Screening of natural products and Alkylating agents for antineoplastic Activity.

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A Thesis Submitted in Partial Fulfilment of the Requirements for the Degree of Masters in Science in the Department of Biotechnology, University of the Western Cape.

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ABSTRACT

Background and objectives

Apoptosis is a process in which a cell programmes its own death. It is a highly organized physiological mechanism in which injured or damaged cells are destroyed. Apart from physiological stimuli however, exogenous factors can induce apoptosis. Many anti-cancer drugs work by activating apoptosis in cancer cells. Natural substances have been found to have the ability to induce apoptosis in various tumour cells and these substances have been used as templates for the construction of novel lead compounds in anticancer treatment. On the other hand, alkylating agents such as cisplatin, cis- [PtCl2 (NH3) 2] have been widely used as antineoplastic agents for a wide variety of cancers including testicular, ovarian, neck and head cancers, amongst others. However, the use of cisplatin as an anticancer agent is limited due to toxicity and resistance problems.

The aim of this present study was to screen the leaves of *Rhus laevigata*, a South African indigenous plant, for the presence of pro-apoptotic and anti-proliferative natural compounds and also to screen newly synthesised palladium based complexes (15 and 57) and a platinum based complex (58) for their antineoplastic activities tested against a panel of cell lines.

Results.

The results showed that crude methanol extracts from *Rhus laevigata* as well as the newly synthesised palladium based complexes (15 and 57) and a platinum based complex (58) induced apoptosis in the cell lines tested, as demonstrated by the externalization of phosphatidylserine, mitochondrial membrane permeabilization, caspase-3 activation, and DNA fragmentation. Caski (cervical cancer) and H157 (non small cell lung carcinoma) cell lines treated with the methanol extract from *Rhus laevigata* however, were more resistant to apoptosis induction. Among the metallo-complexes, complexes 15 and 57, palladium based complexes, were the most active.

Conclusion

The methanol extract from the leaves of *Rhus laevigata* contain pro-apoptotic and anti-proliferative natural compound(s), which need to be characterised and elucidated as they could provide the much-needed lead compounds in the fight against cancer. On the other hand the newly synthesized palladium complexes also need further evaluation to see if they can be used as anticancer agents that can overcome the problems associated with cisplatin.

Key words: *Rhus laevigata;* cisplatin; palladium; antineoplastic; apoptosis*;* natural products; alkylating agents; novel lead compound; screen; cytotoxicity; resistance.

DECLARATION

I declare that "Screening of natural products and alkylating agents for antineoplastic activity" is my own work and 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.

STONARD SOFIEL ELISA KANYANDA

……...…… **September 2007**

Signed **Date**

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DEDICATION

This work is dedicated to my family

TABLE OF CONTENTS

CHAPTER FIVE

LIST OF TABLES AND FIGURES

CHAPTER ONE FIGURES

CHAPTER ONE TABLES

CHAPTER TWO FIGURES

CHAPTER TWO TABLE

CHAPTER THREE FIGURES

CHAPTER THREE TABLES

CHAPTER FOUR FIGURES

CHAPTER FOUR TABLES

ABBREVIATIONS

7-AAD	7-amino actinomycin D
ANT	Adenine nucleotide translocator
AO	Acridine orange
ATP	Adenosine triphosphate
AIDS	Acquired immune deficiency syndrome
Anexin-V PE	Annexin-V phycoerythrin
Apaf-1	Apoptosis activating factor -1
AIF	Apoptosis-inducing factor
ATM	Ataxia telangiectasia mutated
ATP	Adenosine triphosphate
ATR	Ataxia telangiectasia related
Bax	Bcl-2 associated x protein
Bcl-2	B cell leukaemia-2
BH	Bcl-2 Homology domains
Bid and tBid	(Truncated) BH3 interacting domain
CAD	Caspase-activated deoxyribonuclease
CARD	Caspase recruitment domain
Caspase	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
Cisplatin	Cis-dichlorodiammineplatinum(II) complex
CLM	Confocal laser microscopy
D4-GD1	Inhibitor of small G protein
DD	Death domain
DED	Death effector domain
DIABLO	Direct IAP binding protein with low pI
DISC	Death-inducing signalling complex
DMEM	Dulbeco's modified medium

DMSO	Dimethylsulphoxide
DNA	Deoxyribonucleic acid
$DiOC_6$ (3)	3,3′-diehexyloxocarbocyanine iodide
DcR	Decoy receptor
DR	Death receptor
DR-3	Death receptor-3
EDTA	Ethylene diamine tetra acetic acid
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
IAPs	Inhibitors of apoptosis;
ICAD	Inhibitor of caspase-activated DNase.
ICE	Interleukin-1-β-convertin caspase enzyme
IM	Inner membrane
ICE	Interleukin-1-beta converting enzyme (now known as caspase-1)
JC-1	5,5′, 6,6′-tetrachloro-1, 1′3,3′-tetraethylbenzimidazolycarbocyanineiodide/choride
LMW	Low molecular weight
MAPK	Mitogen-activated protein kinase
Mdm2	Murine double-minute 2
Myc	Mylocytoma
NFκB	Nuclear factor κB
NRU_{50}	Concentration at which 50% of cells take up Neutral Red dye
OM	Outer membrane
OPG	Osteoprotegerin
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
PI3K/Akt	Phosphatidylinositol 3-kinase **(**serine/threonine-protein kinase)
PKC	Protein kinase c
PS	Phosphatidylserine
Rb	Retinoblastoma
ROCKI	Rho-associated coiled-coil forming kinase
RNA	Ribonucleic acid
RPMI	Roswell park memorial institute
SARs	Structural activity relationships
SMs	Secondary metabolites
Smac	Second mitochondria-derived activator of caspase
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end labelling
TMRE	Tetramethylrhodamineethylester
TNF	Tumour necrosis factor
TRADD	Tumour necrosis factor receptor-associated death domain
TRAIL	TNF-related apoptosis-inducing ligand
TEM	Transmission electron microscopy
TE	Tris-EDTA [Ethylenediamine tetracetic acid]
VDAC	Voltage -dependent anion channel
XRCC1	X-ray repair complementing defective repair in Chinese hamster cells 1

CHAPTER 1: LITERATURE REVIEW

1.1 Natural Products

1.2 Traditional medicine

1.3 Methodology in plant drug discovery

1.4 Apoptosis

1.5 Cancer and the cell cycle

1.6 *Rhus* species

1.7 Metallo-compounds in anticancer drug discovery

1.8 Aims of this project

1.1 NATURAL PRODUCT

1.1.1 Definition

Natural products have been defined by Singh (2004), and Cannell (1998), as biological molecules, which are not necessarily essential or are not directly implicated in the normal growth, reproduction or survival of an organism. They are said to be secondary metabolites which have a molecular weight of less than 1500 atomic mass unit (amu) approximately and are often species dependent with restricted occurrence in nature. Verpoorte (2000), also said that natural products play a role in the interaction of the organism with its environment, so as to ensure the survival of the organism in its ecosystem.

1.1.2 Primary Metabolites

On the other hand primary metabolites have been described by Buchanan *et al*., (2002), as being compounds which are essential for the growth and metabolism of the organism, and these include proteins, lipids, nucleic acids, phytosterols, polysaccharides, and organic acids. These compounds are mostly ubiquitous.

1.1.3 The boundary between Primary and Secondary Metabolism

Buchanan *et al*., (2000), further said that there is no clear cut distinction between primary and secondary metabolites on the basis of precursor molecules, chemical structures, or

biosynthetic origins as both, primary and secondary metabolites are found among the diterpenes (C_{20}) and triterpenes (C_{30}). Buchanan *et al*., (2000) , gave the example of the diterpene series, in which both kaurenoic and abietic acids are formed by a very comparable sequence of related enzymatic reactions. They reported that kaurenoic acid is an essential intermediate in the production of gibberellins, which are growth hormones found in all plants. On the other hand, abietic acid is a resin constituent which is largely restricted to members of the Fabaceae and Pinaceae. On the other hand, the essential amino acid proline is said to be a primary metabolite, whereas the C_6 analog pipecolic acid is regarded as an alkaloid and therefore a natural product. The basic structural polymer in wood is lignin and is second only to cellulose as the most plentiful organic substance in plants. Lignin however, is considered a natural product rather than a primary metabolite. Based on either structure or biochemistry between primary and secondary products there is no clear distinction. For this reason Buchanan *et al*., (2000) have therefore described primary products as products participating in nutrition and secondary metabolic processes inside the plant. Secondary products have been described as those products that influence ecological interactions between the plant and its environment.

1.1.4 Roles of Secondary Metabolites (SMs) to the organism

Koskinen (1995), stated that the real role of secondary metabolites (SMs) is chiefly unclear. Nevertheless, the function or importance of these SMs to the organism is usually of ecological nature (Verpoorte, 2000), since they are used as defense against predators (herbivores, pathogens etc), or for territorial defense by producing antibiotics agents and

mycotoxins, which prevent insect predation. Secondary metabolites are also used as allelopathic agents, antifeedants (chemicals that inhibit feeding), attractants for pollinators and seed-dispersing animals, sex hormones as sex attractants and enhancers of the reproductive processes (Koskinen, 1995; Croteau *et al.,* 2000; Cotton, 2002; Singh, 2004). Even though plants are better known as a source of secondary metabolites, however, bacteria, fungi and many marine organisms (sponges, tunicates, corals, and snails) have also been reported to be sources of secondary metabolites (Cannell, 1998; Wink, 1999).

1.1.5 Uses of Secondary Metabolites to man

Man has used secondary metabolites for thousands of years. Some uses of secondary metabolites to man include the following: dyes (e.g. shikonin, and indigo), flavors (e.g. vanillin, mustard oils, and capsin), fragrances (e.g. rose and, lavender oils), stimulants (e.g. nicotine, ephedrine and caffeine), polymers, waxes, hallucin ogens (e.g. morphine, and cocaine), waxes, fibers, glues, insecticides (e.g. coniine, strychnine, and aconitine), and as therapeutic agents (e.g. atropine, quinine, carenolides, codeine, etc), (Cannell, 1998; Wink, 1999; Buchanan *et al*., 2000).

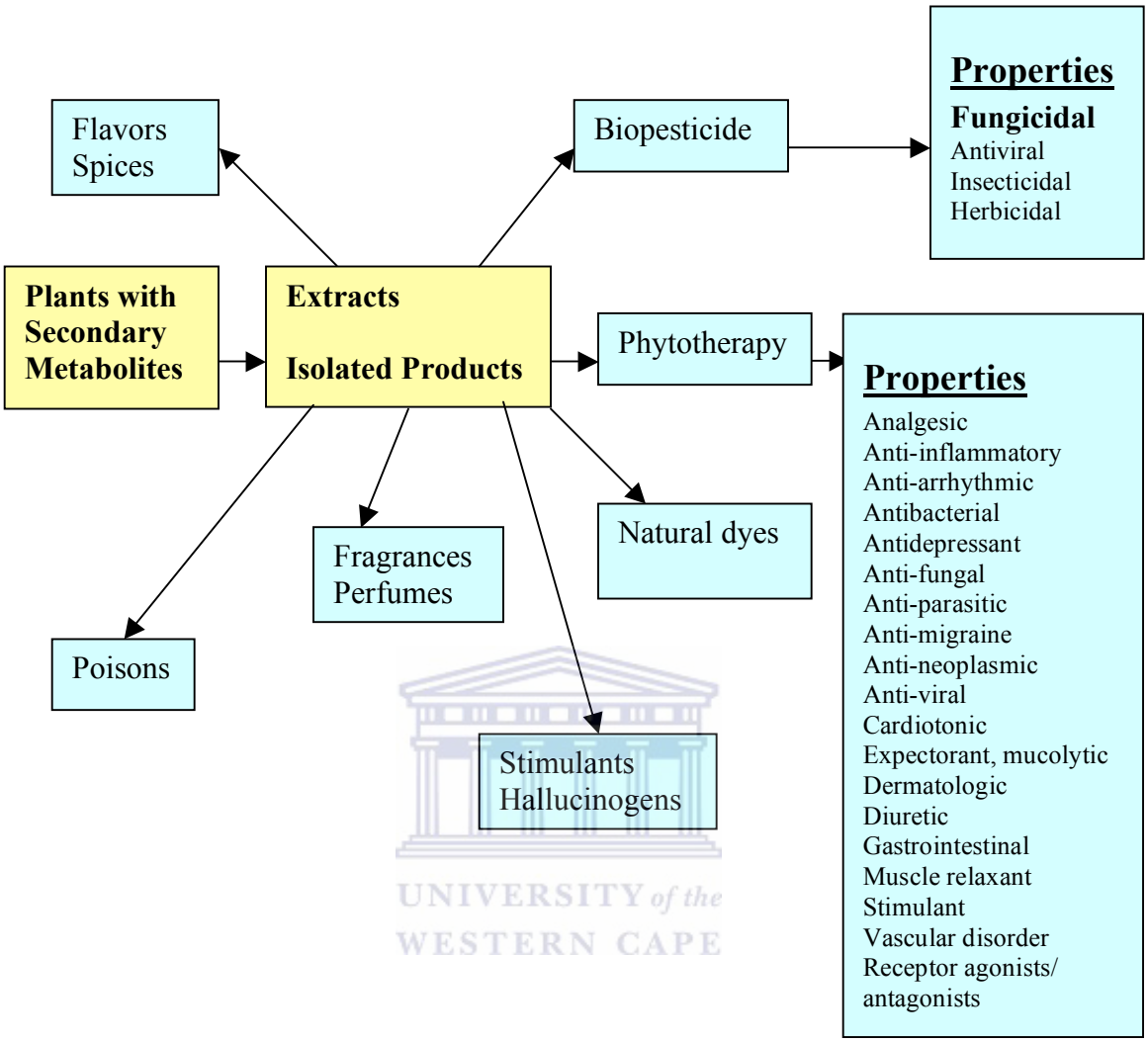


Figure 1.1: Schematic diagram on the utilization of secondary metabolites in biotechnology (From Wink, 1999).

1.1.6. Metabolism of secondary metabolites.

Carbohydrates are primary metabolites, which are produced by the action of photosynthesis in plants and these carbohydrates provide the carbon skeleton for the secondary metabolites. Dennis and Turpin (1995) and Wink (1999), estimated that higher plants

produce more than 100,000 secondary metabolites out of which 15,000 have reportedly been characterized as drugs. Such secondary metabolites may include: alkaloids, phenolics, steroids, tannins, terpenoids, iridoids, saponins, and polyketides. The recognition of the biological properties of many natural products has encouraged the search for new drugs, antibiotics, insecticides, and herbicides. With regards to their biosynthetic origins of natural products, Dennis and Turpin, (1995); Buchanan *et al.,* (2002), divided the plant natural products into three major groups and these were: tepenoids, alkaloids and phenylpropanoids.

1.1.7 The Shikimic acid pathway

The shikimic acid pathway was named after one of the key intermediates, shikimic acid. The shikimic acid pathway has been described by Mann *et al.,* (1994); and Dewick, (2003), as a universal process whereby aromatic metabolites are produced, but in particular the three amino acids, phenylalanine, tyrosine, and tryptophan. Herrmann and Weaver, (1999), showed that this pathway changes simple carbohydrate precursors developed from glycolysis and the pentose phosphate pathway to the aromatic amino acids. In a sequence of seven metabolic steps, phosphoenolpyruvate and erythrose 4-phosphate are converted to chorismate, the precursor of the aromatic amino acids (figure 1.2) and many aromatic secondary metabolites (Herrmann, 1999; Dewick, 2003). All pathway intermediates are also considered branch point compounds that may serve as substrates for other metabolic pathways. One of the pathway intermediates is shikimic acid, which has given its name to this whole series of reactions. The shikimic acid pathway occurs in plants and

microorganisms but not in animals. Herrmann, (1999) and Dewick, (2003), have further stated that animals have no way of producing the three aromatic amino acids, phenylalanine, tyrosine, and tryptophan, which are essential nutrients in animal diets and they have said that the pathway provides important targets for herbicides, antibiotics, and vaccines. They said that the chemical compound(s) that obstruct with any enzyme activity in this pathway are regarded as "safe" for humans when handled within reasonable concentrations and therefore the shikimate pathway is important in the biosynthesis of many compounds of commercial interest.

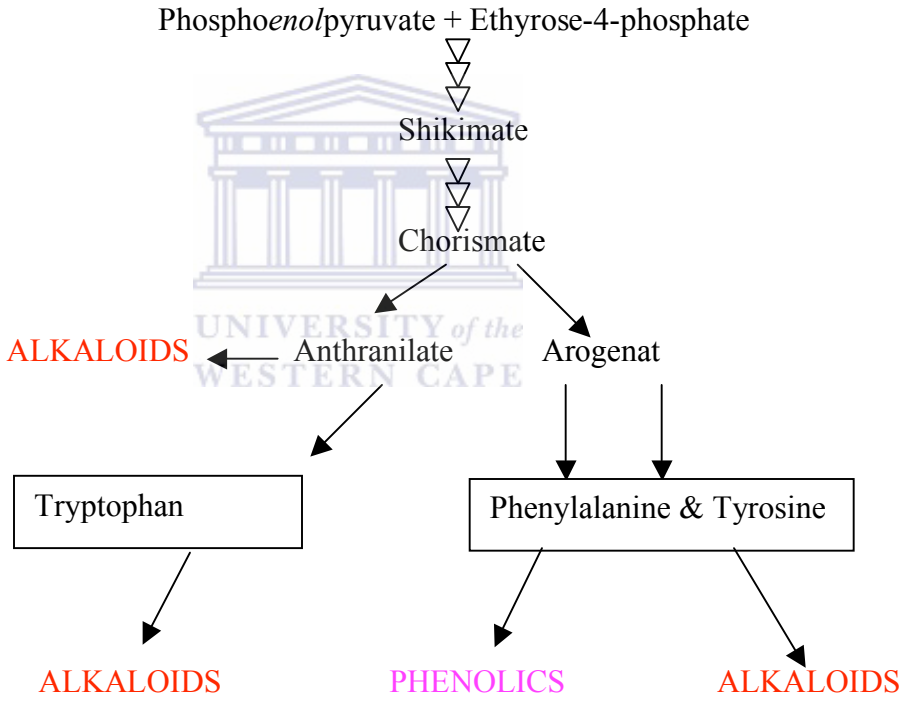


Figure 1. 2: Schematic representation of the Shikimic acid pathway. The condensation of phospho*enol*pyruvate (PEP) from glycolysis and ethyrose-4-phosphate from the pentose phosphate pathway is catalysed by 3-deoxy-D-arabino-heptulosonate-7-phosphate (DAHP) synthase. Through a series of reactions shikimic acid is produced, which is converted to chorismate, the final precursor for the synthesis of phenylalanine, tyrosine and tryptophan. Phenylalanine, tyrosine and tryptophan are the precursors for alkaloids (From Edwards and Gatehouse, 1999).

1.1.8 The four major classes of secondary metabolites briefly explained

1.1.8.1 Alkaloids

The term alkaloid is reported to have been coined in 1819 in Halle, Germany, by Carl Meissner (Buchanan *et al*., 2000). It is derived from alkali. The word alkali is derived from the Arabic *al qalay,* the plant from which soda was first obtained (Kutchan, 1995), and it means to roast (Koskinen, 1995). All alkaloids are basic compounds containing nitrogen in a heterocyclic ring and they are common to about 15 to 20% of all vascular plants but are found to a lesser extent in microorganisms and animals **(**Aniszewski, 2007). The degree of their basicity however, varies depending on the structure of the alkaloid molecule, and the presence and location of other functional groups. Some alkaloids are essentially neutral (Dewick, 2003). In plants, both true and protoalkaloids are precursors and alkaloids are synthesized mainly from aromatic amino acids, phenylalanine, tyrosine, and tryptophan. Pseudoalkaloids however, are synthesized from other compounds, e.g. acetate and non-protein amino acids for instance ornithine. A variety of classes of alkaloids are distinguished, depending on the ring system or skeleton that is present (Aniszewski, 2007). Most alkaloids are reported to be toxic and are said to have a wide range of different pharmacological effects. Alkaloids are mostly used as drugs, medicines and poisons (Kutchan, 1995; Koskinen, 1995; Cotton, 2002).

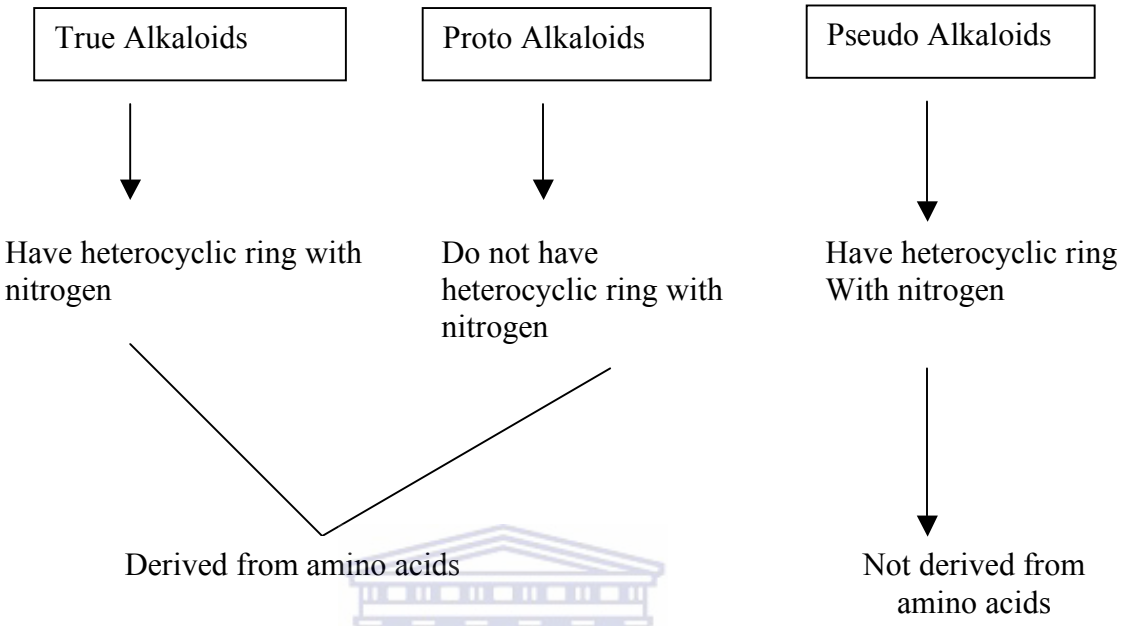
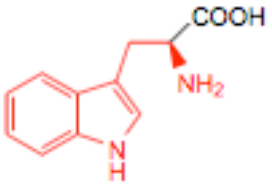
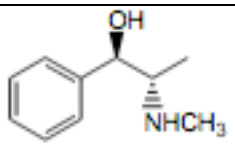
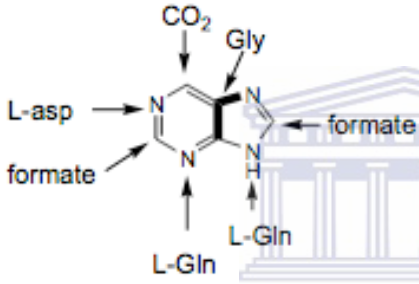


Figure 1.3: The three main types of alkaloids (Aniszewski, 2007).

Table 1.1: Skeleton structures of alkaloids

SKELETON STRUCTURE	DESCRIPTION	ALKALOID TYPE
	Nitrogen is part of the heterocyclic ring system	True alkaloids
	Nitrogen atom is outside the ring system	Protoalkaloids
	Purine ring is pieced together with small components from primary metabolites	Pseudoalkaloids

1.1.8.2 Flavonoids

Flavonoids are compounds composed of a three-ring structure with various substitutions. These compounds are phenolic and are generally found as water-soluble pigments in plants. This basic structure is shared by tocopherols (vitamin E). Flavonoids can be subdivided according to the presence of an oxy group at position 4, a double bond between carbon atom 2 and 3, or a hydroxyl group in position 3 of the C (middle) ring (Middleton *et al*., 2000). Different types of flavonoids are distinguished, such as flavones, flavonols, isoflavone, flavanones, chalcones and anthocyanidins (van Wyk *et al*., 2000).

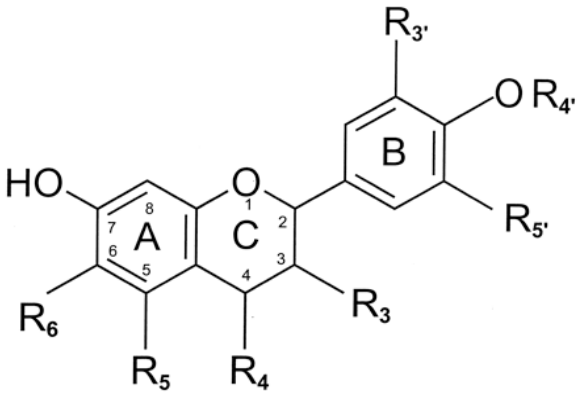


Figure 1.4a: Basic structure of a flavonoid (Crozier, *et al*., 2000**;** Beecher, 2003; Taylor and Grotewold, 2005). General structure and numbering pattern for common food flavonoids. For most food flavonoids, R4=H, R5=OH, and R6=H. Exceptions include, biochanin A, R4=CH3; formononetin, R4=CH3, R5=R6=H; glycitein, R5=H, R6=OH; and hesperitin, R4=CH3. Aditional individual flavonoids within each subclass are characterized by unique functional groups at. R_3, $R_{3'}$, and R.

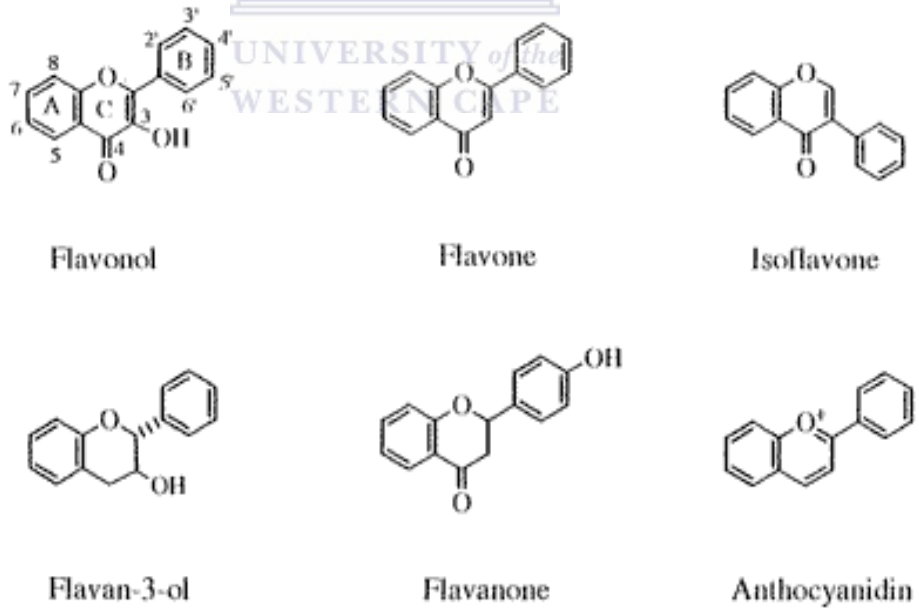


Figure 1.4b: Structures of the six main classes of flavonoids

1.1.8.2.1 Functions of flavonoids

There are many functions of flavonoids. One major function of flavonoids is that they act as signaling compounds, in the attraction of pollinating and seed-dispersing animals. These compounds have shown that they have anti-inflammatory, anti-oxidant, anti-allergic, antibacterial and antiviral effects, (Wink, 1999; Casctaldo and Capasso, 2002). It is also reported that they contribute to plant colors (Shirley, 2002). Even though various flavonoids have been reported to slow down the development of chemically induced cancers in animal models such as lung, oral, esophageal, stomach, colon, skin, prostate, and mammary cancers, epidemiological studies do not however, give convincing evidence that high intakes of dietary flavonoids are connected with significant reductions in human cancer risk (Benbrook, 2005).

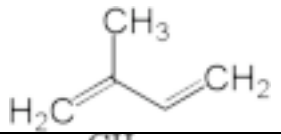
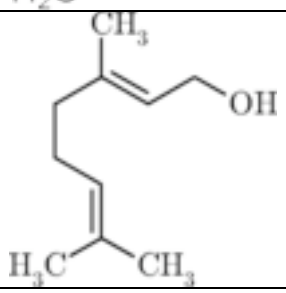
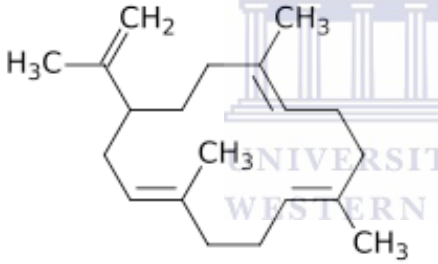
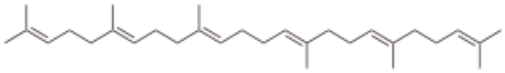
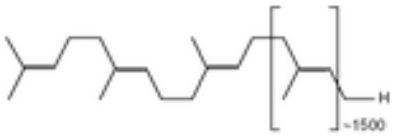
1.1.8.3 Terpenoids

The name "terpene" is derived from "turpentine**"** (Buchanan *et al*., 2000). Terpenes are derived from isoprene C_5H_8 units and have the basic formula of multiples of it, i.e., $(C_5H_8)_n$. This is called the isoprene rule. The isoprene units may be arranged in a linear or forming rings. When terpenes are further altered, for example by addition of hydroxyl groups or removal of a methyl group, the resulting compounds are called terpenoids, e.g. menthol and thujone (Dubey *et al*.,2003). Moss *et al.*, (1995), said that some authors also call these compounds terpenes.

Terpenoids, are also called isoprenoids, and are produced by the head-to-tail joining of C5 isoprene units. Isoprenoids are classified according to the number of C5 units, (table 1.2) for example hemiterpenes (C5), monoterpenes (C10 and 2 isoprene units, like limonene, carvone or carveol), sesquiterpenes (C15 and 3 isoprene units), diterpenes (C20 and 4 isoprene units and these may include the retinoids), sesterterpenes (C25), triterpenes (C30 and 6 isoprene units), tetraterpenes (C40 and 8 isoprene units and these include all different carotenoids like α-and β-carotene, lutein, lycopene, zeaxanthine and cryptoxanthine). Polyterpenes have a large number of isoprene units e.g. rubber (Wagner and Elmadfa, 2003; Dubey *et al*., 2003). Triterpenoids and steroids are usually present in plants in the form of saponins. It means therefore that they have more than one or more sugar groups attached to them and this makes them water-soluble.

Terpenoids play diverse roles in plants such as hormones, photosynthetic pigments, electron carriers, mediators of polysaccharides assembly, structural components of membranes as well as communication and defense. They provide a range of commercially useful natural products for instance, the anticancer drug taxol, fragrances, insecticidal pyrethrins, carotenoids and antioxidants. (McGarvey and Croteau, 1995). For this reason, Dewick, (2003), stated that probably terpenoids are the most promising class of natural products, with the potential of discovering of a wide variety of compounds that are of economic importance.

Table 1.2: Classification of terpenes based on number of isoprene units

Tepernoid structure	Composition	Molecular Formula	Example
	Hemiterpene Single isoprene unit	C_5H_8	Isoprene
	Monoterpenes Two isoprene units	$C_{10}H_{18}O$	Geraniol
	Sesquiterpenes Three isoprene units	$C_{15}H_{26}O$	Farnestol
	Diterpenes Four isoprene units	$C_{20}H_{32}$	Cambrene
	Triterpenes Six isoprene units	$C_{30}H_{50}$	Squalene
	Tetraterpenes Eight isoprene units	$C_{40}H_{56}$	Lycopene
	Polyterpenes Many isoprene units	$(C_5)_n$*	Rubber (Gutta-percha)

- Where "n" may be 9-30,000

1.1.8.4 Phenylpropanoids

Phenylpropanoids are compounds that are produced through the shikimic acid pathway. This group includes phenylalanine-derived chemicals that have a diverse group of secondary metabolites (Noel *et al*., 2005), and this includes flavonoids, lignin, coumarins and phenolic molecules, that have a variety of functions such as structural support, pigmentation, defense and signaling (Douglas, 1996; Gómez *et al.,* 2004). The structural variety of phenylpropanoids is due to the action of enzymes and enzyme complexes, which bring about regio-specific condensation, aromatization, acylation, hydroxylation, glycosylation, cyclization prenylation, sulfation, and methylation reactions (Noel *et al*., 2005). These compounds are distinguished by the presence of a phenol group (hydroxylated aromatic ring).

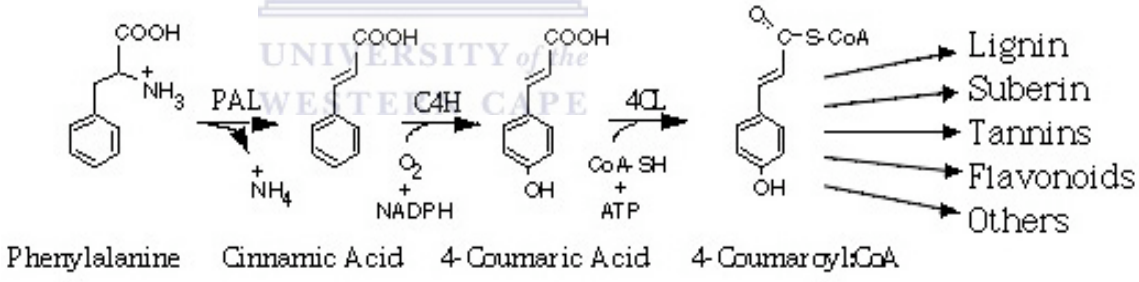


Figure 1.5: Biosynthesis of phenylpropanoids (from Douglas, 1996). Environmental stress e.g. wounding, pathogen infection, and/or UV irradiation in specific tissues and cell types can activate the production of phenylpropanoid compounds in plants. Phenylpropanoids are obtained from cinnamic acid by the action of the enzyme phenylalanine ammonia-lyase (PAL) which catalyzes the gateway metabolic step from primary metabolism into phenylpropanoid metabolism. The de-amination of phenylalanine then produces cinnamic acid. Cinnamic acid is further modified by the actions of hydroxylases and O-methyltransferases, and most

phenylpropanoid compounds are derived from such hydroxycinnamic acids. (Hahlbrock and Scheel, 1989; Dixon *et al.,* 2002; Achnine *et al*., 2004; Noel *et al*., 2005;).

1.2 OVERVIEW OF TRADITIONAL MEDICINE: DEFINITIONS AND TERMINOLOGY

1.2.1 Traditional medicine

Definition:

The term "Traditional Medicine" has been described by Sofowora (1982), as being the total combination of knowledge and practices of indigenous people which they may either understand or not. The knowledge and practices are used to diagnose, prevent or eliminate a physical, mental or a social disease and this may rely entirely on the past knowledge and careful observation as handed down from generation to generation either verbally or in writing.

1.2.2 Herbal medicine

Definitions:

The term "Herbal medicine" has been described by Kayne, (2002) as being unrefined or refined and labelled products from plants that contain effective ingredients for the remedy of illness. These ingredients could come from the aerial or underground parts of plants, or other plant material, or could just be a combination of different parts of the plant. The herbal preparation could also be combined with other ingredients such as juices, gums, fatty oils, essential oils and any other substances of this kind. Sofowora (1982), has described herbal medicine as the use of any plant either in whole or in part or the use of more of its parts, that contain substances that can be used for healing purposes or can be

used in combination with other ingredients with the aim of making useful drugs for healing purposes.

1.2.3 Brief history of traditional medicine

Kayne, (2002), indicated that the exact origins of traditional medicine are not known. He said that there is a possibility that several different primitive people discovered that some herbs were good to eat, and yet others had healing powers. Newman *et al*., (2000) further stated that opium poppy, *Papaver somniferum*, was perhaps the earliest medicinal plant, because it was well known in ancient Greece. In addition, Kayne, (2002) pointed out that Hippocrates mentioned the use of poppy juice as cantharic, hypnotic, narcotic and styptic. Sofowora, (1982), also reported that seeds of the opium poppy and castor oil (*Ricinus communis*) were unearthed from some ancient Egyptian tombs an indication that those seeds were used in that area most likely as far back as 1500BC. One obvious thing is that nature has provided humans with basic needs such as food-stuffs, protection, clothing, fertilizers, flavors and of course, medicines for thousands of years (Gurib-Fakim, 2006). The knowledge of herbal treatment by indigenous people was probably developed through trial and error over several thousand years. This knowledge was passed on verbally from one generation to the next generation

1.2.3.1 Isolation of pure active compounds from medicinal plants.

Table 1.3 shows some of the pure active compounds that have been isolated from medicinal plants through bioassay-guided isolation (Gurib-Fakim, 2006). Numerous other

methods have been utilized to acquire compounds for drug discovery including synthetic chemistry, combinatorial chemistry, and molecular modeling (Lombardino and Lowe, 2004). It was indicated by Newman *et al.,* (2000; 2003), that in spite of the fact that in recent times there has been a lot of interest in molecular modeling, combinatorial chemistry, and other synthetic chemistry techniques by pharmaceutical companies and organizations which provide financial support to research institutions, natural products, and particularly medicinal plants, remain an important source of new drugs, new drug leads, and new chemical entities (NCEs). It has been shown that known compounds isolated from medicinal plants act on newly confirmed molecular targets, e.g. kamebakaurin, which has been shown to inhibit NF-κB (Balunas and Kinghorn, 2005) and β-lapachone, which is reported to selectively kill cancer cells over normal cells because it targets an enzyme elevated in various tumors (Reinicke *et al*., 2005).

Pieters and Vlietinck (2005) and Kim, (2005), have reported that most of the world's developing countries still depend on traditional medicine for their primary health care needs. They said this is because most developing countries cannot afford to import western medicine, therefore developing countries are encouraging the use of traditional medicines as an essential component in their public health care programs. Additionally, Pieters and Vlietinck (2005), have said that traditional are locally available and the local population eagerly accepts them. However, there is the danger that some of the traditional drugs are not safe for human consumption and that sustainability might not be possible due to over exploitation of plant population in the wild.

