.17 Annexin/PI Assay

The Annexin V-FITC apoptosis kit (BD Pharmingen, USA) was used to calculate early and late apoptosis with modification to reduce false positive events by fixing the cells with paraformaldehyde and addition of RNase to remove cytoplasmic RNA (Rieger et al., 2011). The Annexin/PI assay determines if cells are viable, apoptotic and necrotic depending on the state of plasma membrane integrity and permeability. Cells undergoing apoptosis are known to externalize PS which binds strongly with Annexin V allowing for the evaluation of apoptosis (Vermes et al., 1995). PI does not stain live cells with intact plasma membranes, but stains cells with compromised plasma membranes in late apoptotic and necrotic states (Darzynkiewicz et al., 1992; Kroemer et al., 1998). PI passes through the membrane and intercalates into nucleic acids to display red fluorescence (Faleiro and Lazebnik, 2000; Rieger et al., 2011). Cells (2 ml) at a density of 5 x 10^4 were grown in 40-mm petri dishes and allowed to attach for 24 h.

The cells were treated after 24 hours with MLE test compounds at different time points (12, 24 and 48 hours at 200 µg/ml extract; 15 µg/ml pure compounds for 12 and 24 h). After the treatment, cells were harvested and centrifuged at 335 x g for 10 min. The supernatants were washed in 1% PBS and resuspended in Annexin V binding buffer. The cells were centrifuged at 335 x g for 10 min and supernatants discarded. Cell extracts were suspended in 100 µl Annexin V binding buffer and 5 µl Annexin V Alexa Fluor 488 added and allowed to incubate in the dark for 15 min. PI (4 µl) diluted in 1 x Annexin V binding buffer (1:10) was added and allowed to incubate for 15 min in the dark at room temperature. Annexin V binding buffer (500 µl) was added to wash the Annexin/PI stained cells. The cells were centrifuged at 335 x g for 10 min and the supernatants were discarded. The cells were resuspended in 500 µl Annexin binding buffer and 500 µl 2% paraformaldehyde to make a 1 % final solution of paraformaldehyde on ice for 10 min. PBS (1 ml) was added to the cell, centrifuged at 425 x g for 8 min and the supernatant discarded. The latter step was repeated twice. RNase A (16 µl) at a concentration of 50 µg/ml was added to the cell for 15 min at 37°C. PBS (1 ml) was added to the cell, gently flicked and centrifuged at 425 x g for 8 min. The supernatants from the extract treated cells were discarded and the cells were suspended in the residual PBS and analyzed on a Becton Dickinson FACScan instrument (BD Biosciences Pharmingen, San Diego, CA, USA) fitted with a 488 nm argon laser while a Tali® Image-Based cytometer was used for cells treated with pure compounds. A minimum of 10,000 cells per sample were acquired and analyzed using CELLQuest PRO software.

3.3.18 Cell Cycle Analysis

Cells were seeded at a density of 2 x 10^5/well in 6-well plates and incubated for 24 hours at 37°C in a CO_2 incubator to form a monolayer. The cells were treated with 200 µg/ml MLE for 12 and 24 h. After treatment, the cells were washed with 2 ml PBS and the cell pellet resuspended in 1 ml (1%, w/v) paraformaldehyde in PBS (pH 7.4) on ice for 30 min. The cell pellets were washed twice in 5 ml PBS. Slowly, 70% of ethanol was added to the cell while vortexing to reduce cell clumping. The cells were stored at -20°C for 48 h, after which they

were pelleted at 4000 rpm for 10 min. Cells were washed in 2X PBS and 1 ml of PI master mix containing 100 µg/ml RNase and 40 µg/ml in PBS. Cell cycle phase distribution was determined using a FACSCalibur Flow Cytometer (BD Biosciences Franklin Lakes, NJ, USA). DNA content of 50,000 events was determined by ModFit software (Verity Software House, Topsham, ME), which provided histograms to evaluate the cell cycle distribution.

3.3.19 Bromodeoxyuridine (BrdU) Incorporation Assay

The BrdU incorporation assay measures cell proliferation by detecting *de novo* DNA synthesis. The assay is based on the incorporation of BrdU as a thymidine analog into nuclear DNA that can be tracked with an antibody probe. Cell proliferation was determined by the ELISA-BrdU (5-bromo-2'-deoxyuridine) chemiluminescence assay kit (Roche, Germany). Briefly, after incubating the cells with IC_{50} concentrations of the compounds for 12, 24 and 48 hours in a 96-well microtiter plate, 10 µl BrdU labeling solution (100 µM, final concentration) was added, and the plate was incubated for 2 h. The labeling solution was removed and the cells were fixed with FixDenat™ solution for 30 min. After removal of the FixDenat solution, anti-BrdU-POD (peroxide dismutase) solution was added and further incubated for 90 min. This was followed by 3 rinses with washing buffer provided with the kit. Then substrate was added for 5 min and the chemiluminescence intensity was determined according to the manufacturer's instruction using GloMax Multi detection system (Promega, USA). Cell proliferation was expressed as a percentage relative to the untreated control.

3.3.20 Evaluation of Reactive Oxygen Species (ROS)

Detection of reactive oxygen species (ROS) activities within cells can be evaluated using the fluorogenic molecular probe 5- (and 6)-chloromethyl-2', 7'-dichlorofluorescein diacetate actyl ester (CM-H_2 DCFDA) (Invitrogen). CM-H_2DCFDA permeates freely into cells where its acetate groups get cleaved by intracellular esterases to a highly fluorescent compound 2, 7-dichlorofluorescin (DCF). The fluorescent DCF can be detected by flow cytometry or fluorescence microscopy and spectroscopy. Briefly, cells were cultured in a 6-well plate at a

density of 2 x 10^5/ml. The cells were treated with 200 µg/ml extract and 15 µg/ml of the pure compounds for 12 and 24 hours. After the treatment, cells were washed with PBS and stained with 7.5 µM of CM-H_2 DCFDA prepared in PBS from a DMSO stock solution and incubated for 30 min at 37°C in a humidified CO_2 incubator. The cells were washed twice with ice-cold PBS following which the cells were acquired and 10,000 events analyzed on a Becton Dickinson FACScan instrument (BD Biosciences Pharmingen, San Diego, CA, USA) fitted with a 488 nm argon laser.

3.3.21 Mitochondrial ToxGlo™ Assay

This assay evaluates biomarkers associated with changes in cell membrane integrity and cellular ATP levels relative to vehicle control cells during short exposure periods (Promega, 2011). Integrity of cell membrane is first assessed by measuring the presence or absence of distinct protease activity associated with necrosis using a fluorogenic peptide substrate bis-AAF-R110 (bis-(Ala-Ala-Phe)-rhodamine 110) to measure dead cell protease (Niles et al., 2007). ATP is measured by adding ATP detection reagent, resulting in cell lysis and generation of a luminescent signal proportional to the amount of ATP present (Promega, 2011). Breifly, 100 µl cells were plated in a 96-well white walled plate at 5 x 10^4 /ml in galactose fortified glucose and FBS free medium. The cells were treated with the log increase concentration of compounds (0.01, 0.1, 1 and 10 µg/ml) for 2 hours before evaluation as described above.

3.3.22 Florescence Microscopic Evaluation of Mitochondrial Status

The status of mitochondria was analyzed by fluorescence microscopic examination of cells stained with the fluorochrome Mito Tracker Red (CMXRos, Lonza Walkersville, USA). Mito Tracker Red (CMXRos) is an aldehyde fixable cationic lipophilic fluorochrome that passively diffuses through the plasma membrane of viable cells and is selectively sequestered in mitochondria with an active membrane potential which allows for the evaluation of mitochondrial functional state in adherent cells (Poot et al., 1996). Briefly, to assess the

effect of the compounds, the cells were cultured in 6-well plate containing coverslips. Twenty minutes to the end of the treatments (3, 6 and 12 h), DAPI (4',6-diamidino-2-phenylindole) stain (5 µl) was added to each treatment and 100 µl of CMXRos (250 nM) in medium was added and incubated for 30 min at 37°C. At the end of the incubation period, the media and the CMXRos were aspirated, washed in PBS and coverslips removed for visualization by mounting on glass microscope slides. The resultant images were taken using a Zeiss Axio-plan 2 fluorescent microscope (Zeiss, Germany)

3.3.23 Immunofluorescent Staining of Actin Filaments

The actin cytoskeleton is a network of dynamic assembly of actin polymers and a variety of associated proteins. Actin performs essential functions for intra- and extra-cellular movement and support in eukaryotic cells. The associated proteins are important for the signalling pathways controlling actin assembly (Jung et al., 2012). Therefore, a change to its normal functions, organization and regulations may lead to cancer. The immunofluorescent staining of actin filaments in the cytoskeleton, focal contacts and nucleus of the cells were evaluated using TRITC-conjugated phalloidin, anti-vinculin and DAPI staining kit according to manufacturer's instructions (Merck Millipore, Darmstadt Germany). Briefly, cells were cultured on cover slides in 6-well plate and treated for 3 hours and 6 hours. Cells were fixed with 4% paraformaldehyde for 15 min followed by washing twice in wash buffer (0.05% Tween-20 in PBS). Cells were permeabilized with 0.1% Triton X-100 (in 1% PBS) for 5 min followed by washing twice with wash buffer (0.05% tween-20 in 1 x PBS). Blocking solution (1% BSA in PBS) was applied for 30 min. Primary antibody (anti-vinculin) (100 µl) was added for 1 hour followed by three washes with wash buffer. The resultant images were taken using a Zeiss Axio-plan 2 fluorescent microscope (Zeiss, Germany).

3.3.24 Fluorescence Microscopic Evaluation of ROS

The cell permeant reagent chloromethyl-2', 7'-dichlorofluorescindiacetate (CM-H_2DCFDA) fluorogenic dye was used to measure the induction of ROS in cells. Cells were treated in 6-

well plates containing coverslips for 3, 6 and 12 h. After treatments, 2 µl of Hoechst 33342 (NucBlue Live ReadyProbes™ Reagent, USA) was added to the medium and incubated for 20 min. After the medium was removed, cells were washed with Hank's balanced salt solution (HBSS). CM-H_2DCFDA (2 µM) was droppered onto coverslips and incubated at 37°C for 30 min in the dark. After incubation, cells were washed twice with HBSS and the coverslips mounted on the microscope slides. The resultant images were taken using a Zeiss Axio-plan 2 fluorescent microscope (Zeiss, Germany) at blue and red fluorescent channels.

3.3.25 Topoisomerase-I Relaxation Assay

This assay is based on the inhibition of the relaxation of supercoiled circular DNA by topoisomerase-I (Osheroff et al., 1983). Topoisomerase (1 U) will relax 0.5 µg of supercoiled pBR322 when incubated in assay buffer in a total reaction of 30 µl at 37°C for 30 min. However, any test compound that inhibits the relaxation of the supercoiled plasmid in the presence of topoisomerase is considered a topoisomerase inhibitor. Gels were run in the absence of ethidium bromide or chloquine. Supercoiled DNA moves faster than relaxed/nicked DNA on 1-1.2% agarose gel electrophoresis. A master mix containing doubled distilled water (ddH2O), 1X assay buffer (20 mM Tris-HCl (pH7.5), 200 mM NaCl, 0.25 mM EDTA and 5% glycerol), 0.5 µg supercoiled DNA, 1 U topoisomerase-I enzyme and the test compounds were prepared as summarized in the Table 3.2. The stock solutions (5 µl) of test compounds were added to 30 µl of the total reaction volume to give 15 µg/ml concentrations. Preparation was done on ice before incubating the mixtures at 37°C for 2 h. After 2 hours of incubation, reactions were terminated with 6 µl 6X Stop buffer (3% SDS, 60 mM EDTA, 50% glycerol, 0.25% bromphenol blue). The reaction products were determined by electrophoresis on 1% agarose gel in TAE (Tris-acetate-EDTA) running buffer at 6.5 V/cm for 2 h. The gels were stained with ethidium bromide (0.5 µg/ml) for 30 min, and then destained in distilled water for 30 min. Gels were directly scanned with an image analyzer (Biometra, Germany) for visualization.

Table 3.2: Master mix preparation of topoisomerase-I

Reagent	**Stock Concentration**	**1X Concentration**	**Volume (µl)/assay**	**X 40 Reactions**
H_2O			21.4	856
10X assay buffer	10 X	1 X	3	120
Supercoiled DNA	50 µg	0.5 µg	0.3	12
Topo I enzyme	100 U	1 U	0.3	12
	Reaction volume without test fraction:		25	
	Reaction volume with test fraction:		30	
Master Mix with Topo I + 9.3 mg/ml Camptothecin (Prepared on Ice)				
Reagent	**Stock Concentration**	**1X Concentration**	**Volume (µl)/assay**	**X 3 Reactions**
H_2O			19.4	58.2
10X assay buffer	10 X	1 X	3	9
Supercoiled DNA	50 µg	0.5 µg	0.3	0.9
Topo I enzyme	100 U	1 U	0.3	0.9
	Reaction volume without test fraction:		23	
	Reaction volume with test fraction:		30	

3.3.26 Extraction of Protein

The treated and untreated cells grown in T-25 cell culture flasks were washed twice with 1X PBS following the addition of 500 µl Cytobuster (Novagen) for 5 min at room temperature. Cells were harvested with a cell scraper and transferred to a microcentrifuge tube and centrifuged for 5 min at 16,000 x g at 4°C. The supernatant was transferred to a sterile microcentrifuge tube and stored at -20°C.

3.3.27 SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

3.3.27.1 Preparation of Protein Samples

The Bradford assay was used to evaluate protein concentration of cell samples. The protein samples were loaded onto gels at 1 µg/µl following heating at 95°C for 10 min.

3.3.27.2 SDS Gel Preparation

Proteins were separated by denaturing SDS-PAGE under reducing conditions (Laemmli, 1970). Briefly, gels were prepared from 40% stock of premixed 37:5:1 acrylamide: bisacryamide and consisted of separating and stacking gels which were poured between two assembled glass plates separated by a 1.5 mm thick comb spacer. The gels were prepared in 1.5 mm Hoeffer dual gel caster and about 10 ml were enough for one gel. The separating gel was poured about 1 cm below the wells of the comb and about 1 ml water saturated 1-butanol was overlaid on top of the separating gel and left to set. Afterwards, the butanol was poured off the gel and rinsed with deionized water. Stacking gel (≈ 5 ml) was poured over the running gel. The comb was immediately inserted into the stacking gel to make wells and allow stacking gel to set. After, the comb was removed, the gel transferred to the Mighty Small apparatus (Hoeffer) containing running buffer.

3.3.27.3 Loading and Electrophoresis of Samples

Two equal volumes of samples were loaded into two different gels and electrophoresed at 100 V/cm until the loading dye barely leaked out of the gel into the running buffer. One of the gels was blotted onto nitrocellulose paper membrane while the other was stained with Coomasie stain.

3.3.27.4 Staining and Destaining of SDS-PAGE Gel

The gel was stained with Coomasie blue solution for 30 min. Destaining was done by several washes with the SDS-PAGE destaining solution on a shaker.

3.3.28 Western Blot Analysis

A ‘wet’ system of transfer proteins separated by SDS-PAGE onto nitrocellulose membrane was used. Gels, sponges, and Whatman filter paper were equilibrated in pre-chilled transfer buffer for 30 min. Cellulose membranes were immersed in 100% methanol for 15 to 20 sec and 30 min allowed for equilibration. Transfer apparatus was assembled by placing the

sponge on the black side of transfer cassette followed by Whatman paper, the gel, cellulose membrane, Whatman paper and lastly the sponge at the second end and making sure bubbles were squeezed out. The cassette was placed into a wet tank with the black side of the cassette correspondingly placed on the black side of the tank. Pre-chilled transfer buffer was added with a magnetic stirrer to mix the buffer to maintain constant buffer temperature. The protein was electro-blotted onto the membrane at 100 V for 90 min. After blotting, the membrane was blocked with 5% milk in TBST (Tris, NaCl and Tween 20; pH 7.8) at room temperature for an hour. The membrane was immediately incubated in 1:1000 dilution of the primary antibody in TBST overnight at 4°C on a shaker. The membrane was later rinsed thrice with TBST for 5 min each. Thereafter, the membrane was incubated with secondary antibody (1: 10,000 in TBST) at room temperature for an hour. The membrane was rinsed with TBST twice at 5 min each. Then, Super Signal West Pico chemiluminescent substrate was prepared by mixing solutions A and B (1:1, v/v) according to the manufacturer's instructions (Thermo Scientific) and the substrate mixture was placed on the membrane. The blot was stripped for another primary antibody. The stripping was performed by pre-wetting the membrane in methanol for a few seconds, then washed in distilled water for 5 min and incubation of the membrane in 0.2 M NaOH for 5 min. This was followed by a 5 min wash in distilled water and finally in TBST for another 5 min. The membrane was then blocked with blocking buffer (5% milk in TBST) for another round of protein evaluation (actin antibody).

3.3.29 Oxygen Radical Absorption Capacity (ORAC)

The ORAC assay is used to measure the scavenging potential of antioxidant compounds against the peroxyl radical (Cao et al., 1993). ORAC generates both qualitative and quantitative measures which present fast- and slow-acting antioxidant activity of a test compound (DeLange and Glazer, 1989). The assay is based on the inhibition of the peroxyl-radical-induced oxidation initiated by thermal decomposition of azo-compounds like 2, 2'-azobis (2-amidino-propane) dihydrochloride (AAPH) (Prior et al., 2003). Briefly, 12 µl of test sample was combined with 138 µl of fluorescein working solution. The reaction was

started by addition of 50 µl AAPH (150 mg/6ml of phosphate buffer) in a 96-well plate. The absorbance was measured with a Fluoroskan Spectrum microplate reader with excitation wavelength set at 485 nm and emission wavelength 530 nm at a temperature of 37°C against a reagent blank prepared with phosphate buffer. Trolox solution was used as a standard calibration curve with concentration ranges from 83-417 µM. ORAC values were calculated using a regression equation ($y=a+bx+cx^2$) and expressed as micromoles of Trolox equivalents (TE) per milligram of test sample. In this assay, three different reactive species were used to assess antioxidant and prooxidant activity of the antioxidants: (1) AAPH (4 mM) as a peroxyl radical generator, (2) H_2O_2-Cu^{2+} (H_2O_2, 0.6%; Cu^{2+} as [$CuSO_4$] 18 µM) as mainly a hydroxyl radical generator ($ORAC_{OH}$ assay) or (3) Cu^{2+} as ($CuSO_4$) (18 µM) as a transition metal oxidant (Cao et al., 1997).

3.3.30 Ferric Reducing Antioxidant Power (FRAP)

FRAP evaluates the potential of a test compound to reduce ferric iron (Fe^{3+}, oxidant in system) to ferrous iron (Fe^{2+}). This redox-linked colorimetric assay incorporates antioxidants as reductants, where the reduction of Fe^{III}-Tris (2-pyridyl)-S-triazine) (TPTZ), which is known to have a deep indigo/blue colour (Benzie and Strain, 1996). FRAP is a single electron transfer reaction from the antioxidant molecule to the oxidant. The change in absorbance value of either oxidant or antioxidant is a measure of reducing capability of the antioxidant (Ou et al., 2002). A working FRAP reagent was prepared as required by mixing 30 ml of 300 mM acetate buffer (pH 3.6) with 3 ml of 10 mM 2,4,6 tripyridyl-S-triazine (TPTZ) solution in 40 mM HCl and 3 ml of 20 mM $FeCl_3 \cdot 6H_2O$ solution. Trolox standard was used as illustrated in Table 3.3. The standard and samples (100 µl) were prepared in triplicate followed by the addition of 3 ml freshly prepared FRAP reagent. Then, reaction mixture was incubated at 37°C for 4 min. Absorbance was read at 593 nm against a blank prepared with distilled water.

Table 3.3: Preparation of FRAP assay standard

Tube	Trolox (µM)	Volume of Stock (µl)	Phosphate Buffer (µl)	Final Volume (µl)
S1	0	0	750	750
S2	83	125	625	750
S3	167	250	500	750
S4	250	375	375	750
S5	333	500	250	750
S6	417	625	125	750

3.3.31 Trolox Equivalent Antioxidant Capacity (TEAC)

The TEAC assay estimates inhibition of radical cation production by the antioxidant in the sample. The concentration of antioxidant in the sample is inversely proportional to the absorbance of the radical cation produced by 2, 2'-azo-bis- (3-ethyl benzothiozoline-6-sulfonate) (ABTS) (Gupta et al., 2009).

The TEAC assay is useful assay for tracking unknown antioxidants in a complex mixture (Arts et al., 2004). Potassium peroxodisulphate solution (8 µl) was added to 5 ml of 7 mM of ABTS and kept in the dark for 24 hours before use. Trolox (6-hydroxy-2, 5, 7, 8-tetramethyl chroman-2-carboxylic acid) (1 mM) was dissolved in 50 ml of ethanol. Trolox concentration ranged between 50-500 µM. Briefly, 25 µl of test sample was added to 300 µl of ABTS (1 ml of ABTS in 20 ml ethanol) in triplicate. The reaction mixture was incubated for 30 min. Absorbance of the reaction was measured with a Multiskan Spectrum Plate Reader at a wavelength of 734 nm and temperature set at 25°C against a blank prepared with ethanol. The TEAC values were estimated using a linear equation ($y=mx+c$) between ABTS scavenged (y in mM) and the ABTS concentration (x in µM). Results were expressed as µmole Trolox equivalents per milligram dry weight of test sample.

3.3.32 Inhibition of Fe (II)-Induced Microsomal Lipid Peroxidation

Endogenous and exogenous factors such as metabolism, chemicals and ionizing radiation are linked to the induction of free radical in biological tissues (Iqbal et al., 2003). Iron is known to be involved in the generation of reactive oxygen species (ROS) *in vivo* such as H_2O_2 which may attack lipids to form damaged products (Imlay and Linn, 1988; Aruoma et al., 1989). Lipid peroxidation is an important product of the reaction between free radicals and lipid (D'Souza and Prabhu, 2006). The ability of an agent to protect lipid from peroxidation by ROS can be related to the antioxidant capacity of the agent. Liver microsomes were prepared from male Fischer 344 rats. Rat livers were homogenized in 0.15 M KCl containing 10 mM potassium phosphate buffer (pH 7.4) and centrifuged at 9000 x g. The supernatant fraction (S9) was stored at -80°C until ready to use.

Microsomes were purified on a Sepharose-2B column as described previously (Gelderblom et al., 1984). Inhibition of Fe^{II}-induced microsomal lipid peroxidation was determined by the method described by (Snijman et al., 2009). The reaction mixture contained microsomes (1 mg of protein/ml in 0.01 M potassium phosphate buffer pH 7.4, supplemented with 1.15% KCl). The samples (100 µl) were pre-incubated with 500 µl microsomes at 37°C for 30 min in a shaking water bath. KCl buffer (200 µl) was added, followed by 2.5 mM ferrous sulphate (200 µl) and incubated for an hour in a shaking water bath. The reaction was terminated with 1 ml 10% trichloroacetic acid (TCA) containing 0.01% butylated hydroxytoluene (BHT) and 1 mM EDTA. Samples were centrifuged at 2000 rpm for 15 min. Supernatant (1 ml) was mixed with 1 ml of 0.67% thiobarbituric acid (TBA) solution and incubated in a water bath at 90°C for 20 min. The absorbance was read at 532 nm. The percentage of inhibition of TBARS formation relative to the positive control was calculated by:

$$\% \text{ Inhibition of TBARS formation} = \frac{A_{control} - A_{sample}}{A_{control}} X\ 100\%$$

3.4 Statistical Analysis

Data are expressed as means ± SD of experiments performed in triplicate. The values were analyzed by Two-Way ANOVA, followed by Tukey's multiple comparism test using GraphPad Prism version 6.05 for Windows (GraphPad Software, La Jolla California USA, www.graphpad.com).

CHAPTER 4

ANTI-PROLIFERATIVE AND APOPTOSIS INDUCTION POTENTIAL OF THE METHANOLIC LEAF EXTRACT OF *HOLARRHENA FLORIBUNDA*

4.1 Introduction

Cancer remains the leading cause of mortality and morbidity in the world. Newly diagnosed cancer cases are about 10 million with 6 million deaths reported worldwide annually (Parkin et al., 2001). In addition, about 1.6 million new cases and 6 hundred thousand deaths are predicted for 2013 in the USA alone (Siegel et al., 2013). Chemotherapy is a major mode of treatment of various cancers, but its success is marred by the attendant toxicity and drug resistance (Yaacob et al., 2010; Leonard et al., 2009; Wonders and Reigle, 2009). Traditional uses of natural plants to treat different diseases, including cancer, and scientific isolation of effective drugs against cancer from plants give credence to their importance. The reason for the viable source of therapeutic agents from nature might not be unconnected with the structural diversity inherent in over a million of species of plants and microorganisms that still remain beyond the comprehension of man (da Rocha et al., 2001).

Drugs currently in use as anticancer agents have over 60% of their origin from natural products (Cragg et al., 2009). Vinblastine (Velban®), vincristine (Oncovin®), vinorelbine (Navelbine®), etoposide (VP-16®), teniposide (VM-26®), paclitaxel (Taxol®), docetaxel (Taxotere®), topotecan (Hycamptin®), and irinotecan (Camptosar®) are some of the examples of anticancer drugs that had passed through different stages of pre-clinical and clinical trials and hence approved for clinical use (Wang et al., 1997; Lee, 1999). *Holarrhena floribunda* (G. Don) is a tree that belongs to the *Apocynaceae* family, a family of plants

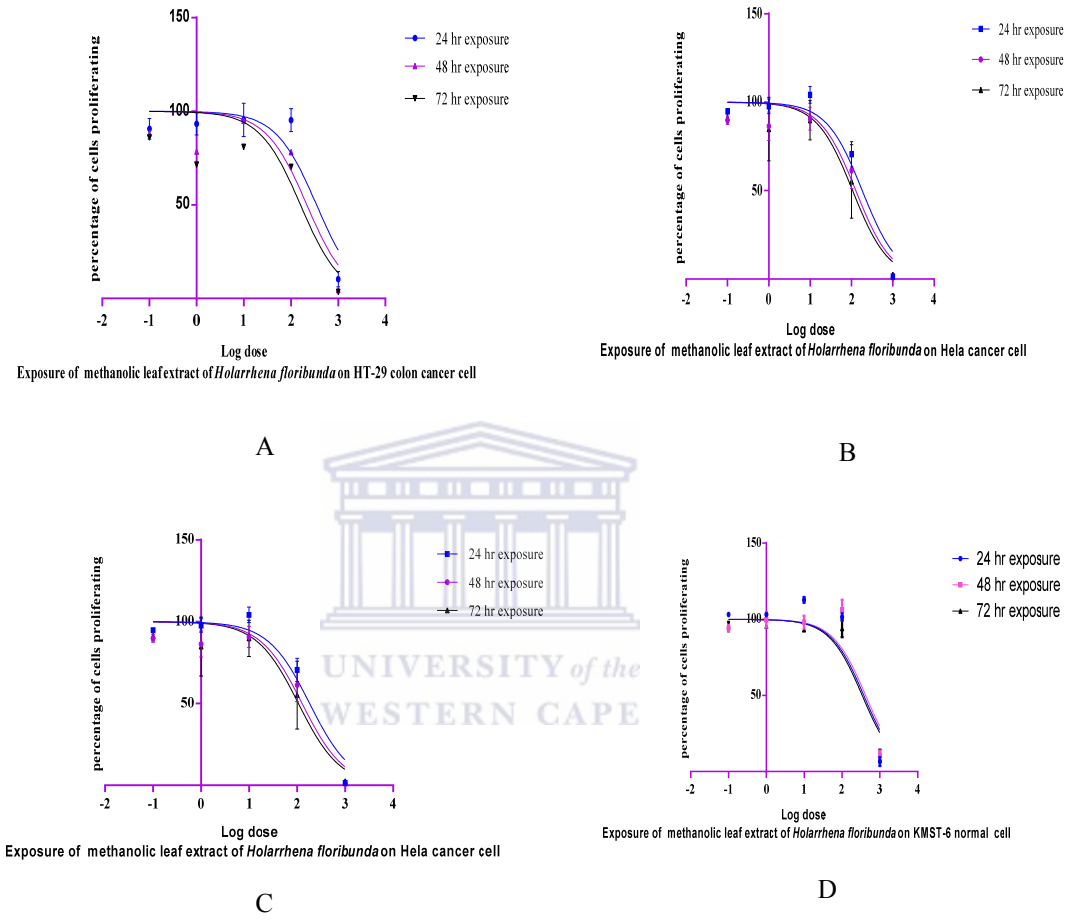
known to be rich in alkaloids. Alkaloids are compounds found to be responsible for the anticancer activity of the *Camptotheca acuminata* plant from which one of the currently used anticancer drugs (camptothecin) is isolated.

The leaves of the plant—*Holarrhena floribunda*—evaluated in this study, are used in folklore medicine as an anti-malarial in Ghana (Bouquet and Debray, 1974). The bark is put to use in the Ivory Coast as a treatment for diarrhoea and the leaves for the amenorrhea (Kerharo and Adam, 1974). The root is boiled in milk and used to bathe boys attaining puberty and it is also used as a cure for snakebites and venereal disease (Iwu, 2014). It has been shown to exhibit significant cytotoxicity activity out of some plant extracts screened (Abreu et al., 1999). Its antioxidant, antimutagenic and lipid peroxidation inhibition properties have been reported (Badmus et al., 2010). The present study aimed to evaluate the antiproliferative, apoptosis and reactive oxygen species inducing activities of the MLE of *Holarrhena floribunda* in breast cancer carcinoma cell (MCF-7), colon cancer (HT-29), cervical cancer (Hela) and normal fibroblasts (KMST-6) as control.

4.2 Cytotoxicity of the Methanolic Leaf Extract

The cytotoxic effect of the MLE of *Holarrhena floribunda* was evaluated on HT-29, HeLa, MCF-7 and KMST-6 using MTT assays. Figure 4.1 shows a log dose-response curve to calculate half-maximal (IC_{50}) cytotoxic effects of the MLE on the cell lines. Table 4.1 depicts IC_{50} values for 24-, 48- and 72-hour treatments. The results showed that all the cells responded to the cytotoxicity effect of the MLE in a dose- and time-dependent manner. The HeLa cancer cells, however, were more sensitive to the plant extract as shown by its IC_{50} values for 24, 48 and 72 hours (182.6, 127.4 and 106.7 µg/ml, respecively). Moreover, the extract exhibited a selectively lower cytotoxicity to normal KMST-6 fibroblasts with higher IC_{50} values of 376.9, 428.2 and 342.3 for the 24-, 48- and 72-hour exposure times, respectively.

Cell viability in respect of MLE doses ranging from 100 - 500 µg/ml was evaluated using the trypan blue dye exclusion assay. The assay further reaffirms the sensitivity of the cancer cell lines compared to the non-cancerous KMST-6 cells (Figure 4.2).



Log dose (µg/ml) effects of extract after 24, 48 and 72 hours exposure of cells specified. MTT assay was employed to assess the cytotoxic effect of the extract and the graphs were prepared using Graphpad Prism 6 statistical software.

