BIOACTIVITY GUIDED FRACTIONATION OF *Sutherlandia frutescens* EXTRACTS FOR THE INDUCTION OF APOPTOSIS

By

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Submitted in partial fulfillment of the requirements for the degree of Magister Scientiae (M.Sc.) in the Department of Biochemistry, University of the Western Cape.

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PREFACE

"I declare that, BIOACTIVITY GUIDED FRACTIONATION OF *SUTHERLANDIA FRUTESCENS* EXTRACTS FOR THE INDUCTION OF APOPTOSIS, is my own work, that it has not been submitted for any degree or examination in any university, and that all the sources I have used or quoted have been indicated and acknowledged by means of complete references".

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ABSTRACT

Sutherlandia frutescens popularly known as "cancer bush" or "kankerbos" is indigenous to South Africa and claimed by traditional healers to have wide therapeutic potential, most specifically against cancer. To verify these traditional claims we used apoptosis-based bioassays, organic solvent extraction, TLC (thin layer chromatography) and HPLC (high performance liquid chromatography) to evaluate extracts from *Sutherlandia frutescens* microphylla from different geographical populations and from selected *S. frutescens* subspecies.

The data demonstrate that a specific *Sutherlandia frutescens* extract has the ability to induce apoptosis in cultured cells. This investigation has suggested that the induction of apoptosis by the extract shows some specificity for transformed cultured cells. In addition, biological activity was traced by chemical fractionation of the crude extract to the chloroform and ethyl acetate fractions. Extracts obtained from *S. frutescens* microphylla from different geographical regions and subspecies were compared, there was variation in apoptotic activity between the extracts. This suggests that the apoptotic activity and hence possible anti-cancer activity of this plant depends on external environmental factors.

In summary, the presented findings are supportive of the claims made by traditional healers that *S. frutescens* has anti-cancer activity. However we have found that apoptotic activity was not present in all the plants, even of the same species, and hence further investigations are required to identify which factors cause certain plants to have greater apoptotic activity than others. Furthermore the extract analyzed in this study must be further characterized to identify compounds with anti-cancer activities.

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ABBREVIATIONS

7-AAD	7- amino actinomycin D
Annexin-V PE	Annexin-V Phycoerythrin
Apaf-1	Apoptosis activating factor 1
ATP	Adenosine Triphosphate
Bax	Bcl-2 associated x protein
Bcl-2	B cell leukemia -2
Caski	Cervical epidermoid carcinoma
Caspase	Cysteine aspartic acid –specific proteases
CD95	Cluster of Differentiation 95
c-FLIP	Cellular fas associated death domain-like ICE inhibitory protein
CHO	Chinese Hamster Ovary cells
DMEM	Dulbeco`s Modified Eagles medium
DMSO	Dimethylsuphoxide
DNA	Deoxyribonucleic acid
DR	Death Receptor
ELISA	Enzyme Link Immunosorbent Assay
FACS	Fluorescence Activated Cell Sorter
FADD	Fas-associated Death Domain
FCS	Foetal Calf Serum
GABA	Gamma hydroxyl Amino Butric Acid
HELA	Cervical epithelial carcinoma
HEPES	N-[2-hydrocyethlyl] piperazine-n-[ethanesulphonic acid]
HNMR	Proton nuclear magnetic resonance
HPLC	High Performance Liquid Chromatography
ITS	Internal Transcribed Spacer
kD	Kilodalton
LDH	Lactate Dehydrogenase
MAPkinase	Microtubular Associated Proteins Kinase
Mdm2	Murine double-minute 2
MS	Mass Spectroscopy
Myc	Mylocytoma

NFkB	Nuclear factor kB
NHF	Normal Human Fibroblast
NK-cells	Natural Killer
p53	Phosphoprotein 53
PARP	Poly (ADP) Ribose Polymerase
PBS	Phosphate Buffered Saline
PS	Phosphatidylserine
RPMI	Roswell Park Memorial Institute
TLC	Thin Layer Chromatography
TNF	Tumour Necrosis Factor
TTE	Tris- EDTA [Ethylene Diamine Tetracetic acid]
UV	Ultra violet light

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CHAPTER ONE

1. Introduction to traditional medicine

The current global incidence of cancer is about 10 million cases each year (Larsen *et al.,* 2003). Cancer in simple terms is the abnormal proliferation of cells beyond normal cell growth regulatory mechanisms. As the cells become cancerous they take an abnormal form in addition to continuous replication. In the later stages dividing cells invade parts of the body beyond their original locations. Genetic defects, chemicals and viruses, are some of the possible causes of cancer. However the actual mechanism of the disease is far from being fully understood (Lewin, 1997).

Medicinal plants, which were discovered in the traditional medicines practises of our societies, are the oldest known health care products. They are not only used locally to treat diseases, but also form basic materials for the synthesis of drugs or as sources for pharmacologically active compounds used in drug development (Harvey, 2000). In South Africa, known medicinal plants have been used locally for the management of cancer (Van Wyk *et al.*, 1997). These include *Sutherlandia frutescens*, which is the plant of interest in this study. The South Africa Medical Research Council is currently conducting research in plant extracts from *S. frutescensv var* microphylla to test efficacy in AIDS patients (Matsabisa, 2002). Extracts are traditionally administered as a tonic and it is claimed that it may be able to increase energy, appetite and body mass in people suffering from diseases like HIV (Bagozzi, 2002).

1.1 Traditional medicine and disease

Coronary heart attack, diabetes, hypertension, AIDS and cancer are some of the known problem diseases. It is of interest to note that they may also be partly prevented by utilizing compounds from plants which are biosynthetically derived from primary plant metabolites. The practise of utilizing traditional medicines played a key role for the protection and the restoration of health before the arrival of modern medicine (Mann, 2002). Acupuncture, traditional birth attendants, mental healers and herbal medicines are some of the practises that fall in this category (Abramov, 1999).

Herbal drugs gained prominence in the Western world because of their medicinal potential (Ramawat and Merillon, 1999a). South Africa and the Cape region in particular are rich in terms of biodiversity. *S. frutescens* commonly known as "cancer bush" or "kankerbos" is distributed along much of the western part of S. Africa (Figure 1.1) and was used by the Khoi San and the Nama people (Van Wyk *et al.*, 1997). Extracts from this plant has been used to treat stomach cancer, as a blood tonic and against other ailments like coughing, uterine disease and eye infection (Moshe *et al.*, 1998). Traditional healers and herbalists claim that this plant treats cancer and other diseases while, Thomson (2002), described *S. frutescens* as a poisonous herb. Folklore on this plant implies that the consumption of some of the decoctions are curative (Bieseubach, 1998) and these claims necessitate detailed investigations into the activity of this plant. *S. frutescens* plants throughout its range must therefore

first be tested for activity and plant populations which show biological must be identified.

Figure 1.1: Regional distribution of *Sutherlandia. frutescens* within South Africa. The plant is predominantly distributed along the western part of S. Africa, particularly along the west coast and parts of the Northern province and Namibia, the distribution is indicated in red (Van Wyk *et al*, 1997).

1.2 Therapeutic potential of medicinal plants

Plants have traditionally been a valid and good source of compounds for the treatment of different diseases (Bum *et al.,* 2001). Plant cells produce primary and secondary metabolites. Secondary metabolites are alcohols, phenolics, steroids, lignans, tannins phytosterols and flavonoids. These molecules are

produced by plants in response to abiotic and biotic stimuli such as pathogens (Wink, 1999a). Secondary metabolites are biosynthetically produced from primary metabolites synthesised in specialised cells at a particular developmental stage (Bourgaud *et al.*, 2001). Synthesis of these products can be very specialised and localized to specific organs or cells. Many of theses metabolites have effects on mammalian cells and tissues hence their medicinal and/or toxic properties (Ramawat and Merillon, 1999a).

Many modern drugs presently on the market have their origin from medicinal plants, among the most familiar and common are morphine, codeine, atropine, cocaine, caffeine, quinine, ergotamine, vincaleucoblastine and taxol. Some of these plant-derived compounds are distributed in the whole plant while others are concentrated in specific areas either in leaves, roots, seeds, pods, flowers or the stem (Wink, 1999c).

2. The role of medicinal plants in cancer and apoptosis

2.1 Introduction

Valuable medicines have been obtained from plants that show anti-neoplastic and neoplastic activity. Drugs belonging to the former catergory prevent the formation of new cancer cells while drugs belonging to the latter category destroy existing cancer cells. Taxol, which is obtained from the bark of the yew tree *Taxus brevifolia* and *Taxus baccata* demonstrates good anti-neoplastic activity (Ramawat and Merillon, 1999b). *Cantharanthus roseus*, which contains

viblastine, is a well-known neoplastic drug (Dark *et al.*, 1997; He *et al.*, 2001). Previous work on garlic to demonstrate anti-cancer activity showed that, certain extracts from garlic have anti-proliferative effects on cancer cells (Pinto and Rivlin, 2001). It was speculated that certain compounds in garlic might inhibit the enzyme cytochrome p450 when this enzyme activates carcinogens such as nitrosamines, hydrazines and halogenated hydrocarbons. The compounds from garlic may also inhibit the formation of DNA adducts which are associated with carcinogenesis. Pinto and Rivlin (2001) further showed that extracts of garlic could block cell cycle progression through signal transduction pathways. This can lead to the expression of the nuclear transcription factor NF-kB and promote apoptosis (Ueda *et al.*, 2003). NF-kB is a member of the oncogene family that regulates genes that encode proteins associated with the immune function, inflammation and cell proliferation (Ageciras *et al.*, 2002; Adams, 2003).