Table 1.3: Botanical drugs used in traditional medicine which have given useful modern drugs (adapted from Gurib-Fakim, 2006).A summary of some of the traditional medicines, which have given useful modern drugs. It can be seen that plants used for medicinal purposes by indigenous peoples has provided novel compounds or lead compounds that are in use in this modern society.

Botanical names	English names	Indigenous use	Origin	Uses in biomedicine	Biologically active compounds
Adhatoda vasica	–	Antispasmodic, antiseptic, insecticide, fish poison	India, Sri Lanka	Antispasmodic, oxytocic, cough suppressant	Vasicin (lead molecule for Bromhexin and Ambroxol)
Catharanthus roseus	Periwinkle	Diabetes, fever	Madagascar	Cancer chemotherapy	Vincristine, Vinblastine
Condrodendron tomentosum	–	Arrow poison	Brazil, Peru	Muscular relaxation	d-Tubocurarine
Gingko biloba	Gingko	Asthma, anthelmintic (fruit)	Eastern China	Dementia, cerebral deficiencies	Ginkgolides
Harpagophytum procumbens	Devil's claw	Fever, inflammatory conditions	Southern Africa	Pain, rheumatism	Harpagoside, Caffeic acid
Piper methysticum	Kava	Ritual stimulant, tonic	Polynesia	Anxiolytic,mild stimulant	Kava pyrones
Podophyllum peltatum	May apple	Laxative, skin infections	North America	Cancer chemotherapy, warts	Podophyllotoxin and lignans
Prunus africana	African plum	Laxative, 'Old man's disease'	Tropical Africa	Prostate hyperplasia	Sitosterol

1.3 METHODOLOGY IN PLANT DRUG DISCOVERY

The process in plant drug discovery involves a multidisciplinary approach and is a complex one. Pieters and Vlietinck, (2005), reported that the process can begin with a botanist, ethno botanist, ethno pharmacologist, or plant ecologist who collects and identifies the plant(s) of interest. Plant species, which are known to treat specific ailments but their active compound(s) have not yet been isolated may be collected, or the plants maybe randomly collected for a large screening program. After the plants are collected, a phytochemist

(natural product chemist) prepares extracts from the plant materials and subjects the extracts to biological screening in pharmacologically relevant assays. If a plant extract shows that it contains active compound(s) the process of isolating and characterizing the active compound(s) through bioassay-guided fractionation commences. As these plants may be collected from different countries, Baker *et al*., (1995), have said that it is important to protect the intellectual property rights of those given countries from where the plants were collected.

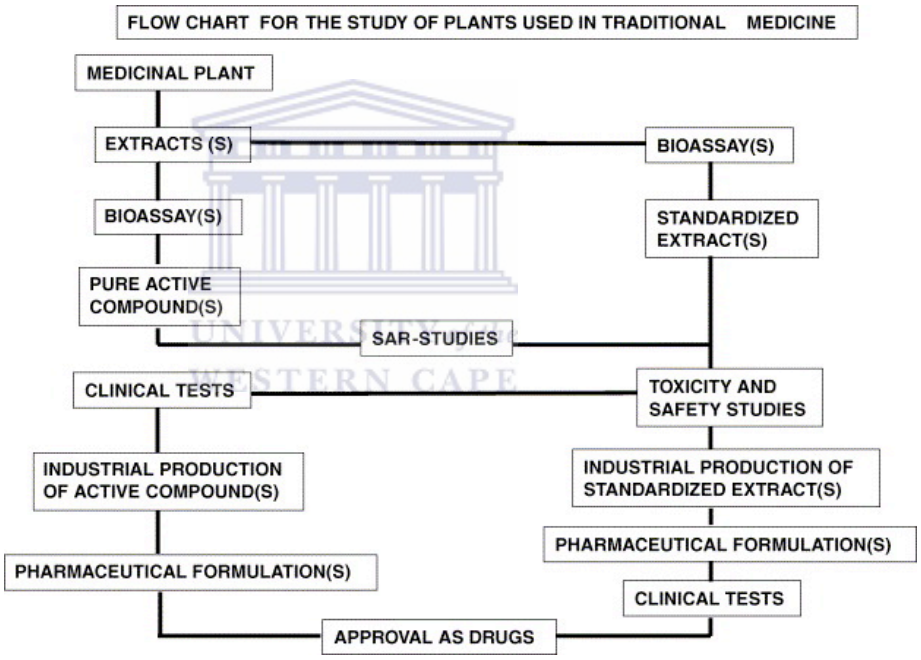


Figure 1.6: The study of plants as used in traditional medicine (adapted from Pieters and Vlietinck, 2005). The flow chart shows that in a "standard" drug discovery procedure active compounds are obtained by a bioassay-guided isolation. Crude extracts of medicinal plants, are subjected to structure–activity relationship studies (SAR). Toxicity and safety studies as well as clinical tests are later carried out. Active compounds are then prepared on an industrial scale, where appropriate pharmaceutical formulations are developed before the compound(s) are approved as a drug(s). Careful quality control procedures are done at every step. In the

"traditional" medicinal system, however, pharmacological evaluation of extracts may only lead to the establishment of standardized extracts. Toxicity and safety studies can be carried out on the standardized extracts and thereafter, industrial production of these standardized extracts can start. If clinical tests are good, formulation of the standardized extracts may lead to approval as drugs.

1.3.1 Medicinal plants in anticancer drug discovery

As reported by Butler, (2004), drug discovery from medicinal plants has played an important role in the treatment of cancer. Newman *et al*., (2003) reported that between 1940 and 2002, 40% of cancer drugs were natural products as such, or were natural product-derived, with about 8% considered to mimic natural products. Presently anticancer agents from medicinal plants which are in clinical use have been categorized into four main classes of compounds and these are: vinca (or *Catharanthus*) alkaloids, epipodophyllotoxins, taxanes, and camptothecins. Ngan *et al*., (2001); Okouneva *et al*., (2003), have reported that vinca alkaloids and their semi-synthetic derivatives block mitosis with metaphase arrest by binding specifically to tubulin resulting in its depolymerization. The epipodophyllotoxins are reported to bind tubulin, causing DNA strand breaks during the G_2 phase of the cell cycle by permanently inhibiting DNA topoisomerase II (Gordaliza *et al*., 2004; Cragg and Newman, 2004, 2005; Balunas and Kinghorn, 2005;). The taxanes, as well as paclitaxel and their derivatives, are reported to act by binding tubulin and once this is done, depolymerization of the microtubule does not take place. Camptothecin acts by inhibiting topoisomerase I, which is involved in the removal of supercoils of DNA during replication (Cragg and Newman, 2004, 2005).

Table 1.4: Summary of mode of action of some of the commonly used natural product anticancer drugs.

Drug Class	Mechanism of Action
Anthracyclines	Topoisomerase II inhibitors
Camptothecin	Topoisomerase I inhibitors
Epipodophyllotoxins	Topoisomerase II inhibitors
Taxanes	Tubulin- binding agents
Vinca alkaloids	Tubulin- binding agents

1.3.2 Plant drug discovery for cancer chemoprevention

Khan and Partin, (2003); De Flora and Ferguson, (2005), have defined cancer chemoprevention as the administration of agents, which prevent the induction, restrain, or delay the progression of cancer. Greenwald *et al*., (2002), mentioned that for a normal cell to be transformed into a cancerous cell it goes through several stages. Firstly is the initiation stage, which is normally caused by DNA damaging agents. Secondly is the promotion stage, in this stage the cell increasingly proliferates. Thirdly the cell is genetically altered and it still proliferates. Chemoprevention therefore targets each of these steps including anti-initiation strategies for example, DNA repair, removal of toxic substances, removal of free radicals, metabolism of cancer causing agents and also involvement of anti-promotion/anti-progression strategies, example inflammation reduction, increase in apoptosis, altered gene expression, and decrease in angiogenesis. It has been suggested by Liberman *et al*., 2001; Khan and Partin, 2003; Kinghorn *et al*., 2004), that herbal medicines, dietary supplements, edible plants, fruits and vegetables are

important in cancer chemoprevention because they contain compounds, which can prevent the onset of cancer and some chemopreventive agents are in clinical trials. Some of such potential cancer chemopreventive agents include: curcumin which is in Phase I, for colon cancer, genistein in Phase I, for breast and endometrial cancers, soy isoflavones in Phase II, for prostate cancer, indole-3-carbinol in Phase I, for breast recurrence cancer, perillyl alcohol in Phase I, for breast cancer and green tea/epigallocatechin gallate in Phase II, for breast cancer. These chemopreventive agents are said to work by targeting initiation, promotion, and progression stages of carcinogenesis. Dorai and Aggarwal, (2004), have also reported that some chemopreventive agents have been found to reverse chemoresistance and radioresistance in patients that are undergoing cancer treatment.

1.3.3 Problems and challenges in drug discovery from medicinal plants

Although plants have provided useful drugs, there are however, many problems that go with drug discovery from medicinal plants. Reichert, (2003), stated that the first major problem is that the process is lengthy and he estimated that it takes an average of 10 years from the time of collecting the plants to the time the drug is ready for use. The second problem is the cost involved. The general cost for all drug discovery research for an average of one new "blockbuster" drug to mature has been estimated to be more than US$ 800 million pre-tax in 2000 dollars (Dickson and Gagnon, 2004; Verkman, 2004). A recent survey by DiMasi *et al*., (2003), showed that 68 randomly selected new drugs from medicinal plants, totalled 802 million US dollars for research and development costs which also included unsuccessful projects. The survey showed that the cost was exorbitantly high

because much time was spent on numerous leads that were disposed of during the drug discovery process. The survey further showed that out of 5000 lead compounds probably only one successfully advanced through clinical trials stage and approved as a drug with New Chemical Entity (NCE). Balunas and Kinghorn (2005), have also pointed out other problems faced with drug discovery from medicinal plants. Some of such problems included: dereplication of the active compound, purification of the new compound by chromatographic methods, structure determination methods, and scale-up of the production. In spite of the many problems that face plant drug discovery, Pieters and Vlietinck, (2005), and Balunas and Kinghorn, (2005), have however, pointed out that plants could still be envisaged to have a major role in the search for new medicines.

1.4 APOPTOSIS

Definition:

The term "apoptosis" has been defined by Kroemer *et al*., (1998) and Tsujimoto and Shimizu, (2000), as a cell process whereby the cell programs its own death. This process involves activation of catabolic processes and enzymes, which occurs before the cell dies. The process is an important physiological mechanism that selectively removes unwanted cells. It plays a very vital part in a variety of biological events, which include morphogenesis and elimination of harmful cells.

1.4.1 History of the Name "apoptosis"

The term apoptosis was introduced into modern scientific writing by Kerr, Willey and Currie (1972), in which they described it as a cell death (programmed cell death/cell

suicide) different from necrosis. The term was chosen for its meaning for it represented the falling of leaves as used in ancient Greek (Kerr *et al*., 1972; Cruchten, 2002). Since then this term has been in accepted use in biomedical sciences. It is a process whereby unwanted cells in a multicellular organism deliberately give up life i.e. deliberately die. The term was also used in philosophical writings of the orthodox world, mainly in political and social contexts in which it was used to mean "failure, ruin, refuse, decay or dissolve" (Hetts, 1998; Maysinger, 2006).

1.4.2 Cell membrane and mechanisms of apoptosis

The mammalian cell membrane was described by Singer and Nicolson, (1972), as a semi-fluid mosaic structure made of phospholipids, proteins and some cholesterol. Phospholipids are the major components of the membrane and are arranged in a form of a 'bi-layer' each arranged in an amphipathic structure (Singer and Nicolson, 1972; Fadeel, 2004). The outer leaflet of the lipid membrane is mostly made of the choline-containing phospholipids, (phosphatidylcholine and sphingomyelin). This layer makes contact with the out side matrix, (*in vivo*), or with cell culture medium, (*in vitro*). The inner membrane is mostly made of phosphatidylethanolamine and phosphatidylserine molecules and is in contact with the cytoplasm. Phospholipids contain non-polar, hydrophobic fatty acid tails, which in both leaflets make up the interior volume of the membrane, giving the characteristic bi-layer structure. It is this bi-layer structure composition and its essential phospholipids that maintain a 'viable' cell. The membrane, and its component parts, provide a semi-permeable barrier and generates a concentration gradient between the

inside of the cytoplasm and the extracellular environment (Avers, 1982). The major feature associated with apoptosis is the loss of the phospholipid asymmetry and exposure of the negatively charged phosphatidylserine from the inner to the outer surface of the cell membrane. This change is necessary for recognition and engulfment of the apoptotic cell by macrophages (Fadok *et al*., 2001). The plasma membrane asymmetry in viable cells is said to be maintained by the activity of an aminophospholipid translocase, which is believed to be a 120-kDa Mg^{2+}-dependent adenosine triphosphatase (ATPase) (Fadok *et al*., 2001). This adenosine triphosphatase (ATPase) transfers any phosphatidylserine (and, to a lesser extent, phosphatidylethanolamine) that may have reached the outer leaflet back to the inner leaflet of the plasma membrane. However, the rapid appearance of phosphatidylserine on the cell surface during cellular activation and during apoptosis is mainly due to the activation of a lipid-nonspecific membrane phospholipid scrambling activity, which moves phospholipids bidirectionally across the plasma membrane, in so doing increasing the surface expression of phosphatidylserine (Fadok *et al*., 2001). Therefore phosphatidylserine is commonly used as a marker for apoptosis (Barrett *et al*., 2001).

The enzymatic scramblase activity depends on calcium concentration present inside the cell (Wyllie *et al*., 1984). Under normal condition the calcium concentration inside the cell is very low. An increase in calcium concentration however, triggers the phospholipid transportation mechanism, which results in a symmetric distribution of negatively charged phospholipids between both leaflets of the plasma membrane. Since transportation of scramblase does not require energy, therefore ATP is not involved. Fadeel, (2004), said

that in healthy cells scramblase activity is inactive and activation only occurs under stressful conditions.

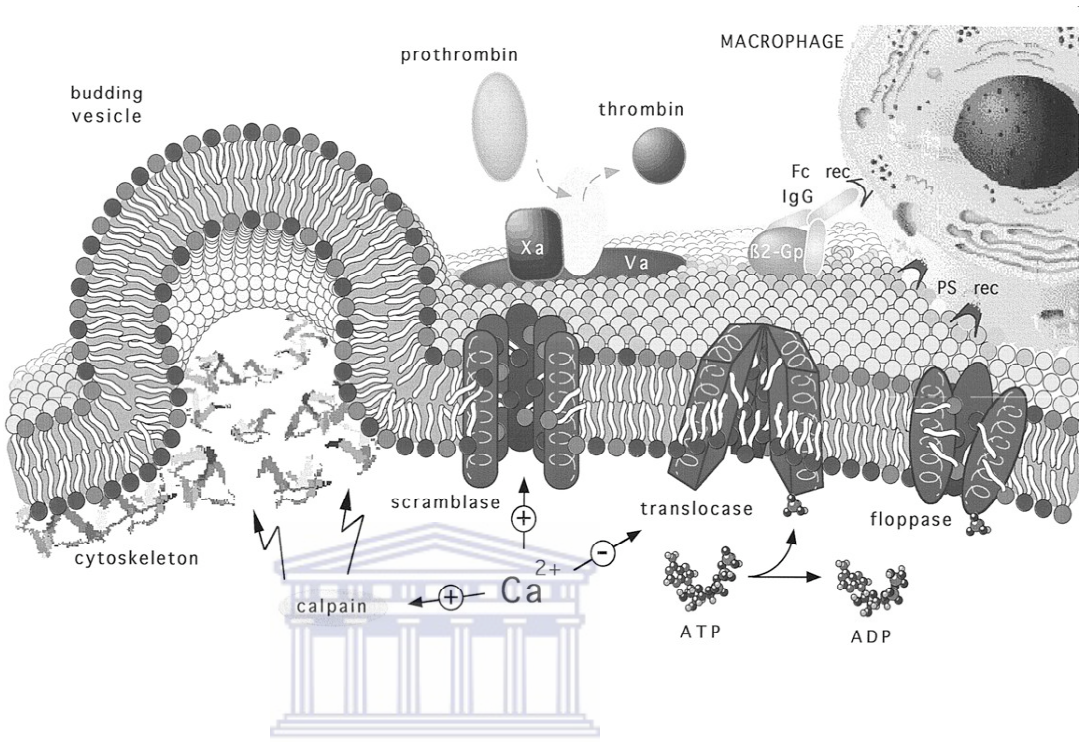


Figure 1.7: The regulation and physiology of membrane phospholipid asymmetry (from Zwaal and Schroit, 1997).

Membrane lipid asymmetry is regulated by the cooperative activities of three transporters. The adenosine triphosphate (ATP)-dependent aminophospholipid-specific translocase, which rapidly transports phosphatidylserine (PS) and phosphatidylethanolamine (PE) from the cell outer-to-inner leaflet; the ATP-dependent nonspecific lipid floppase, which slowly transports lipids from the cell's inner-to-outer leaflet; and the Ca^{2+}-dependent nonspecific lipid scramblase, which allows lipids to move randomly between both leaflets.

1.4.3 Characteristics of apoptosis and necrosis

An apoptotic cell has characteristic sequence of biochemical and physical changes. These changes affect the cytoplasm, nucleus and plasma membrane. Frédérich *et al.,* (2003), said that during apoptosis the cell shrinks and loses contact with neighboring cells. In the cytoplasm, the endoplasmic reticulum dilates and the cisternae swell to form vesicles and vacuoles. In the nucleus, the chromatin condenses and activation of an endonuclease cleaves genomic DNA into nucleosomal DNA which collects into multiples of internucleosomal 180-bp fragments which upon agarose gel electrophoresis give rise to characteristic DNA ladder. The nucleus becomes convoluted and buds off into several fragments, which encapsulate within the apoptotic bodies. In the plasma membrane, cell junctions disintegrate, whereby the plasma membrane becomes convoluted and eventually blebs. Finally, the cell itself fragments with all its cellular contents inside thereby forming apoptotic bodies of various sizes (Lawen, 2003). The apoptotic cell/bodies are then easily engulfed by macrophages (Duke and Ojcius, 1996: Hetts, 1998). Since the apoptotic bodies are surrounded by an intact plasma membrane, apoptosis usually occurs without leakage of cell contents and usually without inflammation. On the other hand, in cell oncosis the cell swells and disintegrates in an unordered manner, eventually leading to the destruction of the cellular organelles and finally rupture of the plasma membrane and leakage of the cell contents (Frohlich and Madeo 2000**;** Skulachev, 2002; LaCasse *et al*, 2005).

It was stated by Zamzani and Kroemer, (1999), that when normal cells are induced with death-inducing stimuli (figure 1.8) they react by initiating several other molecular

pathways, which lead to cell death. If cells fail to die, embryogenesis and organ dysfunction results and this contributes to the onset of cancer. Apoptosis pathways lead to cell death by activating initiator caspases, which in turn activate effector caspases to cleave cellular substrates. Zamzani and Kroemer, (1999), further said that apoptotic cells show cytoplasmic and nuclear condensation, the DNA is damaged, the cells form apoptotic bodies, and they have intact plasma membranes. These cells also expose surface molecules which phygocytic cells target for phagocytosis. In addition to that Otsuki *et al*., (2003), said that in the absence of phagocytosis, apoptotic bodies may proceed to lysis and may result in secondary or apoptotic necrosis.

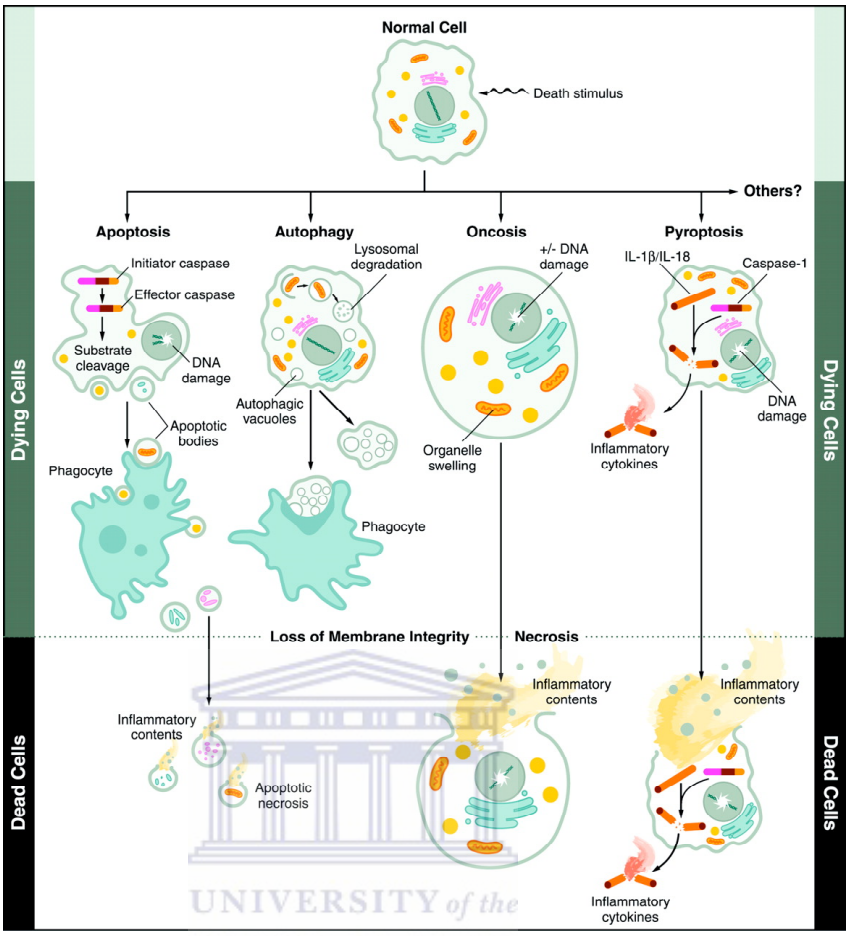


Figure 1.8: Pathways leading to cell death (from Fink and Cookson 2005).

Lysosomes may initiate cells to digest their own contents in autophagic vacuoles. Morphologically autophagic cells are vacuolated, cytoplasmic contents are degraded and the chromatin is slightly condensed. Autophagic cells can also be taken up by phagocytes. Oncosis is the prelethal pathway leading to cell death accompanied by cellular and organelle swelling and membrane breakdown, with the eventual release of inflammatory cellular contents. Pyroptosis is described as the cell death pathway caused by the activation of caspase-1, a protease that also activates the inflammatory cytokines, IL-1ß, and IL-18. This pathway is therefore naturally proinflammatory. Pyroptosis also involves cell lysis and also the release of inflammatory cellular contents. There is a possibility that other pathways exist but have not yet been described.

1.4.4 Apoptosis detection methods

When the term "apoptosis" was introduced by Kerr et al., (1972), its study mainly focused on cell morphology by the use of transmission electron microscopy (TEM). In recent times however, there has been noticeable progress in the study of apoptosis. Sciences such as biochemistry, molecular cell biology and genetics have provided more information on apoptosis and this has equipped researchers with a range of apoptosis detection methods. Most of these detection methods have mainly focused on apoptotic cell signaling (Schultz and Harrington, 2003; Jayaraman, 2005). The table 1.5 shows some of the features measured in apoptosis cell research and some of the detection methods used to measure those features. One feature associated with apoptosis is DNA fragmentation. Not all cells that die by apoptosis however show DNA fragmentation, which can be visualized by DNA laddering. This feature has been proven in cells which are deficient of Caspase-3 activation (Yuste et al., 2001), and in cells lacking cell nuclei e.g. red blood cells and lens fiber cells (Nagata, 1997, 2005).

Table 1.5: Some features measured in apoptosis studies and some of their detection methods.

Feature measured	**Detection method**
Morphological evaluation	Light microscopy/TEM/Phase contrast microscopy Fluorescence microscopy
Phosphatidylserine exposure	Annexin V/ APOPercentage™ Apoptosis assay
Caspase activity/cleavage	Flow cytometric/colorimetric/western blot
DNA fragmentation	Gel electrophoresis/TUNEL
PARP-1 cleavage	Western blot/Flow cytometric
Mitochondrial depolarization	TMRE/JC-1/$DiOC_6$ /Mito Flow
Bad/Bax/Bak	Western blot (anti-Bad/ -Bax/-Bak antibodies)
p53	Western blot (anti-p53 antibodies)
DNA perturbations	Propidium Iodide/Acridine orange (Cell cycle analysis)
IC_{50}	Neutral Red/Annexin V /MTT assay
Bcl-2	Western blot (anti-Bcl-2 antibodies)
Bcl-xL	Western Blot (anti-Bcl-X_L antibodies)

1.4.5 Diseases associated with apoptosis

Several pathways are involved in order for apoptosis to be achieved. First when stimulated to undergo apoptosis, initiator caspases are activated and secondly the initiator caspases activate effector caspases to cleave cellular substrates. If there are any defects in the apoptotic pathways however, this may result in complications and diseases. Reed, (2002), reported that many diseases are due to too much cell death or too little cell death. Some of the diseases which he said are due to defective apoptosis regulation are: (i) cancer, restenosis, autoimmune diseases and persistent infections (these are due to *insufficient apoptosis*) (ii) ischaemia, heart failure (due to loss of myocytes), neurodegenerative diseases such as (Alzheimer's, Parkinson's, Huntington's and myotrophic lateral sclerosis), osteoarthritis involving chondriocyte depletion, Human immunodeficiency virus which depletes T lymphocytes (Badley and Dockrell, 1997), and Type 1 diabetes which involves destruction of immune-mediated islets of Langerhans, (these diseases are due to excessive apoptosis).

1.4.6 Pathways of apoptosis

Apoptosis is induced through two main pathways involving either the mitochondria (the intrinsic pathway) or the activation of death receptors (the extrinsic pathway). Both pathways converge to induce the activation of caspases, which are the final executioners of

cell death. It should be well known however, that caspase-independent forms of apoptosis have been reported (Leist and Jaattela, 2001; Yuste *et al*., 2001; Reed, 2002).

1.4.6.1 The extrinsic pathway

According to Farrow (2000), all cells of a multicellular organism interact with and become accustomed to the environment they are in. He said that this responsiveness comes about largely through the expression of a range of specific receptors at the cell surface. The receptors transmit extracellular signals across the plasma membrane to the inside of the cell through intracellular signaling pathways (Reed and Pellecchia, 2005). Once the signal is received, the suitable functional response is carried out. The signals could be soluble factors, which are produced locally by the cell or the signal could be produced distantly for example hormones and growth factors. In order to achieve this, cells have a range of receptors on their surfaces which respond specifically to each stimulus. These receptors are divided into families, according to the way in which they generate the intracellular signals that give rise to the particular functional response. Some cell surface receptors are needed to transmit apoptotic signals initiated by ligands (for example, specific antibodies) or by natural ligands called death receptors (DRs) (table 1.6). These death receptors play a central role in instructive apoptosis (Schultz, and Harrington, 2003). Apoptotic cell death happens by the coordinated action of many different proteins.

It is said that cells maintain asymmetry of the inner and outer leaflets of the plasma membrane by continuously translocating phosphatidylserine (PS) to the inner leaflet

(Zwaal and Schroit, 1997; Vermes *et al.,* 1995). But if the cell translocates phosphatidylserine (PS) on the outer leaflet of the plasma membrane, then it is a signal for removal of the cells (Schlegel and Williamson, 2001). Death receptors are part of the tumor necrosis factor (TNF) gene super-family and these supply a speedy and efficient route to apoptosis (Ekert and Vaux, 1997; Kumar *et al.,* 2005). These receptors can activate caspases within seconds of ligand binding, causing an apoptotic destruction of the cell within hours (Ekert and Vaux, 1997; Walczak *et al.,* 1997). The death receptors best described to date are listed in Table 1.3 together with their various similar names (Ekert and Vaux, 1997; Walczak *et al.,* 1997; Ashkenazi and Dixit, 1998; Kumar *et al.,* 2005).

In addition to death receptors, the TNF super family contains decoy receptors (DcR), which restrain death signaling through the sequestration of the ligand. Decoy receptors include DcR1, DcR2 and osteoprotegerin (OPG), which bind to TRAIL and DcR3 particularly binds to Fas ligand and inhibits FasL activity (Ashkenazi and Dixit, 1999; Müllauer *et al.*, 2001). Signalling by death receptor is also controlled by cellular FLICE-like inhibitory protein (c-FLIP) an endogenous inhibitor that interacts with (Fas associated death domain) FADD so as to prevent apoptosis from taking place (Irmler *et al.,* 1997).

Table 1.6: Summary of the best-characterized death receptors and their related ligands (Kumar *et al*., 2005).

ACTIVATING LIGAND	DEATH RECEPTOR
TNF and Lymphotoxin alpha	TNFR1/DR1/CD120a/p55
FasL/CD95Land DAXX	Fas/CD95/Apo1/DR2
Apo3L/TWEAK	DR3/Apo3/WSL-1/TRAMP/LARD
TRAIL/Apo2L	TRAIL-R1/DR4
	TRAIL-R2/DR5 (Trail-R2, TRAIL-R2/Apo-2/TRAILCK2/KILLER
TRADD	DR6

1.4.6.2 The intrinsic pathway (Mitochondrial mediated apoptosis)

The intrinsic pathway is activated by mitochondrial disruption following cytochrome c release (Reed and Pellecchia, 2005). This pathway is initiated by growth factor withdrawal, UV irradiation, ischaemia, oxidative stress and cytotoxic drugs (Gupta, 2002; Ashe and Berry, 2003;). An 'apoptosome' is an approximately 1MDa oligomeric structure, which is formed by the interaction of cytochrome c, Apaf-1, d-ATP/ATP and procaspase-9 with the consequence of initiating a caspase cascade (Lawen, 2003). Members of the Bcl-2 family control this pathway (Budihardjo *et al*., 1999; Gupta, 2002; Schultz and Harrington, 2003; Lawen, 2003). Mitochondria contain a voltage-dependent anion channel (VDAC) through which molecules of ≤ 1.5 kD pass (Green and Reed, 1998) and their intermembrane space also contain several molecules such as cytochrome c, certain pro-caspases, adenylate kinase 2, Endo G, Diablo/Smac (Second Mitochondria-

Derived Activator of Caspases/direct IAP-Associated binding protein with low pI) and apoptosis-inducing factor (AIF) (Zamzami *et al*, 1996; Srinivasula *et al*., 2001). When the outer membrane (OM) is permeabilized, these molecules get released into the cytoplasm and inner membrane (IM) permeabilization leads to changes in mitochondrial membrane potentials ($\Delta\Psi m$). The release of cytochrome c is one of the major steps in the mitochondrial apoptosis pathway that is associated with permeabilization of mitochondrial outer membrane. As mentioned earlier, the release of cytochrome c results in the formation of the apoptosome, which in turn activates executioner caspases to induce apoptosis (Green and Reed, 1998; Regular *et al.,* 2003).

A third major pathway of apoptosis induction that does not primarily involve DRs or mitochondria is the p53 pathway. According to Mak and Yeh, (2002), the p53 is activated in response to DNA damage. Mak and Yeh, (2002); Ashe and Berry, (2003), have said the pathway blocks cells with damaged DNA in the G1 and G2 phases of the cell cycle. They further said that if the DNA damage is severe, and depending on cell type and oncogene composition of the cell, p53 initiates apoptosis by mechanisms that to some extent depend on the transcription of apoptosis executionary genes like Bax and genes whose products produce reactive oxygen species.

Müllauer *et al.*, (2001) and Budd, (2003), have pointed out that some human diseases are due to mutations in genes that are associated with cell surface receptors. Different genes are involved at different levels in the apoptosis pathway and if these genes are mutated, they may be the causative or contributing factors for some of the human diseases.

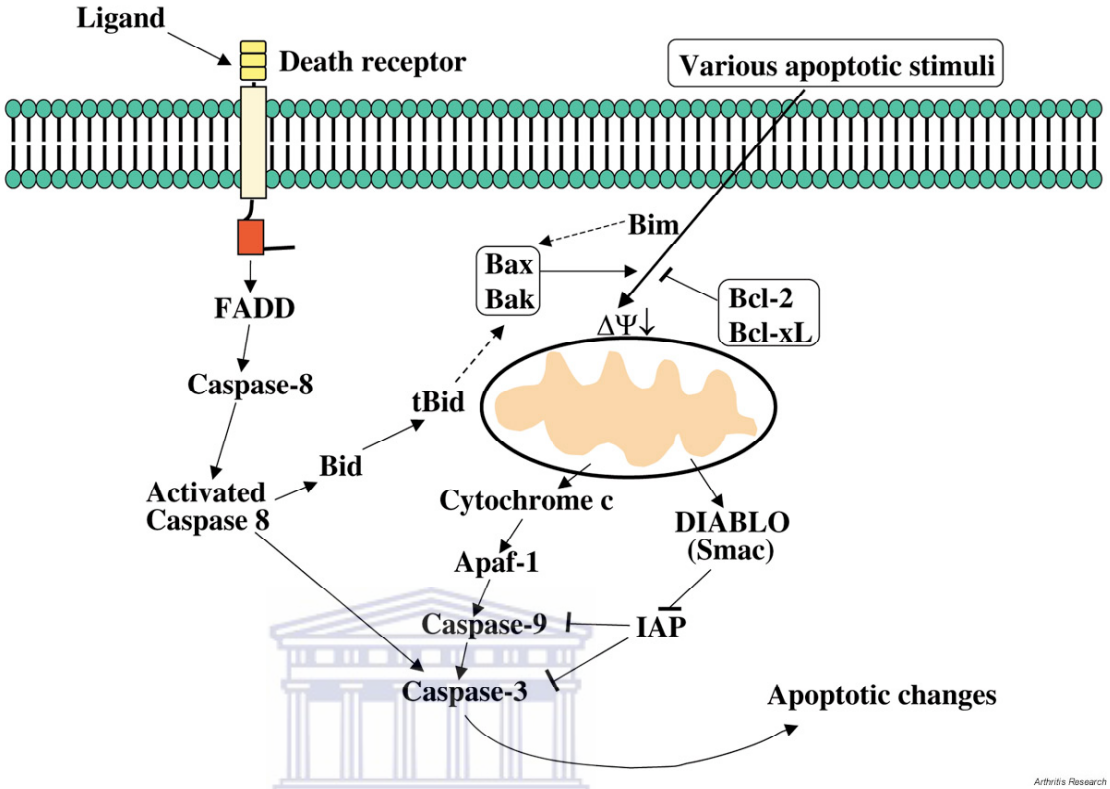


Figure 1.9: Diagrammatic representation of intrinsic and extrinsic pathways of apoptosis (from Mak and Yeh, 2002).

The extrinsic pathway is triggered by death receptor stimulation, which starts a signaling cascade. This cascade results in caspase-8 activation. The activation of caspase-8 leads to the activation of caspase-3 and also stimulates the release of cytochrome c by the mitochondria. The activation of caspase-3 leads to the degradation of cellular proteins, which are necessary for the maintenance of the cell survival and integrity. On the other hand, the intrinsic pathway arises when various apoptotic stimuli trigger the release of

cytochrome c from the mitochondria. Cytochrome c interacts with Apaf-1 and caspase-9 to promote the activation of caspase-3, which leads to the degradation of cellular proteins.

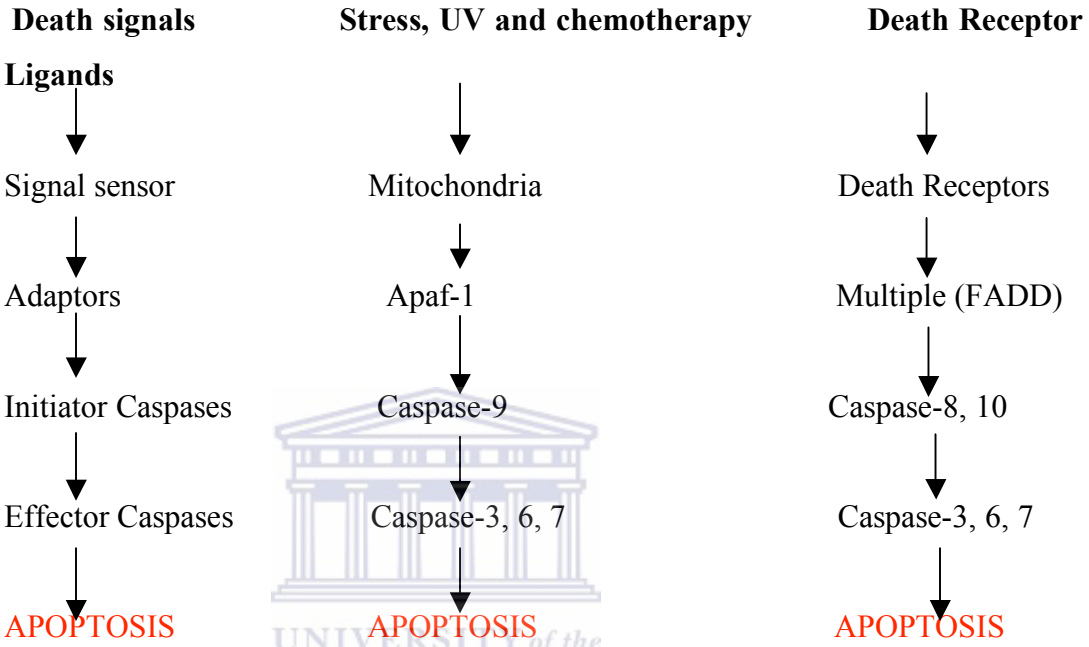


Figure 1.10: Diagrammatic summary of steps in signaling pathways of apoptosis (Gupta, 2002).

