Biochemical investigation of anti-cancer activity of *Tulbaghia violacea*

Gbemisola Morounke Saibu

A thesis submitted in fulfilment of the requirements for the degree of Doctor Philosophiae in the Faculty of Sciences, University of the Western Cape

Supervisors: Dr Mervin Meyer

Co-supervisor: Prof David Katerere

November 2012

ABSTRACT

Natural products have been a source of many pharmaceutical drugs and a number of drugs that are currently used in the treatment of cancer are derivatives of compounds originally isolated from natural products. There is evidence that extracts of *Tulbaghia violacea* can be used to treat cancer. The activation of apoptosis in cancer cells is a target for the development of novel anti-cancer drugs since one of the characteristics of cancer cells is resistance to apoptosis due to the deregulation of biochemical pathways leading to apoptosis. In fact, many current anti-cancer drugs exert their effects through the activation of apoptosis. Previous studies showed that extracts of *T.violacea* induce apoptosis in cancer cells and one study reported on the isolation of a compound (methyl-α-D-glucopyranoside), which is responsible for the pro-apoptotic activity of the *T.violacea* extract. Therefore the aim of this study was to investigate the anti-cancer activity of methyl-α-D-glucopyranoside and extracts prepared from *T.violacea*. In this study the pro-apoptotic activity of methyl-α-D-glucopyranoside and extracts prepared from *T.violacea* were investigated on a panel of human cancer cell lines, which included HepG2, MCF7, H157, HT29 and the non-cancerous cell line, KMST6. The induction of apoptosis was evaluated by flow cytometry using several bioassays which measures biochemical events (caspase activation, phosphatidylserine externalisation and reactive oxygen species (ROS) production that is associated with the induction of apoptosis. The results demonstrated that the effects of methyl-α-D-glucopyranoside on cultured cells are transient and that the cells recover from the effects of methyl-α-D-glucopyranoside. This suggested that methyl-α-D-glucopyranoside is not the compound responsible for the pro-apoptotic bioactivity in the *T.violacea* extract. This study also showed that cytotoxic and pro-apoptotic bioactivity of the leaf-extract was significantly higher in comparison to the tuber-extract. The bioactivity of the organic solvent extracts (dichloromethane, hexane, methanol and 50% methanol/water) of

T.violacea leaves was also significantly higher than water extracts of *T.violacea* leaves. A comparison of the different organic extracts prepared from the *T.violacea* leaves showed that the highest activity was observed for the dichloromethane and hexane extracts. In an effort to identify the bioactive compound(s) the dichloromethane extract was subjected to Versaflash® column chromatography. However, due to problems experienced with the solubility of the dichloromethane sub-fractions, these compounds could not be tested for their bioactivity. Palmitone (16-hentriacontanone) was identified as one of the major compounds present in the dichloromethane sub-fractions. This compound was previously shown to have anticonvulsant bioactivity but there is no evidence in the literature that it has anti-cancer or pro-apoptotic activities. Fingerprinting of the methanol extract showed the presence of long chain fatty acid derivatives, flavonoids and allicin derivatives in the methanol extract. Although, this study failed to isolate the pro-apoptotic bioactive compound(s) present in the extracts of *T.violacea*, it confirmed that extracts of this plant induce apoptosis in cultured human cancer cell lines.

Key words: *Tulbaghia violacea*, Apoptosis, Cancer, Cytotoxicity, Bio-assay guided fractionation, Flow cytometry, Natural products, Palmitone, Methyl-α-D-glucopyranoside

DECLARATION

I declare that "Biochemical investigation of anti-cancer activity of *Tulbaghia violacea"* is my own work, that it has not been submitted for any degree or examination in any other university, and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Saibu Gbemisola Morounke November 2012

DEDICATION

To God almighty, the author and finisher of my faith, to the memory of my father- Late Michael Adegboyega Osituga, to the jewel of my inestimable value (My mum) - Mrs Beatrice Adefoluke Osituga and to my wonderful family, Mr Olusola Saibu, Ayomide and Tanitoluwa

The best academies were your love, arms and knees!

Table of contents

LIST OF FIGURES

CHAPTER ONE

CHAPTER TWO

CHAPTER THREE

CHAPTER FOUR

CHAPTER FIVE

CHAPTER SIX

CHAPTER SEVEN

CHAPTER EIGHT

ABBREVIATIONS

ANT	Adenine nucleotide translocator
APAF-1	Apoptotic protease-activating factor-1
ATP	Adenosine triphosphate
Box	Bcl-2-related death promoter
Bax	Bcl-2 associated x protein
Bcl-2	B cell leukaemia-2
BH	Bcl-2 Homology domains
Bid and tBid	(Truncated) BH3 interacting domain
CAD	Caspase-activated deoxyribonuclease
CARD	Caspase recruitment domain
Caspases	Cysteine aspartic acid-specific proteases
CDKs	Cyclin dependent kinase
CDKIs	Cyclin dependent kinase inhibitors
CD-95	Cluster of differentiation
CED-3	Cell-death abnormality-3
CHO	Chinese Hamster Ovary cells
^{13}C	Carbon-13
COSY	Correlated spectroscopy
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
DcR	Decoy receptor
DR	Death receptor
DR-3	Death receptor-3
FACS	Fluorescence 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-1β-converting enzyme
GC-MS	Gas chromatography- Liquid Chromatography
^{1}H	Hydrogen ion
HRS	Hour(s)

IAPs	Inhibitor of apoptosis
ICAD	Inhibitor of caspase-activated DNase.
ICE	Interleukin-1-β-convertin caspase enzyme
IM	Inner membrane
IR	Infrared
LD_{50}	The dose that kills (50%) of cells (LD- lethal dose)
LC-MS	Liquid-Gas mass chromatography
MeOH	Methanol
Mg/ml	Milligram per millilitre
MDG	α-methyl-D-glucopyranoside
MDR	Multidrug Resistance
MPTP	Mitochondrial permeability transition pore
MS	Mass spectroscopy
Myc	Mylocytoma
NaOH	Sodium Chloride
NMR	Nuclear Magnetic Resonance
NFκB	Nuclear factor κB
NK-cells	Natural killer
p53	Phosphoprotein 53 (tumour protein/TP53)
E2F	Family of transcription factors involved in cell

	cycle
PARP	Poly (ADP) ribose polymerase
PT Pore	Mitochondrial permeability transition pore
PBS	Phosphate buffered saline
PCD	Programmed cell death
PKC	Protein kinase c
PS	Phosphotidylserine
Rb	Retinoblastoma
SI	Selectivity index
Smac	Second mitochondria-derived activator of caspase
TRADD	Tumour necrosis factor receptor-associated death domain
TLC	Thin layer Chromatography
TRAIL	TNF-related apoptosis-inducing ligand
VDAC	Voltage -dependent anion channel

ACKNOWLEDGEMENTS

I wish to thank my God, the Almighty, who is the keeper and the sustainer of my life. For He made everything beautiful in His own time and paved a way when none seemed to exist. Glory be unto Him in the highest.

My appreciation goes to Dr. Mervin Meyer, my wonderful supervisor. Thank God for sparing your life to see to the completion of my academic pursuit in South Africa. You have been a great pillar, teacher, and a mentor. Thank you for your patience, dedication, intellectual generosity and constructive criticism in making this research work a reality. I also want to thank my co-supervisor, Prof David Katerere, for his guidance, support and invaluable intellectual input and for pushing me so hard to get the best out of me. God bless you sir. Prof Jasper Rees (grandfather), thank you for your support and encouragement.

Much thanks to Medical Research Council, South Africa, for funding this project and also to Dr. Gilbert Matsabisa, Dr. Dudu Molefe and Dr. Mamello Sekhoacha, Myolis and Asandra of Medical Research Council IKS Unit as well as Pamela, Nonthu and Thandeka who are members of PROMEC Unit for allowing me to make use of resources in your laboratory and for those wonderful moments we shared during my visits to your lab.

Prof Mabusela, and Dr. Mohammed of Chemistry Department, thanks for always finding solutions to my questions and queries on NMR and Mass Spec interpretation and for many invaluable pieces of advice and guidance.

My gratitude also goes to my mentors in Nigeria, Prof Magbagbeola, Dr Odesanmi, Prof Babajide Elemo, Dr Olawale Raimi, Dr Tony Ojekale, Mr Adeola and Dr Bamidele Iwalokun for their words of encouragement and mentoring me all along.

Dr Bello, God bless you for always being there for me and my family especially on health related matters. You are a brother I wish I also had in South Africa.

To you Dr Dare Iyapo thanks for your never-ending love and assistance. God bless you, you are an angel !.

There have been many people in my life that have encouraged me, but none more than my husband and my children. Words cannot express the debt and love I owe my crown, Olusola Saibu and my wonderful boys; Ayomide and Tanitoluwa. You guys proved to me again that, nothing compares to the love from ones' own family. I cannot thank you all enough for the sacrifice you made while I am in this foreign land, thousands of miles away from home. I promise to make it up for you guys!

I want to thank the jewel of my inestimable value, my Mum -Mrs Beatrice Adefoluke Osituga. Thank you my mum. Your prayers have kept me going since dad's demise in 1981. I thank the Almighty for sparing your life to witness the fulfillment of your desires for me to have good education. Thank you mum for your immeasurable supports during my difficult times and for pushing me to be at my best. You are the best mother in the world! May you live to reap the fruit of your labour. My life and accomplishments would be meaningless without you, my siblings: Seyi and Shina Osituga and your families.

To my lab members of Biochemistry Postgraduate lab and Apoptosis/NIC group both past and present, thank you guys for the wonderful moments we have shared together, especially my best friend, Late Stonard Kayanda, the memories will linger forever. It is unfortunate that death snatched you away from us, RIP my dear friend. Not leaving out Mustapha Drah, Marious, Nteve, Nichole, Paul, Cleo, Rose, Sife, Nosipho, Maggie, Charlene, Zukile, Lauren, Dr Andrew Faro, and the members of staff (Academic and non-academic) Biotechnology department University of the Western Cape- Ms Bridget Daniel, Mr Andrew, Ms Frances, Dr. Amanda Skepu, Dr. Rudo Ngara,

Dr. Ashley Pretorius, Dr. Abraham Madihe, Dr. Kudzayi Ngara and Ms Eloise Braff, thank you all for your care, advice and everything you have shared with me during this learning phase of my life. To department of postgraduate Studies under the leadership of Prof Lorna Holtman, thanks for making the post graduate lab available for us and for the services rendered by the coaches and the stasticians to analyze this work. Azeez Fatai, thank you for assisting me with use of graph pad to analyze this work.

I am also indebted to my spiritual fathers and mothers in the Lord- Pastor Lawani, Pastor and Pastor Mrs Oduwole, Pastor and Dcns Fatoba, Past and Dcns Adelusi, Pastr and Mrs Tolu Balogun, Pastor and Dcns Akinyeye, Pastor Buki, Pastor and Dcns Honour, Adewunmi's family and the Ayodele's, the Cameroonians brethren, the children department and Good Women Christian fellowship of Household of God parish RCCG, Cape Town. Thank you all for your prayers and encouragement.

I appreciate LASU, UWC, and HPR colleagues, Curtis Lanre Cole-Showers, Dr Solomon Adekola, Mr Alegbe, Mr Ademola Abidoye, James Ayorinde, the Kappos, James Onyemata (I owe you a lot), Mr Ademola Adesina, Mr Mukadas Akindele (my wonderful brother), Mr Wunmi Olalekan, Williams, Kemi Tovide, Dr Wale Olowu, Dr Chinwe, Dorcas Kubru, Moni Olaiya, Bunmi, Fred, Deji Adebiyi, Ogunseye's, Babajide's, Rufai Raji, Seun Fadipe, Deji Adebiyi, Mr Jelili Badmus, Mr Amosu Albert, Paul Sewa and Pastor Ranti Samuel, thanks for making my journey to South Africa a worthwhile one.

To you Mr Bolaji Akinyemi, you are more than a cousin to me. God bless you for always being there when needed. Dr Omodele Ibraheem and Dr Seyi Aboyade, thanks a lot for your prayers, for assisting with the editing and for always there when needed. The Oladejo's, Popoola's , Akiode's and Fashola's families thank you for taking care of my boys while away. God bless you all.

CHAPTER ONE: INTRODUCTION AND LITERATURE REVIEW

Table of contents/ Outline

1.5 Treatment of Cancer

1.6 Drug discovery from natural products

1.6.1 Ethnobotanical bioprospecting for anti-cancer drugs

1.6.1.1 Vinca alkaloids

1.6.1.2 Epipodophyllotoxins

1.6.1.3 Taxanes

1.6.1.4 Camptothecin

1.6.1.5 Homoharringtonine

1.6.1.6 Elliptinium

1.7 *Tulbaghia violacea*

1.7.1 Taxonomy and geographical distribution of *Tulbaghia violacea* plant

1.7.2 Medicinal uses, reported chemistry and biological activities of *Tulbaghia violacea*

1.8 Research questions

1.0 Cancer Overview

Cancer is a hyper proliferative disorder which is characterised by deregulation of apoptosis, increased cell proliferation, cell invasion, angiogenesis as well as metastasis (Bharat, *et al.*, 2006). Cancers are also characterised by uncontrolled growth of cells (Weinberg, 2007). Cancer is the second leading cause of death in both developed and developing countries (Michael, *et al.*, 2010; Ahmedin, *et al.*, 2011). Through metastasis, cancer cells can leave the place of origin and invade surrounding tissue and organs. It is often the failure of vital organs due to cancer cell invasion that result in the death of the cancer patient. According to the World Health Organisation (WHO), over 11 million people were diagnosed with cancer in 2005, and this figure may increase to 16 million by the year 2020 (World Health Organisation, 2009).

South Africa has one of the highest incidence rates of cancer in Africa (Mqoqi, *et al.*, 2004), where one out of six males and one out of eight females are at a risk of developing cancer (Carl, 2006). Common human cancers affecting female South Africans include cancers of the breast, cervix, uterus, colorectal and oesophagus. However, in males, cancers of the prostate, lung, oesophagus colon/rectum and bladders are the most common (National Cancer Registry, 2004).

It is estimated that a mature adult human consist of about 10^{15} cells (John, 2001). These cells undergo constant division and differentiation. Cells possessing capacity for division and replenishment are known as stem cells (Kuo-Chen, 2002). Nearly 10^{12} cell divisions occur daily in the stem cell compartments. However, the human body maintains a

constant weight over a significant length of time in spite of this massive generation of new cells. A complex network of molecular mechanisms that regulate cell proliferation on one hand and cell death on the other is responsible for the control of cell multiplicity. Any factor that results in the alteration of the balance between these two opposing mechanisms in favour of increased cellular multiplication has the potential to alter the total number of cells in a particular organ or tissue if not promptly and adequately halted. This increased cellular multiplicity is clinically detectable as neoplasia (John, 2001).

Neoplasia is described as being benign when the cell mass or the tumour remains localized, encapsulated, and does not spread to other sites (Aguiar, *et al.*, 2011). This kind of tumour is generally indolent and often amenable by local surgical removal. However, a neoplasia is described as malignant when the mass invades and destroys surrounding structures and is also capable of metastasis (i.e. able to spread to distant sites) (Stedman's, 2003; World Health Organisation, 2009).

Cancer cells can be classified according to the type of tissue from which they originate, as well as their resemblance to the particular normal cell type. Generally, they may be classified as: carcinomas (cancers of epithelial tissues), sarcomas (cancers of smooth muscles), lymphomas (cancer of lymphoid tissues), leukaemia (cancer of blood tissues), germ cells tumours and blast tumours (cancers of embryonic tissues or stem cells) (Pascal & Jurg, 2000).

1.1 Carcinogenesis

Development of cancer involves multistage processes, which includes initiation, promotion and progression. During the initiation stage, there is an initiator agent that causes physical or chemical changes to the cell leading to DNA damage (Kang, *et al.*, 2005). Cells may respond to the damage and repair such damage by shutting down cell division and activating cell repair mechanisms. Tumour suppressor genes such as *p53* play a role in sensing DNA damage, blocking the expression of genes involved in the regulation of cell growth and activating cell repair mechanisms. If the damage to the cell is too severe the cell will commit suicide through the activation of apoptosis. However, if these two processes (sensing damage and activating cell death) do not happen, the damaged cell will survive and replicate. The result is that the daughter cells, which are referred to as initiated cells, will carry the same damaged DNA (John, 2001). The result is the uncontrolled growth of these cells. Several mutations in tumour suppressor genes such as *p53* have been identified. These mutations result in the loss of *p53* function. The etiopathogenesis of up to half of all cancers has been linked to the loss of *p53* function (Bridonneau, *et al.*, 1998).

The promotion stage of cancer involves accumulation of pro-neoplastic cells and the process is reversible at various points. At this stage, the initiated cells go through selective clonal expansions due to the acquisition of a proliferative advantage, and/or the ability to evade growth inhibitory or apoptotic signals (DiGiovanni, 1992). This clonal expansion of initiated cells may enhance the probability of additional genetic mutations that could result in the development of malignant lesions (Xi, 2003).

The progression stage involves the growth of mutated cells to a malignant cell population with increased metastatic and invasive potential (Kang, *et al.*, 2005; Lee & Surh, 2005). This phase occurs as a result of cellular adaptations to harsh micro environmental conditions including acidosis and hypoxia (Robert & Robert, 2004). This condition has a potent proliferative advantage due to the acidic environment it creates via an unregulated glycolysis (Vincent & Gatenby, 2008). The rate of proliferation of tumour cells varies widely and depends on the cell type. For example, normal intestine mucosa and lymphoma tumour cells proliferate faster than solid tumours while acute leukaemia proliferates slower than the corresponding precursors in normal bone marrows (Kang, *et al.*, 2005; Lee & Surh, 2005). In summary, tumour cells are characterised by uncontrolled proliferation of cells, resistance to anti-growth signals, self-sufficiency in the absence of growth signals, sustained angiogenesis, tissue invasion, metastasis and resistance to apoptosis (Jorgen, *et al.*, 2011).

1.2 Apoptosis Overview

Apoptosis is a process of cell death that is induced by a tightly regulated suicide programme in which cells destined to die activate enzymes capable of degrading the cells nuclear DNA, and cytoplasmic proteins (Bahr & Groner, 2004). Portions of the apoptotic cells then break off, resulting in the appearance of apoptotic bodies. The dead cells or cell fragments are engulfed and eliminated by phagocytes. This death signalling mechanism does not evoke inflammation reaction which is often associated with another form of cell death called necrosis (Gopinath, *et al.*, 2006). Necrosis is a type of cell death with no features of apoptosis and the death is usually considered to be uncontrolled. It is also referred to as the premature death of cells in living tissue (Pierre & Guido, 2006). The morphological

characteristics of apoptosis include cell shrinkage and membrane blebbing, disappearance of microvilli on plasma membrane, the fragmentation of chromosomal DNA into nucleosome size units of about 200bp and the formation of apoptotic bodies (figure 1.1). All of these changes occur before the loss of plasma membrane integrity. These morphological characteristics are distinct from that of necrosis (Lawrence & Jonathan, 2001). Necrosis is characterized with unwarranted cell loss in human pathologies and it always results in inflammation (Pierre & Guido, 2006). Cell death through the induction of apoptosis is characterized by controlled autodigestion of the cell, but this characteristic is different from necrosis through distinct morphological and biochemical features. This includes chromatin condensation, membrane surface blebbing, oligonucleosomal DNA fragmentation as well as the breakdown of the cell into a series of smaller units also known as apoptotic bodies, they are phagocytosed through by adjacent cells in most tissues (Thompson, 1995). However, apoptosis processes are associated with activation caspases and loss of membrane phospholipid asymmetry leading to externalization of phosphatidylserine (Fabisiak, *et al.*, 1998).

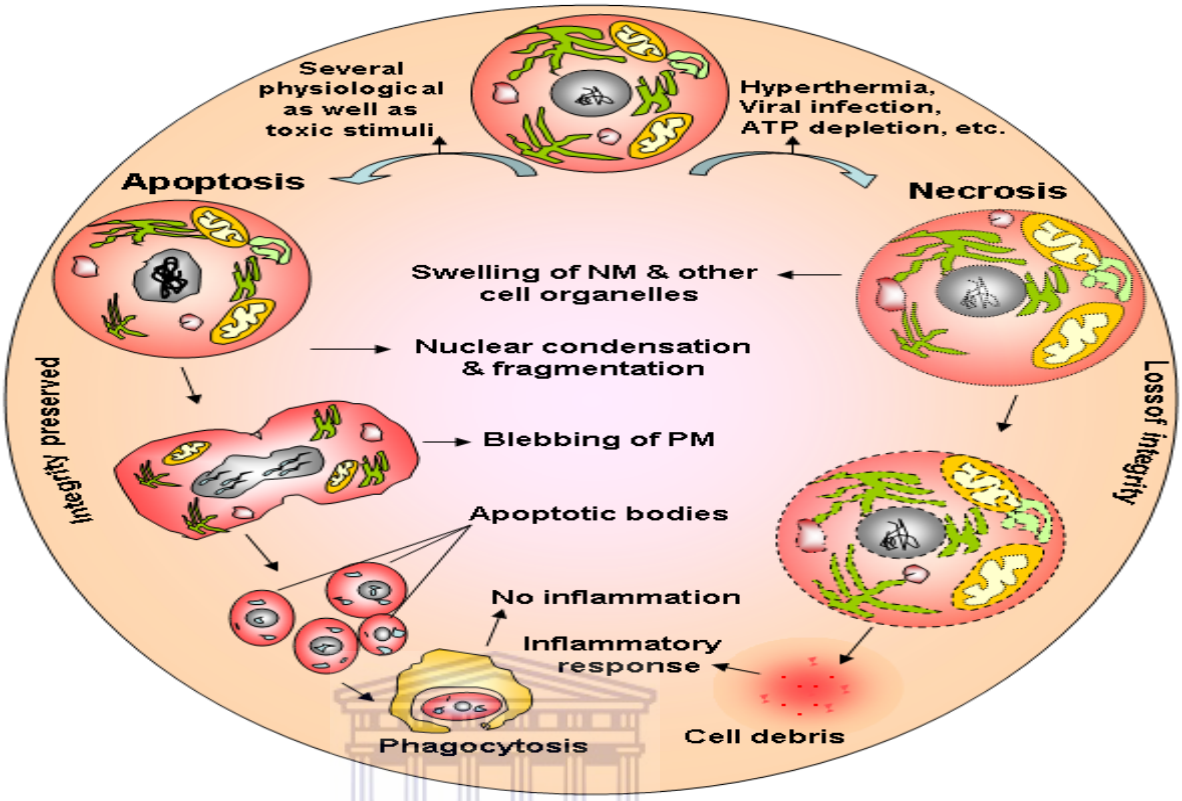


Figure 1.1 Hallmarks of the apoptotic and necrotic cell death processes.
PM=Plasma membrane, NM= Nuclear membrane. Adapted from (Rajesh & Rajeshwar, 2009).

Apoptosis forms a part of biological processes of multi-cellular organisms; and is present in diverse organisms from worms to human (Gregory, *et al.*, 1999). It is a mechanism that enables multi-cellular organisms to tightly regulate or control cell growth in order to prevent pathological processes such as immune deficiency, auto immunity, and cancer (George, 1999). The major role of apoptosis is to remove cells that have incurred irreparable DNA damage or replication defects that might enhance proneness to or development of pathological conditions such as autoimmune diseases and cancer (Aguiar, *et al.*, 2011). In other words, apoptosis is involved in the maintenance of cell homeostasis and prevention of tumour development. It counter-balances cell division and cell differentiation

and ensures proper functioning of different tissues such as the immune system as well as reproductive organs. The balance between cell proliferation and programmed cell death is maintained by controlling the number of differentiated cells (Bridonneau, *et al.*, 1998). Apoptosis is thought to be confined to multi-cellular organisms, however, some bacteria are also able to control bacterial colony numbers through similar mechanisms (Lewis, 2000).

Apoptosis is triggered by death signal which could either be a physiological messenger molecule such as hormone or cytokine, a stress stimulus such as deprivation of growth factors or a harmful condition such as radiation, pharmacological and chemical substances (Hengartner, 1996). Therefore, apoptosis is a protective response in cells exposed to stress stimuli and damaging conditions, thus preventing the appearance and proliferation of potentially dangerous cells (Kensler, 1997). The induction of apoptosis forms the basis of therapeutic effects of most of the antitumour drugs, including the drugs that cause DNA damage or inhibit DNA replication such as anthracyclines.

Defects in apoptosis may also lead to drug resistance (Scott & Athena, 2000). Moreover, excessive apoptosis could lead to diseases such as Alzheimer's, Parkinson's, amyotrophic lateral sclerosis and Huntington's disease, Acquired Immune Deficiency Syndrome (AIDS) and ischemia (Thompson, 1995).

Flemming in 1885 proposed that cell deaths are involved in rabbit graafian follicles. He later developed the theory of apoptosis involvement within the cell in the same year. He observed a similar mechanism in the degeneration of testicular germ cell populations, and

coined the term “chromatolysis” to describe the phenomena. Flemming documented the morphological features of apoptosis, but the concept was not fully understood by many scientists including him (Susan, 2007). Councilman, a pathologist studying liver diseases in 1890 also observed this form of cell death, which he referred as “hyaline” or acidophilic bodies’. Councilman described hyaline bodies in the liver of dying patients of yellow fever and subsequently, after studying the cells under electron microscope concluded and named the single dying hepatocytes “Councilman bodies”. Sixty years after, an Australian pathologist, John Kerr during his PhD study proposed the term “apoptosis” to describe a relatively conserved set of morphological features observed in various cell types during physiological episodes of cell death (Kerr, *et al.*, 1972). Apoptosis was known to have resulted from coagulative necrosis of single cells and it was also observed that the remains of these cells called apoptotic bodies were phagocytosed by macrophages. The word “apoptosis” is a Greek word meaning “falling off” or “dropping off” and is used in reference to the formation of apoptotic bodies. Thus, apoptosis can be referred to as a manifestation of a process or programme of events leading to cell death which is recognized by distinct morphological and biochemical features (George, 1999).

1.2.1 Molecular mechanisms of apoptosis

Most of the discoveries on apoptotic death machinery in mammalian cells were obtained from work done on the nematode worm, *Caenorhabditis elegans* as a model to study apoptosis (figure 1.2). At mitochondrial level, there are two different survival mechanisms in mammalian system (i) One resembles the CED-9/CED-4/CED-3 pathway this was described in *Caenorhabiditis elegans*, and it proposed that Bcl-xL binds APAF-1 leading to

prevention of activation of caspase and subsequent apoptosis (figure 1.2) (Green & Reed, 1998), (ii) The second one implicates anti-apoptotic members which includes Bcl-2 and Bcl-xL and these are used for maintenance of the integrity of the mitochondrial, regulation of membrane permeability to small pro-apoptotic molecules such as cytochrome c, (Kluck, *et al.*, 1997; Yang, *et al.*, 1997) and finally apoptosis inducing factor (AIF) (Susin, *et al.*, 1996) (figure (1.2). However, it has not been known how mitochondrial integrity were being controlled by Bcl-2 family but because of the structure of Bcl- xL which are similar to pore-forming bacterial toxins and since Bcl-2, Bcl-xL and Bax do form ion channels in lipid bilayers, therefore it is assumed that by regulation of the small conductance ion channels through these molecules could assist in preservation of mitochondrial physiology (Adams & Cory, 1998; Green & Reed, 1998)

The major impact on the study of apoptosis has been on the aspect of identification of different genes that encode the proteins that are responsible for the various stages of apoptosis, such as initiation, processing and execution of cell death. *C. elegans* is a free living non-parasitic, non-hazardous, non-infectious and non-pathogenic multi-cellular organism that is widely used as a model to address fundamental questions in developmental biology, neurobiology and behavioural biology (Donald, 1997). In the *C. elegans*, 131 out of the 1090 somatic cells that are usually produced during development undergo apoptosis. The analogues of *Ced* genes which control apoptosis in *C. elegans* was found in humans and these genes have been observed in directly activating the actions of anti-apoptotic factors which were found to be capable of suppressing the actions of these proteins (such as Bad and Egl-1) (Imawati, *et al.*, 1999). This programmed cell death depends on the actions of three key genes which includes *ced* -3, *ced* -4 and *ced* -9 (CED, cell-death abnormal),

(Hengartner, 1996). The cloning of these three genes has revealed the existence of homologues (Bcl2, Apaf-1 and caspase) in other species including humans (figure 1.2). The cell-death genes *ced-3* (a caspase), *ced-4* (an Apaf-1 homolog), and *ced-9* (a Bcl-2 homolog) are all capable of controlling cells autonomous in order to control apoptosis (Chinnaiyan, *et al.*, 1997). Worms that are deficient in these genes are either *ced* -3 or *ced*-4 or both contain at birth 131 superfluous cells, which shows that these gene are involved in killing processes (Ellis & Horvitz, 1986; Shaham & Horvitz, 1996).

However, in contrast, *ced* -9 is a negative regulator of PCD. *ced*-4 binds to *ced* -3 and thus leads to *ced* -3 activation, that is *ced*-3 and *ced* -4 function to kill cells and *ced-9* gene helps in preventing the death-promoting action of these genes and it is however homologous to the Bcl-2 family (Chinnaiyan, *et al.*, 1997).

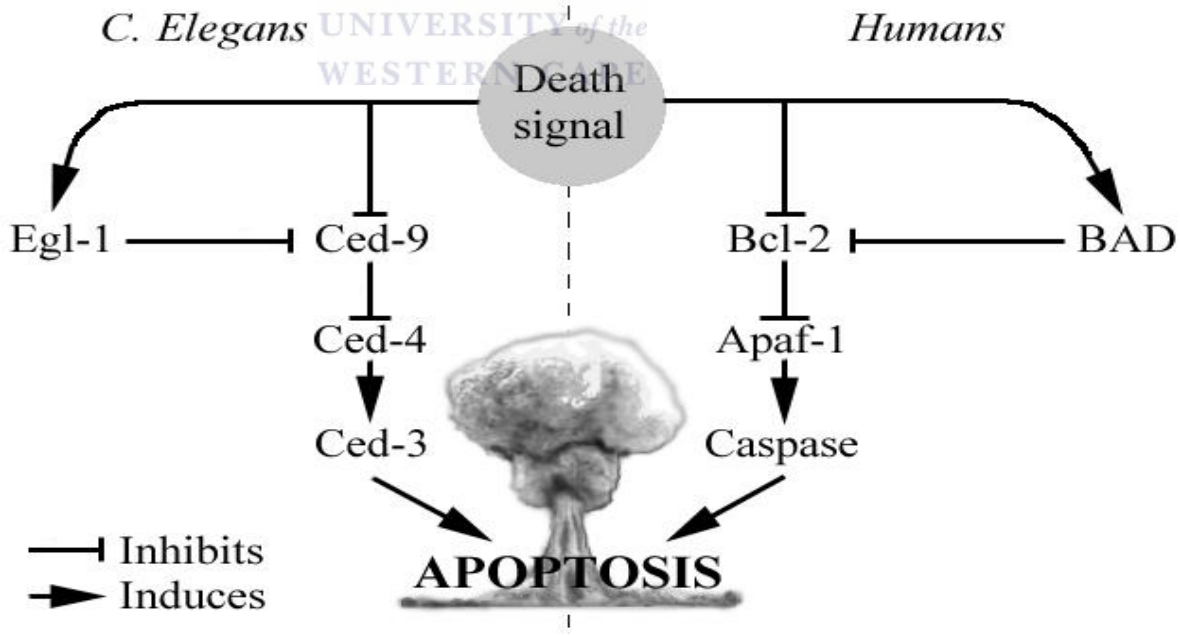


Figure 1.2: Highly conserved programmed cell death pathways. Adapted from (Lewis, 2000).

ced -4 plays a vital role in the cell death pathway through linking of *ced*-9 and the Bcl-2 family biochemically to *ced* -3 as well as the ICE family of pro-apoptotic cysteine proteases. The protective nature of *ced-9* and also the killer nature of *ced-3* and *ced-4* are usually present in normal cells (Seshagiri & Miller, 1997). Death by overexpression of *ced-3* does not require endogenous *ced-4* function, while killing by overexpression of *ced-4* is dependent on endogenous *ced-3* function. Therefore, *ced-4* acts upstream of *ced-3* and *ced-4* function can be bypassed by high levels of *ced-3* activity or it may be that *ced-3* and *ced-4* act in parallel, with *ced-3* leading to a greater chance to kill (Spector, *et al.*, 1997; Wu, *et al.*, 1997; Imawati, *et al.*, 1999). Inhibition of Ced-9 or Bcl-2 leads to activation of the next stage in the suicide programme while the subsequent activation of Ced-4 or Apaf-1 factors initiate the final executors of apoptosis figure 1.2) (Amarante-Mendes & Green, 1999). The analogous gene that regulates apoptosis in *C. elegans* has been observed in directly activating the actions of anti-apoptotic factors which were found to be capable of suppressing the actions of these proteins (such as Bad and Egl-1) (Imawati, *et al.*, 1999).

Apoptosis in mammalian cells is mediated through two major pathways, namely, the intrinsic pathway and extrinsic pathway. The intrinsic pathway is associated mitochondrial dysfunction while the extrinsic pathway is associated with activation of the death receptors (DRs) on the cell membrane. These two distinct pathways ultimately result in activation of a family of cysteine aspartyl specific- proteases, (caspases) (Thompson, 1995).

The intrinsic pathway involves utilization of mitochondria to initiate cell death through the opening of mitochondrial permeability transition pore (MPTP) or rupture of the outer mitochondrial membrane. The intrinsic apoptotic mechanism can also be initiated by

cellular stress, specifically mitochondrial stress caused by several factors such as DNA damage, free radicals, and deprivation of growth factors or trophic hormones (Weinberg, 2007). This leads to the activation of the pro-apoptotic members of the Bcl-2 family known as Bad and Bax, which dimerise and insert into the mitochondrial outer membrane, and form channels through which cytochrome *c* and other mitochondrial proteins escape into the cytosol (Gopinath, *et al.*, 2006; Johnstone, *et al.*, 2008; Papenfuss, *et al.*, 2008). In the cytoplasm, cytochrome *c* combines with Apaf-1 to form a large oligomeric complex known as the apoptosome, that activates procaspase-9 (Wilson, *et al.*, 2009). The active caspase-9 known as the initiator caspase, activates the executioner caspases, caspases- 3, 6 and 7 which execute the full implementation of the apoptotic programme (Scaffidi, *et al.*, 1999).

The extrinsic pathway is initiated by the interaction of certain members of the tumour necrosis factor (TNF) receptor family, often referred to as 'death receptors' with their cognate death ligands such as TNF-α, CD95L, or TRAIL (Fischer & Schulze-Osthoff, 2005). Once activated by ligand binding, the death domains (DD) of these receptors bind to and activate a protein called Fas-associated death domain protein (FADD) in the cytoplasm, to form the death inducing signalling complex (DISC) which activates the initiator caspase-8 and less commonly, caspase-10. The activated initiator caspases activate the executioner caspases (caspases-3, -6 and -7) (Weinberg, 2007). Activated caspase-3 trigger events that will result in the death of the cell. Activated caspase-8 can also activate the intrinsic pathway. Active caspase-8 can cleave and activate a protein in the cytosol known as Bid (figure 1.3). Bid is a pro-apoptotic member of the Bcl-2 protein family. The truncated Bid

(tBid) translocates to the mitochondrial outer membrane and opens the mitochondrial channels resulting in release of mitochondrial apoptotic factors into the cytosol.

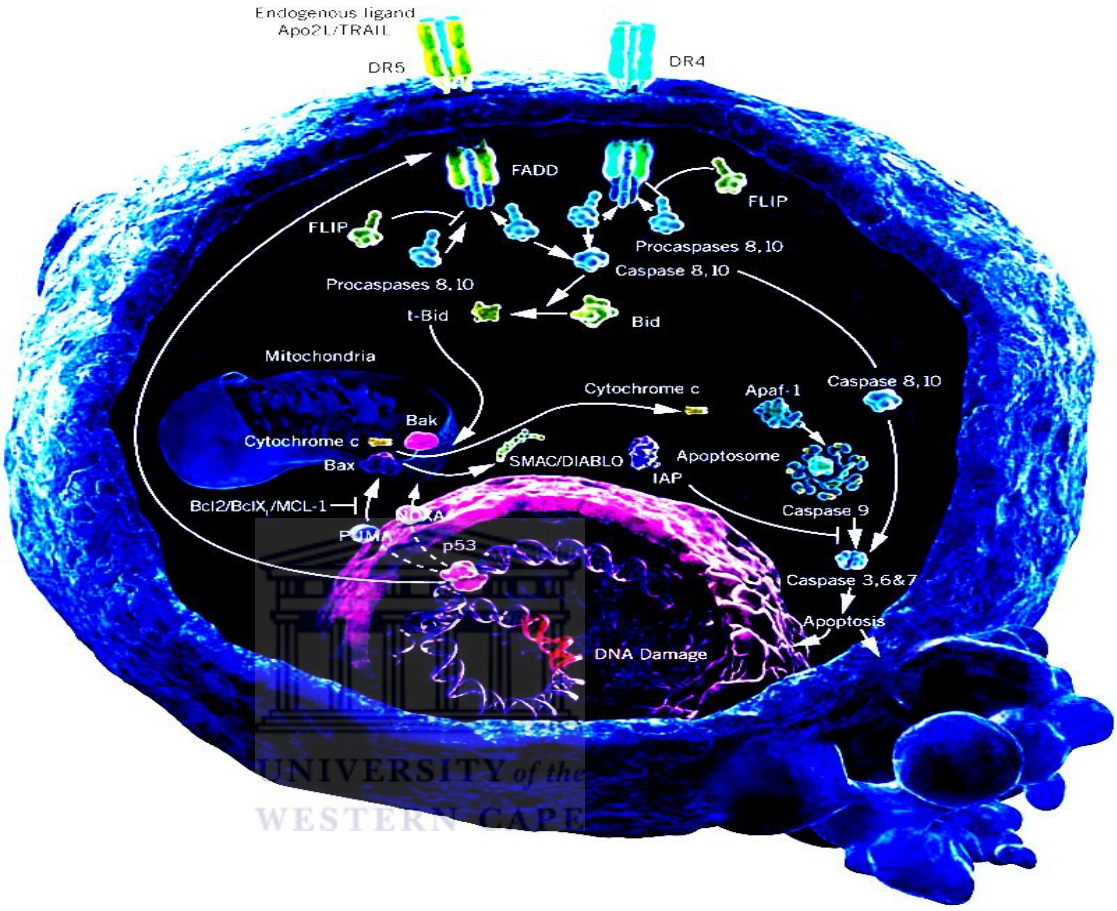


Figure 1.3: Schematic diagram showing intrinsic and extrinsic mechanisms of apoptosis. Bid is cleaved by the initiator caspase-8 to form truncated Bid (tBid) which translocates into the mitochondrial outer membrane and opens the mitochondrial channels to release pro-apoptotic factors including cytochrome *c*. Taken from (Kumar, *et al.*, 2007).

Other pro-apoptotic proteins of the Bcl-2 protein family, such as Bax and Bak can also translocate to the mitochondrial outer membrane resulting in the formation of pores or mitochondrial apoptosis-induced channels (MAC). This leads to release of pro-apoptotic factors like cytochrome *c* which are released into the cytosol through these pores. Cytochrome *c* combines with ATP, Apaf-1 and pro-caspase 9 to form the apoptosome (figure 1.3). Anti-apoptotic proteins of the Bcl-2 family such as Bcl-2, Bcl-X_L and MCL-1

can inhibit the formation of MAC (Gopinath, *et al.*, 2006; Kumar, *et al.*, 2007). The apoptosome activates pro-caspase-9 (Wilson, *et al.*, 2009) and the active caspase-9 activates the executioner caspases such as caspase-3 which execute the full implementation of the apoptotic programme (Scaffidi, *et al.*, 1999).

1.2.2 Regulation of apoptosis

Cells require extracellular growth factors and mitogens to continue to grow and divide and also survival factors to escape cell death. Insufficient levels of survival factors could result in the activation of apoptosis (Boets & Goethals, 2011). Inappropriate activation or inhibition of apoptosis may lead to diseases such as cancer and other types of disorders (Barr & Tomei, 1994). The universality and incidence of apoptosis in the multi-cellular organisms stress the importance of its regulation. Several stimuli are capable of inducing apoptosis. Some could be universal and can generate or produce apoptosis in nearly all cells, but most apoptosis-inducing factors have shown selectivity of their targets (Avci-Adali, *et al.*, 2010). If the pro-apoptotic signals predominate, a dedicated death program will then be activated and the cell will undergo apoptosis. These signals could either positively or negatively (i.e. trigger or repress) affect apoptosis. The induction involves binding and subsequent initiation of apoptosis by a signal molecule while the active repression or inhibition of apoptosis by a transducer molecule is referred to as the negative induction.

In addition to physiological control mechanisms of apoptosis, different variety of pathological insults can trigger apoptosis by various stimuli either from outside or within the cell such as the ligation of cell surface receptors, DNA damage due to defects in DNA repair

mechanisms, treatment with cytotoxic drugs or irradiation, lack of survival signals, contradictory cell cycle signalling or developmental death signals (Christopher, 1996).

Most cells initiate intracellular apoptotic signalling in response to a stress such as hypoxia, nutrient deprivation, radiation, infections, free radicals, heat and so on (Cotran, *et al.*, 1998). Death signals of such diverse origins ultimately activate common cell death mechanism which could later lead to the characteristics features of apoptosis (Morita & Tilly, 1999). Depending on the damage and the cell type, p53 could either cause cell cycle arrest or activate the apoptotic self-destruction sequence (Balint & Vousden, 2001). Figure 1.4 shows the four stages leading to apoptosis as proposed by Morita and Tilly in 1999. The first stage involves various harmful stimuli that interact with a cell. The second stage, involves early activation of a signalling molecule. This signal is produced by a regulatory mechanism that assesses the strength of the apoptosis inducing signal over anti-apoptotic signals in the third stage. If the death inducers prevail, the cell commits to apoptosis and enters the fourth and final stage that involve specific executionerr proteins which responsible for the organized destruction of the cell (Morita & Tilly, 1999).

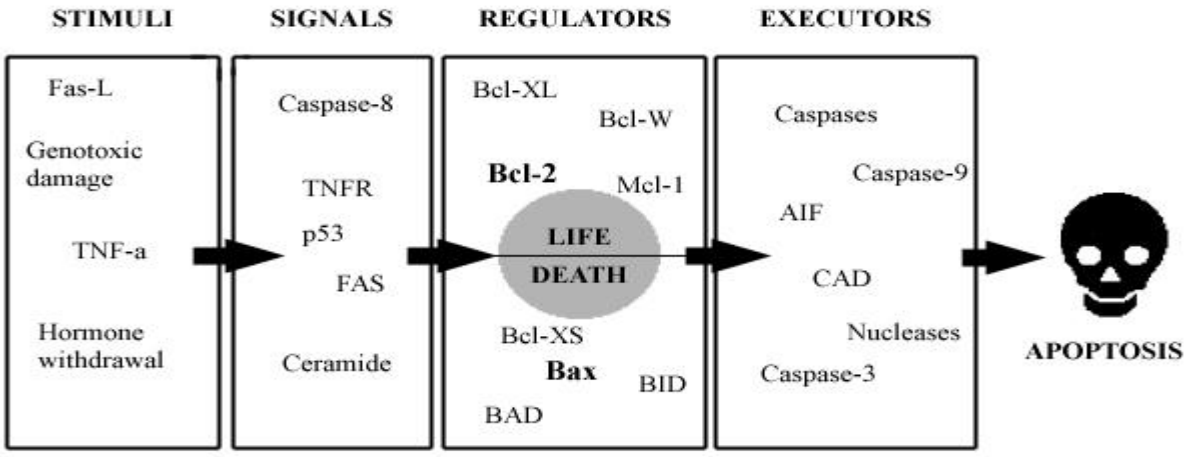


Figure 1.4 : Four stages leading to apoptosis.
Adapted from (Morita & Tilly, 1999)

Increased activities of caspases-8 and 9 have been noticed in peripheral blood mononuclear cells of patients with neurodegenerative disorders such as Alzheimer's disease (Tacconi, *et al.*, 2004) and Parkinson's disease (Viswanath, *et al.*, 2001). Apoptosis-regulating genes are found in every metazoan organism and are conserved throughout evolution (Liu & Hengartner, 1999).

Mitochondria are important in eukaryotic organisms without which the cells will cease to respirate aerobically and eventually die. This has been exploited by some apoptotic pathways and some of the associated proteins target mitochondria affecting them in several ways. This may lead to swelling of the mitochondria forming membrane pores, or increasing the permeability of the mitochondrial membrane which could later lead to the leakage of apoptosis signalling molecules (Cotran, *et al.*, 1998). For example, nitric oxide induces apoptosis by aiding the dissipation of mitochondria membrane potential thus making it more permeable (Brüne, 2003). Second mitochondria-derived activator of caspases, (SMACs) proteins when released into the cytosol cause an increase in the mitochondrial permeability

(Fesik & Shi, 2001). SMAC binds to IAPs to prevent its inhibition of apoptosis. In most cases, IAP represses the activity of caspases that degrade the cells (Fesik & Shi, 2001). This suggests that the activities of caspases are indirectly regulated by mitochondrial permeability.

The dissipation of the mitochondrial inner transmembrane potential ($\Delta\psi$) and the permeability transition (PT) also lead to a loss of the biochemical homeostasis of the cell. ATP synthesis is stopped, redox molecules such as NADH, NADPH, and glutathione are oxidized, and reactive oxygen species (ROS) are increasingly generated (Kroemer, *et al.*, 1997). An increase in the level of ROS directly leads to the oxidation of lipids, proteins, and nucleic acids, therefore enhancing the disruption of $\Delta\psi$ (Marchetti, *et al.*, 1997). However, there appears to exist a consensus that a permeability transition pore (PTP) is formed and it consist of the adenine nucleotide translocator (ANT) as well as the voltage-dependent anion channel (VDAC) as its core components (Bruno & Kiel, 2002). ANT has been the most abundant protein of the inner mitochondrial membrane as well as a transmembrane channel which is responsible for the export of ATP in exchange with ADP (antiport). Overexpression of ANT-1 in human cancer cell lines exclusively induces apoptosis by means of its characteristic features through mediation of RIP which affects the interaction between both cyclophilin D and ANT. This allows the binding of zVAD.fmk (zVAD) to ANT, thus preventing ANT from adopting the conformational c-state and later results in inhibition of ADP/ATP exchange, the reduction of cellular ATP as well as and necrotic cell death (Flierl, *et al.*, 2005). They are closely conserved homologue ANT-2, but they do not induce apoptosis, which suggests a specific mechanistic role of ANT-1 in mitochondrial

apoptosis events (Bauer, *et al.*, 1999). VDAC, is the most abundant protein of the outer mitochondrial membrane, it forms a non-selective pore via the outer membrane (Christopher, *et al.*, 2004). Through direct protein-protein interactions, VDAC-ANT complexes likely connect both the inner and the outer mitochondrial membranes together to a site called "contact sites", which is similar to an association of the two membranes and thereby constituting the PT pore (Beutner, *et al.*, 1998). Since PT, the loss of $\Delta\psi$, as well as the release of mitochondrial proteins is of central importance in mediating and enhancing apoptosis pathways, these mitochondrial events must therefore be kept under strict control which in most cases are dependent on members of the Bcl-2 family.

1.3 Anti-cancer therapies targeting the extrinsic and intrinsic apoptosis pathways

Apoptosis is one of the build-in mechanisms used by organisms to prevent the development of cancer. One of the characteristics of cancer cells is resistance to apoptosis. The goal of an effective anti-cancer therapy is to selectively kill cancer cells without harming the normal cells. In some cases, resistance to apoptosis may explain why cancer therapies fail. Many anti-cancer strategies involve the reactivation of apoptosis in cancer cells or sensitising cancer cells to apoptosis. Physiological differences between normal and cancerous tissue is often exploited to develop such anti-cancer treatment strategies (Nguyen & Wells, 2003; Mashima & Tsuruo, 2005). For example, the microenvironment of a solid tumour is characterized by low pH, hypoxia, and growth factor deprivation (Mashima & Tsuruo, 2005).

Most of the key regulators of apoptosis signalling pathways have been identified, and many have emerged as main targets for design of anti-cancer therapeutic strategies. These include the death receptors, Bcl-2 proteins (also referred to as the gatekeepers of the intrinsic apoptosis pathway), caspases, caspase inhibitors, and various transcriptional regulators of proteins paying a role in apoptosis (Fischer & Schulze-Osthoff, 2005). Several novel therapeutic approaches are being developed. For instance, death receptor ligands are being mimicked by recombinant ligands or agonistic antibodies; caspases are being activated by small molecule drugs; pro-survival factors such as inhibitors of apoptosis (IAPs) in cancer cells are being specifically down-regulated by antisense therapy or other strategies (Fulda, *et al.*, 2002; Chen, *et al.*, 2007; Higuchi, *et al.*, 2008; Barcic & Ivancic, 2010).

An in-depth understanding of apoptosis signalling pathways led to the understanding of how several human cancers develop and progress. For example, the relatively slow growing nature of basal cell cancers, in spite of their high mitotic rate, can be explained by their associated high rate of apoptosis (Kerr, *et al.*, 1972). Studies on knockout and transgenic mice demonstrated strong association between over-expression of Bcl-2 protein and B cell lymphomas, leukaemia, melanoma and some myelomas (Strasser, *et al.*, 1993; Leiter, *et al.*, 2000). Expression of a *bcl-2* transgene alone in mice did not result into a fast tumour development, but, when it was combined with a c-*myc* transgene, leukaemia developed extremely rapidly (Strasser, *et al.*, 1990). This synergy between an oncogene and an apoptosis inhibitor suggested that apoptosis is necessary for preventing a survival of cells that have acquired activating mutations in growth-promoting genes. Studies have

demonstrated positive correlation between the expression of nearly all of the anti-apoptotic proteins and various types of cancer. For instance, elevated levels of X-linked Inhibitor of Apoptosis protein (XIAP) have been observed in small cell carcinoma of lung (Hofmann, *et al.*, 2002) and Melanoma inhibitor of apoptosis (ML-IAP) expression was noted in melanoma cells (Kasof & Gomes, 2001).

1.3.1 Death receptor ligands

The discovery of death ligands known as tumour necrosis factor-related apoptosis-inducing ligands (TRAILs) raised optimism for anti-cancer therapy because they target the tumour cells and spare the normal cells. That is, they selectively induce apoptosis of various cancer cells leaving the normal cells unharmed (LeBlanc & Ashkenazi, 2003). Trail receptors, TRAIL-R1/R2 are selectively expressed on tumour cells. TRAIL binds and then activates its receptors and then in turn triggers death receptor-mediated apoptotic tumour death. Five members of the human TNF receptor (TNFR) super-family have been identified that bind TRAIL, these include, the death receptors DR4 (death receptor 4, also known as TRAIL-R1 or TNRFIOA) and DR5 (death receptor 5, also known as TRAIL-R2 or TNFR10B) (Walczak, *et al.*, 1997). Both DR4 and DR5 contain a conserved cytoplasmic death domain, which is capable of binding TRAIL as well as initiating death signals. Two decoy receptors, DcR1 (also known as TRAIL-R3 or TNFR10C) (Degli-Esposti, *et al.*, 1997b) and DcR2 (also known as TRAIL-R4 or TNFR10D) (Degli-Esposti, *et al.*, 1997a), have close homology to the extracellular domains of DR4 and DR5. However, DcR1 does not have a transmembrane domain or a death domain, while DcR2 has a truncated, non-functional death domain.