2.2 The role of medicinal plants triggering apoptosis as anti-cancer agents.

The possibility of apoptosis in cells to be influenced by plants remains a crucial issue and needs investigation. Cellular mediated complexes and processes can possibly be modified by extracts that contain complex compounds from medicinal plants (Khan *et al.*, 2000). This can potentially be of significant therapeutic value to combat cancer (Chakrabarty *et al.*,2002). Nutraceuticals, which are dietary compounds from plants like soya bean, garlic, ginger, green tea have also been suggested by epidemiological studies to reduce the incidence

Medicinal plants like *Ochrosia* and *Aspidospema* species contain secondary metabolites called ellipticines, which inhibit topoisomerases and have the ability to intercalate DNA (Wink, 1999b). They cause selective inhibition of phosphorylation of p53 protein and this results in higher accumulation of unphosporylated protein leading to the expression of apoptosis-inducing genes and consequent cell death. Zhao *et al.* (2003) showed that boswellic acid acetate extracted from *Boswellia carteri* can induce apoptosis in metastatic melanoma and fibro-sarcoma cells causing chromatin condensation.

However, not all plant extracts cause programme cell death in animal cells for therapeutic benefit (Steenkamp, 2003). Certain plant groups may contain very toxic compounds. Metabolic poison manifested by necrosis can be differentiated from apoptosis. Necrosis is a form of pathological injury that causes groups of cells to swell, disintegrate and causes exudative inflammation (Gerschenson and Rotello, 1992). The damaging inflammatory effect of necrotic cells can even cause the loss of life of the organism (Proskuryakov *et al.*, 2003).

There are a variety of plants that contain compounds that cause injury or death to mammalian organism if administered. Steenkamp *et al.* (2001 and 2003) showed that there are a variety of South. African plants used in traditional medicine that contain toxic compounds. *Senecio latifolius* is toxic as it contains pyrrolizidine alkaloids. Doses above 4.5mg / 1ml cause necrosis and chronic

fever, lower doses of up to 350 ng / 1ml treatment cause teratogenic or carcinogenic effects in human hepatoma cell lines. *Adenia gummifera* contains modecin, which causes acute centrilobular necrosis and hypoglycemia. *Pteridium aquilinum* contains ptaquiloside, a sesquiterpenoid, which can be carcinogenic, capable of destroying the bone marrow and cause internal bleeding. *Clivia miniata* contains lycorine (isoquinoline alkaloid), which causes paralysis. *Bridelia micrantha* contains dephinidin methylsalicylate, which causes death within 4 hours of ingestion.

3. Apoptosis and the development of cancer

3.1 The phenomena of apoptosis and cell death

Apoptosis is a Greek word for "falling apart" (apo-, apart; ptosis, falling) and the term for the biological phenomenon was proposed by Kerr *et al.* (1972) When apoptosis is orchestrated there is a complete collapse of cells characterized by membrane blebbing, cell shrinkage, protein fragmentation, chromatin condensation and DNA degradation followed by rapid engulfement of cell debris by neighbouring cells (Christop, 2003). Impairment of the apoptotic mechanism ultimately generates pathological conditions via developmental defects like auto-immune diseases, neurodegeneration or cancerous neoplasia (Reed *et al.*, 2001).

Any pathological or physiological stimuli including hormones, growth factor deprivation, chemotherapeutic agents and ionising radiation can trigger

apoptotic stimuli, initiating a cell death pathway (Schwartz and Osborne, 1993). Cells that get caught in this process are removed from the system to avoid disrupting tissue architecture or normal function (Gerschenson and Rotello, 1992). The stage in a cell cycle at which a cell is programmed to die is critical and depends on intrinsic and extrinsic signals that can activate its cell death pathways (Los *et al.*, 2003). Activation of this apoptotic signal can be caused by aging, defective proteins or mutation. The apoptotic process causes exposure of the inner cell membrane phospholipid molecule phosphatidylserine to the outer membrane of cells and this molecule can be detected by phagocytic lectins as a cell death markers (Martin *et al.*, 1994; Savill and Fadok, 2000).

However, the apoptotic machinery may not always be efficient or protective. Some cells evade this immune surveillance and this can be devastating to the life of the organism and is a chief characteristic of cancer cells. However, this cellular failure can sometimes be corrected by an extrinsic (i.e. therapeutic) induction of apoptosis.

Physiological mechanism of cell growth, embryogenesis, synaptic interactions, oncogenesis and immune deficiency are crucially linked to apoptosis. During embryogenesis apoptosis is crucial in the sculpture of body cavities and structures which are needed at different stages through the embyrogenic process. Developmental stages are also dependent on apoptosis, the tadpole losing its tail to become a frog is an example (Collins and Rivas, 1993;

Jacobson *et al.,* 1997). Cells undergo cytoplasmic shrinkage when they die and are rapidly engulfed and removed out of the system by phagocytes. This happens before they have time to burst and spill their cytoplasmic content, which can elicit a damaging inflammatory response in the form of necrosis (Savill and Fadok ; 2000 Proskuryakov *et al.,* 2003).

3.2 Mechanism of Apoptosis

There are several mechanisms for apoptotic activation. Two general and well-researched mechanisms will be discussed. The first mechanism uses CD 95 ligands to initiate the process (Figure 1.2). The mechanism of apoptosis in this pathway involves a proteolytic cascade of caspases (Leblanc, 2003; Rami, *et al* 2003). These are enzymes with aspartate specificity that get amplified as a result of their proteolytic cleavage of one another in sequence.

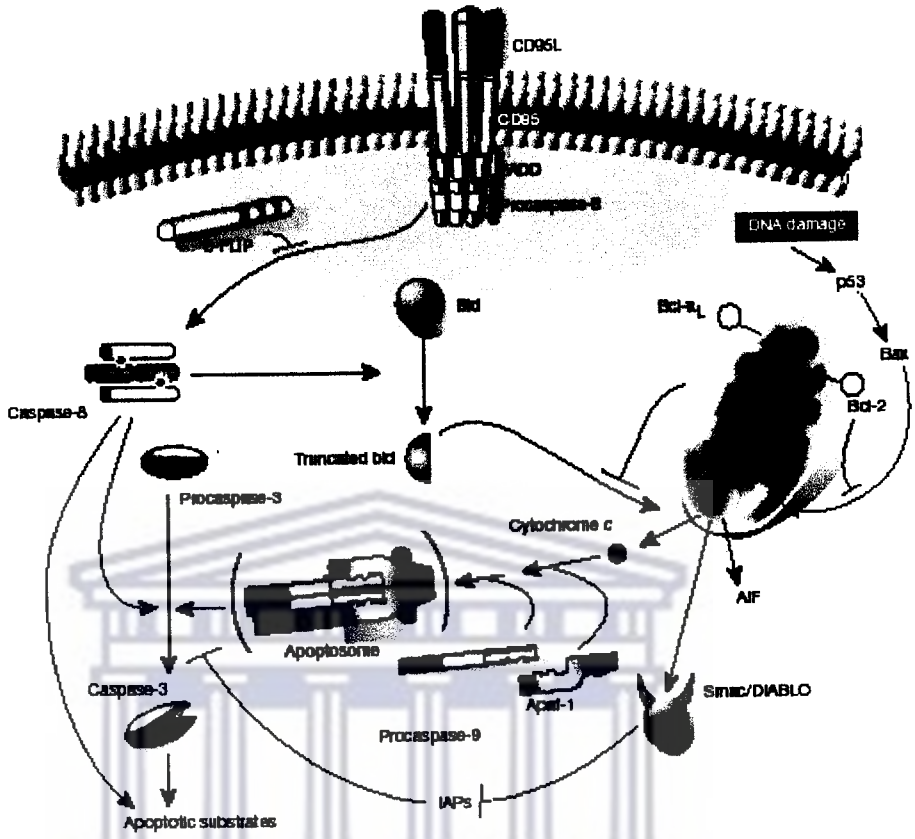


Figure 1.2: The activation of apoptosis through two major pathways utilizing the CD 95 ligands or TNF (Hengartner, 2000). CD 95 lingand binds to CD 95 receptor causing receptor clustering. Clustered receptors initiate a death complex by the activation of several other enzymes through c-FLIP. The CD 95 ligand receptor complex generates the activation of central death caspase 3 which is regulated by the apoptosome complex. DNA damage causes activation of a TNF ligand to bind to the TNF receptor. This complex activates the p53 pathway that causes cytochrome c release from the mitochondria and promotes apoptosis by the formation of apoptosome complex (Hengartner, 2000).

The binding of CD 95 ligand to the receptor causes receptor clustering and the initiation of a death signalling complex. FADD is an adaptor molecule that carries a death effector domain which binds to CD95 and Fas is a 45 kD transmembrane protein capable of binding to its death associated domain to form FADD (Lautrette *et al.,* 2003). This causes multiple procaspase-8 molecules to aggregate, which results in caspase-8 activation. Caspase-8 activity is in turn controlled by c- FLIP. If caspase-8 is not regulated, it allows procaspase-3 to form activated caspase-3, this is a central caspase, and activation of caspase-3 indicates that the death process is certain. The regulation of capsase-3 is controlled by the apoptosome complex, which promotes the apoptotic event.

The second mechanism of apoptosis is through TNF activation which results from DNA damage and acts through the p53 pathway. When DNA is damaged, the damage is indicated by activation of the tumor necrosis factor (TNF) which binds to its receptor to activate the p53 pathway (Vogelstein *et al.*, 2000; Work and Kaye, 2002). The p53 protein in turn activates the Bax protein. The Bax protein causes changes in the mitochodrial membrane by de-polarising the membrane, which in turn causes the release of cytochrome c. Cytochrome c then acts with procaspase-9 to form the apoptosome complex (Harris, 1993). This complex is directly connected to caspase-3 that ultimately leads to cell death (Hengartner, 2000).