Figure 4.1: Cytotoxic effects of the MLE on HT-29, HeLa, MCF-7 and KMST-6 cells

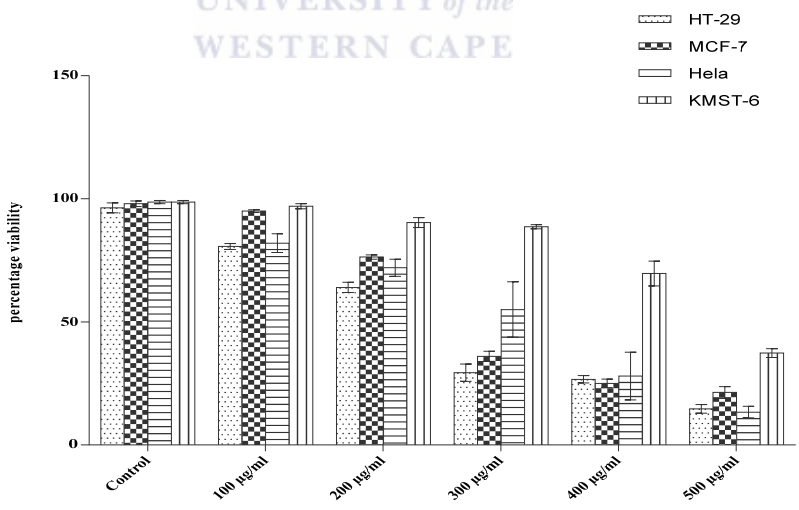
The antiproliferative activity of the extract was further evaluated using clonogenic survival assay. The assay measures the potential of cells to grow into colonies after treatment in a manner unrestricted by growth contact inhibition as found in normal growing cells.

As presented in Figures 4.3 and 4.4, colony formation decreases with an increase in the concentration of the extract. Remarkably, MCF-7 and HeLa cell lines displayed no colony forming units at MLE concentrations of 400 and 500 µg/ml.

Table 4.1: IC_{50} values of the MLE of *Holarrhena floribunda* for HT-29, Hela, MCF-7 and KMST-6 cell lines for the 24-, 48- and 72-hour treatments

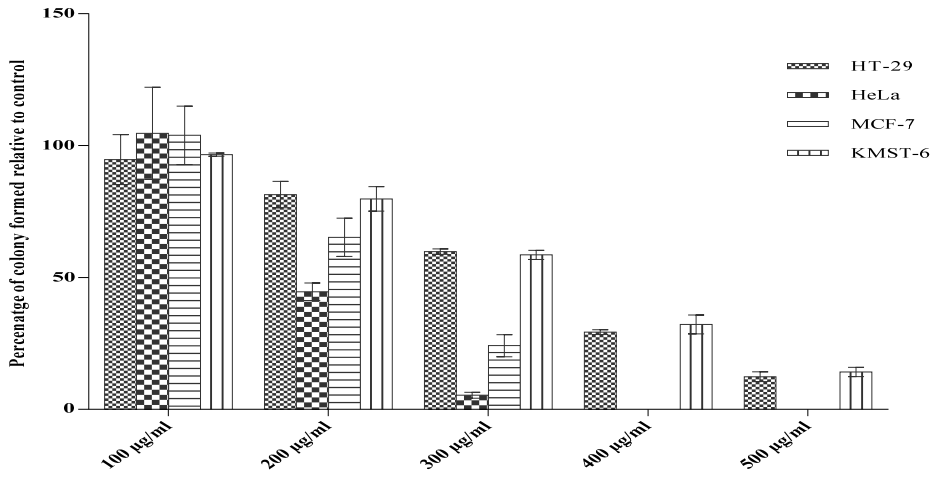
	IC_{50}* (µg/ml) values for cell lines exposed to the MLE of *Holarrhena floribunda*			
Time (h)	**HT-29 colon cancer**	**HeLa cervical cancer**	**MCF-7 breast cancer**	**KMST-6 fibroblasts**
24	349.2	182.6	357.6	376.9
48	217.5	127.4	244.3	428.2
72	159.4	106.7	126.7	342.3

*IC_{50} values were obtained from non-linear regression analysis of the log dose cytotoxic effects of the MLE, using Graphpad Prism 6 statistical software.



The trypan blue dye exclusion assay as assessed with the BioRad TC-20 cell counter was used as described in Chapter 3.

Figure 4. 2: Effects of MLE on the viability of HT-29, MCF-7, HeLa and KMST-6 cells



Paraformaldehyde fixed cells were stained with 0.5% crystal violet dye and colonies formed were counted using a colony counter. Colony formation inhibition at each concentration of the extract is expressed in terms of percent of control and reported as the means ± SD.

Figure 4.3: Percentage colonies formed after a 24-hour MLE treatment of HT-29, MCF-7, HeLa and KMST-6 cells

4.3 Apoptotic Effects of the Methanolic Leaf Extract

Apoptotic effects of the MLE was explored by staining the cells with APOPercentageTM dye and evaluated by flow cytometry. The results showed that the induction of cytotoxicity occurs through the mechanism of apoptosis. The MLE induced apoptosis in a concentration dependent manner (Figure 4.5). The HeLa cell line was significantly ($P<0.0001$) more sensitive to the MLE when compared with other cell lines. The sensitivity of the KMST-6 cell line to the apoptosis inducing ability of the MLE was also low compared to other cell lines. The apoptosis inducing ability of the extract was further tested using the Annexin-V FITC/propidium iodide double staining flow cytometric assay. Cells were exposed to the extract at 200 µg/ml concentration for 12, 24 and 48 h, as presented in Table 4.2. HT-29 colon cancer cells at 24 hours exihibited significant early apoptosis, Hela cervical cancer cells were in the late apoptotic stage at 48 hours while MCF-7 breast carcinoma cells showed significant necrotic cell death at 48 h.

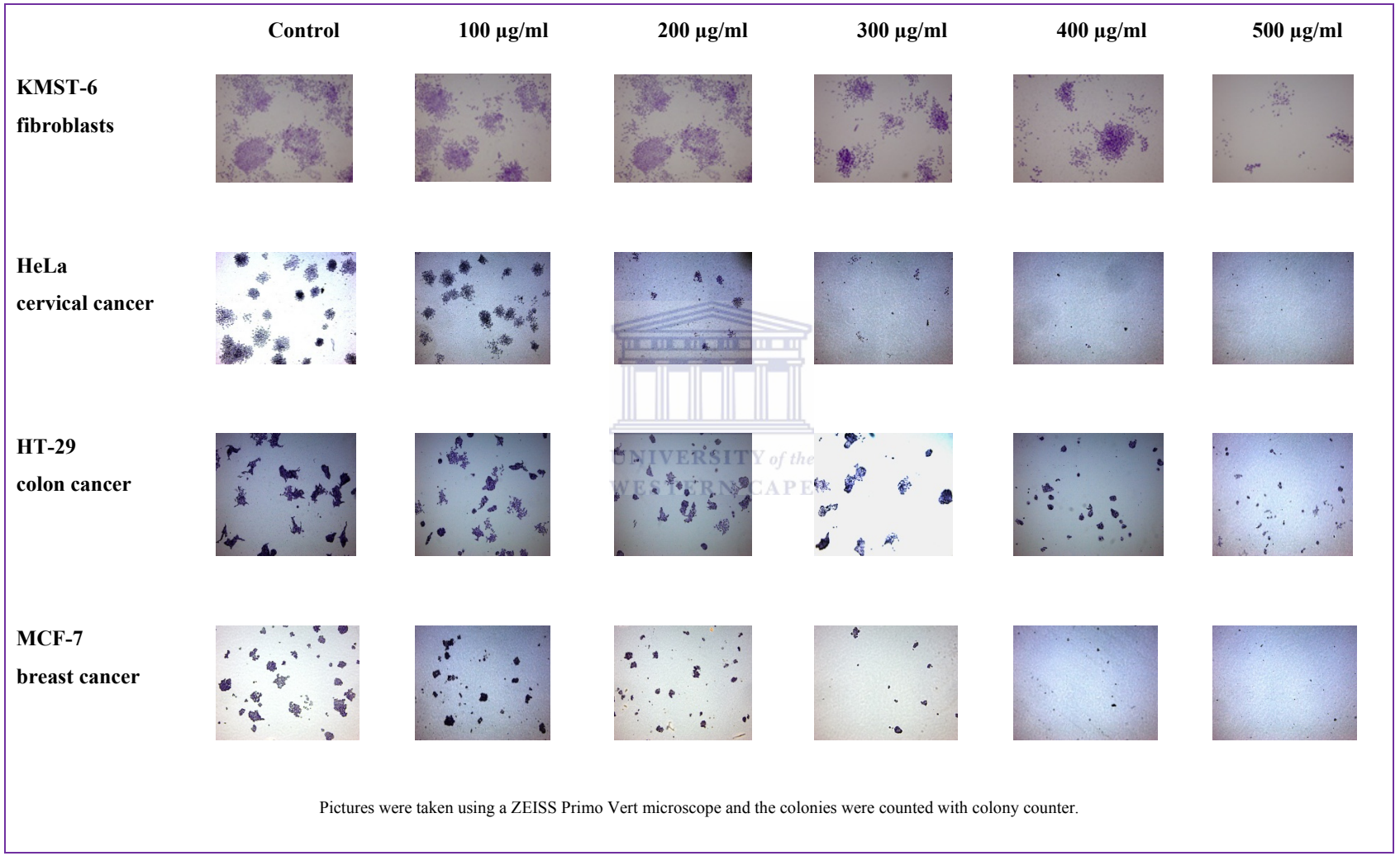
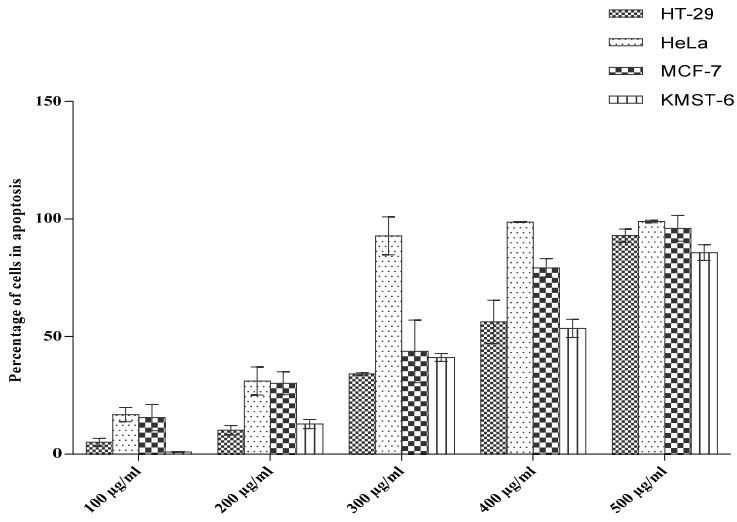


Figure 4.4: Photomicrographs of colonies of cells following 24-hour treatment with the MLE at the indicated concentrations



Cells were stained with the APOPercentage™ dye and evaluated using a flow cytometer.

Figure 4.5: Apoptotic effects of the MLE on HT-29, MCF-7, HeLa and KMST-6 cells

4.4 Effect of Methanolic Leaf Extract on Caspase-3/7 and -9 Activities

The activation of caspase 3/7 and -9 were evaluated to establish the cell death pathway induced by the MLE. As shown in the Figure 4.6, HeLa cells exhibited a concentration dependent fold increase in caspase-3/7 activity. However, MCF-7 and HT-29 cells show a significant concentration dependent decrease in caspase-3/7 activities. Caspase-9 activities in all the cells followed the same pattern. It was observed that the extract caused a decrease in the activities in caspase-9 in all the cells treated (Figure 4.7).

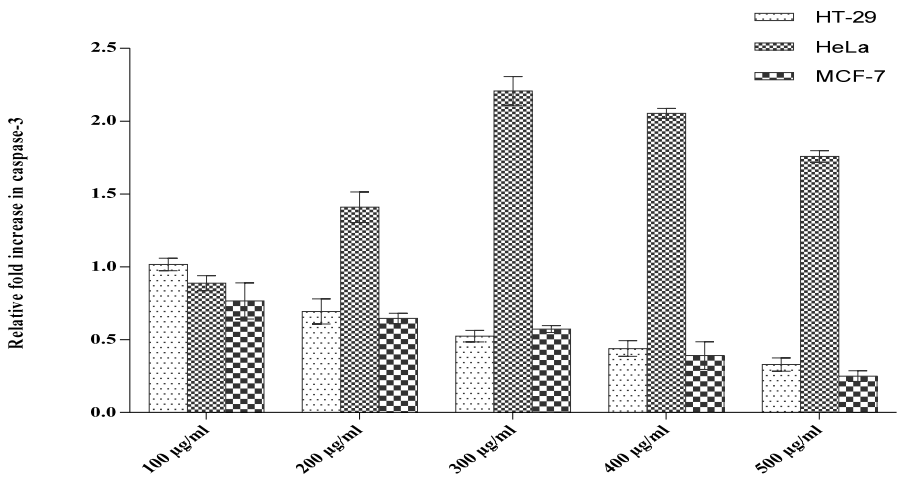
4.5 Effects of Methanolic Leaf Extract on Cell Cycle and ROS Induction

The effects of the extract on the phases of the cell cycle using flow cytometry in cancer cells treated with 200 µg/ml MLE for 12- and 24-hour periods. Representative cell cycle distribution histograms of HeLa cell were presented in Figures 4.8 and 4.9 shows the percentage of cells in different cell cycling stages for HeLa, MCF-7 and HT-29 cells at 12 and 24 h.

Table 4.2: Percentage of live, apoptotic and necrotic cell populations following exposure to the MLE

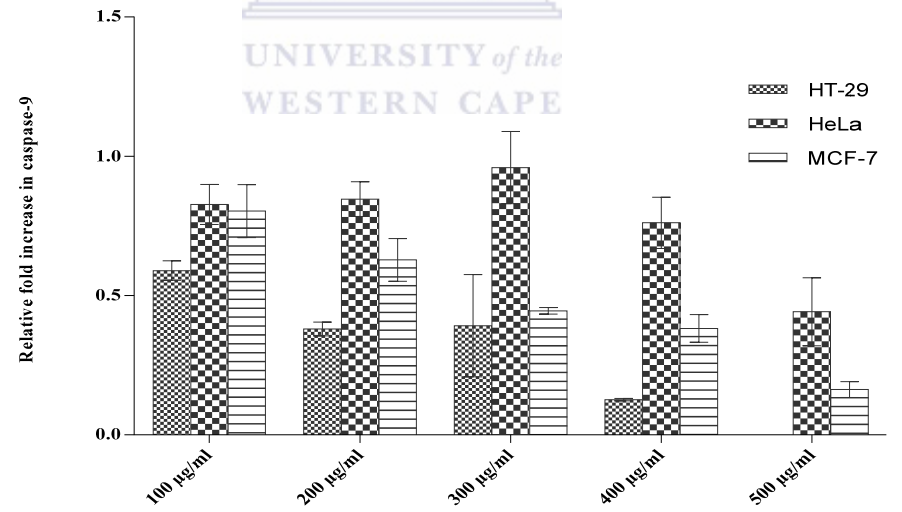
Cell Line	**HeLa**			**MCF-7**			**HT-29**		
Exposure Times	**12 h**	**24 h**	**48 h**	**12 h**	**24 h**	**48 h**	**12 h**	**24 h**	**48 h**
Live	92.41±0.03	84.94±0.69	19.05±0.51	95.22±1.36	92.15±1.77	7.1±1.23	94.22±2.23	78.45±2.25	83.43±4.61
Early Apoptosis	3.41±0.32	6.26±0.50	3.41±0.36	4.69±1.27	6.11±0.34	0.15±0.01	4.56±1.60	16.72±1.64	0.01±0.01
Late Apoptosis	3.53±0.12	6.53±0.78	33.47±1.90	0.18±0.02	0.52±0.19	3.93±1.58	0.26±0.04	4.46±0.70	0.46±0.16
Dead	0.66±0.23	1.38±1.20	43.60±1.75	0.03 ± 0.02	0.16±0.03	87.60±1.17	0.05±0.01	0.07±0.01	16.42±1.73

Cells were evaluated by double staining in Annexin V-FITC/propidium iodide using the flow cytometric assay as described in Chapter 3.



Caspase-3 activity was evaluated after 24-hour exposure of cells to the MLE, using the Caspase-Glo® 3/7 luminescent assay kit (Promega) as described in Chapter 3.

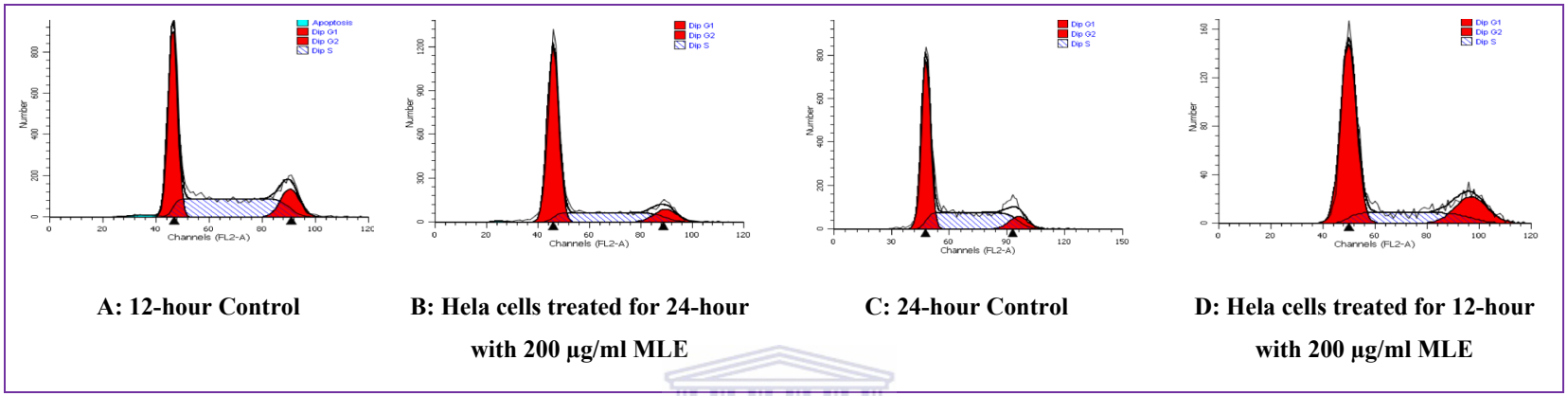
Figure 4.6: Effects of the MLE on caspase-3 activation in HT-29, MCF-7 and HeLa cell.



Caspase-9 activity was evaluated after 24-hour exposure of cells to the MLE, using the Caspase-Glo® 3/7 luminescent assay kit (Promega) as described in Chapter 3.

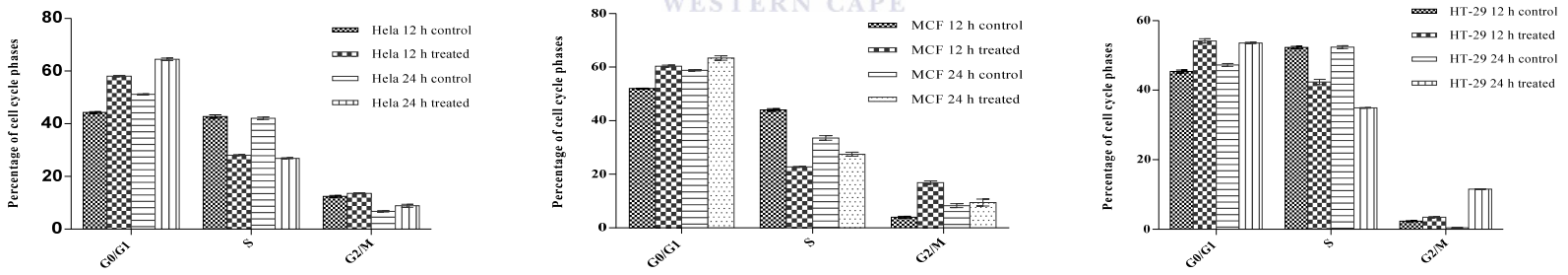
Figure 4.7: Effects of the MLE on caspase-9 activation in HT-29, MCF-7 and HeLa cells.

The results show that the MLE induced a significant accumulation of cells in G0/G1 phases and reduced the number of proliferating cells as shown by low S-phase at both 12- and 24-hours for all the cells. Also, the ability of the MLE (200 µg/ml) at 12- and 24-hours to induce ROS was evaluated using the cell permeant dye chloromethyl-2', 7'-dichlorofluorescindiacetate (CM-H_2DCFDA). As shown in Figure 4.10, MLE-treated cells (black panel) showed an increase in ROS concentration compared to the untreated control (pink panel) in a time-dependent manner. The ROS induction effect of the extract was significantly pronounced in HeLa cells.



A: 12-hour Control **B: Hela cells treated for 24-hour with 200 µg/ml MLE** **C: 24-hour Control** **D: Hela cells treated for 12-hour with 200 µg/ml MLE**

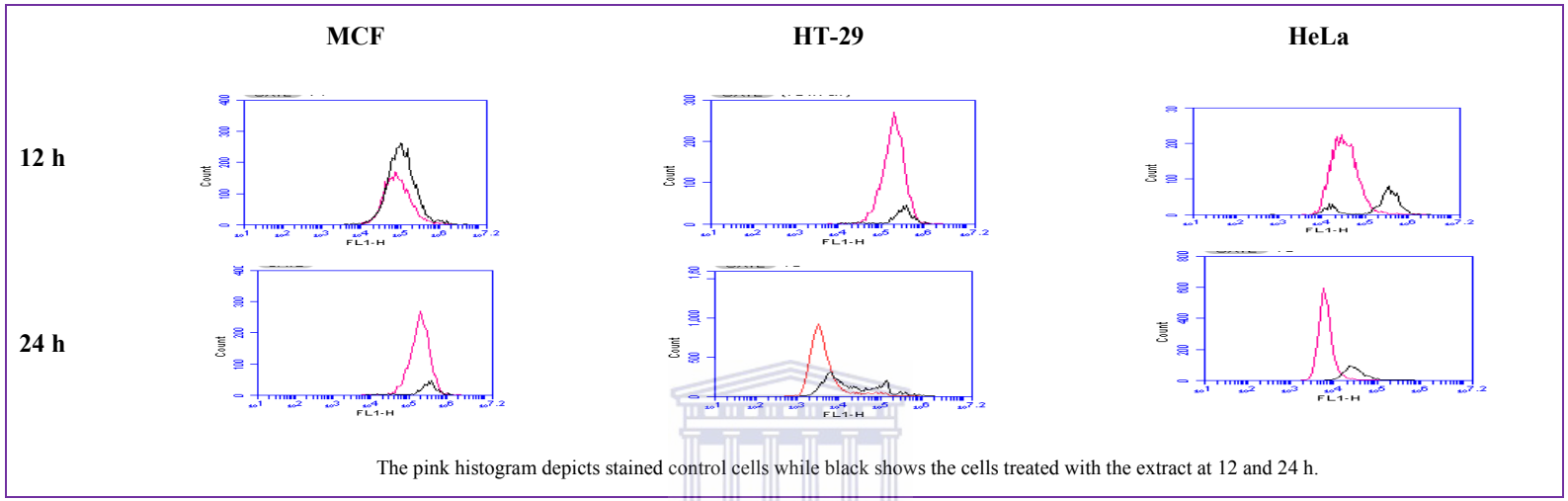
Figure 4.8: Representative histograms of DNA content in cell cycle phases of HeLa cells



HeLa **MCF-7** **HT-29**

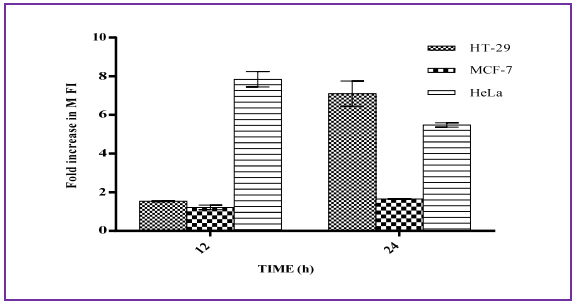
Values are representative of means ± SD of three separate experiments.

Figure 4.9: Percentage of cells in the G0/G1, S and G2/M phases after a 12- and 24-hour exposure to 200 µg/ml MLE



The pink histogram depicts stained control cells while black shows the cells treated with the extract at 12 and 24 h.

Figure 4.10: Representative histograms of cells stained with CM-H_2DCFDA dye and evaluated by flow cytometry



Values are means ± SD of three separate experiments evaluated using flow cytometry.

Figure 4.11: Fold increase in mean florescence intensity (MFI) of cells undergoing induction of ROS following treatment with MLE for 12 and 24h

CHAPTER 5

BIOACTIVITY-GUIDED FRACTIONATION AND CYTOXICITY SCREENING OF THE METHANOLIC LEAF EXTRACT OF *HOLARRHENA FLORIBUNDA*

5.1 Background

Plants provide a reservoir of chemical diversity that provides leads for the discovery of new drugs against human diseases (Newman and Cragg, 2007). The usage of folklore medicine worldwide demonstrates its importance as a reliable source of bioactive antitumour, antioxidant and antimicrobial compounds (Jung, 2014). Assessing plants used in traditional medicine for various biological capabilities is a necessary step in the isolation and characterization of bioactive components inherent in plants for drug development. The African continent lays claim to about 45,000 plant species of which 5,000 are used for medicinal purposes (van Wyk, 2008). Despite the huge potential this number of species presents, only 83 out of 1100 potential drugs coming out of the African continent have been selected as candidates for preclinical development and screening platforms (van Wyk, 2008). Only 1-10% of plants have been scientifically evaluated out of the colossal number of 250,000-500,000 world floral species (Verpoorte, 2000). There is a huge gap between the natural endowment and human potential to stem the tide of avalanche of drugs that present with adverse reactions, especially against cancer. The medicinal constituents derived from plants are not only important as therapeutic agents, but can also serve as templates for the synthesis of active drugs with required therapeutic properties (Mukherjee, 2003). This has opened new vistas for the importance of the structures of bioactive components of plants such as camptothecin, which has been developed into different analogues (topotecan, irinotecan, belatecan and 9-aminocamptothecin).

Holarrhena floribunda is a plant with an approximate height of 17 m and girth of 1 m. The plant belongs to the family of *Apocynaceae* and commonly found in secondary regeneration in deciduous forests, savanna woodlands in Senegal, Nigeria and Congo basin. *Apocynaceae*, the dogane family of flowering plants includes more than 250 genera and 2,000 species of trees, shrubs, woody vines and herbs distributed primarily in tropical and subtropical areas of the world (Wiart, 2006). All the members of the family are known to produce abundant milky latex; simple, opposite and whorled leaves; slightly fragrant, colourful and large flowers with five contorted lobes; and with paired fruits (Wiart, 2006). The most commonly medicinal contents of the family are alkaloids, steroidal alkaloids, cardiotonic glycosides, saponin, and iridoids (Wiart, 2006). Some bioactive natural products from the family are vinblastine, quabain, reserpine and ibogaine. Alkaloids contents of the family has been historically useful to treat cancer with many other compounds awaiting discovery (Wiart, 2006). Some of *Apocynaceae* members reported to have anticancer properties are *Alstonia macrophylla* (Keawpradub et al., 1999), *Nerium oleander* (Siddiqui et al., 1995), *Cerbera manghas* (Chang et al., 2000) and *Calotropis gigantea* (Lhinhatrakool and Sutthivaiyakit, 2006). The objective of this work was to isolate, purify and characterize bioactive components with antiproliferative activity from the MLE of *H. floribunda* using bioactivity-guided fractionation, and to profile cytotoxicity, cell cycle and DNA synthesis effects on HeLa, MCF-7, HT-29 cancer cells and KMST-6 normal fibroblasts of the isolated compounds.

5.2 Bioassay-Guided Fractionation

The cytotoxic potential of the total extract as presented in Chapter 4 led to further interest to isolate the compounds in the leaves responsible for the observed effects. The isolation of compounds involved the use of silica gel and alumina packed column chromatography coupled with cytotoxicity evaluation of sub-fractions using the MTT assay as presented in Figure 5.1.

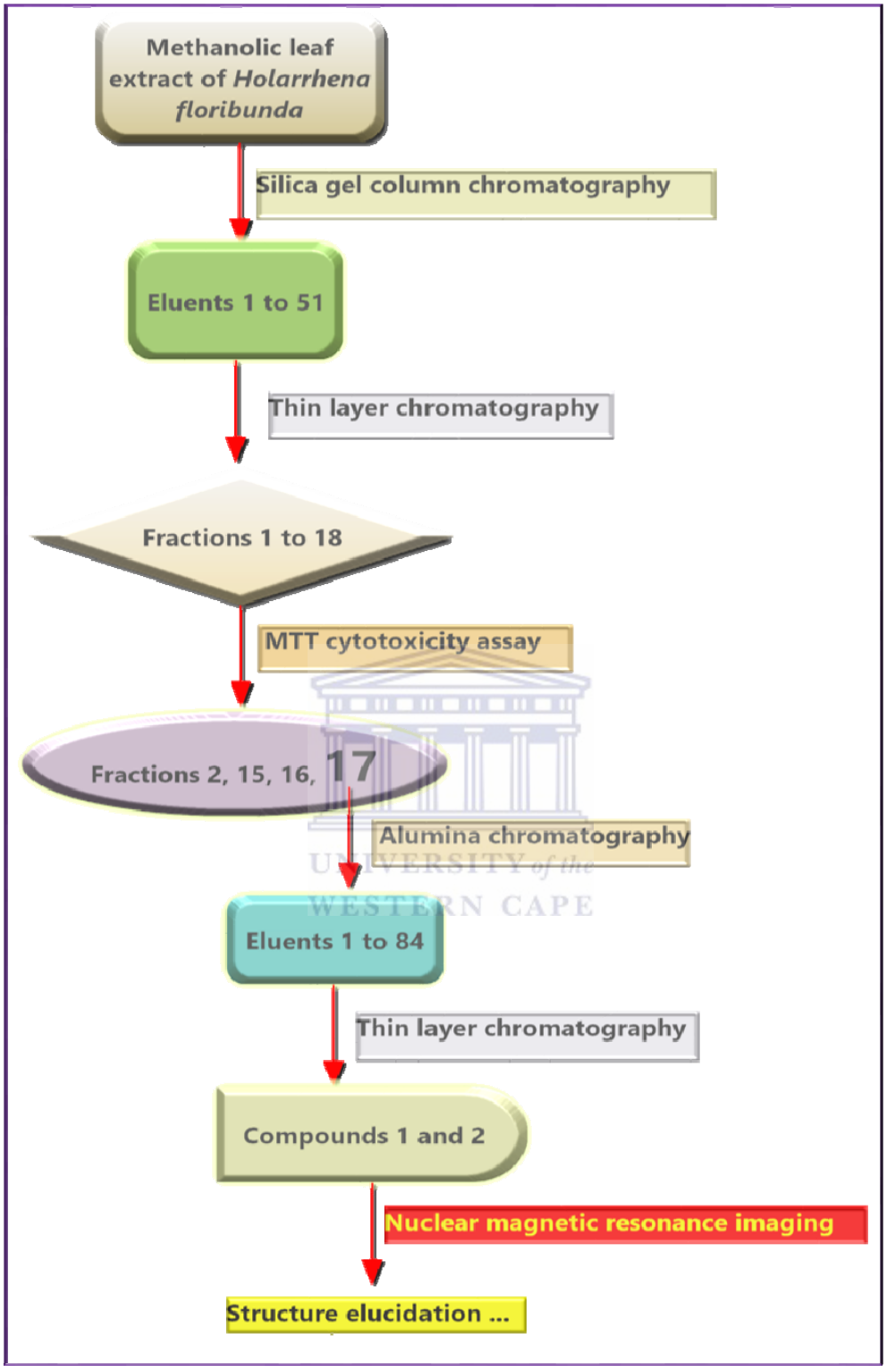


Figure 5.1: Schematic diagram showing purification steps of alkaloids from the MLE

The ground, air-dried leaves, weighing 1.748 kg were soaked in 5.25 L of methanol. The mixture was thoroughly mixed and filtered after 48 hours using a Buchner vacuum filter. The filtrate was evaporated to dryness with Rotary evaporator. The fractionation of the MLE (175 g) was carried out by using a silica gel packed column with gradient mixture of increasing polarity of ethyl-acetate and methanol as mobile phase. The fractions were combined together according to their TLC profiles to yield 18 fractions.