1.3.2 Nuclear Factor κB (NF- κB)

The death receptor mediated apoptotic signalling pathway is also involved in regulation of NF-κB activation (Wen-Hui, *et al.*, 1999). Several cancers are characterized by chronic activation of NF-κB as an adaptation mechanism to their high micro-environmental oxidative stress (Avendano & Menendez, 2008). This result in prolonged cancer cell survival due to increased expression of c-Jun N-terminal kinase (JNK)-phosphatase, which inhibits the activation of pro-apoptotic kinase, JNK (Kamata, *et al.*, 2005). The enhancement of apoptotic signalling mechanism by inhibiting NF-κB activity might offer a prospect for apoptosis targeted anti-cancer therapy. Inhibitors of NF-κB activation are therefore potential anti-cancer drugs. An example of such drug is CHS-282, a potent inhibitor of I kappa B kinase (IKK), which blocks NF-κB activation. (Avendano & Menendez, 2008).

1.3.3 Bcl-2 Proteins

Proteins of the Bcl-2 family are essential checkpoints of the intrinsic apoptotic signalling pathway (Cory, *et al.*, 2003) and focus has been a target for the development of the strategies targeting the intrinsic apoptosis pathway (Letai, *et al.*, 2004). A mechanism was proposed through which Bcl-2 controls antioxidant pathways at sites of free radical generation (Hockenbery, *et al.*, 1993). Studies in non human primates have shown no toxicity at low and high dosages of TRAIL despite *in vitro* study suggesting sensitivity of normal human astrocytes to the drug (Ashkenazi, 2002).

Most conventional anticancer drugs and radiotherapy induce apoptosis primarily by activating the mitochondrial apoptosis machinery, and tumour refractoriness (that is cancers

that resist treatment over time) to these therapies often develop. This can be explained by overexpression of anti-apoptotic Bcl-2 proteins in the tumour which accounts for their drug resistance. Since TRAIL-mediated death signalling does not utilize these proteins, combination treatment of TRAIL with conventional chemotherapeutics or radiotherapy often results in enhanced cytotoxic effects (Degli-Esposti, *et al.*, 1997a). These synergistic effects are due to the activation of the intrinsic and the extrinsic apoptotic pathways, reduced expression of anti-apoptotic proteins such as Bcl-2 or Bcl-X_L, and an up-regulation of pro-apoptotic molecules such as caspase-8 and FADD. Members of this family of protein are categorized as either pro-apoptotic or anti-apoptotic based on their ability to induce or prevent the release of mitochondrial apoptotic factors such as cytochrome *c* into the cytosol to trigger mitochondria-mediated apoptotic death. The stochiometric balance of pro-and anti-apoptotic proteins appears to be a critical event in regulating cell death (Fischer & Schulze-Osthoff, 2005).

The pro-apoptotic members of Bcl-2 include Bax, Bak, Bok, Bad, Bik, Bid, Bim, Noxa and Puma, while the anti-apoptotic proteins include Bcl-2, Bcl-XL and Mcl-1. Over-expression of Bcl-2 proteins has been demonstrated in majority of hormone-resistant prostatic carcinoma, malignant melanoma, and estrogen-positive breast cancer (Avendano & Menendez, 2008). This implies that over-expression of these proteins enhances resistance to anti-cancer therapy that mainly targets the mitochondrial pathway. Oblimersen sodium is an antisense oligonucleotide compound which acts by reducing the expression of Bcl-2 genes. They are specifically designed to bind to human Bcl-2 mRNA, leading to catalytic degradation of Bcl-2 mRNA and further decrease in Bcl-2 protein translation. However,

there has been baseline and inducible expression of Bcl-2 in small cell and non-small cell lung cancer and this may contribute to cancer resistance therapy (Roy & Stanley, 2004).

1.3.4 Mitochondria permeabilization in intrinsic (MMP) pathway

Mitochondrial membrane permeabilization (MMP) is an essential event in intrinsic apoptotic pathway. Several agents have been shown to induce MMP by a direct action on mitochondria, and might therefore trigger apoptosis in cells in which upstream apoptotic signals have been hampered (Debatin, *et al.*, 2002). These agents are either proteins of the permeability transition pore complex, including the peripheral benzodiazepine receptor (PBR) and the adenine nucleotide translocase (ANT), or small molecules such as CD437, a synthetic retinoid that exert a lethal effect due to their physicochemical properties (Fischer & Schulze-Osthoff, 2005). Agents that directly target the mitochondria would be potential anti-cancer therapy since they seem to act independent of the Bcl-2 protein machinery.

1.3.5 P^{53} proteins

P^{53} does not participate directly in the apoptosis signalling mechanism. However, as a transcription factor, it regulates the expression of a variety of pro-apoptotic members of Bcl-2 family (Vousden & Lu, 2002). Damage to the DNA by chemotherapy, radiotherapy or any other agent results in phosphorylation of P^{53}, resulting in the expression of pro-apoptotic proteins such as Bax, Bak, Puma and Noxa (Yu & Zhang, 2008). These proteins alter the permeability of mitochondrial membrane resulting in release of cytochrome *c* into the

cytosol, and ultimately trigger mitochondria-mediated apoptotic cell death (Ashkenazi, 2002). Results from ongoing clinical trials of INGN201 (Ad-p53), a P^{53} gene therapy drug, that utilizes adenovirus as the delivery vector to cancer cells, showed broad-spectrum anti-tumour activity in many models of human cancers (Avendano & Menendez, 2008). The results also showed that, combining INGN201 with chemotherapy or radiotherapy in these models resulted in enhanced activity with no apparent increase in toxicity.

1.3.6 Caspases

Caspase activation would be a potential strategy for cancer therapy since the activation of intrinsic and extrinsic apoptotic signalling pathways culminate in activation of the effector caspases. Several strategies to develop tumour specific inducible caspase activators have been studied. For instance, tumour-selective apoptosis might be triggered by chimeric proteins, such as immunocasp-3 and -6, comprised of anti-erbB2/HER2 antibody (e23sFv) and the translocation domain of *Pseudomonas* exotoxin-A fused to an active caspase (Xu, *et al.*, 2004; Chow, *et al.*, 2011). Tumour specificity of this strategy is due to the over expression of erbB2/HER2 in several cancers such as breast, ovarian, endometrial, gastric, bladder, prostate, and lung cancers (MacCorkle, *et al.*, 1998; Shariat, *et al.*, 2001).

In vivo studies also showed that iCasp9 (inducible caspase-9) triggers apoptosis in xenografted prostate tumours following chemical induction (Xie, *et al.*, 2001). Increased regression of the prostatic tumour and increased host survival were also observed in these mice models. Gene therapeutic approaches have been employed to transfer caspase-6 (regulated by human telomerase reverse transcriptase promoter) into glioma cells (Komata,

et al., 2001). This strategy was expected to trigger tumour-specific apoptosis, since telomerase expression is reactivated in tumour cells but not in normal cell. In addition, apoptotic potential of several small molecule caspase activators such as MX2060 and MX2167 has been evaluated on a variety of tumour cell lines. The MX2060 series is one of more than 40 compound families identified by Maxim Pharmaceuticals through its proprietary caspase-based high-throughput screening system that targets the identification of compounds that modulate apoptosis (Zhang, *et al.*, 2004a). X2167 is the lead member of Maxim Pharmaceuticals MX2060 series of compounds derived from a natural compound, gambogic acid.

The MX2060 series are apoptosis-inducing anti-tumour compounds which triggers apoptosis by a novel molecular mechanism which is different from the actions of traditional anti-cancer drugs (Fischer & Schulze-Osthoff, 2005). MX2167 was found to induce apoptosis in multiple cancer cell lines including prostate, breast, colorectal, lung cancer, and leukaemia cells (Korkolopoulou, *et al.*, 2004; Zhang, *et al.*, 2004b). These studies suggest that caspase activation might be exploited as potential anti-cancer therapeutic strategy.

1.3.7 Inhibitors of apoptosis proteins (IAPs)

Inhibitors of apoptosis (IAPs) are caspase-inhibiting proteins and constitute an important regulatory system in apoptosis pathways (Salvesen & Duckett, 2002). Over-expression of some IAPs has been noted in a number of tumours. For instance, XIAP is over-expressed in acute myeloid leukaemia and this is associated with poor prognosis (Tamm, *et al.*, 2004). There are strong correlations between IAPs expression and oesophageal cancer (Imoto, *et*

al., 2001) and mucosa-associated lymphoid cancer (caused by *Helicobacter pylori*) (Baens *et al*., 2000). Survivin, one of the most studied IAPs, is over-expressed in cancer cells, which makes it a potential target for cancer therapy (Ambrosini, *et al.*, 1997). XIAP inhibits caspase-3, -7, and -9. Antisense targeting of this protein combined with vinorelbine proved effective in a mouse xenograft model of lung cancer (Barcic & Ivancic, 2010). SMAC and Omi/HtrA2 are proteins within the mitochondria that inhibit IAPs and restore caspase activity. SMAC peptides or SMAC-mimetic drugs such as TWX024 and embelin are currently being developed as potential cancer therapeutic strategy (Wu, *et al.*, 2003; Nikolovska-Coleska, *et al.*, 2004). However, SMAC-like peptides often fail to induce apoptosis as single agents, they help sensitize tumour cell lines to other chemotherapeutic agent (Fulda, *et al.*, 2002).

1.4 Reactive Oxygen Species

ROS can induce several cellular effects, depending on the cell type involved and the concentration of ROS (Haendeler, *et al.*, 2004; Okuyama, *et al.*, 2005; Fujino, *et al.*, 2006). Low levels of ROS could act as signal transduction messengers by modulating gene expression (Haendeler, *et al.*, 2004). High levels of ROS have been shown to drive the cell into a state of oxidative stress (Cheng, *et al.*, 2004), oxidative, lipid and protein damages (Fujino, *et al.*, 2006). However this can be overcome through the use of cells that have oxidative enzymes (such as oxidases and peroxidises) which will protect them from oxidative damage so that intracellular ROS would be maintained at appropriate levels (Fujino, *et al.*, 2006). Failure to regulate the levels of ROS can lead to impairment of cellular functions which can result in the induction of apoptosis (Fujino, *et al.*, 2006).

Thioredoxin-2 (Txn2) plays a vital role in protecting the mitochondria against oxidative stress (Pérez, *et al.*, 2008). Thioredoxin (Trx) and glutathione redox systems are intracellular redox systems that helps in controlling cell proliferation and cancer development (Marks, 2006). The thioredoxin (Trx) system consist of thioredoxin (Trx), thioredoxin reductase (TrxR1) as well as nicotinamide adenine dinucleotide phosphate (NADPH) (Arnér & Holmgren, 2006).

Cell viability depends on the nature as well as type of stress exerted on them. Cells still maintain progressive lipid peroxidation after an apoptotic signal, thus oxidative damage and ROS have been linked to apoptosis induction (Amstad, *et al.*, 1994; Czene, *et al.*, 1997). A mechanism was proposed through which Bcl-2 controls antioxidant pathways at sites of free radical generation (Hockenbery, *et al.*, 1993). BcL-2 acts by protecting against apoptosis through blocking of cytochrome c release, but this process only helps to prevent superoxide production when it is over expressed, they can also be used as antioxidant (Cai & Jones, 1998).

Oxidative stress is also a mediator of apoptosis, it acts by decreasing intracellular glutathione, the main buffer of the cellular redox status or through the increase of cellular reactive oxygen species (Bojes, *et al.*, 1997; Suzuki, *et al.*, 1998). Hydrogen peroxide induces apoptosis through the production of hydroxyl radicals as well as changes in oxidant/antioxidant pathway at low concentration (Toledano, *et al.*, 1997). However, at the same low concentrations, hydrogen peroxide increases cell proliferation even in the absence

of serum, but these stimulatory changes do not involve free radicals as they are enhanced by the presence of mannitol or DMSO in the medium (Burdon, *et al.*, 1989). Hence, superoxides as well as hydrogen peroxide are the regulatory signals.

1.5 Treatment of Cancer

Current regimens of cancer therapy include immunotherapy, chemotherapy, radiation therapy, monoclonal antibody therapy and surgery (Hamdy, 2012). The preference of any of these cancer treatments depends on the location and the stage of the disease, as well as the general state of the patient. Total removal of the cancer tissue without injuring the normal tissue is the goal of the treatment. Sometimes this can be achieved by chemotherapy and radiation, however, the effectiveness of this treatment is often limited by toxicity to normal tissues (Patyah, *et al.*, 2010). In severe cases surgery becomes the only alternative to treat these patients (Erhabor & Adias, 2011).

In Western medicine, many chemotherapeutic, cytotoxic and immunomodulating agents are available for cancer treatments. Chemotherapy is the treatment of cancer with cytotoxic drugs. Most of these drugs target rapidly growing cells (Robert 2006). A higher than normal growth rate is often a characteristic of cancer cells and therefore these drugs can target cancer cells. Cancer chemotherapy has been dominated by potent drugs, which either interrupt the synthesis of DNA or destroy its structure once it has formed (Sivalokanathan, *et al.*, 2005). Unfortunately, their toxicity is not limited to cancer cells, since fast growing normal cells (e.g. hair follicles) are also harmed. Apart from the fact that the drugs are expensive, they are also associated with serious side effects and morbidity. Major obstacle

for successful cancer treatment is the development of drug resistance in cancer cells during chemotherapy. The development of multidrug resistance (MDR) to chemotherapy plays a major role in the failure of cancer therapy. MDR is caused by: (i) the up-regulation of membrane proteins which lower intracellular drug concentration by pumping the drugs out of the cell, (ii) changes in phase I and phase II metabolic pathways from activation/detoxification of drugs, (iii) changes in DNA repair response and (iv) changes in apoptosis response (Kumar & Clark, 1990). Generally most of the known cancer therapies cause side effects such as vomiting, nausea, nephrotoxicity (kidney failure), platelet reduction, granulocytopenia, erythemia, exfoliate dermatitis, facial edema, myelo suppression (reduction of bone marrow function), rash, alopecia (hair loss), hypomelanosis, anaphylaxis, gingival discoloration, injection-site cellulites, oral mucosal lesions, diarrhoea, anorexia, loss of appetite and taste, neurotoxicity and emetogenesis (American Cancer Society, 2006). These side effects may be due to poor drug pharmacokinetics profile, low accumulation in cells, increased production of intracellular thiols and increased DNA repair capacity. Many neoplasms exhibit a resistance phenotype either before treatment (*de novo*) and /or during treatment (acquired) (Kumar & Clark, 1990; Oommen, *et al.*, 2004; Brabek & Kasparkova, 2005; Elwell, *et al.*, 2006).

The designs of new drugs are often challenged by physico-chemical properties such as insufficient solubility or hydrolytic instability, making it difficult to control their delivery, stability and ultimately their specific effects *in vivo*. The unresolved problems and side effects of current cancer therapy demand an urgent attention, this can be achieved through

the development of novel drugs with improved efficacy against tumour cells but with less toxicity to normal cells.

1.6 Drug discovery from natural products

Naturally occurring compounds are the end products of secondary metabolism within living organisms. They are biological molecules that are not necessarily essential or directly implicated in the normal growth, reproduction or survival of the organism (Verpoorte, 2000). They are produced by living organisms and may have pharmacological and biological properties that can be used in drug development. The metabolites in plants (also called phytoconstituents) include alkaloids, glycosides, tannins and flavonoids (Ameenah, 2006). Some of these phytoconstituents are used by plants in defence mechanisms against pathogens, herbivores and environmental stress. They are also produced to promote the survival of the plant in its ecosystem. Examples of this include increasing the plant's reproductive appeal through pigmentation (Wink, 1999).

For ages, human beings have relied on nature for shelter, food, clothing, transport and medicine. Plants of medicinal importance have been known since the time of Hippocrates (5th century BC), and have been used by tribes as infusions, decoctions, concoctions and enemas to feed, retard growth, heal, kill, sedate, hypnotise, empower, relieve pain and evoke psychiatric disorders, (Dewick, 2001). They contribute significantly to rural livelihoods. Apart from the traditional healers practicing herbal medicine, many people are involved in collecting and trading medicinal plants to treat a variety of diseases and abnormalities such as diarrhoea, malaria, high blood pressure, cancer and psychiatric

disorders. World health Organization estimates that 80% of the world's population depends on medicinal plants for their primary health care (Gurib-Fakim & Schmelzer, 2007; Mothana, *et al.*, 2008).

Most of the developing countries depend on traditional medicine for their primary health care needs because they cannot afford the cost of Western drugs. This made the developing countries to encourage the use of herbal medicines in the public traditional health clinics (Pieters & Vlietinck, 2005). There has been longstanding scientific and commercial interest in screening medicinal plants with the aim of discovering new drugs that are more effective (Kinghorn, *et al.*, 2003). Examples of drugs that are of plant origin include artemisinins from *Artemisia annua* (used in the treatment of malaria), atropine from *Atropa belladonna* (used in the treatment of cardiac arrest), digoxin from *Digitalis lanata* (used in the treatment of congestive heart failure) and vincristine from *Catharanthus roseus* or rose periwinkle (used in the treatment of leukaemia).

1.6.1 Ethnobotanical bioprospecting for anti-cancer drugs

Medicinal plants such as *Sutherlandia frutescens*, *Cotyledon orbiculata*, *Tulbaghia violacea*, *Taxus baccata* and *Camptotheca acuminate* have a long history of use in the treatment of cancer. Sixty five percent of the widely used anti-cancer agents are derived from natural sources such as plants and marine organism (Balunas & Kinghorn, 2005). Anti-cancer agents from plants currently in use can be categorized into following classes of compounds viz,; vinca alkaloids, epipodophyllotoxins, taxanes, camptothecins, homoharringtonine and elliptinium (Okouneva, *et al.*, 2003).

1.6.1.1 Vinca alkaloids

Vinblastine and vincristine are alkaloids (Figure 1.5) isolated from *Catharanthus roseus*, formally known as *Vinca rosea*. These drugs are used in combination with other cancer chemotherapeutic drugs for the treatment of different types of cancers such as leukaemia, lymphomas, acute leukaemia, advanced testicular cancer, Kaposi's sarcoma, Hodgkin's disease, neuroblastoma, breast cancer and lung cancer (Cragg & Newman, 2005). The drugs act by interfering with glutamic acid metabolism, and by inhibiting cell division (mitosis) in metaphase. The drugs bind to tubulin which later prevents the cells from making the spindles it requires to separate its chromosomes during cell division (Ngan, *et al.*, 2001). Vinblastine and vincristine have a series of side effects such as, headache, stomach pain, constipation, nausea, numbness, mouth sores, hair loss, lowered blood cell counts, and sensory impairment (Engert, *et al.*, 2007).

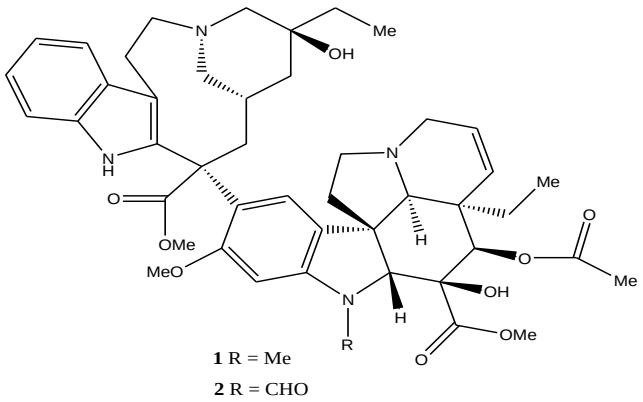
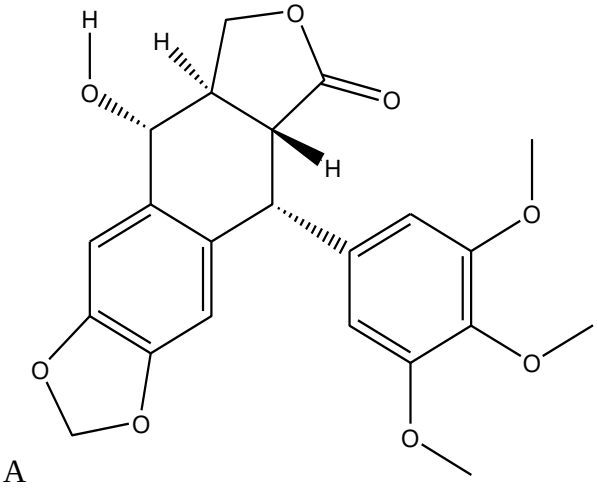


Figure 1.5 Chemical structures of vinblastine and vincristine.

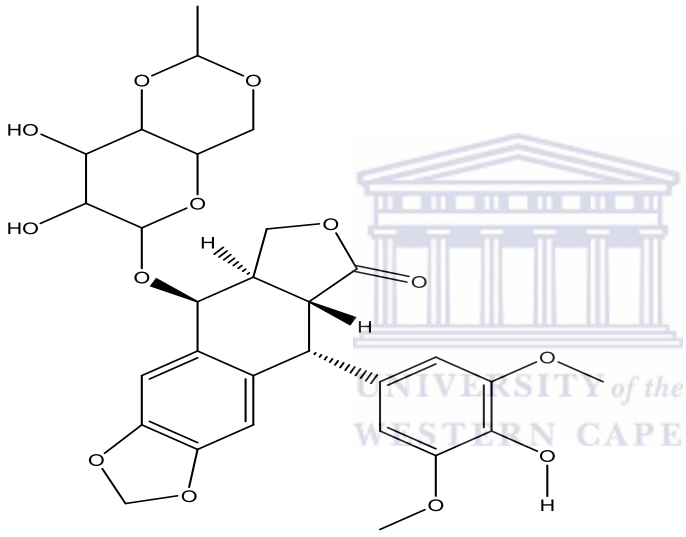
Vinblastine = 1 R (Me), Vincristine = 2 R (CHO).

1.6.1.2 Epipodophyllotoxins

Epipodophyllotoxin is an isomer of podophyllotoxin, it was isolated from the roots of *Podophyllum peltatum* and *Podophyllum emodi.* Podophyllotoxin and its derivatives are anti-mitotic glycosides. They act by blocking the cell cycle at both G1 and S phases by causing damage to DNA through its interaction with DNA topoisomerase II (Clarke, *et al.*, 1993). The two semi-synthetic derivatives of epipodophyllotoxin are etoposide and teniposide (figure 1.6). They are used for treatment of lymphomas and bronchial and testicular cancers, chronic carcinomas, Kaposi sarcoma, malignant melanomas and lymphoma (Harvey, 1999; Cragg & Newman, 2005). Podophyllotoxin is used in creams to treat genital warts which are caused by human papillomavirus (HPV) and this is associated with squamous cell carcinomas (Edwards, *et al.*, 1988). Some of the side effects of these drugs include low leucocytes and platelet count, hair loss, nausea, vomiting and diarrhoea.



A



B

Figure 1.6 Chemical structures of podophyllotoxin (A) and etoposide (B)

1.6.1.3 Taxanes

Taxanes was isolated from *Taxus brevifolia* nutt (Okouneva, *et al.*, 2003; Gordaliza, *et al.*, 2004; Horwitz, 2004). Paclitaxel, an example of taxanes was isolated from the bark of the Pacific Yew, also known as *Taxus brevifolia.* The structure of paclitaxel (figure 1.7) was elucidated in 1971. Different parts of *Taxus brevifolia*, *Taxus canadensis* and *Taxus baccata*

have been used by some native American tribes for treatments other than cancer, while *Taxus baccata* was used in the Indian Ayurvedic medicine for the treatment of cancer (Cragg & Newman, 2005).

Paclitaxel was clinically introduced to the US market in the early 1990s (Rowinsky, *et al.*, 1992; Mohammad, 2006). It has been widely used in the treatment of ovarian cancer, advanced breast cancer, small and non-small cell lung cancer (Rowinsky, *et al.*, 1992). Paclitaxel acts by binding to the microtubules thereby affecting their depolymerisation into tubulin (Schiff & Horwitz, 1980). Paclitaxel exerts its action by preventing the assembly of mitotic spindle which leads to failure of chromosome segregation during cell division (Yoo, *et al.*, 1998). Side effects of taxol include, edema, low platelet count, anaemia and breathing problems.

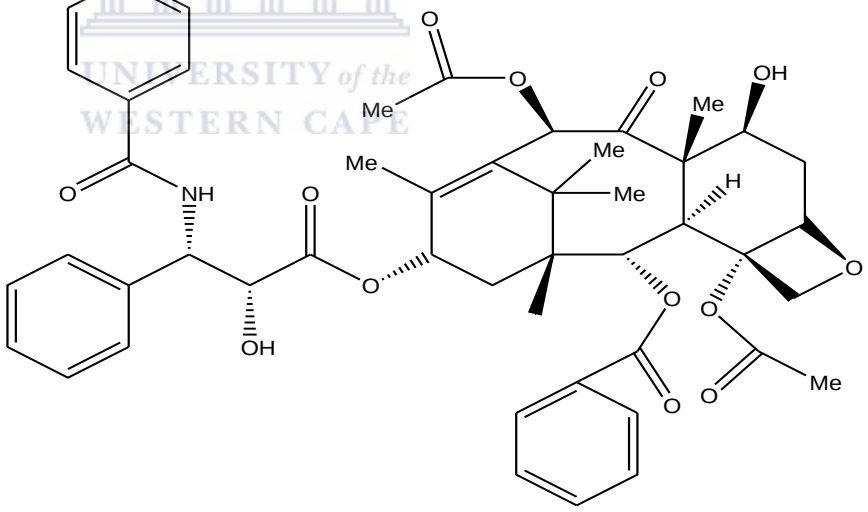
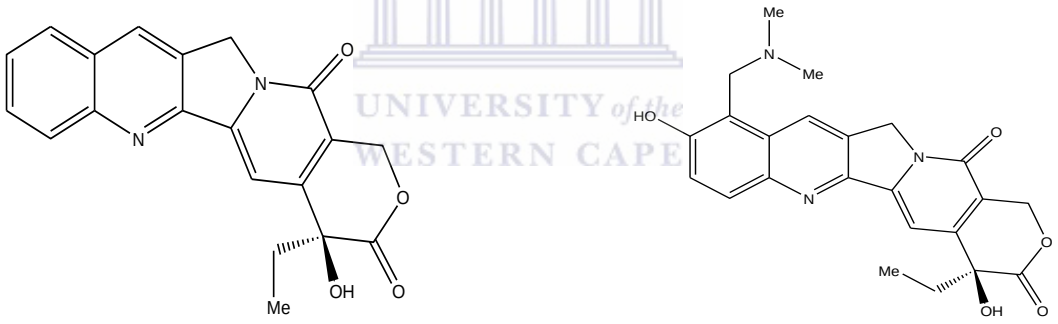


Figure 1. 7 Chemical structure of Paclitaxel (Taxol®)

1.6.1.4 Camptothecin

Camptothecin is a quinoline-based anti-cancer drug (figure1.8A) that was isolated from the Chinese ornamental tree *Camptotheca acuminate* Decne (Nyssaceae) (Okouneva, *et al.*,

2003; Gordaliza, *et al.*, 2004; Horwitz, 2004). Camptothecin induces apoptosis by targeting the nuclear enzyme topoisomerase I. Information on the structure-activity relationships of the parent compound camptothecin has led to the development of a more effective soluble analogues with manageable toxicities (Dancey & Eisenhauer, 1996). The National Cancer Institute (NCI) took the drug into clinical trials in the 1970's but its use was stopped due to severe bladder toxicity (Potmeisel, 1995). Semi-synthetic derivatives of camptothecin, which include topotecan (figure1.8 A and B) and irinotecan, are used to treat ovarian, small cell lung cancers and colorectal cancers, respectively (Creemers, *et al.*, 1996; Bertino, 1997). Anti-tumour activity of irinotecan and topotecan has been confirmed in phase I/II preclinical studies. The side effects of these drugs include nausea, vomiting, lowered blood cell counts and severe diarrhoea.



A B

Figure 1.8 Chemical structures of camptothecin (A) and topotecan (B)

1.6.1.5 Homoharringtonine

Homoharringtonine (figure 1.9) was isolated from the Chinese tree, *Cephalotaxus harringtonia* (Powell, *et al.*, 1970; Itokawa, *et al.*, 2005). Homoharringtonine (HHT) is a cephalotaxus alkaloid plant that was originally isolated in the People's Republic of China. HHT was obtained by alcoholic extraction from the evergreen tree *Cephalotaxus*

harringtonia k. koch var harringtonia. The use of racemic mixture of harringtonine and homoharringtonine was reported for the treatment of acute and chronic myelogenous leukaemia after failure with interferon (Kantarjian, *et al.*, 1996; Cragg & Newman, 2005). They inhibit the synthesis of proteins which later leads to apoptosis. Previous studies have suggested antitumor activity of HHT in many neoplastic diseases such as acute non lymphoblastic leukaemia and there are no record of cross-resistance with conventional antileukemic agents (Warrell Jr, *et al.*, 1985). The activity of HHT against myeloid leukaemias has been through inhibition of protein synthesis, promotion of cell differentiation as well apoptosis induction through a caspase-3-dependent mechanism (Fresno, *et al.*, 1977; Kuliczkowski, 1989; Yinjun, *et al.*, 2004).

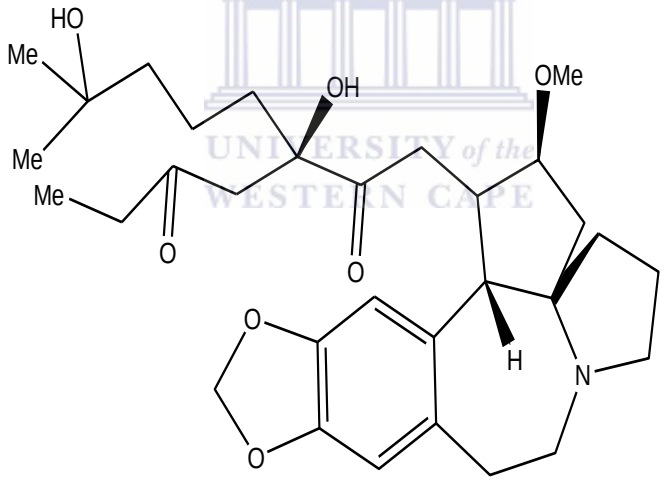


Figure 1.9 Chemical structure of homoharringtonine

1.6.1.6 Elliptinium

Elliptinium is an intercalating agent belonging to the ellipticine family. In France, elliptinium (figure1.10), was isolated from species of the plant family Apocynaceae, including *Bleekeria vitensis*, and it is presently marketed for the treatment of metastatic

breast cancer (Cragg & Newman, 2005). They are topoisomerase II inhibitor and help in stabilizing the cleavable complex of topoisomerase II and induces DNA breakages, leading to inhibition of DNA replication, RNA and protein synthesis (Rouëssé, *et al.*, 1993). Elliptinium can be oxidized to yield a reactive electrophilic form which then covalently binds to a nucleophilic biological molecule. Elliptinium has shown clinical activity in treatment of breast cancer using a weekly regimen. However, some toxicities effects such as xerostomia and immune-mediated haemolytic reactions occurred as a result of development of anti-elliptinium IgM antibodies (Rouëssé, *et al.*, 1993).

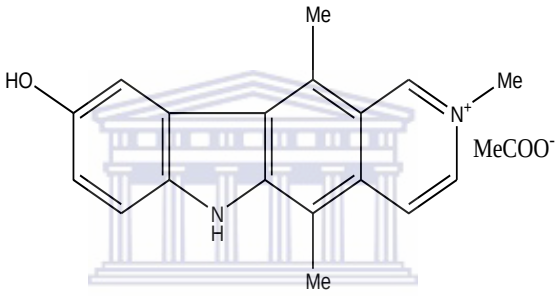


Figure 1. 10 Chemical structure of elliptinium

1.7 *Tulbaghia violacea*

1.7.1 Taxonomy and geographical distribution of *Tulbaghia violacea* plant

Figure 1.11 Photo of Tulbaghia violacea showing the green hairless leaves and purple flowers. Adapted from (Van Wyk, *et al.*, 1997)

T.violacea (figure 1.11) is commonly known as wild garlic, wilde knoffel (Afrikaans), isihaqa (Zulu) or itswele iomlambo (Xhosa). *T.violacea* is indigenous to the Eastern part of South Africa (figure 1.12). It belongs to the subclass monocotyledonae, superorder Liliiflorae, and order – Asparagales, with about 30 genera and 600 species (Dahlgren, *et al.*, 1985) belonging to the *alliacea* family (Fay & Chase, 1996). The subfamily name is derived from the generic name of the genus known as Allium. *Allium sativum* belongs to a member of the *lily* family. Southern African species include *Tulbaghia violacea, Tulbaghia acutiloba, Tulbaghia capensis, Tulbaghia leucantha, Tulbaghia ludwigiana and Tulbaghia natalensis* (Burbidge, 1978). A few species were reported in the UK, such as *T.violacea*, *T.cominsii, T. acutiloba, T.natalensis,* and *T. montana,* although most are rather tender

and are best grown as warm greenhouse plants (Burbidge, 1978). In the USA, it is cultivated as a decorative plant (Watson & Dallwitz, 1992).

Figure 1. 12 Geographical distribution of Tulbaghia violacea in Eastern Cape and KwaZulu-Natal

Tulbaghia species are plants with small flowers and grassy foliage. It is a perennial plant with corm-like rhizomes and narrowly linear, evergreen aromatic leaves with tubular mauve or pale purple flowers, occurring in groups of about ten at the tip of the slender stalk (Burbidge, 1978) (figure1.11). They are characterized by rhizomatous rootstalks with a pungent, skunky or alliaceous scent. The height of *T.violacea* at maturity ranges from 30 cm to 120 cm depending on the environmental conditions. The plant can be grown successfully in a tub and transferred to a greenhouse or a frost free place for the winter

(Watson & Dallwitz, 1992). *T. violacea* produce a strong odour of onion or garlic when bruised (Watt & Breyer-Brandwijk, 1962), hence its common name wild garlic (van Wyk et al., 2000) and/or society garlic (Watson & Dallwitz, 1992).

1.7.2 Medicinal uses, reported chemistry and biological activities of Tulbaghia violacea

T.violacea is an edible plant which has been cultivated for hundreds of years. (Van Wyk, *et al.*, 1997). The leaves and tubers are widely used as herbal remedies for various ailments such as fever, colds, asthma, tuberculosis, rheumatism, paralysis, hypertension, stomach problems and cancer. It can also be used as a snake repellent or consumed as vegetable delicacies (Van Wyk & Gericke, 2000). They are used as decoctions that are either taken orally or as enema (Hutchings, *et al.*, 1996).

Despite its garlic-like flavour, *T.violacea* consumption is not accompanied by the development of bad breath when compared with the consumption of *A. sativum* (Kubec, *et al.*, 2002). Hence it is commonly called "sweet garlic". This suggests that *T.violacea* and *A.sativum* do not contain the same volatile chemical compositions. It was reported that *T.violacea* contain a carbon-sulphur lyase enzyme whose mode of action is related to that of lyases in the other *Allium* species (Jacobsen, *et al.*, 1968). Studies have shown that *T.violacea* have secondary metabolites similar with garlic, thus both show similar biological activities and moreover they both belong to the same family (Order Asparagales, Family Alliaceae) and have the characteristic sulphur smell of garlic (Van Wyk, *et al.*, 1997; Van Wyk & Gericke, 2000; Bungu, *et al.*, 2006; Thamburan, *et al.*, 2006). The medicinal properties of garlic have been attributed to the sulphur compounds, which incidentally have

been isolated in both plants (Burton & Kaye, 1992; Kubec, *et al.*, 2002). These sulphur containing compounds are produced when alliinase, an enzyme present in all plants belonging to the *Allium* species, reacts with alliin when the plant is bruised/ crushed (Burton & Kaye, 1992; Kubec, *et al.*, 2002).

Jacobsen *et al.* (1968) reported the presence of C-S lyase as well as three unidentified S-substituted cysteine sulfoxide derivatives. This was corroborated by Burton and Kaye (1992) who isolated 2, 4, 5, 7-tetrathiaoctane 2, 2-dioxide and 2, 4, 5, 7-tetrathiaoctane from the leaves of *T.violacea.* Similarly, Kubec *et al.* (2002) isolated 2,4,5,7-tine derivatives detected by Jacobsen *et al.* (1968) as (*RSR*C)-*S*- (methylthiomethyl)cysteine-4-oxide (marasmin); (*SSR*C)-*S*-methyl and (*SSR*C)- *S*-ethylcysteine sulfoxides (methiin, MCSO and ethiin, ECSO, respectively). Other classes of compounds reported in *T.violacea* include falonols e.g kaempferol and saponins/sapogenins, which are generally present in *Allium* and *Tulbaghia* (Watson & Dallwitz, 1992).

The crude leaf extracts of *T.violacea* inhibited angiotensin I converting enzyme (ACE) and also blocked the rise in mean arterial pressure (MAP) associated with infusion of exogenous angiotensin I in normotensive male Wistar rats;(Raji, *et al.*, 2012). Mackraj, *et. al*, (2008) observed a reduction in systemic arterial BP associated with decreased renal angiotensin II type 1 (AT1) receptor gene expression in Dahl Salt–sensitive (DSS) rats. Studies have also shown that *T.violcea* has anti-fungal activity against *Candida albicans* (Motsei, *et al.*, 2003) potential benefits in treating coccidial infections (Naidoo, *et al.*, 2008)

and also anti-bacterial activity against *Staphylococcus aureus* and *Bacillus subtilis* (Gaidamashvili & van Staden, 2002; Motsei, *et al.*, 2003).

Lyantagaye,(2005) used the whole plant of *T.violacea*, and did water extraction, he tested the extract on extract on CHO, MCF7, HeLa, H157 and MG63 cell lines using APOP*ercentage*^{TM} assay and found out that *T. violacea* crude water extract induced the apoptotic cell death in CHO, MCF7 and HeLa cells but not in H157 and MG63 cells, he went further to do apoptosis–guided fractionation to further purify the compound responsible for the apoptotic activity of *T.violacea* plant. He used elemental analysis, IR, NMR and single crystal X-ray diffraction to characterize and identify the structures of active compounds and he was able to identify the structures of the active compounds, as methyl-α-D- glucopyranoside (MDG) (figure 1.14), as one of the major compounds responsible for the activity. He tested those three compounds on CHO cell lines using APOPercentage^{TM} assay and found MDG to induce apoptosis within 1hour of induction and that MDG was the most active compound and it is also commercially available. Burton (1990) also identified free sugars including glucose, fructose, sucrose, maltose, arabinose, rhamnose, xylose and galactose, and glycosides from water extract of *T.violacea*. Therefore MDG was used in this study, because it is commercially and readily available.

T. violacea leaves and bulbs have been reported to have anti-proliferative and pro-apoptotic activities on human cancer cell lines (Bungu, *et al.*, 2006; Bungu, *et al.*, 2008). It was observed that *T. violacea* exhibited biological activities which were similar to garlic, suggesting *T. violacea* extract can be substituted for garlic. *T. violacea* also has

anticoagulant (Bungu, *et al.*, 2008), antithrombotic (Bungu, *et al.*, 2008) and antioxidant properties (Naidoo, *et al.*, 2008). These properties play very significant roles towards the prevention and treatment of hypertension as well as some other cardiovascular diseases.

1.8 Research questions

A study by Lyantagaye (2005) reported that MDG was one of the compounds present in the water extract of *T.violacea* that was responsible for the pro-apoptotic activity of the plant extract. This study also proposed that this compound could be further investigated as an anti-cancer agent. The study by (2005) did not investigate the mechanism by which MDG induces apoptosis. Hence the main focus of this study was to further investigate MDG as a novel anticancer agent with pro-apoptotic activity that can be used to treat cancer. The question that this study wanted to address is how MDG induces apoptosis in cultured human cancer cells. Furthermore, to isolate and identify additional compounds with pro-apoptotic activity from *T.violacea* using alternative extraction methods based on organic solvent extraction.

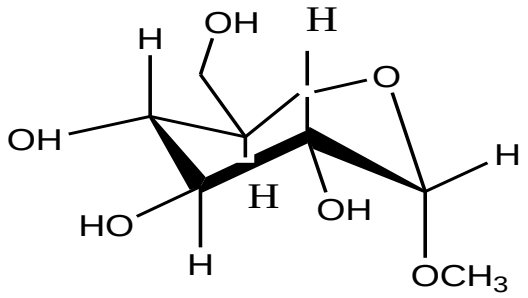


Figure 1. 13 Structure of Methyl- α-D-glucopyranoside

1.9 Objectives:

The objectives of this research are :

1. To confirm the pro-apoptotic activity of MDG both in human cancer and non-cancerous cells.
2. To investigate the mechanism of MDG induced apoptosis.
3. To isolate and characterise pro-apoptotic compounds from different parts of the *T.violacea* plant using bioactivity guided fractionation.

1.10 Hypothesis:

Based on traditional knowledge, the *T.violacea* plant can be used to treat cancer and anti-cancer agents can suppress cancer by inducing cell death through the activation of apoptosis (Harpreet, *et al.*, 2011). Therefore if extracts of the *T.violacea* plant have anti-cancer activity, it is likely that the extracts can induce apoptosis in human cancer cells. This pro-apoptotic activity can be ascribed to the presence of a bioactive compound or compounds in the extract. The hypothesis is that it should be possible to extract these compound(s) using bioactivity bio-guided fractionation. This may lead to the identification of novel anticancer agents.

1.11 Problem Statement:

Cancer is the second leading cause of death globally (World Health Organisation, 2009). Many currently used anti-cancer drugs are associated with serious side effects and resistance (Michael, 2002; Xing-Jie, *et al.*, 2010). Most anti-cancer drugs are derived from medicinal

plants (Harpreet, *et al.*, 2011). There is therefore need for more research to discover new anti-cancer treatments from herbal medicines used in the traditional medicine modality and identify novel compounds and mechanisms of action.

CHAPTER TWO: MATERIALS AND METHODS

Table of contents/Outline

2.3.8 Characterization and identification of the apoptotic compounds

2.3.8.1 Nuclear Magnetic Resonance (NMR)

2.3.8.2 Gas chromatography mass spectroscopy (GC-MS)

2.3.8.3 Liquid chromatography mass spectroscopy (LC-MS)

2.4 Bioassays

2.4.1 Morphological Evaluation treated and untreated cells

2.4.2 xCELLigence System

2.4.3 Measurement of cell surface modifications (externalization of PS) using APOPercentage ™ apoptosis assay measured by Flow cytometer

2.4.4 Measurement of caspase-3 activity

2.4.5 Evaluation of cytotoxicity using MTT Assay

2.4.6 Determination of Reactive Oxygen Species

2.5 Statistical analysis

2.1. GENERAL CHEMICALS

Chemical	Suppliers
Acetone	Sigma
n-Butyl alcohol	Sigma
Chloroform	Sigma
D_2O (Deuterated water)	Sigma
DCM (Dichloromethane)	Sigma
DMSO (Dimethyl sulfoxide)	Sigma
Ethanol	Sigma
Ethyl acetate	Sigma
Hexane	Sigma
Hydrochloric acid	Sigma
Methanol	Sigma
Silica gel 60 F_{254}	Merck
Sulfuric acid (H_2SO_4)	Merck
TLC plates (Silica gel 60 F_{254})	Merck
Toluene	Merck
Vanillin	Merck
Liquid Nitrogen	Merck

The solutions and chemicals used were of HPLC, UnivAR and AnalaR or equivalent grade

2.1.1 Commercial Kits/ Molecular Probes:

APOPercentage™ apoptosis assay (A1000)……Biocolor Ltd

CM-H2DCFDA) molecular probe (C6827)……..…Invitrogen

Active caspase-3-PE apoptosis kit.....BD Biosciences

3-(4, 5-Dimethylthiazol-2-yl)-2, 5-

Diphenyltetrazolium bromide (MTT dye).....Sigma

2.1.2 Tissue Culture Media

Nutrient mixture F-12 (Ham) with L-Glutamine..... Invitrogen

Phosphate Buffered Saline (PBS)

without $CaCl^{++}$ $MgCl,^{++}$..... Gibco

FoetalBovineserum(FBS)..... Invitrogen

Penicillin-streptomycin Invitrogen

Dulbecco's Modified Eagle's Medium (DMEM)..... Invitrogen

Trypsin.....Invitrogen

2.1.3 General Solutions and Buffers

Doxorubicin: 10µM of Doxorubicin in DMSO.

Vanillin-H_2SO_4 spray reagent: 15ml of 10% vanillin in ethanol mixed with 0.5ml H_2SO_4

70% Ethanol: 700ml of ethanol in 300ml of distilled water.

2.1.4 Plant Collections

T.violacea plants were collected from Van Den berg garden village in Stellenbosch, South Africa in May 2009 during winter season. It was authenticated at Biodiversity and Conservation Biology department, University of the Western Cape by Mr Frans Weitz and the voucher specimen numbered 6975 was deposited at the university herbarium.

2.2 METHODS

2.2.1 Tissue Culture

All the cells (table 2.1) used in this study were maintained in a 37°C incubator with 5% CO_2 saturation. All the cells used for this study were kindly provided by Prof Denver Henricks, Department of Clinical and Laboratory Medicine, University of Cape Town, South Africa. HepG2, HT29, MCF7, H157 and KMST6 cells were maintained in DMEM media containing 10% FBS, 10% penicillin-streptomycin, while CHO cells were maintained in Ham F-12 media containing 10% FBS, 10% penicillin-streptomycin.

Table 2.1: Cell lines used for the study

CELL LINE	SPECIES	MEDIA
HT-29 (Colon adenocarcinoma grade II)	Human	DMEM
H157 (Non Small Cell Lung Carcinoma)	Human	DMEM
HepG2 (Human hepatocellular liver carcinoma)	Human	DMEM
MCF-7 (Breast adenocarcinoma)	Human	DMEM
KMST-6 (Non Cancer Human Fibroblast)	Human	DMEM
CHO (Chinese Hamster Ovary)	Chinese Hamster	Hams F-12

2.2.1.1 Thawing of cells.

An amount of 5ml of DMEM media was placed at 4°C to cool. A vial of frozen cultured cells indicated in table 2.1 was taken from the -150^{o}C freezer cells were removed from the -150°C freezer and thawed in a 37°C water bath. The cells were transferred to a 15ml tube which contained the pre-cooled 5ml of DMEM media and centrifuges at 3000g for 3minutes. The supernatant was decanted and the pellet detached. The cells were then resuspended in 5ml media. The latter was put into a 25 cm^2 tissue culture flask. The flask was incubated at 37°C at 5% CO_2 until confluence was reached.

2.2.1.3 Cell count

Cell counts were performed using the countess^{TM} automated cell counter (Invitrogen). The countess^{TM} automated cell counter is a bench top automated cell counter that performs cell counts and cell viability measurements using trypan blue stain. The countess^{TM} automated cell counter uses disposable cell counting chamber slides that contain two enclosed chambers to chambers to hold the samples to allow for measurement of two different samples or perform replicates of the same sample. The cell counting occurs in the central location of the counting chamber and the volume counted is 0.4µL, the same as counting four (1mM x1mM) squares in a standard hemocytometer.

2.2.1.4. Trypsinization of cells

The cells were trypsinized once confluency was reached. The DMEM media in the flask was discarded and cells were washed with phosphate buffered saline (PBS). The PBS was

discarded and cells were trypsinized with the addition of 1 x trypsin and allowed to trypsinize at 37°C for 2–3minutes. DMEM media was added to the flask to stop trysinization. The cells were collected by centrifugation at 3,000g.

2.2.1.5 Seeding of cells

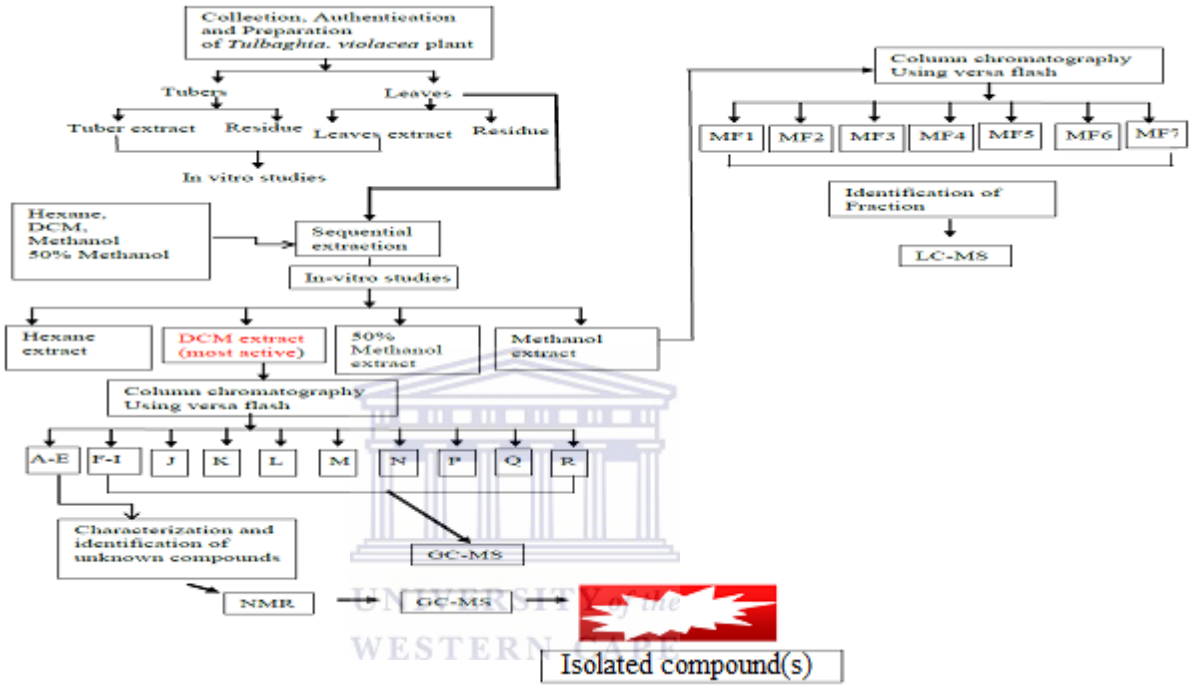
Once cells were thawed and incubated in a 25cm^2 tissue culture flask, cells were incubated at 37°C until confluency was reached. Cells were then trypsinized and centrifuged to remove the supernatant and resuspended in 5ml of DMEM media. Cells were counted with the use of the countess^{TM} automated cell counter (Invitrogen) and seeded at the following densities: 5ml of 2.4 x 10^4 cells/ml in 25 cm2 flasks, 1 ml of 1.0 x10^5 in 24 well plates, 2ml of 2.5 x 10^4 cells/ml/well in 6-well plates or 100µl of 0.25 x 10^5 cells/ml/well in 96-well plates. When 6, 24 or 96 well plates were used, 2ml, 1ml, 500µl or 100µl of cells were seeded respectively. Cells were incubated at 37^{o}C until ready for testing.

2.2.1.6 Freezing of cells

For long term storage the cells were trypsinized and centrifuged. The cell pellet was resuspended in DMEM media containing 10% DMSO. The latter was aliquot into 2ml cryo-vials and stored at -150°C.

2.3 BIOGUIDED FRACTIONATION OF ACTIVE COMPOUNDS

2.3.1 Plant Extraction Flowchart



KEY:

A-E – 90% HEX/ DCM
F-I - 25% DCM/HEX
J - 75% DCM/HEX
K -75% DCM/HEX
L -75% DCM/HEX
M -90% DCM/HEX
N=100%EA
P=90%EA/MEOH
Q=50%EA/MEOH
R =50%EA/MEOH TO 100% MEOH
TVL- *Tulbaghia violacea* leaves

KEY:
MF1 - 100% Hex
MF2 – 50% Hex /DCM
MF3 – 100% DCM
MF4–70% DCM/MeOH
MF5-50% DCM/MeOH
MF6-20%DCM/MeOH
MF7-100% MeOH

Figure 2.1: Flow chart of activity-guided fractionation of *Tulbaghia violacea* leaves and tubers.