3.3 Mitochondrial control of apoptosis

Cytochrome c is a nuclear-encoded mitochondria protein that is involved in the electron transfer chain. It plays an important role in oxidative phosphorylation as electron shuttle between complex cytochrome c reductase and cytochrome c oxidase. Cellular stress can cause cytochrome c release into the cytosol during apoptosis (Harris, 1993). Cytochrome c interacts with apoptotic protease-activating factor (Apaf-1) and procaspase-9 to form an apoptosome complex shown in Figure 1.2. The complex activates caspase-9 that subsequently activates caspase-3 to induce the fragmentation and degradation of genomic DNA. Other proteins like Bcl-2 can also regulate the release of cytochrome c through direct interaction with each other (Preston *et al.*, 2001).

Cells that undergo mitochondrial rupture risk death through the apoptotic mechanism. There is a loss of electrochemical gradient across the inner membrane causing the production of reactive oxygen species and decline in ATP production and the release of cytochrome c which ultimately leads to cell death (Green and Reed, 1998; Hengartner, 2000).

3.4 Activation of Apoptosis

3.4.1 Lymphocyte activation

Lymphocytes become activated in response to viral infection of other cells. In order to prevent the virally infected cell from multiplying and spreading the infection to other cells the lymphocytes can induce cell death. This happens

when the lymphocyte secretes various proteins onto the surface of the infected cells. Peforin is one such protein, this protein aligns along a cellular transmembrane channels to allow other proteins such as granzyme B, which is involved in apoptosis, to enter. Perforin perforates the cell wall and due to the change in the osmotic gradient causes cell swelling. Swelling in turn causes proteolytic cleavage and activates other pro-caspases to start a proteolysis of the death cascades (Leblanc, 2003).

Lymphocytes also induce cell death by producing a protein, Fas ligand, that binds to Fas receptors on the surface of the target cell causing receptors to aggregate. Aggregated receptors recruit adapter proteins from the cytoplasm, which in turn recruit procaspases-8 molecules into the aggregate (Dianzani *et al.,* 2003). The cluster of pro-caspase molecules cleave and activate each other to begin the suicide sequence.

3.4.2 Extracellular Activation

Through cell surface interaction extracellular survival factors may bind to cell surface receptor proteins such as Bax (White and Strayer, 2002). Bax protein is a Bcl-2 homologue that resides in the cytoplasm (Adams and Cory1998; Beere and Green, 2003). In response to stimuli it generates pores in the lipid membrane and causes the release of cytochrome c from the mitochondria leading to *Bax* gene phosphorylation (Bernstein *et al.,* 2002). *Bax* gene

phosphorylation in turn causes activation of protein kinase and promotes apoptosis (Raff, 1998).

A major apoptosis pathway involves cell surface 'death receptors' (DR). After DNA damage, an apoptotic signal gets transmitted through the specific 'death ligand' CD 95 (Pfeffer, 2003). The CD 95 ligand binds to the Fas CD 95 death receptor or the TNF receptor. Members of these receptor families are TNF-R1, TNF-R2, and the Fas CD 95 death receptors. The extracellular ligand binds to the receptor causing the receptor to interact with a homologous domain of the adapter protein (Gulbins, 2003) and as a result, caspases get recruited. This upstream event subsequently initiates a cascade of downstream effector caspases, which cleave cellular proteins and ultimately cause cell death (Ashkenazi and Dixit, 1998).

3.5 Mutations of genes involved in apoptosis and cancer development

3.5.1 Mutations in the *p53* gene

The p53 gene is involved in apoptosis and it acts as a tumour suppressor (Krall *et al.*, 1994; Lebedeva *et al.*, 2003). A functional p53 protein is therefore necessary so that the cell death programme is always operational (Mullauer *et al.*, 2001). Mouse embryo fibroblasts derived from p53 knock-out mice show excessive induction of apoptosis by c-Myc (mylocytoma) leading to neurodegeneration. Reintroduction of the *p53* gene in *p53* negative cells expressing the c-Myc protein rapidly triggers normal apoptosis.

Furthermore, *c-Myc* transgenes (overexpression of c-Myc protein) induce tumours in mice more effectively in a p53 null background. This implies that the p53 confers an advantage to cells expressing c-Myc (Klefstrom *et al.*, 1997) and suggests that loss of p53 may substantially increase the mutation rate that will generate clonal variants and enhance uncontrollable growth (Gerard and Trevor, 1998). Malignancy of cancer cells increases as they accumulate mutations in the p53-encoding gene (Lane and Lain, 2002).

3.5.2 Mutations in the *TNF* gene

The Tumour Necrosis Factor, (TNF), is a cytokine, which is secreted during immune cell (microphage) stimulation in response to infection (Pfeffer, 2003). The TNF causes endothelial cells to express adhesion molecules for leukocyte binding and exert a pyrogenic effect. The TNF ligand binds to membrane receptors and triggers pathways that have a cytotoxic effect (Luo *et al.*, 2003). The TNF pathway leads to the activation of NF-kB protein (nuclear factor kappa beta), which in turn causes the periodic fever syndrome (Mullauer *et al.*, 2001; Haefner, 2002).

A mutation of the *TNF* gene results in the reduction of the ligand binding activity of cytokines. This causes cancerous cells to escape leukocyte binding and these cancerous cells have damaging metastatic (invasive) properties.

3.5.3 Mutations in the *Fas* gene

The Fas molecule is a transmembrane receptor protein. Binding of its natural ligand, Fas ligand (Fas L) can trigger apoptosis (Green and Ware, 1997). The apoptosis signal is transmitted via an intracellular death domain that interacts with a homologous motif which is the adapter protein FADD (Yonehera, 2002). This in turn recruits procaspase-8 and causes aggregation of procaspase-8 within the Fas signalling complex. Creation of this complex triggers activation by autocleavage leading to a downstream event of caspase activation (Luo *et al.*, 2003). Mutation of the *Fas* gene in tumour cells may help them to escape immune tracking from T- and NK-cells by making the cells resistant to the Fas ligand binding to the Fas receptor (Mullauer *et al*, 2001).

3.5.4 Mutations in the *Bax* gene

The Bax protein in response to stimuli generates pores in the lipid membrane and causes the release of cytochrome c (Adams and Cory, 1998; Beere and Green, 2003, Bernstein *et al.*, 2002). A mutation in the *bax* gene causes human colon carcinomas. The mutation occurs in the microsattellite region (short nucleotide repeat sequence) due to faulty DNA mismatch repair (Mullauer *et al.*, 2001). The microsatellite mutator phenotype leads to genetic instability due to accumulations, deletions and insertions in this region (Rampino *et al.*, 1997).

4. Elements of the Apoptosis mechanisms as therapeutic targets for anti-cancer drugs

4.1 Targets for anti-cancer drugs

Natural products remain one of the richest sources of drug leads (Raskin *et al.*, 2002). Of the 520 new approved drugs between 1983 and 1994, 39% were derived from natural products (Harvey, 1999). Drugs to treat cancer are designed to interact with tumour cells at specific sites, this interaction controls cell proliferation by creating negative and positive cell signalling pathways with minimal cytotoxic side effect that are associated with conventional cancer chemotherapy (Chen and Fang, 2002). It is hoped that novel molecular targets are to be cancer specific, however to-date there are no "smart bullet" drugs to target cancer cells selectively (Thacker, 2003).

Potential molecular targets for anti-cancer drug discovery can be grouped into the following categories: Growth factor receptors; (tyrosine kinases and serine/theronine kinases); signal transduction pathway targets; apoptosis related targets, tumour angiogensis, metastatic and cell span targets (Buolamwini, 1999).

4.2 Apoptosis targets

Apoptosis related targets include ligands, such as CD95, tumour necrosis factor and related apoptosis inducing ligands as well as downstream molecules such as caspases and Bcl-2 family members (Los *et al.*, 2003). In addition, *Mdm2*, a

cellular oncogene is transcriptionally induced by p53 protein and regulates p53-induced apoptosis (Lane and Lain, 2002). Therefore interference with the *Mdm2* gene function may enhance the apoptotic action of p53 (Buolamwini, 1999).

Mitochondria contribute significantly to cancer cell physiology (Preston *et al.*, 2001). In the event of cytochrome c release from the mitochondria it causes the activation of caspases with aspartate specificity (Glaser and Weller, 2001). Larger numbers of these specific substrates includes poly-ADP ribose polymerase (PARP), which is a 116-kD-repair enzyme that gets cleaved during activation of apoptosis. The activation of PARP generates cleavable moieties that can be potential targets for drug development (Vermeulen *et al.*, 2002).

Angiogenesis is the mechanism of blood vessel formation in cells. Vascular targeting of these vessels can suffocate tumours by cutting their supply of oxygen (Ravi, 2001). The response to this creates an anti-angiogenic effect. (Matter, 2001). Molecules or compounds that antagonise the angiogenic action could also acts as potential therapeutic agents (Dark *et al.*, 1997; Hanahan and Weinberg, 2000).

5. Description of *Sutherlandia frutescens*

5.1 Systematics of *S. frutescens*

S. frutescens is a small shrub of a metre in height with small dense hairy leaves. This gives it a silvery appearance (Van Wyk *et al.*, 1997). The leaves branch from a common stem and each contains numerous leaflets. Depending on the nature of the species some bear pink flowers and others bear white flowers, which are followed by small balloon-like pods, see Figure 1.3.