The eighteen fractions (Table 5.1) obtained from the methanolic extract were subjected to bioassay-guided fractionation using tetrazolium salt 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide (MTT) cytotoxicity assay to check for potential fractions that can be further purified for anticancer activity. Bioassay-guided fractionation led to identification of three sub-fractions with strong cytotoxicity. The three cytotoxic sub-fractions were found to be similar on TLC plate and one of the three (sub-fraction 17) was selected for further purification by alumina packed column chromatography.

The purification of sub-fraction 17 (0.6 g) on alumina column chromatography afforded 21 sub-sub fractions. The NMR characterization and identification of the isolated compounds were done on 200 MHz Unity Inova spectrometer (Mercury 200) as stated in Chapter 3. Compound 1 obtained weiged 49.5 mg and compound 2 weighed 20.2 mg.

5.3 NMR Data of the Isolated Alkaloids

The NMR spectra of the purified alkaloids is shown in the Figures 5.2 to 5.5.

Compound 1: colourless crystals; ^{1}H NMR (200 MHz, $CDCl_3$): δ_H 5.38 (H-6); 3.12 (H-3); 2.54 (H-17); Me 2.10 (Me-21); 0.97 (Me-19); 0.63 (Me-18). ^{13}C NMR (200 MHz, CD_3OD): δ_C 209.6 (C-20); 139.0 (C-5); 122.8 (C-6); 63.6 (C-17); 56.8 (C-14), 50.2 (C-9); 46.7 (C-3); 43.9 (C-13); 40.0 (C-7); 38.7 (C-12); 37.3 (C-10); 33.0 (C-1); 31.78 (C-8); 31.74 (C-7); 31.5 (C-21); 29.4 (C-2); 24.4 (C-15); 22.7 (C-16); 20.7 (C-11); 18.8 (C-19); 13.1 (C-18).

Table 5.1: The weight and the IC50 cytotoxicity activities of sub-fractions from MLE on HeLa cells

Fraction	Weight (g)	IC_{50} (µg/ml)
1	7.50	>500
2	2.12	100
3	5.38	>500
4	1.59	>500
5	1.92	>500
6	0.91	>500
7	0.24	>500
8	0.44	>500
9	0.84	>500
10	0.31	>500
11	0.16	>500
12	6.10	>500
13	14.45	>500
14	9.04	>500
15	39.63	<30
16	23.80	<30
17	16.40	<30
18	6.17	>500

Compound 2: colourless crystal: ^{1}H NMR (200 MHz, $CDCl_3$): δ_H 3.15 (H-3), 2.48 (H-17); 2.06 (Me-21); 0.73 (Me-19) and 0.55 (Me-18). ^{13}C NMR (200 MHZ, CD_3OD): δ_C 209.8 (C-20); 63.8 (C-17); 56.7 (C-14); 54.2 (C-9)?; 45.2 (C-3); 44.2 (C-13) 39.0 (C-5, -12); 36.3 (C-10); 35.9 (C-8); 35.4 (C-4); 32.0 (C-1); 31.9 (C-21); 29.8 (C-2); 28.8 (C-7); 28.5 (C-6); 24.3 (C-15); 22.7 (C-16); 20.7 (C-11); 13.4 (C-18); 11.3 (C-19).

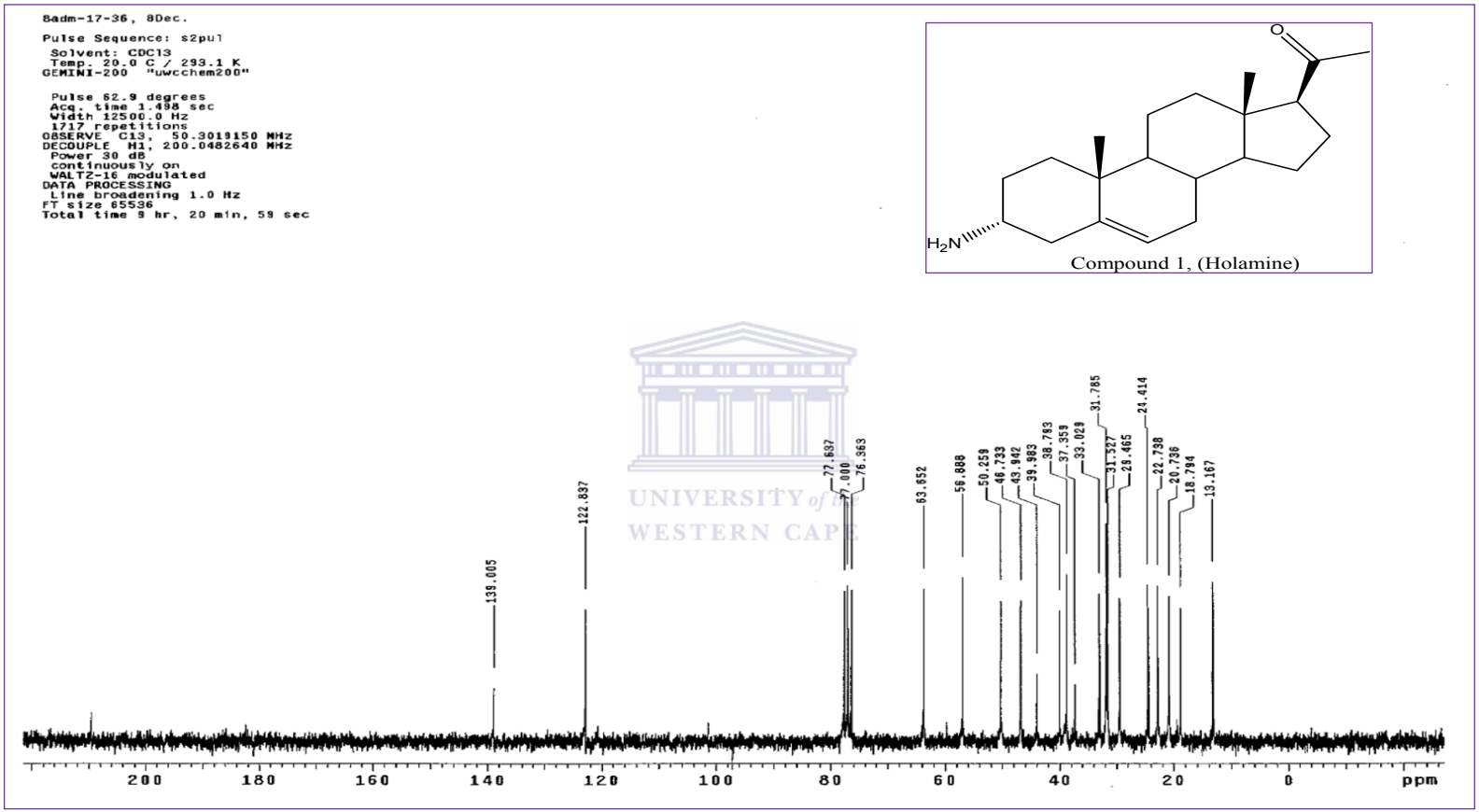


Figure 5.2: [1]H NMR spectrum of isolated alkaloids (compound 1) from the MLE

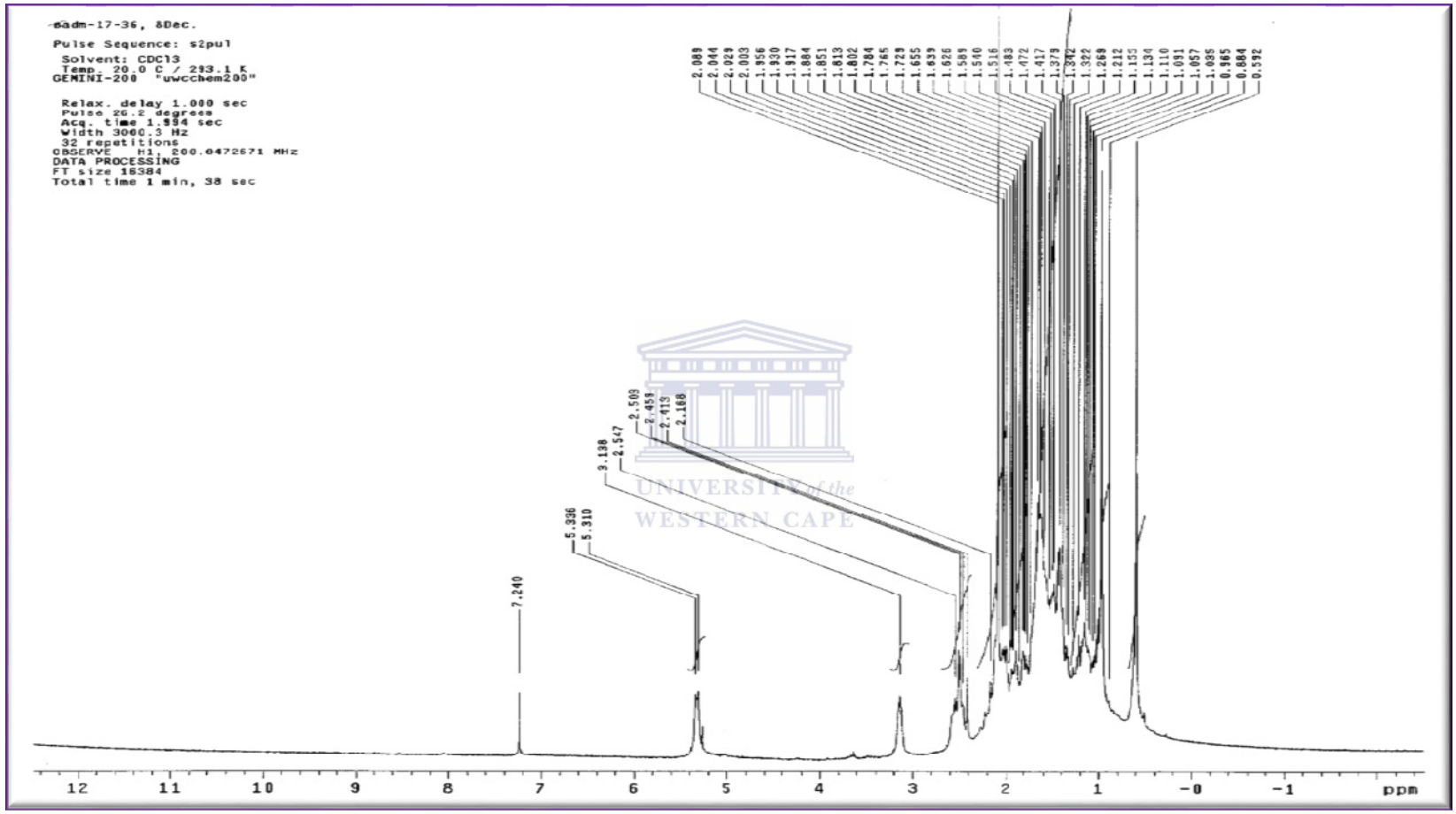


Figure 5.3: ^{13}C NMR spectrum of isolated alkaloids (compound 1) from the MLE

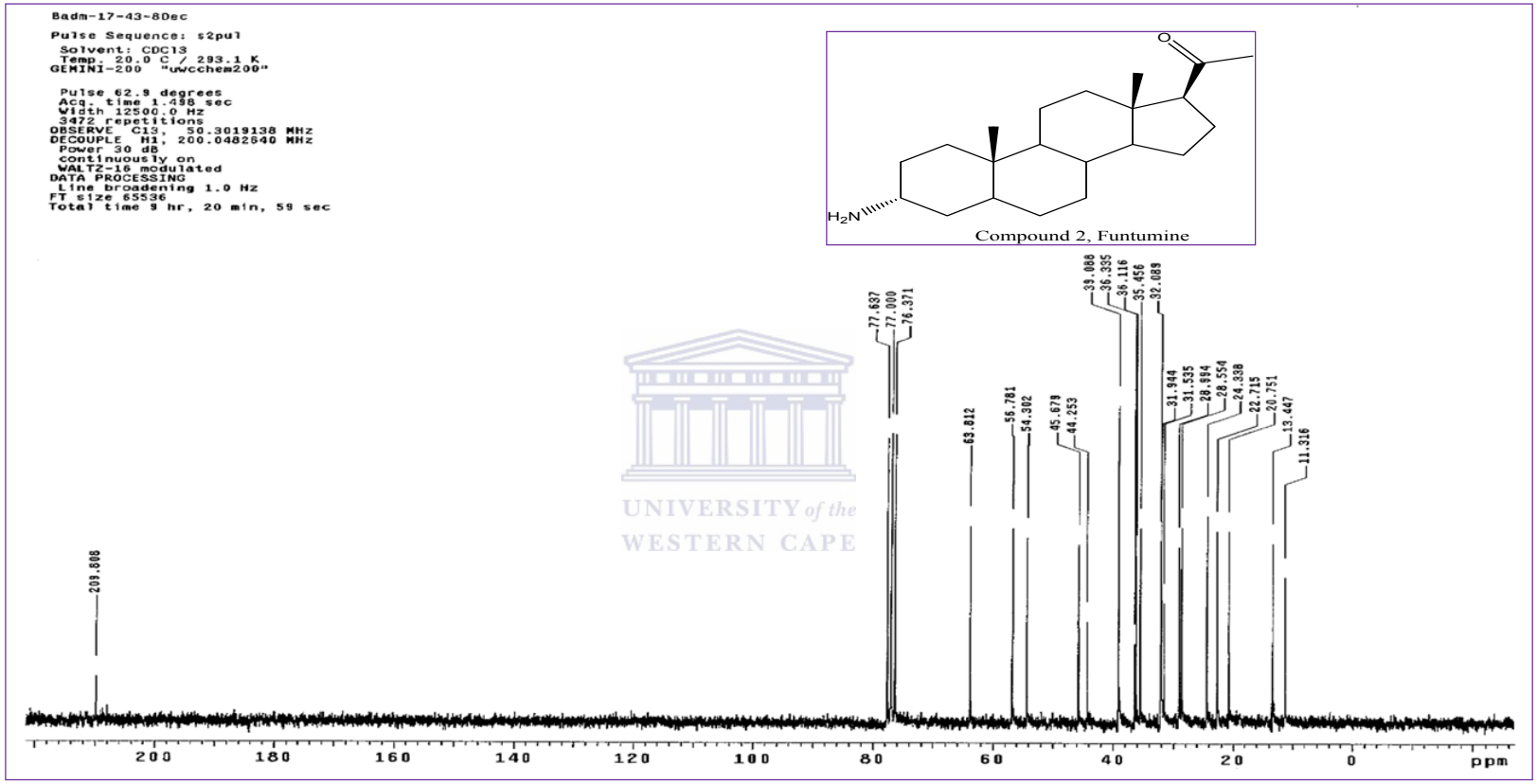


Figure 5.4: [1]H NMR spectrum of isolated alkaloids (compound 2) from the MLE

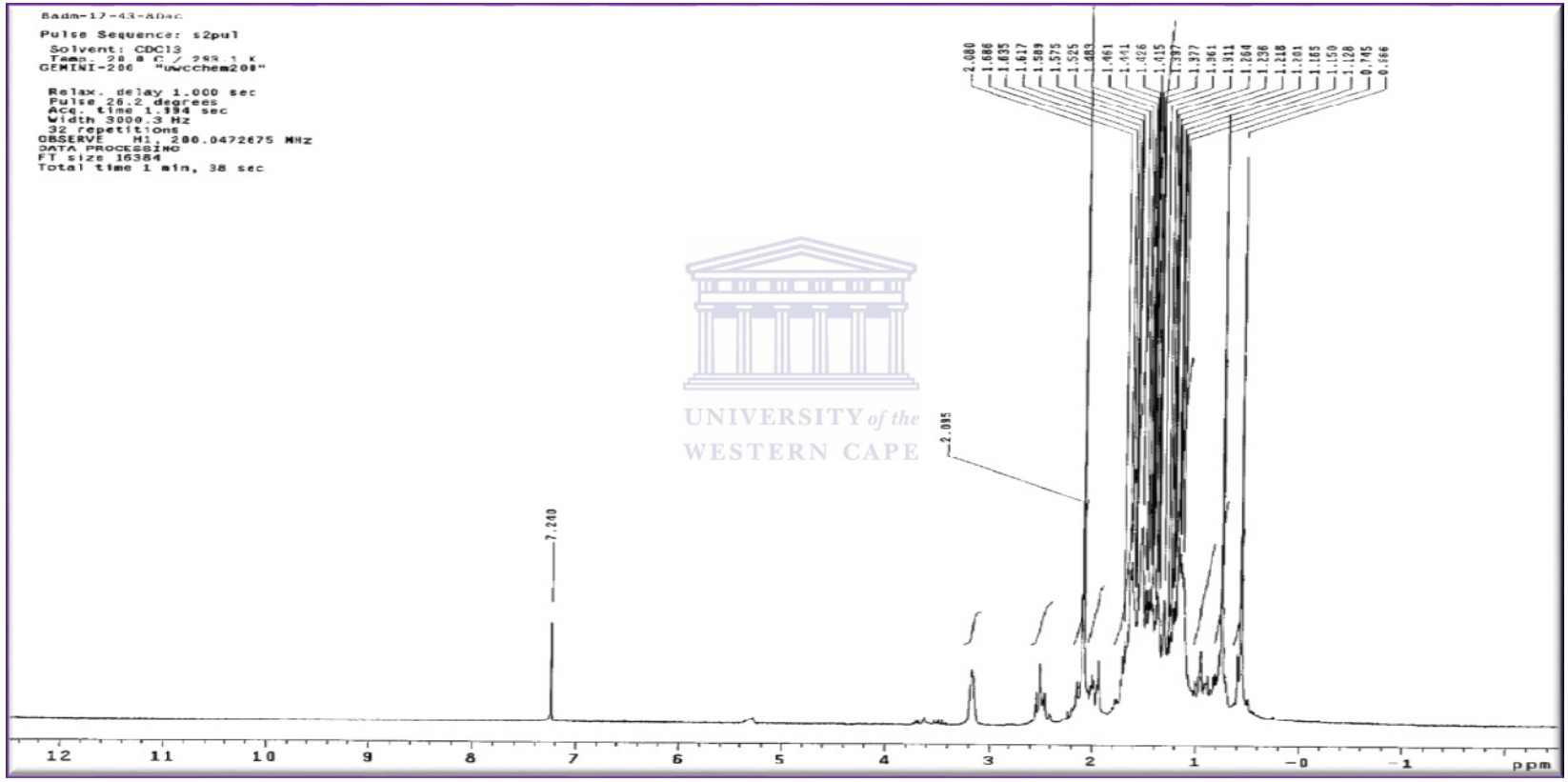


Figure 5.5: ^{13}C NMR spectrum of isolated alkaloids (compound 2) from the MLE

5.4 Cytotoxicity of Isolated Compounds

The cytotoxicity of compounds 1 and 2 were tested on HT-29 (colon cancer), MCF-7 (breast cancer), HeLa (cervical cancer) and KMST-6 (normal fibroblast cell). Figure 5.6 shows the log dose-response curve of the isolated compounds and standard anticancer drugs (cisplatin and doxorubicin) in the cancer and normal cell lines. Table 5.2 depicts the IC_{50} values of the isolated compounds and standards while Table 5.3 shows the selectivity index. The cytotoxicity result shows that compound 1 induced significant cytotoxicity on HT-29 cancer cell compared with other cancer cell lines with lowest IC50 of 31.06 µM followed by MCF-7 (42.82µM), HeLa (51.42 µM) and KMST-6 (102.95 µM). The cytotoxicity effect of compound 1 is closely similar to that of compound 2. Compound 2 also shows cytotoxicity against HT-29 as the most sensitive with IC_{50} of 22.36 µM followed by HeLa (46.17 µM), MCF-7 (52.69 µM) and KMST-6 (85.45 µM). Cisplatin and doxorubicin showed significant indiscriminate cytotoxicity activities against cancer cells and normal cell line as presented in Table 5.3. The selectivity index as presented in Table 5.4 shows that the isolated compounds are more selective towards cancer cells than the normal cell line as opposed to the standard anticancer drugs with low selectivity index.

5.5 Cell Cycle Effects of the Isolated Compounds

The effects of the IC_{50} concentrations of the isolated compounds calculated from cytotoxicity assays were tested on the cell cycle phases of the cancer cell lines used in this study. Cancer cells were stained with propidium iodide (PI) and evaluated using flow cytometry. The cell cycle phases (G0/G1, S, G2/M) were evaluated at 12 and 24-hour time points as presented in Figures 5.7 and 5.8. The results showed that the compounds induced significant increase ($P<0.0001$) in cell populations in G0/G1 and G2/M phases with concomitant reduction in S-phase of HT-29 cell at both time points.

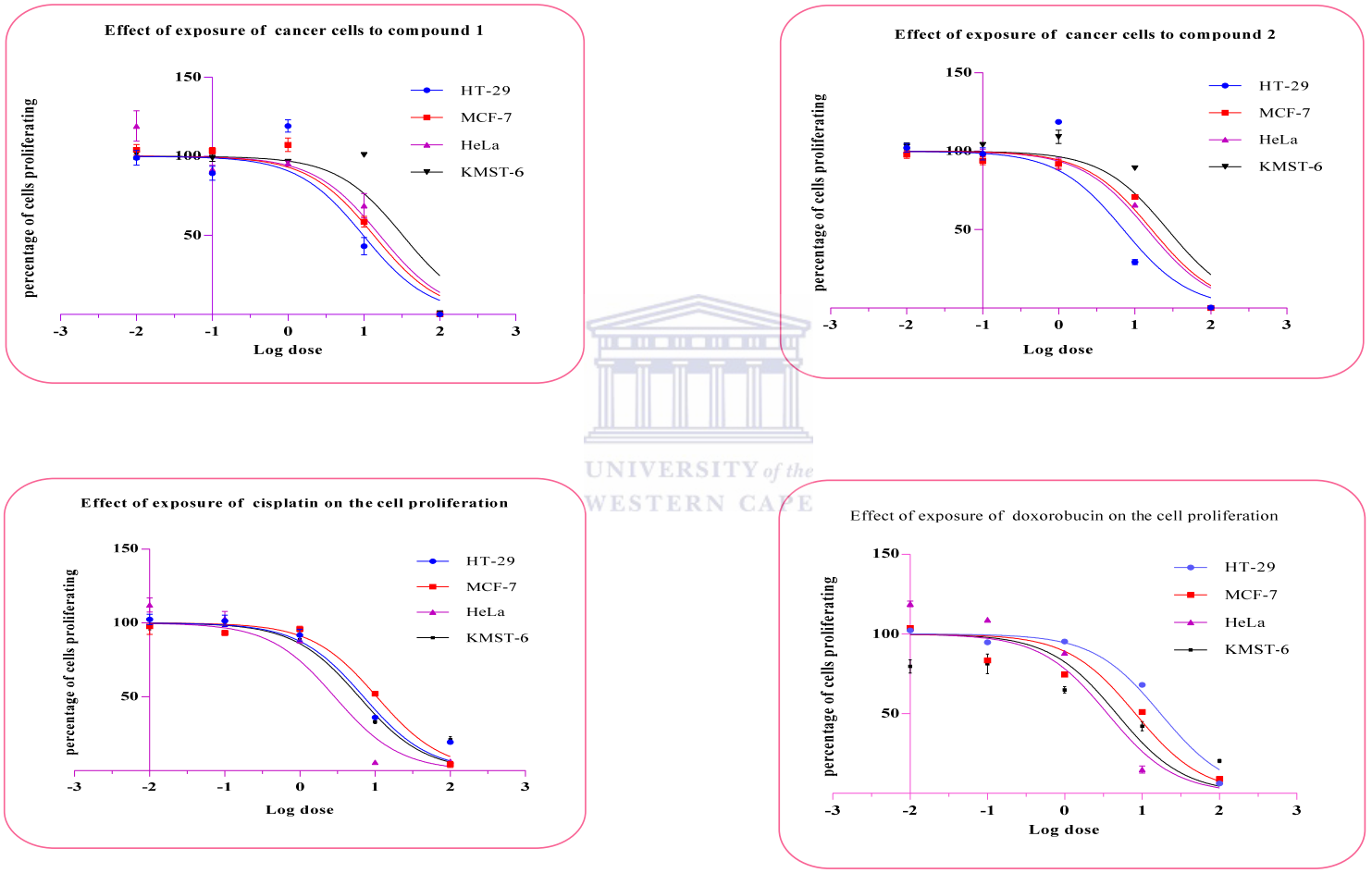


Figure 5.6: Cytotoxic effects of isolated compounds (1 and 2) and standard compounds (cisplatin and doxorobucin) on cancer cells and normal fibroblasts

Table 5.2: IC_{50} values for cancer cell lines treated for 24-hour with compounds 1, 2, cisplatin and doxorubicin

Drug	IC_{50} (µM) determined by non-linear regression of MTT assay dose-response data			
	Cell Line			
	HT-29	HeLa	MCF-7	KMST-6
Compound 1	31.06	51.42	42.82	102.95
Compound 2	22.36	46.17	52.69	85.45
Cisplatin	7.07	2.91	10.58	6.14
Doxorubicin	17.38	3.57	8.20	4.68

Table 5.3: Selectivity index of isolated compounds and standard drugs in normal and cell cancer cells

Drug	Cell Line		
	HT-29	HeLa	MCF-7
Compound 1	3.31	2.00	2.00
Compound 2	3.82	1.62	1.85
Cisplatin	0.87	2.11	0.58
Doxorubicin	0.27	1.31	0.57

A significant increase was observed in the G0/G1 phase with reduction in S-phase in HeLa cell treated with both compounds while significant increase ($P<0.01$) in G2/M-phase was only observed at 24-hour for the compound 1. MCF-7 cells showed a significant increase ($P<0.0001$) in cell populations at G0/G1 phase and reduction in S-phase. Treatment of MCF-7 cells with compound 2 resulted in a significant increase ($P<0.0001$) in the G2/M-phase at both time points while compound 1 only caused a significant increase ($P<0.0001$) at the 12-hour time point.

5.6 Effects of Isolated Compounds on DNA Synthesis

The effects of the isolated compounds on DNA synthesis was tested using BrdU chemiluminescent ELISA kit by treating the cells (HeLa, MCF-7 and HT-29) with the respective IC_{50} concentrations determined for the periods of 12, 24 and 48 hours in relation to untreated control cells (KMST-6). The results as presented in Figure 5.9 show that the two compounds significantly reduced DNA synthesis in all the cells. Compound 1 showed significant reduction ($P<0.0088$) between 24 and 48 hours while compound 2 displayed significant reduction ($P<0.0001$) in DNA synthesis between 12 and 24 hours in HeLa cell. MCF-7 cells showed significant reduction ($P<0.0030$) in DNA synthesis when treated with compound 1 between 12 and 24 hours while compound 2 significantly ($P<0.05$) reduced the DNA synthesis between the 24 and 48 hours. Compound 1 only showed significant reduction ($P<0.0001$) at 12 and 24 hours treatment of HT-29 cell while compound 2 showed significant reduction between 12 and 24 hours ($P<0.05$) and between 24 and 48 hours ($P<0.001$).

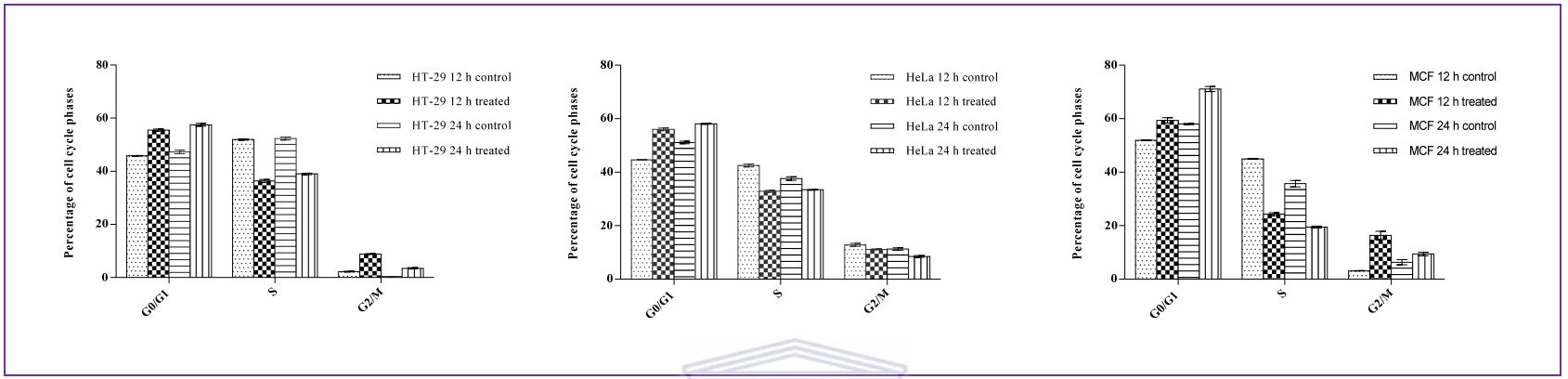


Figure 5.7: Effects of compound 1 on cell cycle progression of HT-29, HeLa and MCF-7 cells stained with PI and evaluated using flow cytometry

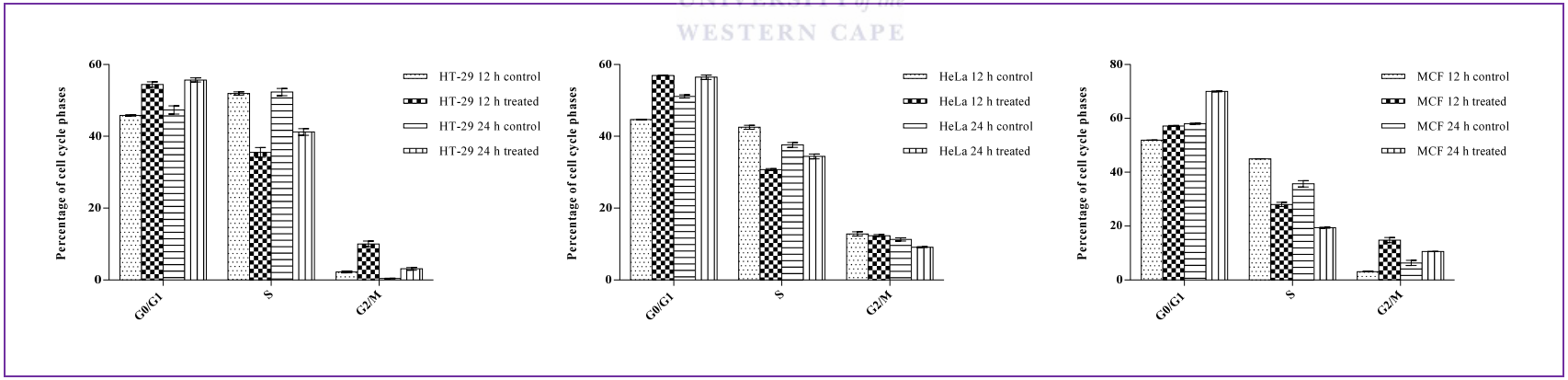


Figure 5.8: Effects of compound 2 on cell cycle progression of HT-29, HeLa and MCF-7 cells stained with PI and evaluated using flow cytometry

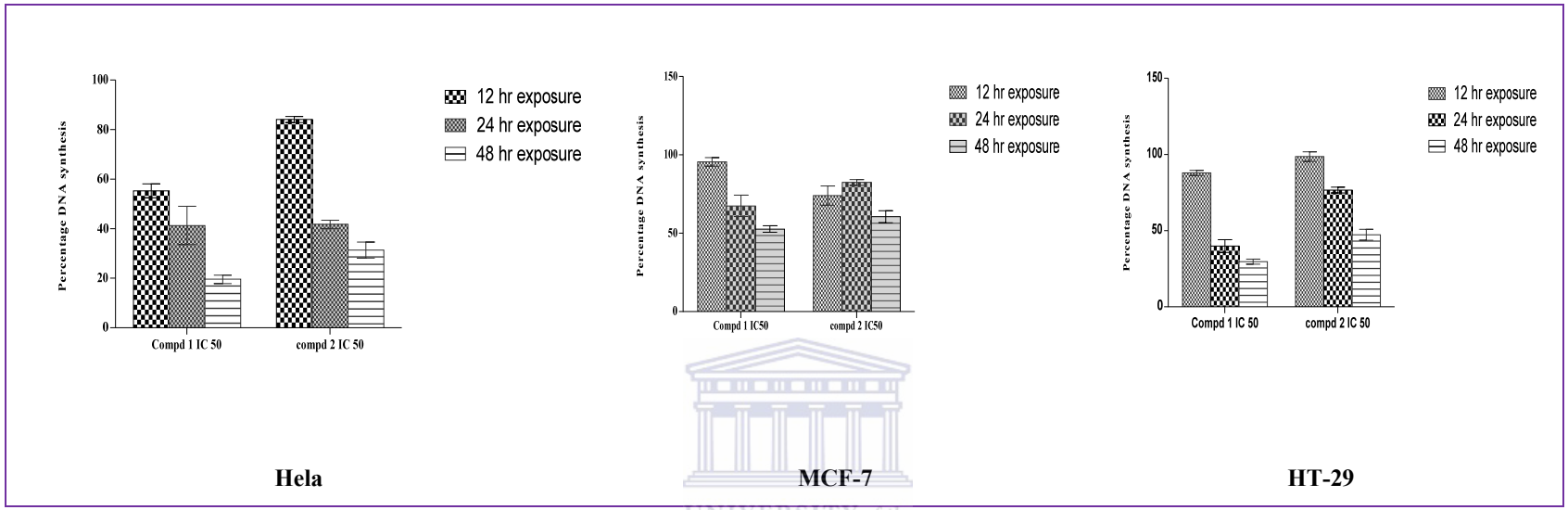


Figure 5.9: Effects of IC_{50} concentrations of compounds 1 and 2 on DNA synthesis at 12, 24 and 48 hours based on the chemiluminescent BrdU ELISA assay

CHAPTER 6

TWO STEROIDAL ALKALOIDS FROM *HOLARRHENA FLORIBUNDA* LEAVES INDUCE APOPTOSIS IN CANCER CELLS

6.1 Background

Apoptosis is a gene-regulated autonomous process of cell component removal without induction of end-point inflammatory effects on surrounding cells (Gallardo-Escarate et al., 2007). It is an important physiological event for the maintenance of tissue homeostasis for all organ systems in the human body (Kumar et al., 2012). Deregulation of apoptosis is linked to several pathogenic conditions such as stroke, neurodegenerative diseases and cancer (Thompson, 1995). Several lines of evidence have shown that chemoprevention and chemotherapeutic agents stimulate induction of apoptosis suggesting its importance in cancer treatment (Al Dhaheri et al., 2013). Cancer cells possess several mechanisms of resisting programmed cell death (PCD) and potential of natural products to induce the process of apoptosis is a crucial step in anticancer activity (Fulda, 2010b) and an important approach to treat cancer (Dixon et al., 1997).