2.3.2 Preparations of the plant material for water extraction

The leaves and the tubers were separated, rinsed with distilled water and dried at room temperature for 7days. The plants parts were cut into pieces and later pulverized and blended using a domestic blender (PHILIPS HR 1737, Cucina, Brazil). About 100g of the leaves and tubers were soaked separately and shaken overnight. This process was repeated thrice. The extract was later filtered with Buchner vacuum filter. After filtration, both leaves and tuber extracts were concentrated using VIRTIS *SENTRY*^{TM} freeze dryer (The Virtis company, Garner, New York), powdered extracts were later obtained and kept in desiccators until further use.

2.3.3 Organic extraction preparation

About 75.5g of *Tulbaghia violacea* leaves (75 g) were extracted with hexane (3 x 7.5liters) for 24hours by continuous stirring and shaking at 37^0C and then filtered. Combined Hexane extracts were evaporated to dryness under reduced pressure. This residue was sequentially extracted with DCM, Methanol and 50% methanol. After solvent extraction, the concentrate was dried under reduced pressure on a rotary evaporator (Rotary Vacuum Evaporator NE-1 Eyela Tokyo Rikakikai co, Ltd). The extracts were kept in desiccators at room temperature till needed and later tested for bioactivity.

2.3.4 DCM fraction preparation

DCM fraction was obtained from the sequential extraction made on the leaves. The fraction was further purified using Versaflash (figure 2.2). The solvents were chosen based on

solvent polarity as follows in this order:– 90% HEX/ DCM(A-E), 25% DCM/HEX (F-I), -75% DCM/HEX (J), 75% DCM/HEX (K), 75% DCM/HEX (L), 90% DCM/HEX (M), 100%EA (N), 90%EA/MEOH (P), 50%EA/MEOH (Q) and, 50%EA/MEOH TO 100% MEOH (R). All the fractions were spotted on TLC to monitor the purification processes.

2.3.5 Methanol fraction preparation

Methanol fraction was obtained from the sequential extraction made on the leaves. The fraction was further purified using Versaflash (figure 2.2). The solvent were chosen based on solvent polarity as follows in this order: 100% Hexane (MF1), 50% Hex/DCM (MF2), 100% DCM(MF3), 70%DCM/Methanol (MF4), 50% DCM/Methanol (MF5), 20% DCM/Methanol (MF6) and finally with 100% Methanol (MF7). The fractions were spotted on TLC to monitor the purification processes.

2.3.6 Determination of extraction yield (% yield)

The percentage yield of dried plant material were calculated as follows

%Yield = (weight of dried extract / weight of dried sample) *100

The yield (%, w/w) from all the dried extracts was calculated as:

Yield (%) = (W1 * 100)/W2

Where W1= weight of the extract after lyophilization of solvent, and

W2 = weight of the plant powder.

2.3.7 Bio-Guided fractionation of *T. violacea* leaves

Bio-guided fractionation of the DCM extract of *T.violacea* leaves was performed on VersaFlash high throughput flash purification (HTFP) (figure 2.3) system eluting first with 100% Hexane and gradually increase polarity by adding DCM, then ethylacetate and finally 100% methanol. (see Figure 2.1 for flowchart). Fractions were dried by rotary evaporator then sealed and kept at room temperature until needed. VersaFlash High Throughput Flash Purification (HTFP) system (Figure 2.2) is the first system to offer all of the capabilities required for flash separations and the versatility to perform unique purification tasks that conventional ash systems do not allow. These capabilities allow scientists to expand beyond the limitations of contemporary flash chromatography, saving time and money while improving their separations.

Figure 2.2 Versaflash High Thoroughput Flash Purification System (HTFP) (image from (www.sigmaaldrich.com)).

2.3.8 Thin layer chromatography (TLC)

TLC was used to monitor the purification process from the crude extract to the final stage. It was done by using silica gel 60 F_{254} analytical TLC aluminium plates, and three different combinations of mobile phase; Hexane: Ethyl acetate (8:2v/v) (HE), Ethyl acetate: Hexane (2:8v/v) (EH) and Butanol: acetic acid: water (5:1:4v/v) (BAW). The developed TLC plates were visualized under UV at 254 nm and then sprayed with anisaldehye or vanillin spraying reagents then heating at 105 ^{o}C for 1-3 min. The relative retention factor (RR_f) values were used to compare the compounds present in different apoptotic fraction from the crude extract.

2.3.9 Characterization and identification of the apoptotic compounds

2.3.9.1 Nuclear Magnetic Resonance (NMR)

A white crystalline compound isolated from DCM extract (Fraction A-E) (Figure 2.2) was characterised on NMR. NMR spectra were recorded, on a 600 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, Stellenbosch University. Software used to run the instrument and generate the spectra is VNMR 6.1C. 1D and 2D. 10mg of the purified fraction (fraction A-E) (Figure 2.2) was dissolved in D-chloroform (1ml). ^{1}H chemical shifts were referenced to the residual signals of the protons of solvent and were quoted in ppm downfield from tetramethylsilane (TMS).

2.3.9.2 Gas chromatography mass spectroscopy (GC-MS)

GC-MS was used to identify the compound (s) present in DCM extract (Fraction A-E) of *Tulbaghia violacea.* The instrument used was Agilent 6890N GC; Agilent 5975MS housed at Stellenbosch University, Central Analytical Facility (CAF). The injector temperature was $260^{0}C$, the column used was HPS (30m, 0.25, mm ID, 0.25 µM film thickness), with injection volume of 1 µl and split ration of ratio 1:10. The constant flow was 1 ml/min, the carrier gas used was Helium while the MS transfer temperature was at $280^{0}C$. The electron energy used was 70eV with scanning mass ranging from 35 to 550m/z with 3.9 minutes solvent delay.

The oven temperature programme was set as follows:

Oven Ramp	°C/minutes	Temp (°C)	Hold (minutes)
Initial		40	5
Ramp 1	10	300	10
Ramp 2	15	310	0

2.3.9.3 Liquid chromatography mass spectroscopy (LC-MS)

The fractions were obtained according to the method described in Figure 2.2. Mass spectrometry was done at Stellenbosch University, Central Analytical facility (CAF). The fractions were introduced into a Waters (Milford, Massachusetts, USA) Time of Flight (TOF) Synapt G2 mass spectrometer by injecting into a Waters UPLC front-end. The chromatographic separation was achieved on a Waters BEH C_{18} column (2.1 x50mm) at a flow rate of 0.4 ml/minute using mobile phase A (water + 0.1% formic acid) and mobile phase B (acetonitrile + 0.1 formic acid). Gradient elution was done as follows: 0-0.5mon 100% A, 0.5-17 minutes linear gradient to 100% B, then return to original conditions in the last 2 minutes. The TOF MS/MS system was operated in electrospray positive ion mode with capillary voltage of 3 kV and cone voltage of 15V. Mass spectra were acquired in the range of 100-2000 m/z. All experiments were conducted under automatic gain conditions.

2.4 BIOASSAYS

2.4.1 Morphological Evaluation treated and untreated cells

Different cell lines shown in table 2.1 were cultured in 6 well culture plates to 90 % confluence. The cells were induced to undergo apoptosis with the plants extracts while the negative control cells were left without treatment and the positive control treated with 10µM of doxorubicin. The cells were incubated for 24hours at 37°C in 5% humidified incubator. Following incubation, the cells were inspected under an inverted (Nikon) light microscope using 20X objective and pictures were taken using a Leica EC3 digital camera. The morphology of the cells was evaluated and recorded (Fink & Cookson, 2005; Kroemer, *et al.*, 2005; Kroemer, *et al.*, 2007).

2.4.2. xCELLigence System

xCELLigence system (see Figure 2.1) was used to evaluate the effect of MDG on cell proliferation using CHO (Chinese Hamster Ovary) cell lines. This system is the predecessor of the new xCELLigence system jointly developed by Roche Applied Science and ACEA Biosciences. These systems utilize an electronic readout called impedance to non-invasively quantify adherent cell proliferation and viability in real-time (com/sis/apoptosis/docs/manual_apoptosis.pdf). The cells were seeded in standard microtiter plates that contain microelectronic sensor arrays. The interaction of cells with the electronic biosensors generates a cell-electrode impedance response that not only indicates cell viability but also correlates with the number of the cells seeded in the well. This impedance technology offers an innovative solution to a number of limitations in current assay systems. First, impedance measurements are non-invasive, so the cells remain in a more normal

physiological state during assays of cell proliferation and cytotoxicity. Secondly, the system is more economical, since it does not require labelling the cells with expensive reagents. It allows real-time, rather than end point, measurement of cell proliferation, viability and cytotoxicity. Real-time and continuous monitoring offers several distinct advantages. For example, it provides constant quality control for the cells allowing the user to make informed decisions about the timing of certain manipulations such as compound addition.

In addition, an actual kinetic record of the cells prior or subsequent to a certain manipulation provides important information about the biological state of the cell, revealing, for example, enhanced cell growth, cell quiescence, morphological changes or cell death. Furthermore, when cytotoxic drugs are tested, real-time monitoring allows calculation of time-dependent IC_{50} values; these are more informative than single time point IC_{50} values, which can vary dramatically depending on the time the experiment was terminated. Traditional methods would require multiple assays and experiments to gather that much information about the mechanism of action of various drugs. In order to show that this new method is superior to traditional methods and suitable for cell proliferation, viability and cytotoxicity studies, and a number of experiments were conducted to assess cell proliferation and drug interaction with different cell lines (https://www.roche- applied-science.com/publications/print_mat/ lab_faqs.pdf).

To assess dynamic apoptosis induction of MDG and ascertain whether cell Index units correlate with the number of cells in the well, increasing numbers of CHO (Chinese hamster ovary) cell lines were seeded at 2500 and 10000 cells per well in triplicate in a

standard microtiter plates that contain microelectronic sensor arrays. CHO cells were grown and cell counts were done according to sections 2.2.1.1 and 2.2.1.2 respectively. Plates were monitored until when the cell reaches 70 to 80% confluence, at which time the cell Index was obtained they were also monitored every 30minutes for the indicated period of time. Each cell type has its own characteristic kinetic trace, based on the number of cells seeded, the overall size, morphology of the cells and the degree to which the cells interact with the sensor surface. Also, each of the cell lines can be characterized by its unique adhesion and spreading kinetics, as well as the time at which it enters the log growth phase.

Figure 2.3: The xCELLigence system

2.4.3 Measurement of cell surface modifications (externalization of PS) using APOPercentage™ apoptosis assay measured by Flow cytometer.

After cells were grown to 90% confluence in 24 well culture plates, they were treated with various concentrations of the plant crude extracts for 24hours. l0µM doxorubicin was used as a positive control for cells induced with the plant crude extracts for 24hours. The cells were induced for 24hours because APOP*ercentage™* showed good positive results at 24hours of induction. This was also consistent with other studies (Jirsova, *et al.*, 2006). Negative control cells were however, left untreated for 24hours so that they could be used to properly distinguish normal cells from apoptotic cells. Following incubation, floating (apoptotic) cells were transferred to 15ml centrifuge tube and the adherent cells were trypsinized and mixed with the tube containing the floating cells. The cells were washed twice with PBS, and resuspended in the residual PBS. APOP*ercentage™* dye in complete culture media diluted 1:160 was prepared. 200 µl of the dye was added to the tube and the cells were incubated for 30minutes at 37^{o}C in a humidified CO_2 incubator. After the incubation period, 500µl of PBS was added to the tube and spun down for 5minutes at 300xg. The pellet was washed one more time with PBS. After which the pellet was resuspended in 400µl of PBS and the cells were acquired and analyzed on a FASCan^{TM} (Becton Dickson) instrument equipped with a 488 nm Argon Laser as a light source within one hour.

Acquisition was done by setting forward scatter (FSC) and side scatter (SSC) on a log scale dot plot to differentiate population of cells and cellular debris. On a linear histogram dot plot, APOP*ercentage™*. (FL-3 channel) was measured against relative cell

numbers. Negative control cells were used to set the cells in the negative quadrant before all samples were acquired. The instrument settings were as follow: voltage set at 470 and AmpGain set at 1. Acquisition was done in Log mode. A minimum of 10,000 cells per sample were acquired and analysed using CELLQuest PRO software (BD Biosciences) by setting the non stained (untreated) cell population in the first quadrant (10^1) of the forward side scatter histogram dot plot and cells which appeared in the second (10^2) or third quadrant were regarded APOP*ercentage*^{TM} positive (apoptotic/necrotic).

2.4.4 Measurement of Caspase-3 activity.

In this assay, cells were seeded at a density of $2.5x10^4$ cells per ml in 24 well culture plates and were incubated at 37^{o}C in a humidified CO_2 incubator for 24hours. The culture media was then removed and replaced with media containing 2mg/ml of water and organic extracts of *T.violacea* leaves extracts. The cells were incubated for 24hours at 37^{o}C in a humidified CO_2 incubator. Positive control cells for the plant extract were induced with 10 µM of doxorubicin for 24hours. Negative control cells received no treatment. The adherent cells were gently washed once with 1000µl PBS trypsinized with 500µl of 0.125% trypsin and incubated for 5-10 minutes at 37^{o}C in a humidified CO_2 incubator. When the cells were detached, 500µl of complete culture media was added to the cells to stop the action of trypsin, and then the cells were transferred into the 15ml tube containing apoptotic cells and centrifuged to a pellet at 300xg for 5minutes. The pellet was washed twice with 1ml cold PBS and resuspended in 0.5ml Cytofix/Cytoperm™ at a concentration of ~$1.0x10^6$ cells/ml then incubated on ice for 20 minutes. After incubation on ice, the cells were spun down in a bench top centrifuge for two minutes at 2000g. The Cytofix/Cytoperm™ was aspirated and discarded and pellet washed twice with 0.5ml Perm/Wash™ buffer at room temperature. Total

number of samples was determined and the amount of Perm/Wash buffer and antibody calculated so that each sample received 100 µl Perm/Wash™ buffer and 20µl Antibody. The cells were incubated for 30minutes at room temperature in the dark. At the end of the incubation period, the cells were washed twice in 1.0ml of Perm/Wash™ buffer. The supernatant was discarded and the pellet resuspended in 0.5ml Perm/Wash™ buffer by flicking the tube (Fujita & Tsuruo, 1998).

The cells were acquired and analyzed on a FASCan™ (Becton Dickson) instrument equipped with a 488 nm Argon Laser as a light source. Using dot plot cell fluorescence was measured by setting the Forward (FSC) and Side Scatter (SSC) to differentiate cell populations and cell debris. On a log histogram dot plot, FL1 channel (Active Caspase-3 FITC) was measured against relative cell numbers. Negative control cells were used to set the cells in the negative quadrant before all samples were acquired. A minimum of 10,000 cells per sample was acquired and analyzed using CELLQUEST PRO Software (BD Biosciences).

2.4.5 Evaluation of cytotoxicity using MTT Assay

Cell proliferation was determined using the MTT assay following the methods described by Mosmann, 1983; Eguchi, *et al.*, (1997) and Freimoser, *et al.*, (1999) with minor modifications (Mosmann, 1983; Eguchi, *et al.*, 1997; Freimoser, *et al.*, 1999). The MTT assay is a method that is technically convenient, it doesn't involve washing steps and it is easy to apply in most experimental designs. Cells were trypsinized and cell counts of 2.4×10^4 were prepared in 50ml cell culture tubes. From this 100 µl was withdrawn and seeded in each well of 96 well plates and were let to grow to the required confluence. Then culture medium was removed

and replaced with 100 µl of fresh medium without or with various concentrations of the test compounds. Triplicate wells were established for each concentration and the cells were incubated for 24hours for 4 hours at 37^0C in a humidified CO_2 incubator. Tetrazolium salt 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) was dissolved in PBS (pH 7.2) to obtain a concentration of 0.5 mg/ml and 10 µl of MTT solution was added to each well. The plates were incubated for 4 hours at 37^0C in a humidified CO_2 incubator. At the end of the incubation period media was removed from each well and replaced with 100 µl DMSO. The plates were shaken on a rotating shaker for 10 minutes before taking readings at 560 nm using a microplate reader. Results of cellular viability were tabulated as mean absorbance of each compound expressed as a percentage of the untreated control and plotted against compound concentration. IC_{50} values were tabulated from the graphs as compound concentrations that reduced the absorbance at 560 nm by 50% of the untreated control wells. To exclude background readings, three wells were seeded with untreated cells in which MTT was not added. Assays were done in triplicate to ensure reproducibility.

The percentage inhibitions of cell proliferation were calculated using the following formula:

$$\% \text{ cytotoxicity} = \left\{ \frac{\text{Abs of negative control} - \text{Abs of treated cells}}{\text{Abs of negative control}} \right\} * 100$$

Dose– response curves were plotted from % IC (Inhibition of cell proliferation values versus concentration of crude extracts (mg/ml) x-axis, log scale. Concentrations that inhibit cells proliferation by 50 % (IC_{50}) were calculated by locating the x-axis values corresponding to 50 % inhibition of cell proliferation Y – axis.

2.4.6 Determination of Reactive Oxygen Species

To evaluate intracellular ROS, we used the molecular probe 5-(and-6)-chloromethyl-2', 7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H_2DCFDA) method as described by Wei *et al*. (2000) with minor modifications. It is a most common probe widely used in *in-vitro* biological research. CM-H_2DCFDA is a non-fluorescent probe that freely permeates into cells, however, it hydrolyzes to DCFH when inside the cell. Recently studies have shown that DCFH2-DA fluorescence is much more likely due to cytosolic redox change because the probe cannot penetrate into mitochondria (Abramov, *et al.*, 2007). The background fluorescence exhibited by DCFH2-DA could also be due to some metal impurities present in the culture media. Oxidation of DCFH2-DA can occur in the presence of intracellular reactive oxygen species which includes H_2O_2, peroxy radicals and peroxynitrite (Royall, *et al.*, 1993a). In the presence of DCFH, correct oxidant oxidizes to highly fluorescent 2', 7'-dichlorodihydrofluorescein (DCF), and can be detected by flow cytometry. Its detection is specific for intracellular ROS.

Briefly, H157, HepG2, HT29, MCF7 and KMST6 cells were cultured in 24 well culture plates and were either left untreated or treated with 2mg of the plant extracts. In addition positive control cells were treated with 1% hydrogen peroxide for 24hours. After the treatments, cells were trypsinised and transferred to 15ml cell culture tubes and washed twice with PBS and stained with 7.5µM of (CM-H_2DCFDA) prepared in PBS. The cells were incubated for 30minutes at 37^{o}C in a humidified CO_2 incubator following which the cells were acquired and analyzed on a FACScan^{TM} (Becton Dickson) instrument equipped

with a 488 nm argon laser. Cell fluorescence (DCF) was measured using (FL-1 channel) against relative cell numbers.

2.5 STATISTICAL ANALYSIS

Results were statistically compared by conducting a one way ANOVA using graph pad program. Differences between the mean ± SEM (standard error of the mean) of samples were considered significant at $P < 0.05$., the IC_{50} values were generated using GraphPad Prism software (GraphPad software, San Diego, CA, USA).

CHAPTER THREE: INVESTIGATING THE PRO-APOPTOTIC ACTIVITY OF MDG

Table of Contents/ Outline

3. 1 Background:

A number of previous studies demonstrated that extracts of *T.violacea* induce apoptosis in cultured cells and more specifically in cell lines generated from human cancer tissue. These studies therefore also propose that the potential anticancer activity of *T.violacea* can be ascribed to this bioactivity. A study by Lyantagaye (2005) isolated three compounds with pro-apoptotic activity from the water extract of *T.violacea.* Two of these compounds were novel and were not commercially available. The third compound was identified as MDG (figure 3.1) and is commercially available. In the Lyantagaye (2005) study a commercial equivalent of MDG was used to demonstrate that MDG induce apoptosis in CHO and MCF7 cells. This study did not investigate the possible mechanism of MDG induced apoptosis. Hence, the objective of this chapter was to carry out an independent evaluation of the pro-apoptotic activity of MDG on MCF7 and CHO cells in an effort to better understand the mechanism of MDG induced apoptosis. The effects of MDG on CHO and MCF7 cells were investigated using theAPOP*ercentage™* assay, morphological evaluation and the xCELLigence system.

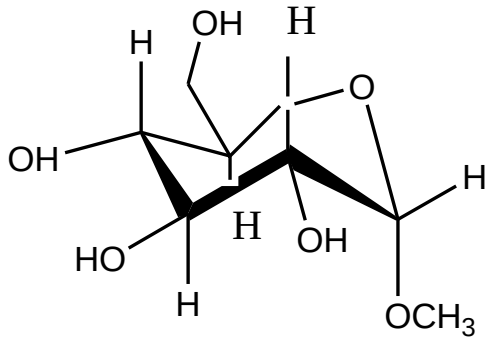


Figure 3.1 Structure of Methyl α-D-glucopyranoside

3.2 A study of the morphological changes induced by MDG in cultured cells.

The induction of apoptosis is associated with changes in cell morphology (reviewed in chapter1). CHO and MCF7 cells were treated for 24hours with MDG and the effects on cell morphology were evaluated. The morphology of MCF7 and CHO cells treated for 24hours with 2mM MDG were compared to the negative control (untreated cells) and positive control (cells treated with 10µM . doxorubicin) cells, respectively (figure 3.2). Doxorubicin is a well known chemical inducer of apoptosis and it also used in the treatment of cancer (Weiss, 1992; Max, *et al.*, 2009). It was observed that there were no discernible morphological changes in the cells treated with MDG (even of the highest dose of 2mM), while there were morphological changes in both cell types (CHO and MCF7) treated with 10µM doxorubicin.

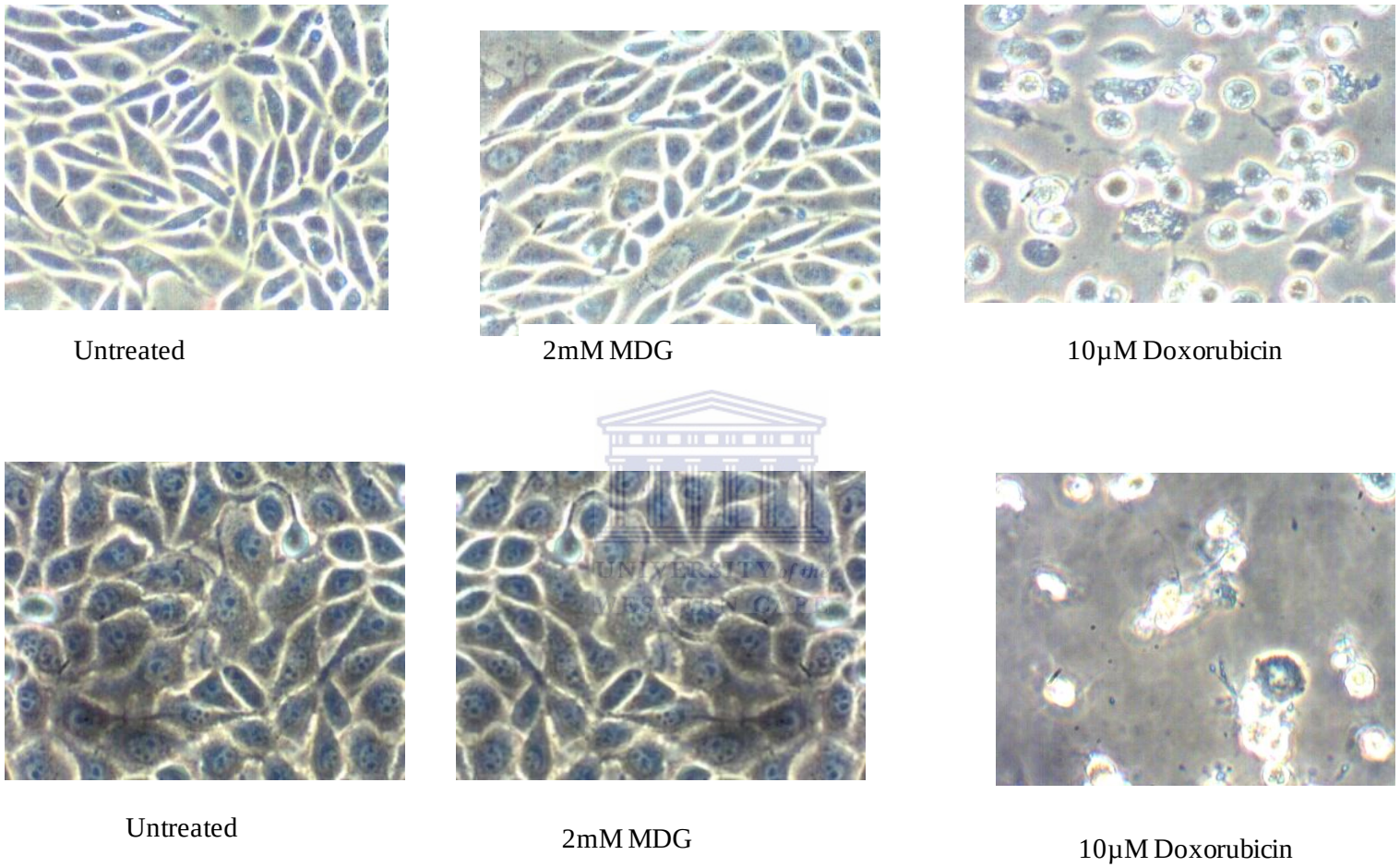


Figure 3.2: Morphologicaevaluation of CHO and MCF7 cells treated with MDG

CHO (panel A) and MCF7 (panel B) cells were treated for 24hours with 2mM MDG or 10µM doxorubicin. The cells were studied by light microscopy using a Nikon microscope. Pictures were taken at x20 magnification using a Leica digital camera. The experiments were done in triplicate.

3.3. Quantification of apoptosis in CHO and MCF7 cells treated with MDG

CHO and MCF7 cells were treated for 24hours with various concentrations (0.06 to 2mM) of MDG. Doxorubicin (l0µM) was used as a positive control. The cells were stained with the APOP*ercentage*^{TM} dye and analyzed by flow cytometry (described in section 2.4.3). Figures 3.3a and 3.3b show that there was little or no significant difference ($P< 0.05$) in the percentage of apoptotic cells between the control cells (untreated) and cells treated with MDG. However, the positive control (cells treated with 10µM doxorubicin) induced significant levels of apoptosis in both CHO and MCF7 cells.

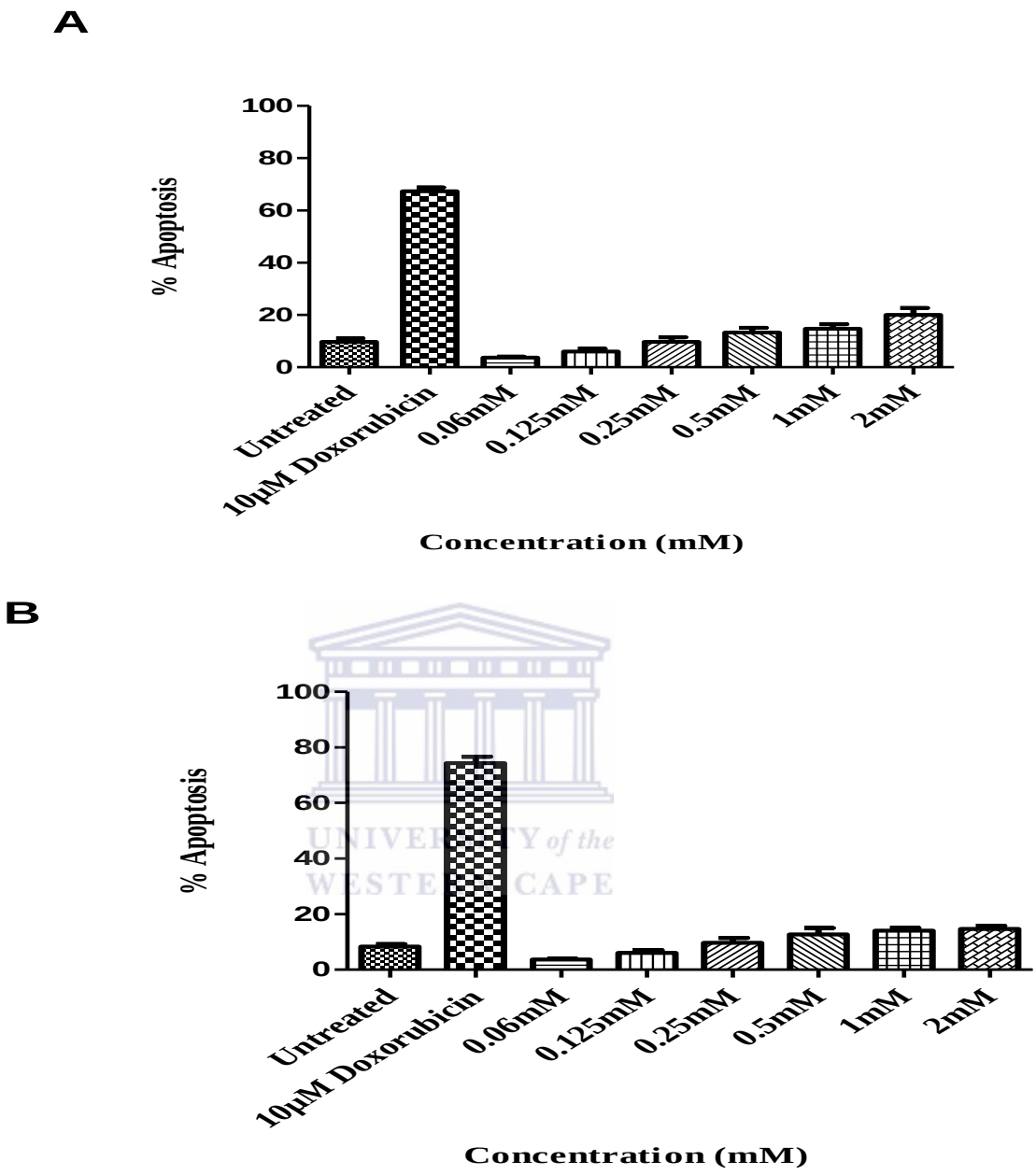


Figure 3.3: Quantification of apoptosis induced by MDG.

CHO (A) and MCF7 (B) cells were treated with various concentrations (0.06 to 2mM) MDG. Apoptosis was quantified by flow cytometry using the APOPercentage^{TM} assay. The graphs indicate the percentage of apoptotic cells observed with the different treatments. The experiments were done in triplicate.

3.4 Evaluating the cell proliferation of cells treated with MDG using the xCELLigence system

The xCELLigence system was used to evaluate the effects of MDG on the proliferation of CHO and MCF7 cells as described in section 2.4.2. The xCELLigence system measures electrical impedance across a microelectrode on an e-plate, thus providing a quantitative measurement on the biological status of the cells in real time. The electrical impedance was measured every minute over 24hours and is expressed as the cell index (figures 3.4a and 3.4b). The cell index reduced immediately after MDG was added to the cell culture. This reduction was observed for a period of 30minutes, after which the cell index increased again. The cell index of the untreated cells continued to increase over time while the cell index of the positive control (cells treated with 10µM doxorubicin) decreased continuously (graph not shown).

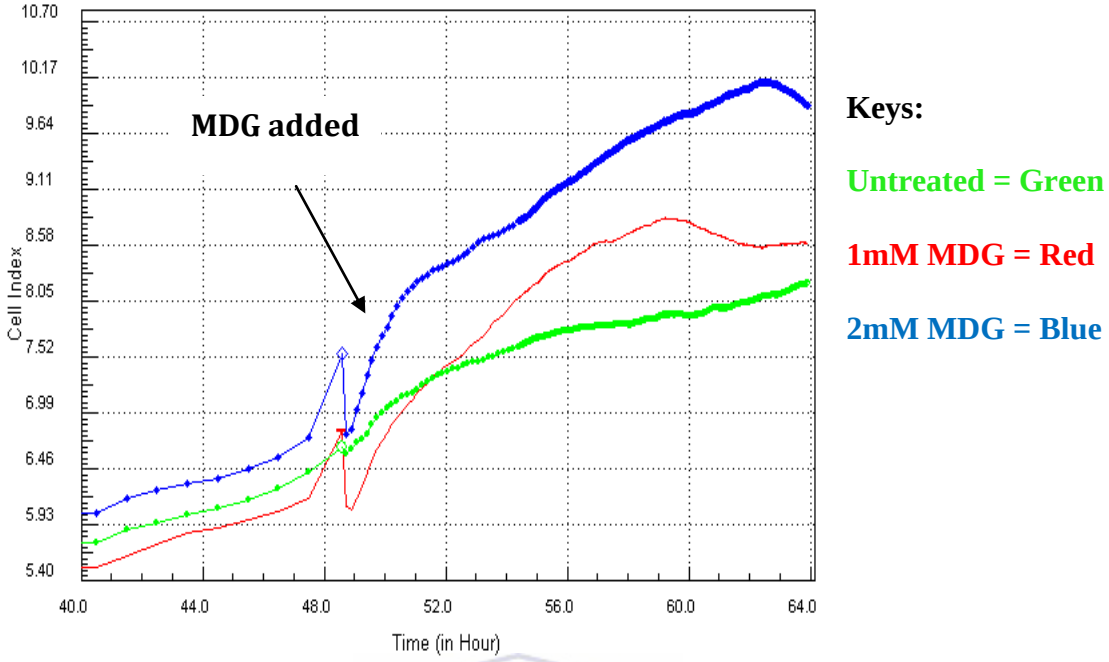
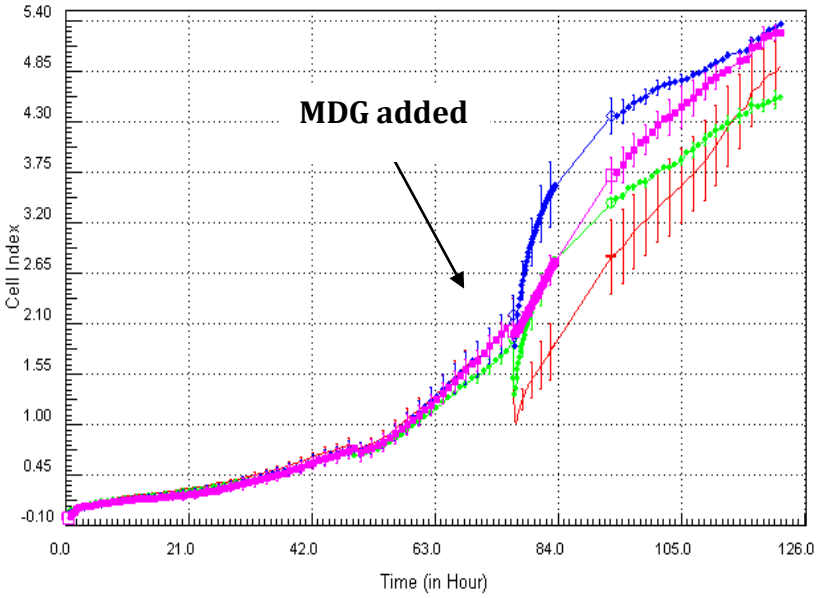


Figure3.4a: Cell proliferation assay showing the effects of 1 and 2mM of MDG on MCF7 cells. The cells were cultured for 24hours and were then either left untreated or treated with 1mM or 2mM MDG. The cells were continuously monitored using the xCELLigence system. The arrow indicates the time point at which MDG was added to the MCF7cells.



Keys:

Untreated = Pink

0.5 mM MDG = Red

1.0 mM MDG = Green

2.0 mM MDG = Blue

Figure 3.4b: Cell proliferation assay showing the effects of 0.5, 1 and 2mM of MDG on CHO cells. The cells were cultured for 24hours and were then either left untreated or treated with 0.5Mm, 1mM or 2mM MDG. The cells were continuously monitored using the xCELLigence system. The arrow indicates the time point at which MDG was added to the CHO cells.

3.5 Discussion

A previous study aimed at isolating and identifying the pro-apoptotic compounds present in *T.violacea* extracts identified MDG as one of the bioactive compounds present in the plant extract. The objective of the current study was to investigate the pro-apoptotic activity of MDG further and to unravel the mechanism by which MDG induce apoptosis in cancer cells.

CHO and MCF7 cells were treated with 2mM MDG and Figure 3.2 shows the morphological features of the cells after 24hours. The cells treated with 2mM MDG shows no discernible morphological changes when compared to the untreated control cells, while the morphology of cells treated with 10µM doxorubicin was clearly affected. The cells appear to be more rounded and lost contact with one another. It can also be observed that a large number of the cells detached from the plate. Although the absence of morphological changes suggested that MDG had no effect on the cells, morphological studies on its own cannot be used to conclude or confirm that the tested compound did not affect the cells at all. This is in agreement with Hacker's report in 2000 that morphological features of apoptosis should be used in combination with other apoptosis assays to evaluate the mechanisms underlying apoptosis (Häcker, 2000). The use of biomarker staining and light microscopy has been one of the methods used to detect apoptosis (reviewed in section 1.3). Apoptosis is associated with a conserved set of morphological features observed in various cell types during physiological episodes of cell death (Kerr, *et al.*, 1972). Kerr used changes in morphological features to develop the concept of cell death as a tool for the disposal of

unwanted cells during embryonic development, normal cell turnover in proliferating tissues as well as during pathological conditions such as cancer (Kerr, *et al.*, 1972).

There was need to confirm the apoptotic activity of MDG using an assay that is specific for apoptosis. The APOP*ercentage*^{TM} assay is a specific assay for apoptosis. To confirm and quantify apoptosis, the assay that takes advantage of the 'flip-flop' mechanism that is activated in apoptotic cells only. This process causes externalization of a phospholipid phosphatidylserine during the apoptotic process (Fadok, *et al.*, 2001). Figure 3.3a and figure 3.2b shows the result for APOP*ercentage*^{TM} assay following the treatment of MCF7 and CHO cells with various concentrations (0.6, 0.125, 0.25, 0.5, 1 and 2mM) of MDG. It was observed that less than 10% of the cells treated with MDG (even of the highest concentration of 2mM) were positive for apoptosis, 80% of the cells treated with 10µM . doxorubicin were positive for apoptosis. The result also indicates that there was little or no significant difference ($P < 0.05$) between the negative control cells (untreated) and cells treated with MDG. This was true for both cell lines.

These findings appear to contradict the work done by Lyantagaye (2005). However, differences in the execution of the experiments may account for the apparent discrepancies. In the study by Lyantagaye (2005) the cells were treated with 0.08 mM MDG for 1hour and apoptosis was then quantified using the APOP*ercentage*^{TM} assay. In contrast, in the current study, the cells were treated for 24hours before apoptosis is quantified. It is therefore possible that the effects observed by Lyantagaye 1hour after the treatment started was in fact a transient affect and that the cells recovered from these effects after time. This demonstrates

the limitations of the APOP*ercentage*^{TM} assay, which is based on end-point analysis. To overcome this, an assay which measures the effects of MDG in real time was required. The xCELLigence system allows for the analysis of cell proliferation in real time.

The xCELLigence system was able to monitor and evaluate the activity of MDG on proliferation of treated cells (CHO and MCF7) every minute (figures 3.4a and 3.4b) in real time compared to APOP*ercentage*^{TM} assay, which provides an end-point analysis. However, in this experiment, CHO cells could not reach 70% confluence until after 72 hours of seeding. The cells were monitored from the time of seeding the cells till the end of experiment and this gave an edge over APOP*ercentage* experiment. Figure 3.4a shows the effects of 1 and 2mM MDG on the proliferation of MCF7 cells and figure 3.4b shows the effects of 0.5, 1 and 2mM MDG on the proliferation of CHO cells. The cell index of both cell lines reduce immediately after MDG was added to the cell cultures. However, this reduction in cell index was a transient event since the cells recovered from the effects of MDG after about 1hour. This is indicated by the increase in the cell index. This transient reduction in cell index may suggest that the cells change shape and possibly shrink within the first hour, but recover after 1hour. It was observed that the cell index of the untreated cells continued to increase over time while the cell index of the cells treated with 10µM doxorubicin continually decreased over time (data not shown).

It can be concluded from this study that MDG does not induce apoptosis in CHO and MCF7 cells at the doses tested in this study. This was confirmed by the apoptosis assay (APOP*ercentage*^{TM} assay) and the cell proliferation assay (xCELLigence assay). This is in

sharp contrast to the findings by Lyantagaye (2005). However, the contradiction is explained by cell proliferation assay which shows that the effects of MDG are only transient and that the cells recover from these effects after 1hour. Considering the fact that the studies carried out by Lyantagaye (2005) was performed within 1hour of treatment with MDG, it can be concluded that MDG is most probably not the bioactive compound responsible for the pro-apoptotic activity that was observed in the water extract of *T.violacea.* Since the other two carbohydrate compounds isolated by Lyantagaye (2005) are not commercially available, these compounds could not be tested. Consequently, the compound(s) that is responsible for this bioactivity (induction of apoptosis) is still unknown. This prompted a renewed study into the chemistry of *T.violacea* to identify these compounds.

CHAPTER FOUR: PRO-APOPTOTIC AND ANTI-PROLIFERATIVE ACTIVITIES OF *T.VIOLACEA* EXTRACTS

Table of Contents/Outline

4.1 Background:

Although the *T.violacea* plant has been used in traditional medicine to treat various ailments including cancer, very little scientific research has been done on this plant to validate the uses of this plant. Although a study by Lyantagaye (2005) suggested that MDG could be one of the bioactive compounds responsible for the anticancer activity of the plant extract, the scientific findings reported in Chapter 3 of this study disputed the findings of the Lyantagaye (2005) study.

In the study by Lyantagaye (2005) water extraction of the whole plant was used to isolate the bioactive compounds. The water extract was subjected to organic solvent extraction to fractionate and isolate the bioactive compounds using bioassay-guided fractionation. Hexane, ethyl acetate/water, chloroform/water and n-butylacetate/water were used as solvents. In the study by Bungu *et al*. (2006), cell growth inhibition of methanol extracts of *T. violacea* leaves and bulbs were tested on MCF7, WHCO3, HT29 and Hela cancer cell lines. Apoptosis activities were investigated using the Hoechst 33342 dye to stain the treated cells for detection of chromatin condensation, nuclear fragmentation and Annexin V-FITC/propiodium iodide staining, they however found out that in WHCO3 treated cell, the leaf extract were more active than the bulb.

This study aimed to use a different approach to isolate bioactive compounds from the plant material. Instead of using the whole plant, the tubers and leaves were separated and tested independently. In addition, solvent extraction was performed directly on the plant

material which thus differs from the previous work done by Bungu *et al.(*2006) and Lyantagaye (2005).

4.2.1 Determining the IC_{50} values of tuber and leave extracts using the MTT assay

The cytotoxic activity of water extracts of *T. violacea* were investigated using 3-(4, 5-dimethylthiazole-2yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay on different cell lines. Various concentrations of the water extracts of *T. violacea* leaves and tubers were prepared and screened against four human cancer cell lines (HepG2, HT29, MCF7 and H157) and a non-cancerous human cell line (KMST6). The cells were treated for 24hours with increasing doses of the extracts and the cytotoxicity was measured using the MTT assay (described in section 2.4.5). The IC_{50} values (concentration that inhibits cell proliferation by 50%) were determined for the extracts using GraphPad prism software (GraphPad software, San Diego, CA, USA) (table 4.1). The water extract of the *T. violacea* leaves exhibited higher cytotoxcity compared to the tuber extracts. The IC_{50} values for the tuber extracts were more than 5 mg/ml on all the cell lines tested in this study, while the IC_{50} values for the leaf extracts were less than 3 mg/ml. MCF7 was the most sensitive cell line with an IC_{50} value of 1.2mg/ml, while the non-cancerous cell line, KMST6 was more resistant to the effects of the leaf extract with an IC_{50} value of 3mg/ml.

Table 4.1: IC_{50} values and selectivity index for *T. violacea* tuber and leave extracts determined by MTT assays.

Cell lines	leaf extract (mg/ml)	Tuber extract (mg/ml)	Selectivity index
MCF7	1.2 ± 0.5	Inactive	2.5
H157	1.5 ± 0.76	Inactive	2
HT29	2.6 ± 1.5	Inactive	1.15
HepG2	2.8 ± 0.45	Inactive	1.07
KMST6	3.0 ± 0.85	Inactive	1

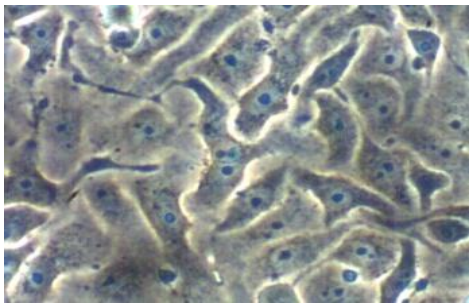
Four human cancer cell lines (HepG2, HT29, MCF7 and H157) and a non-cancerous human cell line (KMST6) were treated for 24hours with various concentrations of water extracts of *T. violacea* leaves and tubers. Cytotoxcity was determined using the MTT assay. The experiments were done in triplicate and IC_{50} values were determined using GraphPad prism software. Data were expressed as the means of the triplicate.

IC_{50} >2.5 mg/ml is considered to be inactive

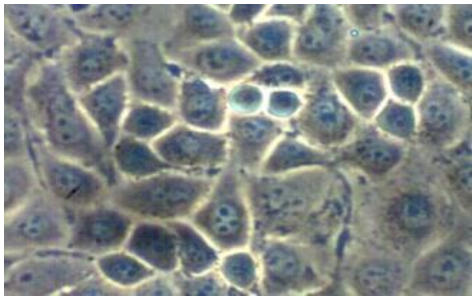
Selectivity index >1 indicates high selectivity

4.2.2 Morphological studies on MCF7 cells treated with leave and tuber extracts of *T.violacea*.

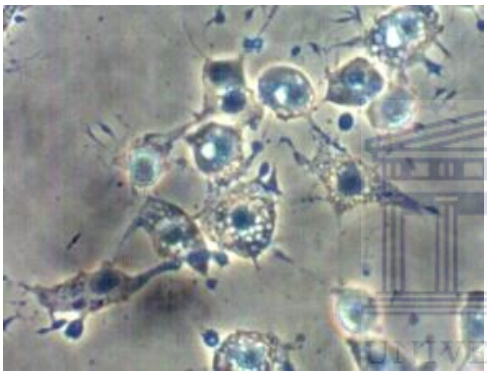
The effects of *T.violacea* leaves and tubers on morphological evaluation of different concentrations of *T.violacea* leaves and tubers were evaluated on MCF7 according to the method described in sections 2.3.1. Based on the IC_{50} values generated using the MTT assay, MCF7 cells were more sensitive to the effects of *T.violacea.* Consequently this cell line was used to evaluate the effects of *T.violacea* leaves and tubers on the morphology of the cells.



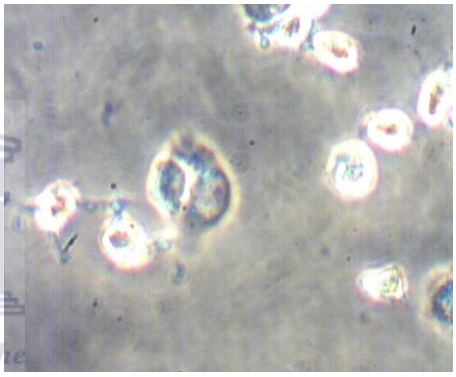
Tuber extract (2.5 mg/ml)



Untreated



Leaf extract (2.5 mg/ml)



. Doxorubicin (10µM .)

Figure 4.1: Morphological changes induced in MCF7 cells by water extracts of *T.violacea* leaves and tubers. MCF7 cells were treated for 24hours with 2.5 mg/ml of a water extract of *T.violacea* or10µM doxorubicin. The cells were studied by light microscope using a Nikon microscope. Images were taken at x20 magnification using a Leica digital camera. The experiments were done in triplicate.

The morphology of MCF7 cells treated for 24hours with 2.5 mg/ml of the tuber and leaf extracts were compared to negative control (untreated cells) and positive control cells (cells treated with 10µM . doxorubicin) (figure 4.1). The water tuber extracts failed to induce any morphological changes in MCF7 cells, while the leaf extract induced morphological changes indicative of apoptotic cell death. The morphological changes include; membrane blebbing, cell shrinkage, vacuolisation and cell detachment. These morphological changes can also be observed in cells treated doxorubicin, a known inducer of apoptosis.

4.2.3 Assessing the induction of apoptosis using the APOP*ercentage*^{TM} assay

Sections 4.2.1 and 4.2.2 demonstrated that the leaf extracts have cytotoxic effects on human cancer cells. These cytotoxic effects are accompanied with morphological changes that are indicative of the induction of apoptosis. However, none of these bioassays are specific for the evaluation of apoptosis. Therefore the APOP*ercenta*ge^{TM} apoptosis assay was used to evaluate the activation of apoptosis in the cells. A panel of 5 human cell lines (the cancerous cell lines; HepG2, HT29, MCF7, H157 and the non-cancerous cell line; KMST6) were treated for 24hours with increasing concentrations (0-2.5mg/ml) of the leaf and tuber extracts. The induction of apoptosis was determined using the APOP*ercentage*^{TM} apoptosis assay (figure 4.2a). The staining of the cells with the APOP*ercentage*^{TM} dye was quantified using flow cytometry.