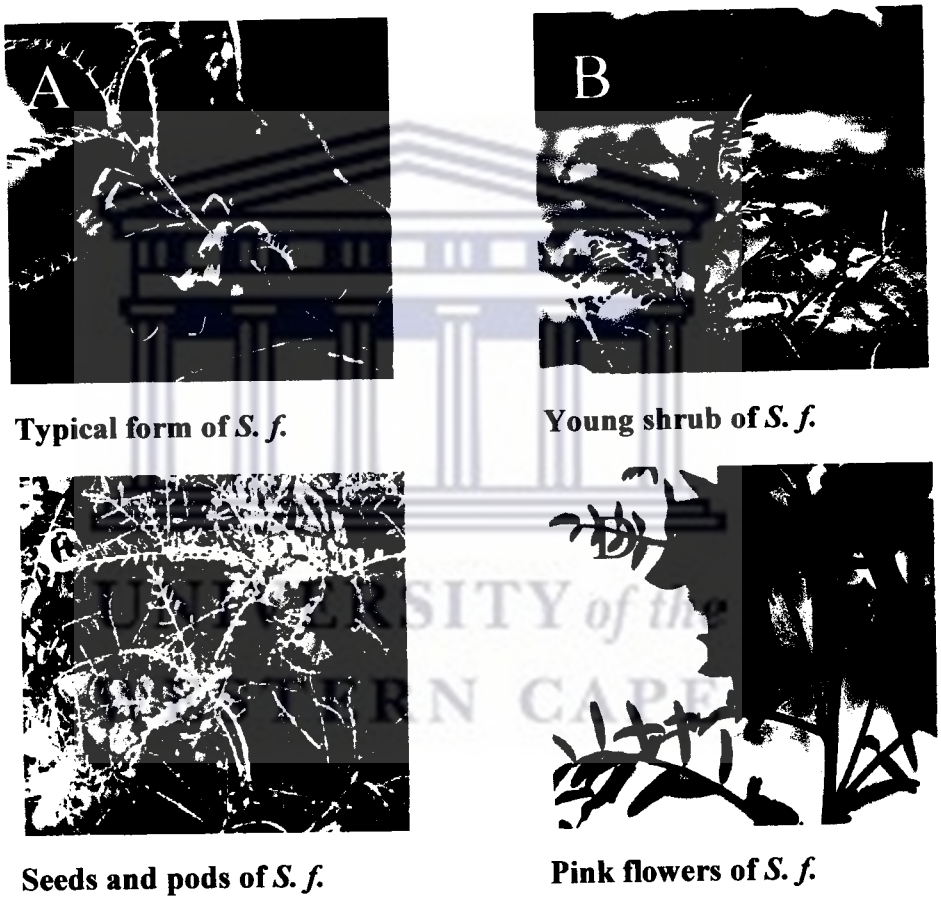


Figure 1.3: Mature form of *S. frutescens.* (A) Branch with flowers, (B) Young *S. frutescens* shrub, (C and D) Balloon pods, seeds and pink flowers.

The genus *Sutherlandia* was named in honour of James Sutherland, an Edinburgh botanist (Salter, 1950). *Sutherlandia frutescens* was reclassified as *Lessertia frutescens*, family *Fabaceae* and subfamily *Papilionoideae* (Goldblatt and Manning, 2000). According to the work by Moshe *et al.*, (1997), *Sutherlandia frutescens* consisted of the following seven taxa all of which are endemic to Southern Africa: *S. frutescens var incana, S. humilis, S. microphylla, S. Montana, S. roboster S. speciosa,* and *S. tomentosa.*

The genus *Sutherlandia* is closely related to *Astragalus,* a genus of cosmopolitan shrubs that is the largest genus of flowering plants. It has diversification of about 2500 species.

The nuclear ribosomal internal transcribed spacer (ITS) region can be sequenced to analyse the different phylogeny of species (Wanntorp *et al.*, 2002). The system gives the opportunity to utilize nuclear ribosomal (nrDNA) regions of 18S-26S subunits for the study of genetic variation amongst different plant species (Simmons and Freudenstein, 2003). According to Baldwin (1995) the two spacers of this region ITS1 and ITS2 each have less than 300bp. This creates the need for amplification by polymerase chain reaction using universal synthetic primers.

Sanderson and Wojeciechowski (1996) used the ITS system to generate a molecular phylogeny (Kelly, 1998) which was used to determine the structural

relatedness and evolutionary divergence of subspecies of *Sutherlandia frutescens* and *Astragalus* (Anderson *et al.,* 1999). Analysis using phylogenetic studies coupled with nuclear ribosomal internal transcribed spacer (ITS) sequences shows a marked diversification rate, between *Sutherlandia frutescens* and species of *Astragalus* (Sanderson and Wojciechowski, 1996).

The divergence of sub families of different plant species can also be analysed using chloroplast DNA (Torrell *et al.*, 1999). This analysis can be used to study the geographical distribution and evolution of closely related plants (Noyes, 1999) that could amount up to some 400-500 specific and sub-specific taxa (Steane, *et al.,* 1999).

5.2 Secondary metabolites in *Sutherlandia frutescens*

According to Van Wyk *et al.* (1997) the active ingredient that may be responsible for the anti cancer activity of *S. frutescens* are canavanine and pinitol. Canavanine is a non-protein amino acid (Jang *et al.,* 2002), which may be incorporated into cellular proteins resulting in functionally defective proteins. Previous work by Swaffar *et al.* (1994) demonstrates that canavanine is capable of inhibiting growth in human pancreatic cancer cells. However, Thomson (2002) described canavanine, pinitol and GABA as very toxic components of *S. frutescens,* based on the observation that there is canavanine toxicity in rats treated with these compounds.

6. Aims

1. To test if plant extracts isolated from *Sutherlandia frutescens* induces apoptosis.
2. Evaluating possible apoptotic activities of *S. frutescens* microphylla extracts collected from different geographical populations and also from selected *S. frutescens* sub-species.
3. Attempt to validate the claim by traditional healers that *S. frutescens* has anti-cancer activity.

CHAPTER TWO

2. Material and methods

2.1 Materials, media and plant samples

Acetic acid (Catalog 1.00063.1000), acetone (Catalog 1.00012.1000), chloroform (Catalog 1.02432.1000), n-butanol (Catalog 1.01988.1000) and ethyl acetate (Catalog 1.10972.1000) were purchased from MERCK (Milnerton, South Africa). Foetal calf serum (Catalog 1640-063), Hams F12 medium (Catalog 3155-023), RPMI medium (Catalog 21875-034) and DMEM medium (Catalog 41965-039) was obtained from Gibco BRL (Laboratory Specialist Services, Cape Town South Africa). Canavanine (Catalog 2219-31-0) and Staurosporine (Catalog 6996-74-1) was supplied by Sigma (Cape Town, South Africa). DMSO (Catalog LDSU 0005) was purchased from B&M Scientific (Cape Town, South Africa). ApoPerecentage was supplied by Biocolor (Belfast, UK).

PBS (Phosphate–Buffered Saline Catalog 10010-015) was obtained from Gibco BRL (Laboratory Specialist Services, Cape Town South Africa)

Molecular grade agarose (Catalog V3125) was purchased from Promega (White Scientific, Brackenfell, South Africa) and 20 cm x 20 cm TLC plates (Catalog 1.11798.0001) were obtained from Merck (Milnerton, South Africa). LDH Kit (Catalog 1644793) was obtained from Roche (Roche Diagnostics, Pinelands South Africa). Fresh and dried plant material was obtained from Kirstenbosch Botanical

Gardens (Cape Town, South Africa) and the UWC Herbarium (Bellville, South Africa- voucher specimen of Dr Gillian Scott number 29, 212, 191 and 206).

2.2 Tissue culture

2.2.1 Cell culture

The cell lines used were CHO 22 (Chinese Hamster Ovary Cells), Caski (Cervical Epidemoid carcinoma cells), Y10 (Variant form of CHO 22), Jurkat T-cell (acute lymphoblastic leukaemia) and Normal Human Fibroblast (NHF-Primary skin culture), see Table 2.1.

Cell Lines	Nature	Foetal Calf Serum	Penicillin/ Streptomycin	Media	Other Conditions	Humidity at 37°C
CHO / Y10	ADHERENT	5 %	0.1 %	HAMS F-12	5 % CO_2	100 %
CASKI	ADHERENT	10 %	0.1 %	RPMI	5 % CO_2	100 %
JURKAT-T	SUSPENSION	10 %	0.1 %	RPMI	5 % CO_2	100 %
NHF	ADHERENT	10 %	0.1 %	DMEM	5 % CO_2	100 %
HELA	ADHERENT	10 %	0.1 %	DMEM	5 % CO_2	100 %

Table 2.1: Cell lines used and their respective growth conditions.

2.2.2 Propagation of cell lines

Cells were grown in appropriate media as indicated in Table 2.1 above supplemented with 5 % FCS and 0.1 % penicillin and streptomycin in 5 % CO_2 at 37° C. When cells were confluent they were washed twice with PBS and treated with 10 % trypsin (adherent) until the cells detached from the bottom. The action of the trypsin was stopped by addition of 2 ml of media, then the cells was collected by centrifugation and the cell pellet re-suspended in the appropriate media (Davis, 1994). Cells were subsequently cultured at dilutions ranging from 1/5 to 1/15 depending on the requirements of the experiment and visualized routinely using a 200 X objective of a conventional inverted light microscope (Nikon/TMS-F Model, Nikon, Japan).