The death receptor pathway involves the interaction of its ligand with adaptor proteins (the most common is FADD, Fas-associated death domain) and initiator caspases (caspase-8 and -10). In the mitochondrial pathway, death signals directly trigger mitochondria resulting in the release of cytochrome c that binds to an adaptor protein Apaf-1 and then recruits the initiator casapse-9. Active caspase-9 triggers effector caspases to induce apoptosis. It can be seen that although there are two distinct initiator caspase pathways; however, both pathways have common effector caspases pathways.

1.4.7 Caspases (Cysteine Aspartate Specific ProteASEs)

The term “Caspases” stands for (cysteine aspartate-specific proteases) (Yuan and Horvitz, 1990; Alnemri, 1996). It is a highly conserved family of enzymes found inside the cell and these enzymes initiate execution of apoptosis and inflammation. All caspases exist within the cell as inactive zymogens. Once the caspases are activated, they signal a cascade of events, which eventually cause the cell to commit its own suicide. Riedl and Shi (2004), further reported that in humans and mice, there are about 14 caspases that have been recognized and these can be sub-grouped depending on how similar their amino acid sequences are or how specific their proteases are.

1.4.7.1 Types of caspases

Functionally, the caspases can be viewed in two ways and these are the upstream (or the initiator) caspases or the downstream (or the effector caspases). According to Kilicc, (2002); Stennicke and Salvesen, (1998), the caspases have been classified as initiator caspases which are categorized by their long prodomains (i.e. having >90 amino acids) and they contain either death effector domain (DED) (for instance caspase-2, -8, -9 a -10 and-12) in mammals or Dronc and Dredd in fruit flies. The executioner or effector caspases contain short prodomains (such as caspase-3, caspase-6 and caspase-7) in mammals and Drice, Decoy, Damm, Dcp1 and Strica in fruit flies. The cell-death abnormality-3 (CED-3) is the only apoptotic caspase which is found in the nematode worm *Caenorhabditis elegans* and works as both initiator and effector caspase and the

remaining caspases-1, -4, and -5 are mainly involved in cytokine maturation but are not necessarily involved in apoptosis.

1.4.7.2 Functioning of caspases

The caspases work in such away that once they have been activated, the prodomains cleave and the large and small subunits separate. The active caspases then form heterotetramers (Lawen, 2003). Initiator caspases cleave and trigger effector caspases. It is these effector caspases that cleave cellular substrates and eventually the cell dies (Liston *et al,* 2003). Effector caspase-3, when activated is able to cleave many important cellular substrates, such as inhibitor of caspase-activated DNase (ICAD), Rho-associated coiled-coil forming kinase (ROCKI), poly (ADP-ribose) polymerase, (PARP) a DNA repair enzyme, actin, fodrin and lamin (Sabraham and Shaham, 2004).

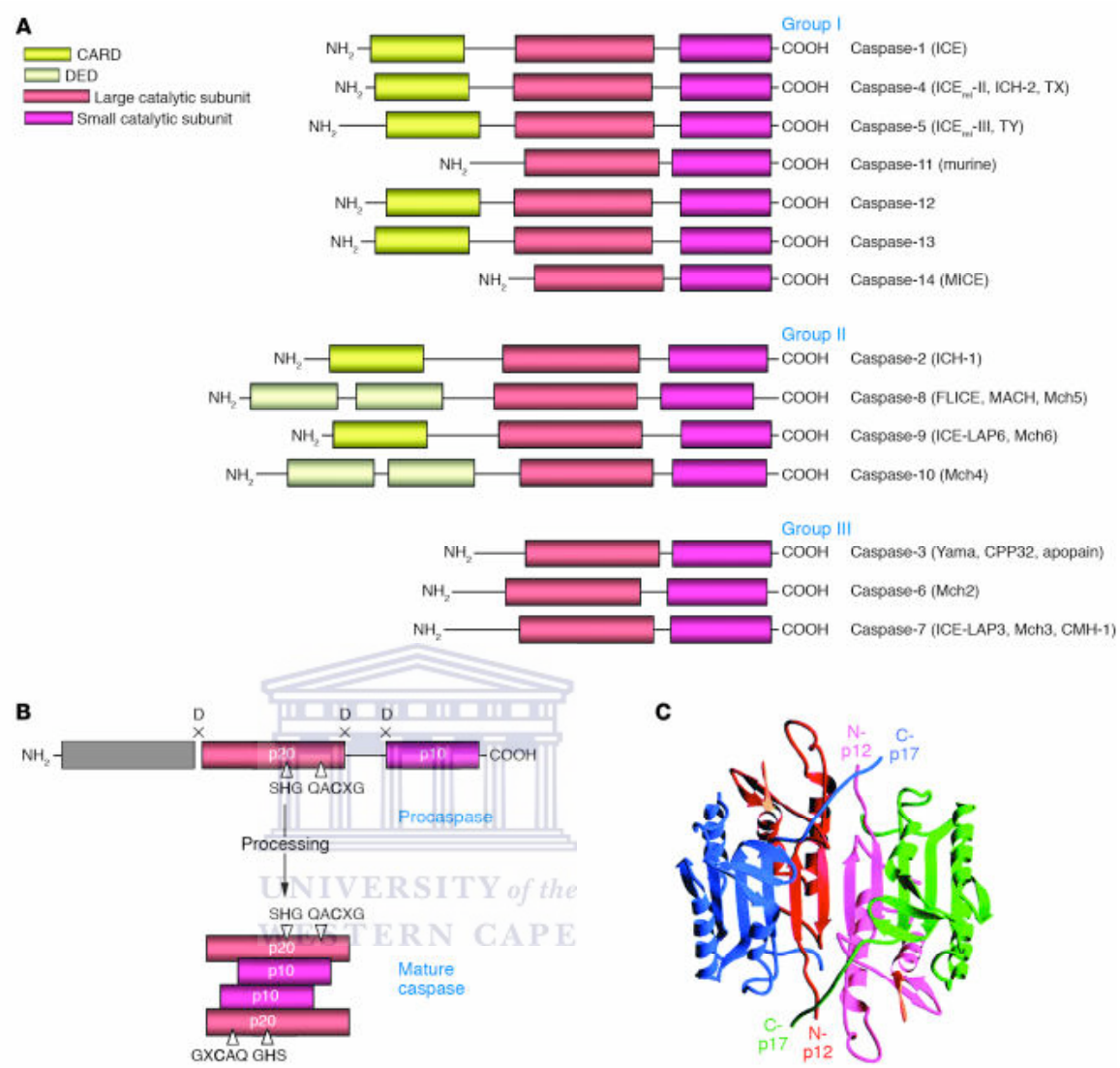


Figure 1.11: Caspase structure (adapted from Lavrik *et al*., 2005).

The caspase family. Three major groups of caspases are shown. Group I: inflammatory caspases; group II: apoptosis initiator caspases; group III: apoptosis effector caspases.

1.4.8 The Bcl-2 family

The Bcl-2 family members have been described by Hu *et al.,* (2003), as the major controllers of the apoptotic process, while caspases carry out the execution process.

According to Hu *et al.,* (2003), the mechanism through which the Bcl-2 family members regulate apoptosis is due to their ability to change the mitochondrial function. The members of this family are divided into pro-and anti-apoptotic molecules and there are more than 20 members of this family, which have been identified in humans and this includes proteins that suppress apoptosis for example, Bcl2, Bclxl, Mc1, A1, Bclw and Bclg and proteins that promote apoptosis such as Bax, Bak, Bok, Bad, Bid, Bik, and Bim1. Hu *et al.,* (2003), further reported that the release of cytochrome c from the mitochondria acts as a switch as it is central in turning apoptosis on or off and is controlled by the pro-apoptotic proteins and the anti-apoptotic proteins of the Bcl2 family.

The Bcl-2 proto-oncogene was discovered at the chromosomal breakpoint of t (14; 18) bearing human B-cell lymphomas (Friend *et al*., 1986; Vaux *et al*., 1988). The gain of chromosome 18q and translocation t(14;18) are commonly found in B-cell non-Hodgkin's lymphomas (B-NHL). Increased transcription and expression of BCL2 have been implicated to be the result of the gain of chromosome 18. (Galteland *et al*., 2005). The study by Vaux *et al*., (1988), showed that when B cells were transfected with Bcl-2 they showed resistance towards apoptosis, which was normally induced in B cells by interleukin-3 (IL-3) withdrawal. It was shown for the first time that the development of tumours depended not only on the capability of the cells to escape growth control but also depended on the capability of the cells to prevent apoptosis. The study found that when there was an excess of pro-apoptotic proteins the cells were more prone to apoptosis, but when there was an excess of anti-apoptotic proteins the cells tended to be more resistant to apoptosis (Vaux *et al*., 1988).

The pro-apoptotic proteins of the Bcl-2 family are often found in the cytosol where they act as detectors of cellular damage or stress. Once the cell(s) have cellular stress these proteins relocate to the surface of the mitochondria where the anti-apoptotic proteins are located. The interaction between pro- and anti-apoptotic proteins disrupts the normal function of the anti-apoptotic Bcl-2 proteins and this leads to the formation of pores in the mitochondria and this causes the release of cytochrome c and also the release of pro-apoptotic molecules from the intermembrane space. Following this activation, the apoptosome is formed, which leads to the activation of the caspase cascade (Gross *et al*., 1999).

1.4.8.1. The members of the Bcl-2 family

The Bcl-2 family proteins have been subdivided into three classes (Gross *et al*., 1999; Breckenridge and Xue, 2004). This subdivision is determined by the functions of the proteins and also the number of Bcl-2 homology (BH) domains that are present: for instance the first subdivision comprises the anti-apoptotic members such as Bcl-2 and Bcl-xL which have four BH domains (BH1 to BH4), the second subdivision comprises of the pro-apoptotic members such as Bax and Bak which have three BH domains (BH1 to BH3), and the third subdivision is the 'BH3-only' pro-apoptotic members such as Bim and Bid which share the homology only within the BH3 domain (Regula *et al.,* 2003). All the proteins in these three classes are able to form either homo-oligomer or hetero-dimer with one another and seem to have well-defined functions in the regulation of mitochondrial

membrane permeabilization (MMP). Gross *et al*., (1999), said that most of the anti-apoptotic members show sequences which are maintained in all the four domains and that the pro-apoptotic molecules mostly display less sequence conservation of the first α-helical segment, BH4. Accordingly, studies using deletion and mutagenesis show that the amphipathic α-helical BH3 domain serves as a significant death domain in the pro-apoptotic members. Gross *et al*., (1999) and Borner, (2003), have further indicated that studies involving separation of "BH3-domain-only" members have shown sequence homology only within the BH3 domain and that to date these are all pro-apoptotic. In addition Korsmeyer, (1999); Igaki *et al*., (2000), said members of the Bcl-2 family have been implicated in a number of cancers, such as melanoma, breast, and lung cancer and said that the members of the Bcl-2 family are involved in resistance to cancer treatment.

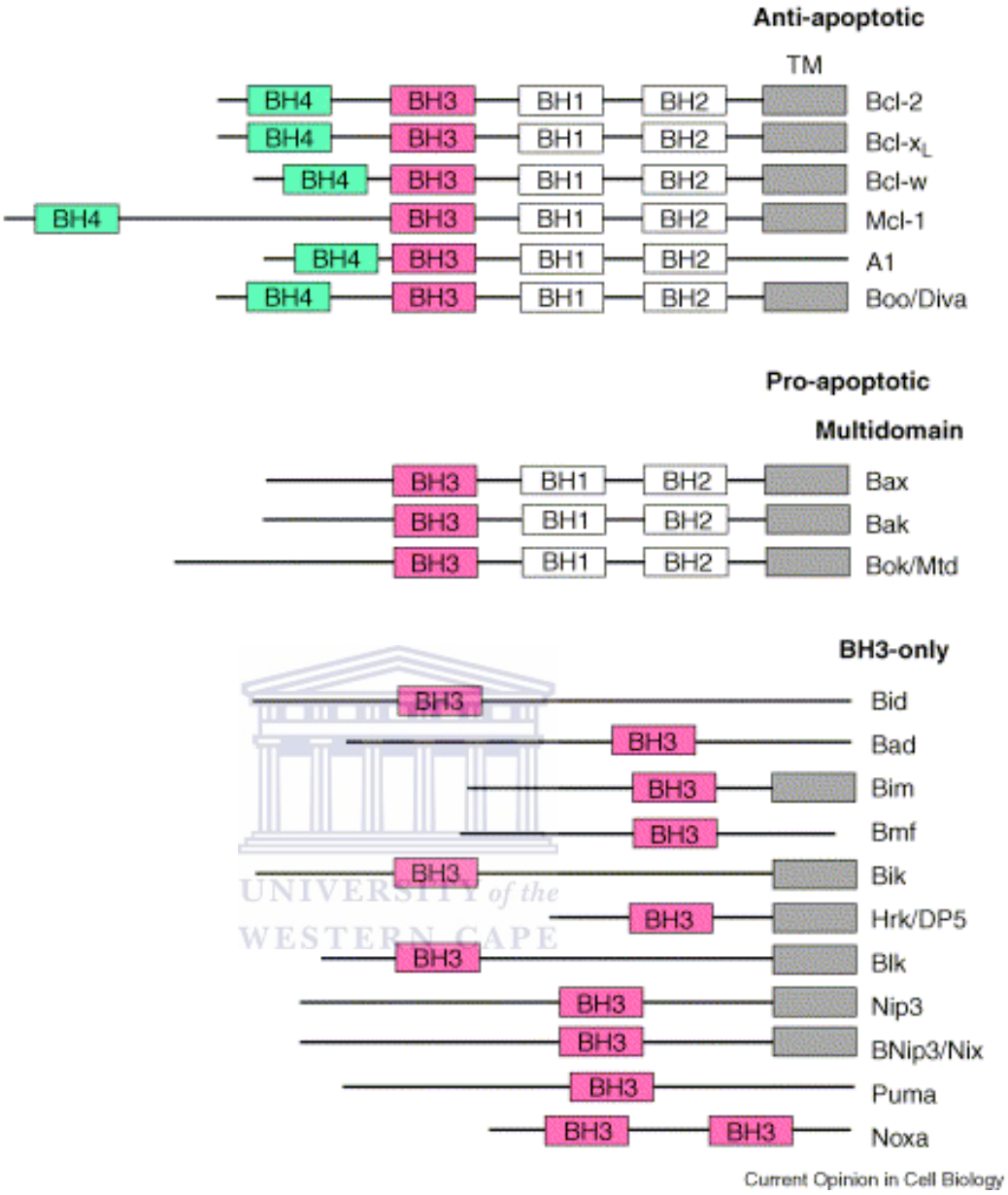


Figure 1.12: Schematic classification of some members of the Bcl-2 protein family (from Kuwana and Newmeyer, 2003).

'TM' refers to a hydrophobic region in the carboxyl terminus of several of these proteins that was originally assumed to be a transmembrane domain. The pro-apoptotic multidomain (BH1–3) proteins might contain a weak BH4 homology (not shown).

1.5 CANCER AND THE CELL CYCLE

Cancer is a disease which results when control of the cell cycle goes wrong and normal cell growth and behavior are completely lost. Generally, cancer can be said to be a compilation of diseases or disorders whereby abnormal cells spread within the body or tissue. The cell division of these abnormal cells is uncontrolled and the cells may have the capacity to invade other cells or tissues (metastasize). The uncontrolled and mostly fast growing cells may cause growths in those tissues in which they are proliferating, which might either form benign or malignant tumors (Rang and Dale, 1991; Lowe *et al.,* 2000). Under normal circumstances, cells grow and divide in an orderly manner, following the regulation of the cell cycle. The cell cycle is therefore a significant controller of cell proliferation and growth, as well as cell division following DNA damage (Shah and Schwartz, 2001). Several proteins are involved in the normal function of proliferative pathway and these proteins may function to sensitize the cells to apoptosis so as to keep a balance between proliferating cells and dying cells. However**,** several disorders such as cancer, viral infections, autoimmune diseases, neurodegenerative disorders, stroke, anemia and AIDS occur due to improper regulation of the cell cycle (Pucci *et al* 2000). In the case of cancer, many factors are involved. Some of such factors may be that cells avoid the apoptotic program through both down-regulation of apoptotic inducers e.g. p53 (Bennett, 1999**),** inappropriate or excessive induction of survival signals e.g. P13K (Dong *et al.,* 1999) or because proto-oncogenes or tumour suppressor genes are mutated thereby allowing a cancerous cell to grow and divide (Dong *et al.,* 1999). Since improper regulation of the cell cycle may lead to diseases such as cancer, it is therefore important to have a basic understanding of the stages of the cell cycle so that novel therapies may

target those genes which are involved in circumventing apoptosis or inhibiting the proper function of the cell cycle for example the cyclin dependent kinase inhibitors (CDKIs) (Pucci *et al.,* 2000; Bertino *et al*., 2003).

The regulation of the cell cycle is a very complicated process because it involves a large selection of factors, in particular the cyclins and cyclin-dependent kinases. Bertino *et al*., (2003), said that there are two general ways by which genes control the cell cycle regulation. Some genes control the production of proteins whose functions are required for the cell cycle to take place, and there are genes that control the initiation of each phase of the cell cycle. Other genes however, serve as negative regulators of the cell cycle and they stop the cell from proceeding to the next step/phase of the cycle.

1.5.1 Cyclin-dependent kinase (Cdks) and cell cycle regulation.

Cyclin-dependent kinase (Cdks), are proteins, which belong to a well-conserved family of serine/threonine protein kinases and they act as control switches in the regulation of the cell as it goes through several stages of the cell cycle (Pucci *et al.*, 2000). Their kinase activity relies on the presence of activating subunits known as cyclins. Specific cyclins are in abundance during the phase of the cell cycle in which they are required and they decrease during phases in which they are not needed (Shah and Schwartz, 2001). They are in fact key to the progression of the cell through the cell cycle. As the cell progresses through the cell cycle it is helped by a number of Cdks, which, when bound to specific regulatory proteins called cyclins, move the cell forward through the cell cycle.

Correspondingly there are cell cycle-inhibitory proteins called cyclin dependent kinase inhibitors (CDKIs), which hinder the cell from moving to the next phase of the cell cycle (Golsteyn, 2005). So far there are at least 9 structurally related CDKs (CDK 1-9), which have been identified, even though the role of some of them in the cell cycle regulation is not clear. A considerable number of cyclins have also been identified to date (cyclins A-T). It is clear however, that as the cells express cyclins D, A, E, and B1, they can subdivide the cell cycle into different clear stages of the cell cycle, (Golsteyn, 2005). For instance, the expression of cyclin D1 can be detected in early G1 of the cell cycle, while the expression of cyclin E can be detected in G1/S peak. Expressions of cyclin A can be detected during G2/M phases and that of B1 can be detected in late G2/M phase (Golsteyn, 2005; Nakayama and Nakayama, 2005).

1.5.2 p53 and cell cycle regulation

One other important protein in the cell cycle regulation is p53, a transcription factor located on human chromosome 17p13 and consists of 393 amino acids that bind to DNA, activating transcription of a protein called p21 $^{Waf1/Cip1}$ which blocks the activity of a cyclin-dependent kinase required for progression of the cell through G1 (Minnella *et al*., 2002; Lozano and Zambetti, 2005). This block allows time for the cell to repair the DNA if damaged before it is replicated. If the DNA damage is so extensive that it cannot be repaired, p53 triggers the cell to commit suicide through the activation of transcription factors. The most common mutation leading to cancer is in the gene that encodes p53. For example, Li-Fraumeni syndrome, an inherited condition whereby a person is susceptible

to multiple cancers, results from a germ line mutation in p53 (Senzer *et al*., 2006). Other proteins that stop the cell cycle by inhibiting cyclin dependent kinases are p16 and p27. It has been shown that a single amino acid substitution can lead to a loss of function of the p53 protein (Hupp *et al*., 1994). Defective p53 can allow abnormal cells to grow, resulting in cancer and it is widely accepted that p53 is the most commonly mutated tumor-suppressor gene linked with human cancers (Vousden, 2000).

Other functions of p53 include the ability to relocalise death receptors like Fas from the golgi to the cell surface (el-Deiry *et al*., 1992; Bennett *et al*., 1998) and the direct involvement of p53 in the mitochondria (Marchenko *et al*., 2000). Despite the fact that p53 protein is a transcription factor, el-Deiry *et al*., (1992) said under normal conditions, it is extremely unstable; therefore it usually does not build up to high enough levels to bind to p53-control elements to initiate transcription. el-Deiry *et al*., (1992), further said that damaged DNA in some way stabilizes p53, leading to an increase in its concentration and that cells with mutations in both p53 alleles do not exhibit delayed entry into the S phase following low levels of DNA damage and do not undergo apoptosis following more extensive DNA damage. If the DNA of such cells is damaged, the damaged DNA can replicate, thereby producing additional mutations that add to the development of an extremely transformed, metastatic cell.

There are three independent pathways that take part in activating p53 network. One pathway is triggered by DNA damage, e.g. ionizing radiation. This pathway depends on two protein kinases: ATM (ataxia telangiectasia mutated) and ChK2 (checkpoint kinase 2)

(Carr, 2000). ATM is activated by double stranded breaks in DNA, and in turn stimulates Chk2.

The second pathway is stimulated by aberrant growth signals, resulting from the expression of the oncogenes Ras and Myc. In humans, the activation of p53 network depends on $p14^{ARF}$ tumor suppressor protein (Lowe and Lin, 2000).

The third pathway is triggered by a wide range of chemotherapeutic drugs, UV light and protein kinase inhibitors. It involves kinases called ataxia telangiectasia related (ATR) and casein kinase II (Meek, 1999).

1.5.3 PARP-1 and DNA repair

Poly(ADP-ribose) polymerase (PARP-1) is an abundant nuclear enzyme that helps in the repair of single-stranded DNA strand breaks through the activation of DNA repair enzymes and check point controls. It is a DNA strand break-sensing molecule, which is involved in response to DNA damage and the safeguarding of genomic stability (Tong *et al*., 2001; Süsse *et al*., 2004). The enzyme typically binds to only one strand of a broken DNA and then recruits XRCC1, DNA ligase IIIα, DNA polymerase β and polynucleotide kinase to the broken end (Caldecott *et al*., 1996). This complex then repairs the broken part. It has been suggested that there is an interaction between PARP and p53. Malanga *et al* (1998) and Tong *et al*., (2001), suggested that PARP can bind to specific domains of the p53 protein and can modify the activity of p53 by poly ADP-ribosylation.

1.5.4 The Retinoblastoma (Rb) gene

The human retinoblastoma (Rb) gene is a nuclear protein that is implicated as a tumour suppressor and regulator of the cell cycle control check point specifically at the G1 phase of the cell cycle (Feakins *et al*., 2003; Mancini *et al*., 1994). The gene contains 928 amino acids and was the first tumor suppressor gene to be identified and was isolated from retinoblastoma tumors (Lee *et al*., 1987).

1.5.4.1 The role of Retinoblastoma (Rb) gene

It can be stated that the major two functions of Rb are the inhibition of cell proliferation and stimulation of cell differentiation (Zhu, 2005). Rb studies have identified two Rb related proteins p107 and p130, which are more closely related to each other than either one of them to Rb. These three proteins (Rb, p107 and p130) have been reported by Zhu, (2005), to be targets for viral onco-proteins that share onco-protein binding sequences and are known as "pocket" domains therefore the three proteins are usually called "pocket proteins". The three pocket domains A,B and C are situated at residues 379-928. According to Wiman, (1993), some studies involving the deletion of both Rb alleles have shown to play a rate-limiting role in retinoblastoma and also in the sarcomas that mainly arise in families that carry the mutated Rb gene. Such sarcomas include; small cell carcinoma of the lung, carcinoma of the breast, bladder, and prostate.

The Rb gene product (Wiman, 1993), binds a number of cellular proteins and one of them is the transcription factor E2F. The transcription factor E2F is needed for the transcription of cellular genes, which take part in growth and DNA synthesis of the cell. There are several genes that contain E2F binding sites and some of such genes are c-myc, cdc2 and Rb. In cells, which are resting, the Rb proteins are present in their least phosphorylated form and Rb interacts with E2F, while in rapidly growing cells, the Rb proteins are greatly phosphorylated. Maximum phosphorylation of Rb proteins is related with S phase of the cell cycle (Mancini 1994; Zhu 2005). The active hypophosphorylated form of the retinoblastoma protein (pRb) binds and blocks the action of the transcription factor E2F, inhibiting transition from the G1 phase to the S phase of the cell cycle (Hanahan and Weinberg, 2000). The increased concentration of Rb protein at promoter site coupled with E2F, binds adjacent transcription factors, preventing their contact with the basal transcription complex, in so doing blocking transcription (Wiman, 2005; Day *et al*., 1997).

Also cyclin D1 stimulates phosphorylation of Rb by associating with cyclin-dependent kinases (CDKs) and p16 binds to CDKs 4 and 6, blocking their association with D-type cyclins. Therefore, preventing the phosphorylation of Rb blocking the activity of E2F (Bertino *et al.,* 2003; Feakins *et al*., 2003**).** Mutations of Rb in retinoblastoma have therefore been implicated in disturbing the ability of Rb to interact and repress E2F, and this therefore suggests that repression of E2F is crucial to Rb tumour suppression activity (Hanahan and Weinberg, 2000; Zhu, 2005).

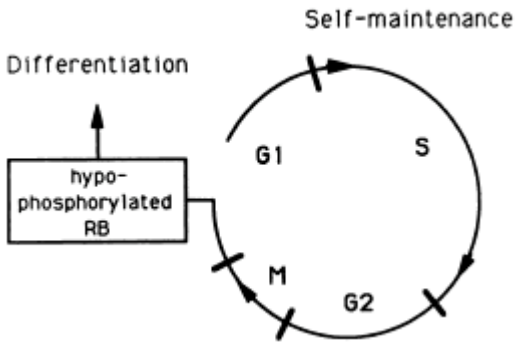


Figure 1.13: Model of Rb in the regulation of cell differentiation (adapted from Wiman, 2005). Phosphorylation of Rb allows progression of the cell through G1 and continuation of a self-maintenance pathway. Un-or hypophosphorylated Rb blocks cell cycle progression in the G1 phase (adapted from Wiman, 2005).

1.5.5. Stages of the cell cycle

The cell cycle has been described by Bertino *et al*., (2003), as a significant controller for cell proliferation, growth and cell division following DNA damage. It is a well-organized series of events, which results in the production of two genetically identical daughter cells. Before the cell divides its genetic material, it goes through several stages. From quiescence (G0) phase, to cell proliferation and as it goes through the stages, there are check points which ensure that all the genetic material are kept intact, not damaged or incomplete or miscopied. Otherwise the checkpoints stop the cell from proceeding to the next stage of the cell cycle until there is a repair to the damage. If the damage cannot be repaired, the cell is eliminated through apoptosis (Pucci *et al*., 2000). According to Schwartz and Rotter (1998), there are four key checkpoints in the cell cycle. At the end of the G1 phase, G2 phase and after DNA has been replicated in the S phase it checks for

damages. Again at the end of the M phase a checkpoint is available, which stops cytokinesis in case the chromosomes are not properly aligned on the mitotic spindle. Bertino *et al.*, (2003) and Schwartz and Rotter, (1998), have further indicated that these check points allow proliferation to occur in the presence of stimulatory signals for instance growth factors and are also activated by DNA damage as well as mis-aligned chromosomes at the mitotic spindle. Accordingly cells that are not in the process of dividing are normally found in the G0 stage, and this includes most of the adult cells. In addition, Pucci *et al*., (2000), said most cells remain in interphase, i.e. the period between cell divisions (comprising G1, S and G2 phases), for at least ninety percent of the cell cycle and that DNA repair genes are active throughout the cell cycle, particularly during G2 after DNA replication and before the chromosomes prepare for mitosis.

Most anticancer drugs are designed to target different stages of the cell cycle so that the cell should not proceed through to the next stage. Some anticancer agents for instance, work in such away that they bind to DNA in the S phase of the cell cycle in order to stop DNA synthesis. Such agents include alkylating, anti-tumor antibiotics, platinum compounds and other miscellaneous agents (Gonzalez *et al.*, 2001; Pasettoa *et al.*, 2006). Since cells in G2 synthesize their components for cell division, other plant-derived anticancer agents in clinical use such as vinca alkaloids (vinblastine, vincristine, vinorelbine), docetaxol and paclitaxel target the microtubules to stop the cells from making spindles needed for mitosis (Jordan and Wilson, 2004). In particular, paclitaxel mainly interferes with the mitotic spindle assembly, which results in the failure of chromosomes to separate (Shah and Schwartz, 2006). For mitosis to take place, the cyclin

B1-CDK 1 complex (which is also called cyclin B1-cdc2 kinase), has to be initiated and once mitosis proceeds however, cyclin B is destroyed by ubiquitin-mediated-proteolysis. Shah and Schwartz, (2001) and (2006), have further reported that cyclin B and Cdc2 kinase activity are directly related to paclitaxel function. Expression of cyclin B and the activation of CDK 1 occur with paclitaxel-induced apoptosis and destruction of cyclin B1 can be inhibited by paclitaxel. On the other hand, the taxanes are known to stabilize the microtubules, and this causes a G2/M arrest, followed by apoptosis (Cragg and Newman, 2004, 2005 and Darwiche *et al*., 2007). Yet other anti-metabolites used as anticancer agents such as 6- mercaptopurine (6-MP), methotrexate, azathioprine, 6-thioguanine (TG) and 5-fluorouracil (5-FU) have been shown to target cells in the G1 phase in order to stop them from synthesizing nucleotides for DNA (Bertino *et al*., 2003). Another drug methotrexate (MTX), which is normally used in mixture with 5-fluorouracil and cyclophosphamide in the adjuvant treatment of breast cancer, works by interfering with the metabolic processes that are involved in the continued existence and reproduction of the cell. Methotrexate mainly targets cells that are rapidly growing and reproducing (Bast *et al*., 2000; Su and Ciftci, 2002), and specifically, the drug binds to and deactivates dihydrofolate reductase (DHFR), a key enzyme necessary for DNA synthesis.

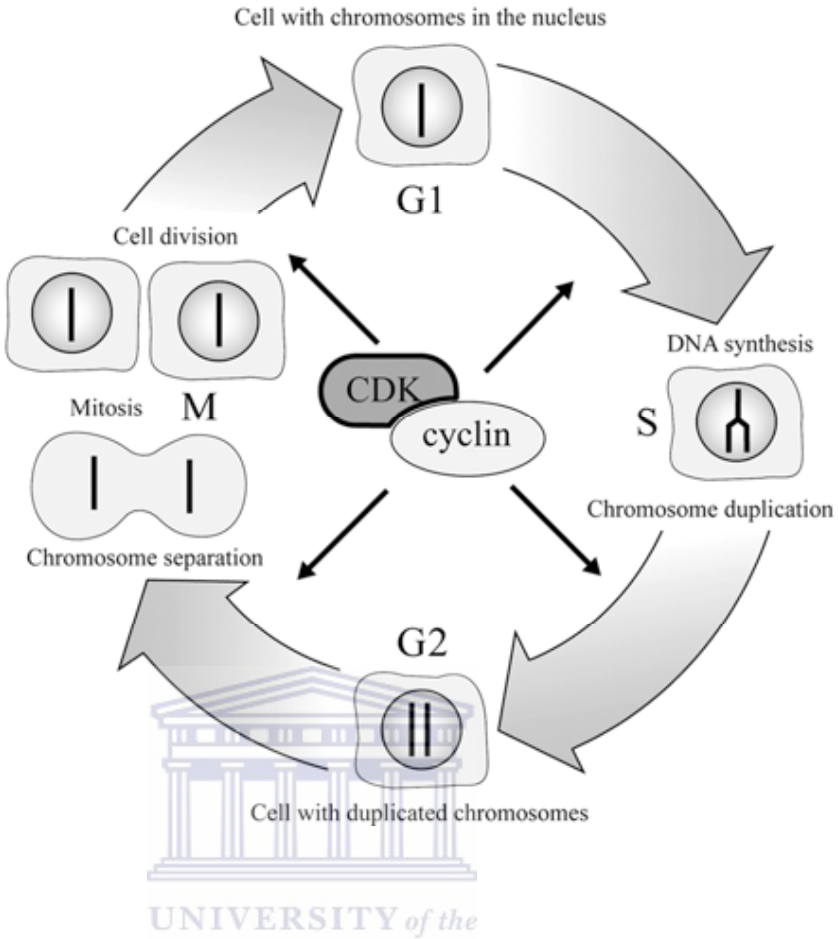


Figure1.14: Stages of the cell cycle (Adapted from Garber, 2001).

1.6 RHUS SPECIES.

Rhus is a genus of woody shrubs and trees, which has spirally and pinnately arranged compound leaves, although some species have three leaflets. The flowers are in thick cluster or spikes 5-30 cm long, and each flower is very small, could be creamy white, greenish or red, with five petals. The fruit form thick clusters of reddish drupes, which are commonly called "sumac" (van Wyk *et al*., 2000). There are said to be roughly 250 species of *Rhus,* which have been placed in the family Anacardiaceae. The name comes

from the Greek name for sumac, rhous, which means red, and this is probably because some species turn red in autumn. According to Moffett, (1993), some species of this genus such as poison ivy, poison oak and poison sumac have been placed in the genus Toxicodendron and have grayish-white fruit.

1.6.1. Rhus laevigata.

Rhus laevigata is a plant mostly found in subtropical and warm temperate regions of the world and is very common in South Africa especially in Western Cape (Watt, 1962). The common names for *Rhus laevigata* are Rankataaibos, Duinetaaibos, Dune taaibos, in Afrikaans, Inhluti and Isihlakoti in Zulu (Low and Scott, 1983; Hutchings *et al*., 1996). This plant is a shrub of about 2 m and produces many branches and is mostly deciduous. Its leaves may either be hairy or smooth and are also divided into three leaflets, the middle one being large (about 40 x 25 mm). The veins of this plant are translucent when held up to the light and its flowers are unisexual, found on separate branches. The plant produces little fruits which are edible and a tasty juice can be made from them (Watt, 1962).

1.6.2. Medicinal usage of *Rhus laevigata*

Traditionally *Rhus laevigata* is used as a medicinal plant. As a medicinal plant, the leaves, bark and roots are used and some of the traditional uses include chewing of its leaves for chest colds and preparation of leaf decoctions for post partem problems (van Wyk *et al*., 2000). Also milk infusions of leaves are administered as enemas for abdominal upsets in children (Hutchings *et al*., 1996). Again, the roots are claimed to be of therapeutic value in

infective disorders of the gastro-intestinal tract and also used as an anti-inflammatory medicine (van Wyk *et al*., 2000).

Presently there is no data that indicates that extracts from *Rhus laeivigata* induce apoptosis in cancer cell lines. There is no data either to show that active compounds from *Rhus laevigata* have ever been isolated and identified. Traditionally reports only indicate that the plant is used for medicinal purposes such as colds, post partem problems and anti-inflammatory medicines but there are no scientific reports, which prove these claims. Other studies have however, shown that extracts from *Rhus verniciflua* have inhibiting activities on human cancer cell lines (Lee *et al.,* 2002, 2004; Lim*. et al.,* 2003; Ko *et al*., 2004; Son *et al*., 2005).

1.7 METALLO-COMPOUNDS IN CANCER TREATMENT.

“Cytotoxic drugs" are drugs that can damage or kill cells specifically by inhibiting cell division and for this reason they are used as antineoplastic agents (Zhang, 2002 Sato *et al*., 2005). The major cytotoxic drugs have been grouped into four general groups, namely, alkylating agents; antimetabolites; natural products and miscellaneous agents. Platinum compounds fit in the group of miscellaneous agents. However, platinum compounds can also be classified as alkylating agents because they act in a similar fashion as alkylating agents. Of the platinum agents currently available today, cisplatin cis-diamminedichloroplatinum (II) is the most effective and extensively used anticancer chemotherapeutic drug (Trzaska, 2005). At the centre of this drug is an atom of the metal platinum. It is these atoms that poison the cancer cells, by forming DNA adducts which are thought to be the primary cause of cisplatin cytotoxicity (Elwell *et al*., 2006).

1.7.1 Platinum (II) Complexes and Cisplatin

The development of metal-containing anticancer drugs started with *cis*-$[PtCl_2(NH3)_2]$ which is often referred to as cisplatin. In spite of the fact that the compound was initially described in 1845, its anti-tumor activities were however, not discovered until 1964 (Rosenberg *et al*., 1969). It is known to be particularly effective against solid tumors such as testicular, ovarian, head, neck and against small-cell lung cancer with a cure rate as high as 90% (Reedijk, 2003). It has been found that cisplatin exhibits anti-tumor activity while its trans isomer does not show any activity (Reedijk and Lohman, 1985). Interestingly, minor variations in the structure of these ligands have shown to have

powerful effect on the anti-tumour activity and toxicities. Many studies with cisplatin trans-compounds have shown to be ineffective against solid tumors, while the cis-counterparts have shown completely opposite results (Ciccarrelli *et al*., 1985; Brabec, *et al*, 2003).

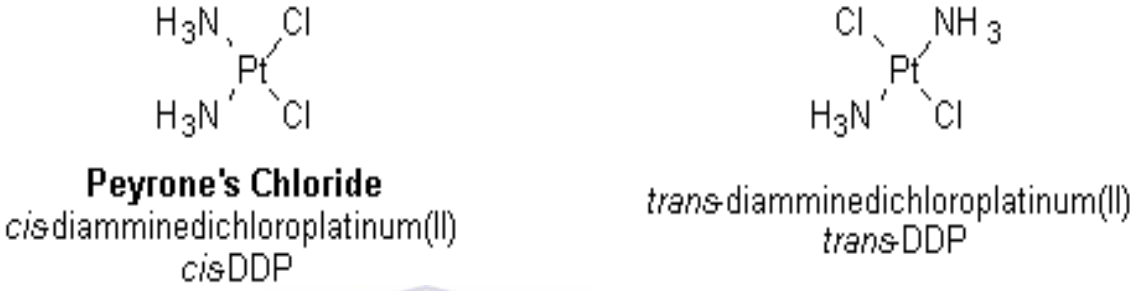


Figure 1.16: Isomeric forms of diamminedichloroplatinum.