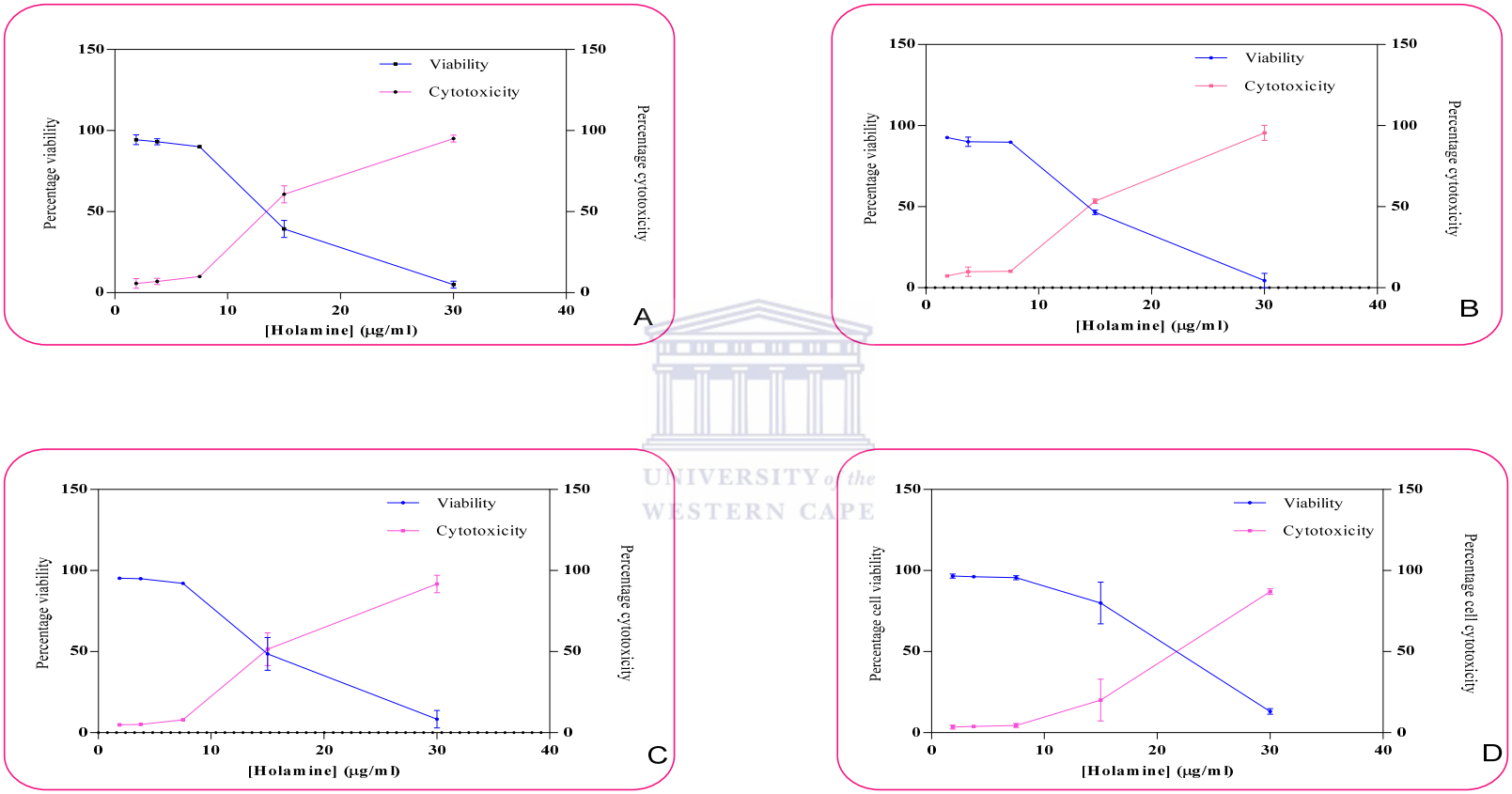
An ideal therapeutic goal for cancer treatment is to trigger apoptotic death selectively in tumour cells (Ganesh et al., 2014). Cytotoxic effects of medicinal plant products can be mediated through the stimulation of apoptotic pathways (Mehta et al., 2010) and a number of them have the ability to induce apoptosis (Parsaee et al., 2013a). It is, however, essential to screen anticancer agents from natural products for their ability to induce apoptosis (Taraphdar et al., 2001). Previous chapters show that the methanolic extract of *Holarrhena floribunda* leaves and

the two steroidal alkaloids (holamine and funtumine) isolated from the leaves have cytotoxicity against the panel of cancer cells used in this study. In addition, *Holarrhena floribunda* used in this study has been extensively reviewed in the previous chapters. Cytotoxic and apoptotic effects of alkaloids in general and steroidal alkaloids in particular are well documented in the scientific literature (Rosenkranz and Wink, 2008; el-Shazly and Wink, 2008; Dassonneville et al., 2000). Holamine and funtumine exert cytotoxic and apoptotic effects on the cell lines studied and their modes of action need to be elucidated. The present study sought to evaluate the effects of the steroidal alkaloids with regard to apoptosis induction, reactive oxygen species production, mitochondrial depolarization, actin filament expression and inhibition of topoisomerase-I.

6.2 Viability and Cytotoxicity of Holamine and Funtumine

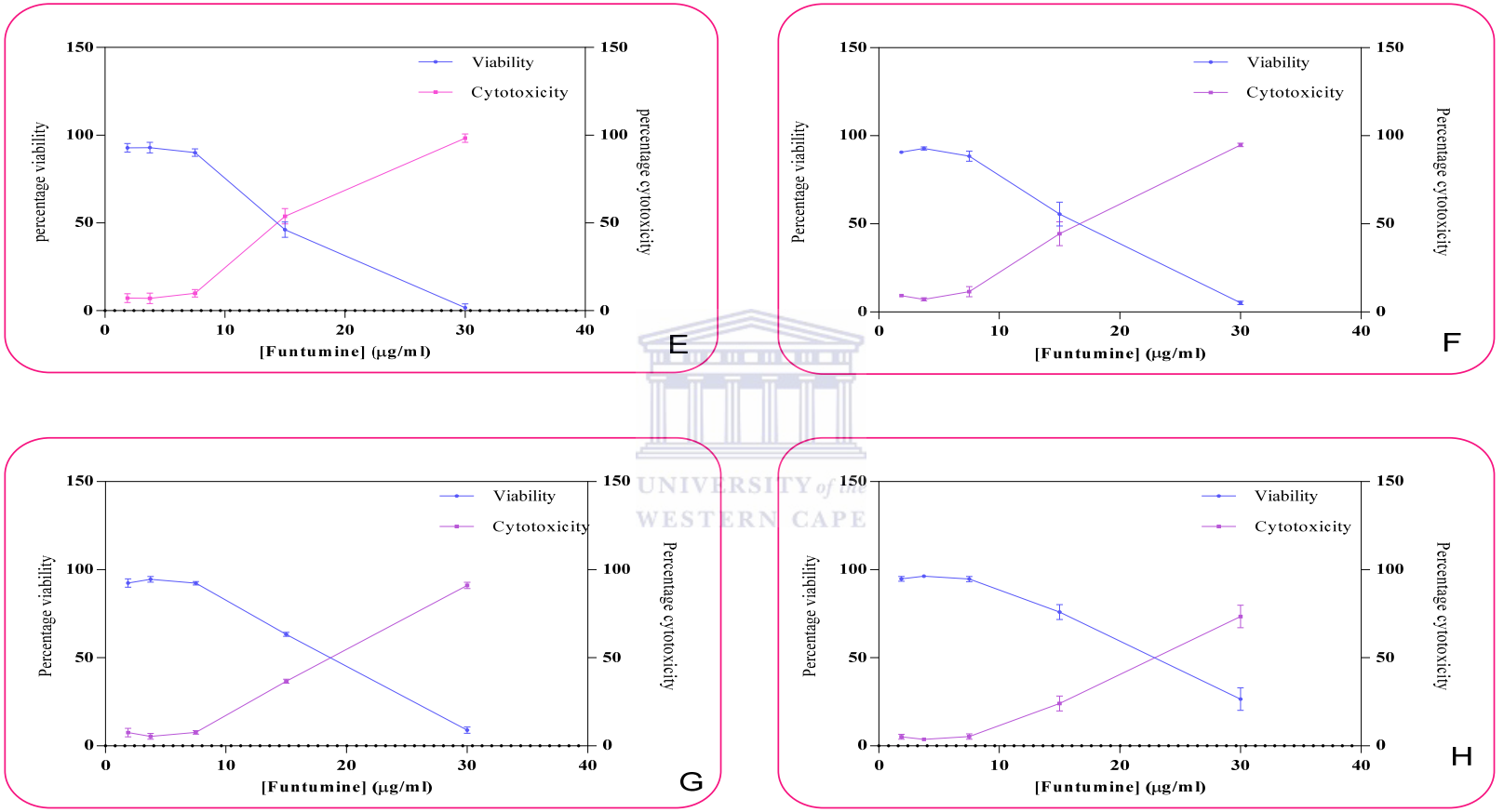
The previous chapter evaluated the cytotoxicity of the isolated compounds using the MTT assay. The present section sought to evaluate the cytotoxicity and viability of the compounds by using the CytoTox-Glo™ cytotoxicity assay (Promega) to further confirm the activities of the compounds against cancer cells and the normal fibroblast cell line (control). Figure 6.1 and 6.2 shows the cytotoxicity and viability effects of the compounds on the cancer and normal cell lines. The results show that the two compounds are significantly more toxic to the cancer cell lines compared with normal fibroblasts.

Holamine and funtumine showed significant cytotoxicity ($P<0.0001$) to MCF-7 and HT-29 compared to KMST-6 while a $P<0.01$ was observed when KMST-6 was compared with HeLa cells treated with holamine and $P<0.001$ when treated with funtumine. There was no significant difference in sensitivity among the cancer cells treated with the holamine. Funtumine, however, showed significant cytotoxicity towards MCF-7 ($P<0.5$) and HT-29 ($P<0.01$) compared with HeLa cells.



A—HT-29, B—MCF-7, C—HeLa and D—KMST-6 cells

Figure 6.1: Effects of holamine on cells evaluated with the CytoTox-Glo™ cytotoxicity assay



E—HT-29, F—MCF-7, G—HeLa and H—KMST-6 cells

Figure 6.2: Effects of funtumine on cells evaluated with the CytoTox-Glo™ cytotoxicity assay

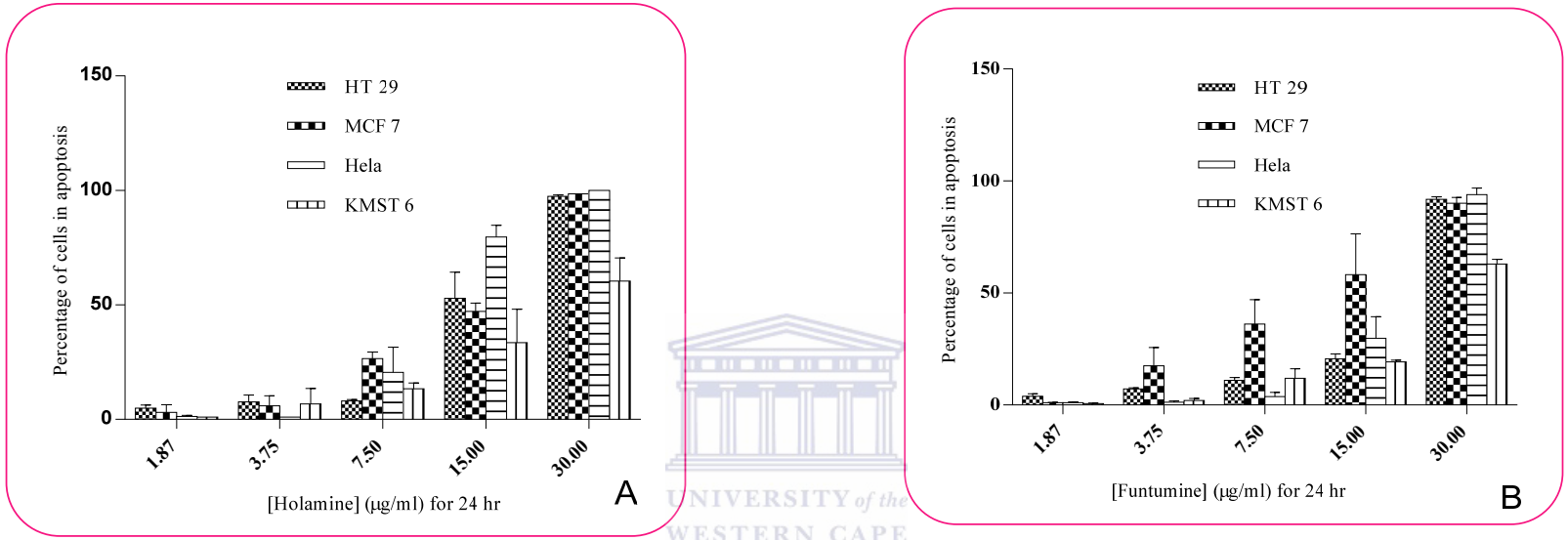
6.3 Apoptotic Effects of Holamine and Funtumine

The apoptotic effects of the compounds were evaluated by the APOPercentage™ dye using flow cytometry and the Annexin-V/PI double staining using Tali® Image-Based Cytometer. Figure 6.3 and 6.4 shows the apoptotic effects of the two compounds on HT-29, HeLa and MCF-7 cancer cells. The Apopercentage assay results show that the cancer cell lines used in this study are significantly ($P<0.0001$) sensitive to the apoptotic effects of holamine when compared with normal KMST-6 fibroblasts.

Likewise, cancer cells were significantly sensitive to funtumine-induced apoptosis compared with KMST-6 control. HeLa cells are more sensitive to the apoptotic effect of holamine while MCF-7 cells are more sensitive to funtumine. The results of Annexin-V/PI staining presented in the Figure 6.4 indicate that the two compounds induced significant late stage apoptosis compared with the early stage. Holamine induced significant late stage apoptotic effects in HeLa cells while funtumine showed a greater effect in HT-29 cancer cells.

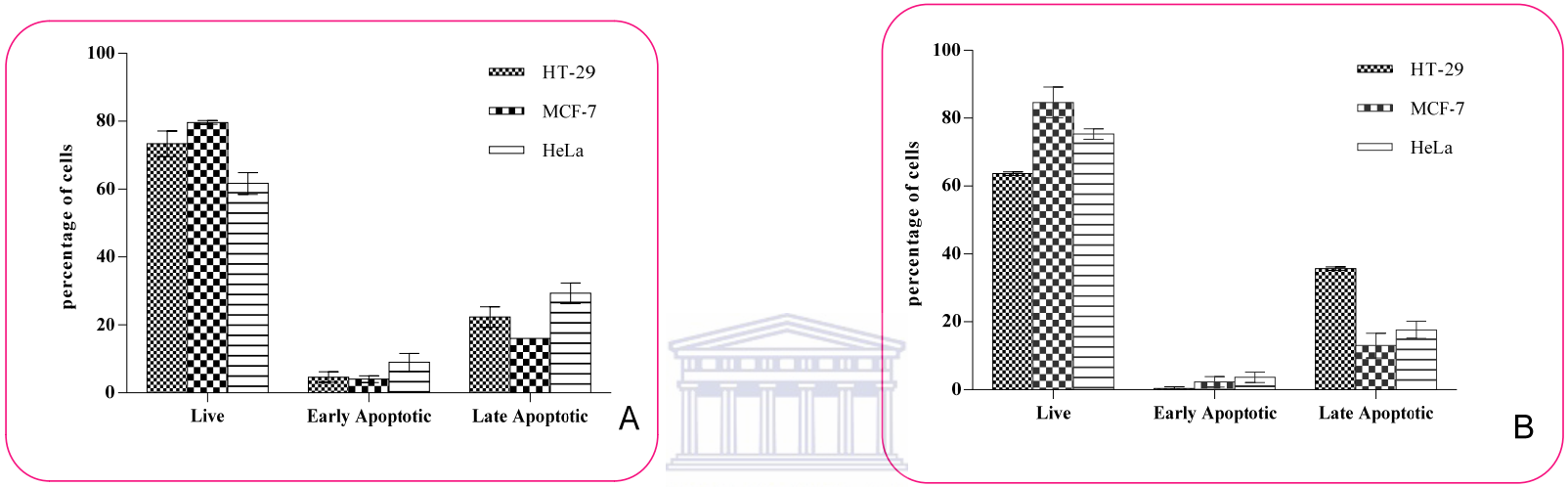
6.4 Effects of Holamine and Funtumine on Caspase-3/7 Activity

The ability of the compounds to induce caspase-3/7 in cancer cell lines (HeLa, MCF-7 and HT-29) treated for 12- and 24-hour periods is presented in Figure 6.5. Caspase-3/7 activity was evaluated using Caspase-3/7 Glo™ assay kit. The results show that the two compounds induced significant caspase-3/7 activity following exposure of cells to concentrations of 15 µg/ml and 30 µg/ml for 24 and 12 hours respectively. The results reveal that the two compounds induced significant caspase-3/7 activity in the HeLa cancer cell line compared with the MCF-7 and HT-29 cancer cell lines ($P<0.0001$).



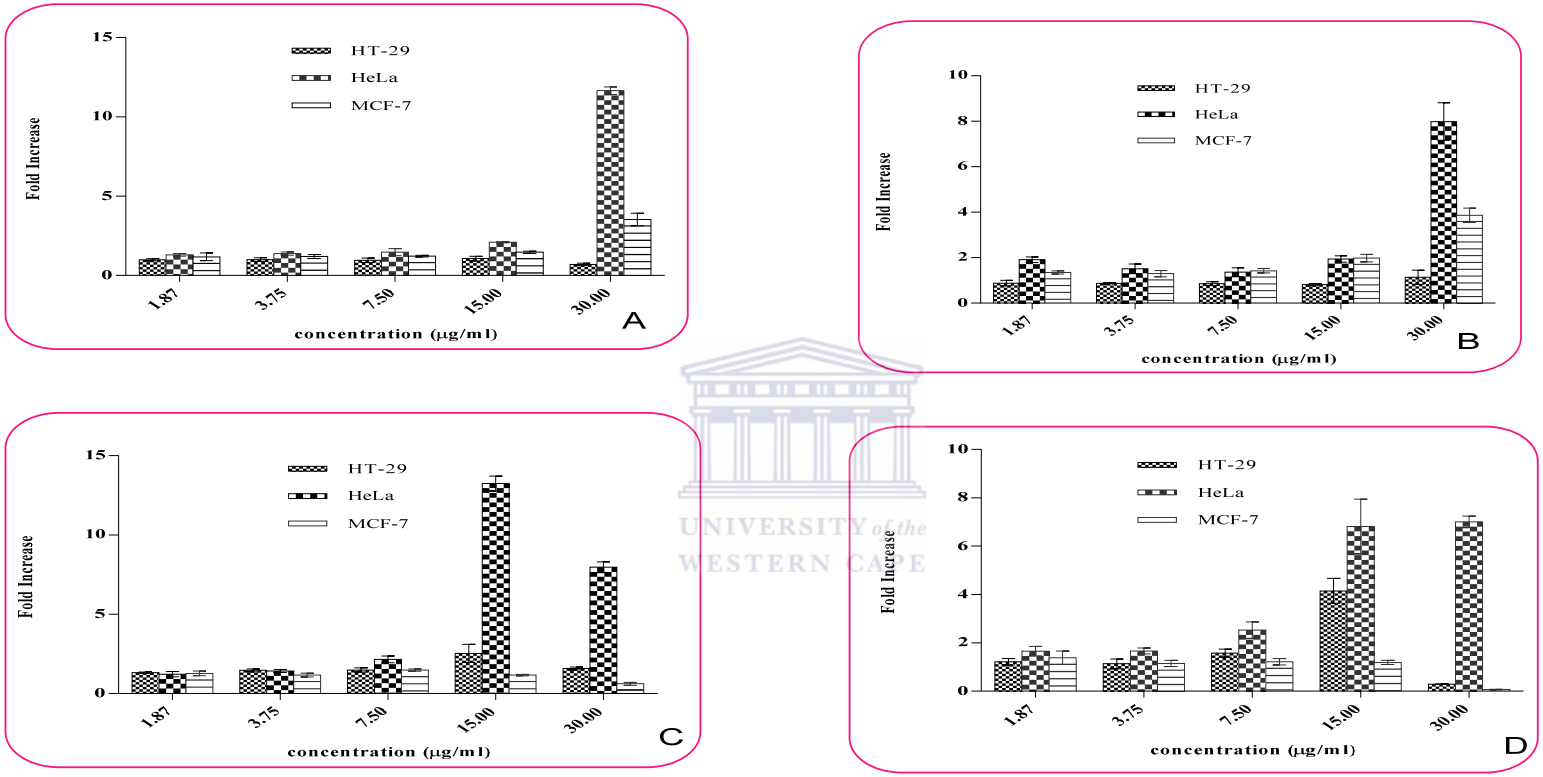
Cells were stained with Apopercentage™ dye and evaluated by flow cytometry. Panel A represents the apoptotic effects of holamine and panel B those of funtumine.

Figure 6.3: Apoptotic effects of holomine and funtumine on HT-29, MCF-7, HeLa and KMST-6 cells



Cells were stained with Annexin-V FITC/PI and evaluated on a Tali® image-based flow cytometer. Panel A represents the apoptotic effects of 15 µg/ml holamine while panel B shows the apoptotic effects of 15 µg/ml funtumine, following exposure of cells for 24 hours to the compounds.

Figure 6.4: Apoptotic effects of each holamine and funtumine on HT-29, MCF-7 and HeLa cell lines



Panel A represents holamine at 12 hours, B represents funtumine at 12 hours while C and D represent compounds 1 and 2 at 24 hours, respectively. Caspase-3 activity was evaluated using Caspase-Glo®-3/7 luminescent assay kit (Promega).

Figure 6.5: Effects of holamine and funtumine on the induction of caspase-3/7 in cancer cell lines (MCF-7, HeLa and HT-29) treated for 12 and 24 hours.

6.5 Effects of Holamine and Funtumine on ROS Induction

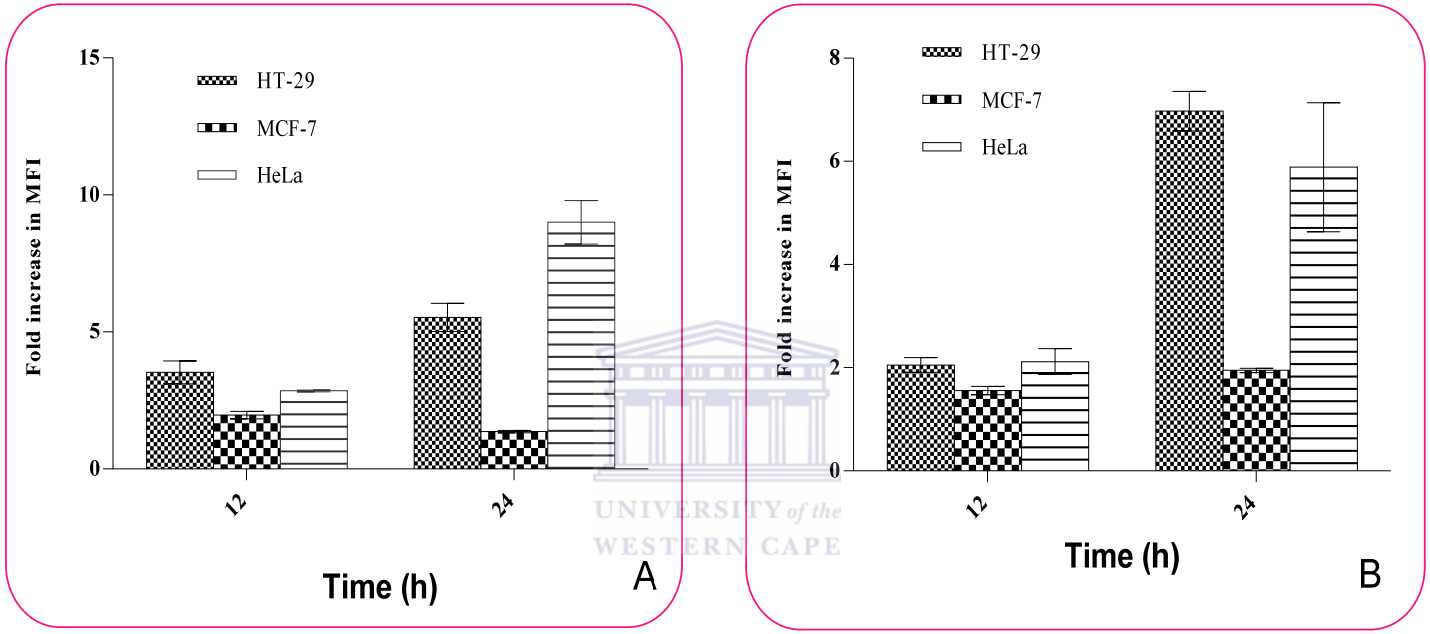
The induction of reactive oxygen species (ROS) by the two compounds were assessed by staining the cancer cells with the cell permeant dye chloromethyl-2', 7'-dichlorofluorescindiacetate (CM-H_2DCFDA) and the results were evaluated by flow cytometry. The cells were treated with 15 µg/ml of the compounds for 12- and 24-hour periods. The concentration used was based on the effective concentration of the compounds that induced caspase-3/7 activity in the cancer cells. The results as presented in Figures 6.6 and 6.7 and show that the two compounds induced significant ROS in all the cell lines at both 12 and 24 hours.

Holamine and funtumine show significant ($P<0.0001$) induction of ROS between 12 and 24 hours for both HT-29 and HeLa cell lines while a significant difference in MCF-7 was not observed for these periods of exposure. Holamine and funtumine induced different levels of ROS in the cell lines. There was, however, no significant difference in ROS induction by funtumine between HT-29 and HeLa cell lines.

6.6 Effects of Holamine and Funtumine on Mitochondrial ATP Production

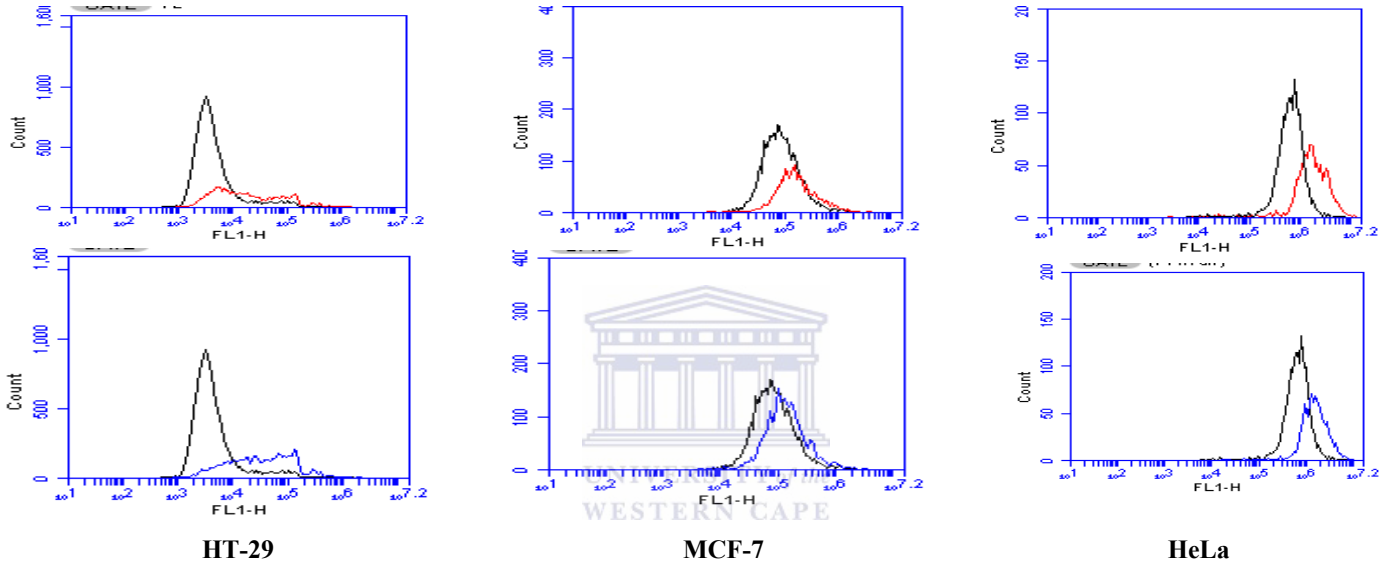
The effects of the compounds on mitochondrial ATP production in cells were evaluated with the mitochondrial ToxGlo™ assay according to the manufacturer's instructions. The cells were treated with log increments in concentration of the compounds (0.01, 0.1, 1 and 10 µg/ml) for 2 hours. The results are summarized in Figure 6.8.

The different cancer cell lines differed in their responses to the effects of the two compounds, suggesting that the cell cytotoxicity induced by these compounds might not be isoeffective or involve the same mechanisms of action. The results show that holamine-induced cytotoxicity is mitochondrial specific in HeLa and HT-29 cells, but not in MCF-7 while funtumine induced mitochondrial cytotoxicity in HeLa and MCF-7, but not in HT-29.