2.3 Preparation of plant extracts

2.3.1 Aqueous extract

Leaves, stems and flowers of the whole plant were collected and washed with distilled water, dried in a ventilated oven for 72 h at 35°C and ground to a fine powder using a Philips Cucina (HR1731/37) domestic blender (Philips, IND/BRAS, Brazil). The plant material was passed through 850 µm pore size sieve and 10 g of powder were extracted in 1 L of boiling water and allowed to cool prior to centrifugation at 1000 x g

The supernatant was freeze-dried for 72 h in the VIRTIS 5L freeze drier (VIRTIS, New York, USA) to obtain a dried powdered plant extract. Extracts were kept in desiccators until needed.

Different doses for testing were freshly prepared from extract stocks of 40 mg / ml stored at – 20° C (449 mg of freeze dried plant material dissolved in 11.225 ml of distilled water to give a final concentration of 40 mg / ml).

2.3.2 Organic solvent extraction

100 ml of acetone: water (4:1 v/v) was added to 10 g of dried plant extract in a 250 ml conical flask and was kept on a shaker at room temperature for 6 h and then afterwards allowed to stand over night. The supernatant was carefully removed and the residue retained in the flask. Extraction of acetone and water was repeated twice to the remaining residue to completely extract the hydrophilic and the lipophilic compounds.

Combined aliquots of 300 ml acetone: water (4:1) was reduced to 50 ml (aqueous extract) by rotary evaporation (Rotary Vacuum Evaporator NE-1, EYELA, Tokyo, Japan). The aqueous solution was extracted successively with equal volumes (50 ml) of solvents with increasing polarity in order of petrol ether, chloroform, ethyl acetate, n-butanol and water in separating funnels. Three subsequent extractions per solvent (50 ml each) were done. Extraction volumes (150 ml) were pooled and reduced to near dryness on a rotary evaporator (< 40° C) and taken up in 5 ml methanol. The residual (extracted) H_2O phase was also reduced to near dryness, weighed and taken up in 5 ml methanol.

2.4 Chromatography

2.4.1 Thin Layer Chromatography

Phase silica gel F_{254} TLC plates (2.5 cm x 10 cm) (Merck, Darmstadt, Germany) were loaded with 2 – 5 µl of 40 mg / ml of the crude extract. Each plate was developed in a chromatographic tank through a saturated volatile mobile phase as monitored by the Whatman filter paper. Plates were examined under UV (254 nm), using various spray reagents. Solvent compositions were chosen with relative mobility (R_f) of 0.5 and 0.2 for the solvent front and the retained spot respectively.

Four fractions were demarcated, scraped and centrifuged at 1400 x g and the supernatant was evaporated to near dryness on a rotary evaporator. The concentrates were resuspended in 1 ml methanol for testing (Cannell, 1998).

2.4.2 High Performance Liquid chromatography

The chromatographic system used was the Beckman HPLC system with management system Gold V 310 (Beckman USA). This consists of the 501 autosampler 340 organiser, 2 x 110 solvent delivery system module and the Knauer variable wavelength monitor. The latter consists of a double pump program solvent model 126, diode array detector, analog interface module 406 and Samsung computer MZ4671. The HPLC column used was the analytical C18 column (250 mm by 10 mm Higgins column) and Hypercarp C18 column (250 mm x 4.6 5 µm; ThermoHypersil column) supplied by Anatech, Cape Town.

Chromatographic conditions include the mobile phase solvent A (1 % acetic acid in water (v/v)) and solvent B (100 % methanol) at a gradient of 25 % to 90 % methanol (flow rate: 0.8 to 1 ml / min.; injection volume 20 µl – 400 µl; detection: 270 nm).

Fractions were collected using the FOXY JR 202F20077 Model fraction collector (ISCO, Nebraska, USA). Sampling time ranged from 0.25 min. to 5 min. (Chang *et al.*, 2001; Theodoridis *et al.*, 2002)

2.5 Apoptotic assays

2.5.1 ApoPercentage™

CHO 22 cells (0.25×10^6) were seeded in a 96-well tissue culture plate. The cells were confluent for 24 h before treatment with the *S. frutescens* extracts. The concentrations varied between 1 mg / ml and 10 mg / ml. For each concentration, wells were set in triplicate and treated for 5 min. to 6 h. Then cells were gently washed with 2 x PBS and 100 µl apoPercentage™ dye was added according to the manufacturers instructions and incubated for a further 60 min. at 37° C. After 60 min. staining, the cells were washed twice with 100 µl PBS to remove un-trapped dye. The cells were visualized under a light microscope and photographs were taken using a 200x objective lens. Dye Release Agent (100 µl) was added (Biocolor, UK) and incubated for 10 min. at room temperature. The cell bound dye recovered into solution was measured using a micro-titreplate colorimeter sero-wel (Bibby sterilin, U.K). Absorbance was read at 550 nm (maximum dye absorbance) and 625 nm (minimum dye absorbance). The difference between these two values was taken as

the real absorbance for the recovered dye that was trapped in the cells. The results were plotted on a line graph with absorbance on the Y-axis and the different concentrations of *S. frutescens* extract on the X-axis. (see manufacturers manual ApoPercentage™ apoptosis Assay, biocolor UK)

2.5.2 Electrophoresis of DNA

Cells were grown in 25 cm^2 flasks and treated for the required time periods with respective apoptosis inducers. The culture medium was removed from the flasks and transferred into 1.5 ml micro-centrifuge tubes. The cells in the medium were collected by centrifugation at 10 000 x g for 5 min. The cell pellet was re-suspended in 0.5 ml TTE followed by vigorous vortexing. Then 0.1 ml ice-cold 5 M NaCl and 0.7 ml ice-cold isopropanol was added to the tube. The tubes were vigorously vortex and placed at - 20° C overnight to precipitate the DNA. Samples were centrifuged at 10 000 x g for 10 min. at 4°C. The DNA pellets were washed once with 0.5 ml 70 % ice-cold ethanol and centrifuged at 10 000 x g for 10 min. at 4°C. The supernatant was removed and the tubes were dried at 37°C. The pellets were dissolved in 45 µl TE containing 5 µl loading buffer. The samples were analyzed by electrophoresis on 1.8 % agarose gels stained with 5 µl (0.5 µg / ml) ethidium bromide (Wyllie, 1980).

2.5.3 Crossmon Trichrome stain

The crossmon trichrome staining procedure was followed as described previously (Anton, 1999). Hydrated smears were stained in a 0.1% acid fuchsin / orange G stain for 5 seconds, rinsed in water and transferred to 1 % light green stain for 30 min.

After staining, the smears were briefly rinsed in water and dehydrated through ascending grades of ethyl alcohol, cleared in xylene and mounted in Canada balsam.

2.5.4 LDH release reaction

CHO 22 and Y10 cells were cultured in 25 cm^2 flasks until confluent. Cells were trypsinized and seeded in a 96-well tissue culture plate at a cell concentration of 0.25×10^5 cells / ml. The cells were incubated for 24 h at 37° C. The media was removed and 100 µl media containing *S. frutescens* extract was added to the wells with concentrations ranging form 1 mg / ml to 10 mg / ml. Triplicate wells were prepared for each concentration. Two controls were also included: the low control (= spontaneous LDH release, 100 µl media was added to each well) and the high control (= maximum LDH release, 100 µl media containing 2 % triton X 100 was added to each well). Cells were again incubated at 37° C for another 24 h. The cells were centrifuged at 250 × *g* for 10 min. and 80 µl of the supernatant was removed and transferred to an optically clear 96-well flat bottom micro-titreplate. To determine the LDH activity in these supernatants 80 µl of the reaction mixture (Roche, Germany) was added to each well and the plate was incubated for 20 min. at room temperature in the dark. The absorbance values of the samples were read at 492 nm using a micro plate colorimeter (Bibby sterilin, U.K.) The reference wavelength was set at 600 nm.

To determine the percentage cell death, the average absorbance were calculated for the triplicates and the resulting values were substituted in the following equation:

% cell death = experimental value - low control / high control – low control (Kim and Sharma, 2003; Paydas *et al.*, 2003)

2.6 FACS system (immunofluoresence)

2.6.1 Annexin-V PE and 7-AAD

CHO and Jurkat T cells (1 x 10^5) were cultured in a 5 ml culture flask and induced with the extracts. Cells were washed twice with cold PBS and then resuspended with in 100 µl annexin –V PE 1 x binding buffer and this was transferred to a 15 ml tube. To this 5 µl of Annexin-V PE and 5 µl of 7AAD was added. Cells were gently vortexed and incubated for 15 min. at room temperature in the dark. The binding buffer (400 µl) was added in each tube and analyzed by flow-cytometry at 488 nm wave length using FACS Calibur™ soft ware and FACS SCAN (Becton Dickinson, California, USA), (Vermeulen *et al.*, 2002; Herzig *et al,* 2003; Nile *et al.*, 2003).

2.6.2 ApoPercentage™ (Immunofluoresence)

Cells (10^4) were seeded in a 6 well plates and allowed to grow to a cell density of 10^6 cells. Cells were induced with 5 µm staurosporine or (0.5 %) chloroform fraction for 6 h. One hour before the end of the incubation time 1 ml apoPercentage™ dye (final. conc. 2.5 µm) was added to the culture. Cells were transferred to 15 ml tubes and washed twice with ice cold PBS and centrifuge at 200 x g for 5 min. and then resuspended in 100 µl Hepes buffer (10mM Hepes / NaOH, Ph 7.4, 140 mM NaCl, 5 mM $CaCl_2$) and incubated at room temperature in the dark for 15 min. Hepes buffer (400 µl) was added to each tube prior to flowcytometry. Forward scatter, side scatter

and orange red fluorescence (FL2) was analyzed for unlabelled (untreated control), labeled (untreated control) staurosporine (positive control) and *S. frutescens* extracts.