A dose dependent increase in the number of apoptotic cells can be observed for the leaf extract, while the tuber extract failed to induce significant levels of apoptosis. An overlay of the relative fluorescence obtained for MCF7 cells treated with 2.5mg/ml tuber

and leaf extract is shown in Figure 4.2b. Also shown in the overlay is the fluorescence for the untreated control and positive control cells. An increase in relative fluorescence, which is indicated by a shift to the right, can be observed for cells that stain positive with the APOP*ercentage*^{TM}

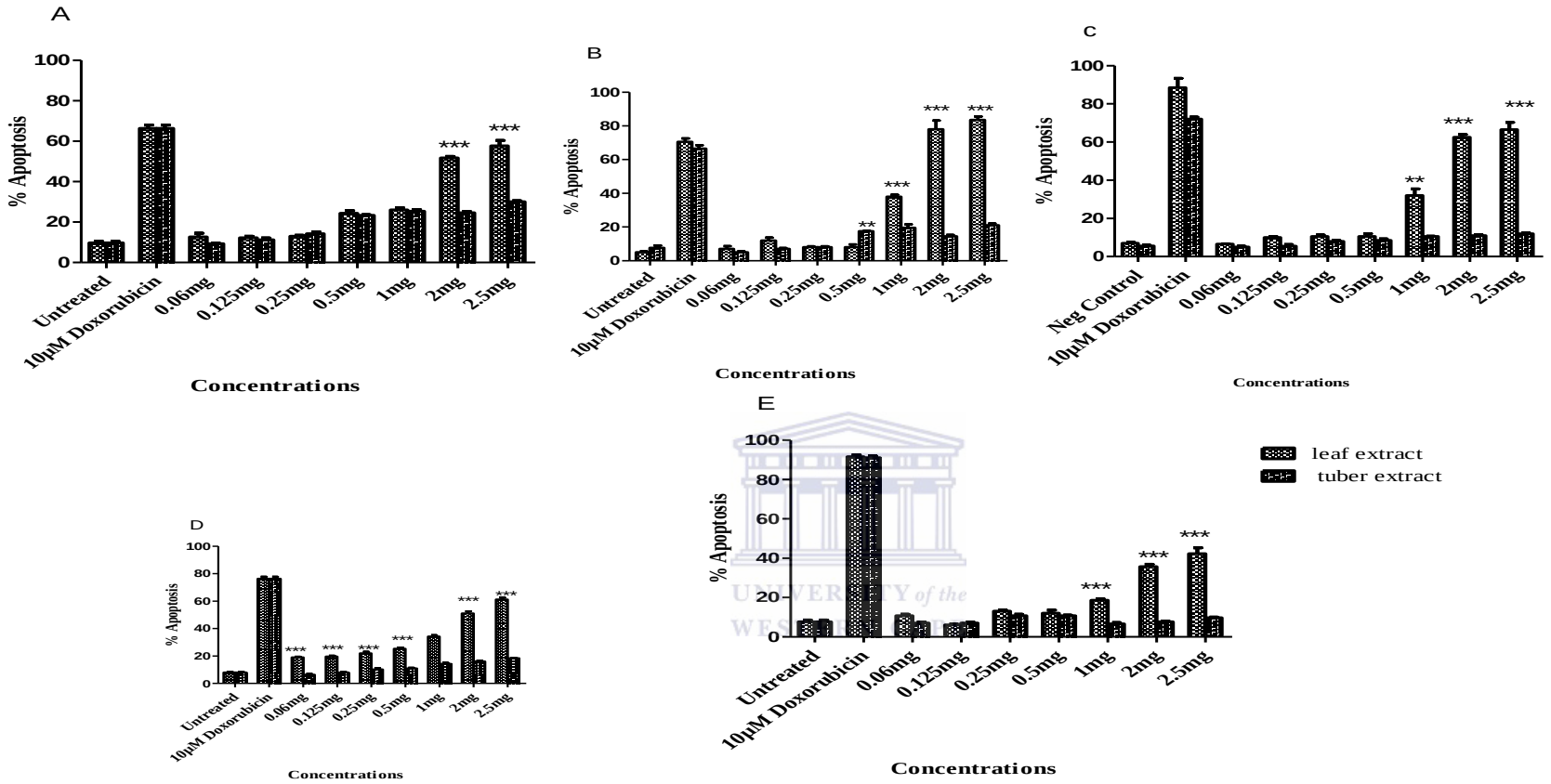
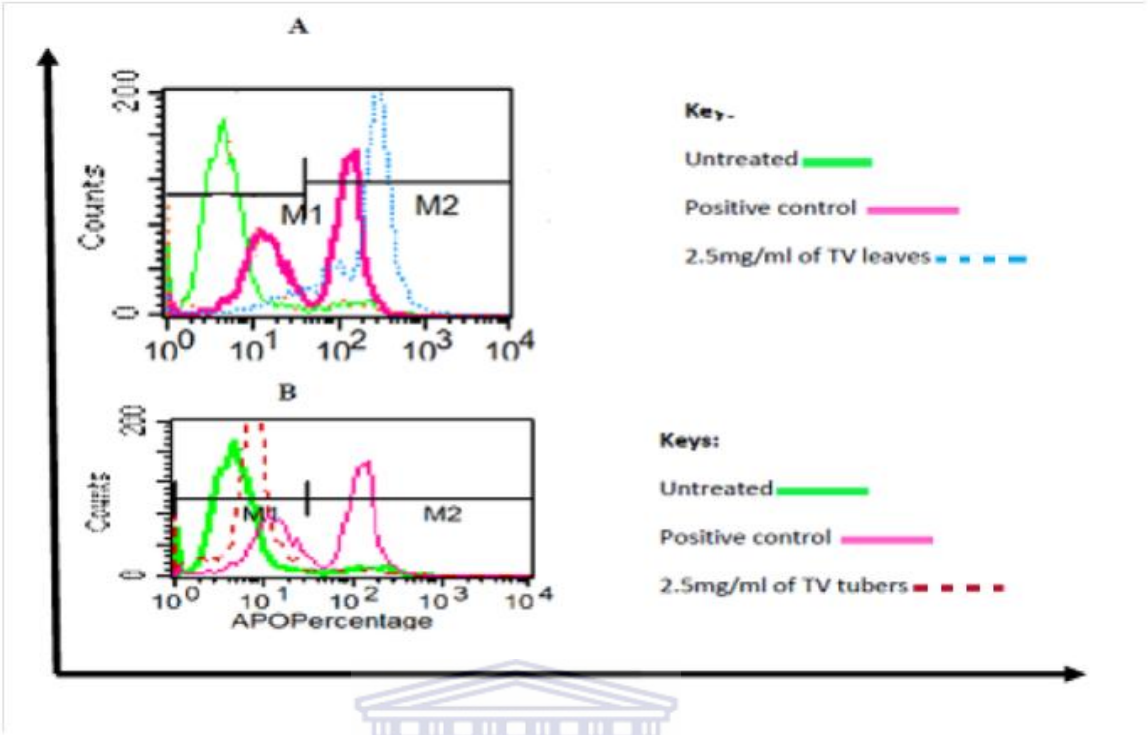


Figure 4.2a: Quantification of apoptosis induced by different concentrations of water leaf and tuber extracts. Four human cancer cell lines (HepG2, HT29, MCF7 and H157) and a non-cancerous human cell line (KMST6) were treated with increasing concentrations (0.06 to 2.5 mg/ml) of water extracts of *T. violacea* leaves and tubers. Apoptosis was quantified by flow cytometry using the APOPercentage^{TM} assay. Each graph indicates the percentage of apoptotic cells observed with different treatments. The experiments were done in triplicate. Graphs A represents HepG2,B represents HT29, C represents MCF7, D represents H157 and E represents KMST6 cell lines. A significance difference between the leave extract and tuber extract ($P < 0.05$) is indicated by ***

The Selectivity Index (SI) which implies the cytotoxicity (safety) selectivity of the water extracts of *T.violacea* was calculated against non-cancerous cell (KMST6) was calculated from the IC_{50} obtained from the induced significant levels of apoptosis in all five cell lines, while the water extract of the tuber induced little leaf The IC_{50} for the extracts against the cell lines are presented in Table 4.2. The result showed that the extracts induced apoptosis at concentrations of 1.21, 0.19, 0.28 and 2.09mg/ml for HT29, MCF7, H157 and HepG2 respectively, while the non cancerous KMST6 cells were less susceptible to the extract with the IC_{50} value of 4.40 mg/ml indicating that the water extracts of *T. violacea* is not toxic this was also confirmed from the result obtained from selectivity index. Moreover, the water extract of the leaves induced apoptosis in a dose and time dependant manner (Figure 4.2a).



Figures 4.2b: Histogram overlay of flow cytometry data obtained for MCF7 cells treated with extracts of *T.violacea* leaves and tubers. The histogram overlays show an overlay of untreated control, positive control, 2.5 mg/ml of leaf and tuber extract on MCF7 cells. A positive control (cells treated ‘;l9 10µM . doxorubicin) was also included. Cellular fluorescence was measured in the FL3 channel on the flow cytometry. The experiments were done in triplicate and it shows the overlay of untreated control, positive control and 2.5 mg/ml of both tubers and leaf extracts on MCF7 cells.

Table 4.2: IC_{50} values for *T. violacea* tuber and leave extracts

Cell lines	*Tulbaghia violacea* (IC_{50} in mg/ml)		
	Leaves	**Selectivity index**	**Tubers**
MCF7	0.19 ± 0.43	23.16	>2.5
H157	0.28 ± 0.10	15.71	>2.5
HT29	1.21± 0.60	3.64	>2.5
HepG2	2.09 ± 1.40	2.11	>2.5
KMST6	4.4 ± 1.36	1	>2.5

Four human cancer cell lines (HepG2, HT29, MCF7 and H157) and a non-cancerous human cell line (KMST6) were treated for 24hours with various concentrations of water extracts of *T. violacea* leaves and tubers. Apoptosis was determined using the APOPercentage^{TM} assay. The experiments were done in triplicate and IC_{50} values were determined using GraphPad prism software.

IC_{50} >2.5 mg/ml was considered to be inactive.

Selectivity index >1 is indicating high selectivity.

4.2.4 Assessing the activation of caspase-3 in human cell lines treated with *T.violacea* leaf extract.

It was demonstrated in Section 4.2.3 that the water extract of the *T.violacea* tubers have very low bioactivity (in particular pro-apoptotic activity) against the human cancer lines used in this study. In contrast the water extract of the *T.violacea* leaves induced significant levels of apoptosis in these human cancer cell lines. Therefore the rest of this study focused on the leaf extract. This was demonstrated using the APOP*ercentage*^{TM} assay. This assay detects apoptosis at the stage of phosphatidylserine exposure. In order to further investigate the activation of apoptosis, two additional markers of apoptosis (the activation of caspase-3 and the production of ROS) was also used to confirm the induction of apoptosis.

Four human cancer lines (HepG2, HT29, MCF7 and H157) and a non-cancerous human cell line (KMST6) cell lines were treated for 24hours with 2mg/ml of water extract of *T.violacea* leaves. The activation of caspase-3 was assessed using a PE-conjugated anti-active caspase-3 monoclonal antibody and flow cytometry as described in section 2.3.4. Figure 4.4 shows that the water extract of *T. violacea* leaves induced significant levels of caspase-3 in the cancerous cell lines HepG*2*, MCF7, H157 and HT29, with the highest bioactivity in HepG2 cells. Close to 70% of HepG2 cells were positive for the presence of activated caspase-3 compared to ~30% of MCF7, H157 and HT29 cells. The water extract of *T.violacea* leaves failed to induce capase-3 activation in KMST6 cells.

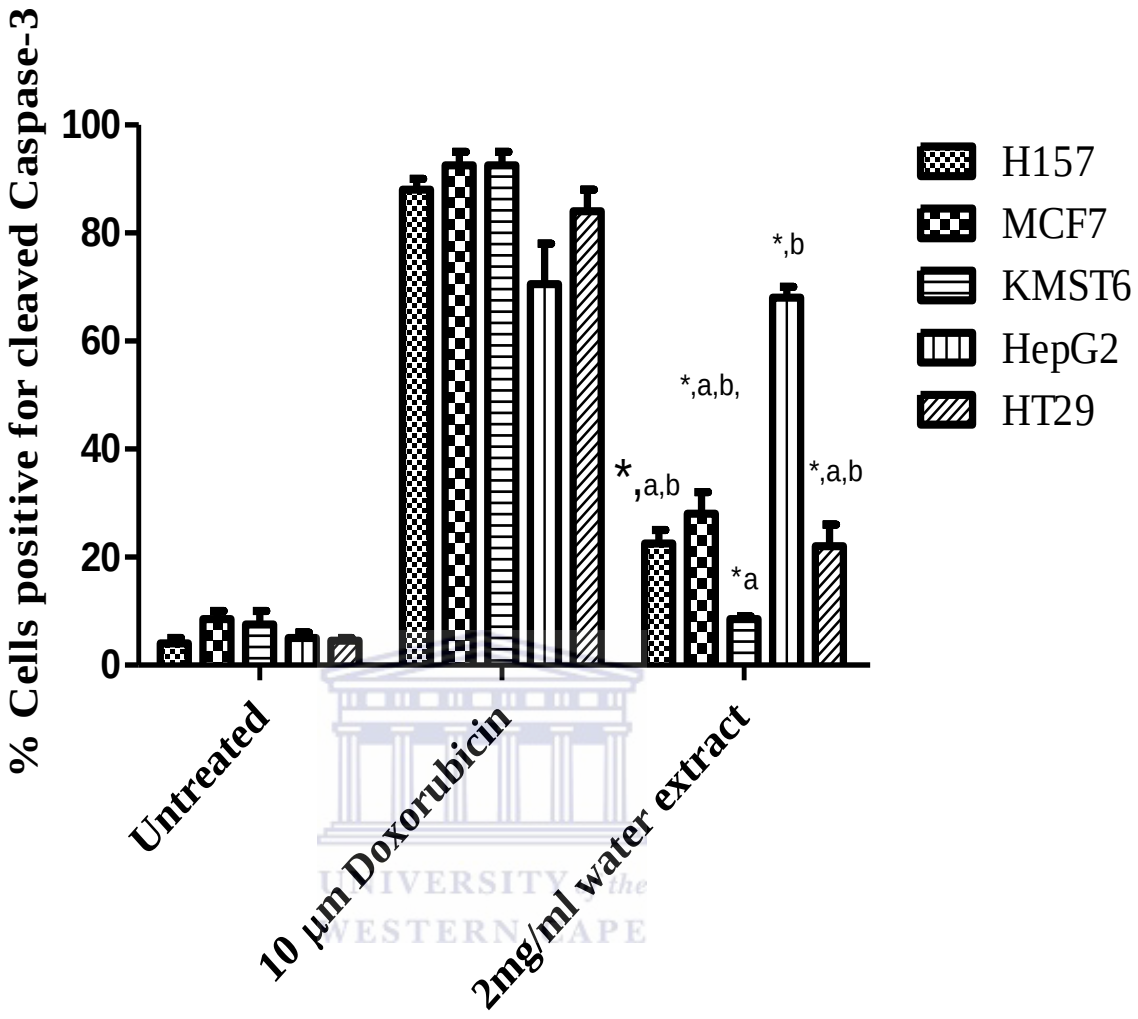


Figure 4.4: Evaluating the activation of caspase-3 on a panel of human cell lines. Four human cancer cell lines (HepG2, HT29, MCF7 and H157) and a non-cancerous human cell line (KMST6) were treated for 24hours with 2mg/ml of water extract of *T. violacea* leaves. The cells were fixed, permeabilized and stained with an anti-active caspase-3-PE antibody. As a positive control, the cells were also treated with 10μM doxorubicin. Cell fluorescence was measured by flow cytometry. The bar graph indicates the percentage of cells staining positive for active caspase-3. The experiments were done in triplicate. A significance difference between untreated and extract ($P < 0.05$) is represented by (*); (a) represent significant difference between the most sensitive cell line and other cell line ($P< 0.05$), (b) represent significant difference between the most resistance cell line and other cell lines ($P< 0.05$).

4.2.5 Assessing the production of ROS in human cell lines treated with *T.violacea*.

A non-fluorescent probe known as 2', 7'-dichlorfluorescein-diacetate (DCFH-DA) can be used to evaluate the production of intracellular Reactive Oxygen Species (ROS) in cultured cells. This probe freely permeates into cells and was used to evaluate the production of ROS in response to treatment with the *T.violacea* leaf extract. The production of ROS was observed in all the cancer cell lines, while no ROS production was observed in the non-cancerous KMST6 cell line (Figure 4.5). A varied response was observed for the cancer cell lines with ~80% of H157 cells and ~50% of HepG2 cells, respectively staining positive for ROS production. Less than 30% of MCF7 and HT29 cells were positive for ROS production.

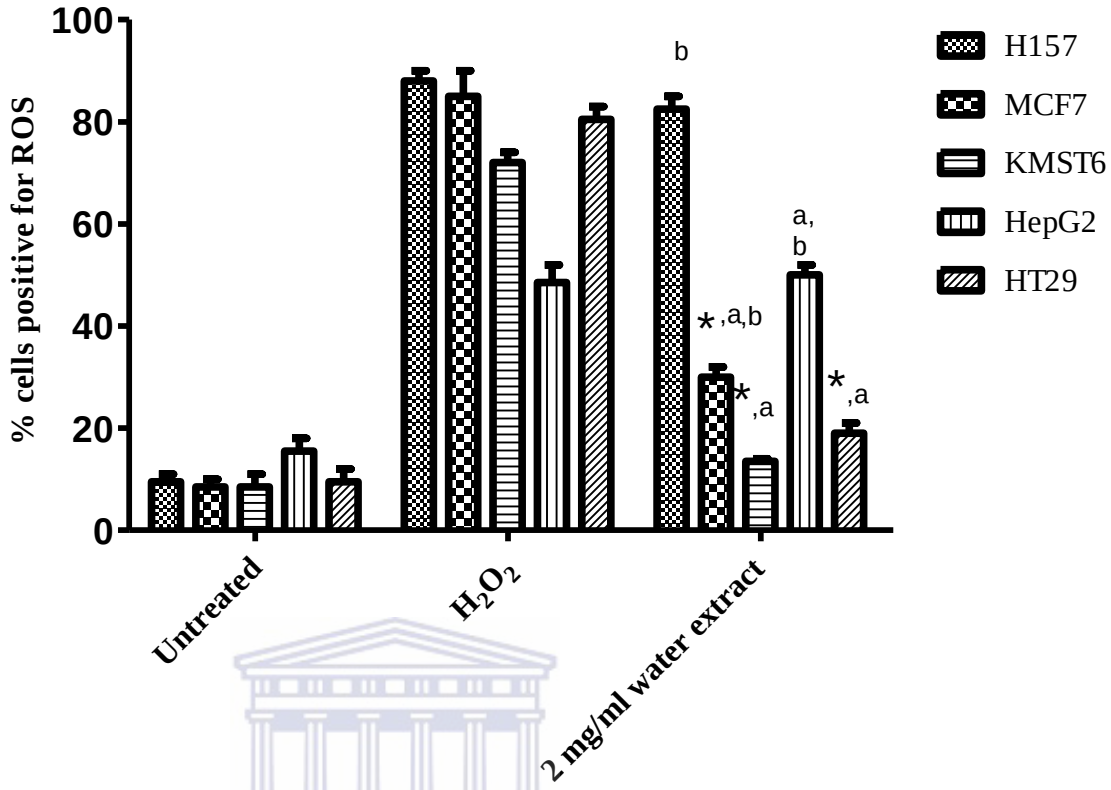


Figure 4.5: Evaluating the production of ROS on a panel of human cell lines. Four human cancer cell lines (HepG*2*, HT29, MCF7 and H157) and a non-cancerous human cell line (KMST6) were treated for 24hours with 2mg/ml of water extract of *T. violacea* leaves. ROS production was measured by flow cytometry using the DCFH-DA probe. As a positive control, the cells were also treated with 1% H_20_2. Cell fluorescence was measured by flow cytometry. The experiments were done in triplicate. * represent significance difference between untreated and extract ($P < 0.05$); a represent significant difference between the most sensitive cell line and other cell lines($p < 0.05$), b represent significant difference between the most resistance cell line and other cell lines ($P< 0.05$)

4.3 Discussion

Based on the IC_{50} values calculated from the MTT results, all the cell lines were remarkably sensitive to the leaf extract of *T.violacea* compared to the tuber extract. MCF7 cells were the most sensitive cells with an IC_{50} of 1.20 mg/ml followed by H157, HT29, HepG2, and KMST6 with IC_{50} of 1.5, 2.6, 2.8 and 3.0 mg/ml, respectively. However, IC_{50} values of the cell lines treated with the tuber extract were greater than 5mg/ml.

Morphologic characteristics of apoptosis include cell shrinkage, nuclear disintegration and cell detachment. This was reported by Kerr *et al.*, in 1972. These morphological changes were also observed in cells treated with doxorubicin, a known inducer of apoptosis. MCF7 cells treated with water extract of *T.violacea* leaves for a period of 24hours also displayed these morphological changes. All the other cell lines (KMST6, HT29, H157 and HepG2) displayed the same morphological changes (data not shown). In contrast the water extract of *T.violacea* tubers failed to induce these morphological changes in all the cells used in this study. This result suggests that the leaf extract of *T. violacea* induced apoptosis while the water tuber extract of *T. violacea* had no effect on the cells. The apoptotic activities could not be quantitated based on the morphological changes. Therefore there is need to do a quantitative assay to evaluate the percentage of apoptosis in the treated cells.

Figure 4.2a showed that a dose of 2mg/ml and 2.5mg/ml of the leaf extract induced significant levels of apoptosis. The leaf extracts induced apoptosis in a dose dependent manner in the cell lines used in this study. The tuber extract failed to induce significant levels of apoptosis in the cell lines used in this study. These results are in agreement with what was

observed in the study on the morphology of cells treated with tubers and leaf extracts of *T.violacea*.

The potency of a drug or a chemical is as the measure of its strength as a poison when compared with other drugs where threshold is the important aspect of dose-response relationship (Kenakin, 2009). Since apoptosis requires involvement of active cell participation and is therefore primarily caused by physiological stimuli, varied doses could result in cell death by apoptosis (Lennon, *et al.*, 1991). Fig 4.2b shows histogram overlays of MCF7 cells treated with different concentrations of water extracts of *T.violacea* leaves and tubers and assayed using APOP*ercentage*^{TM} assay. MCF7 cells were treated with 2.5 mg/ml of water extract of *T. violacea* leaves and tubers, respectively. Doxorubicin (10µM) treatment was used as a positive control and untreated cells served as the negative control. After 24hours, the cells were stained with the APOP*ercentage* dye and analyzed by flow cytometry. An increase in the relative fluorescence of the cells suggests that the cells stained positive for the dye and that the cells are undergoing apoptosis. This increase in fluorescence can only be observed for the positive control and cell treated with 2.5mg of leaf extract.

The results obtained for the APOP*ercentage*^{TM} assay was used to determine the IC_{50} values for the tuber and leaf extracts on all five human cell lines. The IC_{50} values were calculated using GraphPad Prism software (table 4.2). MCF7 was the most sensitive cell line with an IC_{50} value of 0.19 mg/ml followed by H157, HT29, and HepG2 with IC_{50} values of 0.28, 1.21, and 2.09 mg/ml, respectively. KMST6 was the most resistant cell line with an IC_{50} value of more than 2.5 mg/ml. These findings confirm that, water extracts of *T.violacea* leaves

induced significant apoptotic activities in all the treated cell lines while the water tuber extract of the plant lack pro-apoptotic activity at the doses used in this study.

The caspase-3 assay was one of the additional assays used to further characterise the induction of apoptosis in the cell lines. HepG*2*, HT29, MCF7, H157 and KMST6 cell lines were treated with 2mg/ml of water extract of *T. violacea* leaves for 24hours. The cleavage and activation of caspase -3 are known markers of apoptosis (Frankline*, et al.*, 2008). Caspases are crucial mediators of apoptosis. These proteases catalyze the specific cleavage of several cellular proteins and they are indispensable for apoptosis to occur (Jacobson, *et al.*, 1997; Adams & Cory, 1998; Nicholson, 1999). Figure 4.4 shows that the water extract of *T.violacea* leaf induced a significant level of caspase-3 cleavage in all the cell lines used in this study except for KMST6 cells where little or no casapase-3 cleavage was observed. A higher number of HepG2 cells were positive for caspase-3 cleavage compared to MCF7, H157 and HT29 cells. This is an interesting finding since the leaf extract has been shown to induce apoptosis more readily in MCF7, H157, and HT29 compared to HepG2 cells (see table 4.2). This may suggest that the activated caspase-3 is inhibited in HepG2 cells.

ROS serve as signalling molecules in mitochondrial death receptor and P53 modulating apoptosis (Nordberg & Arnér, 2001). ROS production in H157 cells was the highest, while ROS production in KMST6 and HT29 were the lowest compared to H157 and HepG2 cells, ROS production in MCF7 cells were much lower. However, MCF7 were more susceptible to the leaf extract, since higher levels of apoptosis were observed for MCF7. This suggests that an increased level of ROS does not necessarily result in increased risk of

apoptosis. This may be explained by the varying ability of different cancer cell types to suppress the effects of ROS through the production of anti-oxidants.

The findings from this study showed that extracts of *T.violacea* leaves demonstrated higher pro-apoptotic activity compared to tubers in the cancer cell lines used in this study, and therefore validates some of the claims towards the application of this plant for the treatment of cancer. The ability of a compound or a plant extract to induce apoptosis in cancer cells can be used as a test to evaluate its potential to treat cancer. The study has shown that the water extract of *T. violacea* leaves selectively induced apoptosis in human cell lines thus exhibiting a potential as an anti-cancer remedy. It should also be noted that ability of the extract to induce apoptosis in the non-cancerous cell line, KMST6 was much was much lower compared to the cancer cell lines. This suggests that the compounds selectively induce apoptosis in the cancer cells, which is a desired characteristic for anti-cancer treatment.

CHAPTER FIVE: THE BIOACTIVITY-GUIDED FRACTIONATION OF ORGANIC EXTRACTS OF *T.VIOLACEA*

Table of Contents/Outline

5.1 Background:

Medicinal plants are widely used for the treatment and prevention of various diseases including cancer. These plants can be a source of novel bioactive compounds. However, the complex mixtures of compounds present in these plants can act individually, additively or in synergy. (Gurib-Fakim, 2006). As a result separation of these compounds may lead to loss of bioactivity. The previous chapters demonstrated that the water extract of *T. violacea* leaves contains bioactive compounds with pro-apoptotic activity towards some human cancer cell lines. Therefore, the objective of this chapter was to attempt to isolate the active compounds from the leaves through bioassay guided fractionation.

5.2 Sequential extraction of *T.violacea* leaves using different organic solvents

In order to identify the compounds responsible for the pro-apoptotic activity in the leaf extract of *T.violacea,* sequential extraction of the leaves was done using hexane, followed by dichloromethane (DCM) methanol and finally 50% methanol/water. Extraction with each of these solvents was repeated thrice (section 2.3.3). The extracts of each solvent were combined and evaporated to dryness using a rotary evaporator. The final extracts were then weighed and the percentage yield for each solvent was calculated. The percentage yield for hexane, DCM, methanol and 50% methanol as shown in table 5.1 represents~70% of dry leaf mass. This implies that some compounds were not extracted with these solvents and these were probably due to the presence of fibres or complex polysaccharides or plant tissue.

Table 5.1 Percentage yield of the organic solvent extraction of *T.violacea* leaves.

Extract	% Yield of extraction
Hexane	7
Dichloromethane	15
50% Methanol / Water	20
Methanol	25

Powdered leaves of *T. violacea* were sequentially extracted using solvents of increasing polarity (hexane, dichloromethane (DCM), methanol and 50% water/ methanol. The percentage yield of organic solvent of *T. violacea* leaves are calculated as %Yield = (weight of dried extract / weight of dried sample) x 100.

.

5.3 Evaluating the apoptotic activity of organic solvent extracts

Three bioassays, the APOP*ercentage*^{TM} assay (section 2.4.3), the caspase-3 assay (section 2.4.4), and the ROS production assay (section 2.4.6) were used on a panel of 5 human cell lines (KMST6, HepG2, HT29, MCF7 and H157) to investigate the bioactivity of the four organic solvent extracts.The apoptotic activity was used as a guide to purify the active compound(s) present in *T.violacea* leaf extract. Stock solutions (20mg/ml) of each of the fractions were prepared in DMSO and Tween 20 and stored at -20°C. 5% DMSO and 1%TWEEN 20 was used because DCM and Hexane extracts were not soluble in water. However, the effect of the DMSO and TWEEN 20 were tested on the cells (data not shown). A panel of human cell lines (HepG2, HT29, MCF7, H157 and KMST6) was treated for 24hours with a range of concentrations (0.06 to 2mg/ml) of the organic solvent extracts. The induction of apoptosis was measured using the APOP*ercentage*^{TM} apoptosis assay as previously described. Figure 5.1 shows the dose responses of H157 cells treated with four solvent extracts. Figure 5.2 shows the quantification of apoptosis in all cell lines treated with 2mg/ml of four organic solvent extracts

The results obtained from the APOPercentage^{TM} apoptosis assay were also used to determine the IC_{50} values (Table 5.2). The responses of the different cell lines varied based on the solvent extract that was used. In general, the hexane and DCM extracts were more active compared to the methanol and 50% methanol extracts. The MCF7 cell line was more resistant to the effects of the solvent extracts. The IC_{50} values for the hexane, methanol and 50% methanol extracts were more than 2mg/ml when tested on MCF7 cells. The H157 cell line was

the most sensitive cell line. The IC_{50} values for the hexane, DCM, methanol and 50% methanol extracts on H157 cells were 38mcg/ml, 7mcg/ml, 302 mcg/ml and 1289 mcg/ml, respectively.

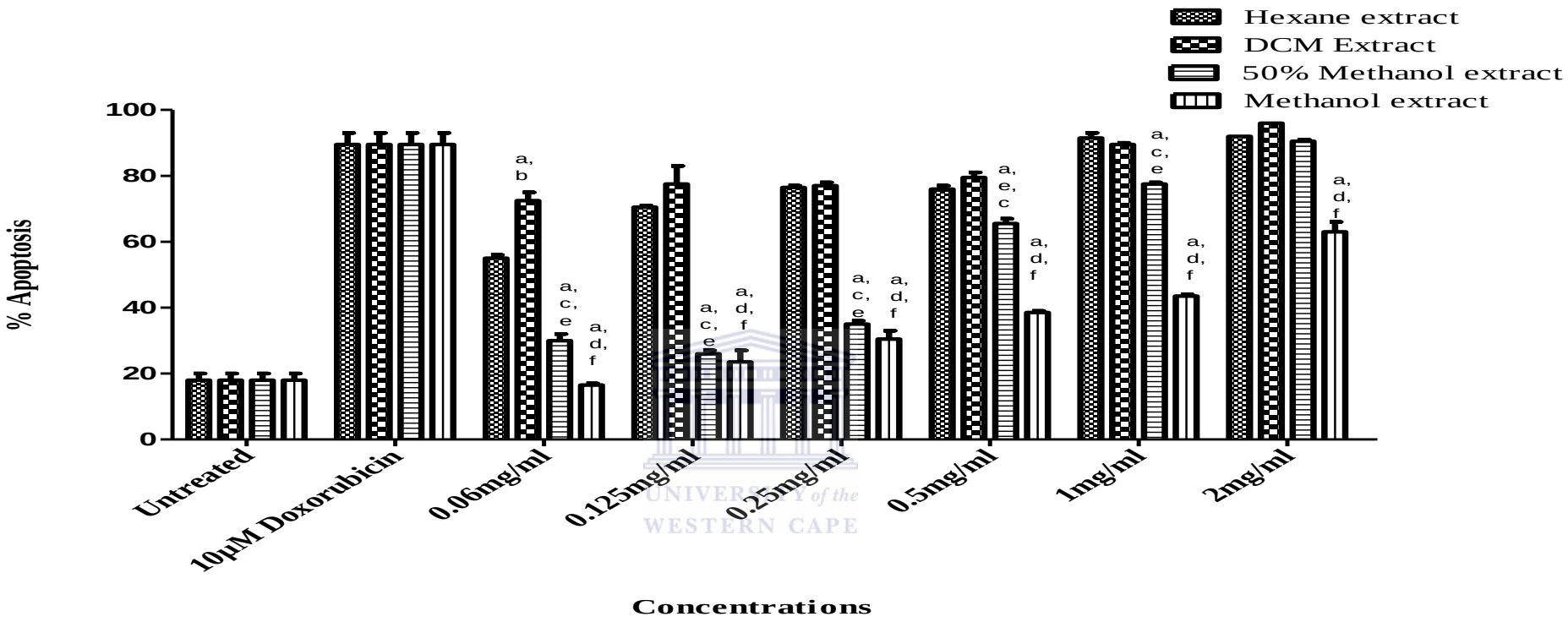


Figure 5.1: Quantification of apoptosis induced by organic solvent extracts of *T.violacea* leaves. H157 cells were treated for 24hours with increasing concentrations (0.06 to 2mg/ml) of the different organic solvent extracts of *T.violacea* leaves. Apoptosis was quantified by the flow cytometry using the APOPercentage^{TM} assay. The graph indicates the percentage of apoptosis cells observed with different treatments. Each bar is the mean ±SEM of three values. Letter a represent significance difference between untreated and the extracts ($p < 0.05$); b represent significant difference between Hexane and DCM extracts ($p < 0.05$), c represent significant difference between hexane and 50% Methanol extracts ($p < 0.05$), d represent significant difference between hexane and Methanol extracts ($p < 0.05$),); e represent significant difference between DCM and 50% Methanol extracts ($p < 0.05$),); f represent significant difference between DCM and Methanol extracts ($p < 0.05$).

Table 5.2: IC_{50} values for organic solvent extracts of *T. violacea* leaves.

	Hexane (mg/ml)		DCM (mg/m)		50% Methanol / Water (mg/ml)		Methanol (mg/ml)	
	Extract	Selectivity index	Extract	Selectivity index	Extract	Selectivity index	Extract	Selectivity Index
H157	0.04	47.75	Cell Line	41	1.09	1.18	0.30	3.27
HT29	0.07	27.29	0.01	41	> 2	ND	>2	ND
HepG2	0.72	2.65	0.15	2.73	>2	ND	1.12	0.88
MCF7	>2	ND	>2	ND	>2	ND	>2	ND
KMST6	1.19	1	0.41	1	1.29	1	0.98	1

The cells were treated for 24hours with increasing concentrations of different organic extracts of the *T .violacea* leaves. Apoptosis was quantified using the APOPercentage^{TM} assay. GraphPad prism software was used to determine the IC_{50} values for the organic solvents extracts on the four human cell lines.

ND means not determined

IC_{50} 2.0mg/ml was considered to be inactive

Selectivity index >1 is indicating high selectivity

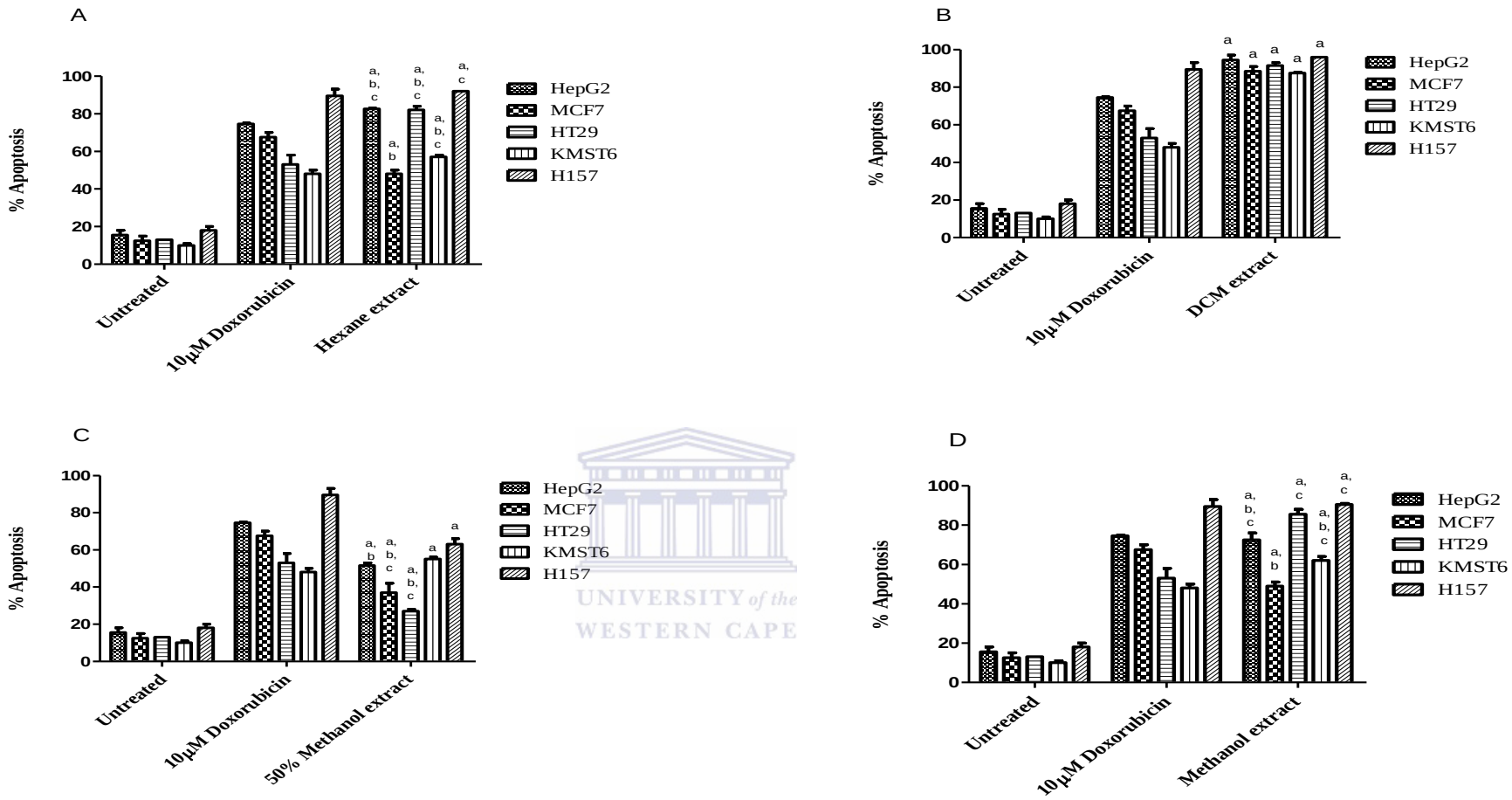


Figure 5.2: Quantification of apoptosis of different organic solvent extracts *T.violacea* leaves treated on different cell lines. Several cancer cell lines (HepG2, HT29, MCF7 and H157) and the non-cancerous human cell line (KMST6) were treated with 2mg/ml of organic solvent fractions of *T. violacea* leaves for 24hours. Apoptosis was quantified by the flowcytometry using APOPercentage^{TM} assay. Each graph indicates the percentage of apoptosis cells observed with different treatments. A: Hexane extract; B: DCM; C; Methanol extract; D: 50 % methanol. Each bar is mean±SEM of three values. Letter a represent significance difference between untreated and extracts ($P<0.05$); b represent significant difference between the most sensitive cell line and other cell lines ($P<0.05$), c represent significance difference between the most resistant cell line and other cell lines ($P<0.05$)

5.4 Assessing the activation of caspase-3 in response to treatment with a DCM extract of *T.violacea* leaves

The APOPercentage^{TM} apoptosis assay showed that the activity of the DCM extract was significantly higher in comparison to the hexane, methanol and 50% methanol extract. To further investigate the pro-apoptotic activity of the DCM extract, the activation of caspase-3 was assessed using a PE-conjugated anti-active caspase-3 monoclonal antibody and flow cytometry as described in section 2.4.4. The results are displayed in Figure 5.3 and show that DCM extracts induced caspase-3 activation in all 5 cell lines tested in this study.

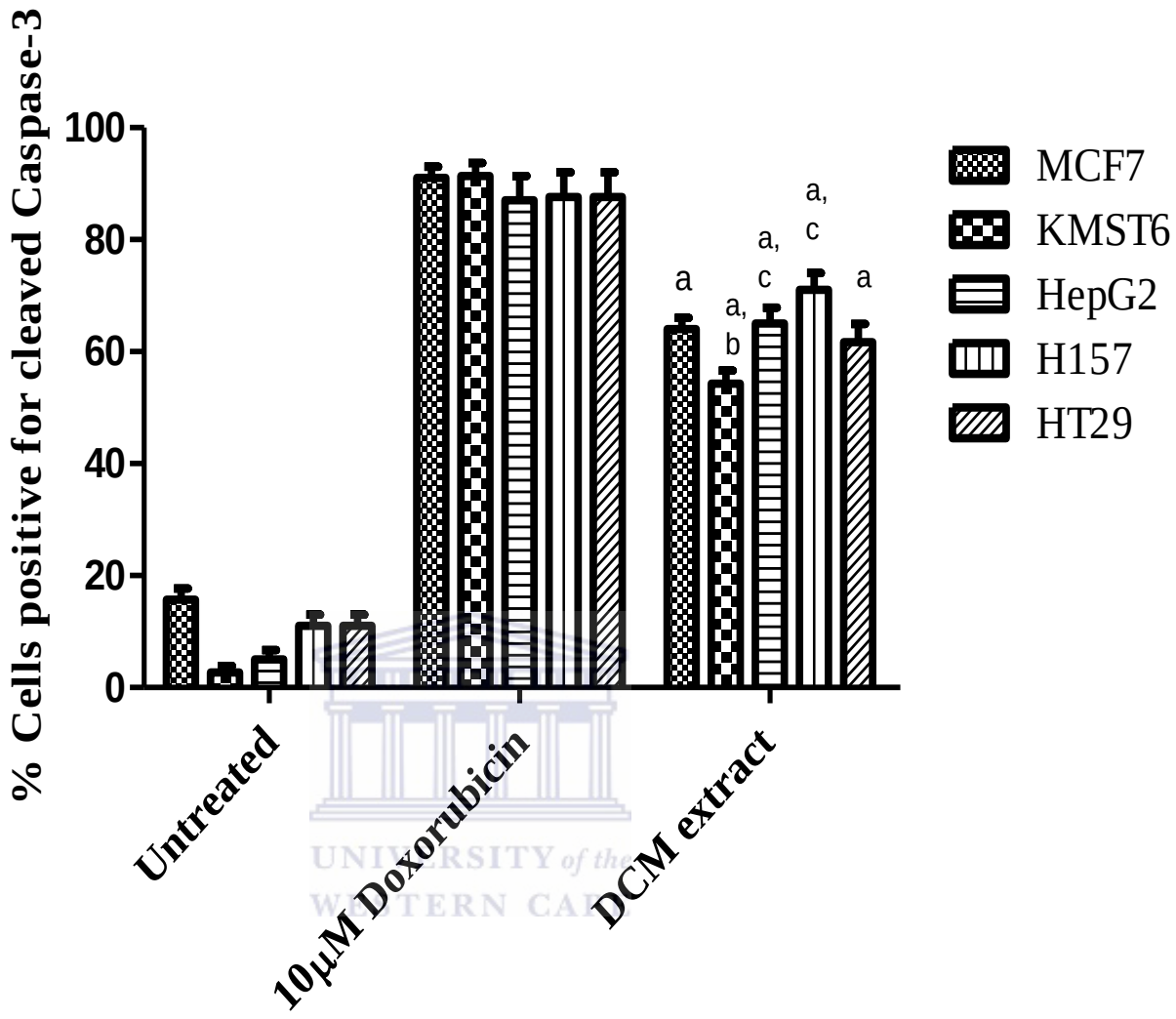


Figure 5.3 The activation of caspase-3 in response to treatment with the DCM extract of *T.violacea* leaves. Four human cell lines (HepG2, HT29, MCF7 and H157) and the non-cancerous human cell line (KMST6) were treated for 24hours with 2mg/ml of DCM extract of *T. violacea* leaves and 10μM . doxorubicin (positive control). The cells were fixed and stained with an anti-active caspase-3-PE antibody. Cell fluorescence was measured by flow cytometry. Each bar is means±SEM of three values. Letter a represent significance difference between untreated and extracts ($P<0.05$); b represent significant difference between the most sensitive cell line and other cell lines ($P<0.05$), c represent significance difference between the most resistant cell line and other cell lines ($P<0.05$)

5.5 Evaluating the production of ROS in cells treated with the DCM extract

The ROS assay was used to evaluate the production of ROS in a panel of cell lines treated with the DCM extract. The DCFH-DA probe was used to measure the production of ROS as described in section 2.4.6. The results showed that the 2mg/ml of the DCM extract induced significant ROS production in HT29, KMST6, HepG2, and H157 cells. MCF7 cells were the most resistant cell line (Figure 5.4).

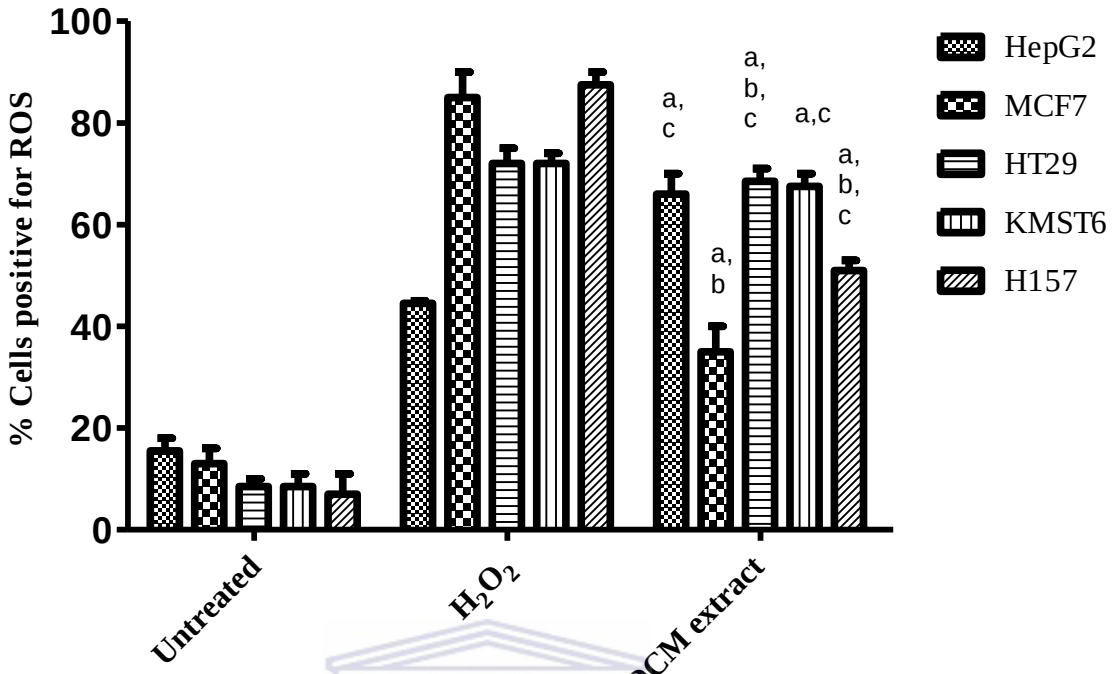


Figure 5.4: The effects of the DCM extract of *T. violacea* on ROS production. Four human cancer cell lines (HepG2, HT29, MCF7 and H157) and a non-cancerous human cell line (KMST6) were treated for 24hours with 2mg/ml of DCM fraction of *T. violacea* leaves and 1% of H_2O_2 (positive control). ROS production was measured by flow cytometry using the DCFH-DA probe. Each bar is mean ±SEM of three values. Letter a represent significance difference between untreated and extracts ($P<0.05$); b represent significant difference between the most sensitive cell line and other cell lines ($P<0.05$), c represent significance difference between the most resistant cell line and other cell lines ($P<0.05$)

5.6. Discussion

The aim of this chapter was to perform bioactivity-guided fractionation of *T. violacea* leaf extract. The fractions containing the bioactive compounds were identified using bioassays that specifically measure the activation of apoptosis. The induction of apoptosis was evaluated using the APOP*ercentage*^{TM} assay, the caspase-3 assay and the ROS production assay. The extracts were tested on four different human cancer cell lines (HepG2, HT29, MCF7 and H157) and a non-cancerous human cell line (KMST6).

As shown in Table 5.1, 50% methanol / water and methanol extracts showed the highest yield 20 and 25 % respectively, while the DCM and hexane fractions yielded 15% and 7%, respectively. A report by Herode, *et al* (2003) showed that percentage of extraction yield could be affected by particle size of the sample, the temperature extraction and the ratio of solvent to sample (Herode, *et al.*, 2003). The average extraction yield in 50% methanol and methanol were 20% and 25%, respectively, indicating that the procedure were efficient in dissolving cellular components, soluble fibres and possibly some high molecular weight polysaccharides. Hexane and DCM had the lowest yield but still showed higher bioactivity. This could support the report by Pineolo *et al.* (2004) that the chemical characteristics of the solvent, the method used during the extraction process as well as different structural and compositional aspects of the natural products may show a distinct behaviour. Higher efficiency in the extraction of solutes is not automatically directly related to the biological activity of the plant species. Differences in the solubility of the active compound(s) present in the plant can account for the variations in the biological activities.

In general, compounds extracted with methanol and 50% methanol solvents were not as active as the compounds extracted with hexane and DCM solvents. This suggests that the nature of the compounds with pro-apoptotic activity tends to be non-polar. H157 cells were susceptible to the effects of the hexane and DCM extracts, while MCF7 and KMST6 cells were more resistant to the effects of the hexane and DCM extracts. This is an interesting finding because MCF7 cells were highly susceptible to effects of the water extract of *T. violacea* leaves (Chapter 4).This may suggest that the compound(s) that were present in the water extract of *T. violacea* leaves and were able to induce apoptosis in MF7 cells have variable polarity and hence extractability, in other words, the compounds in the water extract are polar and those in the hexane and DCM are non-polar such that the MCF7 cells are more sensitive to the polar compounds in comparison with the non-polar compounds in *T.violacea* leaves

Based on the IC_{50} values obtained for *T. violacea* water extract, the hexane extract and the DCM extract, it can be said the less polar fractions exhibited greater potency. As an example, a comparison of the IC_{50} values for *T. violacea* extract (0.28mg/ml) and hexane (0.04mg/ml) on H157 cells show that there is a significant increase in the bioactivity. The IC_{50} value for the DCM extract decreased to 0.01mg/ml. This increase in bioactivity was also observed for HT29, HepG2 and KMST6 cells. It can be concluded that the hexane and DCM extracts contain most of the bioactive compounds with pro-apoptotic activity. As a result, it was decided to use the DCM extract for further study.

The caspase-3 assay (figure 5.3) showed that DCM extracts of *T. violacea* induced significant levels of caspase-3 activation particularly in the four cancer cell lines with the highest bioactivity exerted on the H157 cell lines. This result confirms that DCM extracts of *T. violacea* have significant pro-apoptotic effects. Caspases belongs to a family of cysteine proteases that are involved in the induction of cell death by apoptosis. Caspase-3 has been implicated as a key regulator of apoptosis (Alan & Reiner, 1999). Apoptosis is sometimes accompanied by the activation of caspases (Nicholson & Thornberry, 1997).

ROS play a vital role in the induction of apoptosis in many cells (Barbora & Ales, 2011). During apoptosis process, ROS generation and DNA damage usually occur and the increase of ROS level and DNA damage could act independently or they may be affected by one another (Barbora & Ales, 2011). Studies have shown that an increase in cellular concentration of oxidant species to a level that overcomes the endogenous antioxidant system leads to oxidative stress and this result in protein, lipid as well as DNA damage thus triggering apoptosis mechanism (Barbora & Ales, 2011). Figure 5.4 shows the production of ROS in several cell lines. The result indicates 68.5%, 67.5%, 66%, 51% and 35% ROS production for HT29, KMST6, HepG2, H157 and MCF7 cells, respectively. The bioactivity of DCM extract of *T. violacea* was highest on HT29 cell line and lowest on the MCF7 cell lines.

This study showed that the DCM and hexane extracts of *T. violacea* leaves contain compounds with pro-apoptotic activity in all the cell lines tested in this study. However, DCM extracts appeared to be more bioactive than hexane extracts. Furthermore, it was shown that the bioactivity of the DCM and hexane extracts were higher compared to the

water extracts of *T. violacea*. The bioactivity of methanol and 50% methanol were also higher than water extract. However, the bioactivity of the extract varied depending on the cell line that was used, demonstrating the selectivity of the compounds to induce apoptosis in certain cell.

CHAPTER SIX: CHEMICAL CHARACTERIZATION OF DCM FRACTIONS OF *T.VIOLACEA* LEAVES

Table of Contents/Outline

6.0 Background:

There has been a rapid advancement in the study of plants (phytochemicals) and herbal products for the past few decades. There has also been a vast increase in the sales of herbal products such as *Allium sativum*, *Hypericum perforatum*, *Spirulina*, *Echinacea angustifolia*, *Ginkgo biloba* and *Silybum marianum* (Chauhan, *et al.*, 2011). The chemistry of natural products involves three areas, which includes methods of isolation, structural elucidation and synthetic approaches. Structural elucidation involves determination of chemical structure of an uncharacterized substance such as natural products which is normally determined through chromatographic and mass spectroscopic techniques. Nuclear Magnetic Resonance (NMR spectroscopy is the gold standard for the characterization. In previous chapters, it was concluded that the DCM extract of *T. violacea* leaves contains some pro-apoptotic compound(s). Therefore the objective of this study was to isolate these compound(s) by bio-activity guided fractionation using column chromatography and to characterize the compounds by NMR and GC-MS. The processes involved in isolation of these compounds are illustrated in figure 6.1.