1.7.2. Cisplatin's proposed mode of action

Although cisplatin, cis-diamminedichloroplatinum (II), has been successfully used in chemotherapy treatment for more than 25 years, its biochemical mechanism of action is still unclear (Elwell *et al*., 2006), however, the drug is thought to induce cytotoxicity through binding to nuclear DNA forming cisplatin–DNA adducts (Jamieson and Lappard, 1999), which activate multiple signalling pathways including those involving p53, Bcl-2 family,

caspases, cyclins, cyclin dependent kinases (CDKs), protein kinase c (PKC), retinoblast oma protein (pRb) mitogen activated protein kinase (MAPK) phosphatidylinositol 3 kin ase/protein kinase B (PI3K/Akt) and subsequent interference with normal transcription, and/or DNA replication mechanisms (Demarcq *et al*., 1994; Pasetto *et al*; 2006). If the cell

fails to process the cisplatin–DNA adduct, then cytotoxic processes eventually end up in the death of the cell (Areberg, 1999; Gonzalez *et al*., 2001). It is proposed that chlorine undergoes slow displacement with water molecules forming a positively charged molecule, which then cross links the DNA. Thus the drug needs to be administered in saline solution, to prevent inactivation (Alderdena *et al*., 2006). While the trans isomer does not have an anticancer effect, it is however, toxic and Alderdena *et al*., (2006), said that it is important to test every batch of cisplatin by HPLC to test for the absence of the trans isomer.

Cancers treated with cisplatin however frequently develop resistance to cisplatin and many others show lack of sensitivity (Gonzalez *et al*., 2001). According to Gonzalez et al., (2001) and Alderdena *et al*., (2006), the limited effectiveness of cisplatin is due to many factors including: (a) a poor pharmacokinetic profile, (b) low accumulation in cells, (c) increased production of intracellular thiols (e.g. glutathione and metallothionein) (d) increased DNA repair capacity and (e) as cisplatin is administered intravenously due to its limited solubility in water accumulation of metal ions in the body can lead to harmful side effects including nausea, emetogenesis, hair loss, neurotoxicity (nerve damage), ototoxicity (hearing loss), nephrotoxicity (kidney damage), platelet reduction, myelosuppression, (the reduction of bone marrow function), angioedema, granulocytopenia, erythema, exfoliative dermatitis, facial edema, flushing, hyperhidrosis, rash, alopecia, hypomelanosis, anaphylaxis, gingival discolouration, injection-site cellulites, oral mucosal lesions, diarrhoea, anorexia and loss of appetite and taste which results in a patient finding it difficult to eat, among others (Kumar and Clark, 1990; Zhang

and Lippard, 2003; Brabec and Kasparkova, 2005; Elwell, 2006). These limitations have prompted a search for more effective and less toxic metal-based anti-tumor agents (Brabec and Kasparkova, 2005; Elwell, 2006).

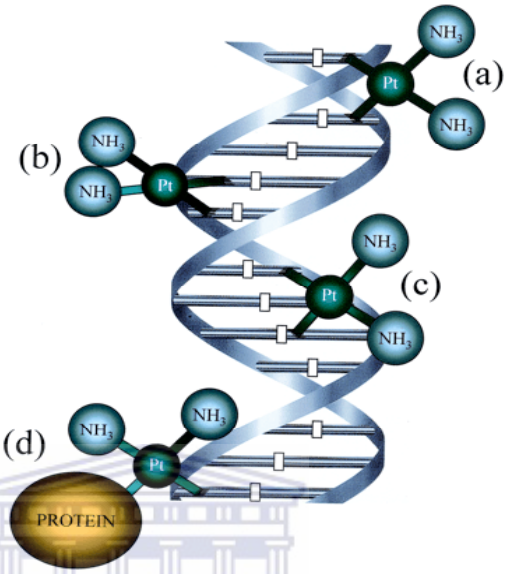


Fig. 1.17: Main adducts formed in the interaction of cisplatin with DNA (adapted from Gonzalez *et al*, 2001).(a), interstrand cross-link, (b), 1,2-intrastrand cross-link, (c), 1,3-intrastrand cross-link, (d), protein-DNA cross-link.

1.8 THE GOALS OF THIS PROJECT

The goals of this project are:

1. As a herbal medicine *Rhus laevigata* is used as an anti-inflammatory. Basing on this claim there is no scientific evidence to show that the plant contains compound (s) which can induce apoptosis. The goal of this project is therefore aimed at carrying out biological assays on the crude extracts of *Rhus laevigata* and test the extracts for compound(s) that can induce

apoptosis in selected human, mouse as well as Chinese Hamster Ovary cell line.

2. Although cisplatin is the most effective agent against most solid tumors, however most cancers develop resistance to cisplatin and many are not sensitive at all to cisplatin treatment. The goal of this project is aimed at synthesizing new platinum and palladium complexes and biologically test them for their antineoplatic activities against a panel of cell lines.

To this end specific objectives will be:

1. To screen the leaves of *Rhus laeivigata* for the presence of pro-apoptotic and anti-proliferative compound(s) (Natural products), with the potential of isolating and characterizing novel lead compound(s) which can be used in anticancer drugs discovery.
2. To screen the newly synthesized platinum and palladium based complexes for their antineoplastic activities against cancer cell lines and test the mechanisms underlying their apoptotic/biological activities with the potential of finding anticancer agents that can overcome the problems associated with cisplatin.

CHAPTER 2: MATERIALS AND METHODS

2. 1 REAGENTS

Chemical	Supplier
Chloroform	Burdick and Jackson
Dichloromethane	Burdick and Jackson
DMSO(dimethyl sulphoxide)	Sigma
Ethanol	Merck
Ethyl acetate	Burdick and Jackson
Foetal Calf Serum	Roche
Ham's F12	Invitrogen
Hexane	Burdick and Jackson
L-glutamine	Sigma
Methanol	Burdick and Jackson
Neutral red	Sigma
PBS without Ca^{2+}/Mg^{2+}	Invitrogen
Penicillin-Streptomycin	Invitrogen
Propidium Iodide	Sigma
Proteinase K	Roche
RNase	Roche
RPMI medium	Invitrogen
Staurosporine	Roche
Trypan blue	Sigma
Trypsin	Invitrogen

2.1.1. General solutions and Biochemical Assay kits

Active caspase-3-FITC assay kit---------- BD Biosciences

Annexin V assay kit---------- BD Bioscience

APO-Direct™ (TUNEL) assay kit. ---------- BD Bioscience

APOPercentage ™ apoptosis assay kit---------- Biocolor Ltd

PARP FITC Kit---------- BD Bioscience

Penicillin streptomycin 1µg/ml in culture media

TMRE (Tetramethyl Rhodamine) kit---------- Invitrogen

Trypan Blue Solution 0.4% in sterile ddH_20

Elution solution (50% EtOH/1% acetic acid)

Neutral red dye 100µg/ml in serum free media pH 6.35

PI (1mg/ml) in 3.8mM sodium citrate pH 7.0

2.2. METHODS

2.2.1. Tissue culture

A vial of frozen cultured cells indicated in table 2.1 was taken from the -150^{o}C freezer. The cells were quickly thawed in a water bath set at 37^{o} C by submerging the lower half of the vial in the water to thaw only until a small amount of ice remained in the vial. The vial was then taken to the class II safety cabinet. Using a tissue paper moistened with 70% alcohol, the outside of the vial was wiped and the lid was loosened. The cells were then tipped into tissue culture flasks containing pre-warmed appropriate culture medium (Absher, 1973) per indicated in table 2.1. The culture media was supplemented with 10% FCS and streptomycin and penicillin was added at a concentration of 1µg/ml of culture media as indicated in general stock solution section. The flasks were properly labelled with the name of cell line and date of culture and incubated at 37^{o} C, 5% CO_2 humidified incubator over night. The following day the cells were inspected under inverted microscope to check for any signs of bacterial or fungal contamination and also to see how confluent the cells were. When the cells were not contaminated and not confluent enough, media was changed and cells were incubated for a further 12 hrs. When the cells were confluent, they were washed 1x with PBS without Ca^{2+}/Mg^{2+} and trypsinized with 0.125% trypsin. Upon addition of trypsin, the cells were incubated for 5 to 10 minutes. To see if the cells were detached and floating, the cells were examined under inverted microscope. If they were not detached, the flask was gently tapped on the side until the cells were completely detached and floating. Action of trypsinization was stopped by

addition of media containing FCS. The cells were then spun down and resuspended in trypsin free media.

Apart from supplementing the media with 10% FCS and 1μg/ml penicillin and streptomycin, RPMI media used for culturing Jurkat cells was also supplemented with 2mM L-glutamine and the rest of the procedure for growing cells from frozen culture as described above was followed.

2.2.2. Cell count

Under sterile conditions, 100-200μl of the cell suspension was removed and mixed with an equal volume of Trypan Blue (dilution factor =2) and mixed by gentle pipetting. A haemocytometer counting chamber cover slip was moistened with water then slid over the chamber back and forth using slight pressure until Newton's refraction rings appeared. Newton's refraction rings are seen as rainbow like rings under the cover slip. Both sides of the chamber were properly filled with 10μl of the sample without overfilling or under filling. The cells were then viewed under a light microscope using the 10X ocular (and a 10X objective) magnification. Cells were counted in each of the 10 squares (1 mm^2 each). If over 10% of the cells were clumped, the entire sequence was repeated. If fewer than 200 or more than 500 cells were present in the 10 squares, a repeat with a more suitable dilution factor was done. Cells that touched the middle lines (of the triple lines) to the left and top of the square were counted, but those which were similarly located to the right and bottom were not counted.

2.2.3. To calculate number of cells

The hemacytometer consists of two chambers, each of which is divided into nine 1.0mm squares. A cover glass is supported 0.1 mm over these squares so that the total volume over each square is 1.0 mm x 0.1 mm or 0.1 mm^3, or 10^{-4} cm^3. Since 1 cm^3 is approximately equivalent to 1 ml, the cell concentration per ml will be the average count per square x 10^4. To calculate the number of cells per ml, and the total number of cells in the original culture: **Therefore cells/ml** = average count per square x 10^4

Total cells = cells per ml (from above) x any dilution factor x total volume of cell preparation from which the sample was taken.

NOTE: The correction factor of 10^4 converts 0.1mm^3 to 1ml (0.1mm^3 = 1mm^2 x 0.1mm).

To calculate the required number of cells per required volume.

The required number of cells was multiplied by the required volume and then divided by the original concentration of the sample. Note that this formula works better if the concentration of the original sample is higher than the required concentration.

E.g. if 10mls of cell culture was required and 2.5x10^4 cells per ml were required but the original sample contained 2.8x10^6 cells per ml:

Required volume=10mls

Required concentration=2.5x10^4 cells per ml

Original concentration=2.8x10^6

Therefore: $\frac{2.5\times10^4 \times 10\text{mls}}{2.8\times10^6} = 8.9\text{mls}$

Which means 8.9mls from the original 2.8x10^6 cells/ml will be withdrawn and made up to 10mls with media to have a concentration of 2.5x10^4cells /ml.

2.2.4. Cryo-preservation of cell lines.

Freezing of cells was carried out as follows: Cells were viewed using an inverted microscope to assess the degree of cell density and confirm the absence of bacterial and fungal contaminants. Once the cells approached the required confluency and that they were free from bacterial or fungal contamination, they were washed with PBS and then incubated for 5-10 minutes with 5ml of 0.125% trypsin if cells were grown in 25 cm^2 flask or 10mls if 75cm^2 flask was used. Trypsinization was stopped by the addition of 1ml culture medium. Cells were recovered by aspiration and transferred into a centrifuge tube. The tube was centrifuged for 2 minutes at 200xg in a bench top centrifuge. The supernatant was discarded and cell pellet was re-suspended in a solution of 90% FCS and 10% DMSO. A small aliquot of cells (100-200µl) was removed and a cell viability count performed. If the cell viability was in excess of 90% the cells were frozen, if not the cells were discarded and a new culture was grown. This was done so as to achieve a good recovery after freezing. Cryo-vials were labelled with date of freezing, name of cell line, media in which the cell line was grown and the name of the person freezing the cells. The cells were then dispensed into 1ml aliquots in the labeled cryo-vials at a concentration of $2.4x10^6$ cells per ml and were frozen at -150^0 C. Before freezing, another aliquot of 50-100 µl was removed from the tube and cultured as a quality control to assess if the vials being frozen were free from contamination. The cells for quality control were grown for 24-48hrs. If the cells were found to be contaminated, the vials were withdrawn from the freezer and discarded.

Table 2.1: Cell lines used, media and supplements used in this study.

CELL LINE	SPECIES	MEDIA	SUPPLEMENTS
Hek 293T (Renal epithelial)	Human	DMEM	Foetal Calf serum, Penicillin/Streptomycin
MG-63 (Osteogenic Sarcoma)	Human	DMEM	Foetal Calf serum, Penicillin/Streptomycin
CaSki (Cervical Cancer)	Human	DMEM	Foetal Calf serum, Penicillin/Streptomycin
CHO (Chinese Hamster Ovary)	Chinese Hamster	F-12Ham	Foetal calf serum, Penicillin/Streptomycin
HeLa (Cervical Adenocarcinoma)	Human	DMEM	Foetal calf serum, Penicillin/Streptomycin
H157 (Non small cell Lung carcinoma)	Human	RPMI	Foetal Calf serum, Penicillin/Streptomycin
MCF-7 (Breast adenocarcinoma)	Human	DMEM	Foetal Calf serum, Penicillin/Streptomycin
Jurkat (Leukemia, cells)	Human	RPMI	Foetal calf serum, Penicillin, Streptomycin, 2mM L-glutamine
3T3 (Embryonic	Mouse	DMEM	Foetal Calf serum,

2.3. STUDY METHODOLOGY

2.3.1. Preparation of plant material and crude extracts

The study methodology on the natural products was done as outlined in figure 2.1

2.3.1.1. Aqueous/Polar Extraction

Plants were collected from the nature reserve of the University of the Western Cape with the help of a botanist who helped to identify and name the plants. The plants were taken to the laboratory where the leaves of the plants were washed properly using distilled water and were divided into two parts (figure 2.2). One part was left to dry while the other part was immediately grounded to powder. To maximize extraction, the ground material was soaked in water for 24 hrs while stirring. After 24 hrs of soaking and stirring the plant/water mixture was filtered and the plant material was discarded. The water extracts were poured in 50ml tubes and then frozen over night at -80 $^{\circ}$C or were frozen using liquid nitrogen. After freezing, the plant extracts were further dehydrated with the freeze dryer. A dry powder was obtained, and weighed. The same procedure was carried out on the dried plant material and the powder obtained from the dried and fresh plant materials were mixed. To avoid the samples from absorbing moisture, the samples were kept in glass-covered bottles with desiccators. Standard concentrations were made from the powder dissolved in appropriate solvent.

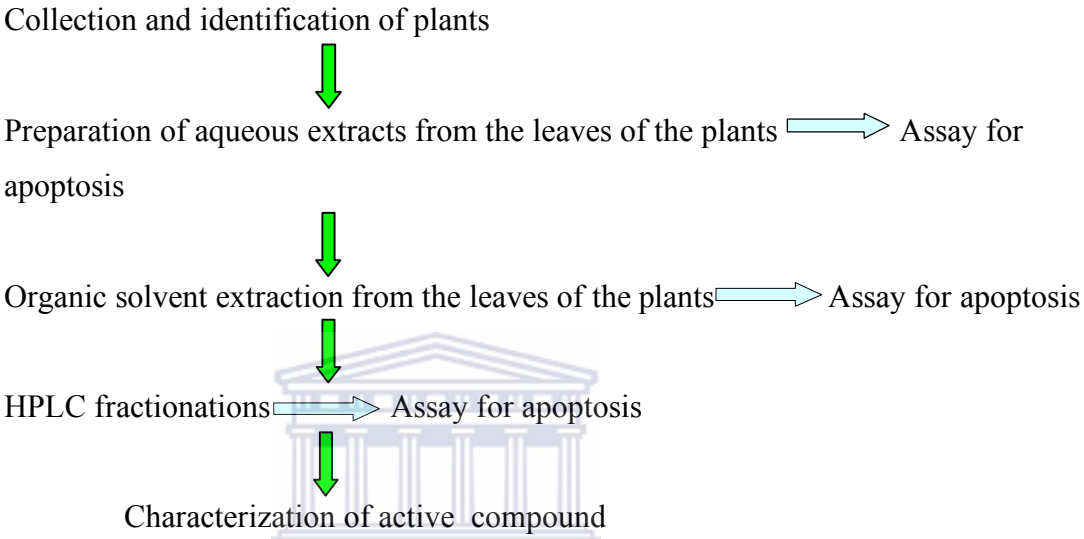


Figure 2.1: Flow chart of the methodology used in the bioassay-guided fractionation.

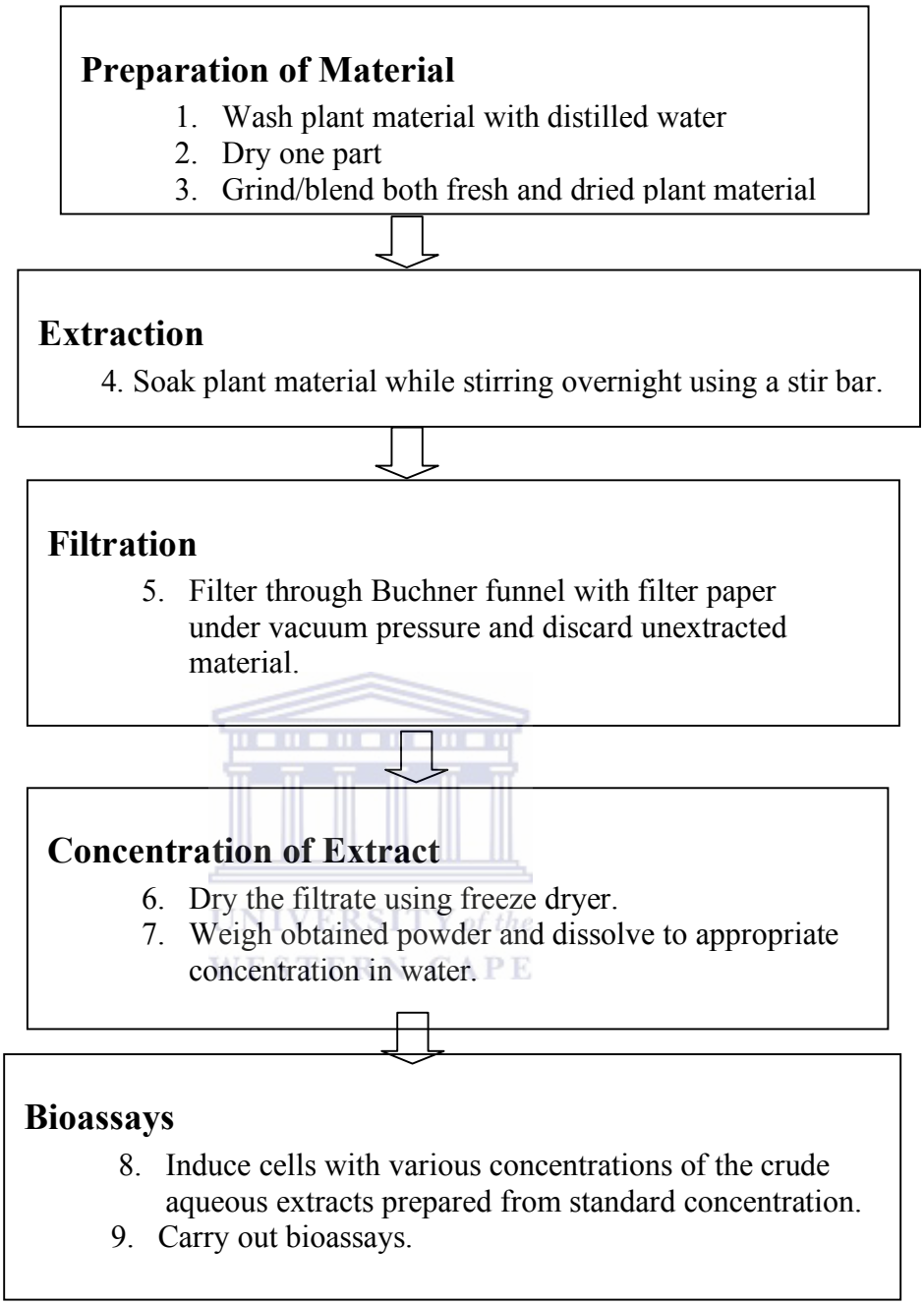


Figure 2.2: Schematic overview of the preparation of aqueous extracts from the leaves of the plants.

2.3.1.2. Organic/Non polar extraction

Organic extraction was performed as shown in figure 2.3 below.

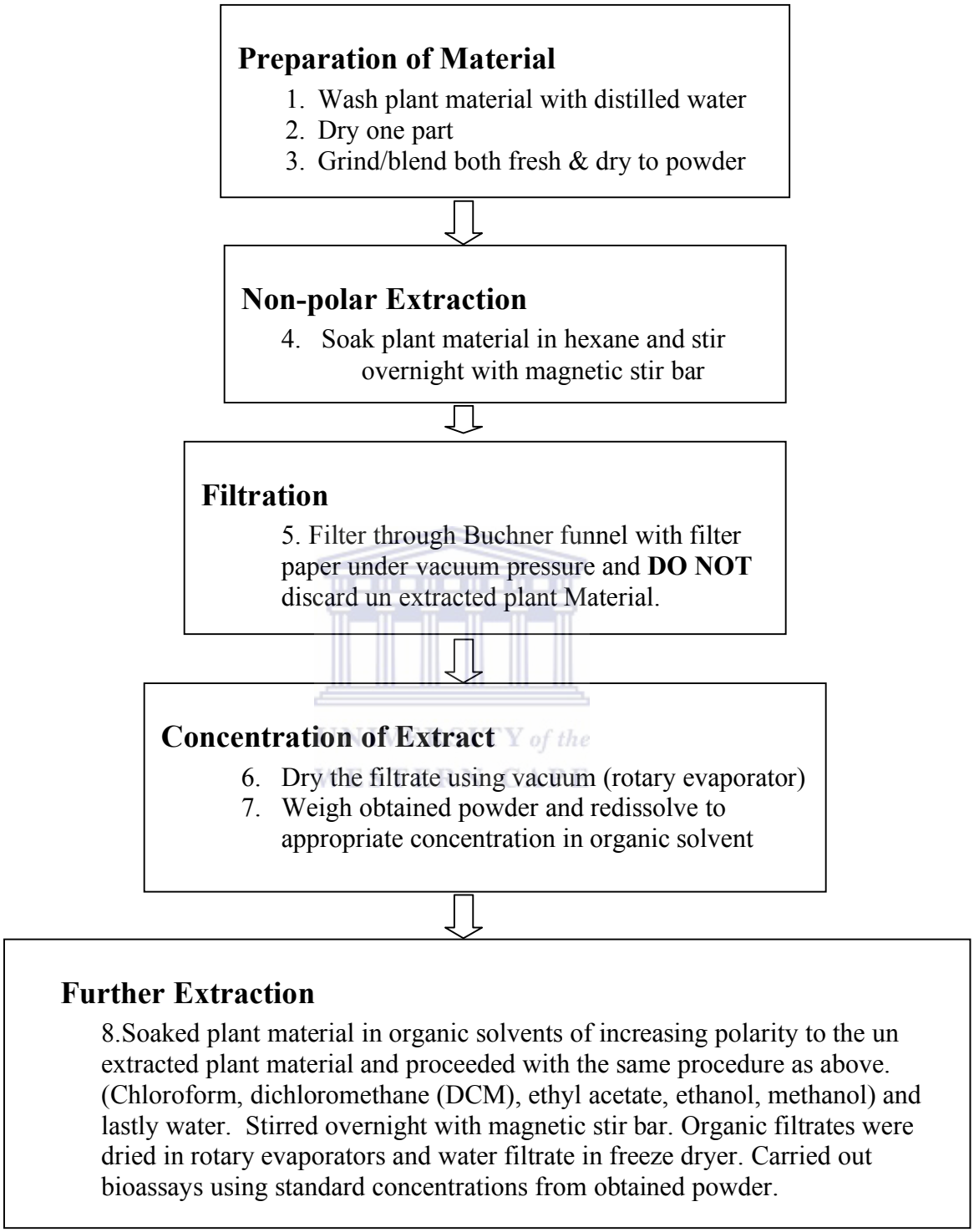


Figure. 2.3: Organic/non polar extraction protocol overview. The ground plant material was first soaked in hexane, which is used to remove non-polar high molecular weight substances/compounds

such as oils, chlorophyll etc. The mixture was stirred overnight. Following soaking, the mixture was then filtered. The procedure was repeated three times and the filtrates obtained were combined. Following the same procedure, chloroform, ethyl acetate, dichloromethane, methanol and lastly water were added in sequence to the plant material and filtered. All filtrates obtained using organic solvents were dried by vacuum dryer while the water filtrates were dried using the freeze dryer (Lyntagaye, 2005).

2.4. METALLO-COMPOUNDS (ALKYLATING AGENTS).

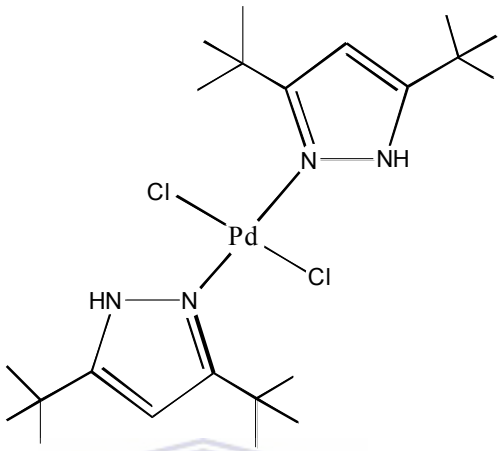
The palladium and platinum complexes used in this study were synthesized by F. Keter University of Johannesburg, Department of Chemistry who prepared the complexes following the procedures described by Darkwa *et al*., (2002) with minor changes. All commercial chemicals and other reagents, other than those described by Darkwa *et al*., (2002), were used as received and manipulations of air and/or moisture sensitive compounds were performed under dry, deoxygenated nitrogen atmosphere, using Schlenk techniques.

The following complexes (figures 2.4, 2.5, 2.6) were synthesized and were assigned numbers 15 (a palladium based complex), 57 (a palladium based complex) and 58 (a platinum based complex).

2.4.1. Dissolving metallo-compounds

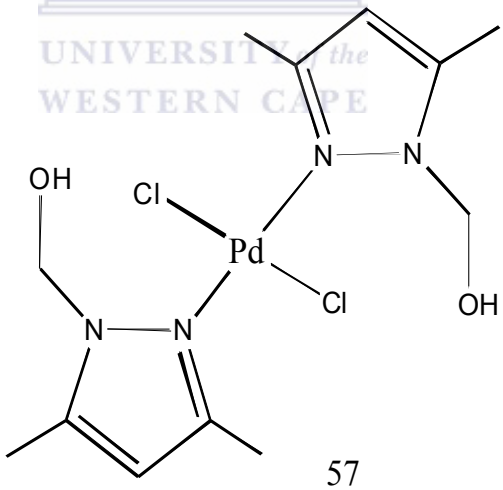
The metallo compounds ɑıɑ dot dissolve in water but were soluble in dimethyl sulfoxide (DMSO). Stock solutions were prepared in DMSO and working concentrations were

diluted in culture media at less than 0.1% DMSO final concentration (Lippard; 1987; Souktani et al., 2000; Ansar and Ansari, 2006).



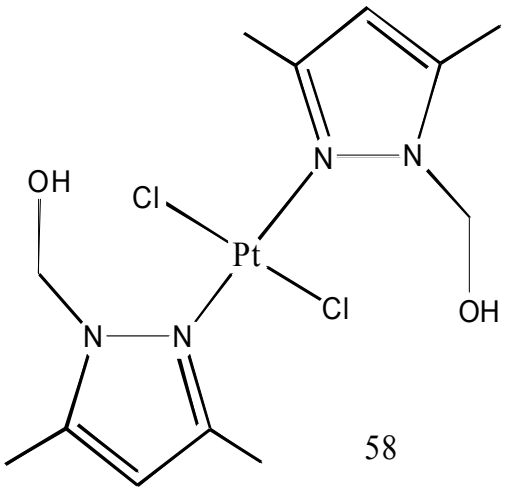
15

Figure 2.4: Synthesized complexes: Dichloro-bis (3,5-di-tert-butyl-1H-pyrazole) palladium (II), a palladium complex assigned **complex 15.**



57

Figure 2.5: Dichloro-bis(3,5-di-methyl-hydroxymethyl-pyrazole)palladium(II) assigned **complex 57.**



58

Figure 2.6: Dichloro-bis(3,5-dimethyl-1-hydroxymethyl-pyrazole)platinum(II) assigned **complex 58.**

2.5. BIOASSAYS

2.5.1. Morphological evaluation

Different cell lines shown in table 2.1 were cultured in 6 well culture plates to 90 % confluency. The cells were induced to undergo apoptosis with 0.5mg/ml and 0.25mg/ml of the crude plant methanol extract. As for the metallo-compounds 0.5mM of the metallo-compounds were used to induce the cells to undergo apoptosis while negative control cells were left without inducement. The cells were incubated for 24 hrs 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 as per recommendations by Fink and Cookson, (2005), Kroemer *et al*., (2005; 2007).

2.5.2. Measurement of cell surface modifications (externalization of PS) using APOP*ercentage*™ apoptosis assay measured by colorimeter.

Cells at a density of $2.5x10^4$ cells per ml were prepared and 100µl was added in triplicate wells of 96 well culture plates and were let to grow to 90% confluence. When the cells reached the required confluency, the culture media was removed and replaced with media containing various concentrations of crude plant extracts, which ranged from 0.5mg/ml to 0.063mg/ml. The cells were incubated for 24 hrs at 37°C in a humidified CO_2 incubator. Thereafter, the cells were gently washed off twice with 200µl of PBS and immediately replaced with APOP*ercentage*™ dye in complete culture media diluted 1:20. The cells were further incubated for 1hr at 37°C in a humidified CO_2 incubator as per manufacture's manual. After a one-hour incubation period, the cells were washed twice with 200µl of PBS then inspected under an inverted Nikon microscope. The dye trapped inside the cells was released by addition of 100µl of dye release reagent as described in APOP*ercentage*™ manual and Johnson *et al*., (2003). The plates were further incubated for ten minutes at room temperature. The bound dye recovered into solution was quantified by reading the plates with a plate reader at 560nm wavelength. The

results were plotted on linear graphs with absorbance on Y-axis and concentrations on X-axis.

2.5.3. Measurement of cell surface modifications (externalization of PS) using APOP*ercentage*™ apoptosis assay measured by Flow cytometer.

After cells were grown to 90% confluence in 6 well culture plates, they were treated with various concentrations of the plant crude extracts and metallo-compounds for 24 hrs. Staurosporine was used as a positive control for cells induced with the plant crude extracts. Positive control cells were treated with 1.0μM staurosporine for 3 hrs. The positive control cells were induced with staurosporine 3 hrs before the end of the 24 hrs of inducing the cells with the plant extracts. This was so because staurosporine induced cells were apoptotic within three hrs of induction. This was consistent with other studies (Belmokhtar *et al*., 2001 Zhang *et al*., 2004). It was within this 3 hrs time period that the flip-flop mechanism of PS occurred (Bratton *et al*., 1997) and APOP*ercentage*™ was positive. Inducing the cells with staurosporine for more than 6 hrs however, did not give good positive results. This was probably because translocation of the PS had already occurred and therefore the results appeared negative for APOP*ercentage*™. On the other hand cisplatin was used as a positive control for the metallo-compound treated cells. The cells were induced for 24 hrs with 0.5mM cisplatin. The cells were induced for 24 hrs because APOP*ercentage*™ showed good positive results at 24 hrs of induction. Hence it was used as optimal time for inducing the cells with cisplatin. This was also consistent with other studies (Choi *et al*., 2001; Jirsova *et al*., 2006;). Negative control cells were

however, left untreated for 24 hrs 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 two times with PBS, and resuspended in the residual PBS. APOP*ercentage*™ dye in complete culture media diluted 1:160 was prepared. 250µl of the dye was added to the tube and the cells were incubated for 30 minutes 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 5 minutes 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™ (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. A minimum of 10,000 cells per sample was acquired and analyzed using CELLQUEST Pro software 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* ™ positive (apoptotic/necrotic).

2.5.4. Measurement of cell surface modifications (externalization of PS) confirmed by Annexin V-PE labeling measured by FACS.

Cells were cultured at a concentration of $2.5x10^4$ cells per ml in 24 well tissue culture plates at 37°C in a humidified CO_2 incubator for 24 hrs. The cells were then induced to undergo apoptosis with various concentrations of the crude plant extract and the metallo-compounds for 24 hrs. Positive control cells for the crude plant extracts were induced to undergo apoptosis with 1.0µM staurosporine for 3 hrs while the positive control cells for the metallo-compounds were induced to undergo apoptosis with 0.5mM cisplatin for 24 hrs as previously indicated. Negative control cells were left untreated for 24 hrs. Following treatment, the culture supernatant containing apoptotic floating cells was transferred into a 15 ml tube. 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°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 trypsinization, and then the cells were transferred into 15ml tube containing the floating apoptotic cells and the tube was subjected to centrifugation. The pellet was washed twice with 1ml cold PBS and resuspended in 1x binding buffer at a concentration of ~$1.0x10^6$ cells per ml. After mixing the cells properly 100µl (~$1.0x10^5$ cells) was transferred into a 5ml FACS tube then 5µl of Annexin V-PE was added to the tube and gently vortexed. The tube was incubated for 15 minutes in the dark at RT. Five minutes before the completion of the

incubation, 5µl of 7-amino-actinomycin D (7-AAD) was added to the tube and also gently vortexed. To the mixture 400µl of 1x Annexin V binding buffer was added and the cells were acquired and analyzed on a FASCan™ (Becton Dickson) instrument equipped with a 488 nm Argon Laser as a light source within one hour as per manufacture's directions and Vermes, (1995). Acquisition was done by setting Forward scatter (FSC) and side scatter (SSC) on a log scale dot plot to differentiate normal, early apoptotic, late apoptotic cells from cellular debris. Cell fluorescence was measured by using both the FL1 channel (7-ADD) and FL2 channels (Annexin-V-PE). On a histogram dot plot, Annexin-V–PE (FL-2) was measured against relative cell numbers on a linear scale. 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.

2.5.5. Measurement of Mitochondria membrane potential (ΔΨ) using TMRE.

Cells were seeded at a density of $2.5x10^4$ cells per ml in 24 well culture plates and were incubated at 37^oC in a humidified CO_2 incubator for 24 hrs. The culture media was then removed and replaced with media containing various concentrations of methanol extract of the plant or the metallo-compounds. The cells were incubated for various time points, in a humidified CO_2 incubator. Thereafter, the cells were gently washed once with 1000µl PBS. The cells were then trypsinized with 500µl of 0.125% trypsin, and incubated for 5-10 minutes at 37^oC 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 trypsinization, and then

the cells were transferred into 15ml tubes and centrifuged for 5 minutes at 300xg. The pellet was washed twice with 1ml cold PBS and resuspended in 1x binding buffer at a concentration of ~$1.0x10^6$ cells per ml. After mixing the cells 100µl (~$1.0x10^5$ cells) of cell suspension was transferred into a 5ml FACS tube and stained with 1ml of TMRE and incubated for 30 minutes at room temperature in the dark. TMRE was reconstituted in DMSO at a stock concentration of 50mM (Jayaraman, 2005). From the stock 1.3µl was withdrawn and added to 13mls complete culture media. (1:10,000). After incubation, the cells were acquired and analyzed on a FASCan™ (Becton Dickson) instrument equipped with a 488 nm Argon Laser as a light source. Acquisition was done by setting the Forward scatter (FSC) and side scatter (SSC) on log dot plot to differentiate population of cells and cellular debris. On a linear histogram dot plot, TMRE (FL-1 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. A minimum of 10,000 cells per sample was acquired and analyzed using CELLQUEST Pro software.

2.5.6. 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 plate and were incubated at 37°C in a humidified CO_2 incubator for 24 hrs. The culture media was then removed and replaced with media containing 0.5mg/ml of crude methanol extract of the plant and 0.5mM of the metallo-compounds. The cells were incubated for 24 hrs at 37°C in a humidified CO_2 incubator. Positive control cells for the plant extract were induced with 1.0µM staurosporine for 3 hrs only since staurosporine induced the cells to

apoptosis within a short time while cisplatin induced positive control cells for the metallo-compounds were induced with 0.5mM cisplatin for 24 hrs. Negative control cells received no treatment. Thereafter, the culture supernatant containing apoptotic-floating cells was transferred into a 15 ml tube. 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 5 minutes. 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 2000xg. 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 100µl Perm/Wash™ buffer and 20µl Antibody. The cells were incubated for 30 minutes 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 and 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).