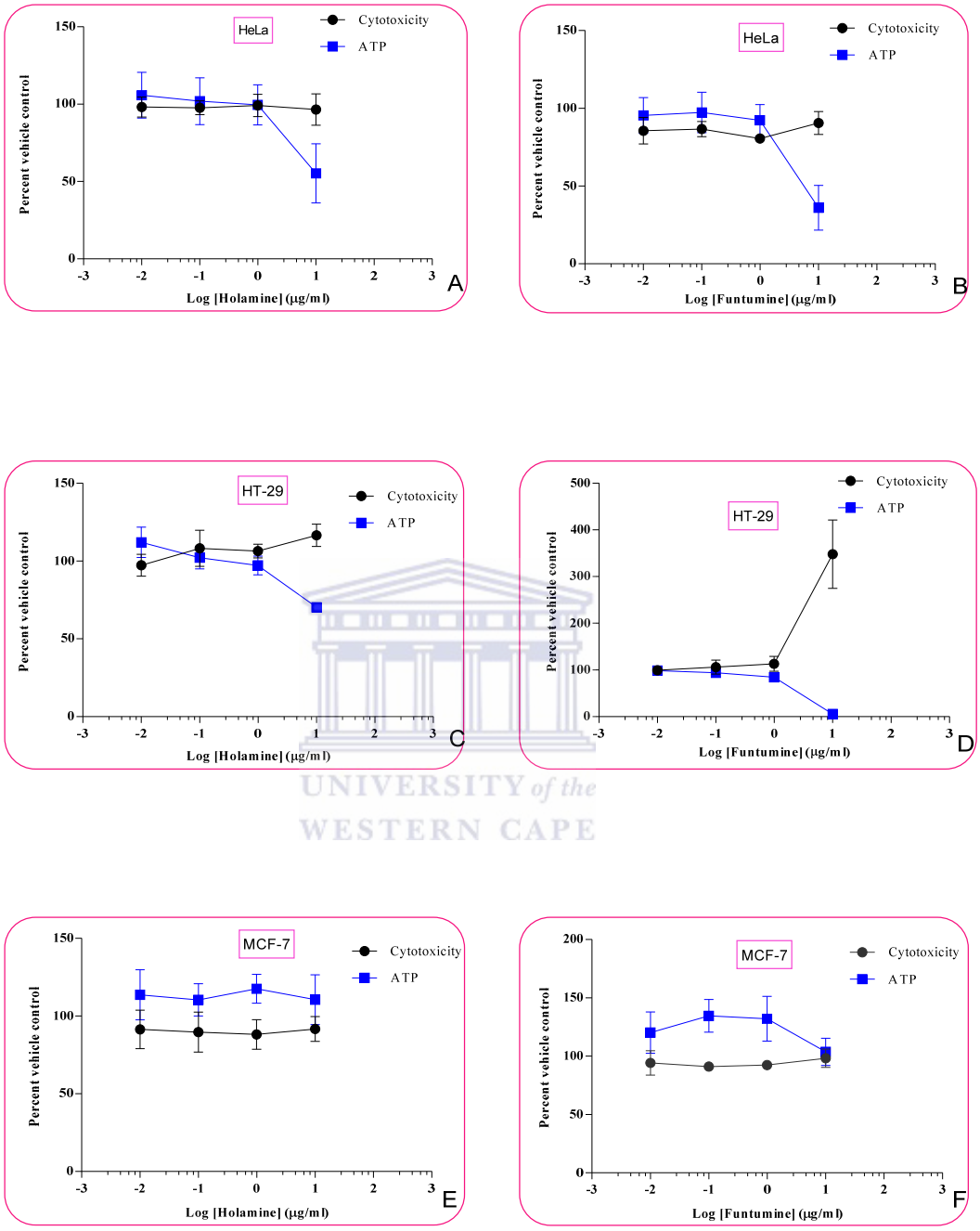
Cells were treated for 12- and 24-hour periods with holamine (A) or funtumine (B), and thereafter stained with CM-H_2DCFDA and the results were evaluated using a flow cytometer. MFI—Mean florescence intensity.

Figure 6.6: Induction of ROS in HT-29, MCF-7 and HeLa cancer cell lines treated with holamine and funtumine



Cells were stained with CM-H_2DCFDA dye. The black histogram depicts stained control cells while the red and blue represent the cells treated with holamine and funtumine, respectively.

Figure 6.7: Representative flow cytometry histograms of ROS production in HT-29, MCF-7 and HeLa cancer cell lines



The effects of holamine and funtumine were evaluated using the mitochondrial ToxGlo assay kit and recorded with a GLOMAX Multi detection system (Promega, USA).

Figure 6.8: Effects of holamine and funtumine on mitochondrial ATP production in HeLa, HT-29 and MCF-7 cancer cells

6.7 Evaluation of Cell Morphology by Fluorescence Microscopy

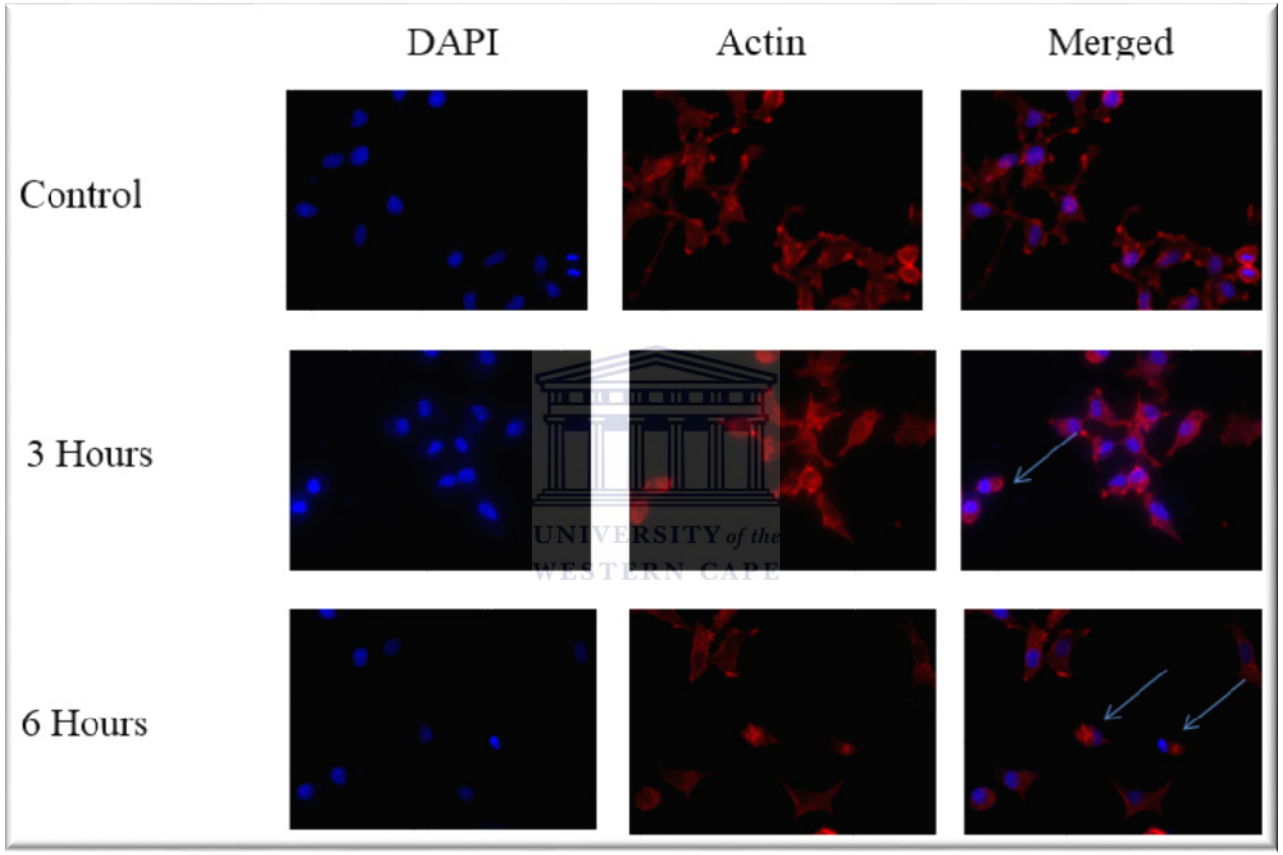
The morphology of HeLa cells was observed under a fluorescence microscope after treatment with the two compounds (15 µg/ml) and staining with tetramethylrhodamine (TRITC)-conjugated phalloidin for the detection of F-actin cytoskeleton, 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) dye for detection of ROS induction and Mitotracker Red™ for the evaluation of cell mitochondrial status.

DAPI (4', 6-diamidino-2-phenylindole) was used to counterstain cell nuclei in each experiment and merged images show co-localization of F-actin (red) and nuclear stain (blue), ROS induced cell (green) and nuclear stain (blue) and mitochondria (red) and nuclear stain (blue) (Figures 6.9 to 6.14). The results show that the two compounds induced alterations of actin organization by decreasing actin at the edges of cell–cell contacts leading to reduced cellular contact between adjacent cells compared with the control with well-defined actin stress fibres as presented in Figure 6.9.

Figure 6.10 shows increased fluorescence intensity of the cells treated with the two compounds after 12 hours treatment compared with the control cells. The increased intensity was not observed at 6 hours treatment with the compounds. Furthermore, the Mitotracker Red revealed evenly distributed mitochondria in the control cells. The fluorescence intensity in the mitochondria of treated cells was lower compared with the treated cells (Figure 6.11).

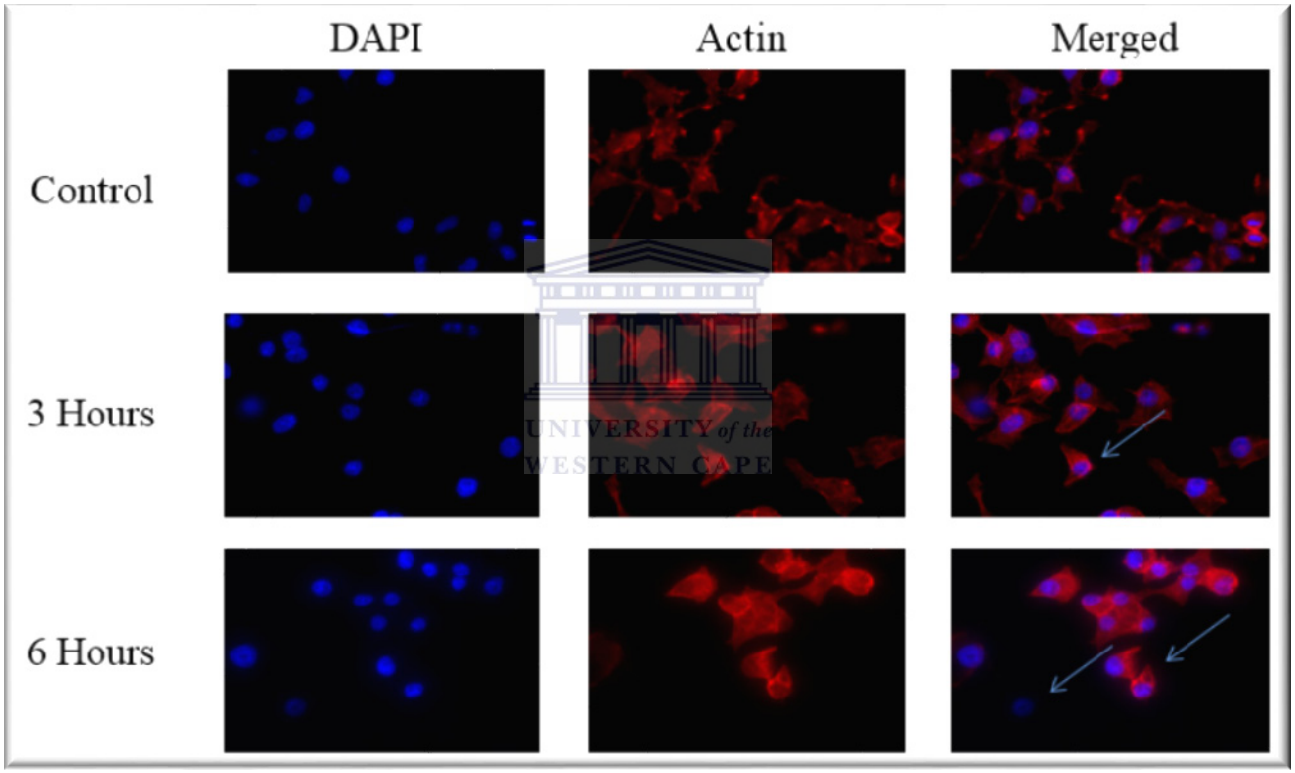
6.8 Evaluation of Cell Morphology by Light Microscopy

The morphological changes in MCF-7, HeLa and HT-29 cells were observed under the light microscope after the cells were exposed to 15 µg/ml of holamine and funtumine for 24 hours. As presented in Figure 6.15, it is clear that untreated control cells exhibit a normal shape while the treated cells show loss of cell adhesion, cell shrinkage, and reduced cell density.



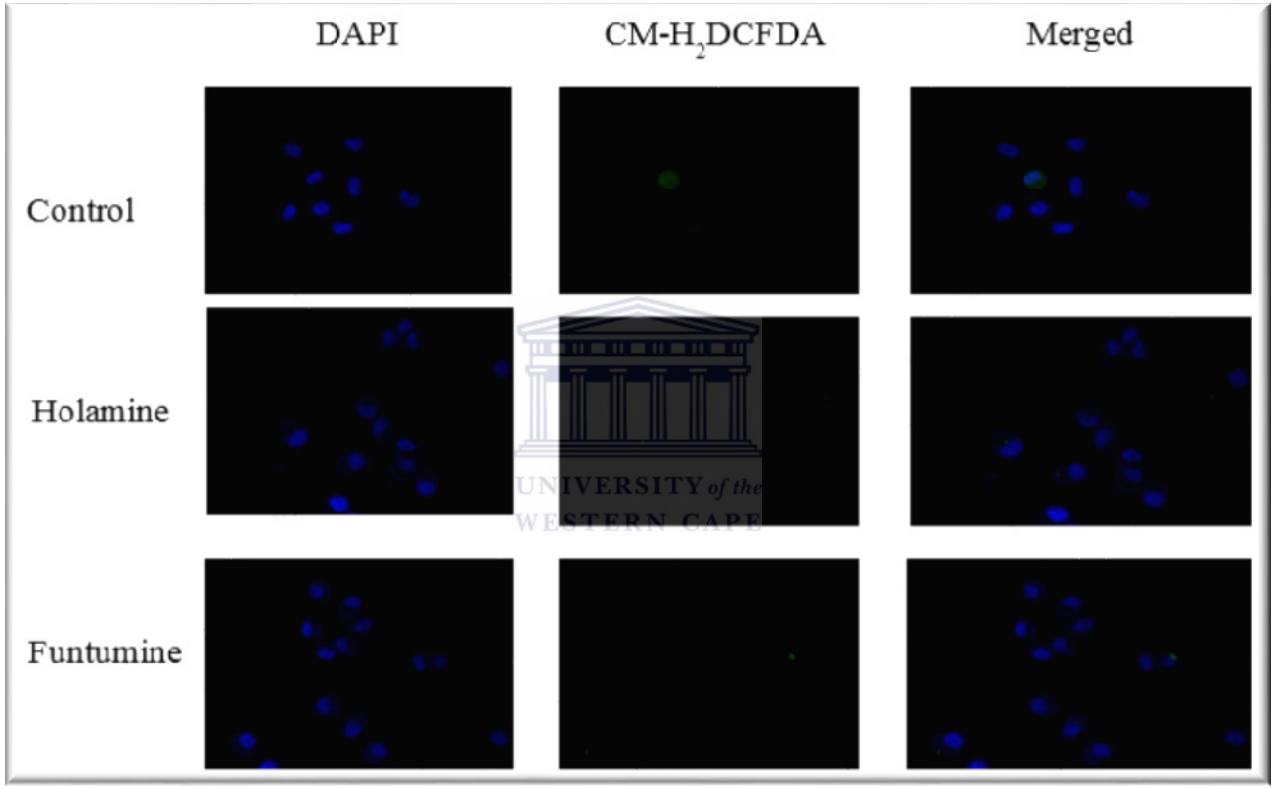
HeLa cells were treated with 15 µg/ml holamine for 3 and 6 hours. Cells were fixed and stained with TRITC-conjugated phalloidin and viewed under a fluorescence microscope (x400) to visualize F-actin cytoskeleton.

Figure 6.9: Effects of holamine on HeLa cell F-actin cytoskeleton



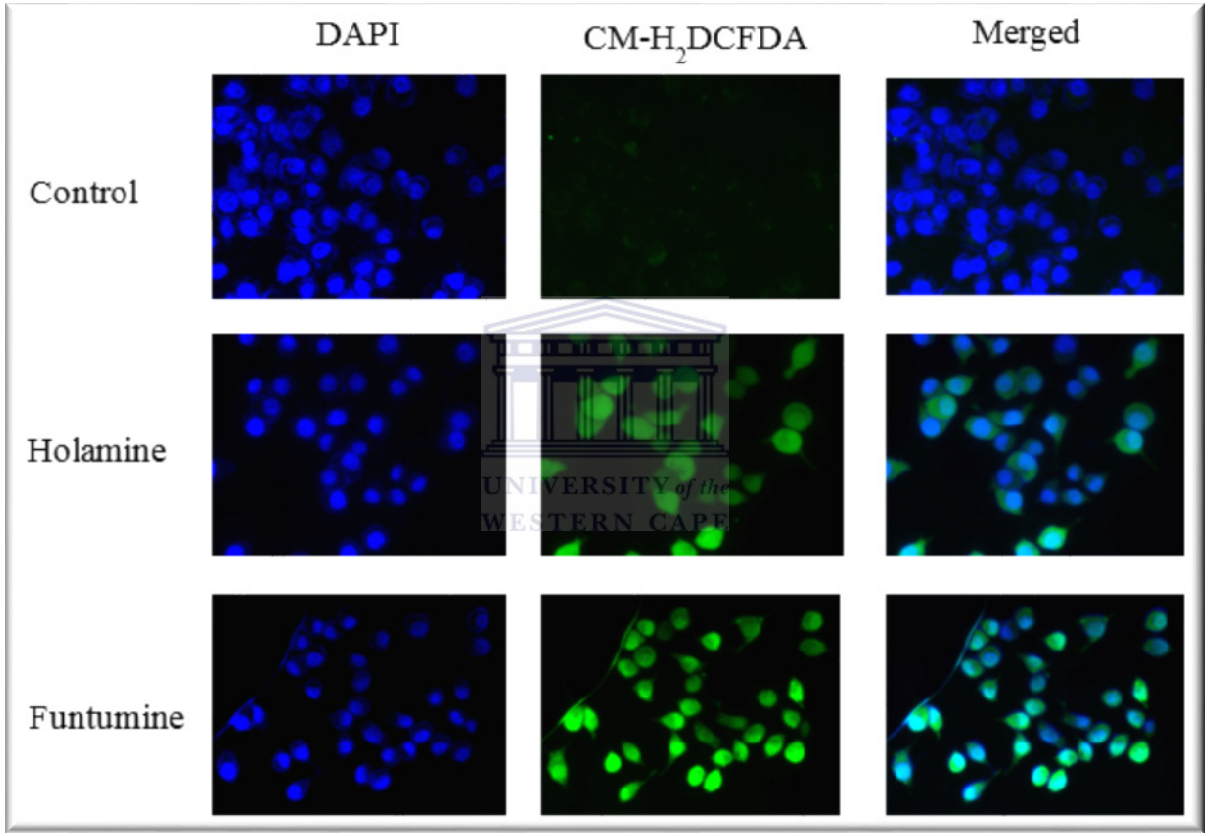
HeLa cells were treated with 15 µg/ml funtumine for 3 and 6 hours. Cells were fixed and stained with TRITC-conjugated phalloidin and viewed under a fluorescence microscope (x400) to visualize F-actin cytoskeleton.

Figure 6.10: Effects of funtumine on HeLa cell F-actin cytoskeleton



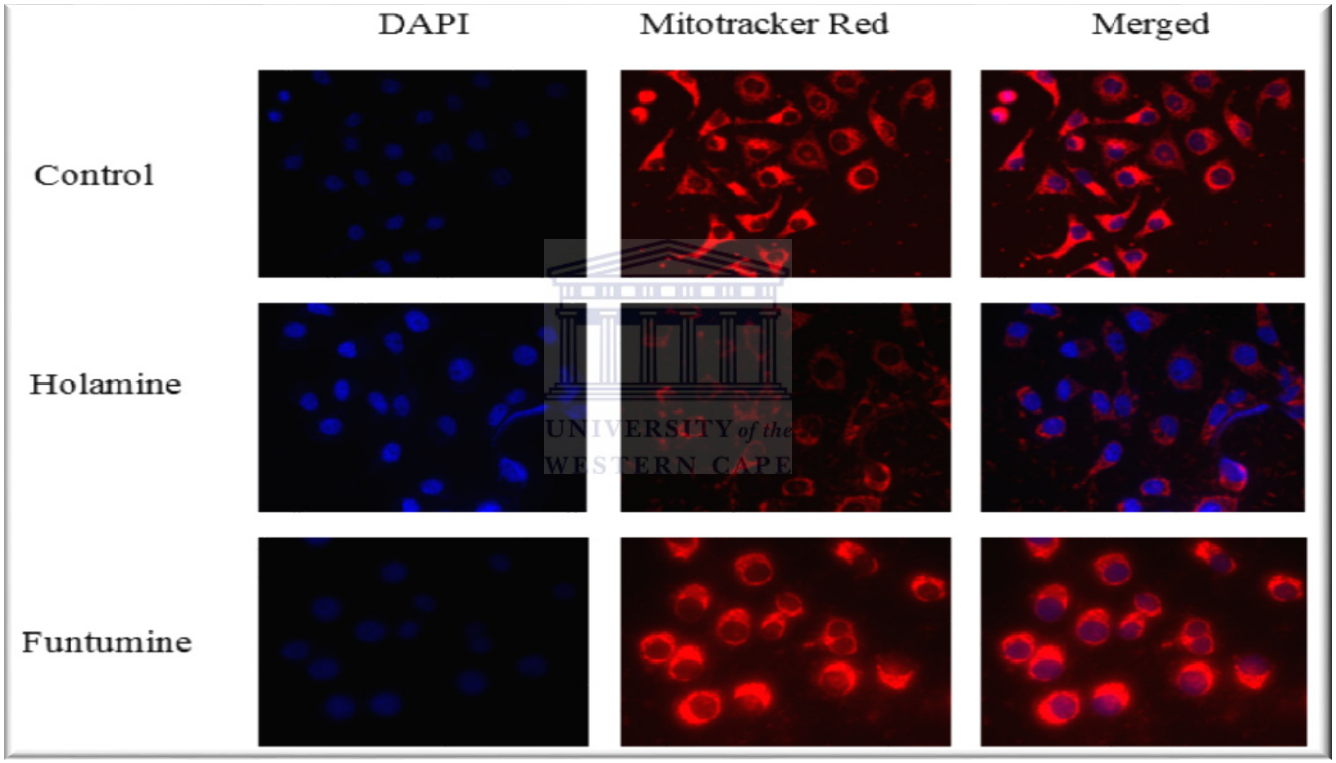
HeLa cells were treated with 15 µg/ml holamine and funtumine for 6 hours to induce ROS production. Cells were stained with 2 µM CM-H_2DCFDA and DAPI dye and viewed under a fluorescence microscope (x400).

Figure 6.11: Induction of ROS production after exposure of HeLa cells to 15 µg/m holamine or funtumine for 6 hours



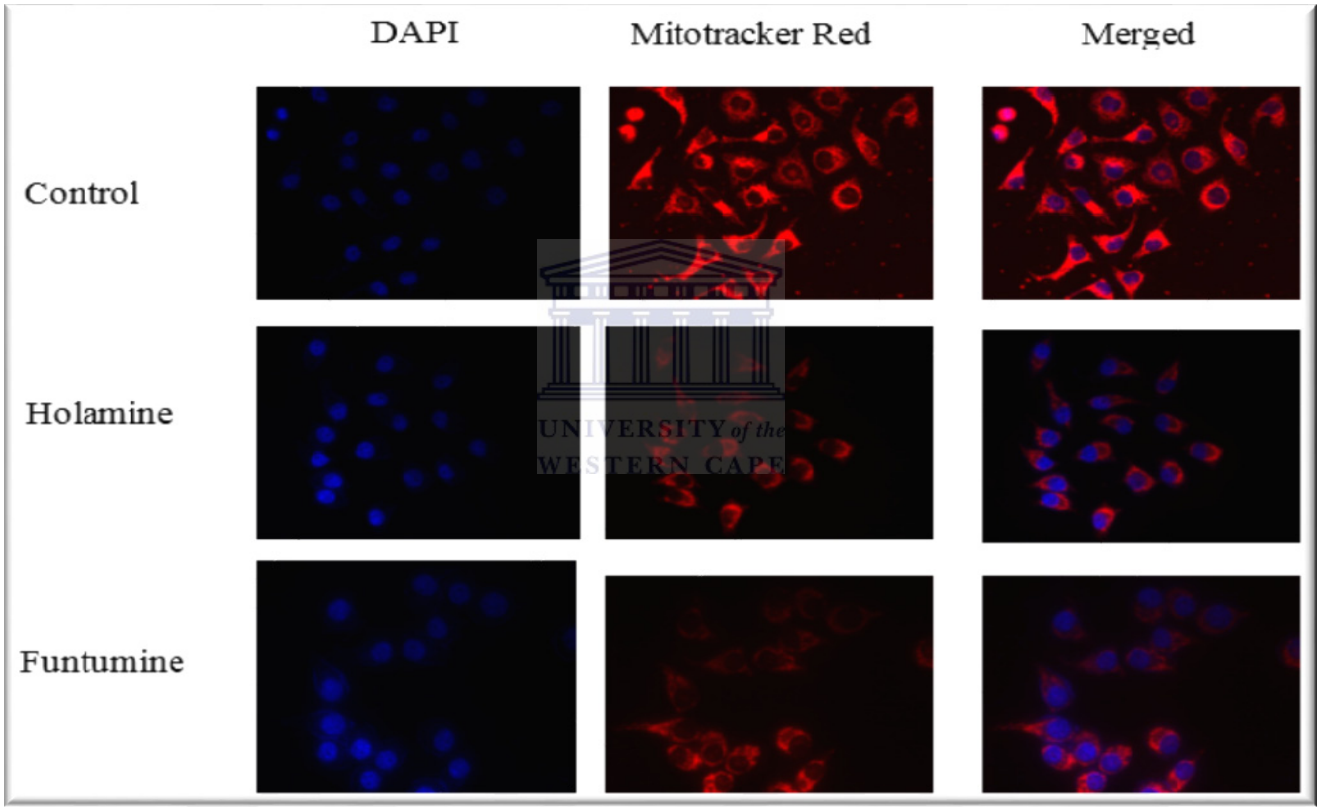
HeLa cells were treated with 15 µg/ml holamine and funtumine for 6 hours to induce ROS production. Cells were stained with 2 µM CM-H_2DCFDA and DAPI dye and viewed under a fluorescence microscope (x400).

Figure 6.12: Induction of ROS production after exposure of HeLa cells to 15 µg/m holamine or funtumine for 12 hours



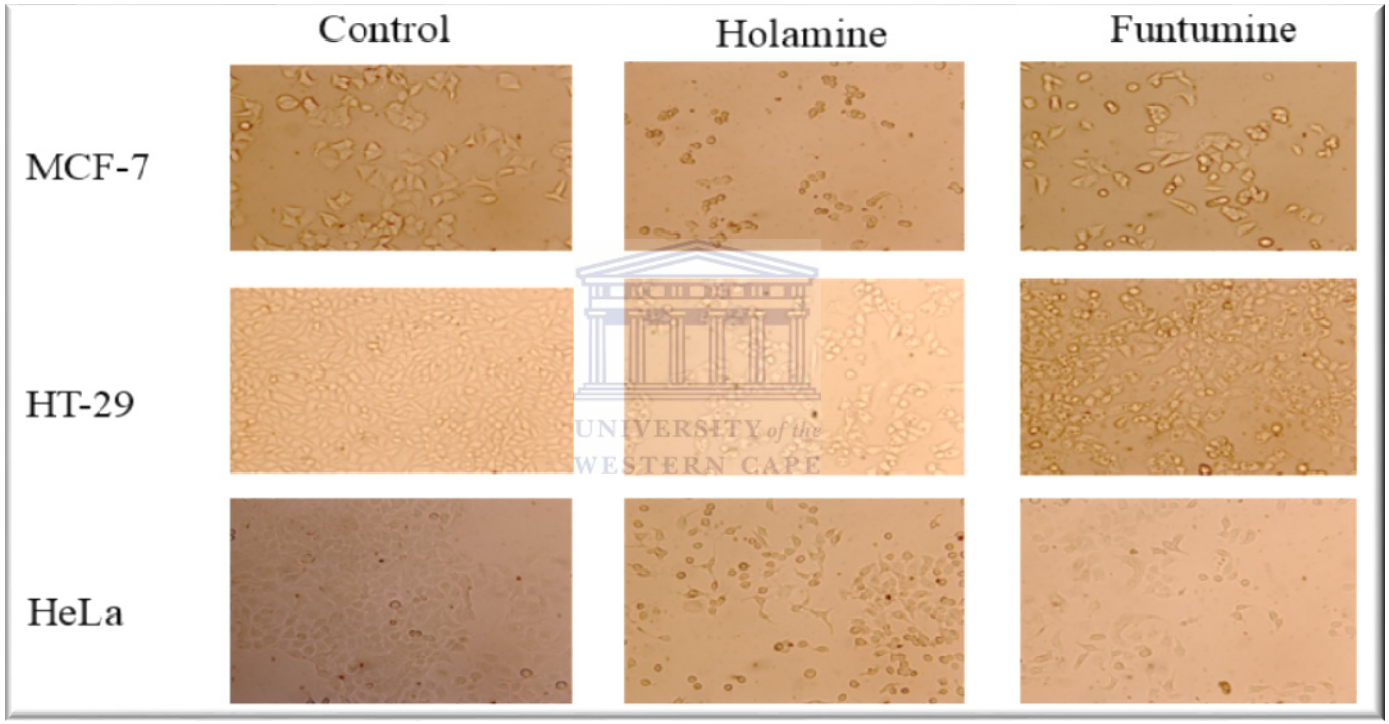
HeLa cells were treated with 15 µg/ml holamine and funtumine for 6 hours to evaluate their effects on cell mitochondria. The cells were stained both Mitotracker red (red) and DAPI (blue) and viewed under fluorescence microscope (x400).

Figure 6.13: Effects of a 6-hour exposure to 15 µg/ml holamine and funtumine on HeLa cell mitochondria



HeLa cells were treated with 15 µg/ml holamine and funtumine for 12 hours to evaluate their effects on cell mitochondria. The cells were stained both Mitotracker red (red) and DAPI (blue) and viewed under fluorescence microscope (x400).

Figure 6.14: Effects of a 12-hour exposure to 15 µg/ml holamine and funtumine on HeLa cell mitochondria

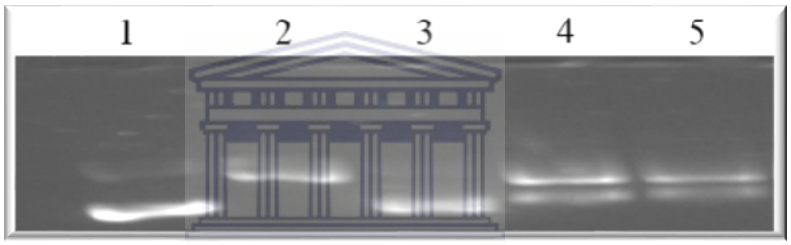


MCF-7, HT-29 and HeLa cells were treated with 15 µg/ml holamine and funtumine for 24 hours and morphological changes assessed. Images were visualized and captured with a Ziess microscope at x200 magnification.