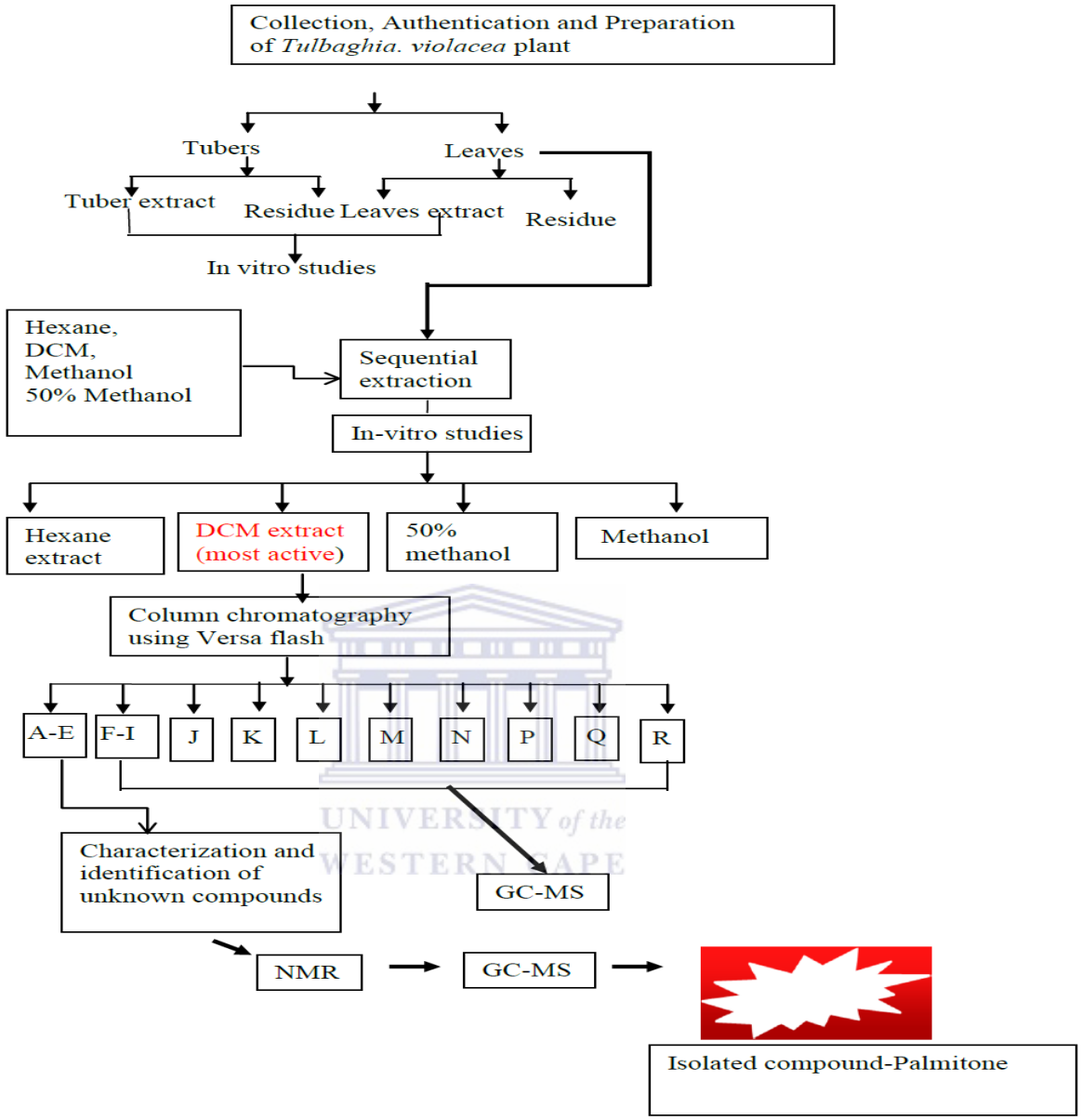


Figure 6.1: Flowchart of the bio assay-guided isolation procedures

KEY S:

A-E – 90% HEX/ DCM; F-I - 25% DCM/HEX; J - 75% DCM/HEX; K -75% DCM/HEX;L -75% DCM/HEX; M -90% DCM/HEX; N=100%EA; P=90%EA/MEOH; Q=50%EA/MEOH; R =50%EA/MEOH TO 100% MEOH

DCM extract were fractionated into several sub-fractions using Versaflash® column chromatography (described in section 2.4.3). The DCM extract was fractionated into 10 fractions (A-E, F-I, J, K, L, M, N, P Q and R). One compound precipitated from fractions A-E as a white amorphous crystalline compound which was coded TVD-001. It was not readily soluble in water but soluble in chloroform. Characterization of TVD-001 was done by a combination of spectroscopic techniques viz: IR, NMR spectroscopy and Mass spectrometry.

6.1 Structural elucidation of TVD-001

The IR spectroscopy was done as described in section 2.3.9 the IR spectrum of TVD-001 is shown in Figure.6.1. The absorption band observed at 1704 cm^{-1} (stretching) confirmed the presence of a carbonyl group (C=O) which may be part of ketone, aldehyde or ester functional groups. The other notable absorption frequencies between 1100 and 900 cm^{-1} (stretching) revealed the presence of C-C bond. The signals at 2923 and 1704 correspond to the presence of alkanes. The spectrum also has an OH at 393cm^{-1}.

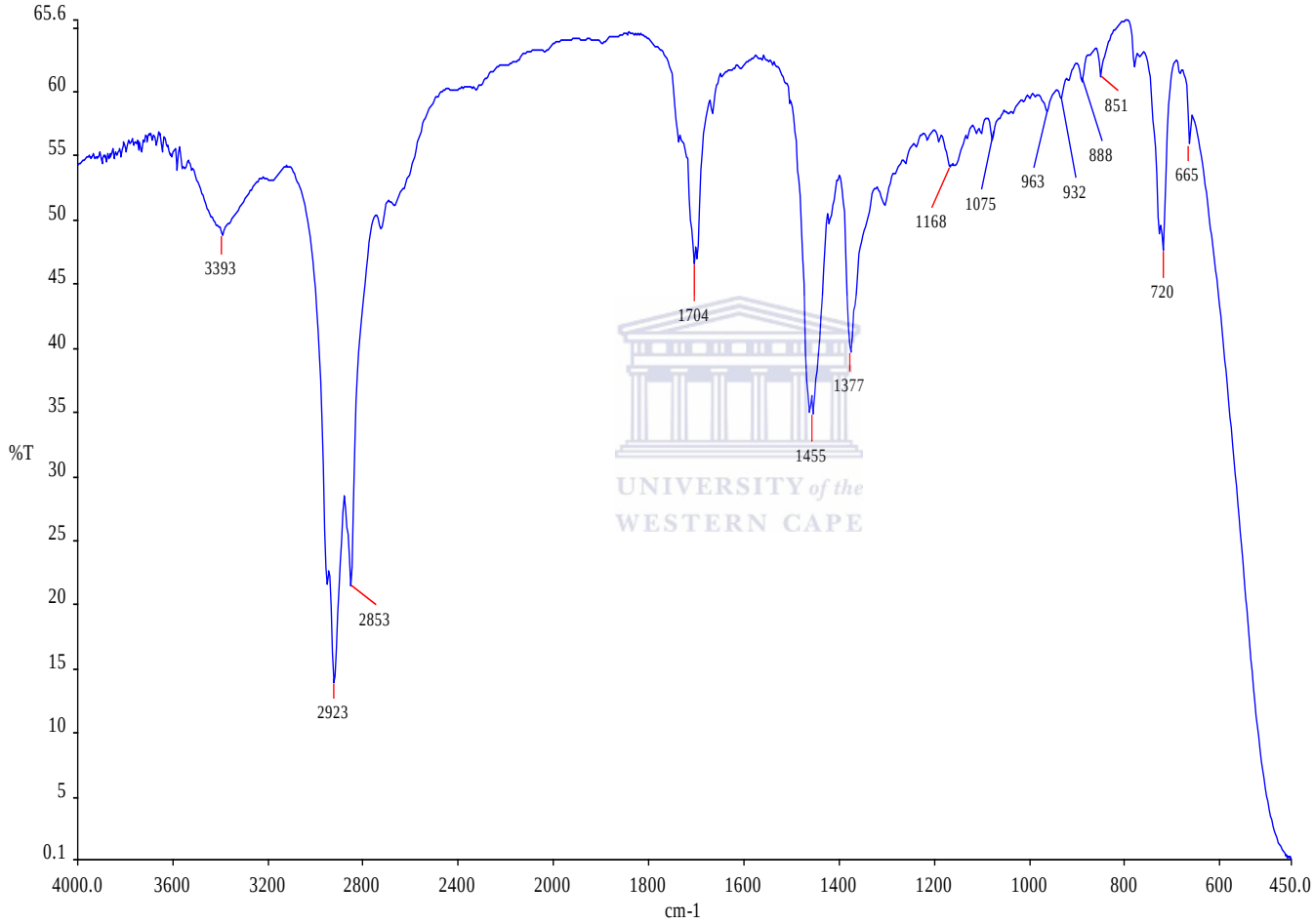


Figure 6.2 IR spectrum of TVD-001

6.1.1 Nuclear Magnetic Resonance

NMR spectra were generated at Stellenbosch University, Central Analytical facility (CAF). The ^{1}H and ^{13}C NMR spectra for the compounds are given in Figures 6.3 and 6.5. The assignment of protons and carbons peaks for the compound as described in section 2.4.6.1 was done using COSY plot (figure 6.4). The summary of the ^{1}H and ^{13}C NMR chemical shifts for TVD-001 is shown in Table 6.1. The proton NMR spectrum of the compound showed a set of four signals. The most downfield signal was a triplet centred at δ2.38, a position characteristic of protons deshielded due to close proximity to a carbonyl group. The splitting pattern suggests the presence of a methylene group on the opposite side to the carbonyl group. The multiplets at δ1.55 and at δ1.25 are suggestive of repeating methylene chains. The most upfield signal appearing as a triplet at δ0.88 is due to terminal methyl protons split by the presence neighbouring methylene protons.

In the ^{13}C NMR spectrum (figure 6.4), the presence of a carbonyl group was confirmed by the appearance of a signal at 211.8 ppm. The other signals of interest included carbon peaks due to the pairs: C-15 and C-17 at 42.8ppm, as well as C-1 and C-31 at 14.1ppm. ^{13}C NMR showed 16 signal peaks between δ14 and δ212. The downfield signal at δ211.7 is typical of ketonic carbon, and the signal at δ76.8 is germinal to it. Methylene carbons were seen between δ22 and δ42 as can be expected (Dudley & Ian, 2007). Many of these appear to be arranged in an alkane chain format as can be seen from their closeness in chemical shifts. A methyl is evident from the signal at δ14.1.

Based on the NMR data, it was apparent that TVD-001 was a long chain alkane with a terminal methyl and a ketone. There was no evidence of the presence of methines confirming the presence of the ketone and absence of aldehyde.

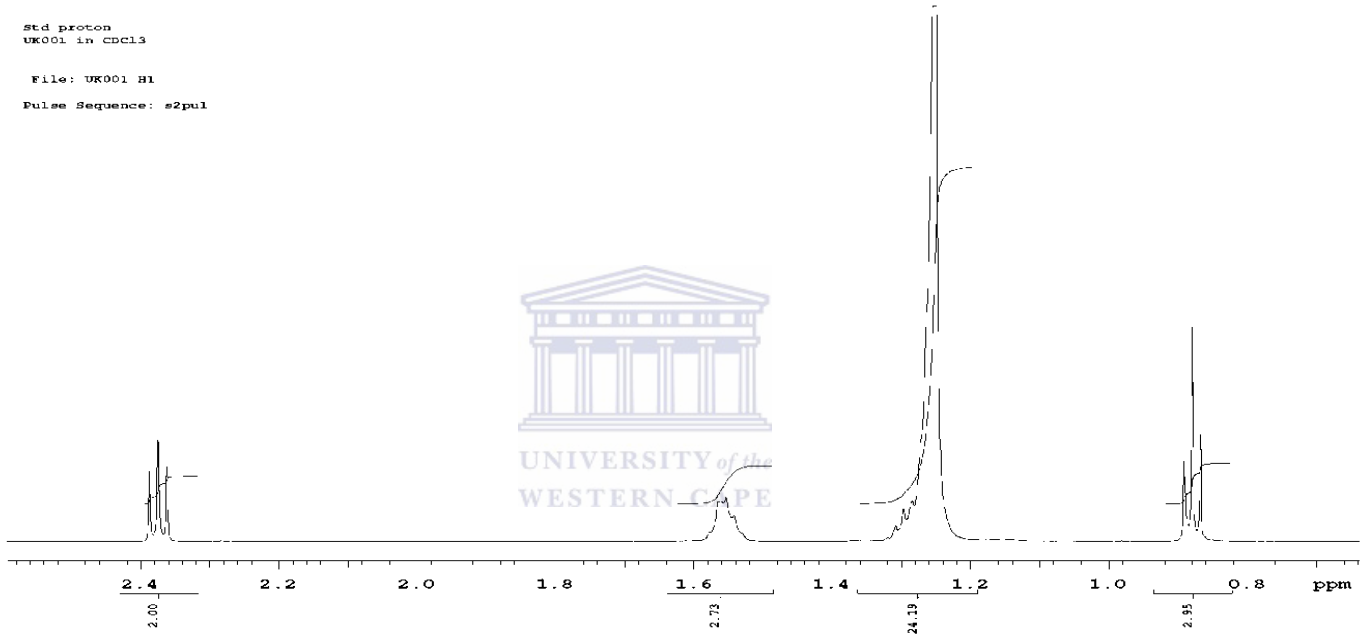


Figure 6.3 **^{1}H NMR spectrum of TVD-001**

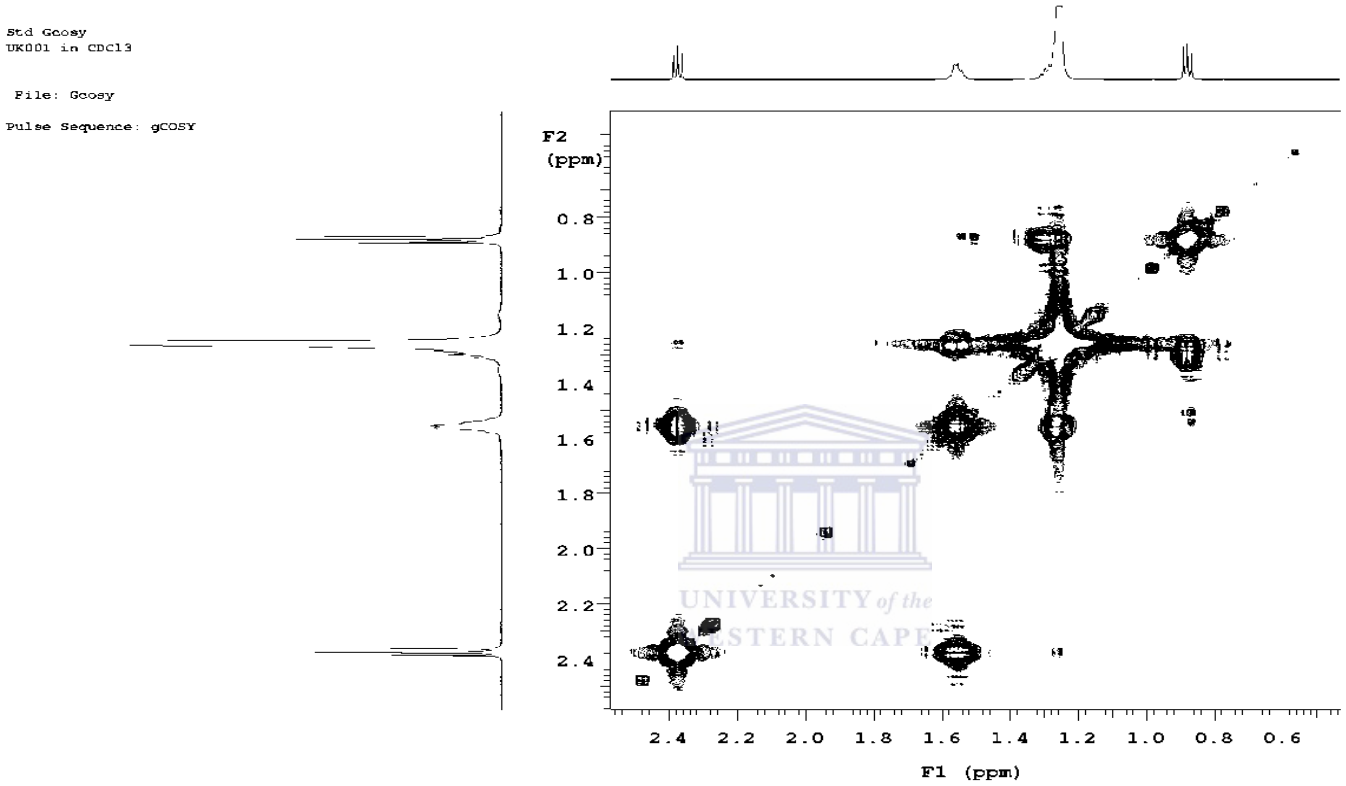


Figure. 6.4 COSY plot for ^{1}H NMR chemical shifts assignment.

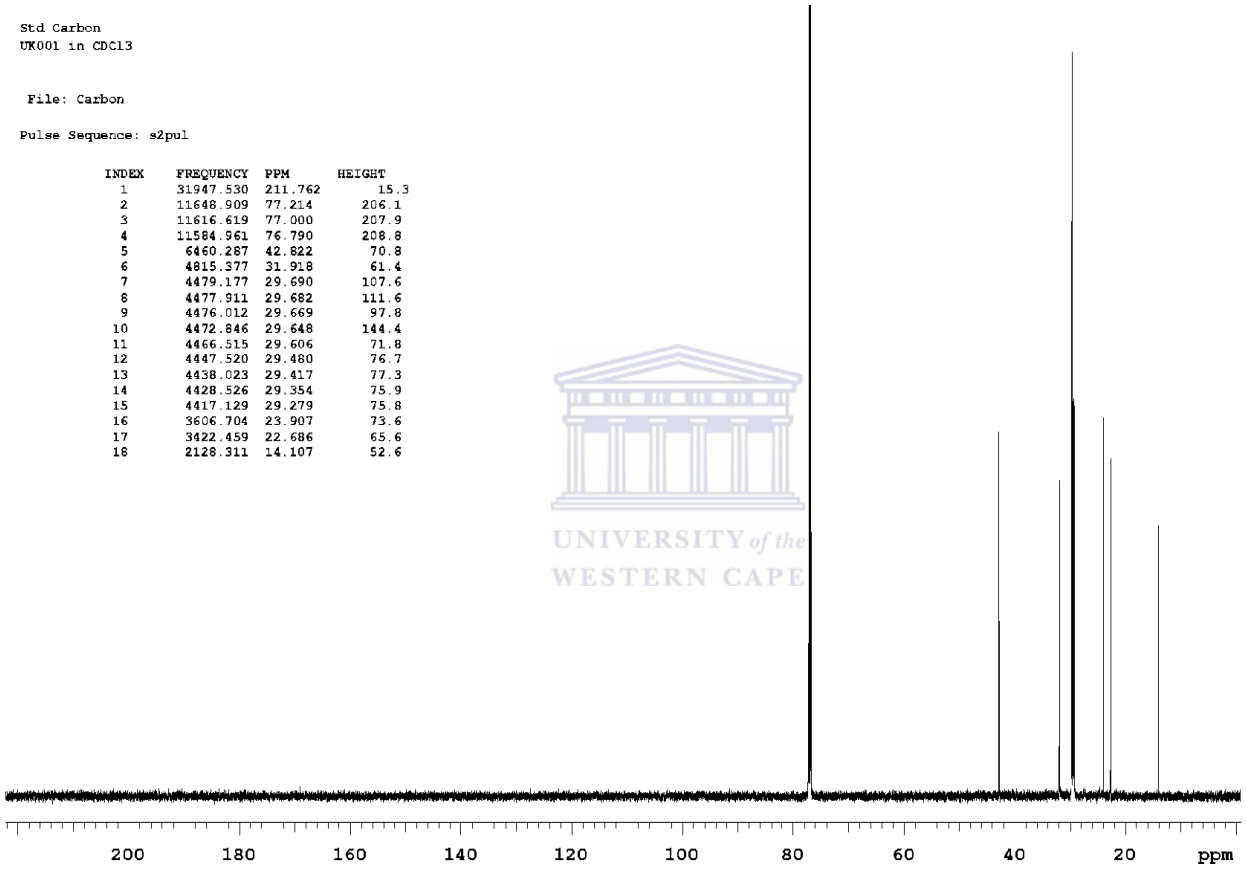


Figure. 6.5 ^{13}C NMR spectrum of TVD-001.

6.1.2 Mass spectrometry

The structure was confirmed by mass spectral analysis (Figures.6.6), which showed the molecular ion peak at m/z 450. Other peaks were at m/z 255, 239 (base peak), 194, 127, 96, 85,71,57 and 43 and 29 (Figure 6.6). The peaks at m/z 255 and 239 are due to the repeated double transfer from different position of the alkyl chain as a result of McLafferty rearrangements (Rontani, *et al.*, 1990). The transition from m/z 255 to 239 was due to loss of CH_4, and from 239 to 194 there was loss of CH_3CHO; from 127 to 96 there is the loss of 31 CH_3O, and 85 to 71, to 57 to 43 to 29 is consistent with the repeated loss of methylenes. The fragmentation pattern confirms the presence of methyl, ketone and a long chain alkane. Further the fracture from a molecule with m/z of 450 to a base peak of almost half is suggestive of a dimeric arrangement.

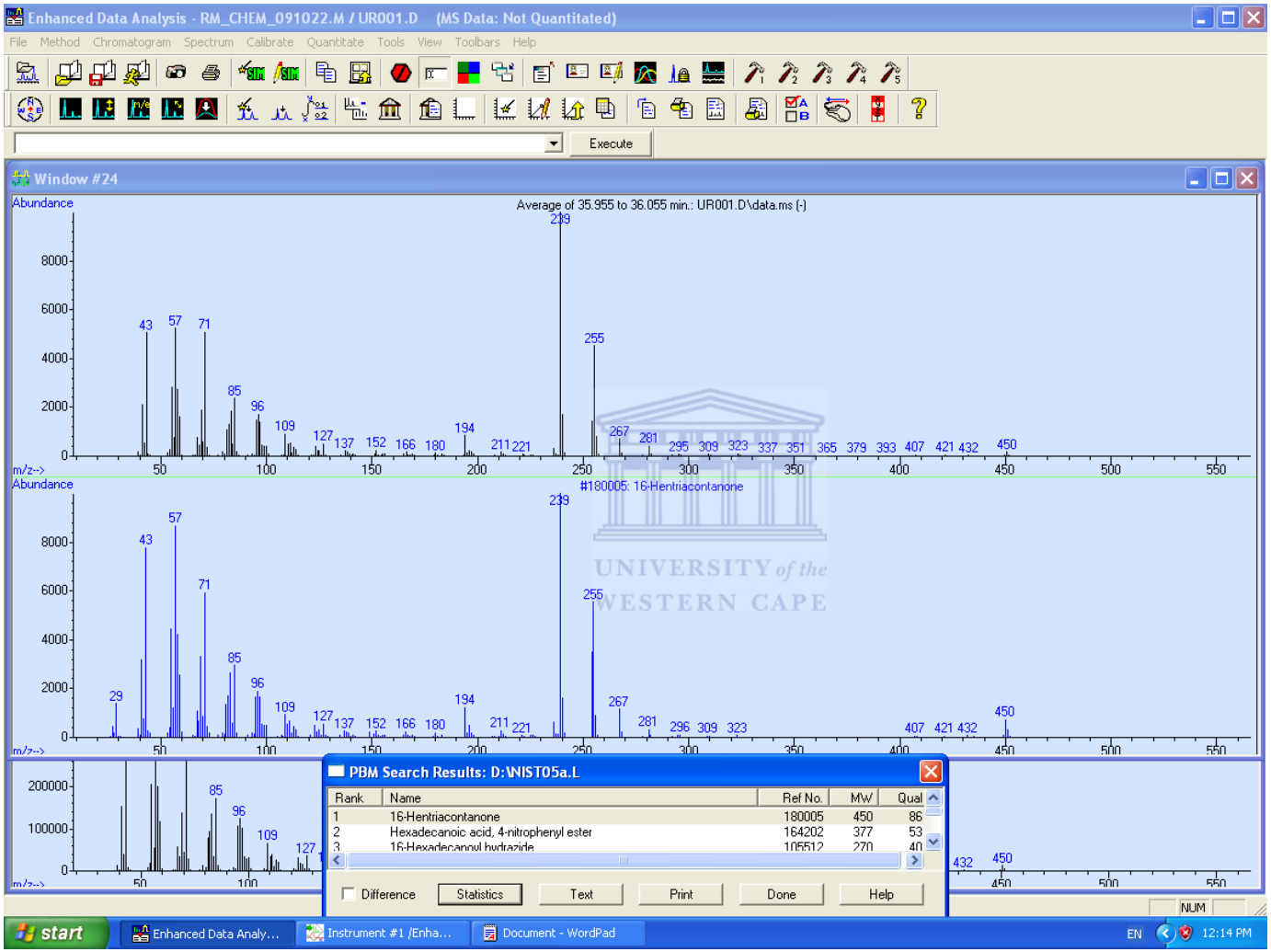


Figure 6.6 From the foregoing, the literature and a search of the Wiley library of the GC-MS, it was concluded that TVD-001 was 16-hentriacontanone. The structure is shown in Figure 6.2 and the NMR results matched to the atomic positions in Table 6.1.

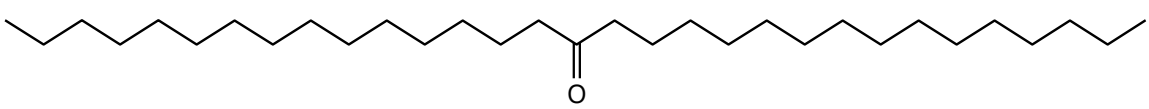


Figure 6.7 Structure of palmitone (16-hentriacontanone) ($C_{31}H_{62}O$)

Table 6.1: Summary of the ^{1}H and ^{13}C NMR chemical shift for 16-hentriacontanone

^{1}H NMR (ppm)	Position	Multiplicity	^{13}C NMR(ppm)	Position
2.3	H-15	*Triplet*	42.8	C-15
1.5	H-14	*Multiplet*	23.9	C-14
1.2	H-2 – H-13	*broad multiplet*	22.7	C-2
0.88	H-1	*Triplet*	14.1	C-1

16-hentriacontanone (more commonly known as palmitone) is a common hydrophobic dialkyl ketone with reported hypnotic and anticonvulsant activity (González-Trujano, *et al.*, 2001). It has previously been isolated from fruits of *Cassia fistula* (Thirumal, *et al.*, 2012), the fern genus *Adiantum caudatum*, (Pan, *et al.*, 2011), *Santalum album* (Pradeep, *et al.*, 2011). This is the first report of its present in this species and genus as far as we could

ascertain. It was present in abundance in the DCM fraction of *T. violacea* leaves as it precipitated from several sub-fractions.

6.2. Discussion

Drug solubility plays a vital role in drug disposition and poor solubility has been known as one of the causes of various drug development failures (Lokamatha, *et al.*, 2010). This is one of the problems that were encountered in this study. Although the DCM extract was soluble and highly active on cancer cells, the sub-fractions of the DCM extract was not soluble and could therefore not be tested. It is possible that the various compounds present in the sub-fraction of the DCM extract are required to maintain the solubility of the DCM extract. However, one compound (16-hentriacontanone) was present in high concentration in sub-fraction A-E. The structure of the compound 16-hentriacontanone was elucidated using NMR spectroscopy and Mass Spectrometry.

The assignment of protons and carbons peaks for the compound was done using COSY and was also assisted by comparison with the literature (González-Trujano, *et al.*, 2001). The proton NMR spectrum of the compound (Figure 6.3) showed a set of four signals. The most downfield signal appeared as a triplet centred at δ2.38, a position characteristic of protons located at a carbon adjacent to a carbonyl group, while the splitting pattern suggests the presence of a methylene group on the opposite side to the carbonyl group. This therefore confirms the presence of methylene protons at H-15 and H-17. The next signal observed as a multiplet at δ1.55 represents the methylene protons located at H-14 and H-18. The methylene protons H-2 to H-13 and H-19 to H-30 all appeared as a multiplet at δ1.25. Finally, the most upfield signal appearing as a triplet at δ0.88 is due to the terminal methyl protons H-1 and H-31, and the triplet is caused by the splitting effect of methylene protons H-2 and H-30

(http://www2.chemistry.msu.edu/faculty/reusch/VirtTxtJml/Spectrpy/nmr/nmr1.htm)

.

The structure was finally confirmed by mass spectral analysis which showed the molecular ion peak at m/z 450 with a molecular formula of $C_{31}H_{62}O$ (González-Trujano, *et al.*, 2001).

Furthermore, the prominence of the base peak at m/z 255 is characteristic of a fragment ion which incorporates the resonance-stabilized and positively charged carbonyl group. However, it is important to mention that palmitone is a hydrophobic aliphatic ketone (Cano-Europaa, *et al.*, 2010). In conclusion, all the spectral data which has been gathered confirm the structure of 16-hentriacontanone. It is not known if this compound has any anti-cancer or pro-apoptotic. However, it is one of the compounds identified in the DCM extract of *T.violacea* leaves.

CHAPTER SEVEN: BIO-GUIDED FRACTIONATION OF METHANOL EXTRACTS OF *T.VIOLACEA* LEAVES

Table of Content/ Outline

7.1 Background:

It was shown in previous chapters that the pro-apoptotic bioactivity of the DCM extracts of *T. violacea* leaves was significantly higher than the activities observed for the hexane, methanol and 50% methanol extracts. In an attempt to isolate the bioactive compound(s), the DCM extract was subjected to column chromatography, which produced 10 sub-fractions. However, these sub-fractions were insoluble in water and DMSO and as result testing could not be done in the bioassays. Since methanol is more polar than DCM, the expectation was that the compounds extracted with methanol might be more water-soluble and suitable for biological testing. Therefore, the objective of this section was to further fractionate the methanol leaf extract using column chromatography and evaluate activity using the APOPercentage^{TM} assay.

7.2 Percentage yield

Methanol fraction was obtained from the sequential extraction made on the leaves (see Figure 7. 1). The fractions were further purified using Versaflash as described in section 2.4.6. The solvents were chosen (see Figure 7.1) based on solvent polarity as follows and in this order: 100% Hexane (MF1), 50% Hex/DCM (MF2), 100% DCM (MF3), 70%DCM/Methanol (MF4), 50% DCM/Methanol (MF5), 20% DCM/Methanol (MF6) and finally with 100% Methanol (MF7). The fractions were spotted on TLC to monitor the purification processes. The percentage yield was determined as previously described (section 2.4.5).

Table 7.1 Percentage (%) recovery for individual methanol sub-fractions from a methanol extract of *T. violacea* leaves.

Solvent fractions	% Recovery
MFI	0.20
MF2	0.61
MF3	25.33
MF4	21.67
MF5	26.3
MF6	18.8
MF7	2.20

The methanol extract obtained from the leaves of *T. violacea* was further separated using different solvent combinations. The percentage yield of organic solvent of *T. violacea* leaves are calculated as %Yield = (weight of dried extract / weight of dried sample) x 100.

7.3 Apoptotic activity of methanol sub-fractions

A dose-response study was performed to establish if sub-fractions (MF3 to MF7) of a methanol extract of *T. violacea* leaves had any pro-apoptotic activity. The IC_{50} values for these sub-fractions were also determined. MF1 and MF2 were pooled together with MF3 because TLC showed that similar compounds are present in these sub-fractions. The cell panel was treated for 24hours with the sub-fractions and apoptosis was measured using the APOP*ercentage*^{TM} assay. The IC_{50} values were determined as previously described (table 7.2). Figure 7.1 shows the result obtained for cells treated for 24hours with 2mg/ml of the sub-fractions. Table 7.2 shows that HT29 cells were the only cells that were susceptible to the effects of sub-fraction. The IC_{50} values for the sub-fractions were higher than 2mg/ml for MCF7, H157 and KMST6 cells. It is only MF3 and MF4, which showed significant activity on HT29 cells

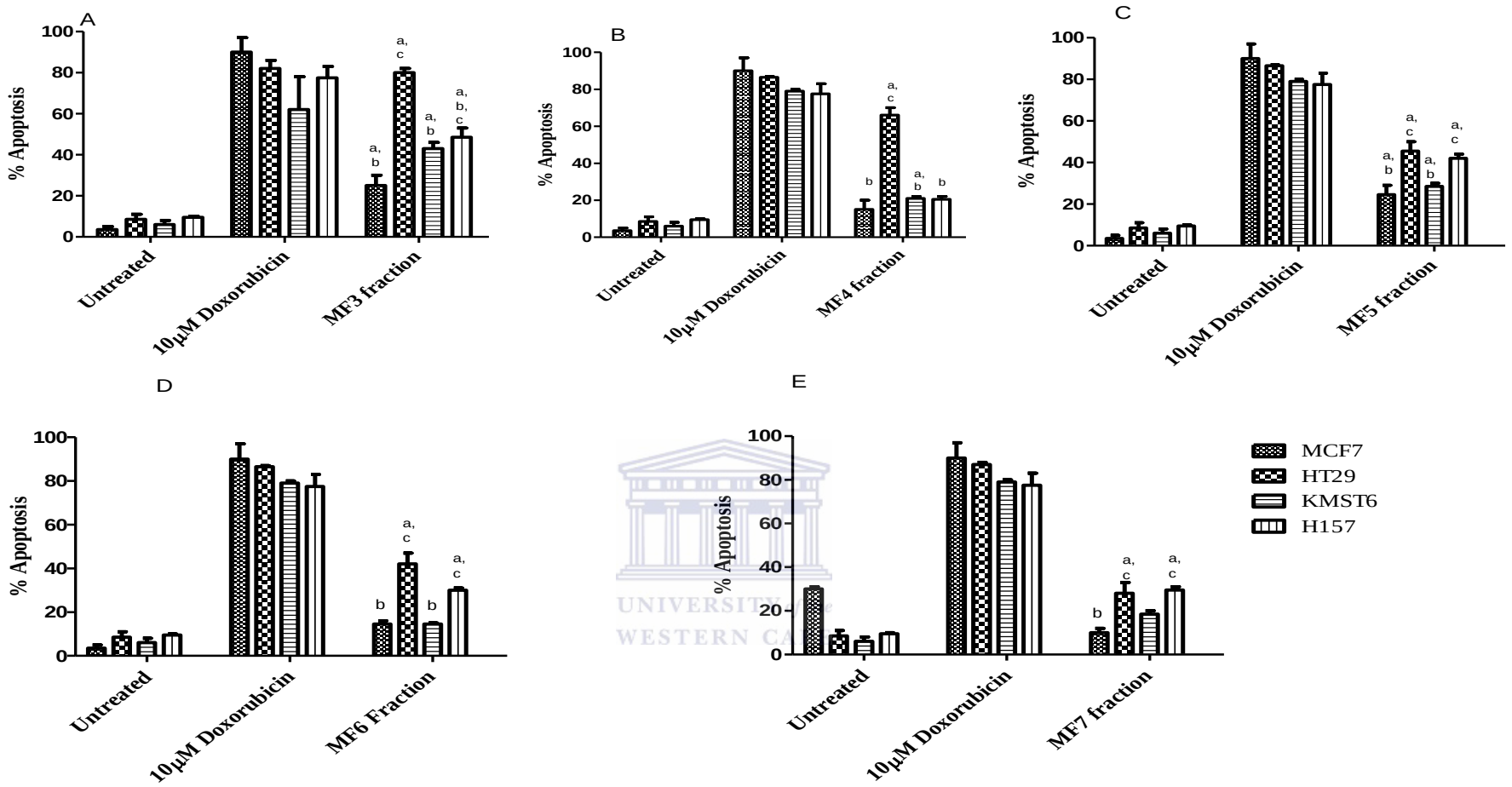


Figure 7.1: **Quantification of apoptosis of different sub-fractions of methanol extract of *T.violacea* leaves in different cell lines.**

A panel of cell lines were treated for 24hours with 2mg/ml of methanol sub-fractions (MF3 to MF7). Apoptosis was quantified by the flow cytometry using APOPercentage^{TM} assay. The graph indicates the percentage of apoptosis cells observed with different treatments. A: MF3; B: MF4; C; MF5; D: MF6; E: MF7 fractions. Each bar is mean ±SEM of three values. Letter a represent significance difference between untreated and extracts ($p < 0.05$); b represent significant difference between the most sensitive cell line and other cell lines ($p < 0.05$) and c represents most resistant cell line and other cell lines ($p < 0.05$)

Cell Line	MF3 (mg/ml)	MF4 (mg/ml)	MF5 (mg/ml)	MF6 (mg/ml)	MF7 (mg/ml)
HT29	0.6	1.6	>2	>2	>2
MCF7	>2	>2	>2	>2	>2
H157	>2	>2	>2	>2	>2
KMST6	>2	>2	>2	>2	>2

Table 7.2 IC_{50} values for methanol sub-fractions on different human cell lines. Several cancer cells (HT29, MCF7 and H157) and the non-cancerous human cell line (KMST6) were treated for 24hours with various concentrations of the sub-fractions (MF3 to MF7) of the *T.violacea* leaf extract. Apoptosis was quantified by APOP*ercentage*™ dye and analyzed using the flow cytometry. The experiments were done in triplicate. The IC_{50} values were calculated using GraphPad prism software.

7.4 Discussion

In this study, the goal was to test sub-fractions (MF3 to MF7) of the methanol extract of *T.violacea* leaves for pro-apoptotic activity in order to identify the sub-fraction that contains apoptotic compound(s). This was done using the APOP*ercentage*^{TM} apoptosis assay. Table 7.1 shows the percentage yield for each of the fractions. MF1 and MF2 produced very low yields. Based on the TLC results the composition of MF1, MF2 and MF3 were very similar. Consequently, MF1, MF2 and MF3 were combined. Yields of 25.33, 21.67, 26.3, 18.8 and 2.61% were observed for MF3, MF4, MF5, MF6 and MF7, respectively (table 7.1).

The APOP*ercentage* data shows that HT29 cells are the only cells that are susceptible to the sub-fraction of methanol extract of *T. violacea*. Furthermore, only MF3 and MF4 were able to induce significant levels of apoptosis in HT29 cells (Figure 7.1). A comparison of IC_{50} values obtained for the methanol extract (Chapter 6) and the sub-fractions of the methanol extract (MF3 to MF7) on HT29 cells shows that the bioactivity was lost during sub-fractionation. The IC_{50} value for the methanol extract on this cell lines was 0.27mg/ml, while the IC_{50} values for the two bioactive sub-fractions, MF3 and MF4 were 0.6mg/ml and 1.6mg/l, respectively.

It can be concluded that the sub-fractionation of the methanol extract resulted in reduced bioactivity. The bioactive compounds were possibly lost during column chromatography. Alternatively, these compounds work in a synergistic fashion in the methanol extract and the separation of the bioactive compounds by column chromatography has a negative impact on the bioactivity.

CHAPTER EIGHT: CHEMICAL FINGERPRINTING OF *T. VIOLACEA* FRACTIONS

Table of Content/ Outline

8.1 Background:

Phytochemical studies usually involve a combination of the use of different analytical instruments, including nuclear magnetic resonance (NMR), X-ray crystallography, mass spectrometry (MS), ultraviolet (UV) and infrared (IR). For the gross analysis (fingerprinting) of complex mixtures, liquid chromatographic separation followed by spectroscopic measurement is suitable. Liquid chromatography coupled to mass spectrometry (LC-MS) in particular has recently emerged as the preferred method of choice as it is more comprehensive and independent of physico-chemical factors, e.g. chromophoric properties (Brandt, *et al.*, 2004). Methanol extract was sub-fractionated into MF1 to MF7 but MF2 to MF7 was used for fingerprinting because MF1 and MF2 were pooled together based on the TLC results, both MF1 and MF2 have similar composition. In this phytochemical study, screening and identification of compounds in the *T. violacea* extract was carried out using LC-MS with a view of generating metabolomic fingerprints. The present study compared the total ion chromatograms (TIC), UV spectra and the LC-MS-MS fragmentation of the four most prominent abundant compounds each of the methanol fractions.

8.2 Metabolic fingerprinting of MF2- MF7

The LC-MS metabolomic fingerprints showed in general a great diversity of compounds eluting between 0.45 min and 14 min. MF2 was the most complex extract with a wide spread of compounds. The early eluting compounds are polar compounds while the late eluting compounds are non-polar. MF3 and MF4 are similar in that they both contain a lot of polar compounds while MF7 is the simplest fraction with fewer compounds. Tables 8.1 and

8.2 show the elution times and molecular mass of the different extracts as well as overlapping constituents. It shows that the fractionation was largely successful in separating out the constituents. MF-2 remained the most complex extracts with a wide number of compounds with masses ranging from 270 to 610. MF5 and MF-6 were the least complex and also showed the most overlap of constituents. Figure 8.1 A to C shows the chromatograms of the extracts from which Table 8.1 has been compiled.

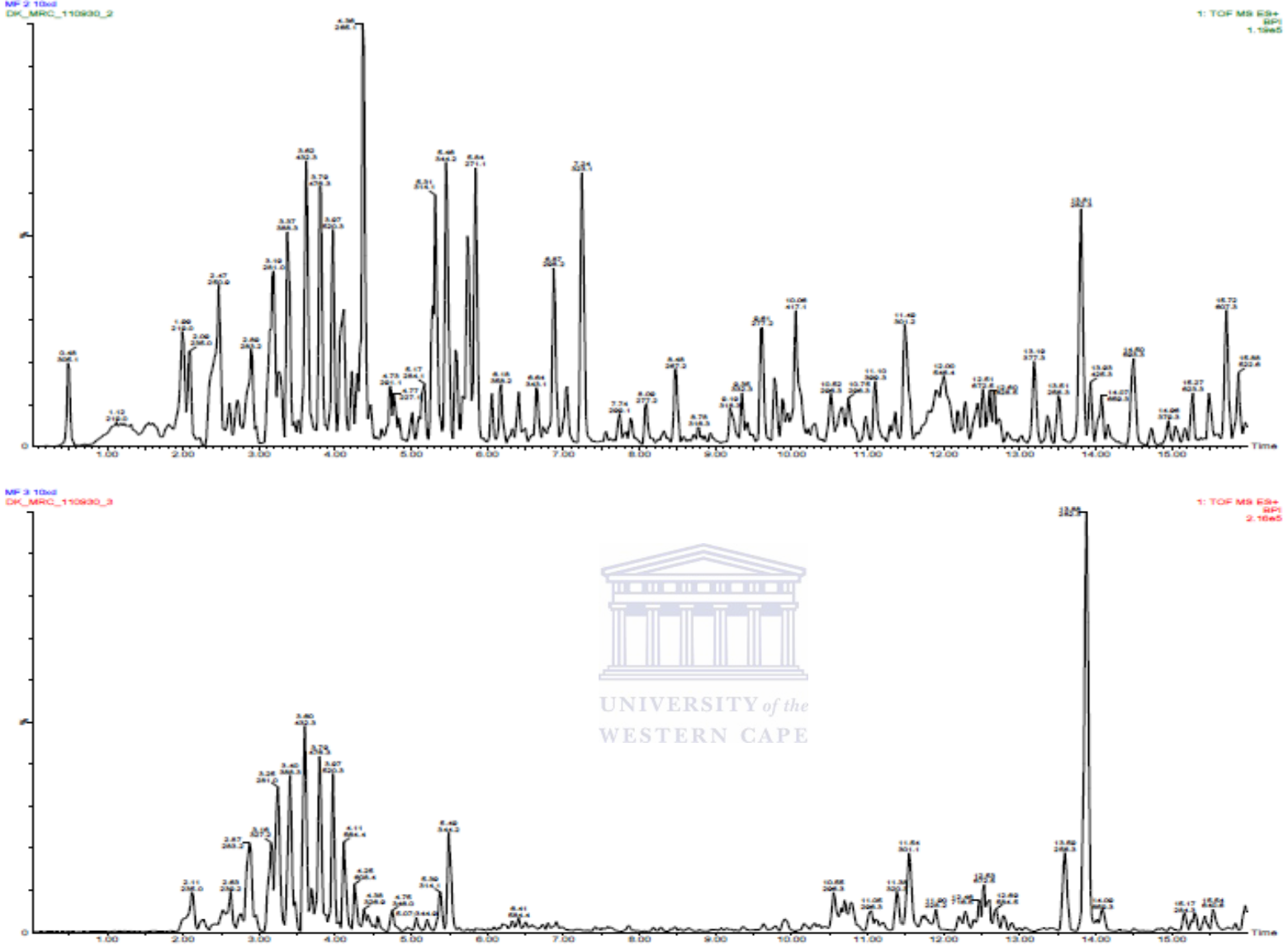


Figure 8.1 A Chromatograms of the methanol fractions (MF2- MF3) of *T.violacea* leaves

Figure 8.1 B Chromatograms of the methanol fractions (MF4—MF5) of *T.violacea* leaves

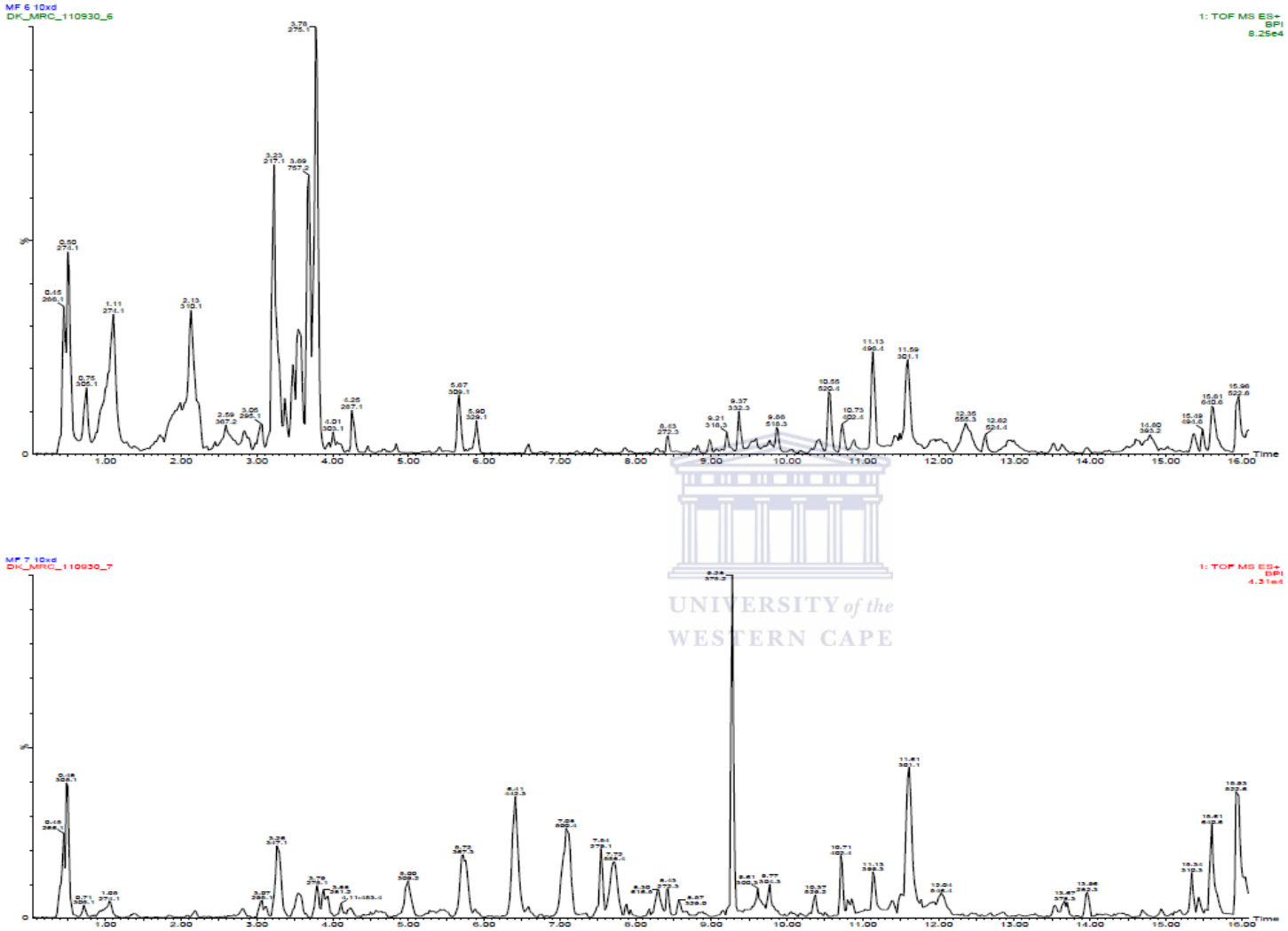


Figure 8.1 C Chromatograms of the methanol fractions (MF6—MF7) of *T.violacea* leaves

Based on the foregoing, attempts were made to elucidate the chemical structures of the four most abundant peaks of each of the fractions (MF2 to MF7) using MS/MS and UV. While this information will not yield a definite answer, it does allow for evidence based speculation on the possible structures within the fractions. Therefore MF2 to MF7 abundance peaks were further analysed to give what was contained in table 8.2. The peaks which were selected in order of their abundance are shown with their retention times, molecular masses and UV max in Table 8.2.

Table 8.2 The retention times, molecular mass and UVmax of selected peaks of the extracts.

Fractions	Retention time(t_R)	M+H amu	UV max
MF2-1	4.32	265.1	230.2, 275.2, 342.2, 405.2
MF2-2	5.46	344.2	237.2, 286.2, 316.2
MF2-3	7.25	323.1	274.2, 364.2
MF2-4	5.86	271.1	241.2, 276.6
MF3-2	3.80	476.3	309.2
MF3-3	5.49	344.2	239.2, 288.2, 317.2
MF3-4	13.88	282.3	does not absorb UV
MF4-1	5.65	319.1	239.2, 286.2, 352.2, 412.2
MF4-2	9.19	318	246.2
MF4-3	9.37	332	247
MF4-4	13.92	282.3	does not absorb UV
MF5-1	3.23	217.1	230.2, 270.2
MF5-2	3.35	231.1	230.2, 271.2
MF5-3	3.69	757.2	266.2, 348.2
MF5-4	4.26	287	265.2, 347
MF6-1	3.23	347.1	230.2, 270.2
MF6-2	3.67	757.2	270.2, 348
MF6-3	3.78	275	230.2, 270.2,348.2
MF6-4	5.67	309.1	242.2, 273.2
MF6-5	11.74	494.4	265.2, 347
MF7-1	3.28	347	274.2, 361.2
MF7-2	6.38	447.2	245.2

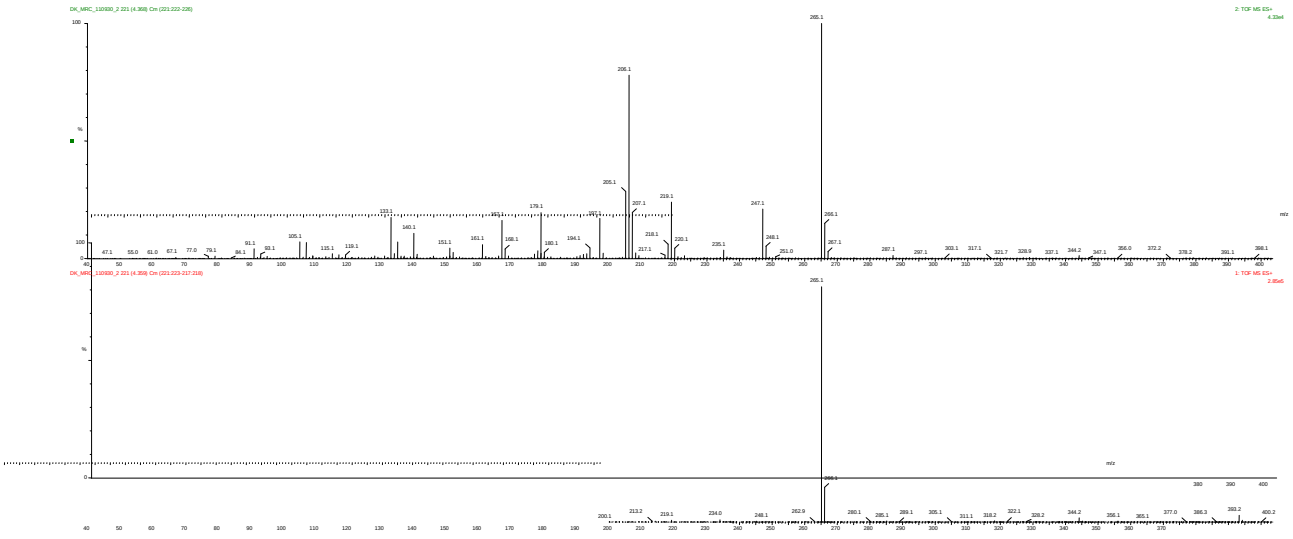


Figure 8.2A showing MS/MS fragmentation of MF2-1

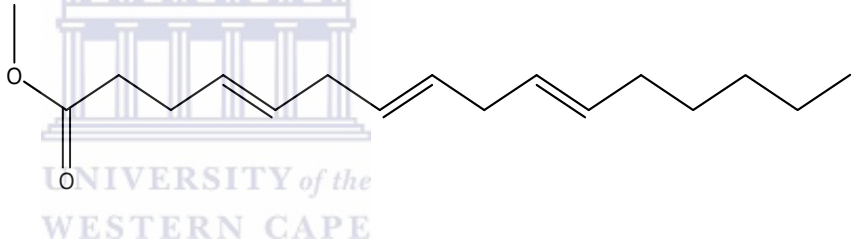


Figure 8.2B showing methyl hexadeca-4,7,10-trienoate which matches with MF2-1.