2.5.7. DNA Fragmentation detected by APO-DIRECT™

Cells were seeded at a density of $2.0x10^6$ cells per ml in 6 well culture plates and were let to grow to 80% confluence incubated at 37°C in a humidified CO_2 incubator. When the cells had reached the required confluency, the culture media was removed and replaced with media containing 0.5mg/ml of crude methanol extracts of the plant. The cells were induced with the crude extracts for 36 hrs at 37°C in a humidified CO_2 incubator. The 36 hrs time was chosen because DNA fragmentation is a late event and usually occurs at later time points occurring mostly after loss of cell viability (Collins *et al*., 1997; Pang and Geddes, 1997). Therefore it was necessary to check DNA fragmentation at a later time point and see if apoptosis occurred because at lower time points little or no DNA fragmentation occurred. Again necrosis cell death is usually an early event and can also result in DNA strand breaks. However, morphological evaluations coupled with DNA fragmentation results were necessary in aiding to know whether this was apoptosis or necrosis cell death. Positive control cells for the plant extract were induced with 1.0μM staurosporine for 6 hrs incubated at 37°C in a humidified CO_2 incubator. Positive control cells for the metallo-compounds were induced with 0.5mM cisplatin for 24 hrs 37°C and were also incubated in humidified CO_2 incubator. Negative control cells received no

treatment. Thereafter, the culture supernatant containing apoptotic-floating cells was transferred into a 15 ml tube. The adherent cells were gently washed once with 1000µl PBS, trypsinized with 2000µl of 0.125% trypsin, and incubated for 5-10 minutes at 37^{o}C in a5 % humidified CO_2 incubator. When the cells were detached, 500µl of complete culture media was added to the cells to stop the action of trypsinization, and then the cells were transferred into the 15ml tube containing the apoptotic floating cells and the tube was subjected to centrifugation for 5 minutes at 300xg. The pellet was washed twice with 1ml cold PBS and resuspended in residual PBS. To fix the cells, the cell suspension was resuspended in 5mls of 1% (W/V) paraformaldehyde in PBS and placed on ice for 15 minutes. Following the fifteen minutes incubation on ice, the cells were centrifuged for 5 minutes at 300xg and the supernatant discarded. The pellet was washed twice with PBS and resuspended in 0.5ml PBS. After which the cell suspension was added to 5mls of ice-cold 70% (v/v) ethanol in order to permeabilize the cells. For proper permeabilization, the cells were stored at –20^{0}C for 48 hrs prior to staining. After permeabilization, the cells were pelleted by centrifuging the tube for 15 minutes at 300xg and the alcohol was removed by aspirating being careful not to disturb the cell pellet. The pellet was washed twice with wash buffer as provided for in the kit. Thereafter 50µl of staining solution also provided in the kit was added to the tube and incubated at 37^{o}C for 4 hrs. At the end of the incubation time, 1.0 ml of rinse buffer was added to each tube and centrifuged for 300xg for five minutes. Supernatant was removed and discarded. This procedure was repeated then 1.0ml of PI/Rnase A (which was provided in kit) solution was added to the cell pellet and incubated for 30 minutes at room temperature. The cells were acquired and analyzed in PI/Rnase A solution 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. Two dual parameter and two single parameter displays were created. A gated standard dual parameter display was done by displaying DNA area signal on the Y-axis and the DNA width display on X-axis. Also DNA (Linear Red Fluorescence) was displayed on the X-axis and the FITC-dUTP (Log Green Fluorescence) on the Y-axis. Two single parameter–gated histograms, DNA and FITC-dUTP were displayed. 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).

2.5.8. Cellular viability using Neutral Red Uptake (NRU) assay: Non-Adherent Cells (NAC).

Cells were seeded in 24 well culture plates at a concentration of 2.4×10^5 cells/ml and incubated overnight at 37^{o}C in a humidified CO_2 incubator. The cells were then treated with increasing concentrations of the samples for 24 hrs while negative control cells were left untreated also for 24 hrs following which the cells were microscopically assessed for morphological alterations. Since the cells were non adherent, they were transferred to 15ml tubes and spun down at 300xg for 5minutes. Media containing the test compounds was aspirated and then cells washed twice with Phosphate Buffered Saline (PBS). Neutral red dye was prepared as described in section 2.1.1. Following dye preparation 1ml of the dye was added to each tube and cells were incubated for 2-3 hrs at 37°C in 5% humidified incubator. The cells were then washed twice with PBS and spun down to a pellet. The

supernatant was decanted completely. Then 350µl of elution solution prepared as indicated in section 2.1.1 was added to each tube. The tubes were shaken to solubilize the dye. 100µl from each tube was transferred into triplicate wells of a 96 well culture plate. Using a microplate reader, sample absorbance was read at 540nm. To exclude background reading, three wells were seeded with untreated cells which were not stained with the dye while three other wells were seeded with untreated cells but were later stained with the dye. Results were expressed as a percentage of the untreated controls. Each sample was tested three times. On a linear scale graph, percentage absorbance values of each sample were plotted on Y-axis against concentrations on the X-axis. IC_{50} of each sample was determined from the graphs and averages calculated. Standard error of the mean of the three independent experiments were further calculated (Babich and Borenfreund, 1991; Rixe *et al*., 1996; Fotakis and Timbrell, 2005).

2.5.9. Cellular viability using Neutral Red Uptake (NRU) assay: Adherent cells.

Cells were prepared at a concentration of $2.4x10^5$ in a 50 ml centrifuge tube from which 100µl was withdrawn and transferred into triplicate wells of 96-well plates. The cells were left to form a sub-confluent monolayer. As usual before commencement of the experiment, the cells were inspected under inverted microscope for confluency and any possible contaminations. Cells in triplicate wells were then induced to undergo apoptosis with increasing concentrations of the test compounds and negative control were left untreated for 24 hrs at 37°C in 5% humidified incubator. Following incubation, all wells were washed twice with PBS to remove the test compounds. Then 100µl of Neutral Red

dye in serum free media as indicated in section 2.1.1 was added to each well and plates were incubated for 2-3 hrs at 37^{o}C. Thereafter the cells were washed twice with PBS, and then evaluated microscopically for morphological alterations and dye uptake. PBS was then completely decanted from all the wells by gently blotting the plates over tissue paper then100µl of elution solution was added to each well. The plates were shaken on a shaker for 10 minutes to release the dye. The absorbance values were read at 540nm with a microplate reader. To exclude background reading, three wells were seeded with negative cell which were not stained with the dye while three other wells were seeded with negative cells which were stained with the dye. Results were expressed as a percentage of the untreated controls. Each sample was tested three times. On a linear scale graph percentage absorbance values of each sample were plotted on Y-axis against concentrations on the X-axis. IC_{50} of each sample was determined from the graphs and averages calculated. Standard error of the mean of the three independent experiments were further calculated (Rixe *et al*., 1996; Fotakis and Timbrell, 2005).

2.5.10. Cell cycle analysis

The ethanol-based fixation technique offers an excellent CV for DNA analysis. (Darzynkiewicz, 1990). It is one of the methods used to detect DNA fragmentation. Using cell cycle analysis HeLa cells were tested for DNA fragmentation. HeLa cells were seeded in 6 well plates at $2.0x10^4$ cells per ml and were grown to 90% confluence. Upon inspection and observance that the cells were free from contamination and that they had reached the required confluency, they were treated with 0.5mM of complexes 15

(palladium based complex), and 58 (platinum based complex) and incubated for 3hrs, 6hrs, 12hrs, 24hrs and 36hrs at 37°C in 5% humidified incubator. At the end of each time point, apoptotic floating cells were transferred into a 15ml centrifuge tube and the adherent cells were trypsinized and transferred to the same tube containing floating apoptotic cells. The cells were washed twice with PBS and centrifuged to a pellet. Thereafter the pellet was resuspended in 200µl of PBS and then fixed by the addition of cold 70% ethanol drop wise to avoid cell aggregation. Then the samples were left at -20^{0}C for 48hrs. After that, the samples were centrifuge at 250xg for 10 min at 4^{o}C and washed twice with cold PBS. 250 µl of 500 units/ml Rnase A in PBS with 1.12% sodium citrate was added to the cell pellet. Vortexed gently and incubated at 37^{o}C for 30 minutes. Samples were then stained with 100 µg/mL PI solution. Final PI concentration in the test mixture was 50 µg/mL. The samples were incubated at room temperature in the dark for at least 30 minutes then acquired and analyzed on a Flow cytometer (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 discriminate cell populations and cell debris. Two dual parameter and two single parameter displays were created. A gated standard dual parameter display was done by displaying DNA peak signal on the Y-axis and the DNA width displayed on X-axis dot plot while DNA content was displayed on linear histogram dot plot. 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).

2.5.11. PARP-1 cleavage.

Cells were cultured at a concentration of $2.5x10^4$ cells per ml for 24 hrs in 6 well tissue culture plates at 37°C in 5% humidified incubator. The cells were then treated with the crude methanol plant extract while negative control cells were left untreated and cultured for 24 hrs at 37°C in 5% humidified incubator. Positive control cells were treated with 1.0µM staurosporine for 4 hrs. Caspases are one of the major initiators of the apoptosis pathway components followed by the cleavage of their substrates. As reviewed in section 1.5.2, PARP-1, is mostly involved in DNA repair, stability and other cellular events, and is cleaved by members of the caspase family during early apoptosis. PARP being a DNA-binding protein specifically distinguishes DNA strand breaks, which are produced by genotoxic agents. PARP-1 measurement can be said to be one of the hallmark of apoptosis as it indicates that caspases are activated. If PARP-1 signal can be sustained, it can be measured even in late stages of apoptosis (Tong *et al*., 2001; Süsse *et al*., 2004; Riedl and Shi, 2004). The 4 hrs time period for inducing the positive control cells was chosen as an optimal time to see if members of the caspase family activated PARP-1 early in the apoptosis pathway (an early marker of apoptosis). Upon treatment, cells were washed twice with PBS then fixed with 500µl Cytofix/cytoperm for 30 minutes at room temperature. Thereafter the cells were spun down and cytofix/cytoperm aspirated and the pellet was further resuspended in 250µl of cytofix/cytoperm (Gobeil *et al* 2001). The cells were then stained with 2µl PARP-FITC for 1 hr in the dark at room temperature. At the end of incubation, the cells were washed twice with Perm/Wash buffer, resuspended in 400µl Perm/Wash and analyzed by Flow Cytometry by setting the Forward (FSC) and Side

Scatter (SSC) on a dot plot to discriminate cell populations and cell debris. On a log histogram dot plot, FL3 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. Aminimum of 10,000 cells per sample was acquired and analyzed using CELLQUEST PRO Software (BD Biosciences).

CHAPTER THREE: SCREENING OF EXTRACTS FROM THE LEAVES OF *RHUS LAEVIGATA*

3.1 Screening of various plant extracts for their pro-apoptotic and anti-proliferative activities using CHO cells.

3.1.1 Screening of the methanol extract using CHO cells: Colorimetric

3.2 Morphological evaluation and measurement of apoptosis in CHO cells induced with crude methanol plant extract and methanol using APOP*ercentag*e™ apoptosis assay (methanol control experiment).

3.3 Screening of methanol extract: Annexin V

3.4 Screening of methanol extract: APOP*ercentage* ™

3.5 Caspase-3 activation assay.

3.6 PARP-Cleavage

3.7 Mitochondrial depolarization

3.8 DNA fragmentation by APO-DIRECT™

3.9 Cellular viability

3.10 Morphological evaluation of various induced cells

3.1 Summary

3.1 Screening of various plant aqueous extracts for their pro-apoptotic and anti-proliferative activities.

For screening purposes CHO cells were used as model. Aqueous extracts of various plants with various concentrations were used to induce apoptosis in CHO cells. APOP*ercentage*™ apoptosis assay was therefore employed to measure dye uptake by the cells after treating them for 24 hrs. This was done in order to identify the extracts that contained pro-apoptotic and or anti-proliferative compounds so that they could further be evaluated and discard those which did not induce apoptosis.

In this assay, APOP*ercentage*™ apoptosis assay was used to stain the cells that were undergoing apoptosis. The assay's mechanism of action is that it uses a dye which enters the cells following phosphatidylserine transmembrane movement ('flip-flop' mechanism). Following this event, the dye uptake continues until blebbing of the apoptotic committed cells occur. No further dye can then enter the dead cells and the dye that has accumulated within the cells is not released. The trapped dye can however, be eluted and quantified spectrophotometrically. The amount of dye quantified is proportional to the number of cells with trapped dye i.e. the higher the absorbance readings of the eluted dye, the greater the number of cells which had the trapped dye. Flow cytometer can also be used to measure the cells with trapped dye.

Following treatment of the cells with the aqueous extracts (figure 3.1) the results showed that the crude aqueous extracts from *Rhus laevigata* induced apoptosis more efficiently than the other plant aqueous extracts. The other crude aqueous extracts induced apoptosis at high concentrations i.e. from 2.5mg/ml to 20mg/ml. Aqueous extracts from *C. monilifera* did not induce apoptosis in CHO cells even at the highest concentration of 20 mg/ml. Aqueous extracts from *Rhus laevigata* however, showed that even at lower concentration (0.65mg/ml), the cells became apoptotic. As the interest was to find compound(s), which can induce cells to undergo apoptosis at low concentration therefore the extracts from *Rhus laevigata* were chosen for further evaluation.

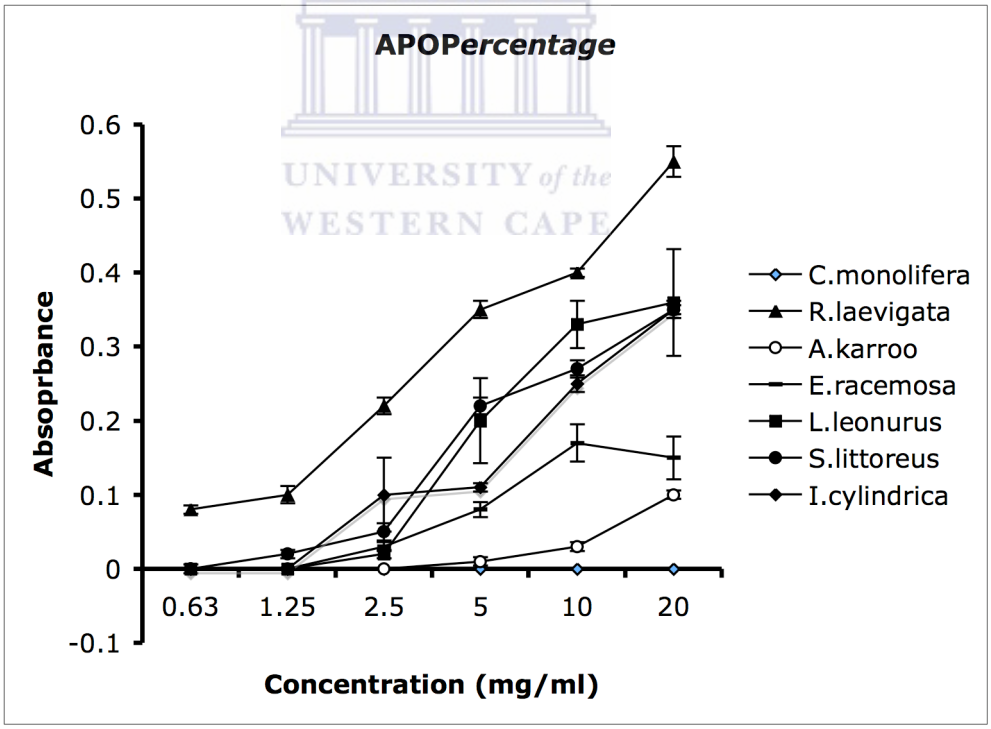


Figure 3.1: Screening of crude aqueous extracts for apoptotic compounds from the leaves of various plants measured by APOP*ercentage*™ dye apoptosis assay.

Aqueous extracts were prepared as indicated in section 2.3.1 and also sub section 2.3.1.1 from the leaves of the following plants: *Chrysanthemoides monilifera*, *Rhus laevigata*, *Acacia karroo*, *Euclea racemosa*, *Leonatis leonurus*, *Senecio littoreus* and *Imperata cylindrica*. From the stock of each plant aqueous extract, concentrations were prepared ranging from 20mg/ml to 0.625 mg/ml as shown and cells were treated for 24 hrs incubated at 37^{o}C in humidified CO2 incubator. The rest of the procedure was as described in section 2.5.3.

3.1.1 Induction of apoptosis in CHO cells using organic and aqueous extracts of *Rhus laevigata* measured by APOP*ercentage*™ apoptosis assay.

Following screening of the various plants for apoptosis induction, leaves of *Rhus laevigata* were further extracted with organic solvents of increasing polarities as described in section 2.3.1.2. The extracts from the organic and aqueous solvents were tested against CHO cells in order to determine the solvent extract that contained the active compound.

The results (figure 3.2) indicate that most of the apoptosis inducing compound(s) were concentrated in the methanol extract followed by the water extract. The other organic extracts had almost no effect on the cells indicating that the apoptosis inducing compound(s) were not contained in them. In order to confirm that the extracts were inducing apoptosis other than necrosis, Annexin V assay was used to discriminate apoptotic cells from necrotic cells.

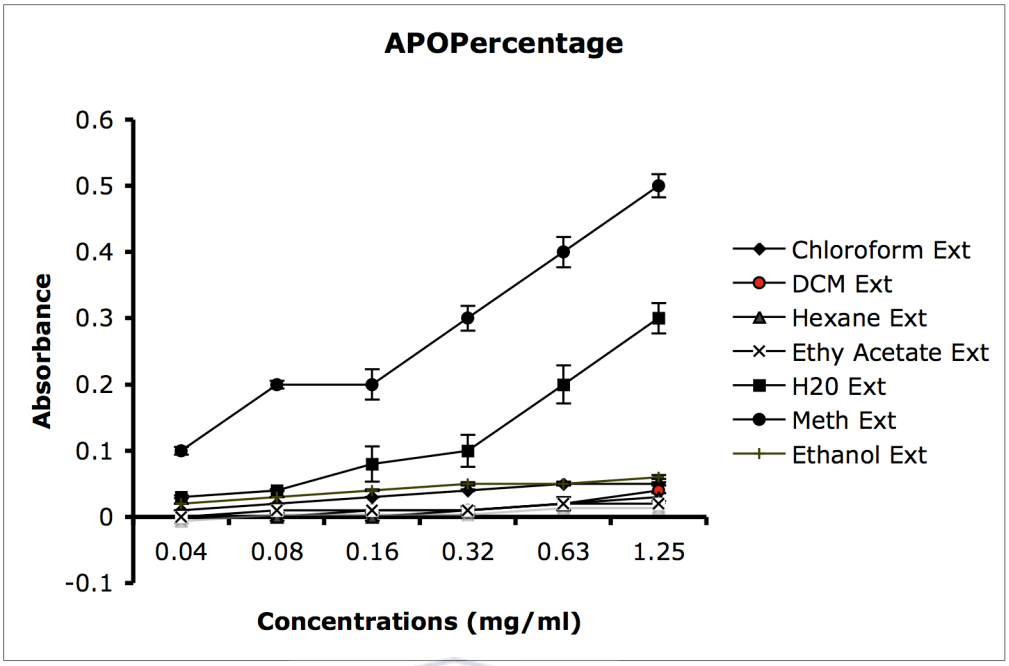


Figure 3.2: Screening of aqueous and organic extracts from the leaves of *Rhus laevigata* tested against CHO cells. CHO cells were induced for 24 hrs with various organic and aqueous extracts from *Rhus laevigata*.

3.2 Morphological evaluation of apoptosis in CHO cells induced with methanol and crude plant methanol extract assessed by APOP*ercentage*™ apoptosis assay (methanol control experiment).

Screening for apoptosis induction was carried out using CHO cells as model cells for subsequent experiments. CHO cells were cultured in 6 well culture plates at 2.5 x 10^5 cells per ml and were allowed to grow to 90% confluency. Two wells were induced with 1% methanol in culture media (20µl methanol in 2mls culture media).

Two other wells were induced with the crude plant methanol extract at a concentration of 0.5mg/ml in which the methanol concentration was less than 1%. The remaining two other wells were left untreated. The cells were then incubated for 24 hrs at 37^{o}C in a humidified CO_2 incubator. Following incubation, cell morphology was evaluated using an inverted Nikon light microscope and pictures were taken using a Leica EC3 digital color camera. Then media from one of each of the wells containing un induced cells, induced cells with methanol and cells induced with crude plant methanol extract was removed and replaced with media containing 1:20 APOP*ercentag*e™ apoptosis dye in culture media and the plate was incubated for 1 hr at 37^{o}C in a humidified CO_2 incubator. Following incubation, the cells were washed twice with PBS and immediately examined under inverted Nikon microscope and pictures were again taken using a Leica EC3 digital camera. From the other remaining wells, 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 then washed twice with PBS, spun down and resuspended in the residual PBS. APOP*ercentage*™ dye in complete culture media diluted 1:160 was prepared. Thereafter, 250µl of the dye was added to the tube and the cells were incubated for 30 minutes at 37^{o}C in a humidified CO_2 incubator. After the incubation period, the cells were spun down and supernatant discarded. The pellet was washed 1x with PBS after which the pellet was resuspended in 400µl of PBS and the cells were acquired and analyzed on a flow

cytometer (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. A minimum of 10,000 cells per sample was acquired and analyzed using CELLQUEST Pro software.

This assay was done as a quality control to assess whether apoptosis induction by the crude plant methanol extract was due to potent compound(s) contained in the crude plant extract and not due to the methanol which was used for extraction. The results (figure3.2) showed that methanol at 1% or less did not by itself induce apoptosis. The results showed that less than 10% of the untreated CHO cells and methanol treated CHO cells took up APOP*ercentage*™ apoptosis dye while 68% of CHO cells induced with the crude plant methanol extract took up the APOP*ercentage*™ apoptosis dye indicating that the methanol extract induced apoptosis in CHO cells. The morphology of CHO cells treated with 1% methanol was unchanged after 24 hrs of induction while the morphology of CHO cells induced with crude plant methanol extract was completely changed following 24 hrs of induction. The cells had rounded off and had lost contact with each another. It was therefore obvious that the apoptotic morphology observed in cells induced with the

crude plant methanol extract was as a result of the potent compound(s) contained in the plant extract, and not due to methanol.

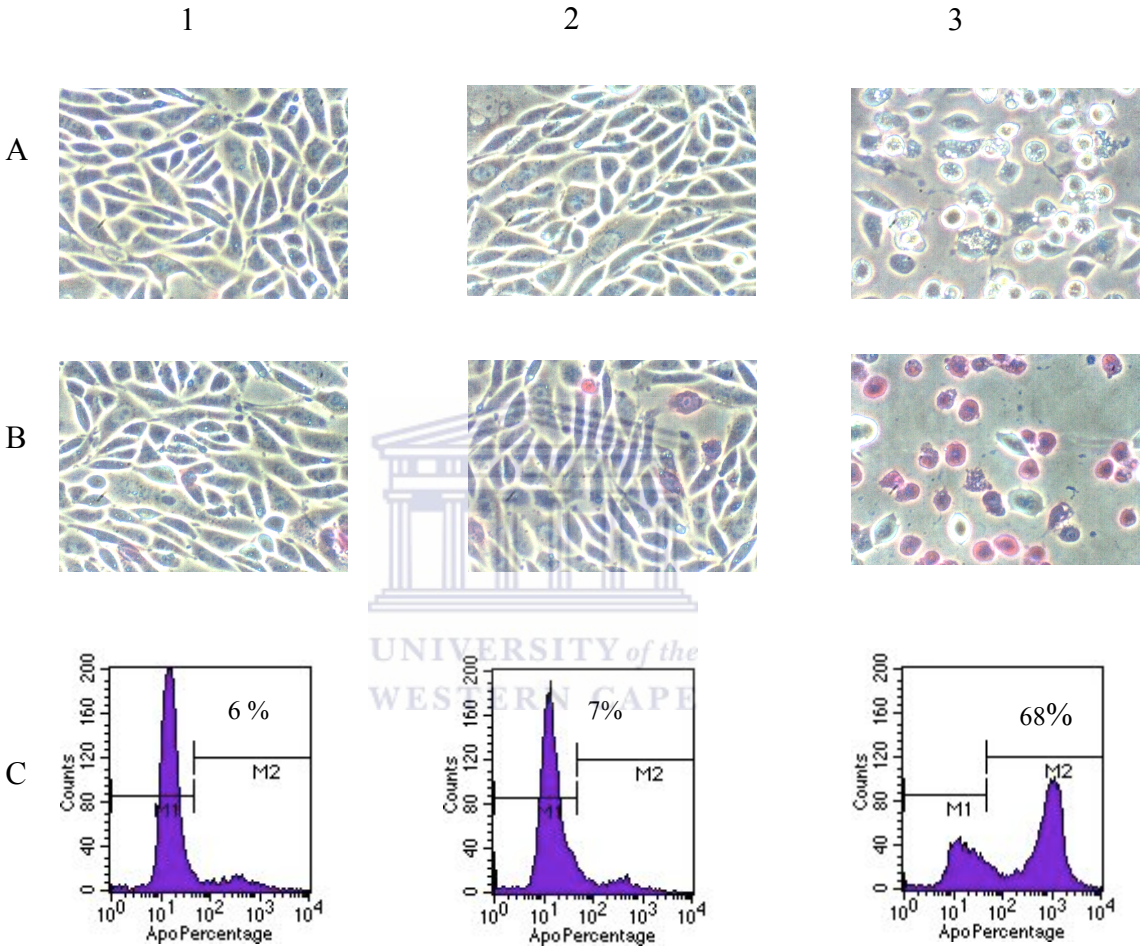


Figure 3.3: Methanol control experiment. The procedure described in section 2.5.1, was followed. Panel 1 (A) shows the morphology of un induced CHO cells not stained with APOP*ercentage*™ apoptosis dye. Panel 1 (B) shows un induced CHO cells but stained with APOP*ercentage*™ apoptosis dye while panel 1 (C) shows the same un induced CHO cells stained with APOP*ercentage*™ apoptosis dye analysed by Flow cytometer. Panel 2 (A) shows the morphology of CHO cells induced with 1% methanol and not stained with APOP*ercentage*™ apoptosis dye. Panel 2 (B) shows CHO cells induced with 1% methanol and stained with

APOP*ercentage*™ apoptosis dye. Panel 2 (C) shows CHO cells induced with 1% methanol stained with APOP*ercentage*™ apoptosis dye analysed by Flow cytometer. Panel 3 (A) shows the morphology of CHO cells induced with crude plant methanol extract and not stained with APOP*ercentage*™ apoptosis dye while panel 3 (B) shows CHO cells induced with crude plant methanol extract stained with APOP*ercentage*™ apoptosis dye and panel 3 (C) shows CHO cells induced with crude methanol plant extract stained with APOP*ercentage*™ apoptosis dye and analysed by Flow cytometer.

3.3. Induction of apoptosis in CHO cells using the crude plant methanol extract measured by Annexin V PE binding assay.

The crude plant methanol extract was further tested against CHO and measured with Annexin V assay to confirm whether the positive results obtained by the APOP*ercentage*™ apoptosis assays were as a result of apoptotic cell death and not necrotic cell death. Annexin V assay was used as a confirmatory assay to the APOP*ercentage*™ apoptosis assays because Annexin V is a Ca^{2+} dependent phospholipid-binding protein with high affinity for PS and can be used to distinguish apoptotic cells from necrotic cells when used in combination with 7-amino-actinomycin D (7-AAD) or propidium iodide. Translocation of PS to the external cell surface is unique to apoptosis however, it also occurs during cell necrosis hence the need to discriminate the two. The difference between these two forms of cell death is that during the initial stages of apoptosis the cell membrane remains intact, while at the very stage that necrosis occurs the cell membrane looses its integrity and becomes leaky. Therefore the measurement of Annexin V binding to the cell surface was performed in conjunction with an exclusion dye to establish integrity of the cell membrane (Wising *et al*., 2005). Apoptosis and necrosis were therefore estimated after double-staining the cells with

phycoerythrin (PE)-labelled annexin V (BD Biosciences, San Diego, CA, USA) and 7-AAD and analysed using flow cytometry. Live cells with intact membranes were distinguished by their ability to exclude 7-AAD which readily penetrates dead or damaged cells. Dual analysis was introduced using a quadrant dot plot, in which 'necrotic cells' were identified as 7-AAD-positive while 'early apoptotic cells' were annexin V-PE-positive only, and 'late apoptotic cells' were recognized as double-positive for annexin V-PE and 7-AAD. Cells that stained negative for both annexin V-PE and 7-AAD were classified as live cells. (Wising *et al*., 2005).

In this assay CHO cells were treated with the methanol extract with concentrations ranging from 0.5mg/ml to 0.063mg/ml. The procedure was as described in chapter two (section 2.5.4).

The results (figure 3.5) showed that the untreated control cells were primarily negative for both Annexin V PE and 7-AAD while the staurosporine treated cells (positive control) were 17% positive for 7-AAD indicating that 17 % of the cells were in their late stages of apoptosis and 15 % were positive for Annexin V. (In total 32 % of the cells were apoptotic). The methanol extract treated cells showed that the extract induced apoptosis in the CHO cells in a dose dependent manner. At 0.063mg/ml, 6% of the cells were in their early stages of apoptosis (Annexin V PE Positive and 7-AAD negative), while 13% were in their late stages of apoptosis. It can be said that 19% of the cells were positive for both Annexin V PE and 7-AAD (apoptotic).

There was no dramatic increase in apoptotic cells induced with an increase in concentration of the plant extract from 0.063mg/ml to 0.125mg/ml. There was however, a big increase in the proportion of apoptotic cells when the concentration was increased from 0.125mg/ml to 0.5mg/ml. At 0.125mg/ml 5% of the cells were positive for Annexin V and 15% were positive for 7-AAD (20% of the cells were apoptotic). At 0.5mg/ml the cells were 20% positive for Annexin V while 22% were positive for 7-AAD (42% of the cells were apoptotic). The picture however, could be different in other cell lines since other cell lines may respond differently. These results indicate that the extracts from *Rhus laevigata* contain pro-apoptotic compound(s), which require further investigations. These results also confirmed that the APOP*ercentage*™ results were positive, as Annexin V is a sensitive assay and can also be used to discriminate apoptotic and necrotic cells.

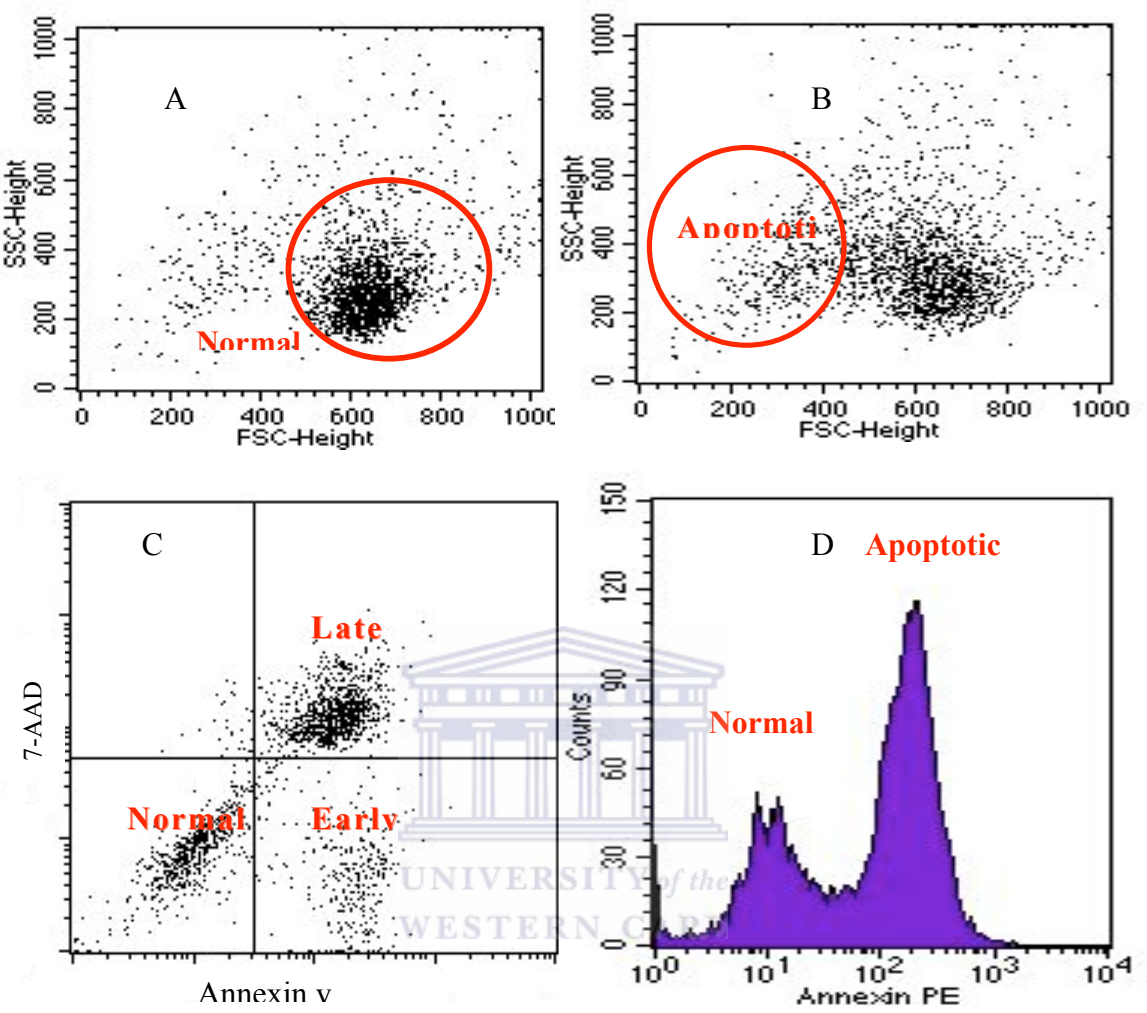


Figure 3.4 An illustration of the principle behind Flow cytometry and how it is used in cell necrobiology and shows flow cytometrically the analysis of Annexin V. (A) FSC normal cells, (B) FSC apoptotic cells, (C) Dot plot Annexin V PE stained cells showing normal, early and late apoptotic cells and (D) Histogram dot plot showing normal and apoptotic cells. When a cell passes through a laser beam in a flow cytometer, it generates light scatter. Forward Side Scatter (FSC) provides information about cell size, while Side scatter (SSC) provides information about the cell's morphological complexity. When a cell dies, its morphology changes, and changes in light scatter may reflect these phenomena.

Necrosis: As shown in figure 3.4, when a cell dies by necrosis, both FSC and SSC tend to increase, likely as a consequence of cell swelling. However, as a consequence of plasma membrane damage and leakage of cell constituents, both FSC and SSC rapidly decrease. (Lower FSC and SSC values), while normal cells have higher FSC and SSC values.

Apoptosis: when a cell dies by apoptosis, its major morphological changes take place in the nucleus. Only in the late apoptotic stages the cytoplasm and the plasma membrane are seriously damaged.

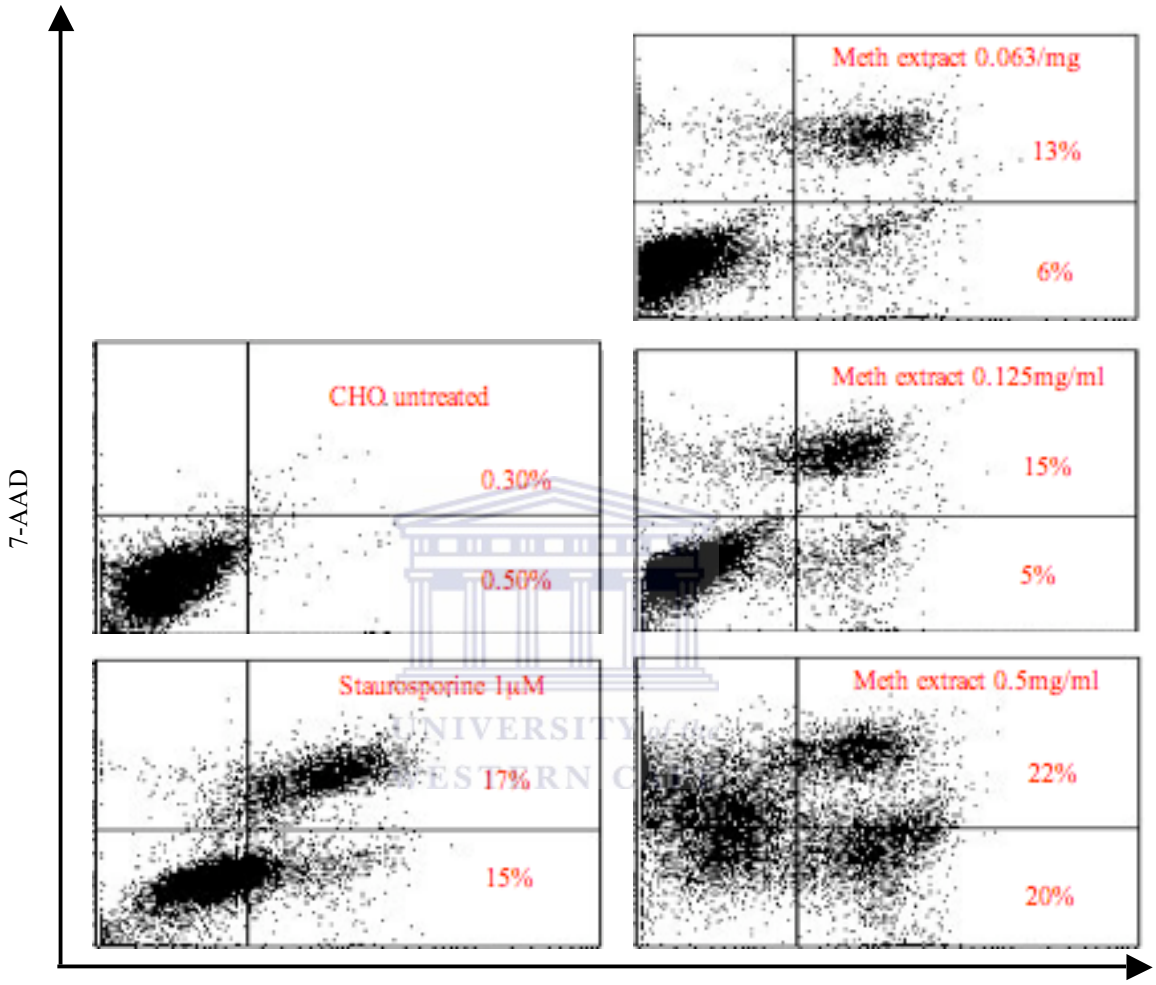


Figure 3.5: CHO cells treated with the methanol extract were tested for Annexin V and were acquired and analysed by flow cytometer.