Figure 6.15: Morphological changes in MCF-7, HT-29 and HeLa cells after 24 hours exposure to 15 µg/ml holamine and funtumine

6.9 Effects of Holamine and Funtumine on Topoisomerase-I Inhibition

The topoisomerase-I inhibition assay is based on the inhibition of relaxation of supercoiled circular DNA as described by (Osheroff et al., 1983). Figure 6.16 shows topoisomerase-I, supercoiled DNA after treatment with the compounds and electrophoresed on 1% agarose gels stained with 0.5 µg/ml ethidium bromide in Tris-acetate EDTA (TAE) at 6.5 V/cm for 2 hours. Gels were directly scanned with an image analyzer (Biometra, Germany) for visualization. The results shows that the two compounds (holamine and funtumine) inhibited the topoisomerase-I enzyme.



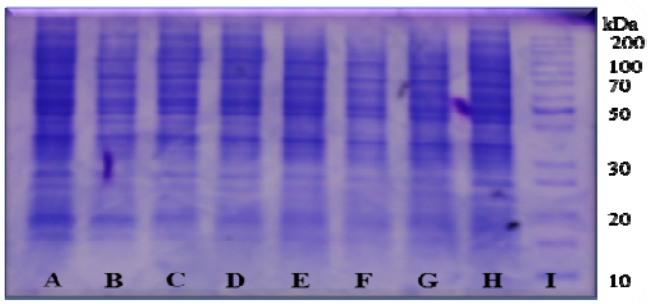
Lane 1 represents supercoiled DNA, lane 2, 3, 4 and 5 represent supercoiled DNA and topoisomerase-I (2 U), camptothecin (9.3 mg/ml; as standard), 15 µg/ml of holamine and funtumine, respectively.

Figure 6.16: DNA relaxation effects of holamine and funtumine on topoisomerase-I

6.10 Effects of Holamine and Funtumine on the Expression of PARP-1

The expression of PARP-1 was evaluated in the HeLa cell line treated with 15 ug/ml each of holamine and funtumine. The cells were treated at different time intervals of 6, 12 and 24 hours with both compounds. Figure 6.17 represents proteins obtained from the treated cells resolved on 12% sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) at a constant voltage of 200 V and stained with Coomassie Blue to determine whether proteins were present in the cell lysates.

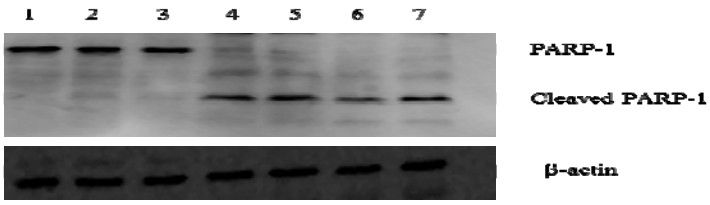
Figure 6.17 illustrates the expression of low and high molecular weight protein profiles of the cells.



Equal amounts of 20 µg were separated on a 12% SDS polyacrylamide gels stained with Comassie brilliant blue and destained. Lanes A and B show 24-hour treatment with funtumine and holamine while C and D, E and F, G and represent 6- and 12-hour treatment with funtumine and holamine respectively. Lane I shows the molecular weight standards. The stained gel was photographed with a UVP-Bioimaging System.

Figure 6.17: SDS-PAGE profile of lysates of HeLa cells treated with or without 15 ug/ml holamine and funtumine for 6, 12 and 24 hours

Figure 6.18 depicts the expression of PARP-1 protein by cells treated with the compounds for 6-, 12- and 24-hour time periods. Electrobloting of the gel onto a nitrocellulose membrane shows that PARP-1 was activated at 12- and 24-hour treatments with both compounds.



Lane 1 represents untreated control while lanes 2, 3, 4, 5, 6 and 7 represent holamine treatment (6 hours), funtumine (6 hours), holamine (12 hours), funtumine (12 hours), holamine (24 hours) and funtumine (24 hours), respectively. The image was taken with UVP-Bioimaging System.

Figure 6.18: Western blot showing the expression of both active PARP-1 and cleaved PARP-1 (inactivated) of HeLa cells treated with 15 ug/ml holamine and funtumine

CHAPTER 7

ANTIOXIDANT ACTIVITY OF FLAVONOIDS ISOLATED FROM *HOLARRHENA FLORIBUNDA* LEAVES

7.1 Introduction

Flavonoids are low molecular weight, structurally related compounds with basic features of the 2-phenyl-benzo-γ-prone nucleus consisting of two benzene rings linked through a heterocyclic pyran ring (Cushnie and Lamb, 2005). The broad pharmacological activity and protective health benefits of natural flavonoids have led to increased interest among scientists with a bias toward functional foods (Kahkonen et al., 1999). Flavonoids and other phenolic compounds with antioxidant activities are reputed to play a preventive role in the development of reactive oxygen species (ROS)-related diseases like cancer (Kahkonen et al., 1999). ROS produced during normal metabolism or induced by exogenous damage have been implicated in several human diseases (Tapas et al., 2008).

ROS is known to contribute to cellular aging, mutagenesis, carcinogenesis and coronary heart diseases (Sastre et al., 2000; Takabe et al., 2001; Kawanishi et al., 2001). The scavenging ability of ROS by flavonoids is essential in preventing impending lesions to the cellular components such as DNA, proteins and lipids (Suganya et al., 2007b). Oxidation of lipid, for example, leads to lipid peroxidation, which is a free-radical-mediated propagation oxidative insult to polyunsaturated fatty acid components of cell membranes (Heim et al., 2002). The potential of the health benefits of these compounds for the prevention and therapeutic use has led to the investigation and identification of a wide range of bioactive principles that include flavonoids

and phenolic compounds in plants (vegetables, fruits, leaves, seeds cereals, roots, spices and herbs) (Suganya et al., 2007b). The protective effects of flavonoids are attributed to their ability to transfer electrons, chelate metals, activate antioxidant enzymes, reduce alpha tocopherol radicals and inhibit oxidases (Heim et al., 2002). Anti-inflammatory, anti-diarrhoeal, anti-ulcer, anti-viral, anti-allergic and vasodilatory actions are also attributed to these compounds (Proestos and Komaitis, 2006). The methanolic extract and sub-fractions isolated from the *Holarrhena floribunda* leaves have been reported to have strong antioxidant activity (Badmus et al., 2010; Badmus et al., 2013). The present work sought to isolate and characterize the flavonoids responsible for the antioxidant activities attributed to the leaves.

7.2 Characterization of Flavonoids from MLE of *Holarrhena floribunda*

Thin layer chromatography (TLC) screening of different fractions from the MLE of *Holarrhena floribunda* showed that sub-fractions 13 and 14 were rich in flavonoids, which were submitted for further chromatographic purification using a combination of silica gel and Sephadex column chromatography and high performance liquid chromatography (HPLC). Figure 7.1 shows the flowchart of the isolation of flavonoids. Sub-fraction 13 was subjected to silica gel chromatography to obtain 46 fractions which were combined according to their TLC profiles (TLC plates were developed using 10% dichloromethane (DCM):methanol and EtOAc:Acetic:formic acid:H_2O (30:2:2:2, v/v). Next, 2 of the 8 sub-fractions were further subjected to Sephadex column chromatography using 100% ethanol.

The flavonoid-rich fractions were purified by HPLC as described in Chapter 3 which led to the isolation of four pure flavonoid compounds (Figures 7.2 to 7.5). The nuclear magnetic resonance (NMR) spectra for the four flavonoid compounds are depicted in Figures 7.6 to 7.13 and summarized in Table 7.1. Sub-fraction 14 was also passed through the same process and led to the isolation of similar flavonoid compounds isolated from fraction 13. Compound 1 yielded

17.6 mg while compounds 2, 3 and 4 yielded 14.7, 12.3 and 17.3 mg, respectively. These flavonoids were further subjected to antioxidant tests: oxygen radical absorbance capacity (ORAC), ferric reducing/antioxidant power (FRAP), Trolox equivalent antioxidant capacity (TEAC) and lipid peroxidation inhibition.

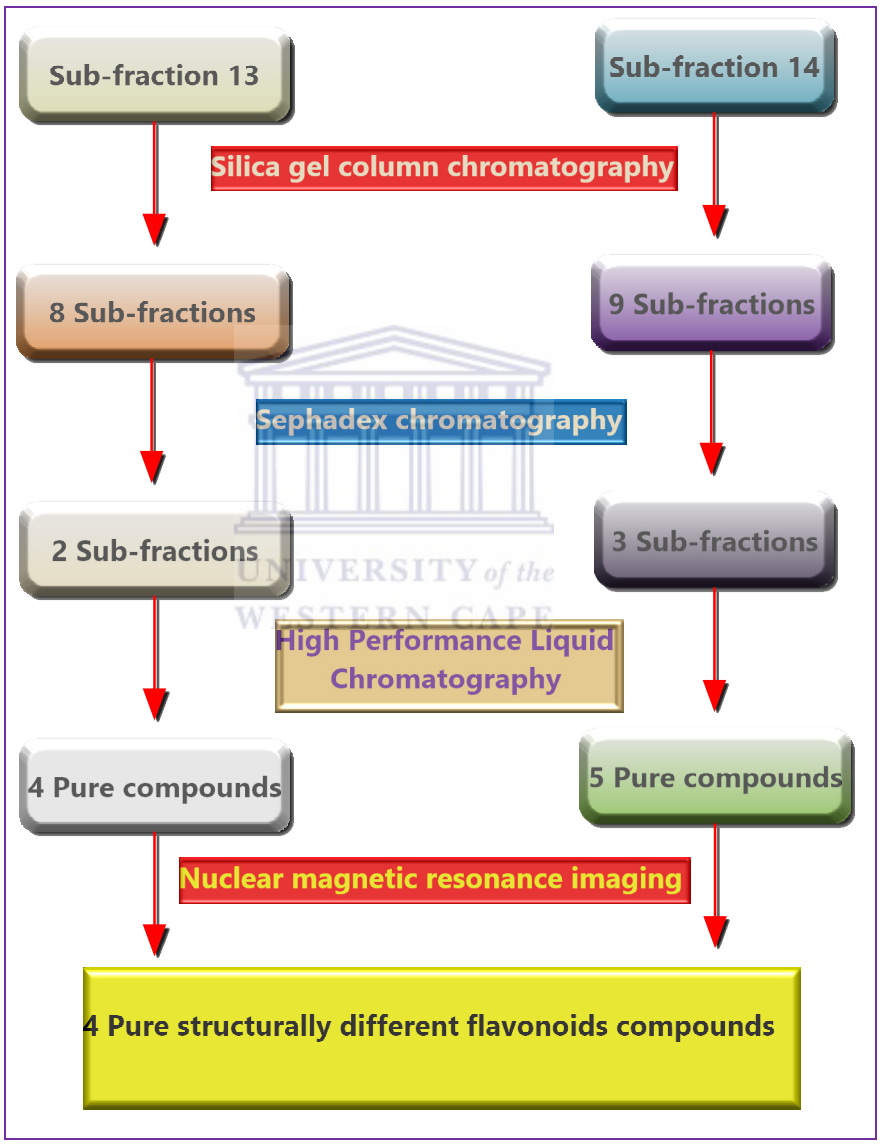


Figure 7.1: Flowchart of the isolation of flavonoids from the methanolic extract of *H. floribunda* leaves

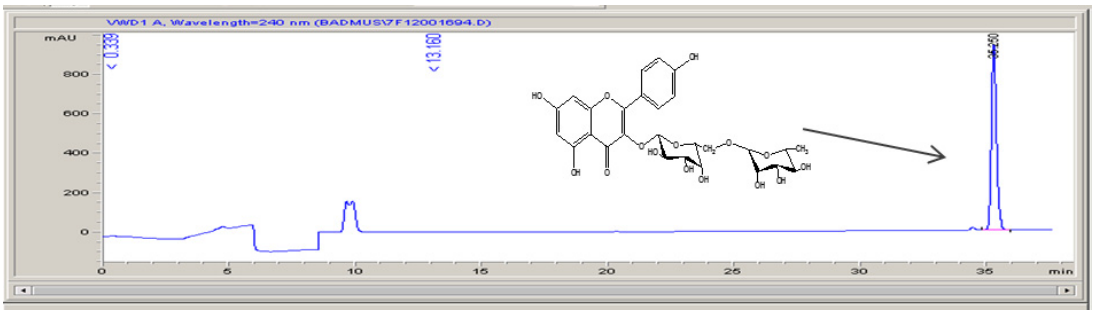


Figure 7.2: HPLC chromatogram of compound 1 at λ=240, Rt=33.169

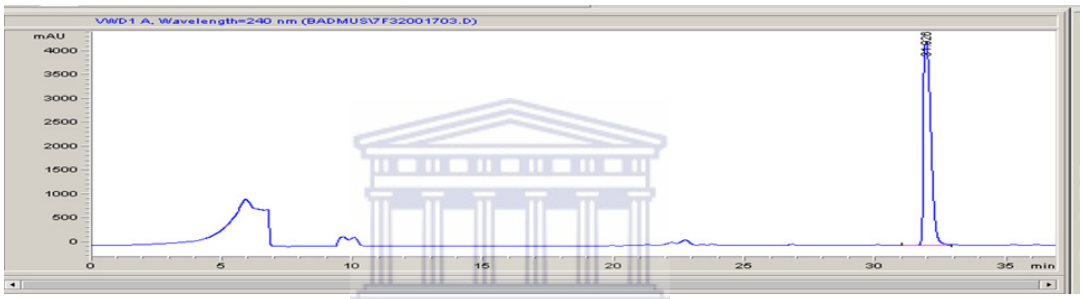


Figure 7.3: HPLC chromatogram of compound 2 at λ=240, Rt=31.926

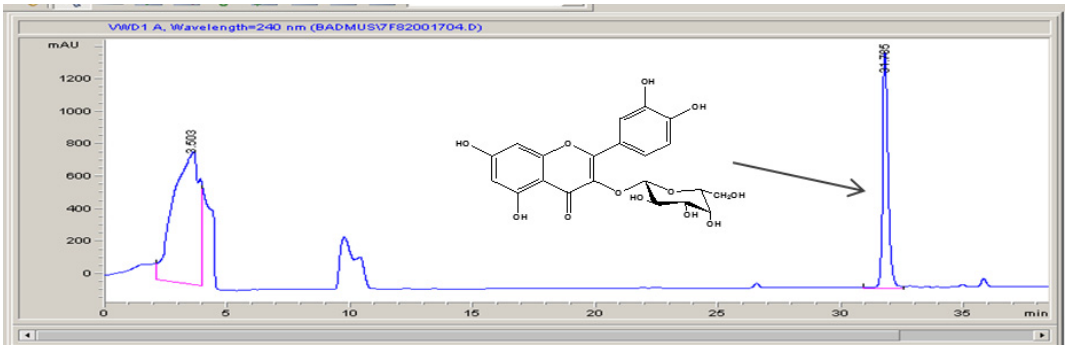


Figure 7.4: HPLC chromatogram of compound 3 at λ=240, Rt=31.765

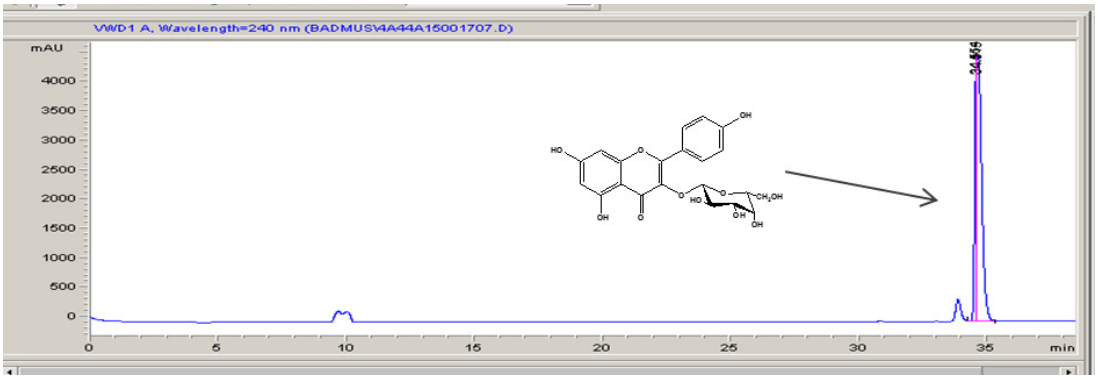


Figure 7.5: HPLC chromatogram of compound 4 at λ=240, Rt=34.565

7.3 Evaluation of Oxygen Radical Absorbance Capacity (ORAC)

The antioxidant evaluation of flavonoids using ORAC assay in the presence of AAPH, Cu^{2+}-H_2O_2 and Cu^{2+} show that the entire isolated flavonoids and total extract have remarkable ORAC activities against the oxidants 2, 2'-azobis (2-amidino-propane) dihydrochloride (AAPH) and Cu^{2+}-H_2O_2 as shown in the Figures 7.14 and 7.15. However, ORAC values for compounds 2 and 3 were significantly higher compared with those for compounds 1 and 4 and the total extract in the AAPH and Cu^{2+}-H_2O_2 systems. Figure 7.16 shows the percentage pro-oxidant ability of the compounds in the presence of Cu^{2+}. The results in Figure 7.16 further reveal that none of the isolated compounds and the total extract act as a pro-oxidant in the presence of Cu^{2+}.

7.4 Evaluation of Ferric Reducing Antioxidant Power (FRAP)

The ability of compounds to reduce Fe^{3+} to Fe^{2+} was evaluated using the established method of FRAP. Figure 7.17 shows the ability of each compound and the total extract to reduce the Fe^{3+} to Fe^{2+} calculated as µmole Trolox equivalent per gram (µmole TE/g). The results show that compounds 2, 3 and extract have significant ($P< 0.0001$) Trolox equivalent per gram when compared with compounds 1 and 4.

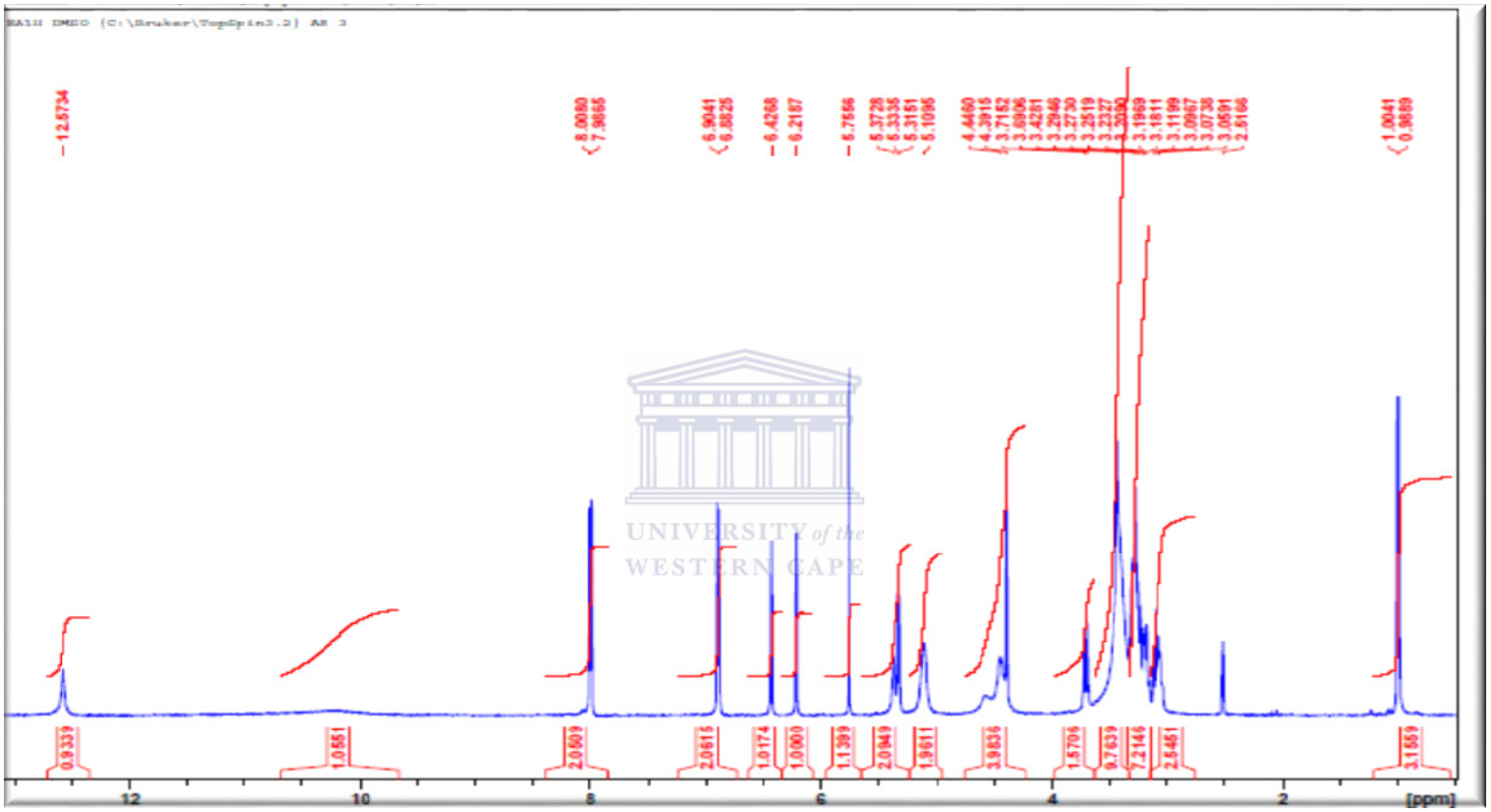


Figure 7.6: [1]H-NMR spectrum of compound 1 isolated from the methanolic extract of *H. floribunda* leaves

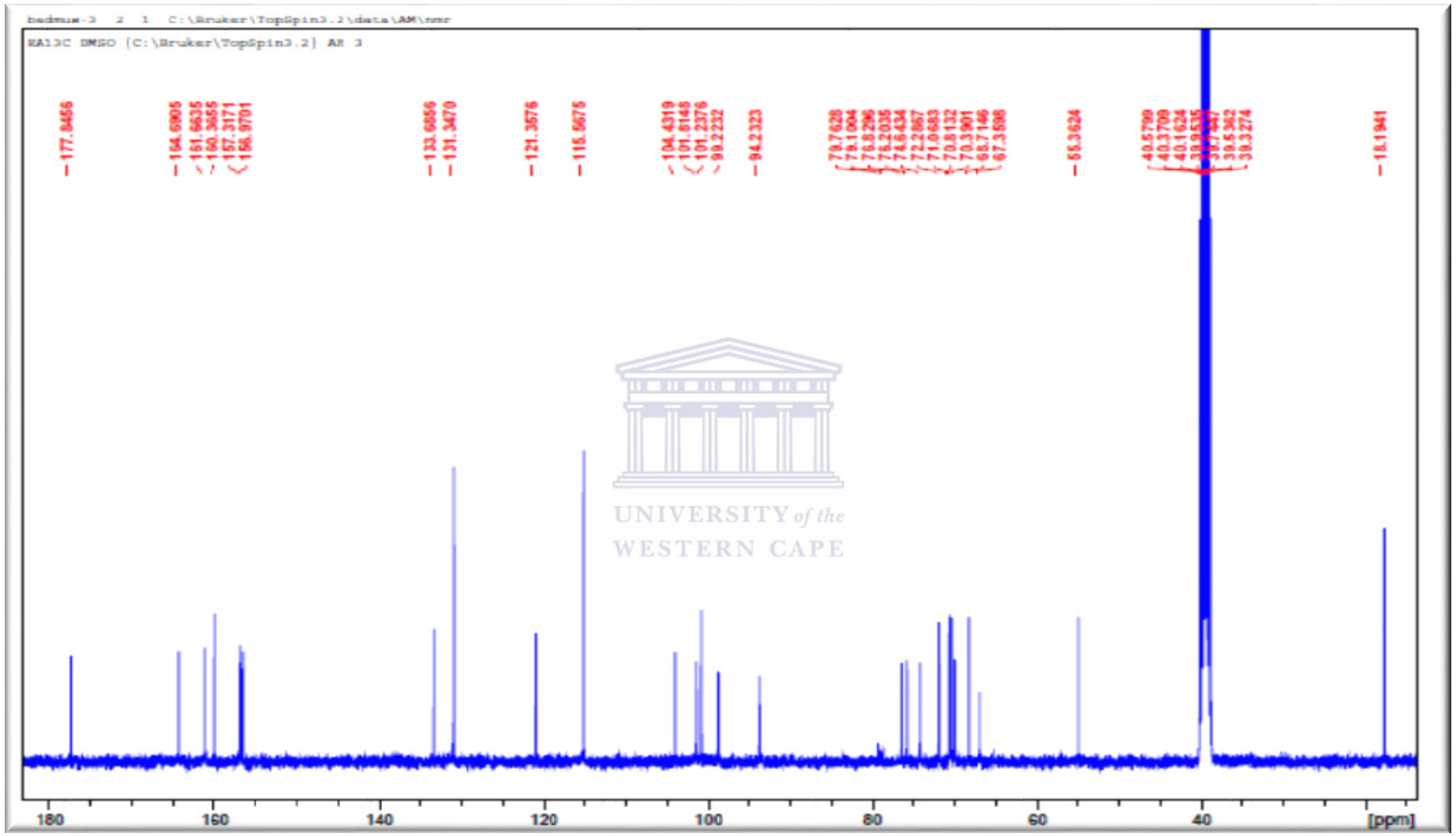


Figure 7.7: ^{13}C-NMR spectrum of compound 1 isolated from the methanolic extract of *H. floribunda* leaves

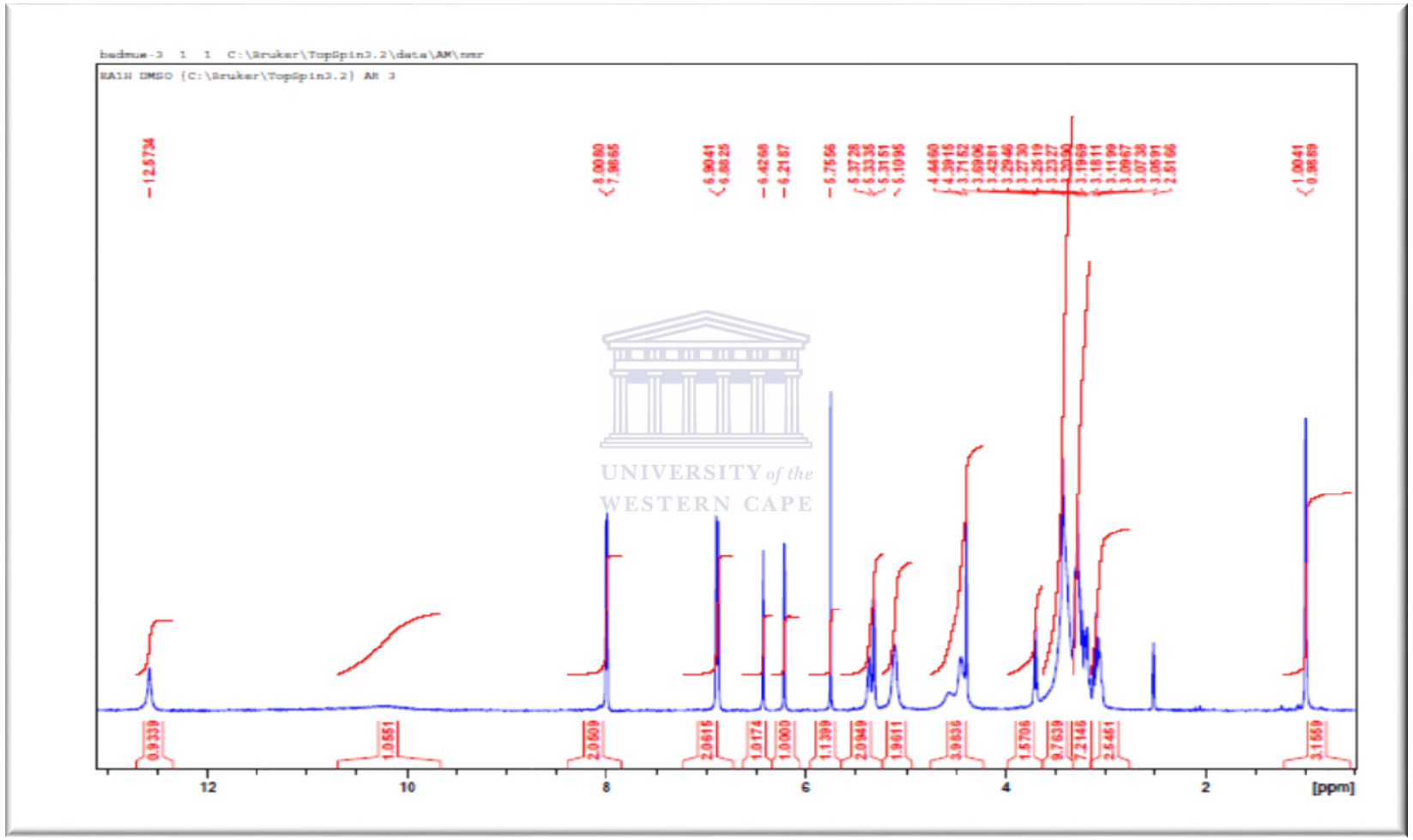


Figure 7.8: [1]HNMR spectrum of compound 2 isolated from the methanolic extract of *H. floribunda* leaves

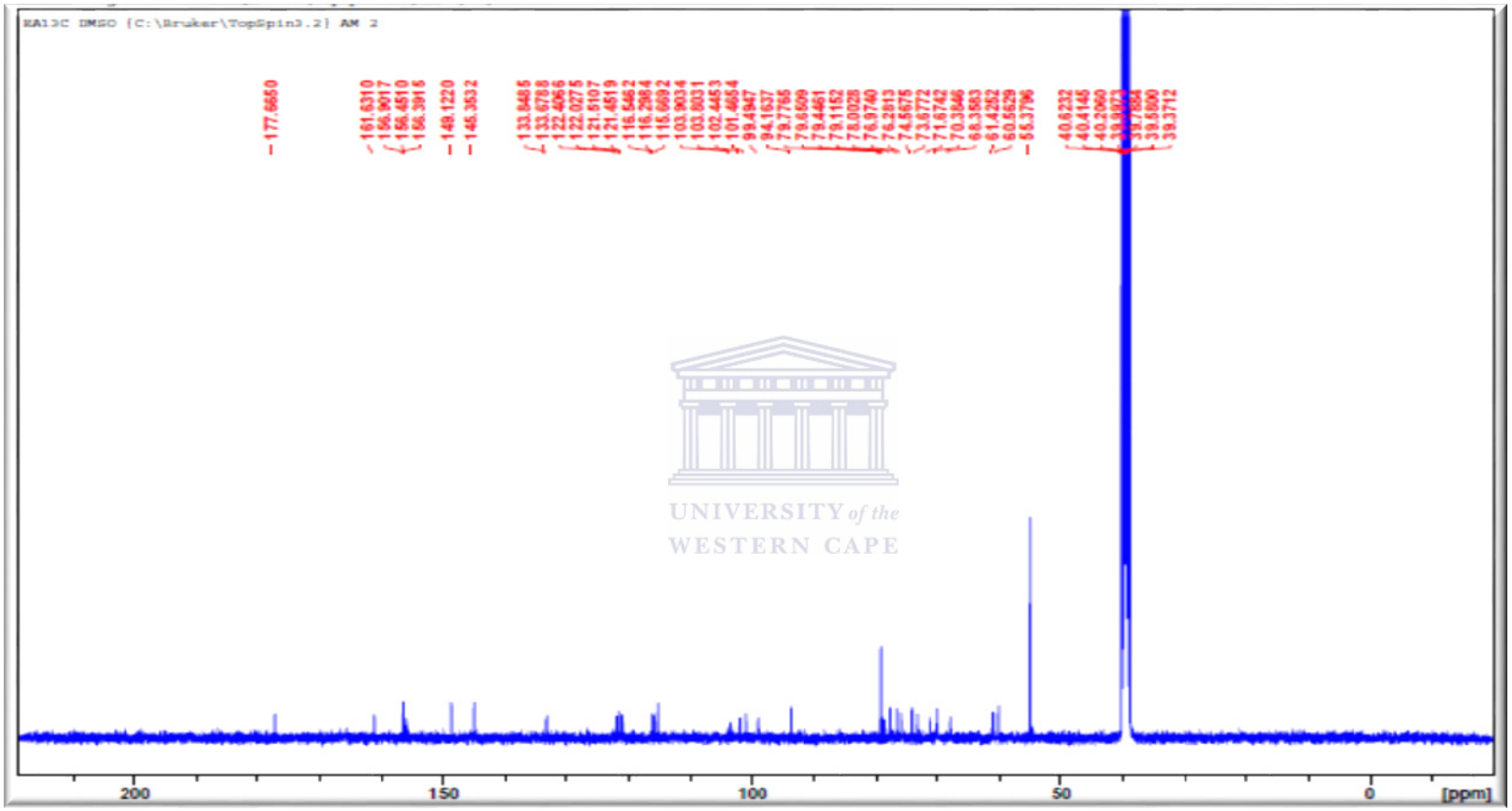


Figure 7.9: ^{13}C-NMR spectrum of compound 2 isolated from the methanolic extract of *H. floribunda* leaves