MF2-1 eluted at t_R 4.32 minute and had a molecular ion at m/z 265 [M+H] which corresponded to $C_{17}H_{28}O_2$. Fragment ions were at m/z 247.1 [M-H_2O], 206.1 [M-C_3H_5], 167.1 [M-C_6H_7], 133.1 [M-C_8H_{11}], 105 [C_8H9] and 91.1 (C_7H_7). The latter fragments at m/z 105 and 91.1 and the repeated loss of 14 (CH_2) or 28 (CH_2–CH_2) is suggestive of a long chain alkane attachment. The UVmax were at 230.2, 275.2, 342.2 and 405.2. The loss of a fragment with 59 amu suggests the existence of a terminal alkyl-methoxyl group (CH_3-CH_2-O-CH_2). MF2-1 is probably a long chain fatty acid or ester. From the literature and as shown for compound in Figure 6.2, hexadecatrienoate compounds also show prominent fragments at 91 and 105 in addition to the loss of CH_2 fragments (http://lipidlibrary.aocs.org/ms/masspec.html).

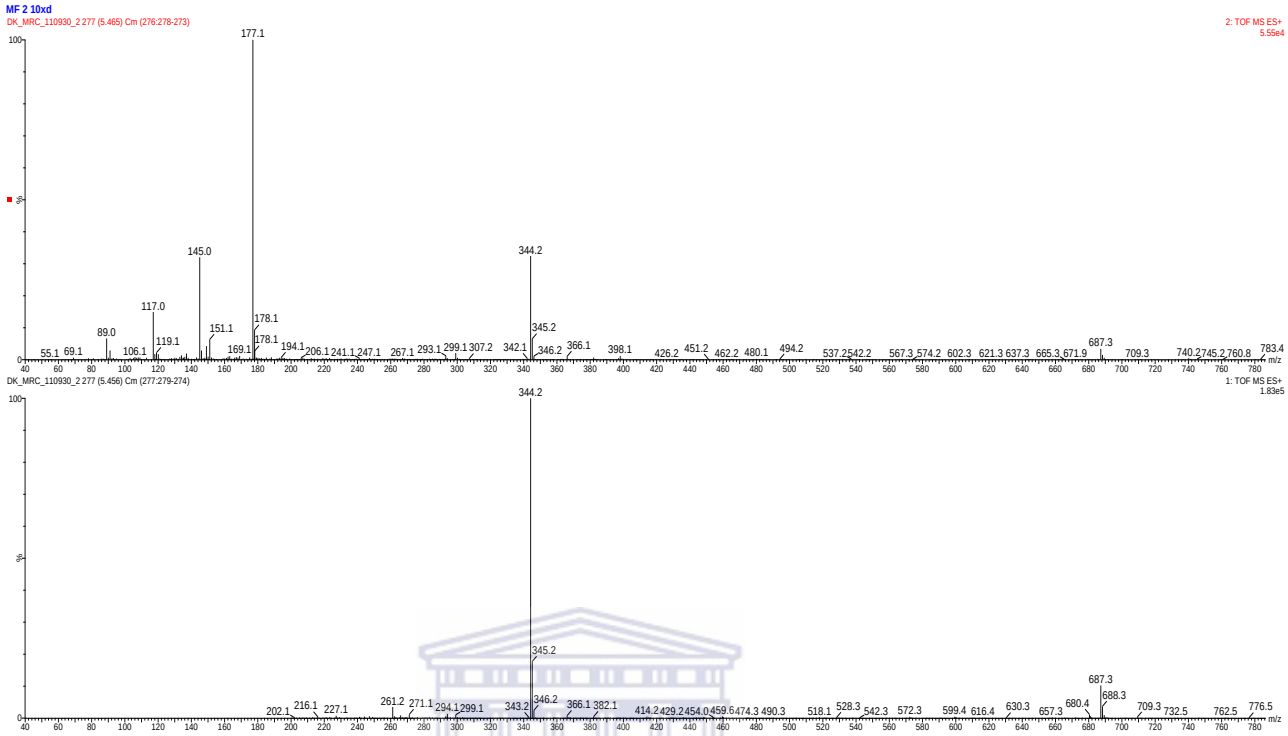


Figure 8.3A showing MS/MS fragmentation of MF2-2

Figure 8.3B showing methyl hexadeca-9, 12-dienoate which matches with MF2-2

MF2-2 eluted at t_R 5.46 minute and a molecular ion of m/z 344.2 [M+H]. The base peak is at m/z 177.1 after the loss of a fragment of m/z 167. This is typical of a fatty acid fragment undergoing McLafferty ion rearrangement. The UV max was 237.2, 286.2 and 316.2. There are also fragments of the loss of 28 amu which is typical of removal of CH_2-CH_2. From the literature, MF2-2 can be predicted as methyl hexadeca-9, 12-dienoate (http://lipidlibrary.aocs.org/ms/masspec.html) (figure 8.3B).

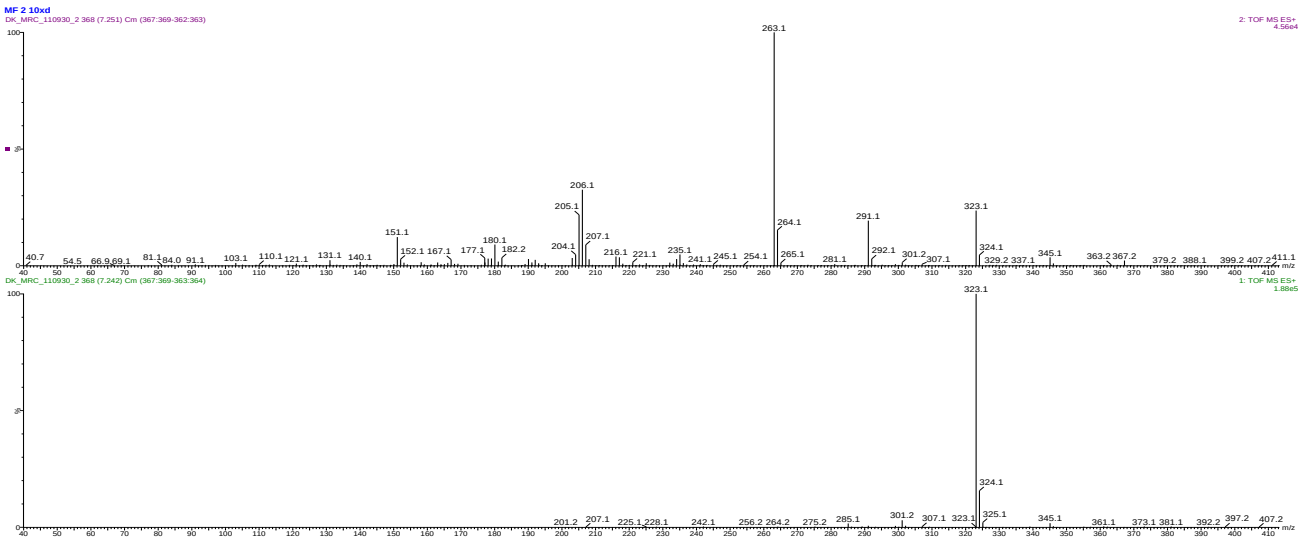


Figure 8.4A showing MS/MS fragmentation of MF2-3

Figure 8.4B showing methyl 8, 11-eicosadienoate which matches with MF2-3

MF2-3 eluted at tR 7.25 minute. It has a molecular ion of m/z 323 [M+H] which correspond to C21H38O2. Major fragments were seen at 291 [M-(CH3OH)], 263.1 [M-C2H4O2], 206 [M-C2H402–C4H9]. MF2-3 is probably long chain fatty acid / ester from the loss of 60 (CH2=C(OH)OH) and 57 (C2H5CO or C4H9). From the literature MF2-3 is likely to be methyl 8, 11-eicosadienoate (methyl icosa-8,11-dienoate) (http://lipidlibrary.aocs.org/ms/masspec.html)**.**

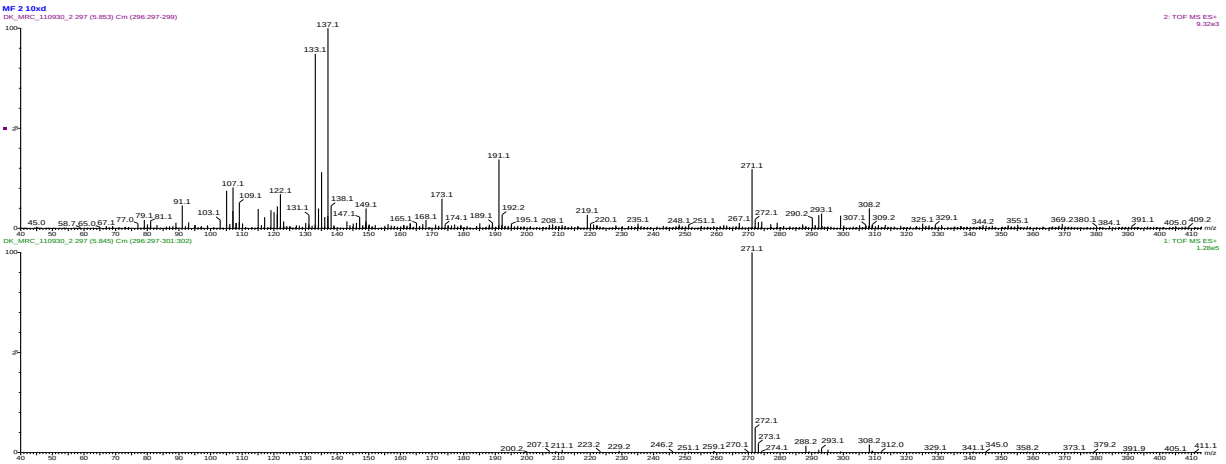


Figure 8.5A showing MS/MS fragmentation of MF2-4

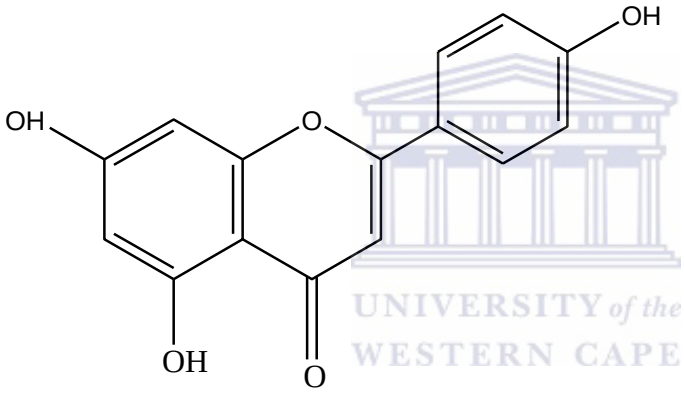


Figure 8.5B showing structure of apigenin which matches with MF2-4

MF2-4 eluted at t_R 5.85 minutes and has a molecular ion of m/ z 271.1 [M+H] which corresponds to $C_{15}H_{10}O_5$ The major fragments are at m/z 271.1 [M+H], 191.1 [M+H-CO] and, 137.1 (base peak), 91.1 [C_7H_7], 107 [C_7H_7O]. The peaks at 91.1 and 107 is indicative of a tropylium and hence the presence of a phenolic ring.

This fragmentation is suggestive of flavonoids and the UV max of 241.2 and 276.2 is typical of these compounds too. Based on the above and comparisons with the literature MF-2.4 was suggestive of apigenin or closely related isomers. Flavonoids have been previously isolated from *T. violacea* (Alfonso, *et al.*, 1996)

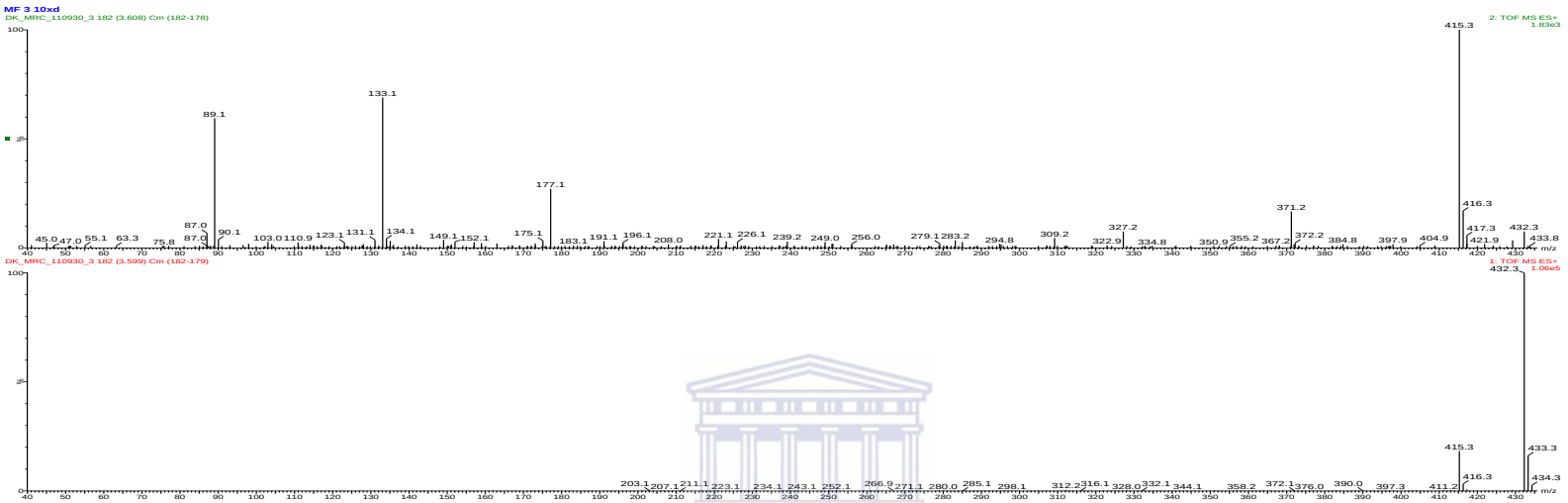


Figure 8.6A showing MS/MS fragmentation of MF3-1

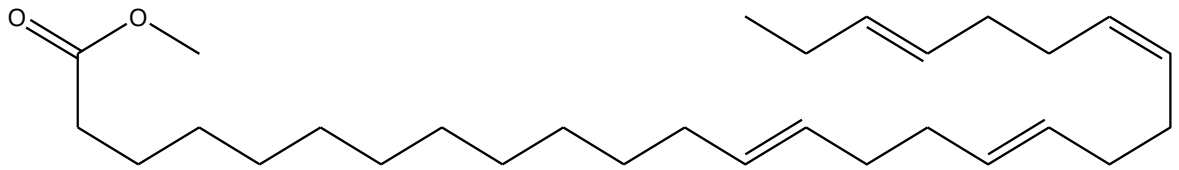


Figure 8.6B showing methyl octacosa-13,17,21,25-tetraenoate which matches for MF3-1

MF3-1 eluted at t_R 3.61 minutes and had a molecular ion of m/z 431 [M+H] which corresponded to $C_{29}H_{50}O_2$. It had poor chromophoric properties. The base peak was at m/z 415. There is repeated loss of fragments of m/z 44 which may be due to CO_2 and also C_3H_8 or aldehydic fragments ($CH_2CH(OH)$). This implies that MF3-1 is probably a long chain fatty acid ester. From the literature the structure can be predicted as octacosa-13, 17, 21, 25-tetraenoate (http://lipidlibrary.aocs.org/ms/masspec.html).

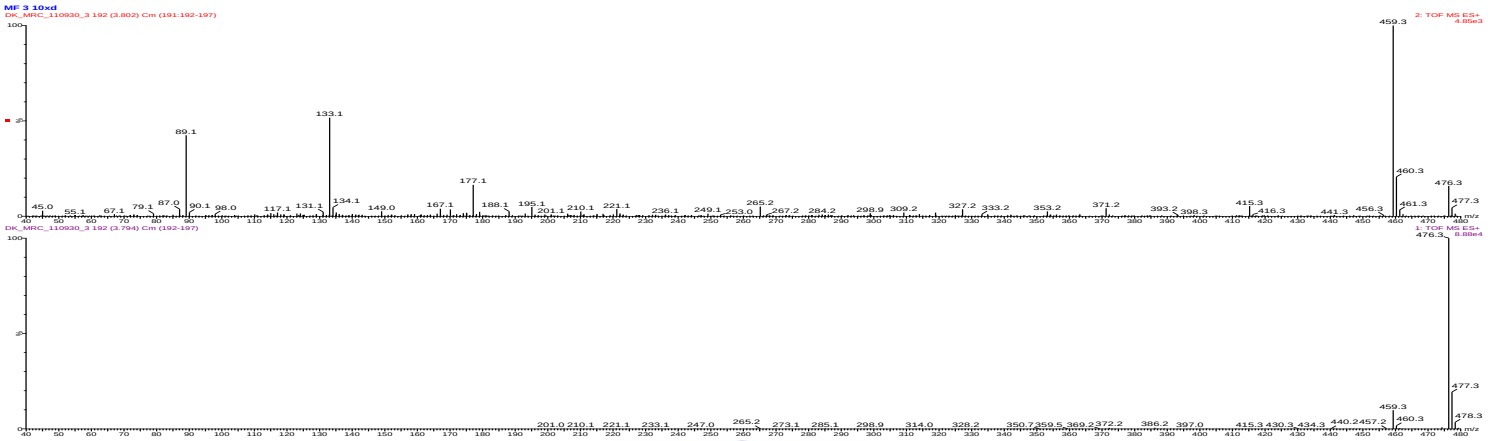


Figure 8.7A MS/MS fragmentation for MF3-2

MF3-2 eluted at t_R 3.80 minutes with a molecular ion of m/z 476 [M+H] and molecular formula of $C_{31}H_{54}O_3$. It appears to be a homologue of MF3-1. Figure 8.7A shows a similar fragmentation pattern with common fragments at m/z 89.1, 133.1, 177.1 and 415.3 (base peak). It differs only in possessing an additional ethoxyl substituent.

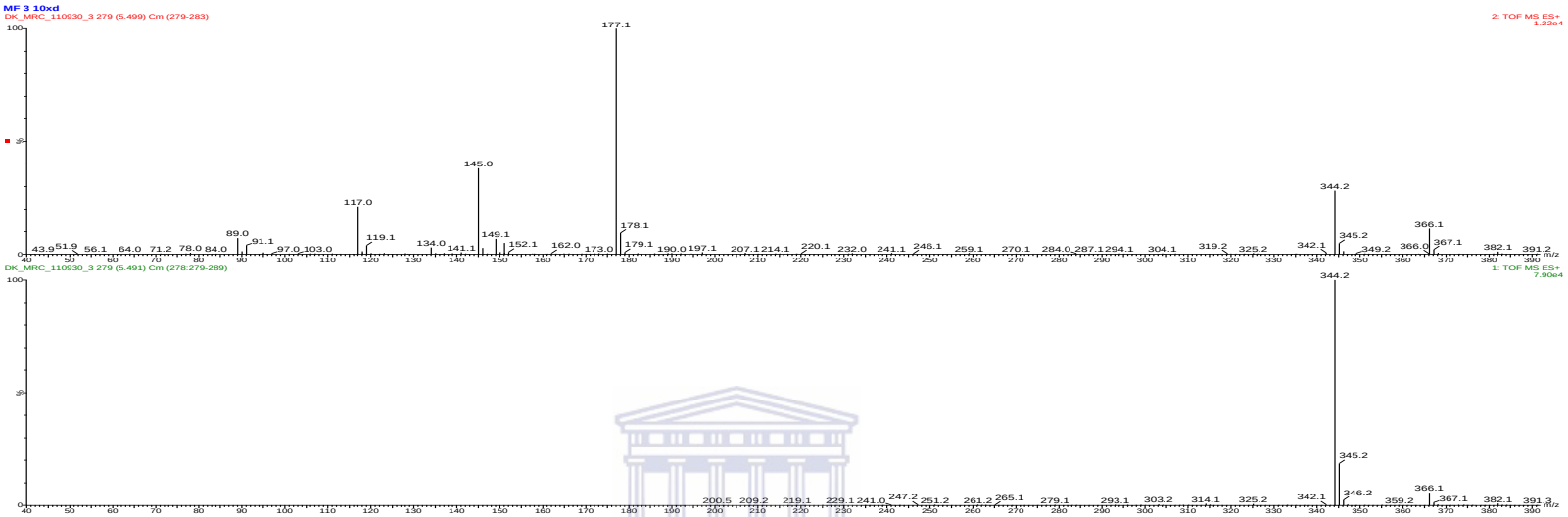


Figure 8.8A showing MS/MS fragmentation of MF3-3

MF3-3 which elutes a at t_R 5.49 minutes with a molecular ion of m/z 344.2 [M+H] and a UVmax 239.2, 288.8 and 317.2. It appears to be a homologue of MF2-2. It shows a similar fragmentation pattern with common fragments at m/z 89, 117, 145 and 177 (base peak). There were also fragments loss of 28 which is typical of removal of 28 (C_2H_4) which is a typical of a fatty acid fragment undergoing McLaffety ion rearrangement

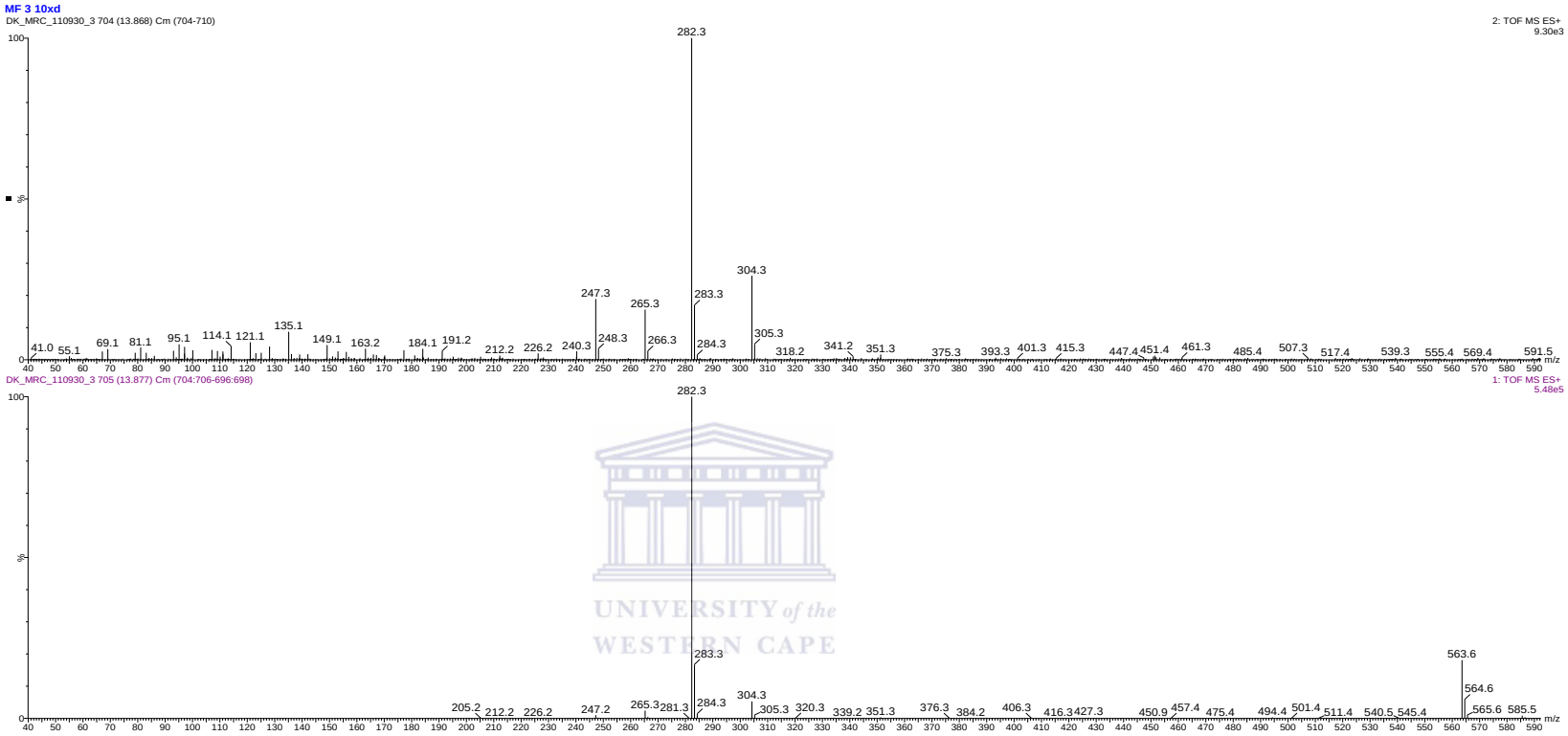


Figure 8.9A showing MS/MS fragmentation of MF3-4

MF3-4 eluted at t_R 13.88 minutes with a molecular ion of m/z 282.2 [M+H]. It has poor UV activity. The major fragments occurred at m/z 247.3 and 265.5 (both due to loss of OH). The fragments at m/z 304 is probably a sodium adduct. The elution time indicates that this compound is non-polar in its nature but the poor fragmentation makes it difficult for further elucidation.

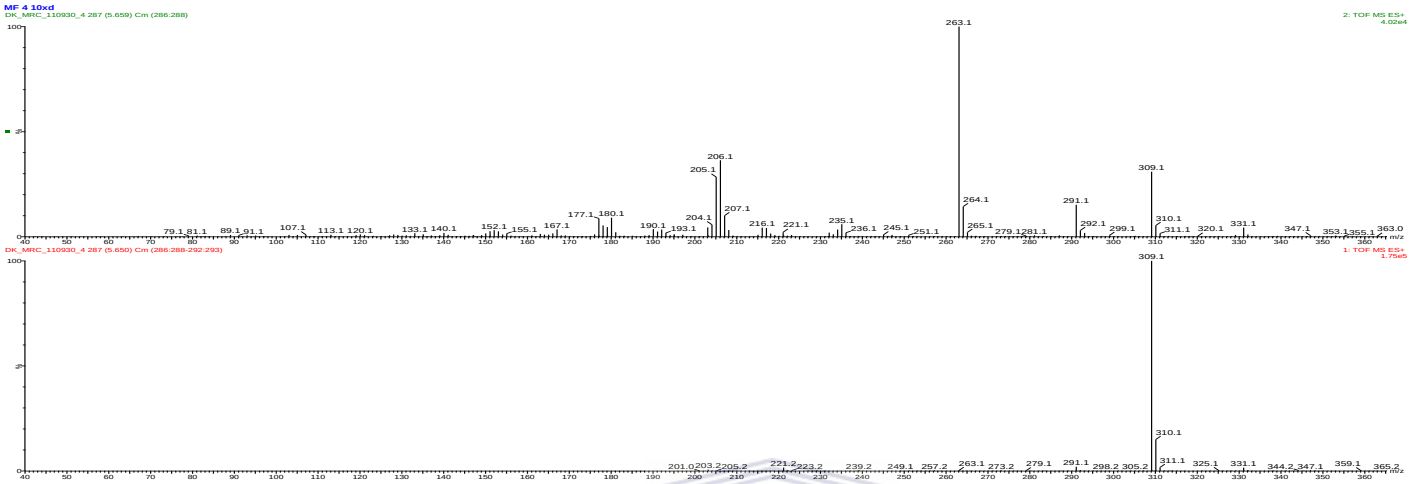


Figure 8.10A showing MS/MS fragmentation of MF4-1

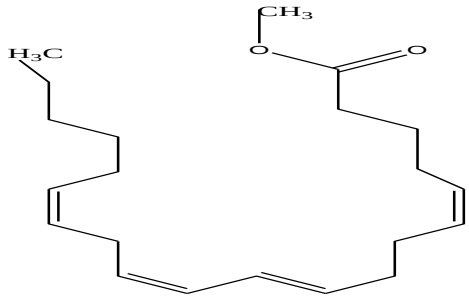


Figure 8.10B showing methyl arachidonate **which matches for MF4-1**

MF4-1 eluted at t_R 5.65 minute with a molecular ion of m/z 319.1 [M+H] giving a molecular formula of $C_{21}H_{34}O_2$. The UVmax were 239.2, 286.2, 352.2 and 412.2. The prominent fragments were at m/z 206.1(loss of 57), 263.1(loss of 28) and 291.1 (loss of 18). The losses of 57(C_4H_9 , 28(C_2H_4) and 18(H_2O) is typical of a long chain fatty ester with ethyl ketone or propionate ester functions. From this information and the literature MF4-1 could be Methyl (5Z,8Z,11Z,14Z)-icosa-5,8,11,14-tetraenoate (methyl arachidonate) or a conjugate isomer.

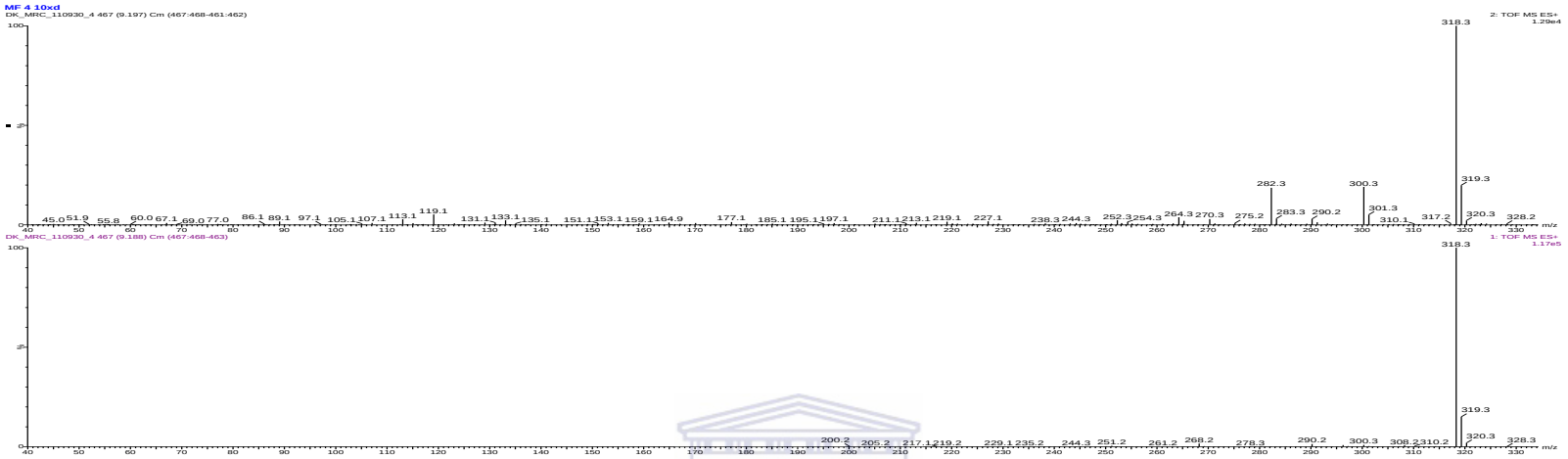


Figure 8.11A showing MS/MS fragmentation of MF4-2

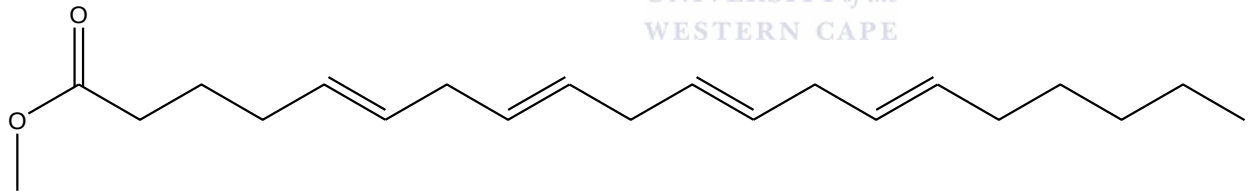


Figure 8.11B showing methyl 5, 8, 11, 14-eicosatetraenoate.which matches with MF4-2

M4-2 elutes at t_R 9.19 minutes and has a molecular ion of m/z 318 [M+H]. The UVmax was 246.2. The compound did not fragment easily and only had fragments at 282.3 (loss of 18) and 300.3 (loss of 18). From the literature MF4-2 was predicted to be methyl 5, 8, 1, 14-eicosatetraenoate (http://lipidlibrary.aocs.org/ms/masspec.html)(figure 8.11B)

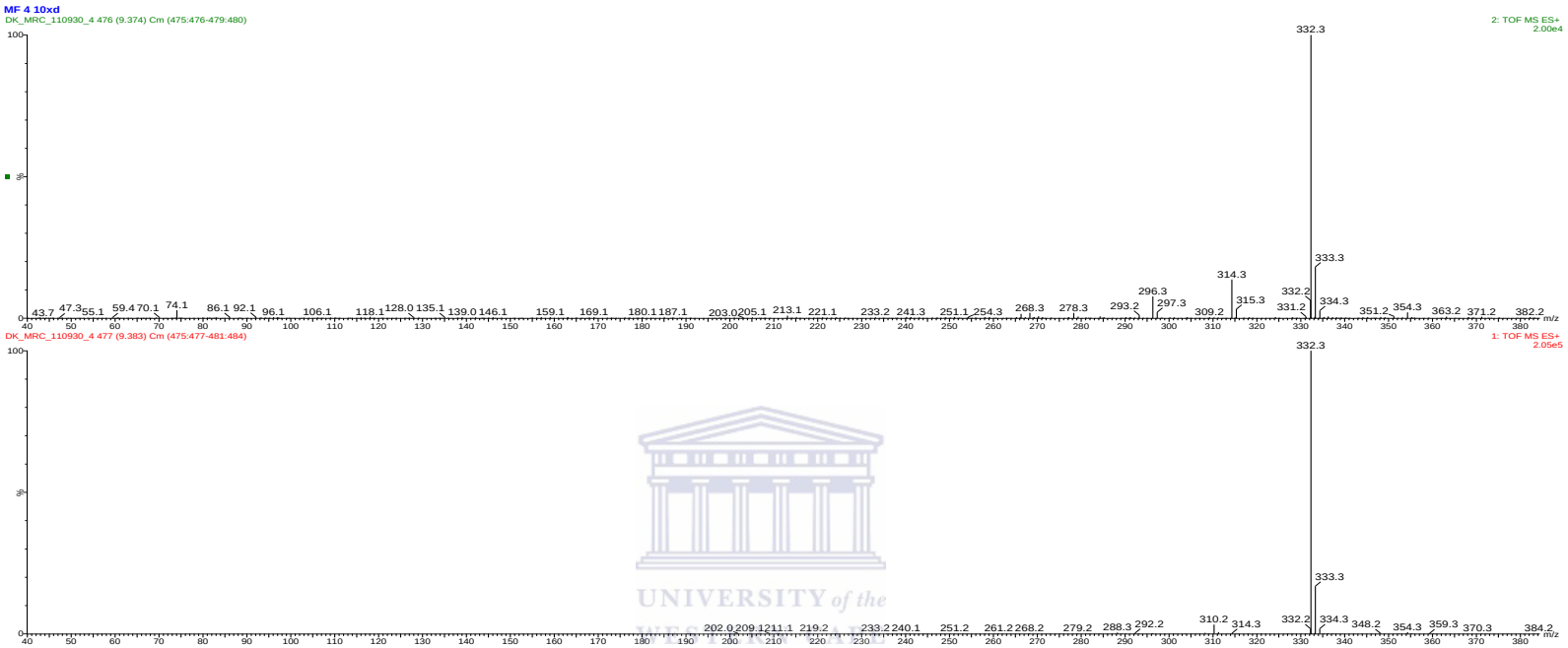


Figure 8.12A showing MS/MS fragmentation of MF4-3

MF4-3 eluted at t_R 9.37 minutes with a molecular ion of m/z 332 [M+H] which gave the molecular formula as $C_{22}H_{35}O_2$.. It has a UVmax at 247 and MS fragment at m/z 296.3 and 314.3. It appears to be a homologue of MF4-1 with an additional CH_2 It may be 7, 10, 13,16-docosatetraenoate or an analogue of the docosatetraenoates.

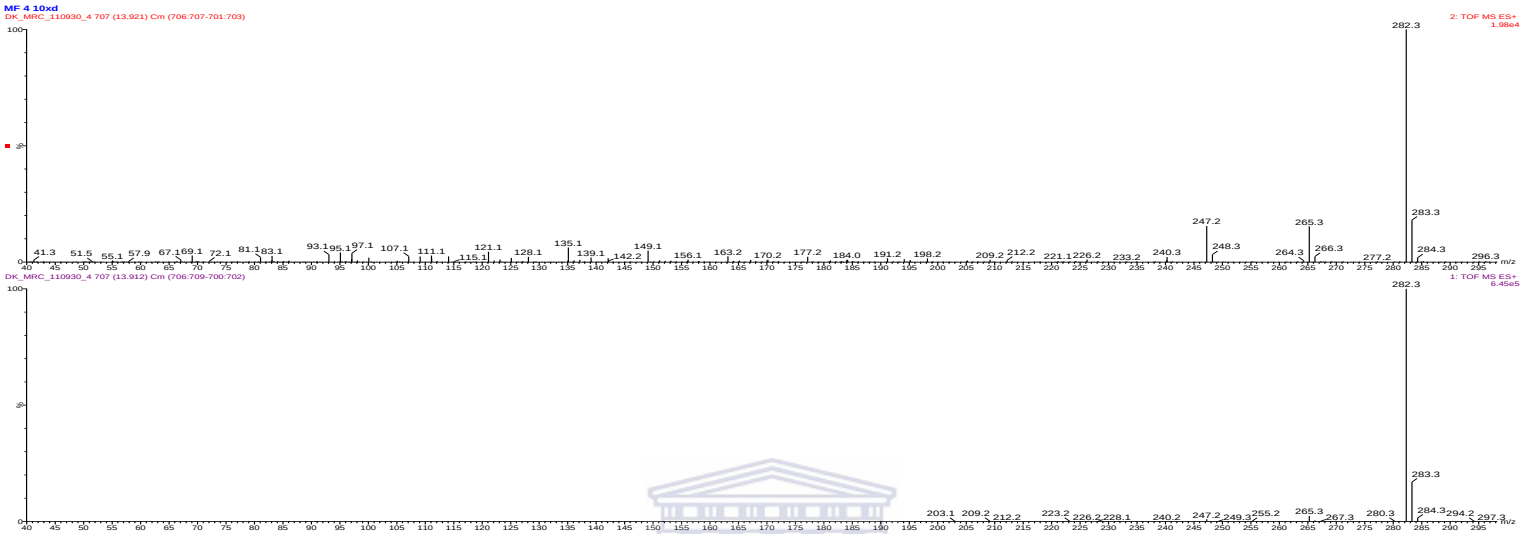


Figure 8.13A showing MS/MS fragmentation of MF4-4

MF4-4 elutes at t_R 13.92 minutes. It has molecular ion of m/z 282.3 [M+H]. The UV properties are poor. The MS fragment patterns were at m/z 93.1, 107.1, 121.1, 135.1, 149.1, 163.2, 177.2, 191.2 (all due to (CH_2) losses), 240.2 (loss of OH), 247.1(loss of H_20), 265.3 (loss of OH) and 282.3 (base peak). It would appear that this is a long chain fatty acid or alcohol.

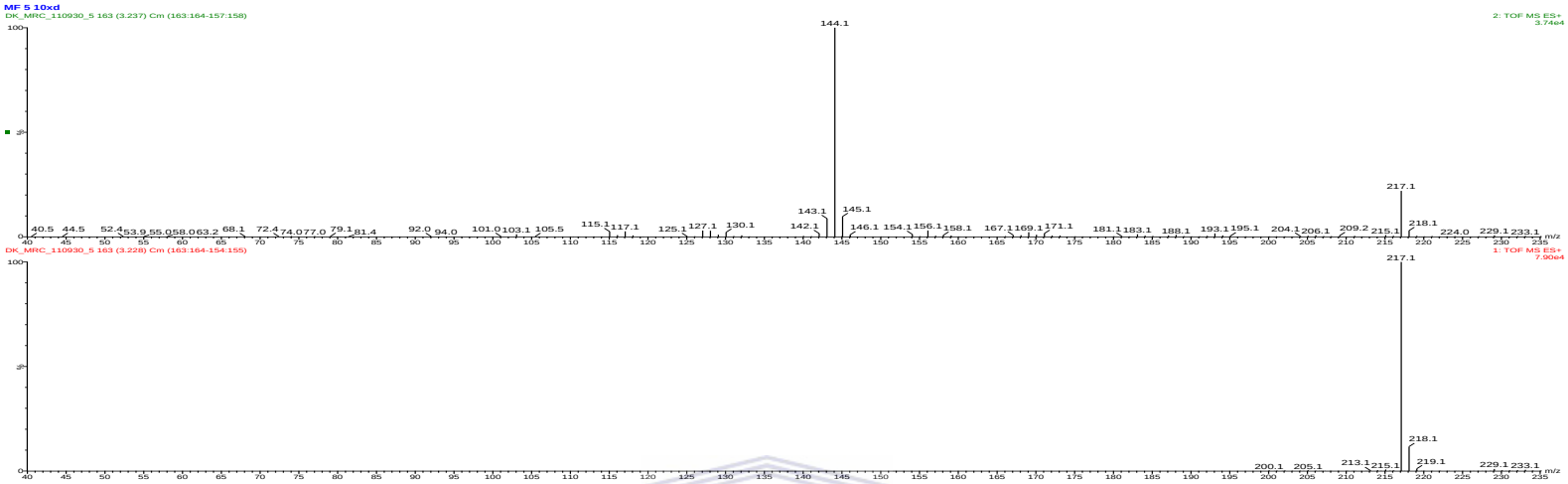


Figure 8.14A showing MS/MS fragmentation of MF5-1

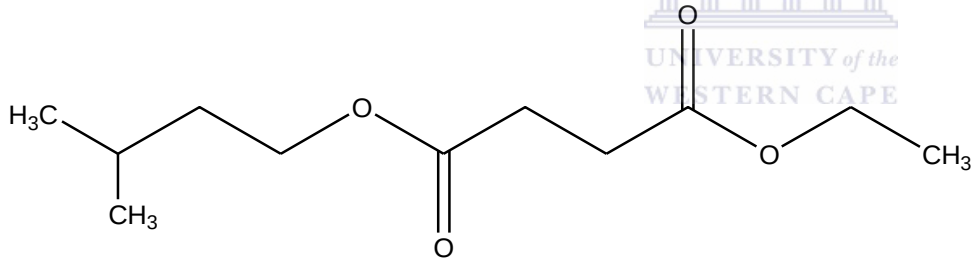


Figure 8. 14B showing methyl 9-(methylperoxy) nonanoate which matches with MF5-1.

MF5-1 eluted at t_R 3.23minutes with molecular ion of m/z 217.1 [M+H] and a molecular formula of $C_{11}H_{20}O_4$. The UVmax were at 230.2 and 270.2. MS fragmentation was generally poor with a single major peak (also base peak) at m/z 144.1 due to loss of 73 ($C_3H_5O_2$) from molecular ion. This implied that this compound is an ethyl ether of a long chain alkane (Williams and Fleming, 1995). This compound appears to cleave near the middle where the ester resides. From the this information and from the literature MF51 can be predicted as ethyl 3-methylbutyl succinate or closely related isomer (figure 8.14B)

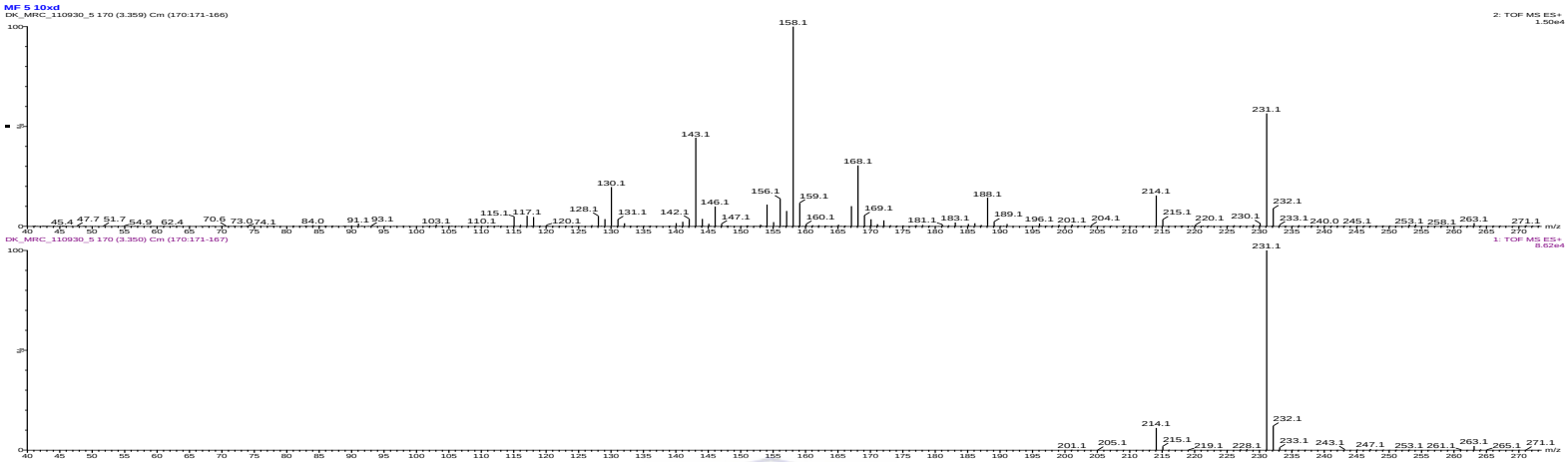


Figure 8.15A showing MS/MS fragmentation of MF5-2

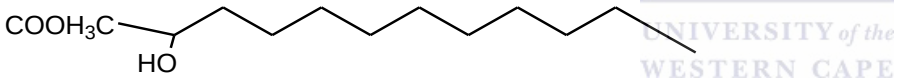


Figure 8. 15B showing methyl 2-hydroxydodecanoate which matches with MF5-2

MF5-2 eluted t_R at 3.35minutes with molecular ion of m/z 231.1 [M+H] and a molecular formula of $C_{13}H_{26}O_3$. The UVmax were 230.2 and 271.2. The MS fragments were at 143.1 (loss of 15 (CH_3)), 158.1 (loss of 73 ($C_3H_5O_2$), 188 (loss of 43 (C_3H_7) and 214 (loss of 17 (OH). From the foregoing and literature MF5-2 was predicted to be methyl 2-hydroxy-dodecanoate (http://lipidlibrary.aocs.org/ms/masspec.html) (figure 8.15B).

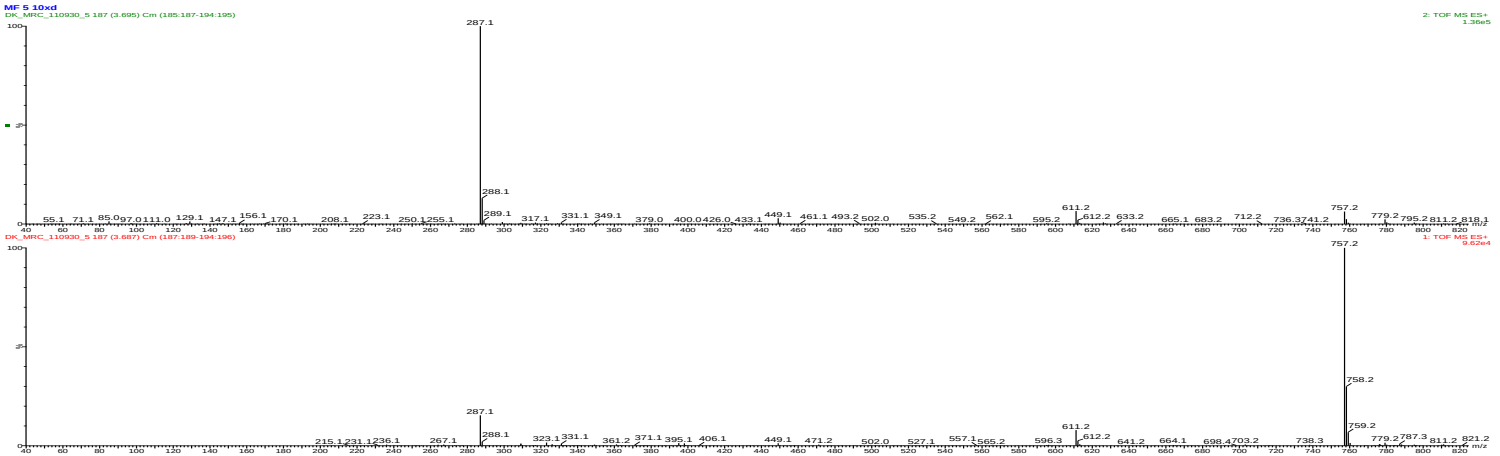


Figure 8.16A showing MS/MS fragmentation of MF5-3

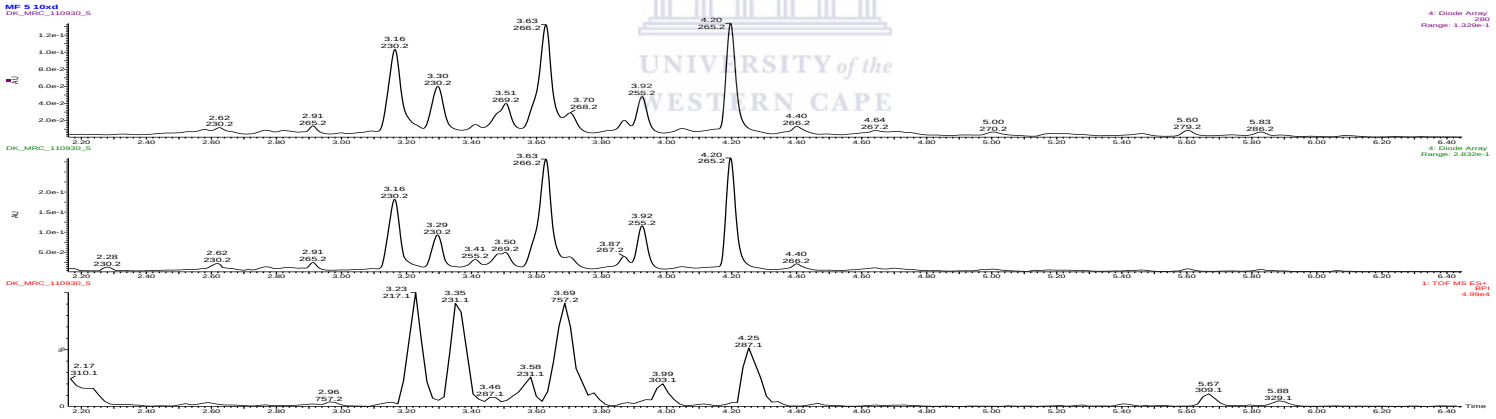


Figure 8.16B showing MS/MS fragmentation and chromatograms of MF5-3 and MF5-4.