3.4 Induction of apoptosis on different cell lines using the plant methanol extract.

The crude plant methanol extract was further tested on human cancer cell lines. As different cell lines may respond differently to apoptosis induction the plant methanol extract at 0.5mg/ml was used to induce MCF, HeLa and Caski human cancer cell lines for 24 hrs. The procedure described in section 2.5.3 was followed. APOP*ercentage*™ assay was used as a screening assay to assess apoptosis and measurement was done by Flow cytometer.

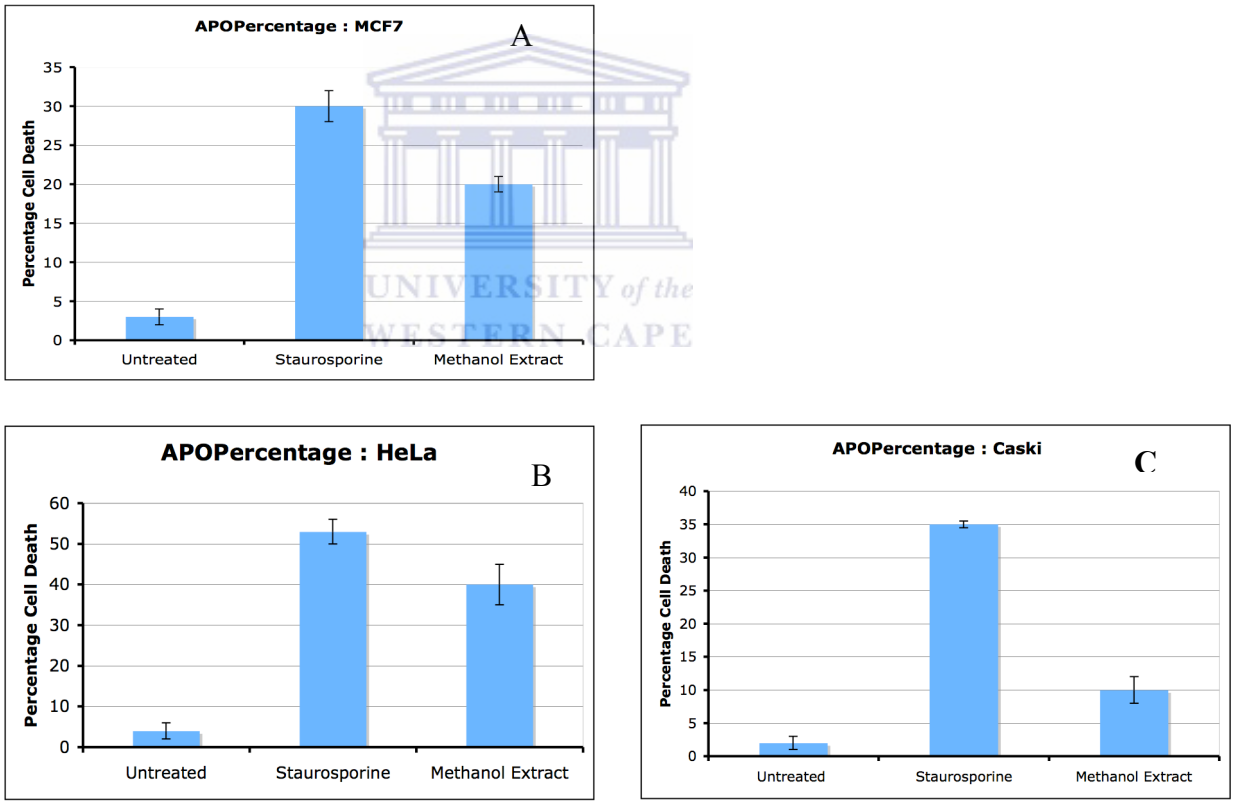


Figure 3.6: Different cells lines treated with methanol extract stained with APOP*ercentage*™ and analysed by flow cytometer. (A) MCF-7 induced cells (B) HeLa induced cells (C) Caski induced cells.

Following induction of apoptosis with the plant methanol extract for 24 hrs, the results (figure 3.6) showed that MCF-7 cells (figure 3.6 A) were 20% positive for APOP*ercentage*™ whereas HeLa cells (figure 3.6B) were 40% positive for APOP*ercentage*™ and Caski (C) were resistant exhibiting only 10% positive for APOP*ercentage*™. The results showed that the pro-apoptotic compounds contained in the plant extract were able to induce apoptosis even in some human cancer cell lines.

3.5 **Caspase-3 activation assay**

CHO cells were further tested for the activation of caspase-3, which is a key protease, implicated during the early stages of apoptosis as reviewed by (Kaufmann *et al.,* 1999; Riedl and Shi, 2004). Its commencement triggers the degradation phase and is the hallmark of irreversible cell death of apoptosis. The cells were treated with 0.5mg/ml of the methanol extract and rest of the procedure described in section 2.5.6 was followed.

The results (figure 3.7) showed that the untreated cells were primarily negative for caspase-3 whereas those induced with staurosporine were 30% positive for caspase-3 and those cells induced with the plant methanol extract were 25% positive for caspase-3. The results showed that CHO cells induced with the plant methanol extract contained compounds which activated caspase-3 activity which is implicated in the early stages of apoptosis and which also proteolytically cleaves and activates other caspases as well as appropriate targets in the cytoplasm e.g., guanine nucleotide dissociation inhibitors (D4-

GD1) and Bcl-2, and in the nucleus, e.g., PARP. It is therefore a good indicator/marker of cells undergoing apoptosis.

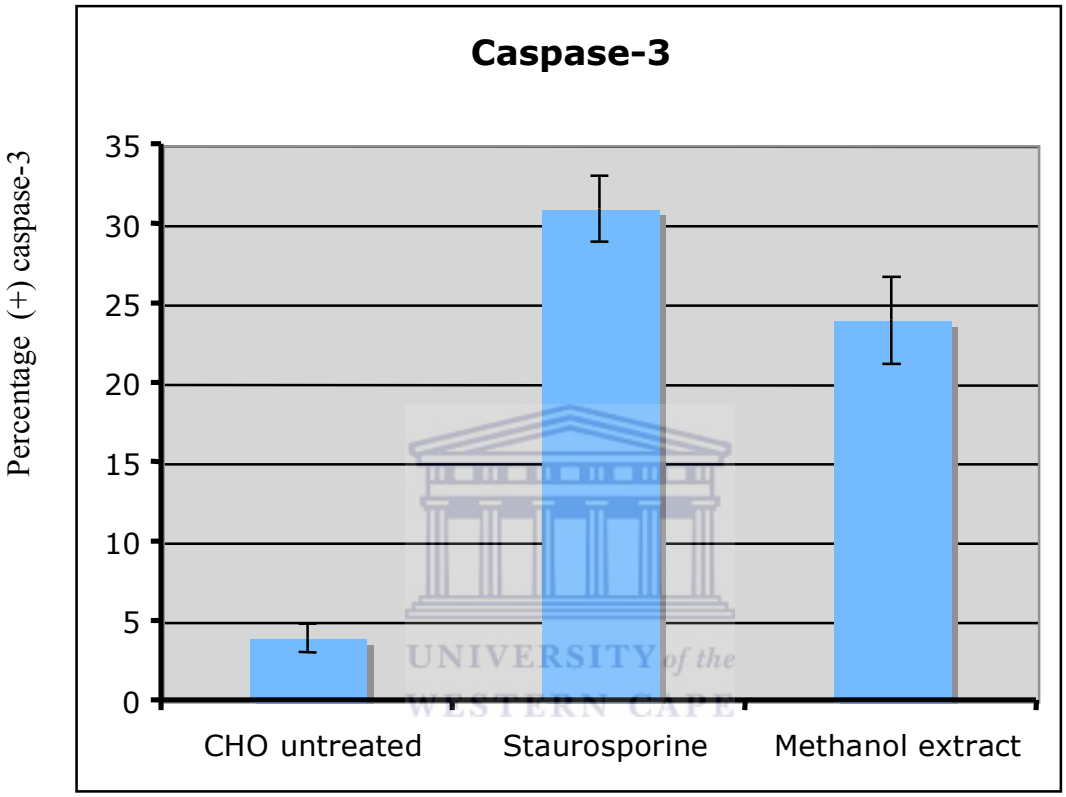


Figure 3.7: Detection of anti-active caspase-3 antibodies following treatment of CHO cells with the crude methanol extract and staurosporine.

3.6 PARP-1 Cleavage

As reviewed in section 1.5.3 PARP-1 plays a critical role in the base excision repair (BER). PARP-1 being a nuclear enzyme is rapidly activated following DNA strand breaks, which finally leads to the modulation of various protein activities in DNA replication, DNA repair and checkpoint control (Beneke *et al.*, 2000; Süsse *et al.*, 2004;

Plummer, 2006). It was therefore necessary to test whether caspase-3 actually activated PARP-1. In this assay, CHO cells were also used as a model for testing PARP activation upon inducing the cells with the plant methanol extract. HeLa cells were used to see if the same phenomenon which can be observed in CHO cells could also be observed in a human cancer cell line such as HeLa when induced with the plant methanol extract. In this assay CHO and HeLa cells were induced with 0.5mg/ml of the plant methanol extract for 24 hrs while positive control HeLa and CHO cells were induced with 1 μM staurosporine for 4 hrs (Zhang *et al*., 2004; Sordet *et al*., 2004). The cells were then fixed and permeabilized for 30 minutes at room temperature and the rest of the procedure was as described in section 2.5.11.

The results (figure 3.8) showed that the untreated HeLa cells were 1% positive for active-PARP-FITC whereas staurosporine treated HeLa cells were 7% positive for active PARP-FITC and HeLa cells treated with 0.25mg/ml methanol extract were 25 % positive for active PARP-FITC and those which were treated with 0.5mg/ml were 60% positive for active PARP-FITC. On the other hand, untreated CHO cells were 2% positive for active PARP-FITC while staurosporine treated CHO cells were 40% positive for active PARP-FITC and CHO cells treated with 0.25mg/ml of the methanol extract were 55% positive for active-PARP-FITC and those treated with 0.5mg/ml were 95% positive for active-PARP-FITC.

The results further indicated that the plant methanol extract caused DNA strand breaks in both HeLa and CHO cells thereby activating PARP. The activation of PARP was due in

part to repair the broken DNA strands. The results were therefore a good indicator that the plant methanol extract contains compound(s) that induce apoptosis.

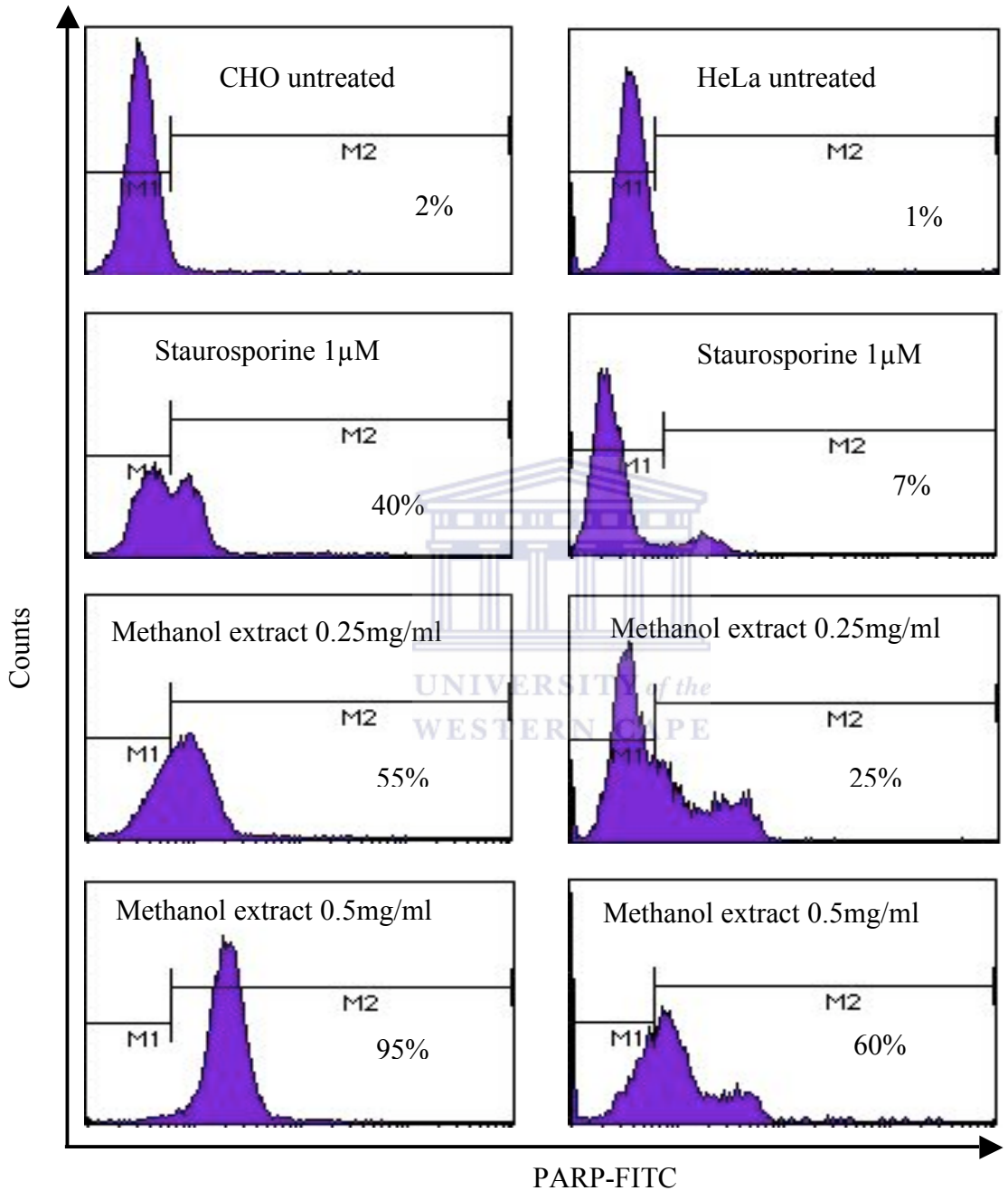


Figure 3. 8: PARP cleavage in HeLa and CHO cells induced with the crude plant methanol extract. HeLa cells and CHO cells were either left untreated or treated with 1µM staurosporine for 4 hours or treated with 0.5mg/ml of the methanol extract.

3.7 Mitochondrial depolarization

One of the hallmarks of the apoptosis process is the loss of the mitochondrial membrane potential ($\Delta\Psi_m$) (Green and Reed, 2000; Iijima, 2006) and as reviewed in chapter one section 1.4.6.2. The crude plant methanol extract was used to induce CHO cells to undergo apoptosis. Since mitochondrial membrane potential ($\Delta\Psi_m$) is an early event, the cells were tested for mitochondrial membrane depolarization after short time exposures to the inducer.

CHO cells were either left untreated or treated with methanol extract at a concentration of 0.5mg/ml for various times of 30 minutes, 1 hr, 2 hrs and 3 hrs. Positive control cells were induced with 1μM staurosporine for 30 minutes. The procedure described in section 2.5.5 was followed.

The results (figure 3.9) showed that 6% of the untreated CHO cells (A) were depolarized while the staurosporine induced cells (B) were 38 % depolarized after 30 minutes of inducement. CHO cells induced with the crude plant methanol extract were 47% depolarized after 30 minutes of exposure to the inducer (C), however as time progressed, at 1 hr 63 % of the cells were depolarized (D) at 2 hrs 82% were depolarized (E) and at 3 hrs 91% (F) of the cells were depolarized. It means that mitochondria depolarization was due to compound(s) contained in the plant methanol extract. It can also be seen that mitochondrial depolarization occurred in a time dependent manner. The results also showed that mitochondrial membrane potential (ΔΨm) started collapsing at an early stage

in the apoptotic pathway as shown by the results obtained following 30 minutes of inducement.

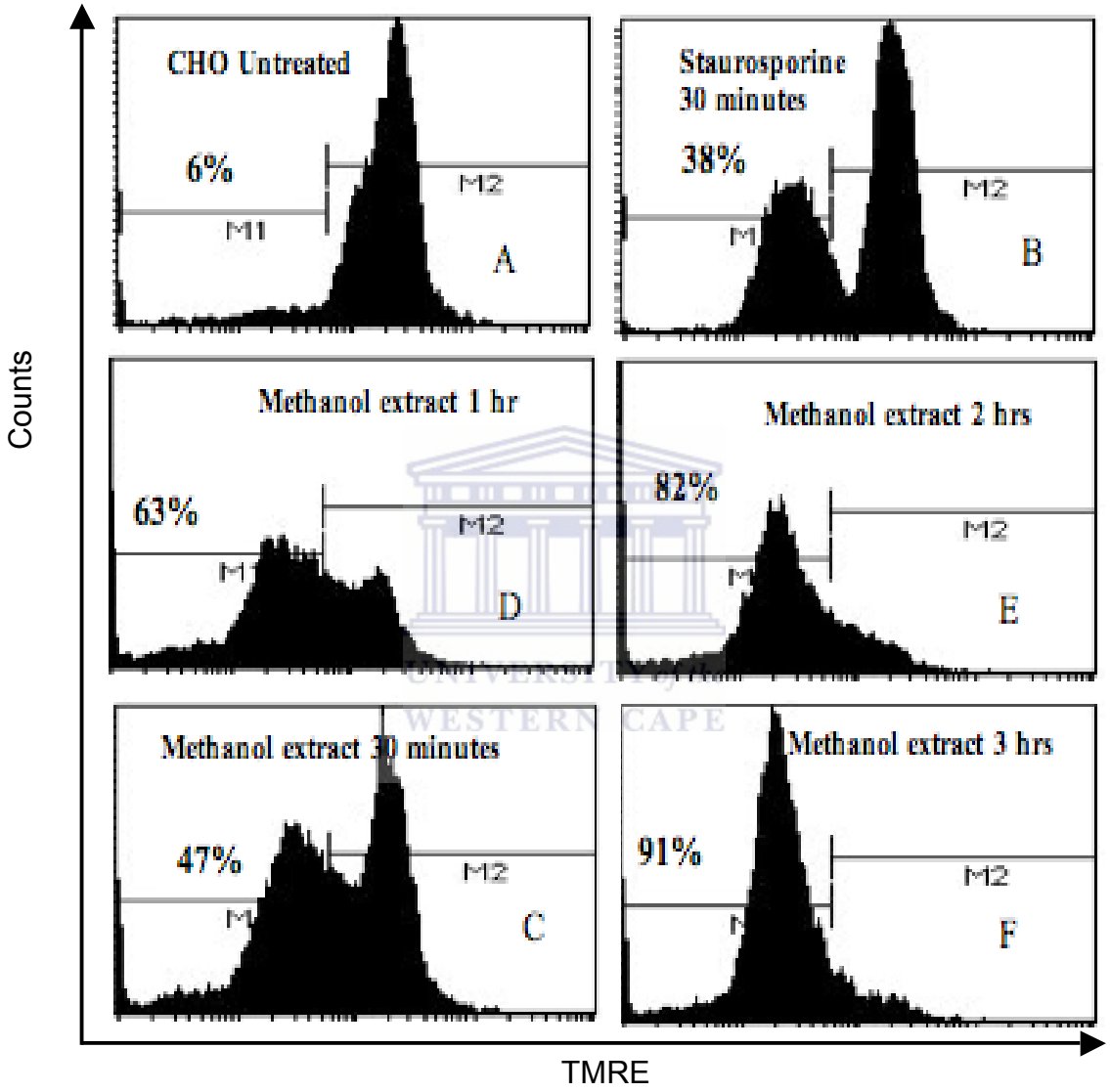


Figure 3.9: CHO cells tested for mitochondrial membrane depolarization. CHO cells were either left untreated (A) or induced with 1μM staurosporine for 30 minutes (B) or were induced with 0.5mg/ml of the plant methanol extract for various times of 30 minutes, 1 hr, 2hrs and 3hrs. (C), (D), (E) and (F) respectively.

3.8. DNA Fragmentation by APO-DIRECT™.

Darzynkiewicz *et al.,* (1997), said that the best methodology to use in cell necrobiology is flow cytometry. This is so because one is able to quantify apoptotic cells (apoptotic index) as compared to alternative methods (analysis of cell morphology, DNA gel electrophoresis). Darzynkiewicz *et al.,* (1997), further said that flow cytometry is rapid, objective and very sensitive. However, since identification of apoptotic cells by flow cytometry is generally based on a single feature which may not necessarily be the marker of apoptosis in every situation, the mode of cell death should normally be confirmed by light or electron microscopy. The crude plant methanol extract was therefore further tested for its effect on DNA fragmentation on CHO cells using the APO-DIRECT™ Flow cytometric method. DNA fragmentation is one of the steps, which occurs at later stages in the apoptosis pathway. It is as a result of activation of endonucleases during the apoptotic program. Degradation of nuclear DNA into nucleosomal units is one of the hallmarks of apoptotic cell death. The nucleases degrade the chromatin higher order structure into fragments of ~300 kb and into smaller DNA pieces of about 50 kb in length and subsequently into 200 bp ladders (Nagata, 2000). APO-DIRECT™ is a method which is used to detect ends of fragmented (broken) DNA. The principle behind the assay (figure 3.10) is that the enzyme terminal deoxynucleotidyl transferase (Tdt) specifically binds and adds dUTPs to 3' –OH ends of fragmented DNA. Fluorochrome labelled antibodies can be conjugated to dUTPs and can be used to detect the broken ends (Gavrieli *et al*., 1992). In this assay fluorochrome FITC was conjugated (labelled) to deoxyuridine triphosphate nucleotides (dUTPs) which labeled the 3'-hydroxyl ends of

double- and single -stranded DNA. The reaction was catalysed by the enzyme terminal deoxynucleotidyl-transferase (Tdt). Identification of DNA break sites was done by staining of DNA strand breaks with FITC-labelled dUTP's analysed by flow cytometer.

CHO cells were either left untreated or treated with 1.μM staurosporine for 6 hrs or treated with 0.5mg/ml methanol extract for 36 hrs and the procedure described in section 2.5.7 was followed.

The results (figure 3.11) showed that the untreated CHO cells were primarily FITC-dUTP negative while CHO induced with staurosporine were 10% FITC-dUTP positive and the methanol extract treated CHO were 20% positive FITC-dUTP. These results also confirm that the methanol extract contains potent compound(s), which induced apoptosis in and eventual DNA fragmentation.

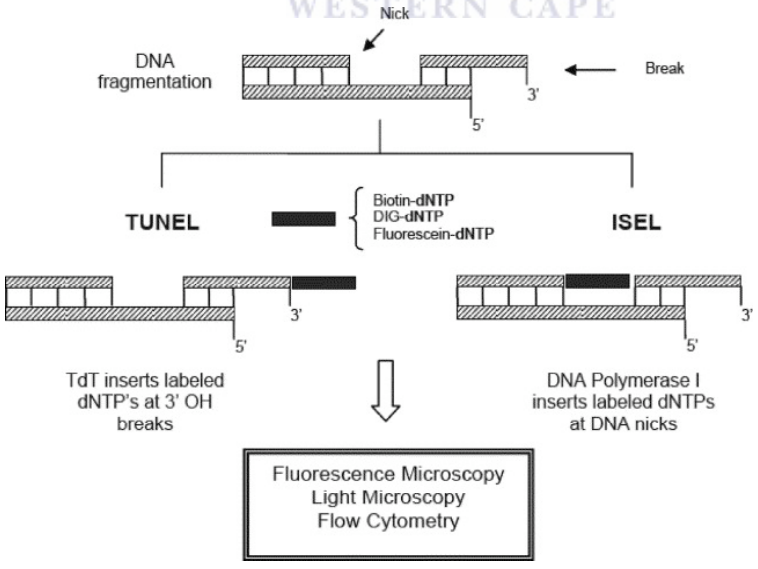


Figure 3.10: An illustration of TUNEL and *In situ* end-labelling (ISEL) techniques (Huerta *et al*., 2007).

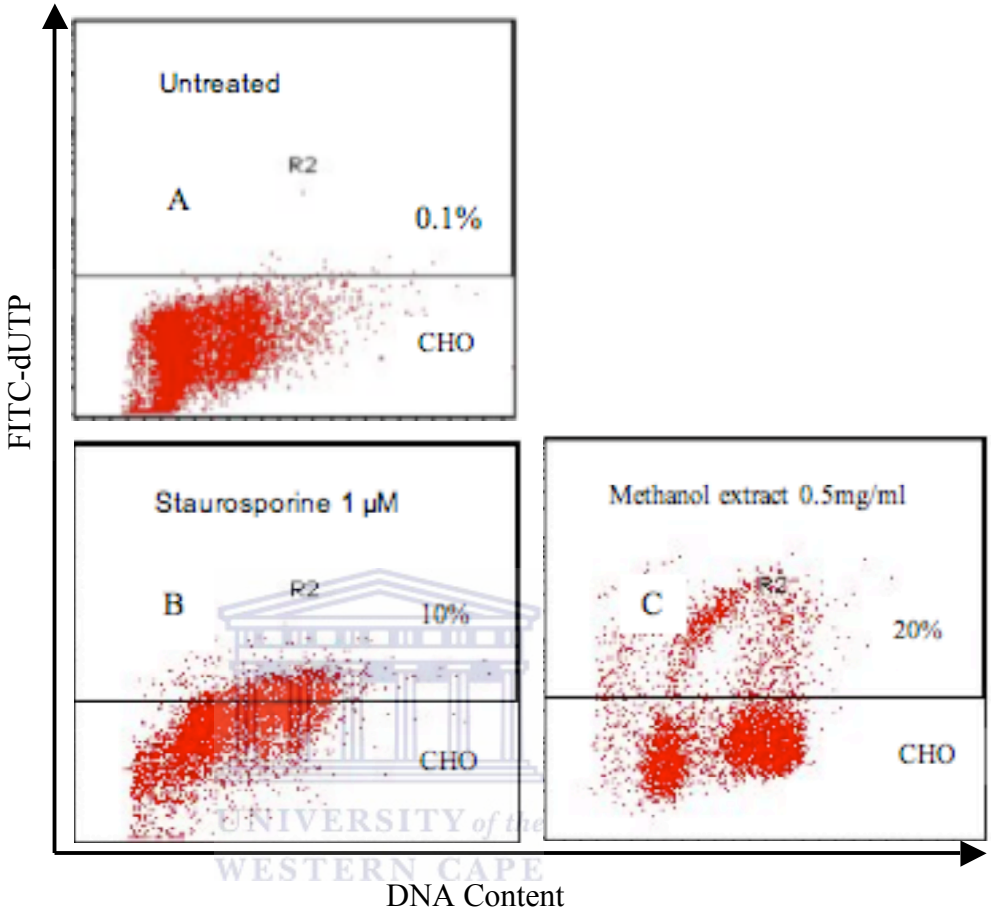


Figure 3.11: DNA Fragmentation of CHO cells. CHO cells were left untreated (A) or treated with 1.µM Staurosporine for 6 hrs (B) or treated with 0.5mg/ml methanol extract for 36 hrs.

3.9 Cellular viability

The Neutral red uptake (NRU) cytotoxicity assay is a cell survival/viability assay which is based on the incorporation of neutral red (NR) to bind to viable cells. NR is a weak cationic supravital dye that readily penetrates cell membrane by non-ionic diffusion and it predominately accumulates intracellularly in lysosomes. If the cell surface or the sensitive lysosomal membrane is altered the lysosomes become fragile and other changes gradually become irreversible. Cytotoxic substances can produce such changes and cause decreased uptake and binding of the neutral red, making it possible to distinguish between viable, damaged, or dead cells via spectrophotometric measurements because viable cells will take up the dye while the damaged or dead cells will not. Cytotoxicity is expressed as a concentration dependent reduction of the uptake of NR following the chemical exposure, thereby providing a sensitive, integrated signal of both cell integrity and growth inhibition (Babich and Borenfreund, 1991; Rixe *et al*., 1996; Fotakis and Timbrell, 2005).

Each cell line was induced to undergo apoptosis with various concentrations of the crude plant methanol extract, which ranged from 2mg/ml to 0.063mg/ml. Cytotoxicity of the crude plant methanol extract was quantitatively measured using the Neutral red uptake assay (NRU).

The numbers (table 3.1) represent the mean NRU_{50} of three replicate experiments and standard error of the mean (SEM).

The results obtained (table 3.1) showed that Jurkat cells were most sensitive with a NRU_{50} of 0.5mg/ml followed by CHO and 293T cells. Caski were most resistant with a NRU_{50} of 1.5mg/ml followed by MCF7 and H157 cells. The results also demonstrated that while the extract was cytotoxic to non-human cell lines such as CHO and 3T3, the extract was also cytotoxic to the human cancer cell lines tested.

Table 3.1: NRU_{50} of different cell lines tested with the plant methanol extract

CELL LINE	**NRU_{50} mg/ml (Means + SEM)**
Caski	1.5 ± 0.7
CHO	0.6 ± 0.1
HeLa	0.8 ± 0.23
H157	1.0 ± 0.23
Jurkat	0.5 ± 0.12
MCF7	1.0 ± 0.64
MG-63	0.85 ± 0.12
3T3	0.95 ± 0.07
293T	0.65 ± 0.12

3.10 Morphological evaluation of different cell lines induced with the plant methanol extract.

As reviewed in chapter one (sections 1.4.3 and 1.4.4) the study of apoptosis was first based on cell morphology (Kerr *et al*., 1972; Otsuki *et al.,* 2003). Various types of cell death have since been defined according to their morphological features without a clear reference to specific biochemical mechanisms (Kroemer *et al*., 2005; Roos and Kaina, 2006). The word 'apoptosis' is simply a morphological description. Häcker, (2000), said that properly defined features of apoptosis provide a reliable basis for the detection of apoptosis, which can be used in combination with other apoptosis assays to properly evaluate the whole mechanisms underlying apoptosis.

Different cell lines were induced to undergo apoptosis as described in section 2. 5.1 and their cell morphology were evaluated microscopically. The cells were induced with 0.5mg/ml of the plant methanol extract, however, most of the cells looked swollen although the nucleus appeared condensed as compared to un induced cells. Classifying this observation as apoptosis was difficult therefore the concentration of the extract was reduced to 0.25mg/ml so that at lower concentration the extract could induce the cells to under go apoptosis and their cell morphology properly described.

When the cells were induced with 0.25mg/ml (table 2.2b), some of the cells (Caski, H157 and MCF7) however, did not show morphological changes in comparison with un induced cells. These cells appeared primarily resistant at this concentration because further incubation (36 hrs) resulted in progressive proliferation of the cells. Morphologically, the

rest of the other cells (CHO, HeLa, Jurkat, MG-63, 293T and 3T3) at 0.25mg/ml, showed characteristic features seen in an apoptotic cell and did not proliferate when they were further incubated for an additional 12 hrs. These morphological observations were important because they helped to determine which cell lines were sensitive or resistant and also to determine the probable concentration required to induce apoptosis in a particular cell line.

Table 2.2 (a): Morphological description of different cell lines upon induction with 0.5mg/ml of methanol extract for 24 hrs.

Cell type	**Morphological Evaluation (0.5mg/ml)**	**Mode of cell death**
CHO	Cells were slightly condensed, and not detached	Apoptosis
Caski	Cells were swollen with vacuolated cytoplasm.	? Apoptosis ? Oncosis
HeLa	Cells were swollen and not detached from plate	? Oncosis
H157	Cells were swollen and some had vacuoles.	? Apoptosis ? Oncosis
Jurkat	Cells were swollen, and detachment from each other (cells were not growing in clusters)	? Apoptosis ? Oncosis
MCF7	Cells were slightly condensed and were not detached from plate	Apoptosis
MG63	Condensed nucleus, not detached from plate surface, and not swollen.	Apoptosis
293T	Cells swollen? Most cells were detached from plate	? Apoptosis ? Oncosis
3T3	Cells swollen? Cells were not detached from plate	? Apoptosis ? Oncosis

Table 2.2 (b): Morphological description of different cell lines upon induction with 0.25mg/ml of methanol extract for 24 hrs.

Cell type	Morphological Evaluation (0.25mg/ml)	Mode of cell death
Caski	No discernible morphological changes seen.	? Resistant
CHO-22	Most cells were condensed and vacuolated.	Apoptosis
HeLa	Few cells were condensed and were not detached from plate surface.	Apoptosis
H157	No discernible morphological changes seen.	? Resistant
Jurkat	Cells were condensed and were detached from each other.	Apoptosis
MCF7	No discernible morphological changes seen.	Apoptosis
MG63	Slight condensed cytoplasm, not lifted from plate surface, not swollen.	Apoptosis
293T	Few cells were detached from plate surface, were not swollen and were not condensed.	? Apoptosis
3T3	Cells were slightly condensed and remained adherent to plate surface (not detached).	Apoptosis

3.11 Summary

Aqueous extracts from the leaves of *Chrysanthemoides monilifera*, *Rhus laevigata*, *Acacia karroo*, *Euclea racemosa*, *Leonatis leonurus*, *Senecio littoreus* and *Imperata cylindrica* were prepared and screened for their anti-proliferative and pro apoptotic activities on CHO cells tested by APOP*ercentage*™ apoptosis assay. The results showed that the aqueous extracts from *Rhus laevigata* were the most active in inducing apoptosis in the CHO cells compared to the other plant extracts. The leaves of *Rhus laevigata* were further extracted with organic solvents with increasing polarity (chloroform, dichloromethane (DCM), ethyl acetate, ethanol, and methanol). The organic extracts were again tested on CHO cells to determine the extract which contained the most active compound(s). The results showed that the crude methanol extract was the most active in inducing apoptosis in CHO cells as compared to the other organic and the aqueous extracts.

A quality control assay on the induction of apoptosis by methanol on CHO was carried out to determine if methanol was the cause of apoptosis induction in the methanol extract. The results showed that methanol at 1% concentration did not induce apoptosis in CHO cells. The concentration of methanol in the plant extract was less than 1% when the plant methanol extract was used to induce apoptosis in the CHO cells. Induction of apoptosis was not therefore due to methanol but was due to compound(s) contained in the leaves of the extracted plant.

Annexin V binding assay was used to assess mode of cell death because Annexin V when used in combination with 7-AAD or propidium iodide can distinguish apoptotic from necrotic cells (late apoptosis). The results showed that increasing the concentration of the plant methanol extract from 0.125mg/ml to 0.5mg/ml also resulted in the proportional increase in both apoptotic (early apoptotic) and necrotic (late apoptotic) cells.

A panel of nine cell lines (Caski, CHO, HeLa, H157, Jurkat, MCF7, MG-63, 293T and 3T3) were induced with the plant methanol extract and their morphology was evaluated. The morphology of the cells was difficult to evaluate when the cells were induced with 0.5mg/ml of the plant methanol extract. When the concentration was reduced to 0.25mg/ml it was possible to describe the morphology of Jurkat, MG-63, 293T, CHO, HeLa, H157, Jurkat and 3T3 cells, however, Caski, MCF7 and H157 did not show any morphological changes at this concentration.

CHO cells induced with the plant methanol extract were tested for the presence of specific active caspase-3 antibodies. The results showed 30% presence of specific active caspase-3 antibodies in the CHO cells following induction with the plant methanol extract.

CHO cells were again induced with the plant methanol extract and tested for collapse of mitochondrial potential. The results showed that mitochondrial depolarization occurred in a time dependent manner following treatment of the CHO cells with the plant methanol extract.

HeLa and CHO cells were tested for PARP cleavage upon induction of the cells with the plant methanol extract. The results showed that CHO cells were 95% positive for PARP while HeLa cells were 20% positive for PARP.

IC_{50} values of the nine cell lines were determined using NRU assay following induction of the cells with the plant methanol extract. The results showed that Caski were the most resistant because they had high NRU values followed by H157 and MCF7. Jurkat cells were the most sensitive because they had the least NRU values followed by CHO cells, indicating that Jurkat cells were the most sensitive to the plant methanol extract.

Finally CHO cells were further tested for DNA fragmentation the final execution of apoptosis following apoptosis induction. APO-DIRECT™, a very sensitive single-step assay for detecting DNA strand breaks was used. The principle behind the assay is described in section 3.8. The results showed that 20% of the cells were positive for FITC-dUTP.

CHAPTER FOUR: **SCREENING ALKYLATING AGENTS FOR ANTICANCER ACTIVITY.**

4.1. SCREENING ALKYLATING AGENTS FOR ANTICANCER ACTIVITY

As reviewed in chapter one (section 1.7), alkylating agents stop cell growth by binding to nuclear DNA thereby forming cisplatin-DNA adducts. The formation of cisplatin-DNA adducts prevents the DNA double helix strands from separating and since this is required in DNA replication, the cells fail to replicate causing tumour damage as well as normal cell damage through the activation of apoptosis (Boulikas and Vougiouka, 2003; Sato *et al*., 2005). For this reason, DNA alkylating agents are a major component of the anticancer drug discovery. Brabec and Kasparkova, (2005), Ciesielska *et al.,* (2006) have said that although alkylating agents have been shown to be effective against most solid tumours, their side effects have been a major concern. That is why for more than twenty-five years a lot of work has involved the modification of the structure of cisplatin in an effort to improve its effectiveness and hundreds of platinum drugs have so far been tested. Ciesielska *et al.,* (2006), further said that although some of the recently designed drugs containing platinum have promising biological properties, there is still a need to develop cisplatin related compounds with novel ligands and test their antitumor action and probably identify compounds, which can overcome the problems faced with cisplatin treatment. For this reason, platinum and palladium complexes with novel ligands presented in section 2.4 were synthesized and in order to establish whether these complexes could induce apoptotic cell death, a number of screening tests were performed in CHO cells and selected cancer cell lines. These screening tests included: (a) morphological evaluation using light microscopy (b) The APOP*ercentage*™ apoptosis

assay (c) The Annexin V binding assay (d) DNA fragmentation (e) cellular viability assay (f) analysis of cell cycle perturbations. (g) Mitochondrial depolarization.