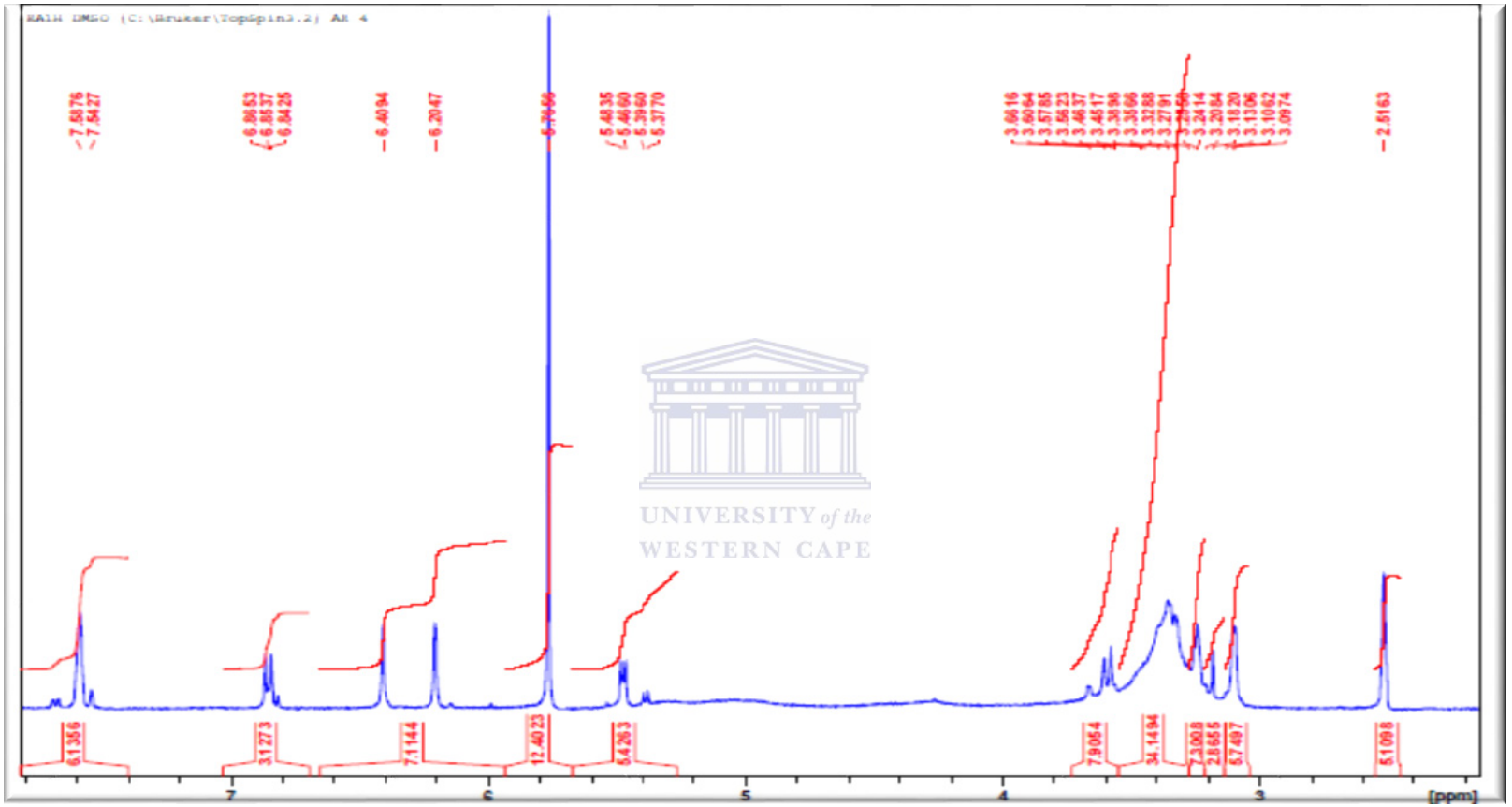


Figure 7.10: [1]H-NMR spectrum of compound 3 isolated from the methanolic extract of *H. floribunda* leaves

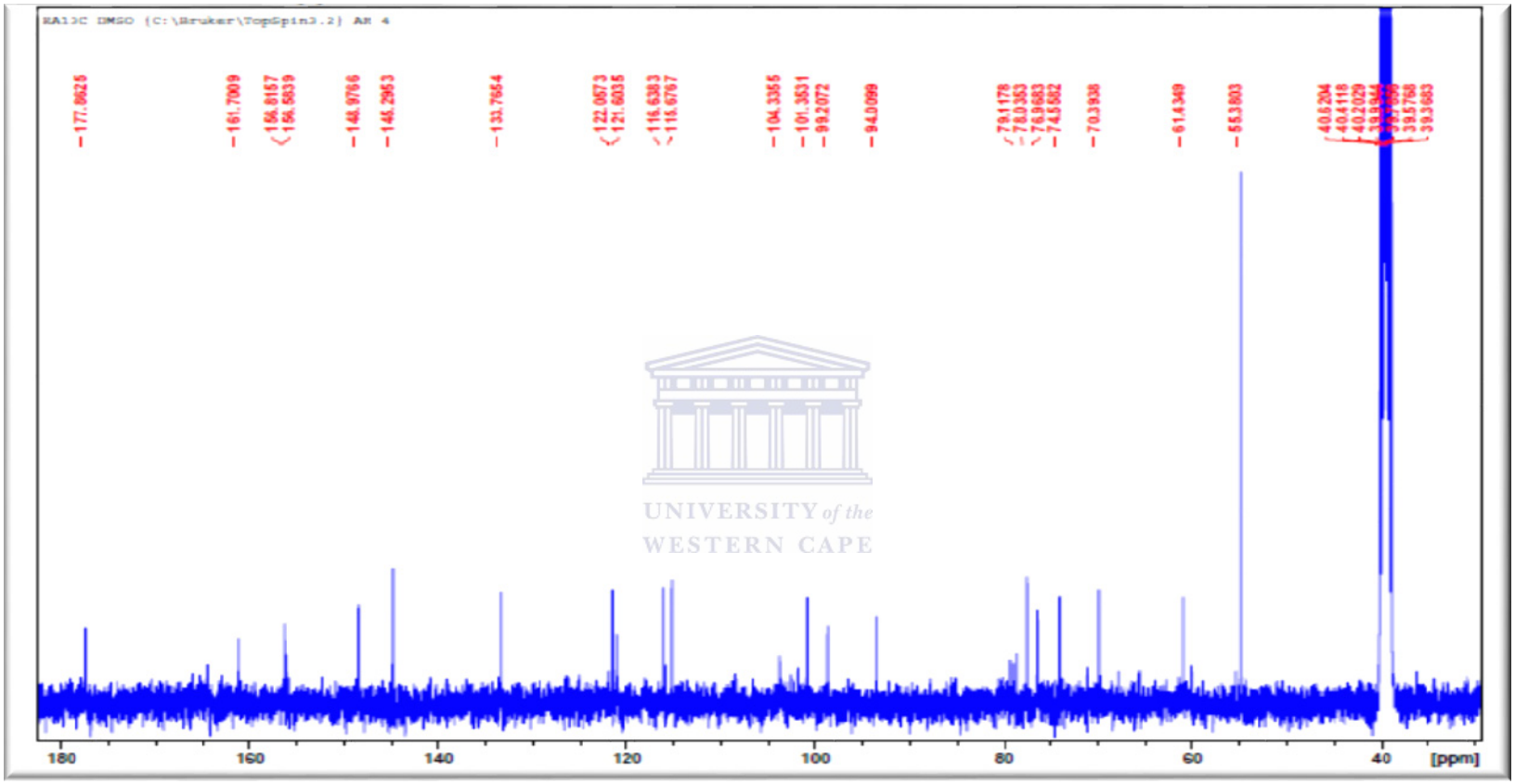


Figure 7.11: ^{13}C-NMR spectrum of compound 3 isolated from the methanolic extract of H. floribunda leaves

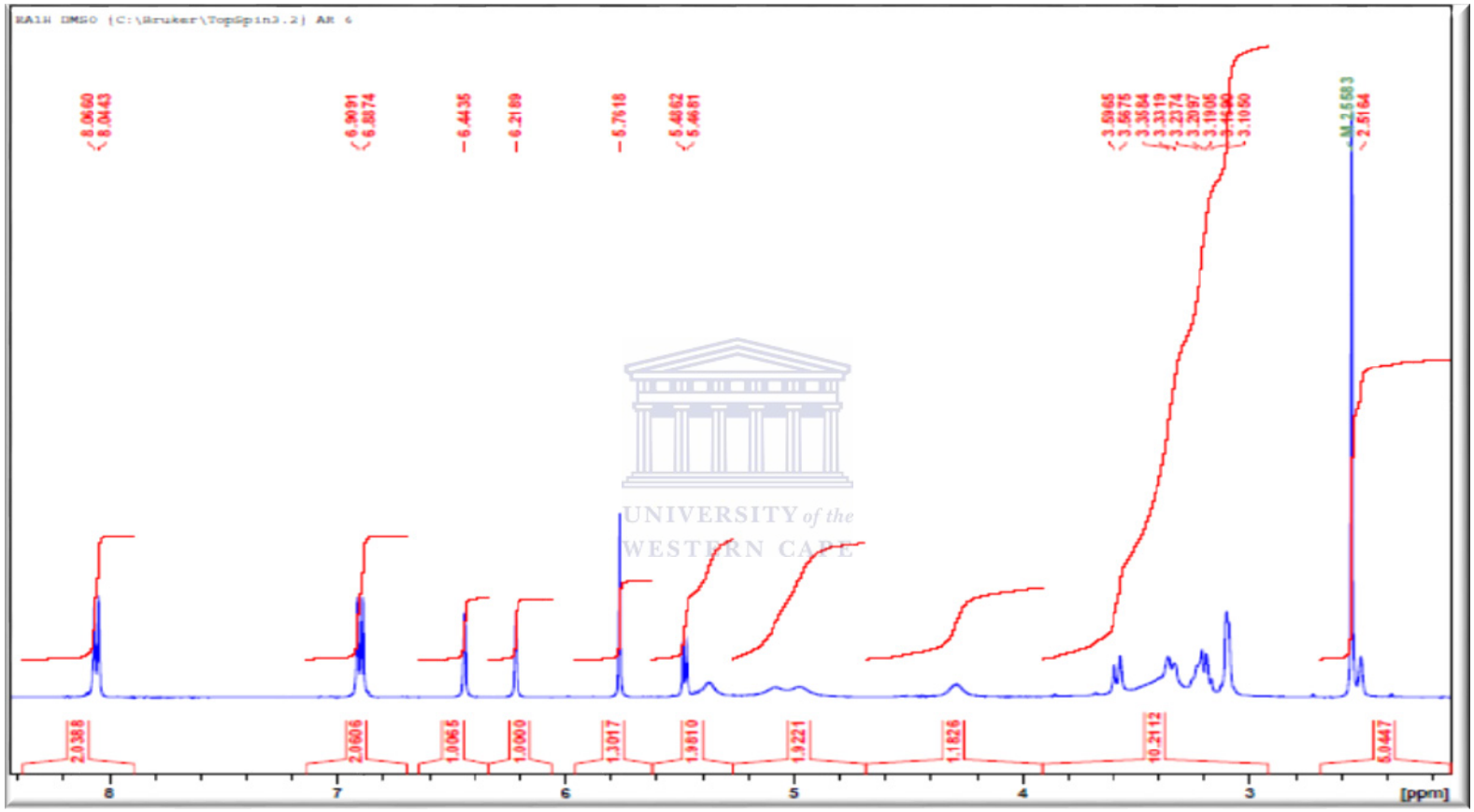


Figure 7.12: [1]H-NMR spectrum of compound 4 isolated from the methanolic extract of *H. floribunda* leaves

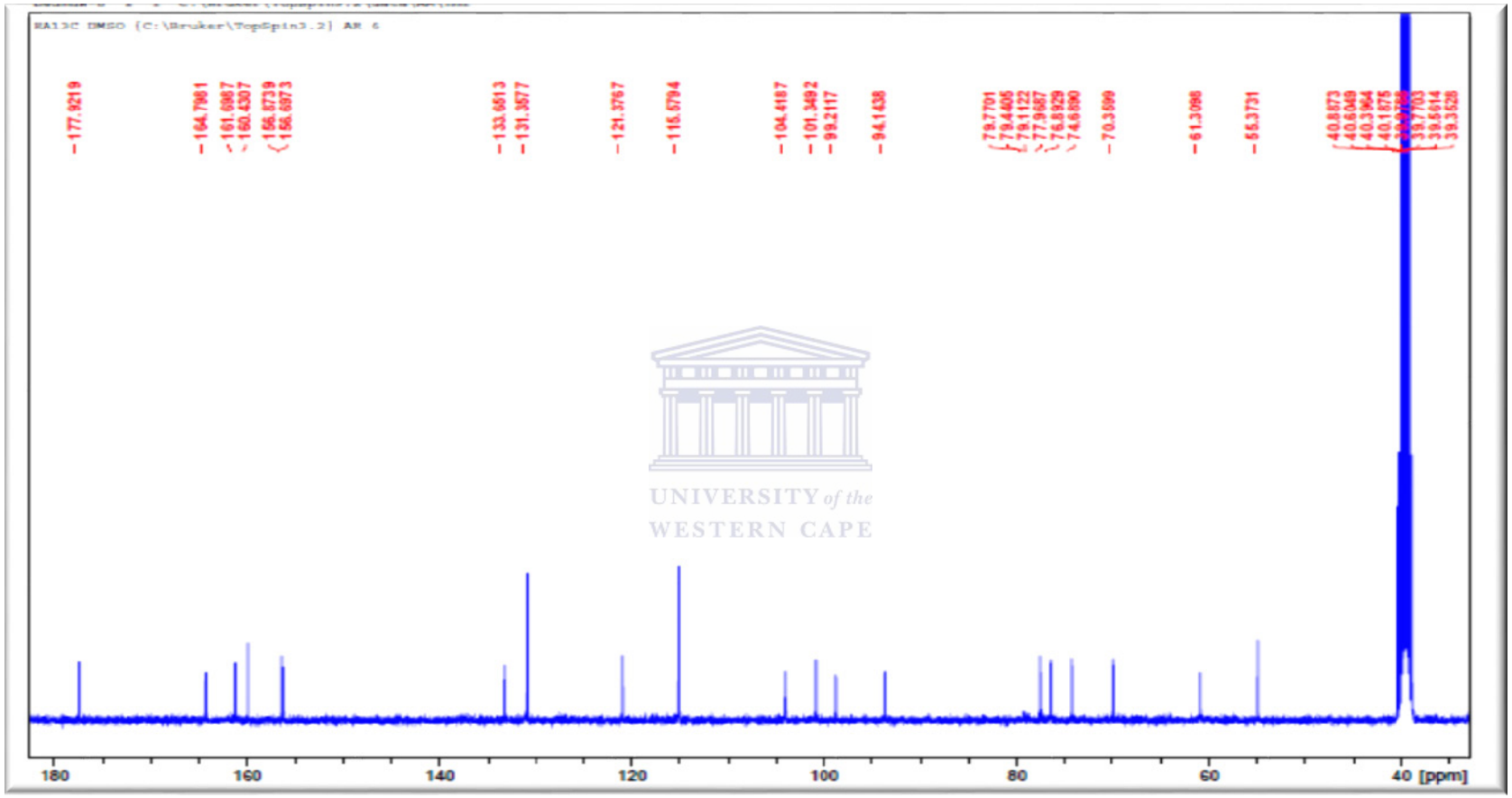
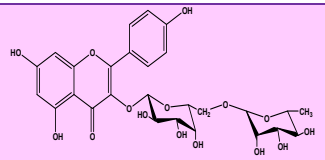
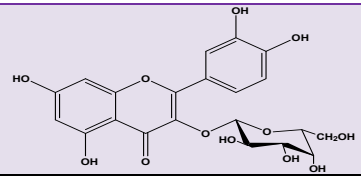
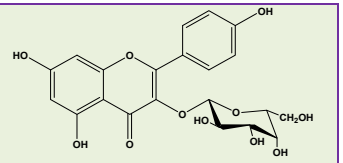
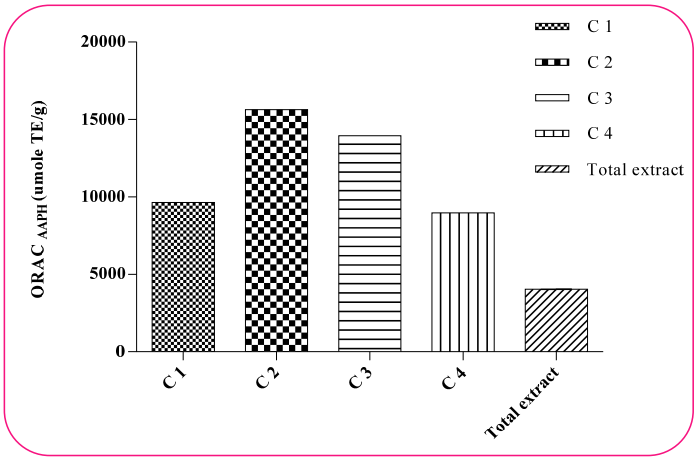


Figure 7.13: ^{13}C-NMR spectrum of compound 4 isolated from the methanolic extract of *H. floribunda* leaves

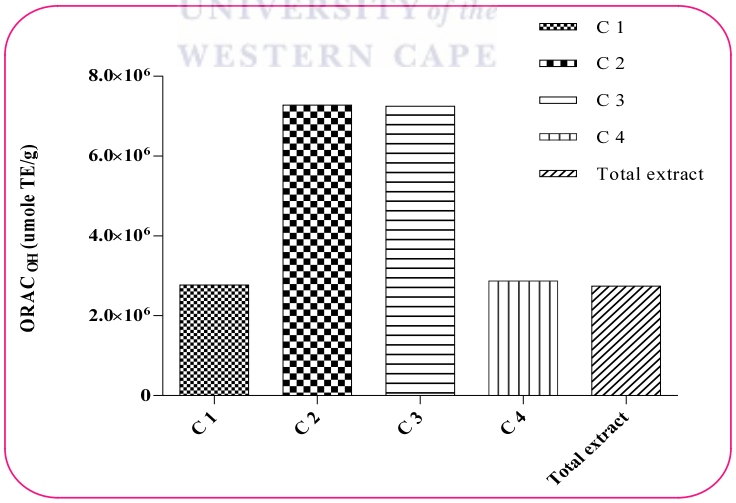
Table 7.1: NMR spectroscopic data (400 MHz, DMSO-d6) of compounds 1-4

			Mixture of quercetin-3-*O*-glucoside and 3-*O*-galactoside					
Position	**C1**		**C2**		**C3**		**C4**	
	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H	^{13}C	^{1}H
2	156.6		156.9		156.5		156.6	
3	133.6		133.8/133.6		133.7		133.6	
4	177.9		177.6		177.8		177.9	
5	161.6		161.6		161.7		161.6	
6	99.2	6.21	99.5		99.2	6.20 s	99.2	6.21 s
7	164.8		165.0		165.5		164.8	
8	94.2	6.44	94.1		94.0	6.40 s	94.2	6.44 s
9	156.8		156.4/156.3		156.8		156.8	
10	104.3		103.9/103.8		104.3		104.3	
1’	121.3		121.5/121.4		121.6		121.3	
2’	131.3		115.6		115.6	7.54 brs	131.3	8.05 d (8.4 Hz)
3’	115.5		149.1		148.9		115.5	6.89 d (8.4 Hz))
4’	160.4		145.3		145.2		160.4	
5’	115.5		116.5/116.2		116.8	6.85 d (4.4 Hz)	115.5	6.89 d (8.4 Hz))
6’	131.3		122.4/122.0		122.0	7.54 brs	131.3	8.05 d (8.4 Hz)
Glucose moiety	**Glucose moiety**		**Glucose/ Galactose moiety**		**Glucose moiety**			
1’’		5.97 d (7.2 Hz)	100.69/102.00		101.3		101.3	5.97 d (7.2 Hz)
2’’	74.6		74.19/71.38		74.5		74.6	
3’’	76.8		76.49/73.30		78.0		76.8	
4’’	70.3		70.19/68.11		70.3	3.00-3.70	70.3	3.19-3.70
5’’	77.9		77.76/75.99		76.9		77.9	
6’’	61.3		61.46/60.33		61.4		61.3	
Rhamnose								
1’’’	73.9							
2’’’	70.3							
3’’’	71.0							
4’’’	72.2							
5’’’	68.7							
6’’’	18.2							
OH-5						12.64 s		



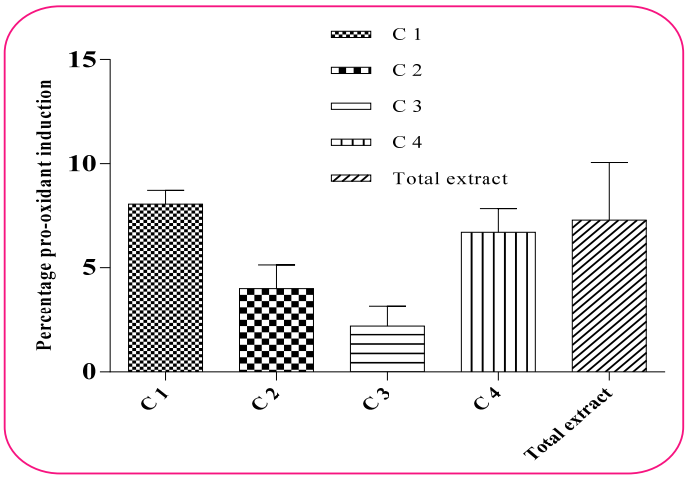
AAPH was used as an oxidant against the compounds. TE/g = Trolox equivalent per gram. Note that standard deviation is very low and as such is not showing in the figure.

Figure 7.14: ORAC values of isolated flavonoids and the total extract from *Holarrhena floribunda* leaves



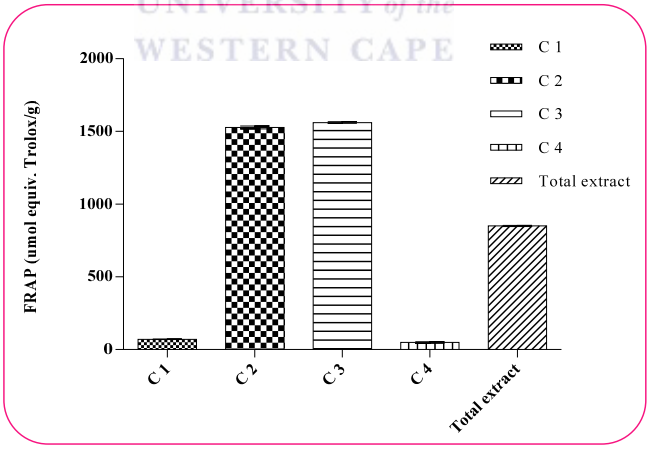
Cu^{2+}-H_2O_2 was used to generate OH radical as an oxidant against the compounds and the extract in the ORAC assay reaction system. TE/g = Trolox equivalent per gram. Note that standard deviation is very low and as such is not showing in the figure.

Figure 7.15: ORAC values of isolated flavonoids and the total extract from *H. floribunda leaves*



Pro-oxidant induction in the presence of Cu^{2+} evaluated using ORAC assay. The values were calculated as percentage decrease of area under the curve of the treated in relation to the untreated control.

Figure 7.16: Pro-oxidant induction in relation to untreated control

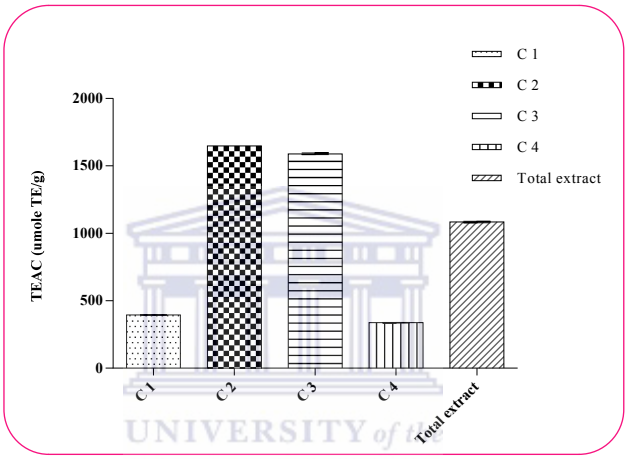


Trolox equivalent per gram (µmole TE/g); Ferric reducing antioxidant power assay (FRAP).

Figure 7.17: Trolox equivalent of the compounds and extract evaluated using FRAP

7.5 Trolox Equivalent Antioxidant Capacity (TEAC)

Trolox equivalent antioxidant capacity of the compounds and the total extract of *Holarrhena floribunda* are presented in Figure 7.18. The results indicate that compounds 2, 3 and the extract expressed significant ($P < 0.0001$) levels of Trolox equivalent capacity compared with the compounds 1 and 4.



Trolox equivalent capacity (TEAC); values of the isolated flavonoids and the total extract in µmole trolox equivalent per gram.

Figure 7.18: Trolox equivalent capacity values of the isolated flavonoids and the total extract

7.6 Inhibition of Fe-Induced Lipid Peroxidation in Rat Liver Microsomes

The potential inhibitory effects of the compounds and the total extract on lipid peroxidation were evaluated in S9 rat liver fractions. The IC_{50} values were calculated using GraphPad Prism 6 statistical software. The IC_{50} values are summarized in Table 7.2 which shows that compound 2 has a value of 9.8 µg/ml while compounds 3 and the extract have values of 10.4 and 7.2 µg/ml, respectively. Values for compounds 1 and 4 could not be determined because their lipid peroxidation inhibitory values did not reach 50% at the concentrations used in this study.

Table 7.2: Inhibition of Fe-induced lipid peroxidation by isolated compounds and the total extract in rat liver microsomes

Compound	Concentration (µg/ml)	% Inhibition	IC_{50} (µg/ml)	Regression Equation
	0.67	4.50±0.37	ND	Y = 0.4353*X + 3.093
	6.67	6.00±1.76		R^2 =0.8370
1	13.40	7.55±1.86		
	16.70	9.27±2.19		
	26.70	16.06±0.98		
	0.67	8.58±0.64	9.8	Y = 2.333*X + 14.05
	6.67	31.11±2.74		R^2 =0.9074
2	13.40	50.67±0.81		
	16.70	61.21 ±1.27		
	26.70	68.34±0.47		
	0.67	11.59±1.62	10.4	Y = 2.202*X + 13.13
	6.67	20.44±9.98		R^2 =0.8193
3	13.40	55.52±0.81		
	16.70	55.37±1.27		
	26.70	63.94±6.45		
	0.67	0.43±0.7	ND	Y = 0.4822*X - 0.8752
	6.67	0.44±0.4		R^2 =0.6700
4	13.40	4.58±2.14		
	16.70	9.75±1.11		
	26.70	11.34±0.55		
	0.67	2.14±1.70	7.2	Y = 2.742*X + 3.333
	6.67	17.33±5.77		R^2 =0.9369
Extract	13.40	50.13±1.68		
	16.70	52.20±0.84		
	26.70	70.70±0.82		
	0.27	5.31±1.73	2.95	Y=3.909*X + 11.80
	0.53	26.00±1.70		R^2 =0.8739
Quercetin	1.13	23.53±1.73		
	2.26	51.23±0.83		
	4.53	65.28±0.99		

CHAPTER 8

GENERAL DISCUSSION

8.1 Introduction

The use of plants as a source of human therapeutic medicine is as old as recorded history. The importance of plant components as therapeutic agents is increasingly being recognized in line with current advances in technology. Globally, natural plant compounds have attracted attention as alternative therapeutic strategies in the fight against diseases, primarily because of their low toxicity and high therapeutic index (Sanchez-Gonzalez et al., 2011; Jung, 2014). Many existing and contemporary drugs in clinical use derive from the natural plants (Cragg et al., 2009). *Holarrhena floribunda* leaves are an important source of drugs used in traditional medicine to cure different diseases, including diabetes, malaria, cancer and oxidant damage-related diseases (Badmus et al., 2013; Fotie et al., 2006).

8.2 Anti-Proliferative and Apoptosis Induction Potential of the Methanolic Leaf Extract of *Holarrhena floribunda*

The present study evaluated the anticancer activity of the *Holarrhena floribunda* MLE in breast cancer (MCF-7), colon cancer (HT-29), cervical cancer (HeLa) and normal human fibroblast (KMST-6) cell lines. The results of this study show that the extract exhibited cytotoxic effects towards all the cancer cell lines in a dose- and time-dependent manner. The IC_{50} values obtained for the various treatment protocols demonstrate that HeLa cells are more sensitive to the cytotoxic activity of the plant while KMST-6, a normal human fibroblast cell line, showed lesser

sensitivity to the extract. The potential of the anticancer activity of the extract to discriminate between normal and cancer cells is an important paradigm in the design and discovery of chemotherapeutic agents. Consistent with this concept, trypan blue dye exclusion and colony formation assays confirm the antineoplastic activities of the extract against cancer cell lines compared to the normal KMST-6 human fibroblast cell line.

Holarrhena floribunda is known to be rich in several phytochemicals like alkaloids, flavonoids, tannins and cardiac glycosides. Some of these phytochemicals have been reported to possess antineoplatic activities against different cancer cell lines. Several reports, for example, underscored that the antiproliferative activities of *Perganum harmala* seeds and *Cassytha filiformis*, respectively, were due to their alkaloids constituents (Lamchouri et al., 2013; Hoet et al., 2004). Flavonoid activities against various cancers have also been reported (Yadegarynia et al., 2012; Matsuo et al., 2005; Li et al., 2014).

To further elucidate the pathways of the cell death induced by the extract, phosphatidylserine (PS) flipping was evaluated using the APOPecerntage™ and Annexin/PI flow cytometric assays. Exposure of PS on the external surface of the cell membrane is generally accepted as one of the biomarkers of apoptosis (Fadok et al., 1992). The results demonstrated the concentration-dependent apoptotic inducing potential of the extract. As a necessary corollary of the results of the cytotoxicity assay, HeLa cells showed a significant sensitivity towards the extract compared to the other cell lines tested. The Annexin/PI assay also confirmed the ability of the extract to induce early and late apoptosis.