MF5-3 and MF5-4 have super imposable UV spectra (table 8.2). MF5-3 eluted at t_R 3.69 minutes and had a molecular ion of 757.2 [M+H]. The fragmentation pattern was poor and only two fragments were produced in the MS-MS experiment i.e. 611.2 [M-146] and 287.1 (M-146 – 324).

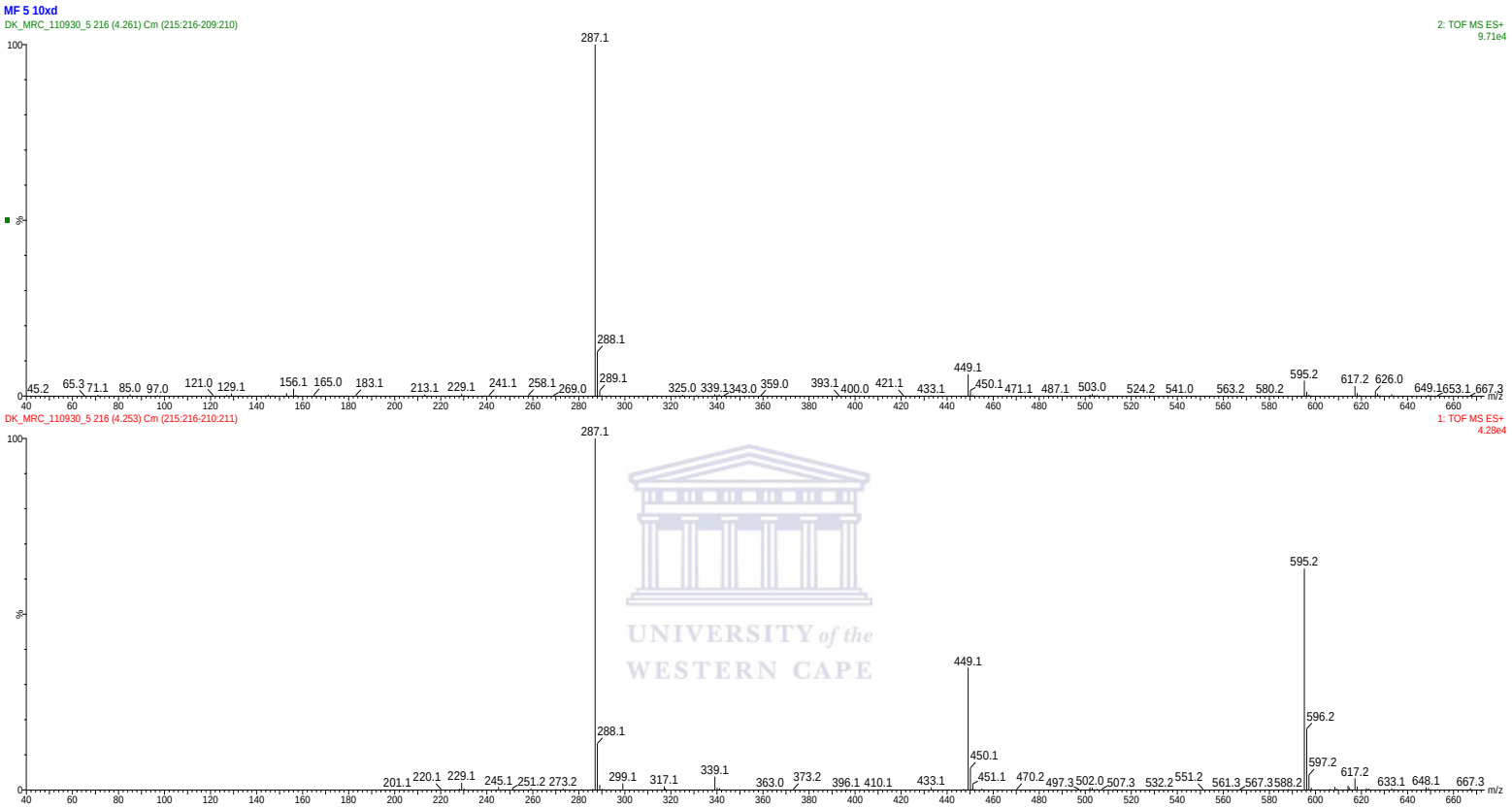


Figure 8.17 showing MS/MS fragmentation of MF5-4

MF5-4 on the other hand eluted at t_R 4.26 minute and had fragment ions at m/z 595 [M+H], 447 [M-146], 287.1 (M–146 –162) (base peak). The loss of similar moieties in both compounds m/z 146 and m/z 162 (or m/z 324 which is double) suggests that these compounds are homologues in a closely related series. The fragment of m/z 162 may result from the loss of allicin and the fragment of m/z 146 is due to the fragmentation of the allicin into a thiol or hydroxyl sugar.

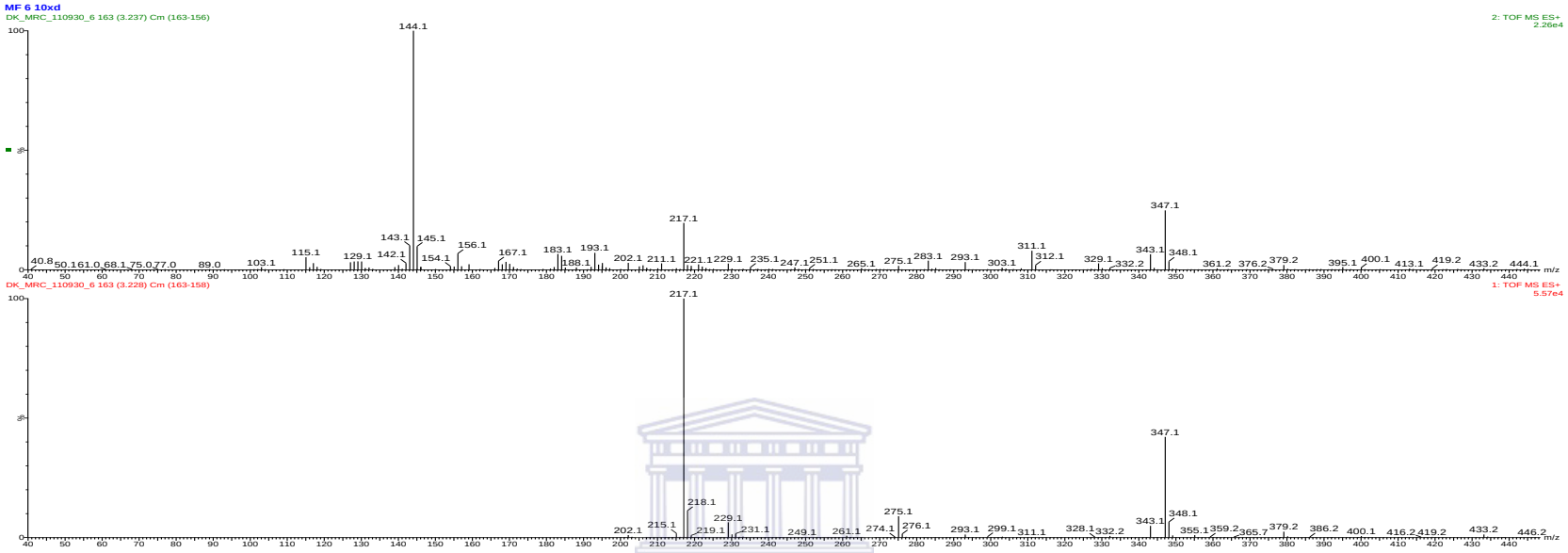


Figure 8.18A showing MS/MS fragmentation MF6-1.

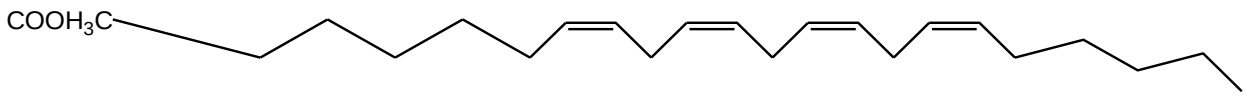


Figure 8. 18B showing methyl docosa-7,10,13,16-tetraenoate which matches with MF6-1

MF6-1 eluted at t_R 3.23minutes with molecular ion of m/z 347 [M+H] and molecular formula of $C_{23}H_{38}O_2$. UVmax are 230.2 and 270.2. The prominent fragments were at 347.1, 329.1 (loss of 18), 311.1 (loss of 18), 293.1 (loss of 18), 217.1 (loss of 76), 144.1 (loss of 73). This fragmentation patterns suggests the presence of ethyl ester groups in a long chain alkane. From the literature MF6-1 was predicted as methyl docosa-7, 10, 13, 16-tetraenoate (figure 8.18B).

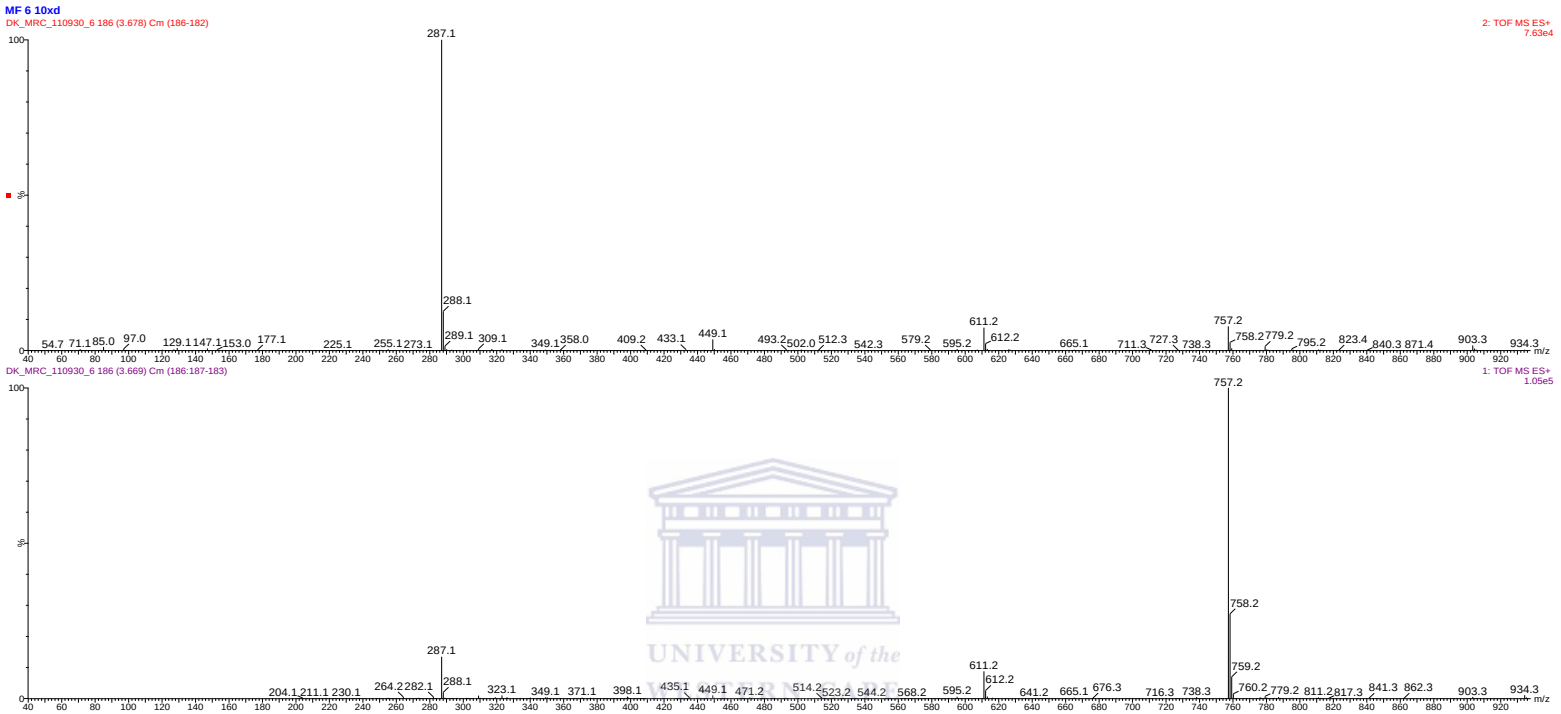


Figure 8.19 showing MS/MS fragmentation MF6-2

MF6-2 eluted at t_R 3.67 minutes and had molecular ion of m/z 757.2 [M+H], the fragmentation pattern is poor, only two fragments were produced in the MS-MS experiment at 611.2 [M-146] and 287.1 [M-146–324] which is similar to both MF5-3 and MF5-4.but their t_R were different figure (8.19A). The loss of similar moieties indicates that these compounds are homologues and probably possess thiol functional groups.

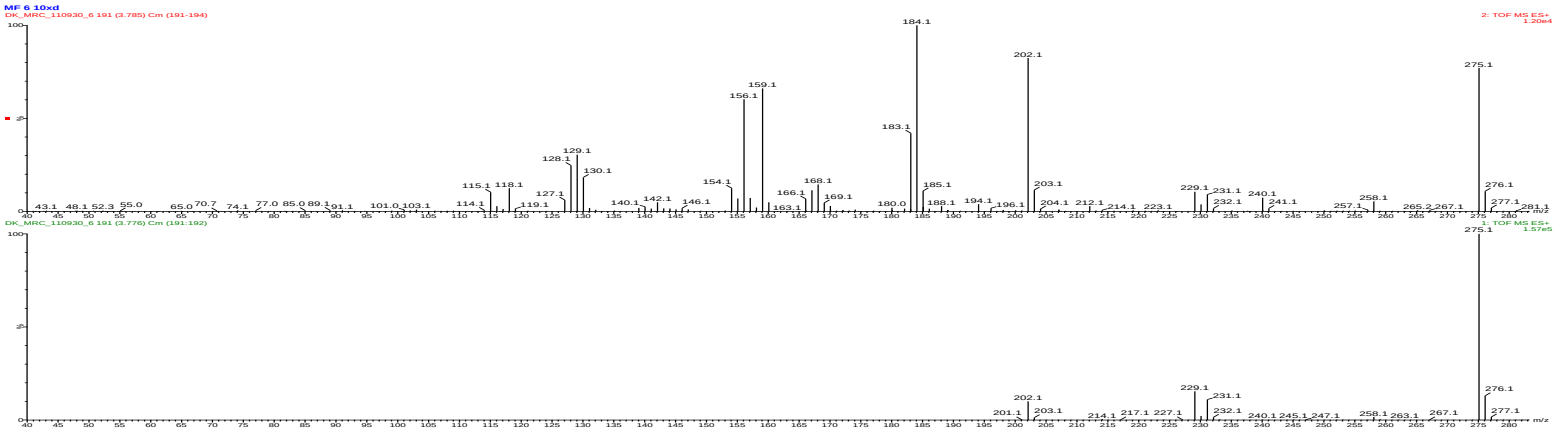


Figure 8.20A showing MS/MS fragmentation MF6-3

MF6-3 eluted at t_R 3.78 minutes with a molecular ions of m/z 275 [M+H]. The UVmax were 230.2, 270.2 and 348.2. The main fragments were 258 (loss of 27), 115 (loss of 14). From this information and from the literature MF6-3 was 2,3-dihydroxypropyl laurate or 2,15-dihydroxypentadecanoic acid.

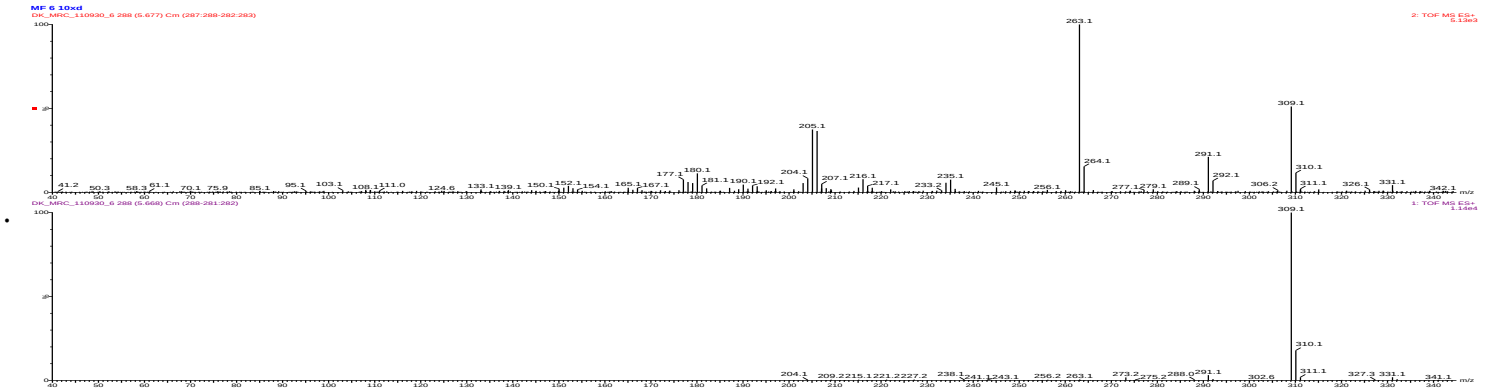


Figure 8.21A showing MS/MS fragmentation MF6-4

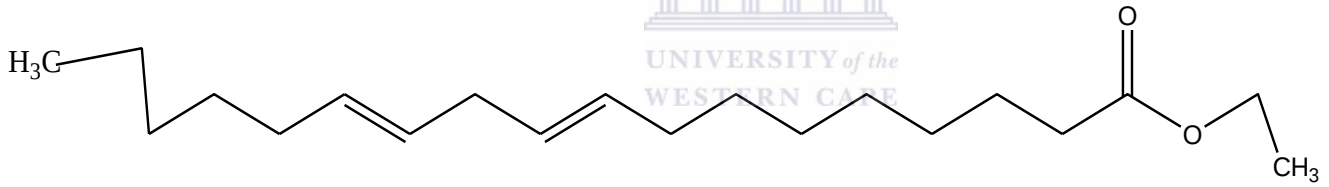


Figure 8. 21B showing Ethyl (9E,12E)-9,12-octadecadienoate which possibly matches with MF6-4

MF6-4 eluted at t_R 5.67minutes and had a molecular ion of m/z 309 (M+H) and molecular formula of $C_{20}H_{36}O_2$. The fragment were 291, 264, 283.1, and 205.1 with loss of 18(H_20), 45(CHCH=OH+) and 28(C_2H_4). The UVmax are 242.2 and 273.2. From the literature MF6-4 could be any of the isomers in Fig 21B

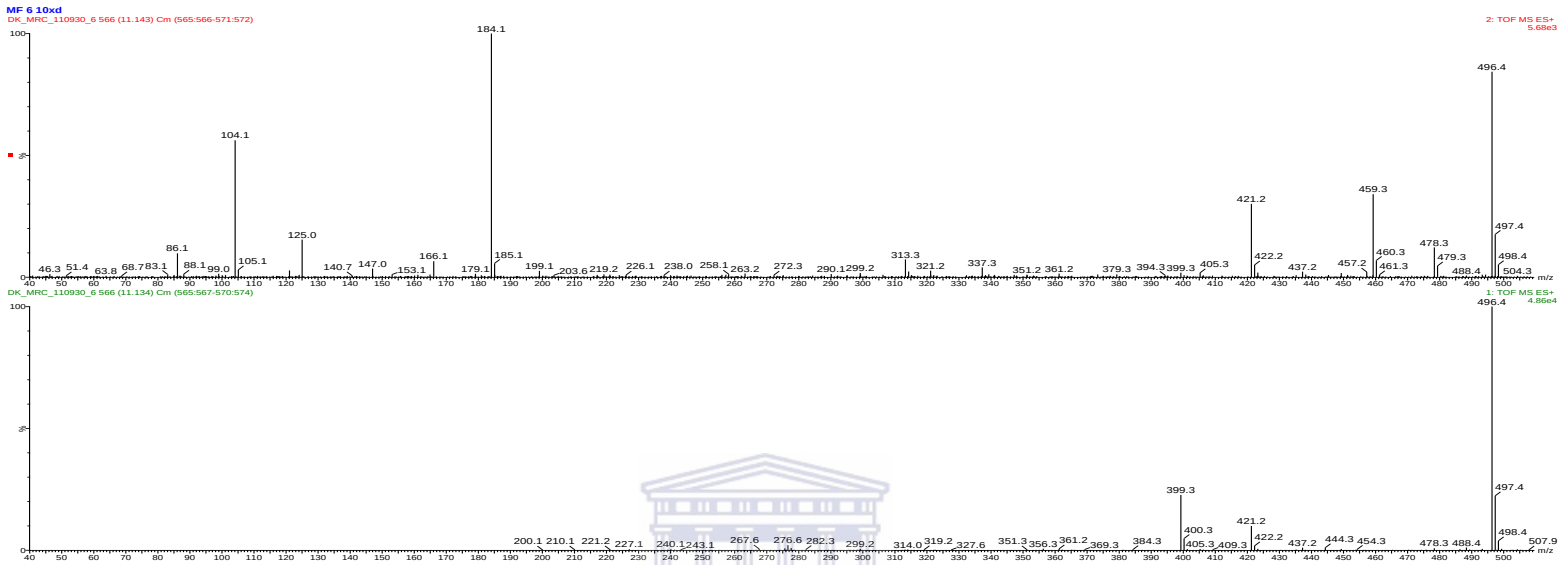


Figure 8.22A showing MS/MS fragmentation MF6-5

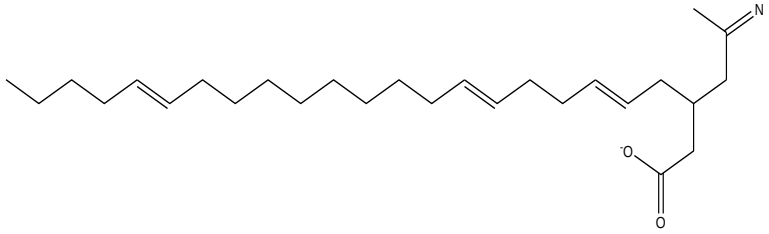


Figure 8. 22B showing 3-pyridylcarbinyl 5,9, 19-heptacosatrienoate which matches with MF6-5.

MF6-5 eluted at t_R 11.14 minutes. It had molecular ions of m/z 496 [M+H]. UVmax were 274.2 and 361.1. The fragments were 478(loss of 18), 421.3 (loss of 75), 184.1(loss of 237 (molecular cleavage)), and 125 (loss of 59).From the literature MF6-5 was predicted as 3-pyridylcarbinyl 5, 9, 19-heptacosatrienoate (http://lipidlibrary.aocs.org/ms/masspec.html) (figure 8.22B).

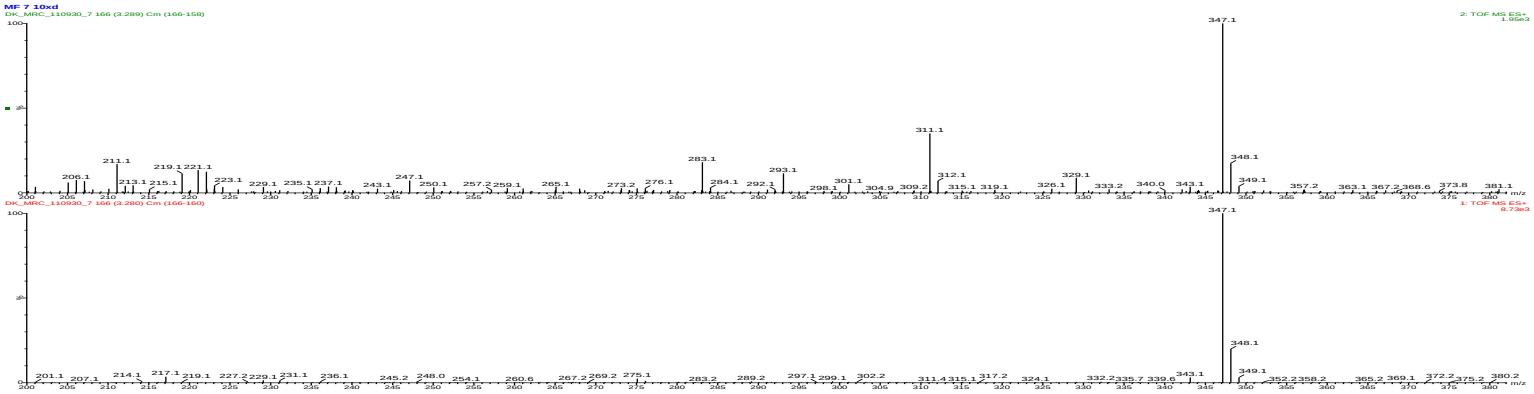


Figure 8.23A showing MS/MS fragmentation MF7-1

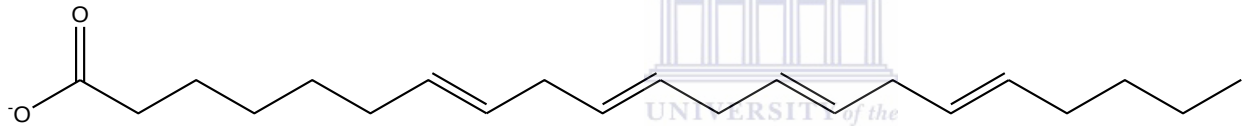


Figure 8. 23B showing 7, 10, 13, 16, -docosatetraenoate which matches with MF7-1.

MF7-1 elutes at 3.28 minutes with a molecular ion of m/z 347.1 [M+H]. The UV max was 274.2 and 361.2. The main fragments patterns were 329 (loss of 18), 311 (loss of 18), 293 (loss of 18), 283 (loss of 28), 265 (loss of 18). This compound is similar to MF6-1

Figure 8.24A showing MS/MS fragmentation MF7-2

MF7-2 eluted at t_R 6.38 minutes and a had a molecular ion of m/z 447 [M+H]. Fragmentation was extremely poor.

8.4 Discussion

Most of the compounds isolated from methanol fractions of *T. violacea* leaves are long chain fatty acid (esters and alkanes). Long chain fatty acids are carboxylic acids consisting of carboxylic head group as well as a long hydrocarbon chain (Lynda & Robert, 2010). Some saturated fatty acid such as palmitic, palmitate, stearic or stearate acids have no double bonds along their hydrocarbon chains while the unsaturated fatty acids contains minimum of one double bond. Consumption of dietary fatty acids in various concentrations is report to result in changes in cell membrane structure and function (Linda, *et al.*, 2006) . Studies have shown that fatty acids play a vital role in biological systems, they reduce most of the risk factors associated with several diseases such as cancer, diabetes and cardiovascular diseases (Richard, *et al.*, 2006), they are the component of both phospholipids and glycolipids. They are linked to the regulation of inflammatory processes such as cancer (Shawn, *et al.*, 2001). Fatty acids have different effects on the production of cytokines, chemotaxis and modulation of blood coagulation pathways.

Studies have shown that dietary n-3 Polyunsaturated fatty acids (PUFA) can inhibit both initiation and promotion stages of carcinogenesis by suppression of arachidonic acid derived eicosanoid biosynthesis which lead to changes in immune response to cancer cells as well as modulation of inflammation, cell proliferation, metastasis and angiogenesis. PUFAs also affect carcinogenesis by influencing the transcription factor activity, gene expression and signal transduction which later result in changes in cell growth and differentiation as well as metabolism, it could also be through either decrease or increase in production of ROS and free radical (Susanna, *et al.*, 2004). In drug development, PUFAs

have been used to either kill or inhibit growth of cancer cells in culture as well as using animal model for effectiveness of chemotherapeutic drugs (Elaine, *et al.*, 2001).

In conclusion from the chemical investigation of *T.violacea*, it was demonstrated that that this plant species contains several long chain fatty acids, allicin-like compounds and flavonoids. These compounds have been shown to have anti-proliferative, and in some cases, pro-apoptotic properties which may explain the biological activity demonstrated in this study, and also explain the rationale behind traditional use of this herb.

CHAPTER NINE:GENERAL DISCUSSION AND CONCLUSION

Table of Content/ Outline

9.1 MDG does not induce morphological changes in human cell cultures.

Two studies previously demonstrated that extracts of *T.violacea* induce apoptosis in human cancer cell cultures (Lyantagaye, 2005; Bungu, *et al.*, 2006). The study from Lyantagaye identified MDG as one of three bioactive compounds in the extract (Lyantagaye, 2005). In the present study the pro-apoptotic activity of MDG was evaluated on MCF7 and CHO cells (section 2.2.1). It was observed that, the positive control (doxorubicin) was able to induce morphological changes (cell shrinkage and cell detachment) in cell cultures that were indicative of apoptosis, while the MDG failed to induce noticeable morphological changes in two cell cultures (CHO and MCF7) (figure 3.2). Previous studies showed that the induction of apoptosis is usually associated with changes in cell morphology (Kroemer, *et al.*, 2005). Hacker's report in 2000 also supported that morphological features of apoptosis would provide a reliable basis for the detection of apoptosis, which could be used in combination with other apoptosis assays for the study of apoptosis (Ross & Brain, 1977; Häcker, 2000; Kroemer, *et al.*, 2005).

9.2 MDG does not induce apoptosis in cultured cells.

Since morphological studies on its own cannot be used to confirm the induction of apoptosis, the APOPercentage^{TM} assay, which is an assay that specifically quantify of apoptosis in cell cultures was used. The results showed that no apoptosis was detected when MCF7 and CHO were treated with different concentrations (0.6, 0.125, 0.25, 0.5, 1 and 2mM) of MDG for 24hours (figure3.3). This study appeared to contradict the previous findings by Lyantagaye in 2005 that MDG induced apoptosis in these cell lines.

9.3 Cell proliferation assay show that the effects of MDG are transient.

The xCelligence system (Roche) provides the biological status of the cell in real time. The xCelligence data showed that both the MCF and CHO cells were transiently affected by the addition of MDG to the culture (figure 3.4). The cell index decreased for a period of 30minutes following the exposure to MDG. After 30minutes, the cell index increased which is an indication that the cells recovered from the effects of MDG. A decrease in the cell index indicates cell shrinkage or cell detachment, while an increase in the cell index suggests that the cells are proliferating. These results explained the apparent contradiction between the Lyantagaye study (2005) and this study in that it demonstrated that the effects of MDG are transient. In the Lyantagaye (2005) study CHO and MCF7 cells were treated for only 1hour before apoptosis was quantified. However, as was demonstrated in the current study, the cells recovered after 1hour and 24hours later, the cells were still viable. This demonstrated that MDG do not induce apoptosis in MCF7 and CHO cells. It also illustrates the limitation of end-point analysis assays, which only gives an indication of what is happening at the time-point when assay is stopped.

9.4 The leaf extract and not the tuber extract of *T.violacea* induce morphological changes in cultured cells

In the Lyantagaye (2005) study the whole plant was used for the extraction of possible bioactive compounds, while the study by Bungu *et al.* (2006) studied the effects of the leafs and the tubers were independently studied. Both these studies showed that organic extracts (hexane, chloroform, ethyl acetate, n-butanol and methanol) of *T.violacea* induce apoptosis in cancer cells.

In the current study the leafs and tubers were separated before the extractions were performed. It is likely that the chemical composition of the tuber and leaf extract will differ; hence the objective was to investigate if there is a difference in the bioactivity of the two plant parts. Water extracts of *T.violacea* leaves induced discernible morphological changes (such as cellular shrinking, chromatin condensation and cell detachments) (Kroemer, *et al.*, 2007) in MCF7 cells. All these morphological changes are features that are associated with apoptotic cell death. However there were little or no discernible morphological changes in the cells treated with the tuber extract. This suggested that the leaf extract of *T.violacea* either contains more of the cytotoxic compounds or that these compounds are completely absent in the tuber extract. This is in contradiction to the study by Bungu *et al*. (2006), which found that the tuber extract was more active than the leaf extract. The Bungu study (Bungu, *et al.*, 2006) found that a crude water extract and a methanol extract of the leafs were more active than an extract of the tubers. This was demonstrated in four human cancer cell lines (MCF7, WHCO3, HT29 and Hela). The cells were treated for up to 48hrs with 250ug/ml of the extracts and cell death was quantified using a cell viability assay and the annexin V assay. It is known that seasonal changes and geographical location can affect the bioactivity of medicinal plants (Sanjay, *et al.*, 2010). It is not known where and when the plant material for the Bungu study was sourced, but it is possible that the location where the plant material was collected may account for the apparent contradiction between the current study and the study by Bungu *et al.* (2006). In the Bungu study MCF7, WHCO3, HT29 and Hela cells were used, while the current study used MCF7, HT29, H157, HepG2 and KMST6 cells. Both studies therefore used MCF7 and HT29 cells. However the current study also

included a noncancerous control cell line (KMST6), which made it possible to determination the selectivity index of the extracts.

9.5 The leaf and not the tuber contains compounds that can selectively induce apoptosis in cancer cell lines

The MTT assay was used to determine the IC_{50} values (the concentration of plant extract that is required for 50% cellular growth inhibition) for water extracts of *T.violacea* leaves and tubers on a panel of 5 human cell lines (table 4.1). Based on the MTT assay the tuber extract of *T.violacea* contained no cytotoxic compounds, while the leaf extract was cytotoxic to all the cell lines tested in this study. The IC_{50} values for the tuber extract were more than 2.5mg/ml on all the cell lines tested and the tuber extract was therefore considered to be inactive. The IC_{50} values for MCF7, H157, HT29, HepG2 and KMST6 cells were 1.2±0.5, 1.5±0.8, 2.6±1.5, 2.8±0.5, 3.0±0.9 mg/ml, respectively. The selectivity index (calculated based on the non-cancerous KMST6 cell line) showed that the MCF7 cell line was the most sensitive cell line.

The MTT assay measures cell viability as a function of the cells active enzymes (mitochondrial succinate dehydrogenase) and does not discriminate between necrotic and apoptotic cell death. Since the objective of this study was to investigate the pro-apoptotic activity of the plant extract, a second assay, the APOP*ercentage*^{TM} assay was used to assess cell death in cells treated with the plant extracts. The principle of the APOP*ercentage*^{TM} assay is based on the externalisation of phosphatidylserine, which is a feature observed only in apoptotic cells. Apoptotic cells take up the fluorescent APOP*ercentage*^{TM} dye and the

cells can be quantified by flow cytometry. The water extracts of *T.violacea* leaves showed a dose-dependent increase in the number cells staining positive for apoptosis (figure 4.2a). This was observed in all the cell lines tested in this study. The tuber extract failed to induce significant levels of apoptosis in these cells. The results of the APOP*ercentage*^{TM} assay was used to determine the IC_{50} values (the concentration of the plant extract that is required kill 50% of the cells by apoptosis) for water extracts of *T.violacea* leaves and tubers on 5 different human cell lines (table 4.2). The IC_{50} values for MCF7, H157, HT29, HepG2 and KMST6 cells were 0.19±0.4, 0.28±0.1, 1.21±0.6, 2.09±1.4, 4.4±1.4 mg/ml, respectively. The Selectivity Index (calculated based on the non-cancerous KMST6 cell line) showed that the MCF7 cell line was the most sensitive cell line. The results for the APOP*ercentage*^{TM} assay are in agreement with the results obtained for the MTT assay. Both assays demonstrated that the MCF7 cell line is the most sensitive cell line and that the non-cancerous cell line, KMST6 was the most resistant cell line. However, the IC_{50} values obtained for the APOP*ercentage*^{TM} assay was much lower than that obtained for the MTT assay. For example, using the MTT assay the IC_{50} value for the MCF7 cell line was calculated to be 1.2±0.5 mg/ml, while the IC_{50} value for the same cell line was 0.19±0.4mg/ml when the APOP*ercentage*^{TM} assay was used. This may be due to the fact that the APOP*ercentage*^{TM} assay is a more sensitive assay and that it detects the externalisation of phosphatidylserine, which may occur well before the mitochondrial enzyme activity diminishes.

These results demonstrated that the water extract of the *T.violacea* tubers do not contain any cytotoxic or pro-apoptotic compounds. As a result the tuber extract was excluded from further investigation and the rest of the study focussed on the leaf extract of *T.violacea.*

Medicinal plants such as *T.violacea* are under threat of extinction due to overexploitation and destructive harvesting methods (Jeremy, 2008). The fact this study demonstrated that the potential anti-cancer bioactivity of the plant is in the leaves of the plant and not the tubers benefits the preservation of *T.violacea*, since the harvesting of the leaves of this plant is less destructive than harvesting the whole plant.

9.6 Apoptosis induced by the water extract is associated with the activation of caspase-3 cleavage and ROS production

The cleavage and activation of caspase-3 are universal markers for the induction of apoptosis (Frankline, *et al.*, 2008) and play a vital role in the initiation and execution of programmed cell death (Fischer & Schulze-Osthoff, 2005). However, the activation of these enzymes is often inhibited in cancer cells. These enzymes are therefore targets for the development of anti-cancer drugs. The activation of cellular caspases via small cell permeable drugs could offer a more efficient way to target cancer cells.

The activation of caspase-3 was assessed in four human cancer cell lines (HepG2, HT29, MCF7 and H157) and a non-cancerous human cell line (KMST6) following treatments with the water extract of *T.violacea* leaves and 10µM doxorubicin (positive control). The results showed that the water extract of *T.violacea* activated caspase-3 cleavage in the five cell lines tested in this study (figure 4.4). However, the cell lines differed significantly in the percentage cells that were positive for cleaved caspase-3. At 68%, the HepG2 cell line exhibited the highest percentage active caspase-3 positive cells, while 28% of MCF7 cells,

23% of H157 cells and 22% of HT29 cells were positive for active caspase-3. This is in agreement with the study by Lyantagaye, which demonstrated that a water extract of *T.violacea* induced the activation of caspase-3 in Chinese Hamster Ovary cells (Lyantagaye, 2005). This study also found that the water extract of *T.violacea* failed to induce caspase-3 cleavage in KMST6 cells, demonstrating the selective killing of cancer cells by the extract.

ROS activate signal molecules that are involved in inflammation and carcinogenesis such as nuclear transcription factor NF-κB, inducible nitric oxide synthetase (iNOS) as well as cyclooxygenase-2 (COX-2) (Ohshima, *et al.*, 2005). Increased levels of ROS can cause oxidative damage of intracellular macromolecules (lipids, DNA, RNA and protein) (Elisa, *et al.*, 2000). This in turn can activate the induction of apoptosis. Chemotherapeutic agents and radiation have been shown to induce ROS in cancer cells, leading to the destruction of the cancer cells through the induction of apoptosis (Stacey & Wei-Xing, 2006). To assess whether the water extract of *T.violacea* induce ROS production in cancer cells a panel four human cancer cell lines (HepG2, HT29, MCF7 and H157) and a non-cancerous human cell line (KMST6) were treated for 24hours with 2mg/ml of the extract. Hydrogen peroxide was used as a positive control and the production of ROS was evaluated using the CM-H_2DCFDA probe. This probe is a general oxidative stress indicator, which diffuses into cells and is oxidised into a fluorescent adduct which is trapped inside cells in the presence of ROS (Kim, *et al.*, 2009). These fluorescent cells can be quantified by flow cytometry. The results demonstrated that the water extract of *T.violacea* induce significant ROS production three of the cancer cell lines (H157, MCF7 and HepG2) (figure 4.5). ROS production in

H157 cells was the highest with 83% of the cells being positive for ROS production, while 50% of HepG2 cells and 30% of MCF7 cells were positive for ROS production.

These results show that the water extract of *T.violacea* induces apoptosis in human cancer cells and that the mechanism most likely involves the activation of caspase-3 and the generation of ROS. However, the varied responses in human cancer cell lines were observed. The non-cancerous KMST6 cell line was very resistant to the effects of the plant extract, with very low levels of apoptosis induced in this cell line. These results support claims that extracts of this plant can be used to treat cancer. The selective killing of cancer cells by *T.violacea* extracts can be exploited for the development of an alternative anti-cancer treatment that has lower levels of bystander cytotoxic effects (i.e. the killing of normal cells).

9.7 The hexane and DCM extracts of *T.violacea* contains the major pro-apoptotic compounds

It was shown that the leaf extract and not the tuber extract of *T.violacea* contain pro-apoptotic compounds. Since the objective of this study was to isolate the bioactive compounds, the next phase was to apply bioactivity guided fractionation to isolate the bioactive (or pro-apoptotic) compound(s). Sequential extraction of *T.violacea* leaves was performed using organic solvents (hexane, DCM, methanol and 50% methanol). This differs from the Lyantagaye study in that a crude water extract of *T.violacea* was prepared of the whole plant, which was then fractionated using organic solvents (Lyantagaye, 2005). One of the limitations of the Lyantagaye study is that the extraction process will only extract

water-soluble compounds. Consequently, non-polar compounds cannot be extracted with this strategy. Sequential extraction using organic solvents should also increase the yield of compounds extracted from the plant material. The organic solvent extracts were tested on a panel of four human cancer cell lines (HepG2, HT29, MCF7 and H157) and a non-cancerous human cell line (KMST6) using the APOP*ercentage*^{TM} assay. An increase in the bioactivity was observed for all four solvent extracts when compared to the water extract of *T.violacea* leaves, suggesting that the solvent extracts contained increased levels of the bioactive phytochemicals. Based on the IC_{50} values obtained for the four extracts, the bioactivity of the methanol and 50% methanol extracts were very low (table 5.2). The hexane and DCM extracts on the other hand displayed significant bioactivity towards most of the cell lines. The H157 cell line was the most sensitive cell line with SI values of 47.75 and 41 for hexane and DCM extracts, respectively. The MCF7 cell line was highly resistant to the effects of all four solvent extracts, with IC_{50} values above 2mg/ml, interestingly this cell line was highly susceptible to the effects of the water extract of *T.violacea* leaves. In fact the IC_{50} and SI values suggested that the MCF7 was more susceptible to the effects of the water extract than any other cell line. It is possible that the compounds that were responsible for the bioactivity in this cell line were lost during the solvent extraction process or that more than one compound was responsible for the bioactivity of the water extract and that these compounds were separated by solvent extraction resulting in the loss of bioactivity. This also suggests that it is not the same compound(s) that is responsible for the bioactivity in the different cell lines. The wide-ranging responses of the different cancer cell lines can be ascribed to the different mutations in these cell lines. It is known that cancer cells have acquired genetic mutations, which make these cells more resistant to apoptosis

(Michael, *et al.*, 2005). It is to be expected that different cancer cell types will have different mutations and will therefore respond differently to pro-apoptotic agents.

The objective was to isolate and identify the bioactive compound(s). Based on the IC_{50} values, the DCM extract was selected for further study. Two additional assays (caspase-3 cleavage and ROS production) were used to confirm the pro-apoptotic activity of the DCM extract. The caspase-3 cleavage assay showed that the DCM extract induced caspase-3 cleavage in all cell lines tested in this study (figure 5.3). Figure 5.4 shows the production of ROS in several cell lines. ROS production in H157 cells was the highest, while ROS production in KMST6 and HT29 were the lowest compared to H157 and HepG2 cells, ROS production in MCF7 cells were much lower. However, MCF7 were more susceptible to the leaf extract, since higher levels of apoptosis were observed for MCF7.

9.8 Palmitone (16-hentriacontanone) is a major component of the DCM extract of *T.violacea*

Palmitone precipitated in several DCM fractions of *T.violacea.* This is the first report of its occurrence in this species. The compound was elucidated using NMR spectroscopy and mass spectrometry as well as comparison with the literature (González-Trujano, *et al.*, 2001). Though it was isolated from DCM, it proved impossible to re-dissolve in that solvent for the purposes of testing it in biological media. An abortive attempt to use cyclodextrin to solubilise palmitone was unsatisfactory, and it is recommended that proper formulation such as encapsulation of the compound in lipid vehicle should be done prior to conducting further biological assays.

9.9 Further fractionation of the methanol extract results in reduced activity

Due to the solubility problems experienced with the DCM extract, the focus of the study was redirected towards the methanol extract. The methanol extract selectively induced apoptosis in H157, HepG2 and KMST6 while HT29 and MCF7 cells were resistant. Five fractions were obtained by Versaflash chromatography of the methanol extract. In general, sub-fractionation resulted in the loss of bioactivity on most of the cell lines tested in this study. This can be ascribed to loss of compounds or the separation of synergistic compounds. Synergy is well-recognized in natural products chemistry (Jeremy, 2008). Interestingly, fractionation of the methanol extract resulted in increase of activity on HT29 cells. While HT29 cells were resistant (IC_{50} value > 2mg/ml) to the effects of the methanol extract, these cells were susceptible to the effects of the methanol sub-fractionations (MF3 and MF4). This may be due to an increase in the concentration of the active compound (s).

9.10 Long chain fatty acid derivatives, flavonoids and allicin derivatives are present *in T. violacea*

Fingerprinting showed the presence of long chain fatty acid derivatives, flavonoids and allicin derivatives in the methanol extract. These were not isolated but they were characterized with reasonable confidence from LC-MS. It was noted however that some of the more unusual compounds seen i.e. the alkaloidal and silylated derivatives were probably synthetic contaminants which might have entered the extracts during the various extraction and drying stages. This finding reinforces the need to conduct further phytochemistry

investigations on this plant species. However the fingerprints do point to a mix of compounds which have all been previously reported to have anti-proliferative and / or anti-apoptotic activity.

9.11 Conclusions and Recommendations and future work

Lyantagaye (2005) reported that MDG was one of the compounds present in the water extract of *T.violacea* that was responsible for the pro-apoptotic activity of the plant extract. In this study it was proposed that this compound could be further investigated as an anti-cancer agent. The study by Lyantagaye (2005) did not investigate the mechanism by which MDG induce apoptosis. Hence the main focus of this study was to further investigate MDG as a novel anticancer agent with pro-apoptotic activity that can be used to treat cancer. The question that this study that wanted to addressed was how MDG induces apoptosis in cultured human cancer cells, however from this study it was found out that MDG does not induce apoptosis therefore, more compounds were isolated and identified from *T.violacea* leaves using alternative extraction methods based on organic solvent extraction.

From the results obtained it can be concluded that

a) MDG does not induce apoptosis in the cell lines tested in this study.
b) The *T.violacea* leaf extract contains pro-apoptotic compounds that selectively induced apoptosis in cancer cells.
c) The bioactivity of organic extracts especially DCM and hexane extracts was higher compared to the water extract of *T.violacea* leaves.
d) Palmitone is the major compound present in the DCM extract of *T.violacea* leaves.
e) Sub-fractions generated from the DCM extract of *T.violacea* are insoluble in water.
f) Sub-fractionation of the methanol extract of *T.violacea* results in loss of bioactivity.
g) Methanol sub-fractions of *T.violacea* are rich in flavonoid borne out by existence of many compounds, which were UV active at 280 nm.

h) The compounds that are responsible for the pro-apoptotic activity of *T.violacea* leaf extract most likely work in a synergistic fashion are still to be identified.

Further work needs to be done on this plant species. The study showed that extracts of *T. violacea* have useful biological activity which appears to be supported by the chemical constituents fingerprinted. The phytochemistry requires further work. In addition isolation, formulation and testing of some of the compounds herein reported will be important. Assembling synthetic analogues of some of the compounds e.g. palmitone is also a viable approach to the development of drugs from this important traditional herb.

Cancer remains a leading cause of morbidity and mortality globally and research into new therapies continues to be necessary and relevant. This study has provided important new evidence which should be followed up.

REFERENCES

Abramov, A. Y., Scorziello, A., & Duchen, M. R. (2007). Three distinct mechanisms generate oxygen free radicals in neurons and contribute to cell death during anoxia and reoxygenation. *J. Neurosci, 27,* 1129-1138.

Adams, J. M., & Cory, S. (1998). The Bcl-2 protein family: Arbiters of cell survival. *Science, 281*, 1322 - 1326.

Aguiar, F. C., Feio, M. J., & Ferreira, M. T. (2011). Choosing the best method for stream bioassessment using macrophyte communities: Indices and predictive models. . *Ecological Indicators, 11*(2), 379-388.

Ahmedin, J., DVM,, Freddie, B., Melissa, M. C., Jacques, F., Elizabeth, W., & David, F. (2011). Global Cancer Statistics. *CA: A Cancer Journal for Clinicians, 61*(2), 69-90.

Alan, G. P., & Reiner, U. J. (1999). Emerging roles of caspase-3 in apoptosis. *Cell Death and Differentiation, 6,* 99-104.

Alfonso, C., Vincenzo, D. F., Ernesto Fattrusso, Virginia, l., Silvana, M., & Carla, C. (1996). The Flavonoids of Allium Ursinum. *Phytochemistry, 41*(2), 531- 535.

Amarante-Mendes, G. P., & Green, D. R. (1999). The regulation of apoptotic cell death. *Brazilian Journal of Medical and Biological Research, 32,* 1053-1061.

Ambrosini, G., Adida, C., & Altieri, D. C. (1997). Anovel anti -apoptosis gene, survivin, expressed in cancer and lymphoma. *Nat. Med, 3*, 917-921.

Ameenah, G.-F. (2006). Medicinal plants: Traditions of yesterday and drugs of tomorrow. *Molecular Aspects of Medicine 27*, 1-93.

American Cancer Society. (2006). Cancer Facts and Figures. *Surveillance Research 1*, pages 52-61

Amstad, P., Moret, R., & Cerutti, P. (1994). Glutathione peroxi- dase compensates for the hypersensitivity of Cu,Zn- superoxide dismutase overproducers to oxidant stress. *J. Biol. Chem., 269*, 1606-1609.

Arnér, E. S. J., & Holmgren. (2006). The thioredoxin system in cancer. *Seminars in Cancer Biology, 16*, 420-426. .

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

Avci-Adali, M., Metzger, M., Perle, N., Ziemer, G., & Wendel, H. P. (2010). Pitfalls of Cell-Systematic Evolution of Ligands by Exponential Enrichment (SELEX): Existing Dead Cells During In Vitro Selection Anticipate the Enrichment of Specific Aptamers. *Oligonucleotides, 20*(6), 317-323.

Avendano, C., & Menendez, J. C. (Eds.). (2008). *Medicinal Chemistry of anticancer drugs* (3rd ed.). U.K: Jordan Hill Oxford.

Bahr, C., & Groner, B. (2004). The insulin like growth factor-1 receptor (IGF-1R) as a drug target: novel approaches to cancer therapy. . *Growth Hormone & Igf Research 14*(4), 287-295.

Balint, E. E., & Vousden, K. H. (2001). Activation and activities of the p53 tumour suppressor protein. *British journal of cancer, 85*(12), 1813-1823.

Balunas, M. J., & Kinghorn, A. D. (2005). Drug discovery from medicinal plants. *Life Sciences, 78*(5), 431-441.

Barbora, B., & Ales, H. (2011). Generation of Reactive Oxygen Species during Apoptosis Induced by DNA-Damaging Agents and/or Histone Deacetylase Inhibitors. *Oxidative Medicine and Cellular Longevity*, 7.

Barcic, D., & Ivancic, V. (2010). Impact of the Prudinec/Jakusevec Landfill on Environment Pollution. *Sumarski List, 1, 34*(7-8), 347-359.

Barr, P. J., & Tomei, L. D. (1994). Apoptosis and its role in human disease. *Biotechnology 12*, 487-493.

Bauer, M. K., Schubert, A., Rocks, O., & Grimm, S. (1999). Adenine nucleotide translocase-1, a component of the permeability transition pore, can dominantly induce apoptosis. *J Cell Biol, 147*(7), 1493-1502.

Bertino, J. R. (1997). Irinotecan for colorectal cancer. *Semin Oncol. , 24*, S18-S23.

Beutner, G., Ruck, A., Riede, B., & Brdiczka, D. (1998). Complexes between porin, hexokinase, mitochondrial creatine kinase and adenylate translocator display properties of the permeability transition pore. Implication for regulation of permeability transition by the kinases. *Biochimica Et Biophysica Acta-Biomembranes, 1368*(1), 7-18.