CHAPTER THREE

3. Results and Discussion

3.1 Evaluation of biological activity of *S. frutescens* aqueous extracts from different geographical populations in South Africa.

The concept that certain plants can treat different forms of diseases is a claim by traditional healers (Bieseubach, 1998). In certain instances plants remain the only source of medicine for rural health care and can provide pharmaceutical companies with the opportunity to generate new drugs. *S. frutescens* locally known as "cancer bush" or "kankerbos" is one plant that has caught the interest of medicinal plant dealers due to the claim that it has a broad therapeutic potential and more specifically, it is perceived by some to have the ability to treat cancer (Van Wyk *et al.*, 1997). With its diverse geographical distribution within southern Africa and given its continuous use by the local people it is advantageous, if not necessary, to investigate the difference between these plant species and search for apoptotic agents (extracts, secondary metabolites and compounds) that can be novel candidates for anti-cancer drugs.

However, some consumers of *S. frutescens* have claim that the use of this plant has no health benefits (Thomson, 2002) while others claim to the contrary (Bieseubach, 1998). Based on these controversial claims we started by screening (bioactivity testing) different collections of the same specie of *S. frutescens* microphylla from

different geographical populations in South Africa. The research methodology for this study is outlined in Figure 3.1.

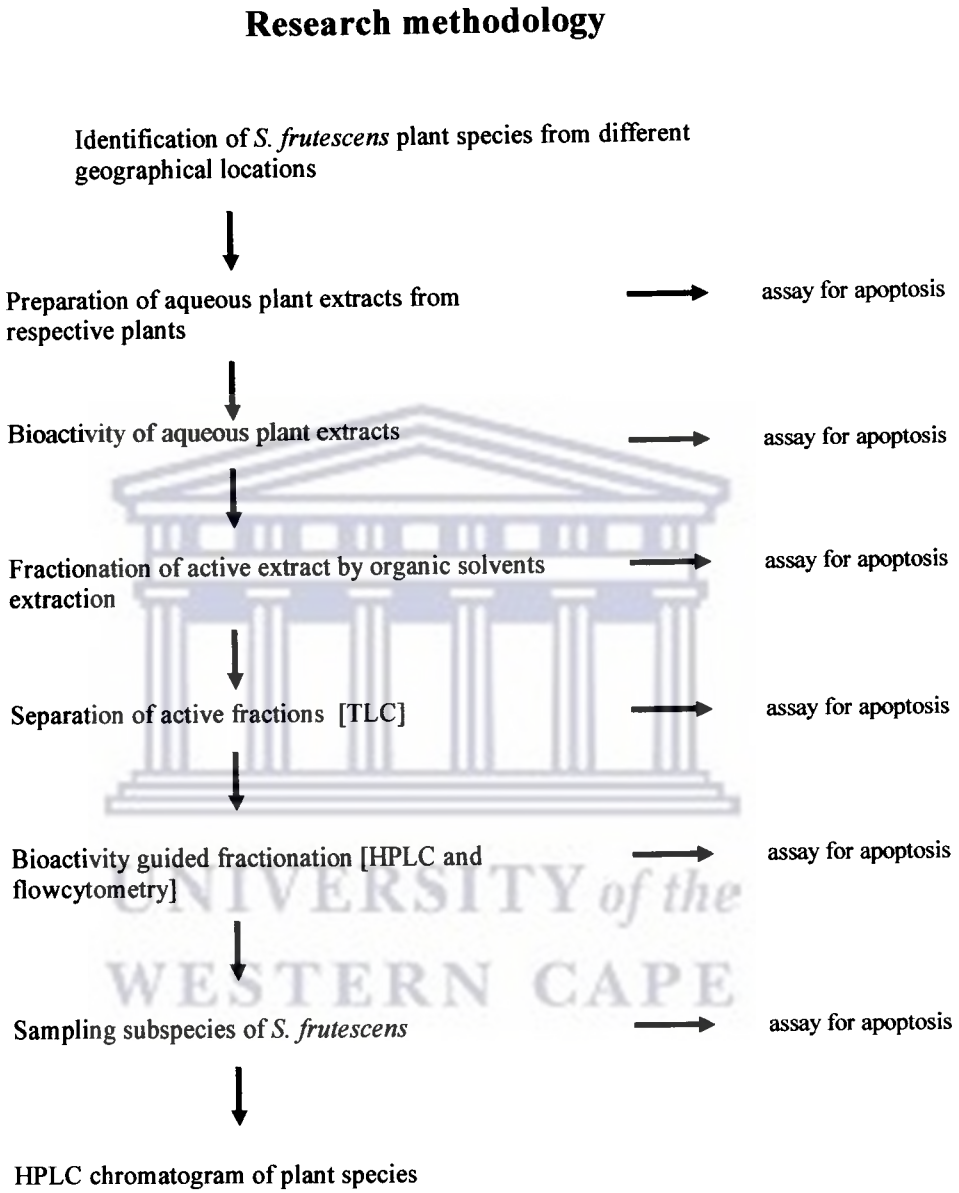


Figure 3.1: Outline of research methodology

The evaluation of the biological activity of *S. frutescens* extracts from different geographical areas was done based on the morphological observation of cell death in CHO cells. In this experiment, an aqueous extract was prepared (see section 2.3.1) from the different *S. frutescens* populations and the activity of these plant extracts was determined. The extracts were tested on CHO cells grown in HAMS F12 (see Table 2.1). The concentrations of the different *S. frutescens* extract ranged from 1 mg / ml to 10 mg / ml. Extract 191 and extract 206 from the Free State and Karoo were not active while extract 212 showed some activity and extract 29 from the Cape Peninsular was the most active. This led to the suggestion that certain populations of this plant species are more active than others (see Table 3.1).

EXTRACT	CELL TYPE	LOCATION	ACTIVITY
191	CHO	Free State	Not Active
206	CHO	Karoo	Not Active
212	CHO	Cape Peninsula	Partly Active
29	CHO	Cape Peninsula	Active

Table 3.1: Bioactivity of *S. frutescens* aqueous extracts from different geographical locations in South Africa. Activity was assayed based on morphological observation of cells. Cells treated with the respective extracts were compared to untreated control cells and observed under a light microscope (Nikon) for cell death at 200X magnification. Cell death was indicated by cell shrinkage, cell disintegration and reduction in cell number.

The following experiment was designed to test extract 29, which was the most active extract from the Cape Peninsula (Table 3.1) on CHO cells and CASKI cells at

different exposure times (8 h and 24 h). The concentration of extract 29 tested was 3.5 mg / ml (Figure 3.2).