Unlike necrosis, apoptosis is an important cell death mechanism that does not trigger an inflammatory response that occasions collateral destruction of normal cells in the surrounding microenvironment (Elmore, 2007). Thus, apoptosis is a protective mechanism that maintains

tissue homeostasis by removing ailing cells (Fan et al., 2005). Cancer cells, however, exhibit resistance to apoptosis in order to sustain their uncontrolled proliferation signature and, therefore, any apoptosis modulating compound is desirable as a plausible chemotherapeutic agent against cancer (Tor et al., 2014).

Two basic processes involved in apoptosis are the intrinsic (mitochondrial) and extrinsic (death receptor) pathways (Hsu et al., 2009). Caspase-3/7 is one of the effector caspases that is involved in the final execution of dying cells while caspase-9 is an initiator caspase that involves the intrinsic pathway (Fan et al., 2005). To understand the mechanism of action induced by the extract, caspase-3/7 and caspase-9 activities were evaluated. The results showed that the extract induced concentration-dependent increases in caspase-3 activity in HeLa cells while in contrast, a concentration-dependent decrease in such activity was observed in MCF-7 and HT-29 cells. The caspase-9 results showed a similar trend of decrease in activities in all the cell lines. The possible reason for these observed results can be explained in two ways. The first is that the increased caspase-3 activity observed in HeLa cells suggests that the extract induced apoptosis is a caspase-dependent manner while the decrease in caspase activities in MCF-7 and HT-29 cells presumably involves degradation of the protease, although the exact mechanisms of apoptosis induction need to be clarified.

However, in agreement with antiproliferative activity of the extract, the results of the cell cycle evaluation confirmed that the extract arrested cell cycle progression by significantly restricting cells in G_0/G_1 phase. This implies that the extract perturbs protein synthesis which is vital to cell progression from G_1 to S-phase. It is known that p53 and MDM2 proteins are important role players in progression through the cell cycle at G_0/G_1 (Plasencia et al., 2005; Tang et al., 2010). It may be possible that the extract interferes with these proteins, but this aspect was not

investigated in this study. Also, the effects of the extract on cell cycle progression may be due to its phytochemical constituents such as flavonoids and alkaloids.

Cells are known to thrive in low levels of reactive oxygen species (ROS), but a relative increase in ROS induces cell cycle arrest and apoptosis (Li et al., 2014). ROS-modulating drugs are, however, being proposed as therapeutic strategies to selectively target the destruction of cancer cells (Lampiasi et al., 2009). The results of this study indicate that the extract induced a time-dependent increase in ROS production. ROS production due to extract (200 µg/ml) treatment for 12 and 24 hours was more evident in HeLa cells, which may explain why this cell line exhibited greater sensitivity to the extract with regard to its antiproliferative, apoptotic and cell cycle arrest effects.

8.3 Isolation, Characterization, Cytotoxicity, Cell Cycle and DNA Synthesis Inhibition of Compounds from the Methanolic Leaf Extract of *H. Floribunda*

Phytochemical studies of the cytotoxic fractions of *Holarrhena floribunda* resulted in two major amino steroidal compounds identified as holamine and funtumine. The two steroidal alkaloids were identified based on their NMR spectra (^{1}H and ^{13}C) which were judged against existing literature profiles (Kam et al., 1998). Holamine is a major steroidal alkaloid from the genus *Holarrhena* because almost all the isolates from the genus led to the identification of the compound (Kam et al., 1998; Dadoun and Cave, 1978; Dodoun et al., 1973). To the best of our knowledge this is the first time funtumine has been isolated from *Holarrhena floribunda* leaves. It has been previously isolated from *Holarrhena* and *Funtumia* genera such as *Holarrhene febrifuga, Holarrhena wulfsbergii* and *Funtumia latifolia* (Dodoun et al., 1973; Nelle et al., 1970; Blanpin and Quevauvillier, 1960).

Alkaloids are widely distributed secondary metabolites derived from plants and well known for their diverse pharmacological efficacies (Patel et al., 2012). Fungicidal, anti-inflammatory and antibacterial activities of aminosteroids have been previously described (Griggs and King, 1978; Szendi et al., 1996). Steroidal alkaloids are useful as starting materials for pharmaceuticals or important semi-synthetic drugs (Kanika et al., 2013). Holamine derivatives were used as starting material for the synthesis of immune stimulating aminopregnenes-I and active adjuvant anaphylactic glycinamide-II (Torelli et al., 1984). Funtumine derivates substituted with a guanylhydrazone moiety have also been shown to interact selectively with the telomeric G-quadruplex *in vitro* (Brassart et al., 2007). The potential for steroidal alkaloids to be used as starting material shows that apart from being candidates for anticancer drug discovery, they can be used as templates for the synthesis of anticancer drugs of desirable characteristics.

The cytotoxic activities of the isolated compounds were evaluated using the thiazolyl blue (MTT) assay. MTT is a colorimetric enzymatic reduction assay in living metabolic competent cells based on the reduction of the tetrazolium salt, but not in compromised dead cells (Ribeiro et al., 2012). It is widely applied to evaluate cytostatic and/or cytotoxic potential of medicinal agents (Karikas, 2010). The cytotoxic effects of these compounds against cancer cells (HT-29, MCF-7 and Hela) were compared with normal fibroblast cells (KMST-6). The IC_{50} values presented in Table 5.2 show that the isolated compounds are significantly selective towards the cancer cells than the normal cell compared with the standard drugs (cisplatin and doxorubicin) used in this study. The assay showed that HT-29 is more sensitive to the cytotoxic effects of both compounds 1 and 2 with IC_{50} values of 9.80 and 7.10 µg/ml, respectively. Furthermore, compound 1 showed IC_{50} values of 13.51 and 16.22 µg/ml and compound 2 16.73 and 14.66 µg/ml for MCF-7 and HT-29, respectively. This is the first cytotoxicity report on the isolated compounds against this panel of cancer cells. The cytotoxic effect of compound 1 isolated from

Holarrhena curtisii have been tested previously on HL-60 and P-388 (Kam et al., 1998) while compound 2 was shown not to display cytotoxicity at a neuro-active concentration (Libor et al., 2004).

Furthermore, most anticancer agents are reported to play roles in cell cycle arrest and cellular demise (Hadfield et al., 2003). Cell cycle transitions were evaluated in this study using flow cytometric analysis of PI stained cells. The results showed that compounds 1 and 2 induced a significant increase in the population of cells in G0/G1 phase and reductions in S-phase in the entire cell treated with corresponding IC_{50} concentration. Moreover, a significant increase was also observed in the population of cells in G2/M phase for both compounds 1 and 2 at 12 and 24 hours for HT-29 cells. The significant increase in G2/M phase was observed only at 12 hours, but not 24 hours for compound 1 while compound 2 produced a significant increase for both time periods in MCF-7 cells.

A significant increase in G2/M phase was observed in HeLa cells treated with compound 1 at 24 hours, but not at 12 hours while no significant increase was observed for compound 2 at both time periods. The induced significant increase in population of cells at both G0/G1 and G2/M phase in HT-29 cells could be related to the low IC_{50} values obtained in the cytotoxicity assays. The cell cycle results imply that the compounds arrest cells substantially at G0/G1 phase of the cell cycle. Cell cycle arrest at G0/G1 stage relates to the induction of cell cycle inhibitory proteins such as p16, p21, p27 related with reduced expression of cyclins required for the G1 to S transition. Liu *et al*. (2003) in their work on molecular mechanisms of G0/G1 cell-cycle arrest and apoptosis induced by terfenadine in human cancer cells showed that terfenadine treatment induced upregulation of p53, p21/Cip1 and p27/Kip1 while simultaneous downregulation was observed for CDK2 and CDK4. Many anticancer drugs from natural origin such as paclitaxel,

docetaxel, vinblastine and vincristine are known cell cycle inhibitors of the G2/M phase. Vinblastine and vincristine affect G2/M phase of cell cycle through inhibition of microtubule assembly while paclitaxel and docetaxel exert their actions through stabilization of microtubules which induces the inhibition of microtubule disassembly and eventual arrest at G2/M phase of cell cycle (Himes et al., 1976; Schiff et al., 1979; Rakovitch et al., 1999). G0/G1 and G2/M arrest might be important pathways by which the isolated compounds induce cytotoxicity in cancer cells.

Agents that cause cell cycle arrest can be categorized into two classes: (1) those that directly inhibit DNA synthesis and (2) those that cause DNA damage leading to G1 or G2 arrest (Hung et al., 1996). The effects of the compounds on DNA synthesis were evaluated using the cell proliferation ELISA chemiluminescent BrdU kit. The results show that the two compounds significantly reduced cellular DNA synthesis within the duration of exposure to IC_{50} levels for the respective cells. This result implies that the compounds can be placed in the category of agents that directly inhibit cellular DNA synthesis, which is consistent with the observed results in the cell cycle analysis with reduction of S-phase.

8.4 Two Steroidal Alkaloids from *Holarrhena Floribunda* Leaves Induced Apoptosis in Cancer Cells.

Numerous anticancer activities of natural products are remarkably correlated with induction of changes in the regulation of target molecules in oncogenic signal transduction pathways implicated in cell growth, replication, angiogenesis, invasion, metastasis and apoptosis in transformed cells (Kuno et al., 2012; Mehta et al., 2010; Neergheen et al., 2010; Amin et al., 2009). In the present study, the mechanistic effects of holamine and funtumine steroidal alkaloids isolated from the methanolic extract of *Holarrhena floribunda* leaves were evaluated.

The two compounds have been isolated before from *Apocynaceae* plant family, but their mechanistic cytotoxic effects remain undetermined.

Cytotoxicity and antiproliferative activities of the compounds were evaluated using the multiplexed CytoTox-Glo™ assay (Promega). The assay offers a convenient and cost-effective manner to address variation (by response normalization), flag non-conforming orthogonal data points and increase per well content (Niles et al., 2009). The cancer cells (HT-29, MCF-7 and HeLa) and non-cancerous cells (KMST-6) were treated with the two compounds with increasing concentrations (1.87, 3.75, 7.5, 15 and 30 µg/ml) for 24 hours. The results, as presented in Figures 6.1 and 6.2 reveal that the two compounds were significantly more toxic towards cancer cells compared to the normal cell line. There was no significant difference in the effectiveness of holamine among the cancer cells while funtumine showed significant cytotoxic towards MCF-7 ($P<0.5$) and HT-29 ($P<0.01$) compared with HeLa cells. Previous work on the cytotoxic activities of steroidal alkaloids is consistent with the present result.

Steroidal alkaloids, tomatidine and solasodine isolated from ground berries of *Solanum aculeastrum* (Srinivas et al., 2007), etioline from *Solanum diphyllum* (Magdi et al., 2009) and epipachysamines B and E, pachystermine A and E isolated from the methanolic extract of *Pachysandra terminalis* stem were reported to have significant cytotoxic effects against a panel of cancer cells (Funayama et al., 2000). Alkaloids are known to induce cytotoxicity as a result of the molecular interaction with one or several targets in a cell, including DNA, RNA and the associated enzymes and processes (Wink, 2007).

Cytotoxicity of holamine and funtumine might be related to the effects on the cell processes associated with survival and resistance to cell demise. Some of these processes include induced external growth factors, intracellular matrix signalling via integrin and Ras protein mutation-

derived constitutive mitogenic signals leading to growing neoplasms that cause destruction and atrophy of the surrounding tissue and adjacent organs (Srinivas et al., 2007; Lukashev and Werb, 1998; Medema and Bos, 1993). The results imply that the compounds might influence on one or more of these processes and can therefore be good lead compounds for anticancer agents in view of their pronounced cytotoxic activities against cancer cells as compared with the normal control cells. Drugs with efficient and selective toxicity in tumour cells are attracting keen interest for the development of new classes of anticancer agents (Phonnok et al., 2010).

Apoptosis is a programmed cell death characterized by morphological features and extensive DNA fragmentation (Collins et al., 1997). Apoptosis plays important role in cell normal survival and maintenance of cell development and homeostasis (Elmore, 2007; Reed and Tomaselli, 2000). Thus, apoptosis induction in cancer cells is an important way to eradicate cancer (Dixon et al., 1997). Agents with apoptosis inducing ability have been used in cancer therapy as apoptotic pathways are frequently impaired in many cancers (Kang and Jang, 2012). In this study, induction of apoptosis by the isolated compounds was evaluated using APOPercentage™ dye and Annexin/PI double staining. The results shown in Figure 6.3 reveal that the two compounds induced significant apoptosis in cancer cell lines compared with the normal cell line. Significant late phase apoptosis compared with early phase was observed with the use of Annexin/PI dye (Figure 6.4) for the two compounds.

A wide variety of apoptosis inducing natural plant products against tumour cells has been identified (Parsaee et al., 2013b). Most of the currently used anticancer drugs are known to induce apoptosis in cancer cells (Rosenkranz and Wink, 2007). The executioners of apoptosis are proteolytic enzymes known as cysteinyl aspartate specific proteases also referred to as caspases. These groups of enzyme function in the two main pathways of apoptosis, which are extrinsic

(caspase-2, -8,or -10) and intrinsic (caspase-9) (Parsaee et al., 2013b). Both initiator caspases activate caspase-3/-7 which eventually commits cells to apoptosis by inactivating PARP protein, which is involved in DNA repair (Looi et al., 2013). The convergence of proteolytic enzymes of both the extrinsic and intrinsic pathways on caspase-3 makes it an important biomarker for the evaluation of apoptosis. Caspase-3 activity was evaluated in this study using the Caspase-3/7 Glo™ assay kit. The results showed that the two compounds induced several fold increased in caspase-3 activity in HeLa cells compared with HT-29 and MCF-7 cells, with holamine the superior inducer. The activation of caspase-3 thus confirms that the induced cell death occurs through the apoptosis. Further to this result, a Western blot assay was carried out to assess the level of PARP-1 protein by treating HeLa cells with 15 µg/ml concentrations of the compounds for 6, 12 and 24 hours. The results, as presented in Figure 6.18 show that the compounds induced deactivation of PARP-1 by 12 hours treatment by cleaving PARP-1.

ROS induction is one of the mechanisms of some forms of conventional treatments such as radiation, etoposide, bleomycin and anthracyclines relied on for the inhibition of cell proliferation (Looi et al., 2013). ROS modulate the delicate cellular redox balance, leading to oxidative stress due to their chemical reactivity. They destabilize mitochondria and attack various components of DNA, leading to generation of oxidized and modified cellular components and eventual induction of apoptosis (Looi et al., 2013; Engel and Evens, 2006). Induction of ROS is attributed to some alkaloids with oxidizing properties (Wink, 2007).

The induction of ROS in this study was evaluated using cell permeant dye (CM-H_2DCFDA) and evaluated using a flow cytometer. The results show that the two compounds induced a significant fold increase in mean fluorescence intensity (MFI) at 12- and 24-hour treatments of HeLa and HT-29 cell lines with 15 µg/ml, while a significant increase between the 12 and 24-hour periods

was not observed in the MCF-7 cell line. This result raises the possibility that induction of apoptosis in both HeLa and HT-29 cells might be due to the increase ROS occasioned by the treatment with both compounds.

The continued rapid proliferation and survival of cancer cells depend, among other factors, on the glycolytic pathways of ATP generation (Gogvadze et al., 2009). The mitochondrion is the site of ATP production which maintains the balance between cell viability and programmed cell death (Koonin and Aravind, 2002). Nuclear functions such as ROS production, modulation of calcium levels and transport of small molecule metabolites are under mitochondrial control (Rizzuto et al., 2012). Mitochondria are attractive target organelles for anticancer agents to restore normalcy and repeal the progression to malignancy (Indran et al., 2011). Drugs with potential to perturb the mitochondrial functions might be an important therapeutic approach to cancer treatment (Gogvadze et al., 2009). Such drugs function by depleting ATP levels, causing the dephosphorylation of pro-apoptotic proteins, mobilization of Bax to the mitochondria, permeabilization of the outer mitochondrial membrane and eventual cell death (Xu et al., 2005).

In this study, the effects of the compounds on the function of the cell mitochondria were evaluated using Mitochondrial ToxGlo™ assay. This assay was carried out to establish if the cytotoxic effects of the compounds occur through mitochondrial perturbation. The results as presented in Figure 6.8 show different cell types responded to the effects of these compounds differently. According to the result interpretation in the assay protocol (Promega, 2011), four different types of mechanistic profiles were given to interpret the results: (1) no change in ATP or membrane integrity equivalent to no mitotoxicity, (2) reduction in ATP with no necrosis implies mitochondrial toxicity, (3) ablation of ATP leads to dose-dependent membrane integrity changes shows mitochondrial toxicity while (4) reduction in ATP with commensurate membrane

integrity gives primary necrosis. In addition, however, a compound can be considered a potential mitochondrial toxin if it decreases the ATP measure with greater than 20% of the average control wells with less than 20% increase in cytotoxicity (Promega, 2011).

According to the profile of results from this study presented in Figure 6.8, holamine induced mitotoxicity without necrosis in HeLa and HT-29 cells, but none in MCF-7 cells. Funtumine induced mitotoxicity without necrosis in HeLa and MCF-7 cells while primary necrosis was found in HT-29 cells. The sensitivity of the different cells to the mitochondrial induced toxicity of the compounds might be related to the differences in ROS generation and mitochondrial dysfunction in each particular cell which make them more vulnerable to oxidative stress induced by the compounds (Wen et al., 2013). Consistent with the results of ROS production in the treated cells, as presented in Figure 6.6, it was observed that both HeLa and HT-29 cells had a significant increase in the ROS induced by the treatment with the alkaloids.

Light and fluorescence microscopy were used to evaluate morphology of the cells while undergoing holamine and funtumine treatments. For fluorescence microscopy, the cells were stained with TRITC-conjugated phalloidin to evaluate the short-term effects of the treatment on the actin filament of cells as presented in the Figures 6.9 and 6.10. CM-H_2DCFDA was used to check the intracellular ROS production and the Mitotracker™ dye was used to evaluate mitochondrial function. F-actin is important for the establishment of cell architecture and integration towards apoptosis (Wink, 2007). It is also plays essential role in cell mobility and contraction during cell division, implicated in the initiation phase of apoptosis in certain conditions also forms important constituents of cell cytoskeleton (Wooley et al., 1998).

In the present study treatment of cells with both holamine and funtumine for 3 and 6 hours caused disruptions of F-actin filaments compared with well-organized stress fibres of actin

filaments (Figures 6.9 and 6.10). It is known that during bleb formation, actin and myosin filaments slide over each other, leading to contraction of the cell border toward the centre as observed in the treated cells (Figures 6.9 and 6.10) (Niero and Machado-Santelli, 2013). This result implies that the compounds can disrupt actin filament organization which can lead to apoptosis. Drugs that induce apoptosis and DNA breakage are known to cause nuclear alteration by disruption of cytoskeletal organization (Niero and Machado-Santelli, 2013; Manelli-Oliveira and Machado-Santelli, 2001).

Intracellular ROS production was evaluated flow cytometrically by staining cells after treatment with compounds for 6 and 12 hours. Figure 6.9 shows that after 12 hours treatment, the compounds induced significant ROS as shown by a bright green colour compared with untreated control. Furthermore, measurement of the effects of the compounds on mitochondrial function showed that the compounds lowered mitochondrial function (Figure 6.10) with low imparted intensity of the mitochondrial stained with Mitotracker red in the treated cells compared with the untreated control. This shows that the effects of the compounds can probably be related to the mitochondrial impairment influenced by the treatment of cells with the compounds for 6 and 12 hours. Morphological analysis of cells treated with the two compounds by observation and capturing of images with a ZEISS Primo Vert (Germany) light microscope shows that the cells demonstrated cellular shrinkage which suggests induction of apoptosis. It further reveals a reduction in cell numbers which was due to cell cycle arrest as previously observed.

In addition to the evaluation of antiproliferative mechanism of the compounds, inhibition of DNA topoisomearse-1 was evaluated. Inhibition of DNA topoisomerase-I is known to induce cell cycle arrest and cell death by apoptosis (Wink, 2007). This is because of the essential roles DNA topoisomerase-I plays by relaxing DNA supercoiling during processes like cell replication,

recombination transcription, chromatin assembly and chromosome partitioning at cell division (Wink, 2007; Ganguly et al., 2007; Wang, 1996). Inhibition of topoisomerase-I can either be through catalytic inhibition or stabilization of transient cleavable ternary complexes by preventing religation (Hofmann and Mattern, 1993). The present work, however, shows that holamine and funtumine inhibited the DNA topoisomerase-I as presented in Figure 6.16. This shows that cytotoxicity and apoptosis activities of these compounds might not be unrelated to the topoisomerase-I inhibition. DNA topoisomerase-I and -II are excellent targets of clinically significant classes of anticancer drugs and an effective strategy for cancer therapy (Salerno et al., 2010). Camptothecin (CPT) and its derivatives topotecan and irinotecan are known topoisomerase inhibitors through which they induced their anticancer activities. Some alkaloids isolated from plants such as pallidine and scoulerine from *Corydalis saxicola* and epibeberine and gloenlandcine from *Coptis* rhizomes have also been found to inhibit DNA topoisomerase-I (Cheng et al., 2008; Kobayashi et al., 1995).

8.5 Isolation, Characterization and Antioxidant Activity of Flavonoids from *Holarrhena floribunda* Leaves.

Dietary antioxidants with potential for therapeutics and prevention have been a major focus of research in recent years. Isolation and characterization of bioactive compounds from plants with health and disease preventing properties have led to the isolation and identification of various arrays of compounds, including flavonoids (Suganya et al., 2007a). The present work isolated flavonoids by subjecting the methanolic leaf fractions of *Holarrhena floribunda* to different chromatography techniques. The flavonoids were isolated, purified using HPLC, identified with NMR spectroscopy and data compared with that reported in the literature. Three pure compounds and an inseparable mixture of two compounds were isolated and identified. The compounds showed a dull spot under UV light, which indicate the nature of flavonoids.

Compound 1 in NMR (1 hour and ^{13}C) showed typical signal of kaempferol (Table 7.1), substituted at C-3 (δc 133.6). It showed two signals at $\delta_H/\delta c$ 6.21/99.2 and 6.44/94.2 of H-6 and -8 respectively; two signals (2H each/d) at 7.99/131.3 and 6.88/115.5 of H-2',6' and H-3',5' of ring B, additionally, two signals at 5.31/101.8 and 4.42/101.2 of two anomeric protons belong to glucose and Rhamnose, the methyl signal of the rhamnose unit appears at 0.99/18.2. The compound was identified as kaemperol-3-O-rutinoside and the structure was confirmed further by comparing the NMR data with the literature.

Compound 3 was purified by HPLC as a yellowish white amorphous powder and its NMR showed signals at 6.20/99.2 and 6.40/94.0, corresponding to ring A H-6 and H-8; ring B showed 1,3,4-trisubtituted pattern and showed signals at 754/115.6; 754/122.0 and 6.85/116.8. The above data, including others in Table 7.1, confirmed the presence of an aquercetin nucleus. The C-2 signal at 156.5 and C-3 at 133.7 indicated the presence of a glucose unit at C-3. The glucose unit showed anomeric proton at 5.47/101.3 in addition to cluster of proton signals at 3.1-3.7 ppm. The above data are identical with quercetin-3-O-glucoside.

Compound 4 was purified using HPLC and showed similar NMR data with the previous compound except for ring B which showed a 1-4 substituted pattern and demonstrated signals at (2H each) 8.05/131.3 and 6.89/115.5. The compound was identified as kaempferol-3-O-glucoside from NMR spectroscopic data and confirmed by comparison with the literature.

Compound 2 was isolated as singlet peak from HPLC and it showed typical data like compound 3 with an additional sugar unit. The anomeric protons at 5.45/101.4 and 5.36/102.4 are counted for as glucose and galactose units. The integration of the two protons is equal to ½ of other protons in the spectra (i.e., H-6). These data indicate two inseparable compounds (viz compound 3 and quercetin-O-galactoside) with the same polarity and found relatively in a ratio of 1:1.

However, further to the isolation of flavonoids from the leaves, isolated flavonoids were subjected to antioxidant evaluation using ORAC , FRAP, TEAC and inhibition of lipid peroxidation. The ORAC assay evaluates the ability of antioxidants to scavenge peroxyl radicals generated by 2', 2'-azobis (2-amidinopropane) dihydrochloride (ABAP) and delay the decay in fluorescence of the fluorescein probe. The ORAC assay takes into account both inhibition time and the degree of inhibition into a single assay by considering the area under the curve (Cao et al., 1995).

In this study, in addition to the commonly used AAPH as an oxidant, Cu^{2+}-H_2O_2 and Cu^{2+} alone were used as a source of oxidants. Cu^{2+}-H_2O_2 is used to mimic the *in vivo* generation of oxidant as H_2O_2 and transition metals are available *in vivo* and are frequently used *in vitro* to induce oxidative damage to proteins and nucleic acids (Cao et al., 1997; Parthasarathy et al., 1989; Sato et al., 1992). In the presence of transition metals like Cu^{2+} alone, it is believed that flavonoids act as a pro-oxidant (Cao et al., 1997). The Cu^{2+} was used in this study to evaluate potential pro-oxidant capacity of the isolated flavonoids. Evaluation of the antioxidant of the isolated compound using ORAC peroxy radical ($ORAC_{ROO\cdot}$) assay presented in Figures 7.14 and 7.15 above shows that all the isolated compounds have a high degree of in $ORAC_{ROO\cdot}$ activity. However, compounds **2** and **3** show significantly higher activities compared with the compounds **1** and **4**. The $ORAC_{OH\cdot}$ activity of these compounds also followed a similar pattern to what is obtained in $ORAC_{ROO\cdot}$ activities (Figure 7.15).

The $ORAC_{OH\cdot}$ activities of the flavonoids were several fold higher than what is obtained for ORAC $_{ROO\cdot}$. This probably shows that the flavonoids are more specific to $OH\cdot$ radical protection. Previous structural-activity relationships studies concluded that phenolic hydroxyls in flavonoids, hydroxyl groups in A and B rings, ortho-dihydroxyl groups in A and B rings are

important to the hydroxyl radical scavenging activity of flavonoids (Chen et al., 2002). Inversely, however, the pro-oxidant activities using Cu^{2+} in ORAC assay show that compounds **1** and **4** were more prone to induce prooxidant in the presence of the metal than the compounds **2** and **3** (Figure 7.16). This was not unexpected because the previous reports have correlated the number of flavonoids' OH group substitutions to their ORAC activity (Rice-Evans et al., 1996). The observed results can be related to the basic structure of the flavonoids as compounds **2** and **3** have quercetin as their basic structures while **1** and **4** have kaempferol. Studies have demonstrated that $ORAC_{ROO\cdot}$ activity of quercetin is higher than that of kaempferol (Cao et al., 1997). The pro-oxidant activity can also be related to the number of substituted OH in the flavonoids—it was shown in this study that the lower the OH, the higher the pro-oxidation in the presence of Cu^{2+}.

FRAP measures reducing capacity based upon reduction of ferric ion in an acidic medium at 593 nm. The present investigation shows that compounds **1** and **4** have significantly lower FRAP activity as compared with compounds **2** and **3**, and the methanolic extract with high activity (1527.63, 15661.37 and 850.06 µmol TE/g, respectively) (Figure 7.17). The TEAC assay involves the evaluation of quenching potential of antioxidant in the presence of long-lived radical cation chromophore 2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulphonate) $ABTS^{\cdot+}$. The results of the TEAC assay follows the same manner as observed in the FRAP assay above. Compound **3** shows (1649.4 µmol TE/g) > compound **2** (1589.9 µmol TE/g) > compound **1** (394.8 µmol TE/g) > compound **4** (337.5 µmol TE/g) (Figure 7.18). The importance of the OH-group, methoxy group and O-dihydroxy substitution in the B-ring have been related to the high activity of the flavonoid using a FRAP assay (Firuzi et al., 2005).

Furthermore, inhibition of iron-induced microsomal lipid peroxidation by the compounds was evaluated. Iron-induced lipid peroxidation is a reliable biological marker of cellular oxidative stress (Dargel, 1992). The results, as presented Table7.2 show that compounds **1** and **4** do not have significant inhibition of lipid peroxidation as the inhibition did not reach 50% inhibition in the concentration used while compound **2** , **3** and the total extract gave IC_{50} values of 15.5, 16.8 and 17.1 µg/ml, respectively. The lipid peroxidation inhibitory activities of flavonoids are shown to be related to the number of hydroxyl group, substitution of the hydroxyl group, cathecol moiety on the B-ring and double bonds between carbon 2 and 3 of the C-ring (Cholbi et al., 1991; Mora et al., 1990). The presence of a sugar moiety, however, affects the activity of the lipid peroxidation inhibition due to steric hindrance between the sugar and adjacent hydroxyl group and likewise the methoxy group (Cholbi et al., 1991).

This can be related to the result obtained in the present study when the inhibition of lipid peroxidation of the isolated glycosylated flavonoids was compared with the quercetin standard. The results showed that IC_{50} inhibition of peroxidation by the standard is about 3-fold lower than the isolated flavonoids. On the other hand, glycosylation of flavonoids increases the hydrophilicity and hence enhances bioavailability better than aglycone flavonoids (Kumar and Pandey, 2013). Despite the lower activity of glycosylated flavonoids compares to their aglycones, bioavailability is a determining factor of their bioactivity *in vivo* (Thilakarathna and Rupasinghe, 2013). Enhancement of bioavailability will thus be an important dynamic in order to exert eventual beneficial effect *in vivo* (Thilakarathna and Rupasinghe, 2013). In addition, the results obtained in this study are in agreement with the number of hydroxyl group substitutions on the B-ring as compounds **2** and **3** with hydroxyl groups in B-ring showed effective dose-dependent lipid peroxidation compared with compounds **1** and **4**.

In summary, the antioxidant activity of flavonoids, which involve neutralization of free radicals initiating oxidative-cascade of reaction or termination of the free radical chain reaction due to hydrogen donating property can be related to their structures (Cao et al., 1997; Suganya et al., 2007b). Compounds **2** and **3** have an *ortho*-dihydroxyl in the B-ring of the flavonoid skeleton (catechol) which plays essential roles in the antioxidant activity of flavonoids (Bors et al., 1990), and is responsible for the observed effects in those compounds. The antioxidant activity of flavonoids is known to depend largely on the functional groups attached to the nuclear structure (Heim et al., 2002). It is noteworthy that the entire antioxidant assay carried out in this study revealed no significant difference between compounds **2** and **3** that are active in the entire assay on one hand and between these compounds (compound **1** and **4**) which are selective in their activities on the other. This implies that the only common factor that is solely responsible for the observed differences in the antioxidant activities of the flavonoids in this study is the *ortho*-dihydroxyl in the B-ring.

8.6 Conclusion

The increasing rates of world cancer incidence and morbidity require every effort to stem the tide. Most of the currently used anticancer drugs derive from natural products because of the inherent phytochemical diversity that is beyond human comprehension. In this view, the current study evaluates the potential anticancer bioactive compounds from the leaf of *H. floribunda*. The study led to the isolation of two steroidal alkaloids—holamine and funtumine—from the methanolic leaf extract of the plant. The cytotoxic activities of the compounds were found to be mediated through induction of apoptosis by activation of caspase-3, reactive oxygen species, mitochondrial toxicity, cell cycle arrest, F-actin filament disassembly and topoisomerase-I inhibition. In addition, from the leaves of this plant, four flavonoids were successfully isolated which might be responsible for the protection of the body's systems against ROS-induced

cancer. However, based on the findings of the present study, the two steroidal alkaloids can be good leads as anticancer drugs and they also hold potential as a template for the synthesis of desirable anticancer agents.

8.7 Future Perspective

This study has shown that the MLE of *H. Floribunda* contains compounds with potential for anticancer drug development and discovery. However, further studies are still needed to decipher the mechanisms of action of the compounds and their safety both in *in vitro* and *in vivo* models. In addition, combinations of the two alkaloids can be rationalized to evaluate their synergistic ability against cancer hallmarks. It also reasonable to combine them with other currently used anticancer drugs to study if they will ameliorate any known side effects.

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