4.2 Induction of apoptosis in various cell lines induced with complexes 15, 57, 58 and cisplatin.

The principal mechanism through which the APOP*ercentage*™ assay works has been reviewed in chapter one (section 1.4.2). In order to screen for apoptosis induction, various cell lines were treated with 0.5 mM of complexes 15, 57, 58 and cisplatin for 24 hours. The cells were acquired using the FACS procedure described in chapter two (2.5.3). Analysis was also done by FACS 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* ™ positive (apoptotic/necrotic). Apoptotic/necrotic cells were reported as percentage of cells positive for APOP*ercentage* ™ over total cells acquired (10,000) multiplied by 100. Two independent experiments were done on each of the cell line.

Table 4.1: A panel of cells lines were treated with cisplatin, complexes 15, 57 and 58 and screened for apoptosis induction.

CELL LINE	INDUCER	% of cells positive for APOP*ercentage*™ (Mean + SEM)
MG-63	**Untreated**	**3 ± 0.82**
	Cisplatin	97 ± 0.41
	Complex 15	99 ± 0.00
	Complex 57	96 ± 0.82
	Complex 58	89 ± 0.41
MCF7	**Untreated**	**8 ± 0.41**
	Cisplatin	95 ± 0.41
	Complex 15	99 ± 0.00
	Complex 57	49 ± 2.45
	Complex 58	45 ± 1.22
HeLa	**Untreated**	**8 ± 1.60**
	Cisplatin	75 ± 2.45
	Complex 15	96 ± 0.41
	Complex 57	92 ± 1.63
	Complex 58	66 ± 0.82
H157	**Untreated**	**9 ± 0.41**
	Cisplatin	80 ± 0.82
	Complex 15	96 ± 0.41
	Complex 57	96 ± 1.22
	Complex 58	60 ± 1.63
3T3	**Untreated**	**8 ± 0.81**
	Cisplatin	84 ± 1.63
	Complex 15	94 ± 0.82
	Complex 57	91 ± 1.63
	Complex 58	97 ± 0.41
CHO-22	**Untreated**	**8 ± 2.45**
	Cisplatin	74 ± 0.41
	Complex 15	98 ± 0.41
	Complex 57	99 ± 0.00
	Complex 58	62 ± 4.08
293T	**Untreated**	**15 ± 2.45**
	Cisplatin	89 ± 3.27
	Complex 15	95 ± 0.82
	Complex 57	99 ± 0.00
	Complex 58	81 ± 2.45
Caski	**Untreated**	**6 ± 0.81**
	Cisplatin	73 ± 1.60
	Complex 15	97 ± 0.00
	Complex 57	97 ± 0.00
	Complex 58	88 ± 0.82
Jurkat	**Untreated**	**8 ± 2.45**
	Cisplatin	95 ± 0.41
	Complex 15	97 ± 0.00
	Complex 57	99 ± 0.00
	Complex 58	95 ± 0.41

The table 4.1 shows the results of APOP*ercentage*™ apoptosis assay following treatment of the cells with various complexes. The results showed that the untreated 293T cells were 15% positive for APOP*ercentage* ™ apoptosis assay while the other un treated cell lines (H157, 3T3, CHO-22, Caski, Jurkat, MCF7, MG-63 and HeLa) were less than 10% positive for APOP*ercentage* ™ apoptosis assay. The results of the treated cells showed that APOP*ercentage* ™ apoptosis assay for cells treated with cisplatin and the newly synthesized platinum and palladium complexes were more than 50% positive tested against the panel of cell lines however, complexes 57 (a palladium based complex) and 58 (a platinum based complex) were not very active against MCF7 cells (49% and 45% positive for APOP*ercentage*™ apoptosis assay respectively). Complex 15 (a palladium based complex) was highly active against all the cell lines tested.

4.3 Morphological evaluation of cells exposed to complexes 15, 57, 58 and cisplatin.

Different cells lines respond differently to apoptosis inducing stimuli because their molecular pathways which lead to cell death may also act differently and therefore may also cause different morphological appearances. As previously indicated in chapter 3 (section 3.10) it is necessary to evaluate the morphological features of a dying cell, as this helps in planning for future experiments.

In this assay, various cell lines were exposed to 0.5 mM of the complexes 15, 57, 58 and Cisplatin for 24 hrs and their morphology was evaluated by an inverted light microscopy as described in section 2.5.1.

Table 4.2: Morphological evaluations of cell lines induced with complexes 15, 57, 58 and cisplatin to undergo apoptosis.

Cell type	Inducer	Morphological Evaluations	Mode of cell death
Caski	Cisplatin	Cells shrunk and were not detached	Apoptosis
	Complex 15	Nucleus shrunk, cytoplasm swollen, not detached	Apoptosis
	Complex 57	Cells shrunk and were not detached	Apoptosis
	Complex 58	Cells shrunk and were not detached	Apoptosis
CHO-22	Cisplatin	Cells moderately shrunk, few detached	Apoptosis
	Complex 15	Nucleus shrunk, cytoplasm swollen, few detached	Apoptosis
	Complex 57	Nucleus shrunk, cytoplasm swollen few detached	Apoptosis
	Complex 58	Cells moderately shrunk, few were detached	Apoptosis
HeLa	Cisplatin	Cells shrunk and were not detached	Apoptosis
	Complex 15	Cells shrunk and were not detached	Apoptosis
	Complex 57	Cells shrunk and were not detached	Apoptosis
	Complex 58	Cells shrunk and were not detached	Apoptosis
H157	Cisplatin	Cells shrunk and most were detached	Apoptosis
	Complex 15	Cells shrunk and were not detached	Apoptosis
	Complex 57	Cells shrunk and were not detached	Apoptosis
	Complex 58	Cells shrunk and were not detached	Apoptosis
Jurkat	Cisplatin	Cells shrunk	Apoptosis
	Complex 15	Cells shrunk	Apoptosis
	Complex 57	Cells shrunk	Apoptosis
	Complex 58	Cells shrunk	Apoptosis
MCF7	Cisplatin	Cells shrunk and were not detached	Apoptosis
	Complex 15	Cells shrunk and were not detached	Apoptosis
	Complex 57	Cells shrunk and were not detached	Apoptosis
	Complex 58	Cells shrunk and were not detached	Apoptosis
MG-63	Cisplatin	Cells shrunk and were not detached	Apoptosis
	Complex 15	Cells shrunk and were not detached	Apoptosis
	Complex 57	Cells shrunk and were not detached	Apoptosis
	Complex 58	Cells shrunk and were not detached	Apoptosis

293T	Cisplatin	All cells shrunk and were completely detached	Apoptosis
	Complex 15	All cells shrunk and were completely detached	Apoptosis
	Complex 57	All cells shrunk and were completely detached	Apoptosis
	Complex 58	All cells shrunk and were completely detached	Apoptosis
3T3	Cisplatin	Cells were condensed and were not detached	Apoptosis
	Complex 15	Cells were condensed and were not detached	Apoptosis
	Complex 57	Cells were condensed and were not detached	Apoptosis
	Complex 58	Cells were condensed and were not detached	Apoptosis

To show the general picture of cells induced with the complexes, CHO cells were cultured in six well culture plates at 2.5 x 10^4 and were allowed to grow to form a monolayer. The cells were then induced with 0.5 mM of complexes 15, 57, 58 and cisplatin for 24 hrs. Following induction, the cells were inspected using an inverted Nikon light microscope and pictures were taken using a Leica EC3 digital color camera.

The pictures in figure 4.1 depict the general morphological observations also seen in the other cell lines induced with the complexes.

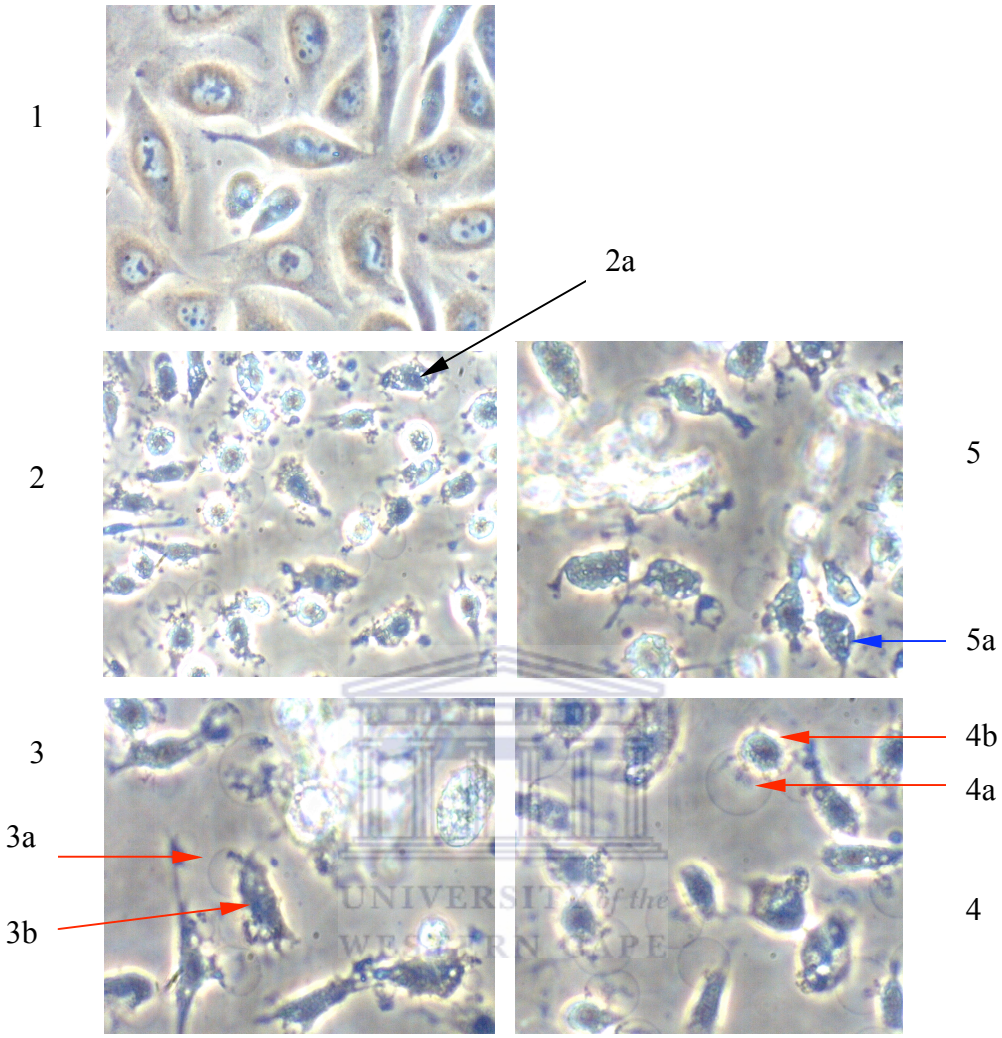


Figure 4.1: Morphology of CHO cells following 24 hrs of exposure to complexes 15, 57, 58 and cisplatin.

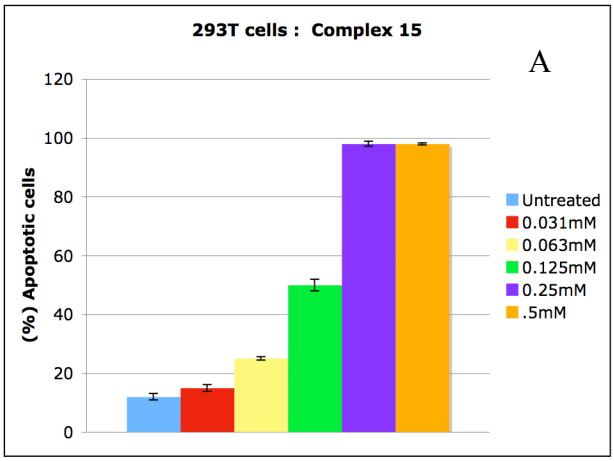
Picture 1 shows untreated CHO cells. Picture 2 shows CHO cells induced with cisplatin and the arrow 2a shows a cell with vacuoles. Picture 3 shows CHO cells induced with complex 15 and arrow 3a shows a cell with a swollen cytoplasm (ballooned shape) while its nucleus is shrunk/condensed shown in 3b. Picture 4 shows CHO cell induced with complex 57. Arrow 4a points to a cell with a swollen cytoplasm while its nucleus

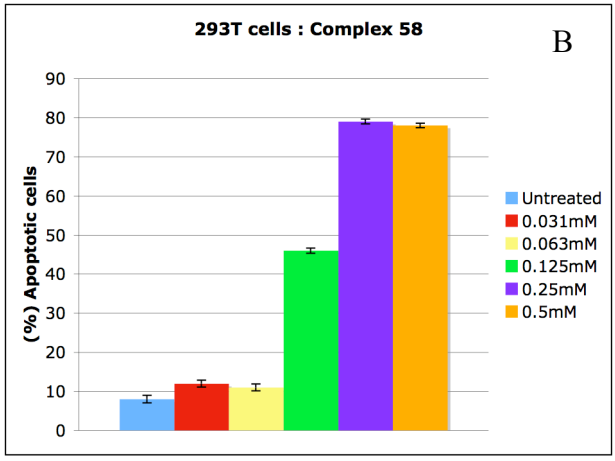
shrunk/condensed 4b. Picture 5 shows CHO cells induced with complex 58 and arrow 5a shows a condensed cell.

4.4. Evaluation for dose dependence

Following the morphological evaluation shown in table 4.2, the dose response of 293T cells was assessed against complex 15 (a palladium based complex) and complex 58 (a platinum based complex). The concentrations of the complexes ranged from 0.5 mM to 0.031mM and the cells were induced for 24 hrs. There after the APOP*ercentage*™ apoptosis assay described chapter (two section 2.5.3) was followed. The cells were acquired and analyzed by FACS as described in chapter two (section 2.5.3). The results of the average of three independent experiments were obtained and then percentage of apoptotic cells (APOP*ercentage*™ positive cells) was plotted on the Y axis against complex concentration on the X axis on bar graph.

The results shown in figure 4.2 showed that in both experiments apoptotic cells were less than 11% positive The unexposed cells were used to define the basal level of apoptosis/necrosis in these cells as there is always a variation in the basal level of apoptosis/necrosis in any given cell population and this also varies from one cell line to another. Even untreated cell population contain a minor percentage of cells that are positive for apoptosis. Therefore there was a need to include a negative control to avoid false positives. As can be seen both complexes i.e. complex 15 (graph A) and complex 58 (graph B) induced apoptosis in 293T cells in a dose dependent manner.





Figures 4.2: (a) and (b) 293T cells tested against various concentrations of complexes 15 and 58 measured by APOP*ercentage*™ apoptosis assay and analyzed by flow cytometer.

4.5 Annexin V binding assay.

Changes in the plasma membrane are one of the earliest features of apoptosis. The Annexin V binding assay was used to confirm the data obtained by APOP*ercentage*™ apoptosis assay and ascertain whether the mode of cell death was due to apoptosis induction. When the cells were treated with the complexes they showed typical morphology of cells undergoing apoptosis and the results of APOP*ercentage*™ apoptosis assay confirmed this observation. A human cancer cell line 293T was used as a model cell line to also confirm the APOP*ercentage*™ apoptosis assay results. In this assay kit, Annexin V conjugated to phycoerythrine (PE) was used to identify cells in their early phase of apoptosis (Annexin V positive, 7-AAD negative) while the vital dye 7-amino-actinomycin D (7-AAD) was used to identify cells that were in their late stages of apoptosis or were already dead/necrotic (Annexin V positive and 7-AAD positive).

293T cells were exposed to 0.5mM of cisplatin, complexes 15, 57, and 58 for 24 hrs and the procedure described in chapter one (section 2.5.4) was followed.

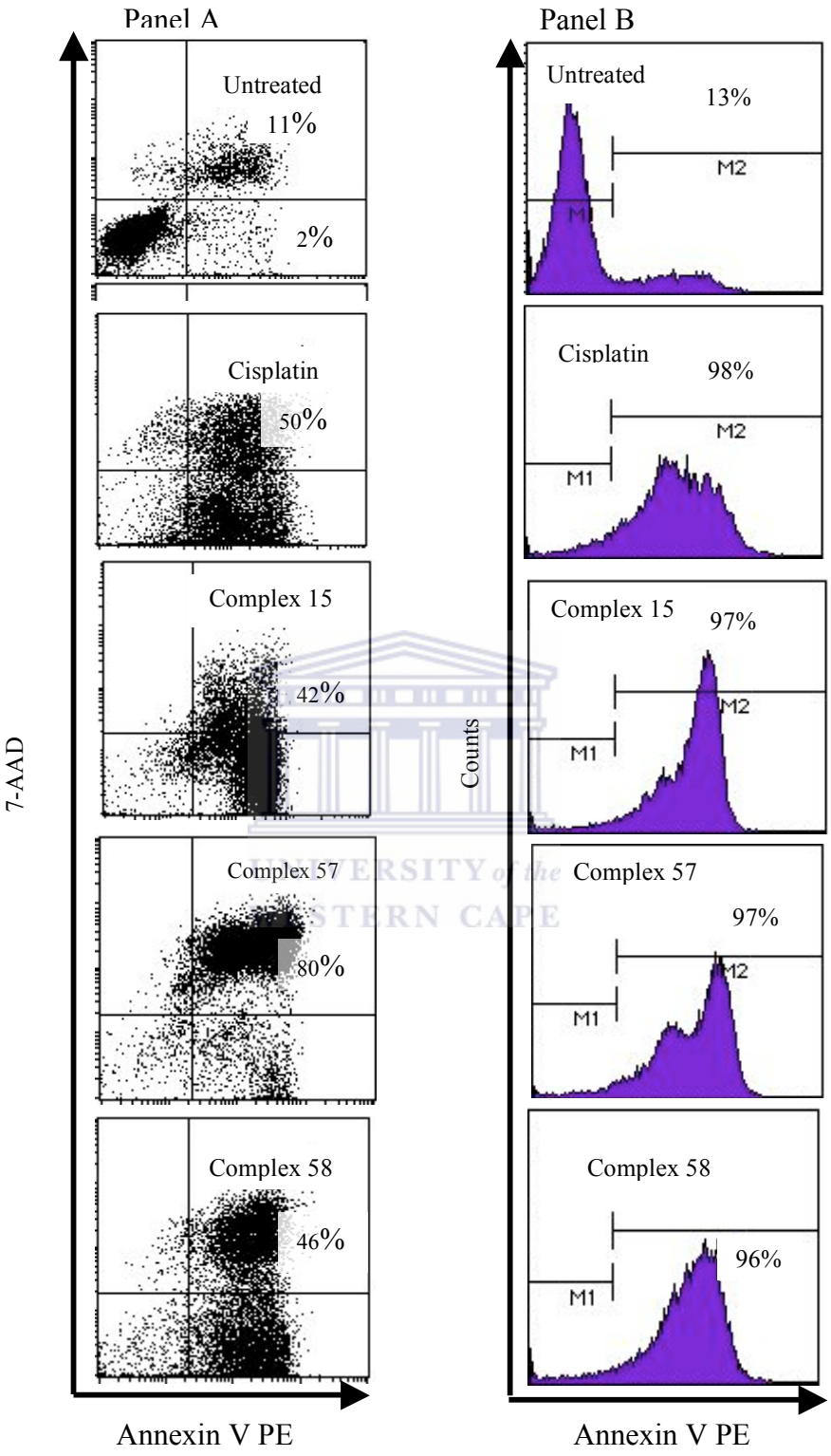


Figure 4.3: Annexin V binding assay of 293T cells tested against complexes 15, 57, 58 and cisplatin.

The results (figure 4.3) showed that the untreated cells were about 13 percent Annexin V positive (panel B 1) and out of the positive cell population about 11% of the cells were in their late stage of apoptosis while 2% were in their early stage of apoptosis as shown in Panel A 1. Cisplatin induced 98% of the cells to apoptosis (panel B 2), and 50% of the induced cells were in their late stage of apoptosis (panel A 2). Complex 15 induced 97% of the cells to apoptosis (panel B 3), and 42% of the induced cells were in their late stage of apoptosis (panel A 3). Complex 57 induced 97% of the cells to apoptosis (panel B 4), and 80% of the induced cells were in their late stage of apoptosis (panel A 4) while Complex 58 induced 96% of the cells to apoptosis (panel B 5), and 46% of the induced cells were in their late stage of apoptosis (panel A 4). The exposed cells showed that that they were very sensitive to all the complexes. All the four complexes induced more than 95% of the cells to apoptosis. These results showed that the screening for apoptosis done by the APOP*ercentage*™ apoptosis assay was compatible with the Annexin V assay.

4.6 Mitochondrial depolarization.

One of the early features in the apoptotic pathway is mitochondrial depolarization as reviewed in chapter one (section 1.4.6.2). Disturbances in the mitochondria may lead to the opening of the mitochondrial permeability transition pore complex, which lead to organelle swelling, and rupture of the outer membrane, which result in the release of cytochrome c. Once cytochrome c is released from the mitochondria, it combines with Apaf-1, and procaspase-9 to form the apoptosome. Caspase-9 processes and activates other caspases, which organize the orderly biochemical execution of the cells. It should

be noted however, that cytochrome c release might depend on the cell type. In some cells cytochrome c is available in excess, and caspases can be activated. However, as pointed out by Green and Reed, (1998), some cells contain large caspase inhibitors, which inhibit the release of cytochrome c, as a result caspase-dependent apoptosis is not induced instead the eventual loss of electron chain may drive the cell towards a necrotic cell death. Since one of the hallmarks of apoptosis is the loss of mitochondria membrane potential ($\Delta\Psi$), its measurement is therefore important, because it provides an early indication of apoptosis in the apoptotic pathway.

293T cells were exposed to 0.5 mM of complexes 15, 57, 58 and cisplatin for 1 hr and 5 hrs and tested for mitochondrial membrane depolarization with TMRE probe. A more detailed procedure is described in chapter 2 (section 2.5.5). TMRE is a highly fluorescent cationic lipophilic dye and is also mitochondria-specific. The dye is rapidly taken up and retained by live cells and for this reason it is used to determine $\Delta\Psi_m$ in intact cells. Its retention is dependent on mitochondrial transmembrane potential (Krohn *et al*., 1999).

The results (figure 4.4) showed that 5% of the untreated cells were positive for TMRE, an indicative of mitochondrial depolarization. Following 1 hr of exposure to cisplatin about 16% of the cells were positive for TMRE, also an indicative of mitochondrial depolarization and at 5 hrs the cells showed 87% mitochondrial depolarization. When the cells were exposed to complex 15, about 28% of the cells showed mitochondrial depolarization and at 5 hrs about 40% of the cells showed mitochondrial depolarisation. Cells that were exposed to complex 57 showed 58 % mitochondrial depolarization after 1

hr of complex treatment and 90% mitochondrial depolarization at 5 hrs. Cells treated with complex 58 showed 7% mitochondrial depolarization after1 hr of treatment and 25% mitochondrial depolarization following 5 hrs of treatment.

The results further showed that mitochondria in the untreated cells were intact as exhibited by the retention of TMRE fluorescence by the cells, as TMRE is mitochondria specific. The treated cells however, showed that the cells lost TMRE fluorescence in a time dependent manner indicating that mitochondria depolarization was as a result of complex treatment.

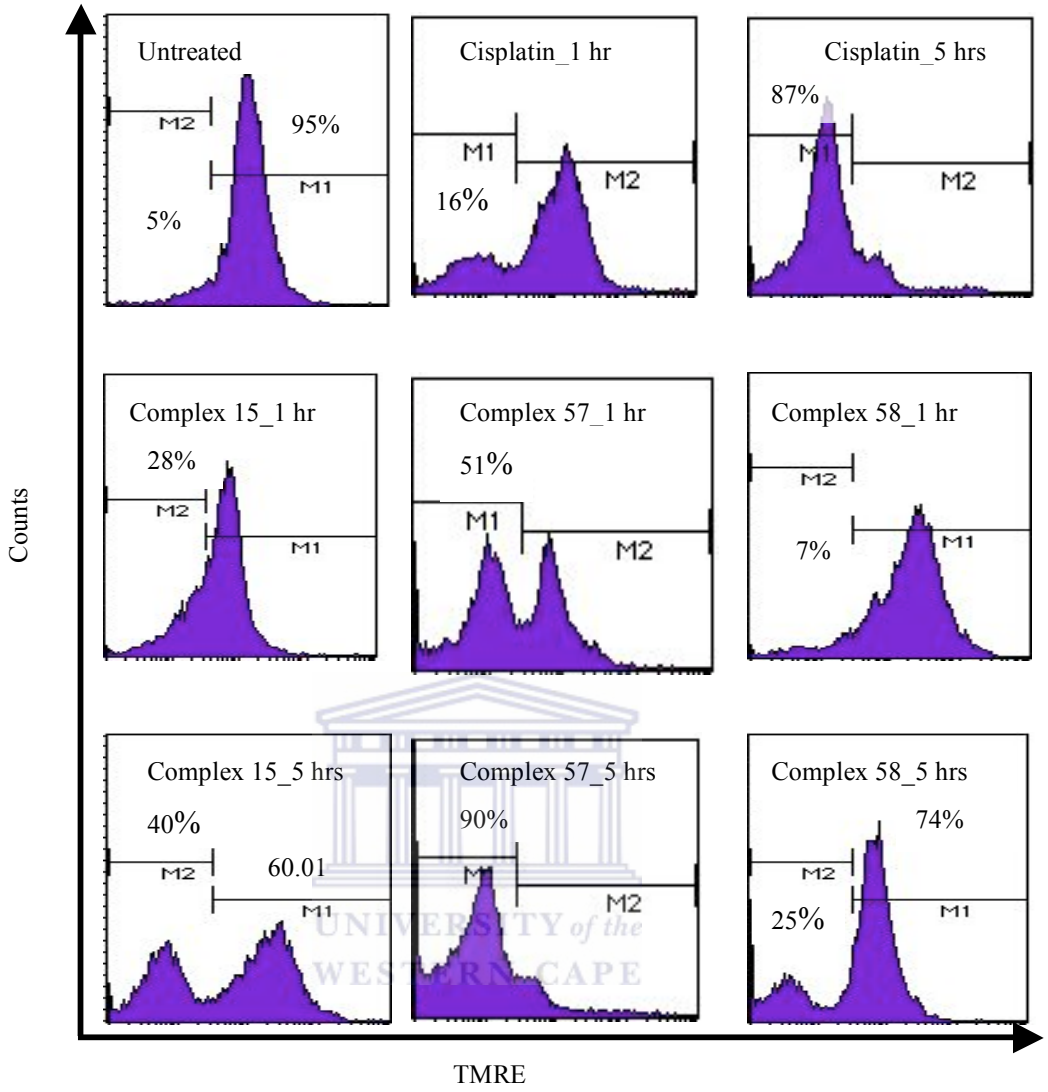


Figure 4.4: Mitochondrial depolarization of 293T cells tested with complexes 15, 57, 58 and Cisplatin measured by tetramethylrhodamineethylester (TMRE).

4.7 Detection of active caspase-3

Complexes 15, 57 and 58 were further screened to determine if their mechanism of action resulted in caspase-3 activation since the caspases play an essential role in the execution phase of apoptosis as reviewed in chapter one (sections 1.4.6.1, 1.4.6.2, 1.4.7 1.4.7.1 and 1.4.7.2). Caspases are the main effectors of apoptosis or programmed cell death (PCD). They are a family of cysteine proteases that cleave proteins after aspartic acid residues and they irreversibly commit cells to die (Riedl and Shi, 2004). When caspases are activated they cause characteristic morphological changes seen in a cell that is undergoing apoptosis such as shrinkage, chromatin condensation, DNA fragmentation and plasma membrane blebbing. As reviewed in chapter one section (1.4.6.1), induction of apoptosis via death receptors results in the activation of an initiator caspase such as caspase 8 or caspase 10. These caspases can then activate other caspases in a cascade. This cascade eventually leads to the activation of the effector caspases, such as caspase 3 caspase-6 or -7. These caspases are responsible for the cleavage of key cellular proteins that leads to the typical morphological changes that are observed in cells undergoing apoptosis. Caspase-3 is a key protease that is activated during the early stages of apoptosis. It is synthesized as an inactive proenzyme that is processed in cells that are undergoing apoptosis by self-proteolysis and/ or cleavage by another protease. Active caspase-3 is therefore used as a marker for cells that are undergoing apoptosis. In this assay the antibodies specific for active caspase-3 were used to recognise cleaved caspase-3 in 293T cells exposed to cisplatin, complexes 15, 57 and 58. Since caspase independent apoptosis has been reported (Shih *et al*., 2003), it was necessary to test whether these

complexes induced apoptosis via caspase activation in these cells or not. Cells were exposed to 0.5mM of complexes 15, 57, 58 and cisplatin for 24 hrs to induce apoptosis. Thereafter the cells were permeabilized, fixed and stained for active caspase-3 as described in chapter two (section 2.5.6).

Following treatment of the cells with the complexes, the results (figure 4.5) showed that the untreated cells were 7% positive for specific active caspase-3 antibodies, whereas cells treated with cisplatin, complexes 15, 57, and 58 were respectively 43%, 36%, 29% and 13% positive for specific active caspase-3 antibodies. The results showed that the complexes induced apoptosis through the activation of caspase-3 in 293T cells.

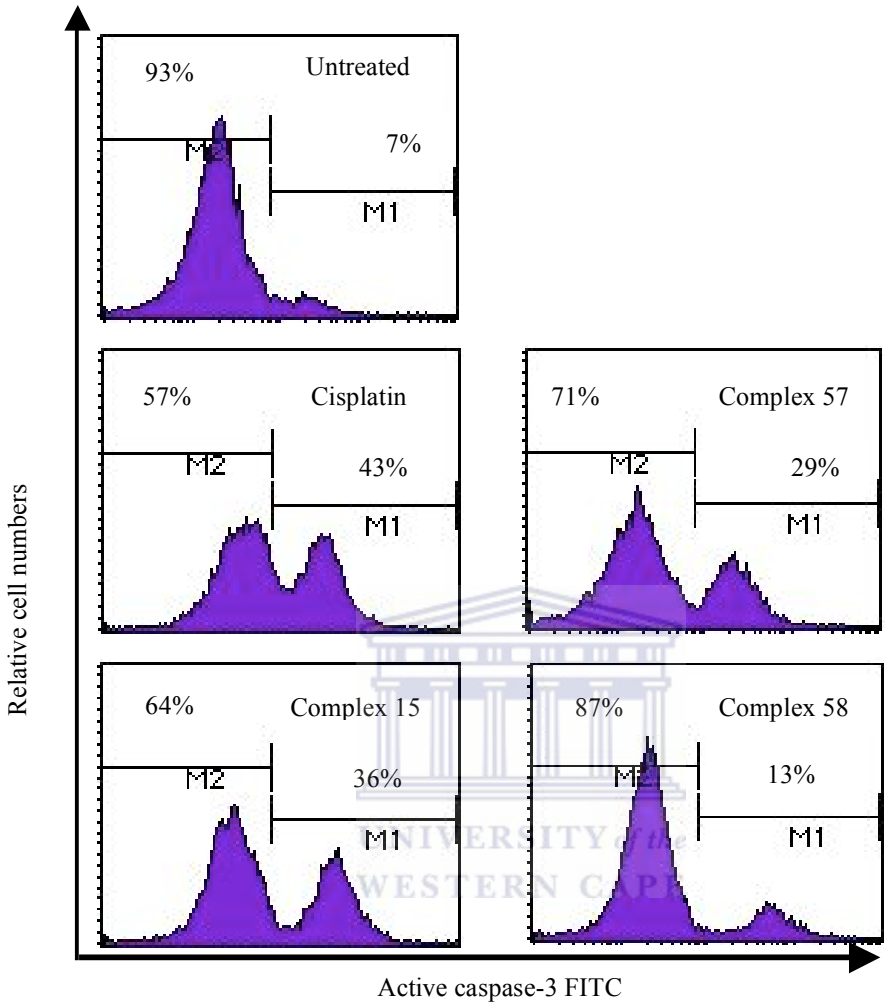


Figure 4.5: Detection of active caspase-3 in 293T cells induced with complexes 15, 57, 58 and cisplatin.

4.8 Detection of chromosomal DNA fragmentation

One of the hallmarks of apoptosis cell death is the digestion of genomic DNA by an endonuclease, which generates a ladder of small fragments of double-stranded DNA. The amount of DNA fragmentation is assumed to be proportional to the cell death and DNA

fragmentation occurs at later stages of the apoptosis pathway. In some cell systems however, Hirata *et al*., (1998), reported that DNA fragmentation is not detected regardless of clear demonstration of characteristic cytoplasmic changes of apoptosis. In this study, all the cell lines tested with the complexes clearly demonstrated typical cytoplasmic changes seen in apoptotic cells (table 4.2). Two cell lines, 293T and Jurkat were treated with 0.5mM of cisplatin, complexes 15, 57 and 58 for 36 hrs because DNA fragmentation usually occurs late in the apoptosis pathway. In order to determine whether these cell lines had their DNA degraded upon exposure to the complexes, the terminal deoxynucleotidyl transferase dUTP nick end-labelling (TUNEL) assay (Vermes *et al*., 1995) was performed as indicated in chapter two (section 2.5.7). The principle of the assay is described in chapter 3 (section 3.8). The assay is suitable for both quantitative and qualitative analysis. In this assay cells were quantitatively acquired and analyzed by flow cytometry.

The results (figure 4.6) showed that the untreated 293T cells were typically negative for FITC-dUTP while cisplatin, complexes 15, 57 and 58 were 11%, 64%, 55% and 61% respectively positive for FITC-dUTP. The insert shows FITC-dUTP against counts.

The results (figure 4.7) showed that the untreated Jurkat cells were 1% positive for FITC-dUTP while cisplatin, complexes 15, 57 and 58 were 48%, 56%, 53% and 61% respectively positive for FITC-dUTP. The inserts show FITC-dUTP against counts. These results clearly showed that the complexes caused DNA fragmentation in the Jurkat cells.

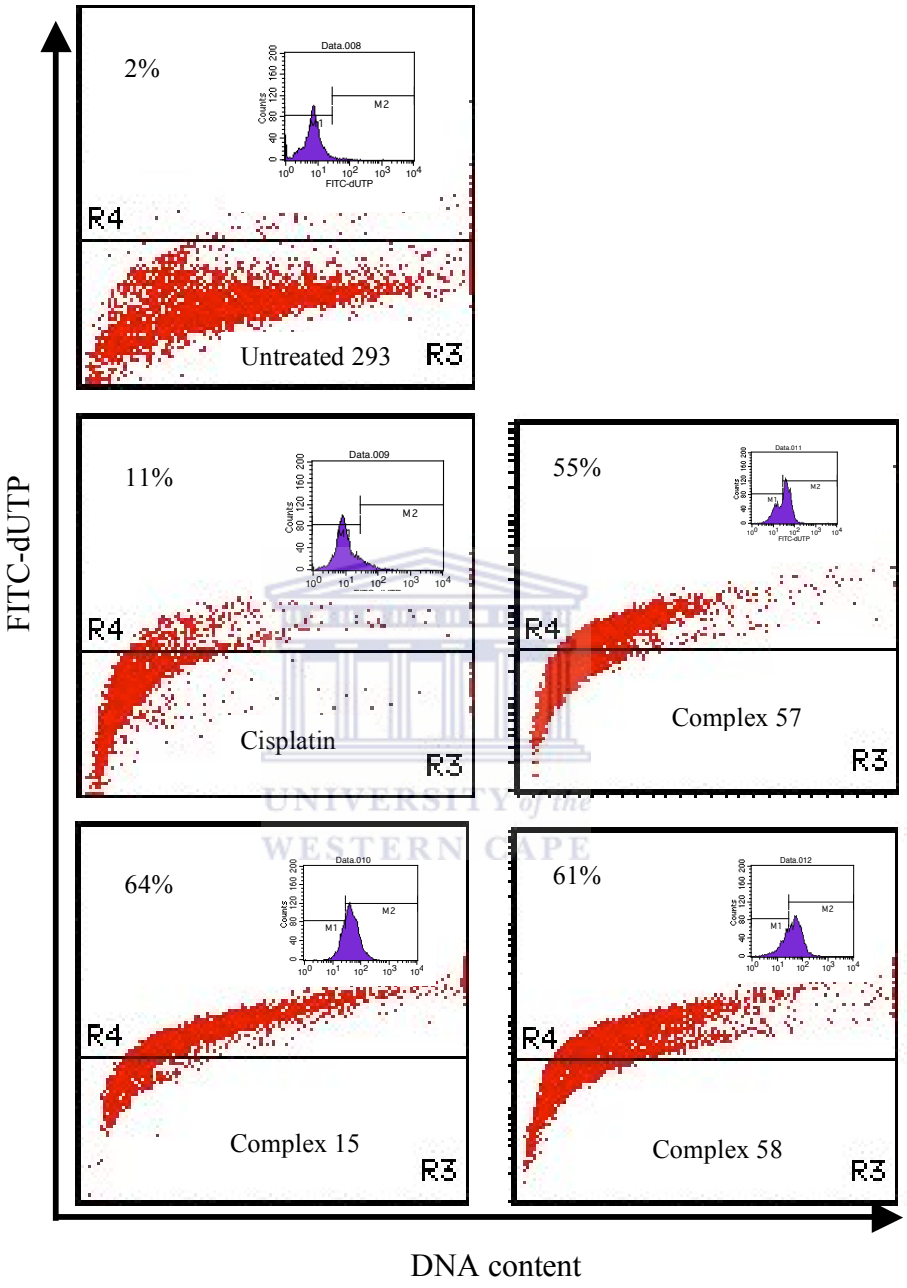


Figure 4.6: DNA fragmentation of 293T cells induced with cisplatin, complexes 15, 57 and 58.