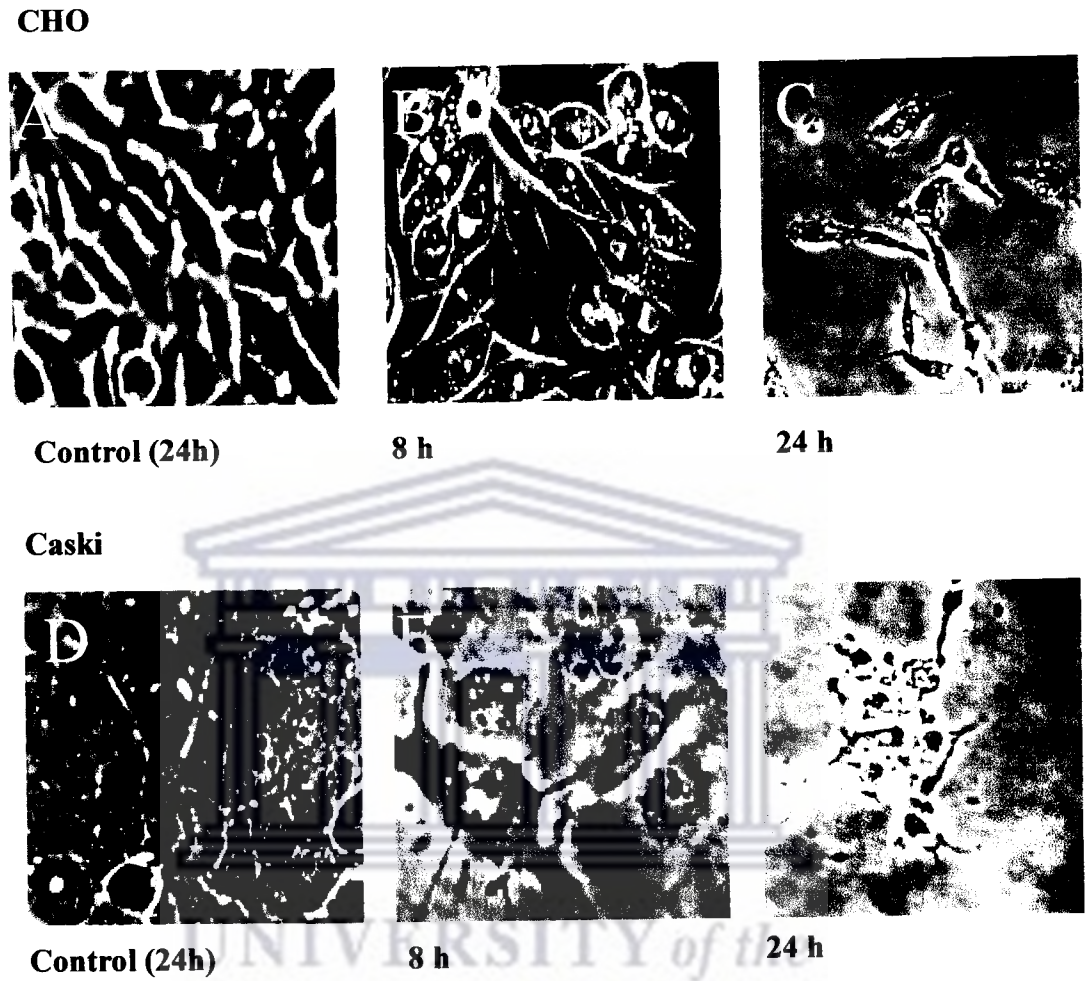


Figure 3.2: Morphological observation of *S. frutescens* extract 29 tested on CHO and cervical carcinoma (Caski) cells at different exposure times. CHO cells and Caski cells (2.5×10^6) were grown in Hams F12 and RPMI media. (A) Untreated control of CHO cells, (B and C) are CHO cells induced with 3.5 mg / ml with *S .f* extract 29 and treated for 8 h and 24 h respectively. (D) Untreated control of Caski cells, (E and F) are Caski cells treated with 3.5 mg / ml of *S. f.* extract 29 and treated for 8 h and 24 h respectively. The morphology was observed with an inverted microscope at 200X magnification (Nikon). Cell death was indicated by cell shrinkage, cell disintegration and reduction in cell number.

Morphological changes indicated cell death in both CHO and CASKI cells (Figure 3.2). The untreated controls in (A and D) cells appear morphologically normal while cells (B and E) treated at 8 h treatments with extract 29 are vacuolated with condensed nuclei and progressive membrane damage. At 24 h the treated cells (C and E) shows complete morphological disintegration. This experiment suggests that there are active constituents in the plant extract that causes morphological changes and cell death.

Canavanine (see APPENDIX) is claimed to be the active constituent of *S. frutescens* (Van Wyk *et al.*, 1997; Thomson, 2002). This claim lead us to investigate the effect of canavanine induce cell death in CHO cells and CASKI cells. Cells were exposed to different concentration of canavanine ranging from 1 mM – 10 mM (Figure 3.3).

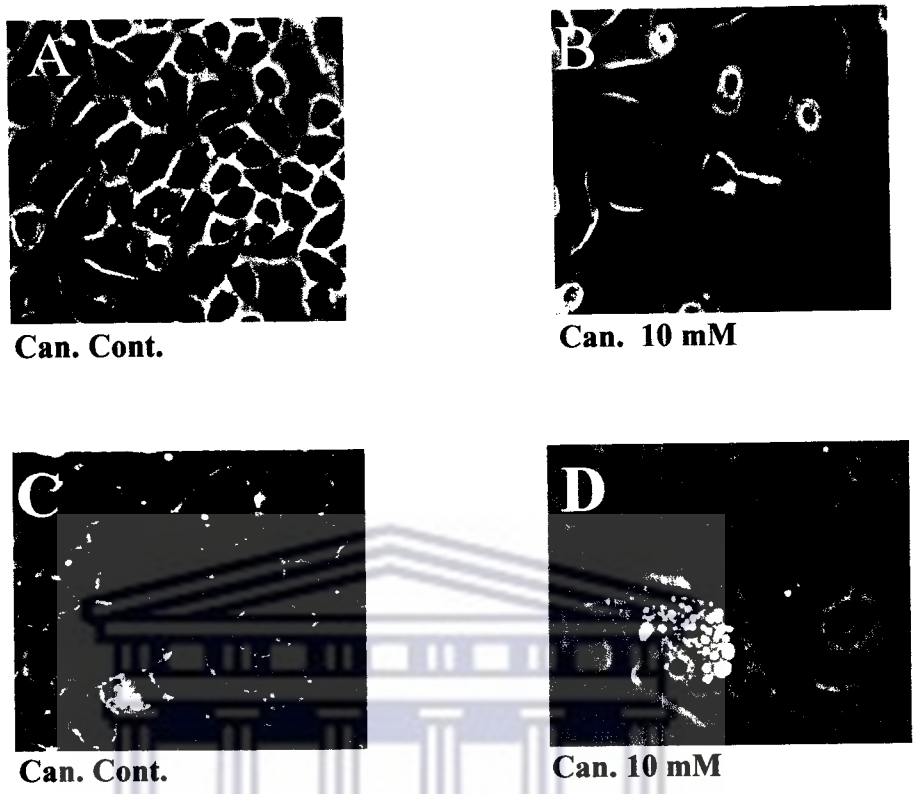


Figure 3.3: Morphological observation of CHO and Caski cells treated with canavanine at 48 h. CHO cells and Caski cells (2.5 x 10^6) were grown in Hams F12 and RPMI. These media were supplemented with 5 % and 10 % FCS respectively, 0.1 % Streptomycin, grown in 5 % CO_2 at 37° C. Cells were treated with 10 mM canavanine (can.) and monitored for cell death at 6 h, 12 h and 48 h. (A) Untreated CHO control cells (cont.). (B) CHO cells induced with 10 mM canavanine. (C) Untreated Caski control cells. (D) Caski cells treated with 10 mM canavanine. At 48 h cells were observed at 200X magnification using an inverted light microscope (Nikon).

Figure 3.3. shows an untreated control CHO and Caski cells (A and C respectively) and CHO and Caski cells treated with 10 mM canavanine and monitored for 6 h, 12

h, 24 h and 48 h (C and D). It was observed that 10 mM canavanine (10 mM) did not cause any observable signs of cell death, cells might be reduced in numbers maybe because of depleted nutrients after being exposed for 48h and a slight effect is seen when the dose of canavanine increases. Canavanine was claimed to be the active constituent of *S. frutescens* in the treatment of cancer. This result suggests that canavanine may not be the active apoptotic constituent of *S. frutescens* or it is active in combination with other compounds.

In order to test that extract 29 from *S. frutescens* causes cell death via apoptosis further assays were carried out.

3.2 Testing *S. frutescens* aqueous extracts for the ability to induce apoptosis

In order to confirm apoptosis we first utilized an assay that takes advantage of the "flip-flop" mechanism in apoptotic cells. This process causes externalization of a phospholipid phosphotidylserine during apoptotic process (Martin *et al.*, 1994; Savill and Fadok, 2000). The apoPercentage™ dye (see APPENDIX) gains entrance into the cells, and when it is trapped, it is a diagnostic tool for apoptosis.

An apoPercentage™ assay was carried out on CHO cells induced by *S. frutescens*.extract 29 (see section 2.5.1). CHO cells were tested with aqueous *S. frutescens* extract 29 (see 2.3.1) at a concentration of 3.5 mg / ml.

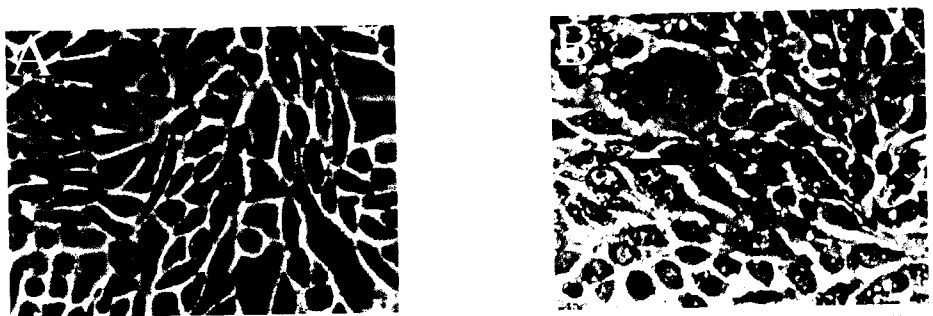


Figure 3.4: The apoPercentage™ assay on CHO cells treated with *S. frutescens* extract 29. CHO cells were grown on HAMS F12 and treated with 3.5 mg / ml of *S. frutescens* aqueous extract 29. Cell death was determined using apoPercentage™ assay. (A) Untreated control CHO cells. (B) CHO cells treated with *S. frutescens* extract 29. The arrow indicates trapped dye within the cellular cytoplasmic matrix. Cells were observed at 200X magnification using an inverted light microscope (Nikon).

The apoPercentage™ assay indicated that CHO cells (control) not treated with extract 29 had no dye within the cells and therefore are morphologically intact (Figure 3.4). CHO cells treated with extract 29 showed cellular shrinkage and contained pink dye visible under the microscope. This trapped dye indicates that the cells are positive for apoptosis and that *S. frutescens* extract 29 activated apoptosis.

The activation of apoptosis by *S. frutescens* extract 29 on CHO cells lead us to investigate the dose response and time of activation of apoptosis by the different *S. frutescens* extracts collected from the various geographical populations.

Dose response and time course experiments was use to determine the difference in potency of extracts from the four different plant collections. The different collections of *S. frutescens* aqueous extracts were again tested in an apoPercentage™ assay on CHO cells to establish a dose response (Figure 3.5).