Bharat, B. A., Haruyo, I., Prachi, G., Priya, W., Gautam, S., Indra, D. B., Manoj, K. P., Shishir, S., & Muraleedharan, G. N. (2006). Oncologic, Endocrine & Metabolic from traditional Ayurvedic medicine to modern medicine:identification of therapeutic targets for suppression of inflammation and cancer. *Expert Opin. Ther. Targets 10*(1), 87-118.

Boets, P., Lock, K.,, & Goethals, P. L. M. (2011). Using long-term monitoring to investigate the changes in species composition in the harbour of Ghent (Belgium). . *Hydrobiologia*, *663*(1), 155-166.

Bojes, H. K., Datta, K., Xu, J., Chin, A., Simonian, P., Nunez, G., & Kehrer, J. P. (1997). Bcl-xL overexpression attenuates glutathione depletion in FL5.12 cells following interleukin-3 withdrawal,. *Biochem. J, 325*, 115-119.

Brabek, V., & Kasparkova, J. (2005). Modification Of DNA by platinum complexes relation to resistance of tumors to anti-tumor drugs *Drug Resistance, 8*(131-14).

Brandt, K., Christensen, L. P., Hansen-Møller, J., Hansen, S. L., Haraldsdottir, J., Jespersen, L., Purup, S., Kharazmi, A., Barkholt, V., Frøkiær, H., & Kobæk-Larsen, M. (2004). Health promoting compounds in vegetables and fruits: A systematic approach for identifying plant components with impact on human health. *Trends in Food Science and Technology 14*, 383-384.

Bridonneau, P., Chang, Y. F., O'Connell, D., Gill, S. C., Snyder, D. W., Johnson, L., Goodson, T., Herron, D. K., & Parma, D. H. (1998). High-affinity aptamers selectively inhibit human nonpancreatic secretory phospholipase A(2) (hnps-PLA(2)). *Journal of Medicinal Chemistry*, *41*(6), 778-786.

Brüne, B. (2003). Nitric oxide: NO apoptosis or turning it ON? *Cell Death Differ*, *10*(8), 864–869.

Bruno, J. G., & Kiel, J. L. (2002). Use of magnetic beads in selection and detection of biotoxin aptamers by electrochemiluminescence and enzymatic methods. . *Biotechniques, 32*(1), 178-188.

Bungu, L., Frost, C. L., Brauns, S. C., & van de Venter, M. (2006). Tulbaghia violacea inhibits growth and induces apoptosis in cancer cells in vitro. *African Journal of Biotechnology, 5*(20), 1936-1943.

Bungu, L., van de Venter, M., & Frost, C. (2008). Evidence for an in vitro anticoagulant and antithrombotic activity in Tulbaghia violacea. *African Journal of Biotechnology, 7*(6), 681-688.

Burbidge, R. B. (1978). A revision of the genus Tulbaghia. *Notes from the Royal Botanic Garden (Edinburgh), 36*, 77-103.

Burdon, R. H., Gill, V., & Rice-Evans, C. (1989). Cell proliferation and oxidative stress, . *Free Radic. Res. Commun*, 149-159.

Burton, S. G., & Kaye, P. T. (1992). Isolation and characterisation of sulphur compounds from *Tulbaghia violacea*. . *Planta Med, 58*, 295- 296.

Cai, J., & Jones, D. P. (1998). Superoxide in apoptosis: Mitochondrial generation triggered by cytochrome c loss. *J. Biol. Chem. , 273*, 11401-11404.

Cano-Europaa, E., González-Trujanob, M. E., Reyes-Ramírezc, A., Hernández-Garcíaa, A., Blas-Valdiviaa, V., & Ortiz-Butróna, R. (2010). Palmitone prevents pentylenetetrazole-caused neuronal damage in the CA^3 hippocampal region of prepubertal rats. *Neuroscience Letters, 470*, 111-114.

Carl, A. (2006). Overview of The South African Cancer Research Environment as a basis for discussions concerning the activation of Cancer Research Initiative of South Africa (CARISA). *Cancer Research Initiative of South Africa*.

Chauhan, G., Sharma, M., Kharkwal, H., & Varma, A. (2011). Pharmacognostic, preliminary Phytochemical studies and anti-cacerous potential of *Trigonella foenum-grecum*. *Pharma science monitor: An international Journal of pharmaceutical Sciences 2*(2).

Chen, C.-Y., Hsu, Y.-L., Chen, Y.-Y., Hung, J.-Y., Huang, M.-S., & Kuo, P.-L. (2007). Isokotomolide A, a new butanolide extracted from the leaves of Cinnamomum kotoense, arrests cell cycle progression and induces apoptosis through the induction of p53/p21 and the initiation of mitochondrial system in human non-small cell lung cancer A549 cells. *Eur J Pharmacol, 574*, 94-102.

Cheng, G. C., Schulze, P. C., Lee, R. T., Sylvan, J., ., Zetter, B. R., & Huang, H. (2004). Oxidative stress and thioredoxin-interacting protein promote intravasation of melanoma cells. *Experimental Cell Research, 300*(297-307).

Chinnaiyan, A. M., O'Rourke, K., Lane, B. R., & Dixit, V. M. (1997). Interaction of CED-4 with CED-3 and CED-9: a molecular framework for cell death. *Science, 275*, 1122-1126.

Chow, L., Xing, Z., Benoy, G., Rees, H. W., Meng, F., Jiang, Y., & Daigle, J. L. (2011). Hydrology and water quality across gradients of agricultural intensity in the Little River watershed area. *Journal of Soil and Water Conservation, 66*(1), 71-84.

Christopher, E. J., Swiatoniowski, A., & Andrew, C. (2004). Lin TJ. Pseudomonas aeruginosa exotoxin A induces human mast cell apoptosis by a caspase-8 and -3- dependent mechanism. *J Biol Chem 279:*, 37201-37207.

Christopher, O. C. B. (1996). p53 and apoptosis. *Bnhih Medical Bulletin, 53*, 522-538.

Clarke, D. J., Johnson, R. T., & Downes, C. S. (1993). opoisomerase II inhibition prevents anaphase chromatid segregation in mammalian cells independently of the generation of DNA strand breaks. *T. Journal of cell science, 105*, 563-569.

com/sis/apoptosis/docs/manual_apoptosis.pdf, h. w. r.-a.-s. Apoptosis and Cell Proliferation Manual.

Cory, S., Huang, D. C. S., & Adams, J. M. (2003). The Bcl-2 family: roles in cell survival and oncogenesis. *Oncogene, 22*(53), 8590-8607.

Cotran, Kumar, & Collins (Eds.). (1998). *Robbins Pathologic Basis of Disease*. Philadelphia: W.B Saunders Company.

Cragg, G. M., & Newman, D. J. (2005). Plants as source of anticancer agents. *J Ethnopharmacol, 100*, 72-79.

Creemers, G. J., Bolis, G., Gore, M., Scarfone, G., Lacave, A. J., Guastalla, J. P., Despax, R., Favalli, G., Kreinberg, R., VanBelle, S., Hudson, I., Verweij, J., & Huinink, W. W. T. (1996). Topotecan, an active drug in the second-line treatment of epithelial ovarian cancer: results of a large European phase II study. *J Clin Oncol., 14*, 3056-3061.

Czene, S., Tiback, M., & Harms-Ringdahl, M. (1997). pH-dependent DNA cleavage in permeabilized human fibroblasts. *Biochem. J, 323*, 337-341.

Dahlgren, R. M. T., Clifford, H. T., & Yeo, P. F. (Eds.). (1985). *The families of the monocotyledons.* . Berlin: Springer-Verlag, .

Dancey, J., & Eisenhauer, E. A. (1996). Current perspectives on camptothecins in cancer treatment. *British Journal of Cancer (1996) 74, 327-338, 74*, 327-338.

Debatin, K. M., Poncet, D., & Kroemer, G. (2002). Chemotherapy: targeting the mitochondrial cell death pathway. *Oncogene, 21*(57), 8786-8803.

Degli-Esposti, M. A., Dougall, W. C., Smolak, P. J., Waugh, J. Y., Smith, C. A., & Goodwin, R. G. (1997a). The novel receptor TRAIL-R4 induces NF-nB and protects against TRAIL-mediated apoptosis, yet retains an incomplete death domain. *Immunity 7*, 813–820.

Degli-Esposti, M. A., Smolak, P. J., & Walczak H, e. a. (1997b). Cloning and characterization of TRAIL-R3, a novel member of the emerging TRAIL receptor family. *J Exp Med., 186*, 1165–1170.

Dewick, P. M. (Ed.). (2001). *Medicinal natural products: A biosynthetic approach.* (2nd ed.). London: John Wiley and Sons.

DiGiovanni, J. (1992). Multistage carcinogenesis in mouse skin. *Pharmacol. Ther., 54*, 63-128.

Donald, D. L. (Ed.). (1997). *C. elegans II*. New York, NY: Cold Spring Harbor Laboratory Press.

Dudley, H. W., & Ian, F. (Eds.). (2007). *Spectroscopic Methods in Organic Chemistry* The Mcgram-Hill Companies.

Edwards, A., Atma-Ram, A., & Thin, R. N. (1988). Podophyllotoxin 0.5% v podophyllin 20% to treat penile warts. *Genitourinary medicine, 64*, 263-265.

Eguchi, Y., Shimizu, S., & Tsujimoto, Y. (1997). Intracellular ATP Levels Determine Cell Death Fate by Apoptosis or Necrosis. *Cancer Research., 57*, 1835-1840.

Elaine, W., Hardman, W.,, Reddy, C., P., Avula,, Gabriel, F., & Ivan, L., Cameron,. (2001). Three Percent Dietary Fish Oil Concentrate Increased Efficacy of Doxorubicin Against MDA-MB 231 Breast Cancer Xenografts. *Clinical Cancer Research., 7*, 2041–2049.

Elisa, C., Jordi, T., & Joaquim, R. (2000). Oxidative stress in bacteria and protein damage by reactive oxygen species. *International Microbiol., 3*, 3-8.

Ellis, H. M., & Horvitz, H. R. (1986). Genetic control of programmed cell death in the nematode C. elegans. *Cell, 44*, 817-829.

Elwell, K. E., Halla, C., Tharkara, S., Girauda, Y., Bennetta, B., Baea, C., & Carper, S. W. (2006). A fluorine containing bipyridine cispatinat inducing apoptosis in cancer cell lines . *Bioorganic & Medicinal chemistry, 15*, 869-8700.

Engert, A., Franklin, J., Eich, H. T., Brillant, C., Sehlen, S., Cartoni, C., Herrmann, R., Pfreundschuh, M., Sieber, M., & Tesch, H. (2007). Two cycles of doxorubicin, bleomycin, vinblastine, and dacarbazine plus extendedfield radiotherapy is superior to radiotherapy alone in early favorable Hodgkin's lymphoma: final results of the GHSG HD7 trial. *J Clin Oncol., 25*, 3495-3502.

Erhabor, O., & Adias, T. C. (2011). From whole blood to component therapy: The economic, supply/demand need for implementation of component therapy in sub-Saharan Africa. *Transfusion Clinique et Biologique, 18,* (5-6), 516–526.

Fabisiak, J. P., Tyurina, Y. Y., Tyurin, A. A., Lazo, J. S., & Kagan, V. E. (1998). Ramdom versus selective membrane phospholipid oxidation in apoptosis: role of phosphatidylserine. *Biochemistry, 37*, 13781-13790.

Fadok, V. A., Bratton, D. L., & Henson, P. M. (2001). Phagocyte receptors for apoptotic cells: recognition, uptake, and consequences. *J Clin Invest, 108*(7), 957-962.

Fay, M. F., & Chase, M. W. (1996). Resurrection of Themidaceae for the Brodiaea alliance, and recircumscription of Alliaceae, Amaryllidaceae and Agapanthoideae. *Taxon, 45*(3), 441-451.

Fesik, S. W., & Shi, Y. (2001). Structural biology. Controlling the caspases. *Science, 294*(5546), 1477-1478.

Fink, S. L., & Cookson, B. T. (2005). Apoptosis, Pyroptosis, and Necrosis: Mechanistic description of dead and dying eukaryotic cells. *Infection and Immunity, 73*, 1907-1916.

Fischer, U., & Schulze-Osthoff, K. (2005). New approaches and therapeutics targeting apoptosis in disease. *Pharmacological Reviews, 57*(2), 187-215.

Flierl, A., Chen, Y., Coskun, P. E., Samulski, R. J., & Wallace, D. C. (2005). Adeno-associated virus-mediated gene transfer of the heart/muscle adenine nucleotide translocator (ANT) in mouse. *Gene Ther., 12*, 570–578.

Frankline, K. K., Stonard, K., Sylvester, S. L. L., James Darkwa, D., Jasper G. Rees, & Mervin Meyer. (2008). In vitro evaluation of dichloro-bis(pyrazole)palladium(II) and dichloro-bis(pyrazole)platinum(II) complexes as anticancer agents. *Cancer Chemother Pharmacol*, 127–138.

Freimoser, F. M., Jacob, C. A., Aebi, M., & Tuor, U. (1999). The MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] Assay is a fast and reliable method for colorimetric determination of fungal cell densities. *Applied and Environmental Microbiology*, *65*, 3727-3729.

Fresno, M., Jimene, A., & Vazquez, D. (1977). Inhibition of translation in eukaryotic systems by harringtonine. *Eur J Biochem, 72*, 323–330.

Fujino, G., Noguchi, T., Takeda, K., & Ichijo, H. (2006). Thioredoxin and protein kinases in redox signaling. *Seminars in Cancer Biolgy, 16*, 427-435.

Fujita, N., & Tsuruo, T. (1998). Involvement of Bcl-2 cleavage in the acceleration of VP-16-induced U937 cell apoptosis. *Biochem Biophys Res Commun, 246*(2), 484-488.

Fulda, S., Wick, W., Weller, M., & Debatin, K.-M. (2002). Smac agonists sensitize for Apo2L/TRAIL- or anticancer drug-induced apoptosis and induce regression of malignant glioma in vivo. *Nature medicine, 8*(8), 808-815.

Gaidamashvili, M., & van Staden, J. (2002). Interaction of lectin-like proteins of South African medicinal plants with Staphylococcus aureus and Bacillus subtilis. *Journal of Ethnopharmacology, , 80*(2-3), 131-135.

George, P. S. (Ed.). (1999). *Apoptosis: A Pratical Approach*. New York: Oxford University Press.

González-Trujano, M. E., Navarrete A, Reyes, B., Cedillo-Portugal, E., & Hong, E. (2001). Anticonvulsant properties and bio-guided isolation of palmitone from leaves of Annona diversifolia Saff. *Planta Med, 67*, 136–141.

Gopinath, S. C. B., Misono, T. S., Kawasaki, K., Mizuno, T., Imai, M., Odagiri, T., & Kumar, P. K. R. (2006). An RNA aptamer that distinguishes between closely related human influenza viruses and inhibits haemagglutinin-mediated membrane fusion. *Journal of General Virology, 87*, 479-487.

Gordaliza, M., Garcia, P. A., del Corral, J. M., Castro, M. A., & Gomez-Zurita., M. A. (2004). Podophyllotoxin: distribution, sources, application, and new cytotoxic derivatives. *Toxicon, 44*, 441-459.

Green, D. R., & Reed, J. C. (1998). Mitochondria and apoptosis. *Science, 281*(5381), 1309-1312.

Gregory, D. P., Sucha, S., Jonathan, B., David, W., & Tony, H. (1999). The protein kinases of Caenorhabditis elegans: A model for signal transduction in multicellular organisms. *PNAS, 96*(24), 13603-13610.

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

Gurib-Fakim, A., & Schmelzer, G. (2007). Medicinal plants in Africa. *PROTA,*, 6.

Häcker, G. (2000). The morphology of apoptosis. *Cell Tissue Res., 301*(1), 5-17.

Haendeler, J., Tischler, V., Hoffmann, J., Zeiher, A. M., & Dimmeler, S. (2004). Low doses of reactive oxygen species protect endothelial cells from apoptosis by increasing thioredoxin-1 expression. Federation of European *Biochemical Societies., 577*, 427-433.

Hamdy, A. A. A. (2012). Cancer therapy and vaccination. *Journal of Immunological Methods, 382*(1-2), 1–23.

Harpreet, S., Leena, P., & Pradeep, P. (2011). Review on cancer and anticancerous properties of some medicinal plants *Journal of Medicinal Plants Research, 5*(10), 1818-1835.

Harvey, A. L. (1999). Medicines from nature: are natural products still relevant to drug discovery. *Trends Pharmacol Sci., 20*, 196-198.

Hengartner, M. O. (1996). Programmed cell death in invertebrates. *Current Opinion in Genetics & Development, 6*(1), 34-38.

Herode, S. S., Hadolin, M., Skerget, M., & Knez, Z. (2003). Solvent extraction study of antioxidants from balm (*Melissa officinalis I.)* leaves. *Food Chem., 80*, 275-282.

Higuchi, A., Siao, Y. D., Yang, S. T., Hsieh, P. V., Fukushima, H., Chang, Y., Ruaan, R. C., & Chen, W. Y. (2008). Preparation of a DNA aptamer-Pt complex and its use in the colorimetric sensing of thrombin and anti-thrombin antibodies. . *Analytical Chemistry, 80*(177), 6580-6586.

Hockenbery, D. M., Oltvai, Z. N., Yin, X. M., Milliman, C. L., & Korsmeyer, S. J. (1993). Bcl-2 functions in an antioxidant pathway to prevent apoptosis. *Cell, 75*, 241-251.

Hofmann, H. S., Simm, A., Hammer, A., Silber, R. E., & Bartling, B. (2002). Expression of inhibitors of apoptosis (IAP) proteins in non-small cell human lung cancer. *J. Cancer Res. Clin. Oncol, 128*, 554–560.

Horwitz, S. B. (2004). Personal recollections on the early development of taxol. *Journal Of Natural Products, 67*, 136-138.

http://lipidlibrary.aocs.org/ms/masspec.html.

http://www2.chemistry.msu.edu/faculty/reusch/VirtTxtJml/Spectrpy/nmr/nmr1.htm.

https://www.roche- applied-science.com/publications/print_mat/ lab_faqs.pdf. Lab FAQs.

Hutchings, A. H., Scott, G. L., & Cunningham, A. (Eds.). (1996). *Zulu Medicinal Plants: An Inventory*. Scottsville,South Africa.: University of Natal Press.

Imawati, B., Holt, O., Micheal, L., Xu, L., & Xiaodong, W. (1999). Biochemical Pathways of caspase Activation during Apoptosis. *Annu. Rev. Cell Dev. Biol., 15*(269-90).

Imoto, I., Yang, Z. Q., Pimkhaokham, A., Tsuda, H., Shimada, Y., Imamura, M., Ohki, M., & Inazawa, J. (2001). Identification of cIAP1 as a candidate target gene within an amplicon at 11q22 in esophageal squamous cell carcinomas. *Cancer research, 61*(18), 6629-6634.

Itokawa, H., Wang, X., & Lee, K.-H. (Eds.). (2005). *Homoharringtonine and related compounds*. Boca Raton, Florida, : Brunner-Routledge Psychology Press, Taylor & Francis Group.

Jacobsen, J. V., Yamaguchi Y, Mann L..K, & Howard F.D. (1968). An alkyl-cysteine sulfoxide lyase in *Tulbaghia violacea* and its relation to other alliinase-like enzymes. *Phytochemistry., 7*, 1099-1108.

Jacobson, M. D., Weil, M., & Raff, M. C. (1997). Programmed cell death in animal development. *Cell, 88*(3), 347-354.

Jeremy, B. C. J. (2008). Ecological extinction and evolution in the brave new ocean *Proceedings of the National Academy of Sciences of the United States of America, 105*, 11458-11465.

Jirsova, K., Mandys, V., Gispen, W. H., & Bär, P. R. (2006). Cisplatin-induced apoptosis in cultures of human Schwann cells. *Neuroscince Letters, 9*, 22-26.

John, S. B. (2001). The molecular biology of cancer. *Molecular aspects of Medicine 21*, 167 -- 223.

Johnstone, R. W., Frew, A. J., & Smyth, M. J. (2008). The TRAIL apoptotic pathway in cancer onset, progression and therapy. *Nature Reviews Cancer, 8*(10), 782-798.

Jorgen, W., Kaisa, H., & Ellen, M. H. (2011). Fibroblast growth factors and their receptors in cancer. *Biochem. Journal., 437*, 199-213.

Kamata, H., Honda, S., Maeda, S., Chang, L., Hirata, H., & Karin, M. (2005). Reactive oxygen species promote TNFalpha-induced death and sustained JNK activation by inhibiting MAP kinase phosphatases. *Cell, 120*(5), 649-661.

Kang, J., Lee, M. S., & Gorenstein, D. G. (2005). The enhancement of PCR amplification of a random sequence DNA library by DMSO and betaine: Application to in vitro combinatorial selection of aptamers. *Journal of Biochemical and Biophysical Methods, 64*(2), 147-151.

Kantarjian, H. M., O'Brien, S., Anderlini, P., & Talpaz, M. (1996). Treatment of chronic myelogenous leukemia: current status and investigational options. *Blood, 87*, 3069-3081.

Kasof, G. M., & Gomes, B. C. (2001). Livin, a novel inhibitor of apoptosis protein family member. *J Biol Chem, 276*(5), 3238-3246.

Kenakin, T., P., (Ed.). (2009). *A Pharmacolgy Primer: Theory, Applications and Methods* (3rd ed.). Califonia: Elsievier academic press.

Kensler, T. W. (1997). Chemotherapy by inducers of Carcinogen Detoxification Enzymes. *Environmental Health Perspectives, 105*, 965-970.

Kerr, J. F., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *British journal of cancer, 26*(4), 239-257.

Kim, A., Kristiansen,, Poul, E., Jensen,, Ian, M., Moller, , & Alexander, S. (2009). Monitoring reactive oxygen species formation and localisation in living cells by use of the fluorescent probe CM-H2DCFDA and confocal laser microscopy. *Physiologia Plantarum, 136*, 369–383.

Kinghorn, A. D., Farnsworth, N. R., Soejarto, D. D., Cordella, G. A., Swanson, S. M., Pezzuto, J. M., Wani, M. C., Wall, M. E., Kroll, N. H., Kramer, R. A., Rose, W. C., Vite, G. D., Fiarchild, C. R., Peterson, R. W., & Wild, R. (2003). Novel Stragedies for the discovery of plants-derived anticancer agents. . *Pharmaceutical Biology, 41*, 53-67.

Kluck, R. M., Martin, S. J., Hoffman, B. M., Zhou, J. S., Green, D. R., & Newmeyer, D. D. (1997). Cytochrome c activation of CPP32-like proteolysis plays a critical role in a Xenopus cell-free apoptosis system. *EMBO Journal, 16*, 4639-4649.

Komata, T., Kondo, Y., Kanzawa, T., Hirohata, S., Koga, S., Sumiyoshi, H., Srinivasula, S. M., Barna, B. P., Germano, I. M., & Takakura, M. (2001). Treatment of malignant glioma cells with the transfer of constitencyactive caspase 6 using the human telomerase catalytic subunit (human telomerase reverse transcriptase) *Cancer Res., 61*, 5796-5802.

Korkolopoulou, P., Goudopoulou, A., Voutsinas, G., Thomas-Tsagli, E., Kapralos, P., Patsouris, E., & Saetta, A. A. (2004). c-FLIP expression in bladder urothelial carcinomas: Its role in resistance to FAS-mediated apoptosis and clinicopathologic correlations. *Urology, 63*(6), 1198-1204.

Kroemer, G., El-Deiry, W. S., Golstein, P., Peter, M. E., Vaux, D., Vandenabeele, P., Zhivotovsky, B., Blagosklonny, M. V., Malorni, W., Knight, R. A., Piacentini, M., Nagata, S., & Melino, G. (2005). Classification of cell death: recommendations of the Nomenclature Committee on Cell Death. *Cell Death and Differentiation, 12*, 1463-1467.

Kroemer, G., Galluzzi, L., Maiuri, M. C., Vitale, I., Zischka, H., Castedo, M., & Zitvogel L. (2007). Cell death modalities: classification and pathophysiological implications. . *Cell Death and Differentiation, 14*, 1237–1243.

Kroemer, G., Zamzami, N., & Susin, S. A. (1997). Mitochondrial control of apoptosis. *Immunol Today, 18*, 44-51.

Kubec, R., Velisek, J., & Musah, R. A. (2002). The amino acid precursors and odor formation in society garlic (Tulbaghia violacea Harv.). *Phytochemistry, 60*(1), 21-25.

Kuliczkowski, K. (1989). Influence of harringtonine on human leukemia cell differentiation. *Arch Immunol Ther Exp (Warsz). 37*, 69–76.

Kumar, P. J., & Clark, M. L. (Eds.). (1990). *Clinical Medicine*: Bailliere Tindall

Kumar, V., Abbas, A. K., & Fausto, N. (Eds.). (2007). *Basic Pathology*. Philadelphia, USA: : Saunders.

Kuo-Chen, C. (2002). Prediction of Protein Signal Sequences. *Current Protein and Peptide Science, 3*, 615-622.

Lawrence, M. S., & Jonathan, D. A. (2001). Methods in Cell Biology. *Apoptosis, 66*, 49.

LeBlanc, H. N., & Ashkenazi, A. (2003). Apo2L/TRAIL and its death and decoy receptors. *Cell Death Differ, 10,* 66–75.

Lee, J. S., & Surh, Y. J. (2005). Nrf2 as a novel molecular target for chemoprevention. *Cancer Letters, 224*(2), 171-184.

Leiter, U., Schmid, R. M., Kaskel, P., Peter, R. U., & Krahn, G. (2000). Antiapoptotic bcl-2 and bcl-xL in advanced malignant melanoma. *Arch Dermatol Res, 292*(5), 225-232.

Lennon, S. V., Martin, S. J., & Cotter, T. G. (1991). Dose-dependent induction of apoptosis in human tumour cell lines by widely diverging stimuli. *Cell Proliferation, 24*(2), 203-214.

Letai, A., Sorcinelli, M. D., Beard, C., & Korsmeyer, S. J. (2004). Antiapoptotic Bcl-2 is required for maintenance of a model leukemia. *Cancer Cell., 6,* 241–249.

Lewis, K. (2000). Programmed death in bacteria. *Microbiol.Mol. Biol. rev., 64,* 503-514.

Linda, M. A., Eileen, A., B., , Hall, B., & Harry, O. (2006). Distribution, interconversion, and dose response of n−3 fatty acids in humans 1' 2' 3' 4. *Am J Clin Nutr, 83*(6), S1467-1476S.

Liu, Q. A., & Hengartner, M. O. (1999). The molecular mechanism of programmed cell death in C-elegans. *Mechanisms of Cell Death, 887,* 92-104.

Lokamatha, K. M., Bharathi, A., Shanta, K., S.,, & Rama, R., N.,. (2010). Effect of PVP-K30 on complexation and dissolution rate of Nevirapine-β-Cyclodextrin complexes. *International Journal of Pharmacy and Pharmaceutical Sciences, 2*(4), 169-176.

Lyantagaye, L. (2005). *Screening Tulbaghia violacea extracts for the presence of poptotic compounds.* University of the Western Cape, Capetown.

Lynda, M., Evans, , & Robert, W., Hardy,. (2010). Optimizing Dietary Fat to Reduce Breast Cancer Risk: Are we there Yet? *The Open Breast Cancer Journal,, 2*, 108-122.

MacCorkle, R. A., Freeman, K. W., & Spencer, D. M. (1998). Synthetic activation of caspases: Artificial death switches. *Proceedings of the National Academy of Sciences of the United States of America, 95*(7), 3655-3660.

Marchetti, P., Decaudin, D., Macho, A., Zamzami, N., Hirsch, T., Susin, S. A., & Kroemer, G. (1997). Redox regulation of apoptosis: impact of thiol oxidation status on mitochondrial function. *Eur J Immunol 27*(1), 289-296.

Marks, P. A. (2006). Thioredoxin in cancer—Role of histone deacetylase inhibitors. *Seminars in Cancer Biology, 16*, 436-443. .

Mashima, T., & Tsuruo, T. (2005). Defects of the apoptotic pathway as a therapeutic target against cancer. *Drug Resistance Updates, 8*, 339-343.

Max, K., Simon, J. v. H., Leonie, S., Anna, C. N., Eva M. Janssen-Megens, & Marion Lohrum. (2009). The novel p53 target gene IRF2BP2 participates in cell survival during the p53 stress response. *Nucleic Acids Research, 37*(2), 322-335.

Michael, D., Tito, F., & Susan, B. (2005). Tumour stem cells and drug resistance *Nature Reviews Cancer, 5*, 275-284.

Michael, J. T., John, O., DeLancey, , Melissa, M. C., Ahmedin, J., & Elizabeth, M. W. (2010). The global burden of cancer: priorities for prevention. *Carcinogenesis, 31*(1), 100-110.

Michael, M. G. (2002). Mechanisms of Cancer Drug Resistance. *Annu. Rev. Med., 53*, 615-627.

Mohammad, S. (2006). Anticancer agents from medicinal plants. *Bangladesh J Pharmacol, 1*, 35-41.

Morita, Y., & Tilly, J. L. (1999). Oocyte apoptosis: like sand through an hourglass. *Dev Biol, 213*, 1-17.

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods, 65*, 55-63.

Mothana, R. A., Abdo, S. A., Hasson, S., & Althawab, F. M. (2008). Antimicrobial, Antioxidant and Cytotoxic Activities and Phytochemical Screening of Some Yemeni Medicinal Plants. *Journal of Evidencebased Complimentary and alternative medicine, 10*, 1093.

Motsei, M. L., Lindsey, K. L., van Staden, J., & Jager, A. K. (2003). Screening of traditionally used South African plants for antifungal activity against Candida albicans. *Journal of Ethnopharmacology, 86(2-3)*, 235.

Mqoqi, N., Kellett, P., Sitas, F., & Jula, M. (2004). Incidence of histologically diagnosed cancer in South Africa, 1998-1999. *National Cancer Registry South Africa, Johannesburg*, 1-96.

Naidoo, V., McGaw, L. J., Bisschop, S. P. R., Duncan, N., & Eloff, J. N. (2008). The value of plant extracts with antioxidant activity in attenuating coccidiosis in broiler chickens. *Veterinary Parasitology, 153*(3-4), 214-219.

National Cancer Registry. (2004). Incidence of Histologically diagnosed cancer in South Africa,1998 – 1999. *14*,

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

Nguyen, J. T., & Wells, J. A. (2003). Direct activation of the apoptosis machinery as a mechanism to target cancer cells. *Proceedings of the National Academy of Sciences of the United States of America, 100*(13), 7533-7538.

Nicholson, D. W. (1999). Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death and Differentiation, 6*(11), 1028-1042.

Nicholson, D. W., & Thornberry, N. A. (1997). Caspses: Killer Proteases. *Trends Biochem. Sci, 22*, 299–306.

Nikolovska-Coleska, Z., Xu, L., Hu, Z., Tomita, Y., Li, P., Roller, P. P., Wang, R., Fang, X., Guo, R., Zhang, M., Lippman, M. E., Yang, D., & Wang, S. (2004). Discovery of embelin as a cell-permeable, small-molecular weight inhibitor of XIAP through structure-based computational screening of a traditional herbal medicine three-dimensional structure database. *Journal of medicinal chemistry, 47*(10), 2430-2440.

Nordberg, J., & Arnér, E. S. J. (2001). Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. *Free Radical Biology and Medicine., 31*, 1287-1312.

Ohshima, H., Tazawa, H., Sylla, B. S., & Sawa, T. (2005). Prevention of human cancer by modulation of chronic inflammatory processes. *Mutat Res, 591*, 110-122.

Okouneva, T., Hill, B. T., Wilson, L., & Jordan, M. A. (2003). The effect of vinflunine, vinovelbine, and vinblastine on centromere dynamics. *Molecular Cancer Therapies, 2*, 427-436. .

Okuyama, H., Nakamura, H., Shimahara, Y., Uyama, N., Kwon, Y. W., Kawada, N., Yamaoka, Y., & Yodoi, J. (2005). Overexpression of thioredoxin prevents thioacetamide-induced hepatic fibrosis in mice. *Journal of Hepatology, 42*, 117-123.

Oommen, S., Anto, R. J., Srinivas, G., & Karunagaran, D. (2004). Allicin (from garlic) induces caspase-mediated apoptosis in cancer cells. *European Journal of Pharmacology, 485*(1-3), 97-103.

Pan, C., Chen, Y. G., Ma, X. Y., Jiang, J. H., He, F., & Zhang, Y. (2011). Phytochemical Constituents and Pharmacological Activities of Plants from the Genus Adiantum: A Review. *Tropical Journal of Pharmaceutical Research, 10*(5), 681-692.

Papenfuss, K., Cordier, S. M., & Walczak, H. (2008). Death receptors as targets for anti-cancer therapy. *Journal of cellular and molecular medicine, 12*(6B), 2566-2585.

Pascal, S., & Jurg, T. (2000). Apoptosis Induced by death receptors. *Pharmaceutica Acta H, 74*, pp281-286.

Patyah, S., Joshi, R., Prasad, B. D. S., Prakash, A., Medhi, B., & Das, B. K. (2010). bacteria in Cancer therapy; a novel experimental strategy. *Journal of Biochemical Science, 17*, 21.

Pérez, V. I., Lew, C. M., Cortez, L. A., Webb, C. R., Rodriguez, M., Liu, Y., Qi, W., Li, Y., Chaudhuri, A., Van Remmen, H., Richardson, A., & Ikeno, Y. (2008). Thioredoxin 2 haploinsufficiency in mice results in impaired mitochondrial function and increased oxidative stress. *Free Radical Biology and Medicine, 44*, 882-892.

Pierre, G., & Guido, K. (2006). Cell death by necrosis: towards a molecular definition. *TRENDS in Biochemical Sciences 32*(1), 0968-0004.

Pieters, L., & Vlietinck, A. J. (2005). Bio-guided isolation of Pharmacologically active plant components, still a valuable strategy for the funding of new lead compounds. *Perspectives, 100*(57-60).

Powell, R. G., Weisleder, D., Smith, C. R. J., & Rohwedder, W. K. (1970). Structures of harringtonine, isoharringtonine, and homo-harringtonine. *Tetrahedron Letters, 11*, 815-818.

Pradeep, K., Madhu, K., & Jagtar, S. (2011). Biochemical characterization of Santalum album (Chandan) leaf peroxidase. *Physiol Mol Biol Plants, 17*(2), 153–159.

Rajesh, P. R., Rich,, & Rajeshwar, P. S. (2009). Apoptosis: Molecular Mechanisms and Pathogenicity. *Excli Jnal, 8*, 155-181.

Raji, I., A..,, Pierre, M., & Kenechukwu, O. (2012). Effect of Tulbaghia violacea on the blood pressure and heart rate in male spontaneously hypertensive Wistar rats. *Journal of Ethnopharmacology (in Press)*.

Richard, J. D., Tilla, S. W., & Toru, S. (2006). n-3 Fatty acids and gene expression. *Am. J. Clin. Nutr., 83*.

Robert, A. G., & Robert, J. G. (2004). Why do Cancers have high aerobic glycolysis? *Nature Reviews, 4*, 891.

Robert , S. K. (2006). Antiangiogenic Therapy: A Universal Chemosensitization Strategy for Cancers? *Science, 312*, 1171-1174.

Rontani, J.-F., Aubert, C., & Giusti, G. (1990). Involvement of double mclafferty rearrangements during the fragmentations of some spiroalkanones. *Journal of Mas Spectrometry, 25*(8), 427–428.

Ross, M. S. F., & Brain, K. R. (Eds.). (1977). *An introduction to phytopharmacy.* Great Britain: Pitman press.

Rouëssé, J., Spielmann, M., Turpin, F., Le Chevalier, T., Azab, M., & Mondésir, J. M. (1993). Phase II study of elliptinium acetate salvage treatment of advanced breast cancer. *Eur J Cancer, 29A*(6), 856-859.

Rowinsky, E. K., Onetto, N., Canetta, R. M., & Arbuck, S. G. (1992). Taxol-the 1st of the texanes, an important new class of anti-tumor agents. . *Semin Oncol., 19*, 646-662.

Roy, S. H., & Stanley, R. F. (2004). Oblimersen Sodium (Genasense bcl-2 Antisense Oligonucleotide) A Rational Therapeutic to Enhance Apoptosis in Therapy of Lung Cancer. *Clin Cancer Res 10*(12), 4245S-4248S.

Royall, J. A., Ischiropoulos, H., & Hiropoulos, H. (1993a). Evaluation of 2',7'-dichlorofluorescin and dihydrorhodamine 123 as fluorescent probes for intracellular H2O2 in cultured endothelial cells. *Arch Biochem Biophys, 302*, 348-355.

Salvesen, G. S., & Duckett, C. S. (2002). IAP proteins: blocking the road to death's door. *Nat Rev Mol Cell Biol, 3*(6), 401-410.

Sanjay, G., Noresah, M., Shariff,, Arvind, B., & Chandra, P., Kala,. (2010). Influence of climate change on production of secondary chemicals in high altitude medicinal plants: Issues needs immediate attention. *Journal of Medicinal Plants Research, 4*(18), 1825-1829.

Scaffidi, C., Schmitz, I., Zha, J., Korsmeyer, S. J., Krammer, P. H., & Peter, M. E. (1999). Differential modulation of apoptosis sensitivity in CD95 type I and type II cells. *J Biol Chem, 274*(32), 22532-22538.

Schiff, P. B., & Horwitz, S. B. (1980). Taxol stabilizes microtubules in mouse fibroblast cells. *Proceedings of the National Academy of Sciences of the United States of America 77*, 1561-1565.

Scott, W. L., & Athena, W. L. (2000). Apoptosis in Cancer *Carcinogenesis, 21*(3), 485-495.

Seshagiri, S., & Miller, L. K. (1997). Caenorhabditis elegans CED-4 stimulates CED-3 processing and CED- 3-induced apoptosis. *Curr Biol, 7*, 455-460.

Shaham, S., & Horvitz, H. R. (1996). An alternatively spliced C. elegans ced-4 RNA encodes a novel cell death inhibitor. *Cell, 86*, 201-208.

Shariat, S. F., Desai, S., Song, W., Khan, T., Zhao, J., Nguyen, C., Foster, B. A., Greenberg, N., Spencer, D. M., & Slawin, K. M. (2001). Adenovirus-mediated transfer of inducible caspases: a novel "death switch" gene therapeutic approach to prostate cancer. *Cancer research, 61*, 2562-2571.

Shawn, A., Ritchie,, Dushmanthi, J., Gerald, F. D., Pearson Ahiahonu1, H. M., & Dayan B Goodenowe. (2001). Human serum-derived hydroxy long-chain fatty acids exhibit anti-inflammatory and anti-proliferative activity. *Clinical Cancer Research 7*, 2041–2049.

Sivalokanathan, S., Ilayaraja, M., & Balasubramanian, M. P. (2005). Efficacy of Terminalia arjuna (Roxb.) on N-nitrosodiethylamine induced hepatocellular carcinoma in rats. *Indian Journal of Experimental Biology, 43*, 264-267.

Spector, M. S., Desnoyer, S., Hoeppner, D. J., & Hengartner, M. O. (1997). Interaction between the C. elegans cell-death regulators CED-9 and CED-4. *Nature, 385*, 653-656.

Stacey, R., M., , & Wei-Xing, Z. (2006). Chemotherapeutic Approaches for Targeting Cell Death Pathways. *The Oncologist 11*(4), 342-357.

Stedman's. (2003). Medical Dictionary. In Edition, t. (Ed.): Lippincott, Williams & Wilkins.

Strasser, A., Harris, A. W., Bath, M. L., & Cory, S. (1990). Novel primitive lymphoid tumours induced in transgenic mice by cooperation between myc and bcl-2. *Nature, 348*(6299), 331-333.

Strasser, A., Harris, A. W., & Cory, S. (1993). E mu-bcl-2 transgene facilitates spontaneous transformation of early pre-B and immunoglobulin-secreting cells but not T cells. *Oncogene,, 8*, 1–9.

Susan, E. (2007). Apoptosis: A Review of Programmed Cell Death. *Toxicologic Pathology, 35:, 2007*, 495–516.

Susanna, C. L., Maria, K., Magnus, I.-S., & Alicja, W. (2004). Dietary long-chain n 3 fatty acids for the prevention of cancer: a review of potential mechanisms. *Am. J. Clin. Nutr., 79* 935-945.

Susin, S. A., Zamzami, N., Castedo, M., Hirsch, T., Marchetti, P., Macho, A., Daugas, E., Geuskens, M., & Kroemer, G. (1996). Bcl-2 inhibits the mitochondrial release of an apoptogenic protease. *Journal of Experimental Medicine, 184*, 1331-1341.

Suzuki, Y., Ono, Y., & Hirabayashi, Y. (1998). Rapid and specific reactive oxygen species generation via NADPH oxidase activation during Fas-mediated apoptosis. *FEBS Lett, 425*, 209-212.

Tacconi, S., Perri, R., Balestrieri, E., Grelli, S., Bernar- dini, S., Annichiarico, R., Mastino, A., Caltagirone, C., & Macchi, B. (2004). Increased caspase activation in peripheral blood mononuclear cells of patients with Alz- heimer's disease. *Exp Neurol 190*, 254-262.

Tamm, I., Richter, S., Oltersdorf, D., Creutzig, U., Harbott, J., Scholz, F., Karawajew, L., Ludwig, W. D., & Wuchter, C. (2004). High expression levels of X-linked inhibitor of apoptosis

protein and survivin correlate with poor overall survival in childhood de Novo acute myeloid leukemia. *Clinical Cancer Research, 10*(11), 3737-3744.

Thamburan, S., Klaasen, J., Mabusela, W. T., Cannon, J. F., Folk, W., & Johnson, Q. (2006). Tulbaghia alliacea phytotherapy: A potential anti-infective remedy for candidiasis. *Phytotherapy Research, 20*(10), 844-850.

Thirumal, M., Surya, S., & Kishore, G. (2012). Cassia fistula Linn - Pharmacognostical, Phytochemical and Pharmacological Review. *Critical Review in pharmaceutical Sciences*(1), 43-65.

Thompson, C. B. (1995). Apoptosis in the pathogenesis and treatment of disease. *Science, 267*, 1456-1462.

Toledano, B. J., Bastien, Y., Noya, F., Baruchel, S., & Mazer, B. (1997). Platelet-activating factor abrogates apoptosis induced by cross-linking of the surface IgM receptor in a human B lymphoblastoid cell line. *J. Immunol., 158*, 3705-3715.

Van Wyk, B.-E., & Gericke, N. (Eds.). (2000). *People's plants: A Guide to Useful Plants of Southern Africa*. Pretoria: Brizza Publications.

Van Wyk, B., Van, O., B.,, & Gericke, N., M., (Eds.). (1997). *Medicinal Plants of South Africa.* South Africa: Briza Publications.

Verpoorte, R. (2000). Pharmacognosy in the New Millennium: lead finding and Biotechnology. *J. Pharmcol. , 52*, 253-262.

Vincent, T. L., & Gatenby, R. A. (2008). An evolutionary model for initiation, promotion, and progression in carcinogenesis. *International Journal of Oncology, 32*, 729-737.

Viswanath, V., Wu, Y., Boonplueang, R., Chen, S., Ste- venson, F. F., Yantiri, F., Yang, L., & Beal, M. F. (2001). Andersen JK. Caspase-9 activation results in downstream cas- pase-8 activation and bid cleavage in 1-methyl-4- phenyl-1,2,3,6-tetrahydropyridine-induced Parkin- son's disease. *J. Neurosci, 21*, 9519-9528.

Vousden, K. H., & Lu, X. (2002). Live or let die: the cell's response to p53. *Nat. Rev. Cancer., 2*, 594–604.

Walczak, H., Degli-Esposti, M. A., & Johnson, R. S., et al.,. (1997). TRAIL-R2: a novel apoptosis-mediating receptor for TRAIL. *EMBO J, 16*, 5386–5397.

Warrell Jr, R. P., Coonley, C. J., & Gee, T. S. (1985). Homoharringtonine: an effective new drug for remission induction in refractory nonlymphoblastic leukemia. *American Society of Clinical Oncology, 3*(5).

Watson, L., & Dallwitz, M. J. (Eds.). (1992). *The Families of Flowering Plants: Descriptions, Illustrations, Identification, and Information*: http://biodiversity.uno.edu/delta/.

Watt, J. M., & Breyer-Brandwijk, M. G. (Eds.). (1962). *The medicinal and poisonous plants of Southern and Eastern Africa*. Edinburgh, London.: E & S Livingstone Ltd.

Weinberg, R. A. (Ed.). (2007). *The Biology of Cancer*. New York, USA: Taylor & Francis Group LLC,.

Weiss, R. B. (1992). "The anthracyclines: will we ever find a better doxorubicin?". *Seminars in Oncology 19*(6), 670–686.

Wen-Hui, H., Holly, J., & Hong-Bing, S. (1999). Tumor Necrosis Factor-related Apoptosis-inducing Ligand Receptors Signal NF-kB and JNK Activation and Apoptosis through Distinct Pathways. *The Journal of Biolgical chemistry, 274*(43), 30603–30610.

Wilson, N. S., Dixit, V., & Ashkenazi, A. (2009). Death receptor signal transducers: nodes of coordination in immune signaling networks. *Nat Immunol, 10*, 348–355.

Wink, M. (1999). Biochemistry of plant secondary metabolism. *Natural product report, 1*(22), 22-27

World Health Organisation. (2009). Cancer Fact sheet. *http://www.who.int/mediacentre/factsheets/fs297/en/print.html*.

Wu, D., Wallen, H. D., & Nunez, G. (1997). Interaction and regulation of subcellular localization of CED-4 by CED-9. *Science, 275*, 1126-1129.

Wu, T. Y. H., Wagner, K. W., Bursulaya, B., Schultz, P. G., & Deveraux, Q. L. (2003). Development and characterization of nonpeptidic small molecule inhibitors of the XIAP/caspase-3 interaction. *Chemistry & Biology, 10*(8), 759-767.

www.sigmaaldrich.com.

Xi, S. (2003). Src kinases mediate STAT growth pathways in squamous cell carcinoma of the head and neck. *J. Biol. Chem., 278*, 31574-31583.

Xie, X. M., Zhao, X. Q., Liu, Y. F., Zhang, J. F., Matusik, R. J., Slawin, K. M., & Spencer, D. M. (2001). Adenovirus-mediated tissue-targeted expression of a Caspase-9-based artificial death switch for the treatment of prostate cancer. *Cancer research, 61*(18), 6795-6804.

Xing-Jie, L., Chunying, C., Yuliang, Z., & Paul, C. W. (2010). Circumventing Tumor Resistance to Chemotherapy by Nanotechnology. *Methods Mol Biol., 596,* 467-488.

Xu, Y. M., Wang, L. F., Jia, L. T., Qiu, X. C., Zhao, J., Yu, C. J., Zhang, R., Zhu, F., Wang, C. J., Jin, B. Q., Chen, S. Y., & Yang, A. G. (2004). A caspase-6 and anti-human epidermal growth factor receptor-2 (HER2) antibody chimeric molecule suppresses the growth of HER2-overexpressing tumors. *Journal of Immunology, 173*(1), 61-67.

Yang, J., Liu, X., Bhalla, K., Kim, C. N., Ibrado, A. M., Cai, J., Peng, T. I., Jones, D. P., & Wang, X. (1997). Prevention of apoptosis by Bcl-2: release of cytochrome c from mitochondria blocked. *Science, 275*, 1129-1132.

Yinjun, L., Jie, J., Weilai, X., & Xiangming, T. (2004). Homoharringtonine mediates myeloid cell apoptosis via upregulation of proapoptotic bax and inducing caspase-3-mediated cleavage of poly(ADP-ribose) polymerase (PARP). . *Am J Hematol, 76,* 199–204.

Yoo, Y. D., Park, J. K., Choi, J. Y., Lee, K. H., Kang, Y. K., Kim, C. S., Shin, S. W., Kim, Y. H., & Kim, J. S. (1998). CDK4 down-regulation induced by paclitaxel is associated with G1 arrest in gastric cancer cells. *Clin Cancer Res, 4*, 3063-3068.

Yu, J., & Zhang, L. (2008). PUMA, a potent killer with or without p53. *Oncogene, 30*(1), S71–83.

Zhang, H. Z., Kasibhatla, S., Wang, Y., Herich, J., Guastella, J., Tseng, B., Drewe, J., & Cai, S. X. (2004a). Discovery, characterization and SAR of gambogic acid as a potent apoptosis inducer by a HTS assay. *Bioorganic & Medicinal Chemistry, 12*(2), 309-317.

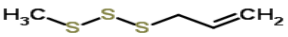
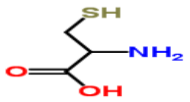
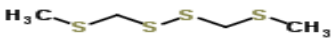
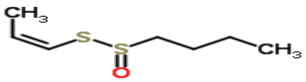
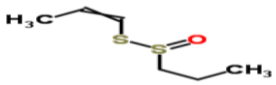
Zhang, X. P., Jin, T. G., Yang, H. M., DeWolf, W. C., Khosravi-Far, R., & Olumi, A. F. (2004b). Persistent c-FLIP(L) expression is necessary and sufficient to maintain resistance to tumor necrosis factor-related apoptosis-inducing ligand-mediated apoptosis in prostate cancer. *Cancer research, 64*(19), 7086-7091.

APPENDICES

Appendix 1: Summary of the major compound peaks found and their molecular masses

Molecular ion (M+)	Retention time (tR)	MF2	MF3	MF4	MF5	MF6	MF7
266	0.45					X	X
274.1	0.5					XX	
305.1	0.71				X		
274	1.11					X	
310	2.13				X	x	
283.2	2.89	X					
327	3.16		X				
281	3.19	XX					
217	3.23				XX	XXX	
281	3.25		X				
347.1	3.26						X
231.1	3.33			X			
388.3	3.37	XX					
388.3	3.4		X				
432.3	3.6		XX				
432.3	3.62	XXX		X			
757.2	3.69				XX	XXX	
278.1	3.78						X
476.3	3.79	XXX	XX	X			
520.3	3.94			X			
520.3	3.97	XX	X				
287.1	4.25				X	XXXX	
265.1	4.36	XXXX					
314.1	5.31	XX					
344.2	5.46	XXX					
344.2	5.49		X				
309.1	5.65			X			
271.1	5.84	XXX					
442.3	6.41						X
298.2	6.87	XX					
500.4	7.08						X
323.1	7.24	XXX					
375.2	9.28						XXXX
318.3	9.19			X			
332.3	9.37			X			
277.2	9.61	X					
417.1	10.06	X					
301.1	11.61				X		X
301.2	11.49	X					
282.3	13.81	XX		XXXX			
607.3	13.81	X					
262.3	13.88		XXXX				
640.6	15.61						X
522.6	15.93						X

Appendix2: Examples of previously isolated compounds from garlic

Compounds	Structures	Formula and molecular weight s
diallyl disulfide (DADS		$C_6H_{10}S_2$ 146.022385 Da
Allyl Sulfide		$C_6H_{10}S$ 114.05
allyl methyl trisulfide		$C_4H_8S_3$ 151.978806 Da
gamma-glutamyl cysteine		$C_3H_7NO_2S$ 121.1582
gamma-glutamyl cysteine		$C_3H_7NO_2S$ 121.1582
Ajoene (1E)-1-(Allyldisulfanyl)-3-(allylsulfinyl)-1-propene		$C_9H_{14}OS_3$ 234.020676
2, 4, 5, 7-tetrathiaoctane		$C_4H_{10}S_4$ 186.3822
S-[(1Z)-1-Propen-1-yl] 1-butanesulfinothioate		$C_7H_{14}OS_2$
S-prop-1-en-1-yl propane-1-sulfinothioate		$C_6H_{12}OS_2$ 164.032959 Da
2,4,6-Trithiaheptane		$C_4H_{10}S_3$ 153.994461 Da