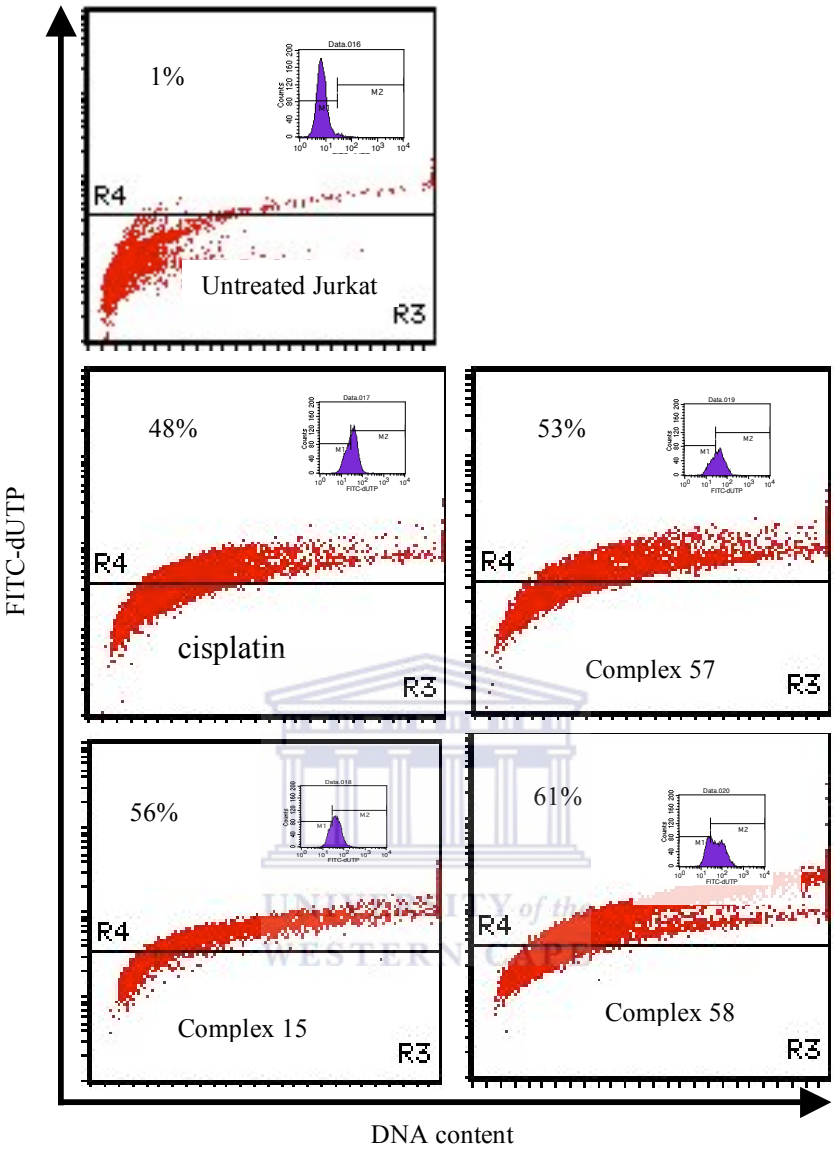


Figure 4.7: DNA fragmentation of Jurkat cells induced with cisplatin, complexes 15, 57 and 58.

4.9 Analysis of cell Cycle

In chapter one (section 1.5) a review of the cell cycle and some of the genes implicated in the cell cycle regulation have been presented. Apoptosis can occur at any stage of the cell cycle because the mechanisms of chemical interactions necessary for apoptosis are

present throughout the cell cycle. Alenzi, (2004), indicated that there is a link between apoptosis and cell proliferation as suggested by some studies that have demonstrated the presence of large numbers of dying cells in proliferating cell populations *in vivo.*

During apoptosis, calcium and magnesium-dependent nucleases are activated resulting in the degradation of DNA (Wyllie *et al.,* 1984). Nicks and fragments within DNA can be caused by exposure of cells to harmful environmental agents such as mutagenic chemicals or radiation (Papamichos-Chronakis *et al* ., 2006) and these can be detected by electron microscopy (EM) (Huerta *et al*., 2007), DNA analysis to look at a Sub-G1 peak as reviewed in chapter one (section 1.5), using strand break labelling (TUNEL) to detect broken DNA (chapter 3 section 3.8), (Huerta *et al*., 2007), using Hoechst binding to detect DNA conformational changes (Ioannou and Chen, 1996), DNA laddering and *In Situ* end labelling and ELISA. (Huerta *et al*., 2007),

The Sub-G1 method relies on the fact that subsequent to DNA fragmentation and following permeabilization, the low molecular weight (LMW) DNA inside the cytoplasm of the apoptotic cells leaks out during the subsequent rinse and staining procedure. The lower DNA content of these cells means they contain less DNA. Following staining with DNA-binding dye, cells that have lost DNA will take up less stain and will appear to the left of the G1 peak (the so- called "sub-G1 peaks, "A0" cells i.e. cells with lower fluorescence level than G0+G1 cells) and are thus considered apoptotic. The major disadvantage of this method is that apoptotic G2-Phase cells that exhibit a reduced DNA content, could represent the DNA content of a G1- cell. Therefore it may not be detected as apoptotic. This would result in underestimation of the apoptotic population.

In this assay, propidium iodide (PI) was used to stain DNA. Propidium iodide (PI) intercalates in the DNA helix and fluoresces red. It has the advantage that it is excited by 488nm light and can be used on most common flow cytometers. However, it requires cells to be fixed or permeabilized and is therefore not a rapid assay. PI also stains double-stranded RNA and this should first be removed with ribonuclease. The intensity of the PI signal is directly proportional to DNA content.

Cell cycle is one of the methods used to detect DNA fragmentation. Analysis of the cell cycle also helps to know the stage at which a prospective drug candidate halts the cells from progressing to the next phase of the cell cycle so that drugs can be designed to target that stage as reviewed in chapter one (section 1.5.5). In section 4.7 of this chapter, 293T and Jurkat cells were treated with complexes 15, 57, 58 and cisplatin and DNA fragmentation was done using the APO-DIRECT™ assay. In the cell cycle analysis assay, HeLa cells were used to analyze DNA fragmentation and to assess the stage at which cell progression was halted. HeLa cells were treated with 0.5mM of complexes 15 and 58 and the procedure described in chapter two (section 2.5.10) was followed.

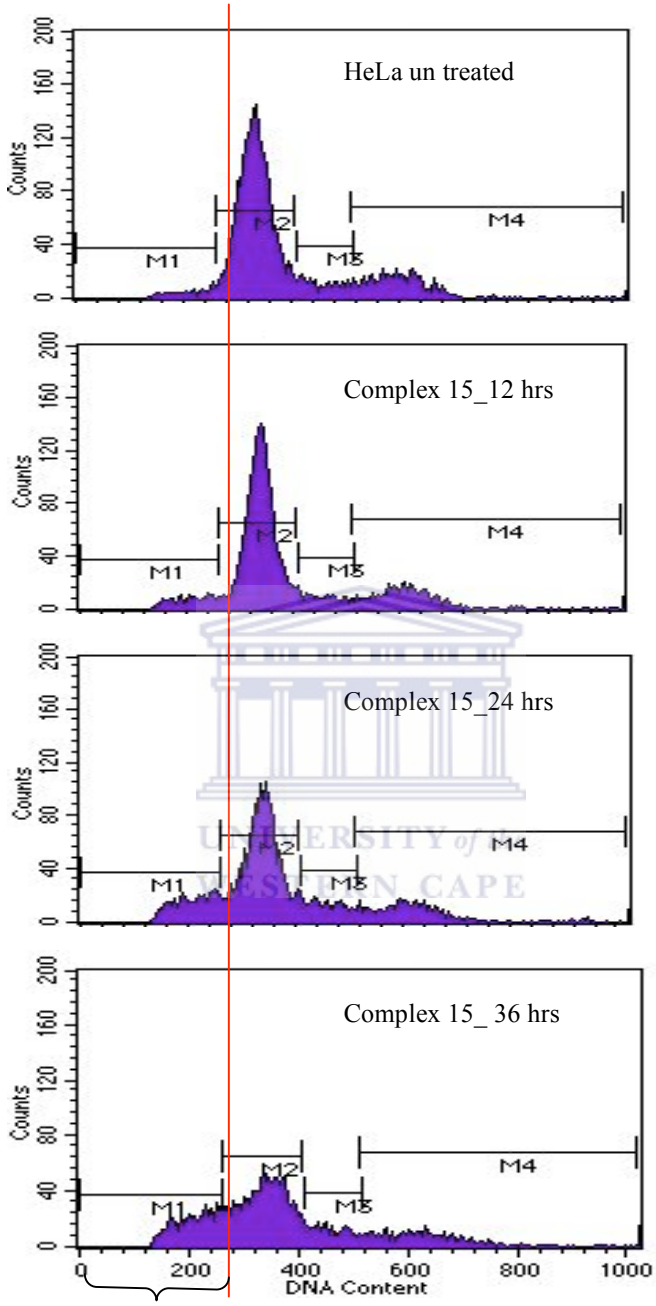


Figure 4.8: Cell cycle analysis of HeLa cells tested against complex 15.

The table 4.3: Cell cycle analysis of HeLa cells treated with complex 15. With reference to figure 4.8, Marker M1 represented A_0 (apoptotic cells, sub G1) (Marker M2 represented G_1 of the cell cycle while Marker M3 represented S phase of the cell cycle and Marker M4 represented G2/M phase of the cell cycle. HeLa cells were induced to undergo apoptosis with 0.5mM of complexes 58 for 12, 24 and 36 hours. The reduction in staining/DNA content of these cells was measured by flow cytometry.

Time	Sub-G1	G1-Phase	S-Phase	G2-Phase
0	**2 %**	74 %	8 %	15 %
12 hrs	**6 %**	70 %	8 %	16 %
24 hrs	**15 %**	60 %	10 %	15 %
36 hrs	**23 %**	51 %	11 %	15 %

The results (figure 4.8) showed that the majority of the cells were typically in the G0/G1 phase of the cell cycle. The results further showed that there was a reduction in the number of cells in G_1 as time progressed and subsequent increases in the number of cells in sub-G_1 (apoptotic cells). It can also be seen that most of the cells were halted at G0/G1 and became apoptotic while a few cells progressed through to S and G2/M phases.

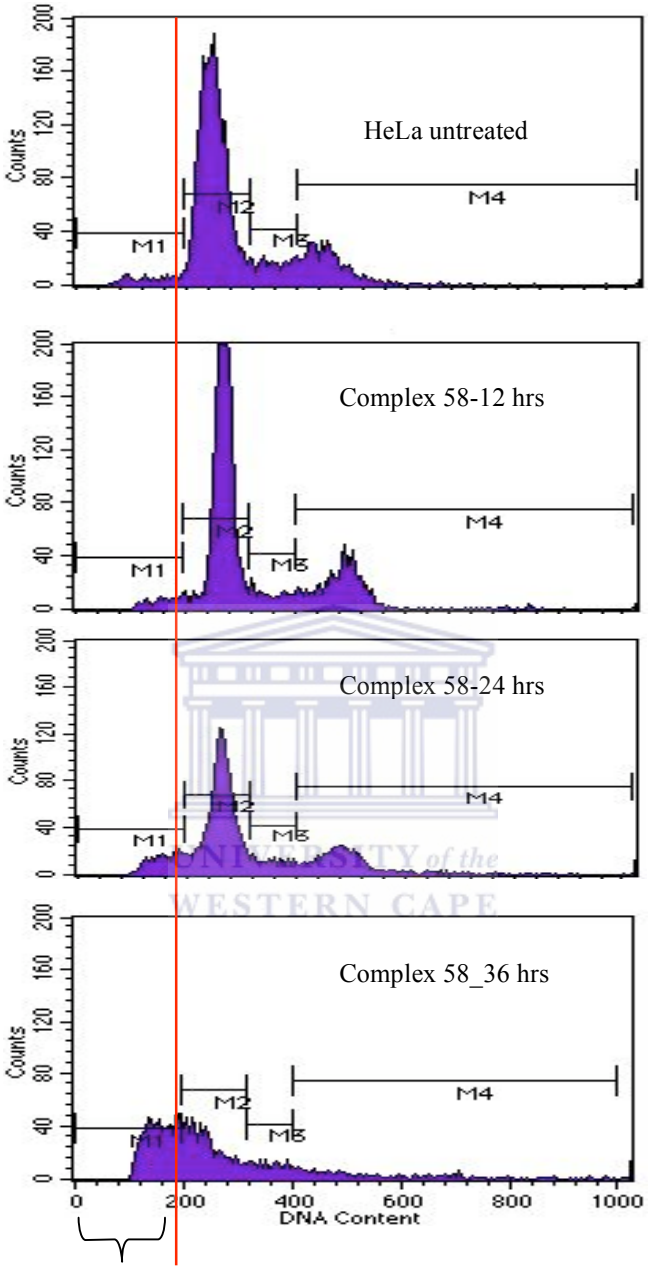


Figure 4.9: Cell cycle analysis of HeLa cells tested against complex 58.

The table 4.4: Cell cycle analysis of HeLa cells treated with complex 58. With reference to figure 4.9, Marker M1 represented A_0 (apoptotic cells, sub G1). Marker M2 represented G_1 of the cell cycle while Marker M3 represented S phase of the cell cycle and Marker M4 represented G2/M phase of the cell cycle.

Time	Sub-G1	G1-Phase	S-Phase	G2-Phase
0	**1 %**	73 %	9%	15 %
12 hrs	**3 %**	70 %	6 %	21 %
24 hrs	**11 %**	58 %	8 %	23 %
36 hrs	**42%**	37 %	10 %	11 %

The results (figure 4.9) showed that the majority of the cells were typically in the G0/G1 phase of the cell cycle. The induced cells showed that there was a reduction in number of cells in G_1 as time progressed and subsequent increases in the number of cells in sub G1 (apoptotic cells). The results further show that as time progressed most of the cells were halted at G0/G1 and became apoptotic while a few cells progressed through to S and G2/M phases.

In both cell cycle experiments cells induced with complex 15 and 58 showed that there was not much difference in the number of apoptotic cells between 0 and 12 hrs, however, the number of cells in the sub-G1 population started increasing at 24 hrs time point and a marked increase in apoptotic cells was observed at 36 hrs time point.

As reviewed in chapter one (section 1.5) there are many factors that can cause cells to be halted in a given phase of the cell cycle. It is possible that the complexes formed complex DNA adducts (section 1.7.2) which halted the cell cycle progression thereby activating several genes, which are involved in cell signalling pathway such as p53, pRb, CDKs and others. The activation of genes, which are involved in the apoptosis pathway can cause

cell cycle arrest at any phase of the cell cycle and also activate genes that are involved in DNA fragmentation. The actual mechanism(s) through which the complexes halted the cell cycle progression and the subsequent DNA fragmentation was not investigated.

4.10 Cellular Viability

Neutral Red Uptake (NRU) cytotoxicity test described in chapter 2 (section 2.5.8 and 2.5.9) was used to evaluate the cytotoxicity of the complexes.

The NRU_{50} results (table 4.5) showed that most cytotoxicity was observed in 293T cells tested against all the complexes under investigation followed by Jurkat as shown by their reduced retention in the uptake of the NR dye (numbers highlighted in red). Less cytotoxicity was observed in H157 exposed to cisplatin and to complex 58, as shown by their increased retention of the NR dye. Complex 58 also showed less cytotoxicity to Caski as also shown by their increased retention of the NR dye (numbers highlighted in blue).

The causes of cisplatin resistance are still not yet clear. (Damia and D`Incalci, 1988; Elwell *et al*., 2006). However, Damia and D`Incalci, (1988), suggested that the mechanisms underlying cisplatin resistance could include the reduction of cisplatin accumulation inside cancer cells, the ability of the cells to detoxify cisplatin, the ability of the cells to repair the cisplatin-DNA adducts faster, modulation of apoptotic pathways, up-regulation in transcription factors, and a higher concentration of glutathione and metallothioneins (MTs). Cisplatin also fails to selectively kill cancerous cells over normal cells (Sakinah *et al*., 2007). Further investigation of the palladium complexes needs to be

done since they are potential anticancer agents. The cells could be treated with the complexes over a period of time and see if they could eventually become resistant. There is need to investigate whether the complexes could selectively kill cancerous cells over normal cells because this study did not carry out this investigation.

Table 4.5: NRU_{50}±SEM (mM) of various cell lines tested with various complexes.

CELL LINE	**NRU_{50} (Cisplatin) Mean + SEM**	**NRU_{50} (Complex 15) Mean + SEM**	**NRU_{50} (Complex 57) Mean + SEM**	**NRU_{50} (Complex 58) Mean + SEM**
Caski	1.10 ± 0.09	0.04 ± 0.02	0.05 ± 0.01	0.05 ± 0.01
CHO-22	0.09 ± 0.02	0.05 ± 0.01	0.06 ± 0.01	0.06 ± 0.02
HeLa	0.04 ± 0.03	0.05 ± 0.04	0.04 ± 0.02	0.03 ± 0.01
H157	2.00 ± 0.40	0.08 ± 0.01	0.08 ± 0.01	1.10 ± 0.10
Jurkat	0.01 ± 0.01	0.01 ± 0.02	0.04 ± 0.01	0.04 ± 0.01
MCF7	0.70 ± 0.40	0.06 ± 0.01	0.06 ± 0.02	0.07 ± 0.03
MG 63	0.06 ± 0.02	0.09 ± 0.01	0.06 ± 0.01	0.09 ± 0.01
293T	0.01 ± 0.01	0.01 ± 0.01	0.03 ± 0.01	0.01 ± 0.02
3T3	0.05 ± 0.01	0.04 ± 0.01	0.05 ± 0.01	0.05 ± 0.01

4.11 Summary

Two palladium based complexes assigned numbers 15 and 57 and one platinum based complex assigned number 58 have been tested for apoptosis induction in human, mouse and CHO cell lines. Selected assays used in the study of apoptosis have been used in this study. One of the earliest features of apoptosis is the translocation of the membrane phospholipid phosphatidylserine (PS) from the inner to the outer surface of the plasma membrane. To test this feature, the APOP*ercentage*™ apoptosis assay was used. The

principle behind this assay is described in chapter 3 (section 3.1) and the results showed that both the palladium and the platinum complexes induced apoptosis in MG-63, MCF7, HeLa, H157, 3T3, CHO, 293T Caski and Jurkat cells as shown by the positive results of APOP*ercentage*™ apoptosis assay.

When the cell membrane phospholipid phosphatidylserine (PS) is undergoing translocation from the inner to the outer surface of the plasma membrane during apoptosis (flip-flop mechanism), it exposes binding sites for Annexin V, a 35-36 kDa which is Ca^{2+}- dependent, phospholipid binding protein with a high affinity for PS. However, translocation of PS also occurs during necrosis. Annexin V binding when used in conjunction with vital dyes such as 7-Amino-actinomycin (7-ADD) or propidium iodide (PI) can be used to test early apoptotic cells and late apoptotic cells. One of the cell lines 293T lost attachment upon treatment with the complexes and Annexin V binding assay was used to test on 293T cells induced with complexes 15, 57, 58 and cisplatin as a positive control. The results for the Annexin V binding assay showed that the cells treated with complexes 15, 57 58 and cisplatin were 97%, 97%, 96% and 98% positive for Annexin V respectively and of these 42%, 80%, 46% and 50% were positive for both Annexin V and 7-AAD (late apoptotic) respectively. The two assays (APOP*ercentage*™ and Annexin V) showed that the complexes induced apoptosis in the cells.

One other feature that is involved in early apoptosis is the activation of aspartate-specific cysteinyl proteases (caspases). Caspases are enzymes that play a critical role in apoptosis

when activated and failure of apoptosis is one of the major contributing factors to tumour growth and autoimmune diseases. Three caspases-3, -6 and -7 are involved in the execution phase of apoptosis. 293T cells were treated with complexes 15, 57, 58 and positive control cisplatin and were evaluated for activation of active caspase-3, which is involved in the early stages of apoptosis. The results showed that the treated cells were positive for active caspase-3 detected using specific antibodies.

Mitochondria play a fundamental role in the regulation of programmed cell death (apoptosis). They contain pro-apoptotic proteins such as apoptosis inducing factor (AIP) and cytochrome C embedded in the mitochondrial membrane. These factors can however be released following pole formation in the mitochondrial membrane called the permeability transition pole (PT). Cytochrome C when released from the mitochondria into the cytosol, is able to interact with Apaf-1 and leads to the recruitment of pro-caspase-9 to form the apoptosome as reviewed in chapter one (section 1.4.6.2). The formation of the apoptosome leads to the activation of caspase 9 and the induction of apoptosis. 293T cells were further tested for collapse of the mitochondria transmembrane potential upon exposure to the complexes 15, 57, 58 and cisplatin. The results showed that the mitochondria depolarization (collapse) increased with time.

The NRU_{50} of the complexes 15, 57, 58 on different cell lines were determined in comparison to the positive control cisplatin. The Neutral red uptake cytotoxicity assay is a cell survival/viability assay based on the ability of viable cells to incorporate and bind neutral red (NR) a weak cationic supravital dye that readily penetrates cell membranes by

non-ionic diffusion and predominantly accumulates intracellularly in lysosomes as reviewed in chapter three (section 3.9). It was shown that cisplatin had less cytotoxicity in Caski, H157 and MCF7 cells. Complex 58 a platinum-based complex, also showed less toxicity against H157 as shown by the increased retention of the dye by the cells. NRU_{50} of complexes 15 and 57 (palladium based complexes) showed increased cytotoxicity against the entire panel of cell lines tested as shown by the reduction in the retention of NR dye by the cells.

Inconsistencies of non-linear dose and response relationships usually cause problems in judging the validity of a drug. 293T cells were treated with different doses of complexes 15 (a palladium based complex) and complex 58 (a platinum based complex) and changes in apoptosis response was evaluated. The results showed that both complex 15 and 58 induced apoptosis in 293T cells in a dose dependent manner.

Apoptotic cells show characteristic features such as shrinkage, loss of contact between neighbouring cells, chromatin condensation and detachment from the growth surface. Saraste, (2002), indicated that these morphological changes occur due to molecular and biochemical events such as activation of caspases which cleave DNA into oligonucleosomal fragments and also cleavage of specific protein substrates which determine the integrity and shape of the organelles or cytoplasm. A panel of cell lines which comprised CHO, Caski, HeLa, H157, Jurkat, MCF7, MG-63 293T and 3T3 cells were respectively treated with complexes 15, 57 58 and cisplatin and their cell morphological was evaluated using light microscopy. The results showed that when

complexes 15 and 57 were exposed to CHO cells and Caski, the nucleus of the cells condensed and surprisingly, the cytoplasm of the cells swelled as shown in figure 4.1. When 293T cells were exposed to complexes 15, 57, 58 and cisplatin, they condensed and completely detached within 24 hrs. In general, the apoptotic morphology observed in all the cells upon exposure to the complexes were characteristic of features of cells undergoing apoptosis. The results of bioassays that were so far performed were in conformity with the observed morphological features.

One of the hallmarks of apoptosis is the degradation of DNA by endogenous DNases, which cleave internucleosomal regions of the DNA into fragments of 180-200 base pair (bp). 293T and Jurkat cells were treated with complexes 15, 57, 58 and cisplatin and were evaluated for DNA fragmentation. The results showed that the complexes 15, 57, 58 and cisplatin caused DNA fragmentation in these cells.

DNA fragmentation can also be evaluated by analysis of the cell cycle whereby cells appearing at the SubG0/G1 peak of the cell cycle are considered apoptotic. This maybe because the cell was halted in a particular phase of the cell cycle and became apoptotic. Following permeabilization fragmented 180-200 base pairs leak out of the cells and this results in a population of cells with reduced DNA content. Staining of such cells with DNA intercalating dye for example propidium iodide produces a DNA profile representing cells in phase G0/G1, S-phase and G-2M phase. Apoptotic cells however, appear as SubG0/G1 population to the left of the G0/G1 peak. In order for the peak to appear in the SubG1 area, cells must loose enough DNA. The disadvantage of the method however is that when cells enter apoptosis from the S phase or G2/M phase of the cell

cycle, or if there is an aneuploid population undergoing apoptosis, they may not appear in the SubG1 peak. Also cells that have lost DNA for any reason, other than apoptosis, will also appear in the SubG1 region. To test for DNA fragmentation cell cycle analysis method was done on HeLa cells. The HeLa cells were treated with complexes 15 and 58 and cell cycle analysis showed that the complexes 15 and 58 caused cell cycle arrest at the G0/G1 phase and subsequent DNA fragmentation.

To put everything together, both the palladium based complexes 15 and 57 and the platinum based complex 58 induced apoptosis in 293T cells in a similar fashion. Morphological observations and NRU_{50} revealed that the complexes also induced apoptosis in Caski, CHO, MG-63, H157, 3T3, MCF7, Jurkat and HeLa cells.

CHAPTER FIVE: GENERAL DISCUSSION

5.1 Herbal Medicine

Plants have been used as medicines for centuries and they have been considered to be potential sources for the screening and isolation of many drugs including anticancer agents. In the traditional system, medicines prepared from plants initially took the form of crude drugs such as tinctures, teas, poultices, powders, and other herbal formulations. It has been suggested by Gurib-Fakim, (2006), that the knowledge of herbal medicines for curing of specific diseases developed through trial and error over many centuries and that the specific plants to be used and the methods of application for particular ailments were passed down through oral history from generation to generation and eventually the information regarding medicinal plants was recorded in the herbals. In more recent history, the use of plants as medicines has involved the isolation of active compounds,

which began with the isolation of morphine from opium in the early 19th century. This discovery prompted more research into medicinal plant drug discovery and led to the isolation of early drugs such as cocaine, codeine, digitoxin, and quinine, in addition to morphine. Balunas and Kinghorn, (2005), said that the search for pharmacologically active compounds from medicinal plants still continues today. Taraphdar *et al*., (2001), Balunas and Kinghorn, (2005), reported that drugs isolated from medicinal plants are more favoured than synthetic drugs because they are isolated from living systems and therefore are biologically friendly than the totally "synthetic drugs," making them good candidates for drug development. The isolation and characterization of active compounds from medicinal plants have provided important anticancer agents that have been used as lead compounds in anticancer drug discovery. Screening of medicinal plants for anticancer compounds is important as it can lead to the isolation of novel lead compounds that can be used in anticancer drug discovery.

5.2 Screening of methanol extracts from the leaves of *Rhus laevigata*

In order to search for new antitumor compounds, methanol extracts from the leaves of *Rhus laevigata* were screened and tested for the presence of pro-apoptotic and anti-proliferative compound(s). The mechanisms underlying inhibition of cell growth and stimulation of apoptosis were evaluated.

The leaves of *Rhus laevigata* were extracted with organic solvents of increasing polarity and the extracts were tested for pro-apoptotic and anti-proliferative activities on CHO

cells. The results showed that the methanol extract induced apoptosis in CHO cells more than the other extracts indicating that the methanol extract contained the active compound(s) which were responsible for apoptosis induction. As a control, CHO cells were treated with 1% methanol only and compared with the plant methanol extract which contained less than 1% methanol. The results showed that cells treated with 1% methanol were not induced to apoptosis while those treated with the plant methanol extract were induced to apoptosis. These results indicated that apoptosis induction was due to compound(s) contained in the plant methanol extract and not due to methanol.

The plant methanol extract was also used to induce apoptosis in different human cell lines, Caski, HeLa, H157, Jurkat, MCF7, MG-63, 3T3 and 293T and mouse cell line 3T3. Morphology of the cells was evaluated following treatment of the cells with the extract in comparison with untreated controls. Morphological evaluation was based on the recommendations by Kroemer *et al*., (2005). The results showed that Caski and H157 did not show discernible apoptotic morphological changes when induced with 0.25mg/ml of the plant methanol extract while the other cell lines showed distinct apoptotic morphological features. It therefore meant that Caski and H157cells were more resistant to apoptosis induction when treated with the plant methanol extract than the other cell lines. This was also demonstrated by the NRU cellular viability/cytotoxicity assay, which also showed that an increase in the concentration of the extract was required in order to achieve 50% killing. To investigate the mechanisms underlying the dynamic morphological changes several tests were done.

MCF7, Caski, HeLa and CHO cells were tested for translocation of phosphatidylserine (PS) from inner to the outer layer of the plasma membrane following treatment of the cells with the plant methanol extract. Apoptosis was measured using the APOP*ercentage*™ apoptosis assay. The results showed that Caski were more resistance to apoptosis induction as compared to the other cell lines.

CHO cells were further tested for translocation of phosphatidylserine (PS) from inner to the outer layer of the plasma membrane following treatment of the cells with different concentrations of the plant methanol extract. Apoptosis was measured using the Annexin V binding assay. The results showed that the extracts induced apoptosis in the CHO cells in a dose dependent manner.

Mitochondrial depolarization, which is one of the early events that occur during apoptosis was investigated in CHO cells following treatment of the cells with the plant methanol extract. The results clearly showed that mitochondrial depolarization started within 30 minutes following exposure of the plant methanol extract to the cells and within three hours there was almost complete mitochondrial depolarization.

Caspase-3 cleavage was also analysed in CHO cells using anti-active caspase-3 antibodies following treatment of the cells with the plant methanol extract. The results showed that the extracts activated caspase-3. The positive results for caspase-3 were a good marker for cells undergoing apoptosis.

Further investigation of Poly (ADP-ribose) Polymerase-1 (PARP-1) was done on CHO cells and HeLa cells following treatment of the cells with the plant methanol extract. The results showed that specific active PARP proteins were more prominent in CHO cells than in HeLa cells, which meant caspase-3 activated PARP-1 to a greater extent in CHO cells than in HeLa cells. It also meant that CHO cells were more susceptible to apoptosis induction with the extract than the HeLa cells. The PARP results also show that treatment of the cells with the plant methanol extract induced DNA fragmentation as PARP is a DNA strand break sensing molecule.

Different cell lines CHO, Caski, HeLa, H157, Jurkat, MCF7, MG-63, 3T3, 293T and mouse cell line 3T3 were induced with the plant methanol extract to undergo apoptosis and their IC_{50} was determined using the NRU_{50} assay. The NRU_{50} assay showed that Caski were most resistant followed by H157 and MCF7. NRU_{50} assay also showed that Jurkat cells were the most sensitive followed by CHO, 293T and HeLa as shown in table 3.1.

Finally TUNEL staining of CHO apoptotic cells, indicative of DNA strand breaks which is one of the hallmarks of apoptosis was observed when the cells were induced with the plant methanol extract for 36 hours.

5.3 Conclusion

Traditionally the leaves of *Rhus laevigata* are used therapeutically as anti-inflammatory. The results provide strong evidence that the plant contains pro-apoptotic and anti-proliferative compound(s), and the results support the traditional use of the plant as anti-inflammatory. The results also mean that the plant contains compound(s) which may be used as lead compound(s) in anticancer drug discovery and therefore needs to be further evaluated.

5.4 Future work

This study has shown that the crude extracts from *Rhus laevigata* contain pro-apoptotic and anti-proliferative compounds. The methanol extracts induced apoptosis in CHO cells as well as in human cancer cell lines MCF7 and HeLa cells. The extracts however, failed to induce apoptosis in Caski cells. This screening work has therefore provided the basis from which more work needs to be done. Future work will therefore involve:

1. Characterization and elucidation of the structure(s) of the compound(s) contained in the crude methanol extract through bioassay guided fractionation.
2. Determination of the pathway through which the compound(s) induce apoptosis.

5.5 Alkylating agents as anticancer agents

The role of anticancer drugs is mainly to slow down and most likely halt the growth and spread of a cancer. Many anticancer drugs target DNA and these anticancer agents are

said to work by damaging the DNA of the affected cancer cells, by inhibiting the synthesis of new DNA strands so as to stop the cell from replicating as the replication of the cell allows the tumour to grow, and by stopping mitosis or stopping the actual cytokinesis (Hurley, 2002). Stopping mitosis stops cell division (replication) and may eventually halt the progression of the cancer. As reviewed in chapter one (section 1.7), alkylating drugs act by covalently modifying bases in DNA, often also resulting in DNA cross-links (Borst and Rottenberg, 2004; Drabløs *et al*., 2004). This causes mispairing of the nucleotides as a result, leading to mutations (Hurley, 2002). This alteration results in the DNA being fragmented by repair enzymes in their attempt to replace the alkylated bases (Drabløs *et al*., 2004).

Cisplatin is one of the alkylating agents, which has been used for many years as an antineoplastic agent. It induces its cytotoxic properties through binding to nuclear DNA (cisplatin–DNA adducts) that activate multiple signalling pathways. These pathways include those involving p53, Bcl-2 family, caspases, cyclins, CDKs, pRb, Protein Kinase C (PKC), Mitogen-Activated Protein Kinase (MAPK) and Phosphatidylinostol 3-kinase (PI3K)/Akt) and subsequently interfering with normal transcription, and/or DNA replication mechanisms (Gonzalez *et al*., 2001; Pasetto *et al*., 2006).

Minor variations in the structure of cisplatin have been found to have great effect on the anti-tumour activity. However, almost all trans-compounds tested have so far been found to be ineffective against tumours, while the cis-counterparts have been found to be active (Farrell**,** 1989).

5.6 Screening of cisplatin and palladium-based complexes for antineoplastic activities

In this study two palladium-based complexes, which were assigned numbers 15 and 57 and one platinum-based complex assigned number 58 were screened for their antineoplastic activity in comparison to cisplatin.

The complexes were tested on nine cell lines namely CHO, Caski, HeLa, H157, MCF7, MG-63, 293T, 3T3 and Jurkat cells for induction of apoptosis. Morphological evaluation using light microscopy showed that all the complexes induced apoptosis in the nine cell lines tested. 293T cells completely detached when they were exposed to complexes 15, 57, 58 and cisplatin.

One of the early events which occur during apoptosis is the translocation of phosphatidylserine (PS) from inner layer to the outer layer of the plasma membrane. APOP*ercentage*™ apoptosis assay is an assay which measures cells that are undergoing apoptosis or are already apoptotic. Each of the nine cell lines was treated with each of the complexes 15, 57, 58 and cisplatin and were evaluated for apoptosis induction using the APOP*ercentage*™ apoptosis assay. The results showed each of the complexes induced more than 60% apoptosis in each of all the nine cell lines.

The translocation of phosphatidylserine (PS) from inner layer to the outer layer of the plasma membrane can also be measured using the Annexin V binding assay which when used in conjunction with vital dyes such as 7-AAD can be used to discriminate early and late apoptotic cells. 293T cells were treated with complexes 15, 57, 58 and cisplatin and analyzed with Annexin V binding assay. The results showed that the cells were positive for Annexin V and it was possible to distinguish early and late apoptotic cells(necrotic cells). The results indicated that the complexes were inducers of apoptosis.

Mitochondrial collapse occurs early in apoptosis and is followed by decreased mitochondrial membrane potential. Changes in mitochondrial membrane potential were examined following treatment of 293T cells with complexes 15, 57, 58 and cisplatin for 1 hr and 5 hrs. The results showed that there was increased mitochondrial depolarization in cells treated with complex 57 and cisplatin at 5 hrs as compared to cells treated with complex 15 and 58 for the same time period.

293T cells were further treated with complexes 15, 57, 58 and cisplatin and examined for activation of caspase-3 which is involved in the execution of cell death. The results showed that all the complexes activated caspase-3 in 293T cells although complex 58 treated cells had reduced caspase-3 activity in comparison with the other complexes.

The NR assay was done following the procedure by Babich and Borenfreund, (1991); Rixe *et al*., (1996); Fotakis and Timbrell, (2005) to determine the NRU_{50} (IC_{50}). Each of the nine cell lines was treated with each of the complexes and examined using the NRU

assay. The NRU_{50} showed that cisplatin had less cytotoxicity to H157 and Caski cells while complex 58 showed less cytotoxicity to Caski cells. This was shown by the high retention of the dye by these cells. The NRU_{50} assay further showed that complexes 15 and 57 both palladium based complexes, were cytotoxic to all the cell lines as shown by the reduction of the cells in the retaining of the NR dye following exposure to the complexes. Jurkat and 293T cells were very susceptible to both the palladium and platinum based complexes.

Jurkat and 293T cells were further treated with complexes 15, 57, 58 and cisplatin in order to determine if the complexes induced DNA degradation in these c ells. DNA fragmentation was assessed by use of the TUNEL assay. The results showed that all the complexes induced DNA fragmentation in these cells.

Complex 15 (palladium-based) and complex 58 (platinum-based) were used to induce HeLa cells to undergo apoptosis and cell cycle analysis followed. Apart from showing DNA fragmentation cell cycle analysis can also be used to show the stage at which a compound can halt the cells from moving to the next phase of the cell cycle. Prospective drug candidates can then be design to target that particular stage. The results for the cell cycle analysis showed that both complexes caused cycle arrest at G0/G1-phase of the cell cycle, and that both induced DNA fragmentation.

5.7 Conclusion

The platinum and palladium-based complexes have been tested for induction of apoptosis in different cell lines derived from hamster, mouse and human. Both the palladium and platinum based complexes induced apoptosis in the panel of cell lines tested in a similar fashion particularly in 293T cells which were properly investigated. Although both the platinum and palladium complexes induced apoptosis in the panel of cell lines in a similar manner, the palladium-based complexes induced apoptosis more than the platinum complexes. Complex 58 however was also more cytotoxic than the parent compound cisplatin.

5.8 Future work

Despite the antineoplastic activity of these complexes, there was still the problem of solubility as the complexes were soluble with the aid of DMSO. Problems such as the ability of cells to detoxify cisplatin, failure of cisplatin to selectively kill cancer cells, solubility of cisplatin in body fluids and faster repair of cisplatin-DNA adducts among others pose a big challenge. That is why there is need to study other anticancer agents such as palladium complexes and see if they can overcome the problems associated with cisplatin as an anticancer agent. The palladium complexes therefore be further evaluated. To this end, future work will involve:

1. Synthesis of more palladium complexes that are soluble in water.

2. Testing of the complexes on normal cells and cancer cells and see if they can selectively kill only the cancerous cells.
3. Determination of the pathways through which the complexes induce apoptosis in different cell lines.
4. Investigation of the mechanisms through which the complexes halted cell cycle progression, which resulted in DNA fragmentation.

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