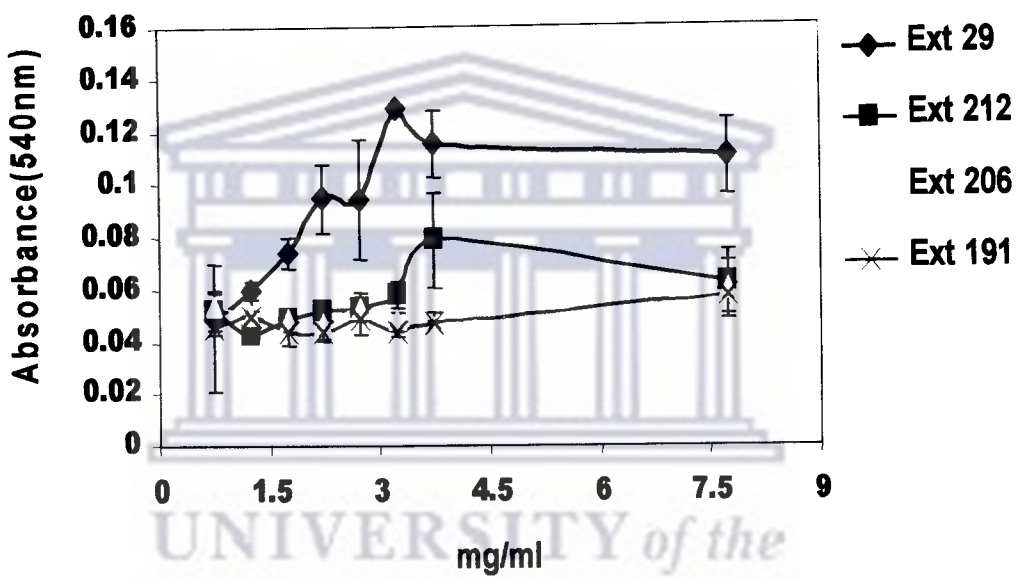


Figure 3.5: Dose response of *S. frutescens* aqueous extracts from different geographical population in South Africa. The percentage of cell death was determined using apoPercentage™ assay. Absorbance was read at 540 nm (maximum dye absorbance) and 625 nm (minimum dye absorbance). The difference between these two values was taken as the real absorbance for the recovered dye that was trapped in the cells. An absorbance reading above 0.06 at 540 nm indicated cell death.

The result shows a rise in absorbance (540 nm) when the dose of extract 29 is increased from 1 mg / ml to 4 mg / ml (A540 > 0.1). There was no rise in absorbance observed with extracts 191 and extract 206 at all concentrations. Extract 212 (A540 < 0.08) showed a slight increase at 3.5 mg / ml and extract 191 (A540 < 0.06) showed slight increase at 4 mg / ml.

The dose response indicated different activities in the different populations tested. The time course experiment was intended to evaluate the shortest possible time for activation and detection of apoptosis using the different collections of *S. frutescens* extracts with the apoPercenatage™ assay (Figure 3.6).

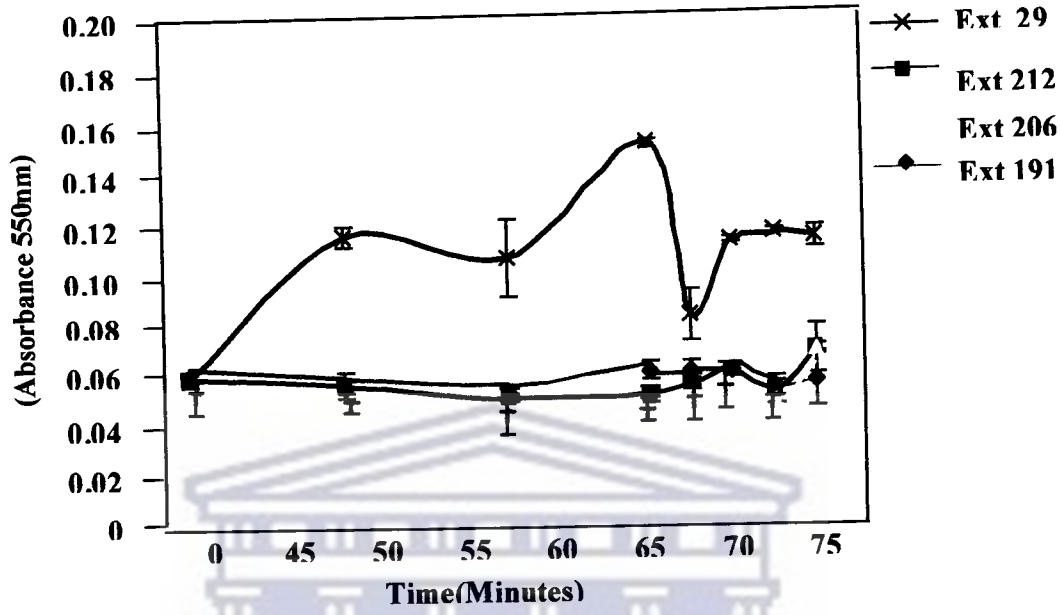


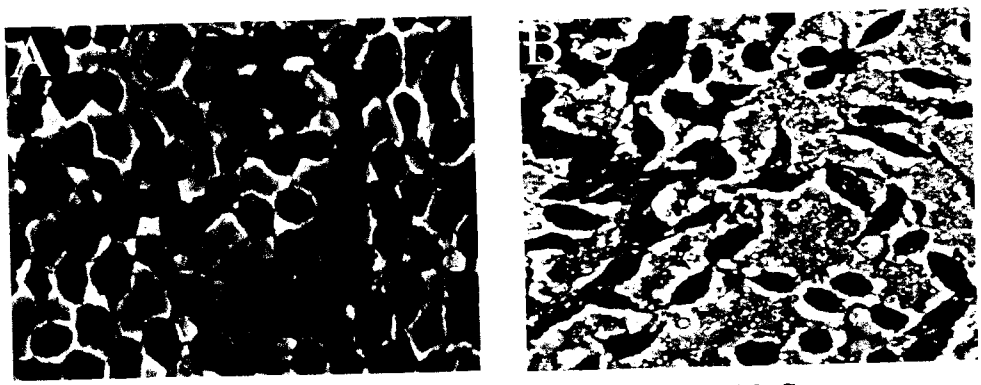
Figure 3.6: The effect of different *S. frutescens* extracts on cell death at specified time-intervals. Cell death was determined using the apoPercentage™. Absorbance was read at 550 nm (maximum dye absorbance) and 625 nm (minimum dye absorbance). The difference between these two values was taken as the real absorbance for the recovered dye that was trapped in the cells. An absorbance reading above 0.06 at 550 nm indicated cell death.

CHO cells were exposed to different *S. frutescens* aqueous extracts at concentrations of 3.5 mg / ml at specified time intervals. Extract 29 showed an exponential increase in activity between 0 - 55 min (A550 > 0.01) and a drop in activity between 55 - 75

min (A550 ≥ 0.10). Extracts 212, 206, 191 showed no change in activity from 0 - 70 min but a slight increase in activity at 75 min (A550 > 0.04).

The results show extract 29 expressing more apoptotic activity than the other three extracts. This result may indicate that the action of *S. frutescens* extract 29 is both time and dose dependent. The observation that the extracts from the other geographical locations did not have apoptotic activity suggests that certain environmental challenges on the different plants may cause them to synthesize compounds and thus making them distinct in different areas. These different compounds present in the same species could be attributed to plants which differ from their area of collection based on soil composition, protection against environmental factors (e.g. high UV) leading to the synthesis and accumulation of secondary metabolites (Wink, 1999b).

Cells dying of apoptosis display condensed chromatin suggested by Anton (1999). There are some characteristic similarities between apoptosis and normal mitosis such as chromatin condensation, dissolution of nuclear membrane and activation of cyclin-dependent kinases. Therefore apoptotic fragments usually contain condensed chromatin. We confirm chromatin condensation induced by *S. frutescens* extracts 29 (3.5 mg / ml) using Crossmon Trichrome stain (see section 2.5.3) (Figure 3.7).



Untreated control

Cells treated with *S. frutescens* extract 29

Figure 3.7: Cytological stain using crossmon trichrome on CHO cells. Cells were grown in HAMS F12 and treated with 3.5 mg / ml extract 29 from *S. frutescens*. Hydrated smears were stained in a 0.1 % acid fuchsin / orange G stain for 5 seconds, rinsed in water and transferred to 1 % light green for 30 min. They were observed at 200X magnification using a conventional light microscope (Olympus, Japan). (A) Control- Untreated CHO cells. (B) CHO cells treated with *S. frutescens* extract 29 at a concentration of 3.5 mg / ml. Chromatin condensation is indicated by pink stain in the cell (Arrow).

The untreated control (Figure 3.7 A) did not pick the primary stain but the CHO cells treated with extract 29 was able to pick up this stain (Figure 3.7 B). The stain in the treated cells indicates that the nuclear material stained by fuschin is undergoing apoptosis. This in turn suggests extract 29 from *S. frutescens* has the ability to induce apoptosis by nuclear condensation, which is in conformity with the observations obtained by Anton (1999).

3.3 *S. frutescens* induced apoptosis by DNA fragmentation

Previously it has been suggested that the morphological appearance of chromatin fragmentation and condensation is a direct result of DNA cleavage. It occurs as result of activation of endogenous Ca^{2+} and Mg^{2+} endonucleases which are enzymes that selectively cleave DNA at sites located within the nucleosomal subunits generating strand breaks of multiple integers of 50-200bp fragments (Wyllie, 1980). There is a further proposition that during apoptosis there is DNA cleavage at the matrix attachment region where loops of chromosomal DNA are attached to the loops of the nuclear matrix resulting in chromatin condensation (Anton, 1999).

We used *S. frutescens* extracts from the different geographical populations to treat cultured CHO cells and then analyze DNA fragmentation to investigate apoptosis and compare the different extracts. CHO cells were exposed to *S. frutescens* aqueous extracts from the different geographical population at concentration of 3.5 mg / ml (Figure 3.8).

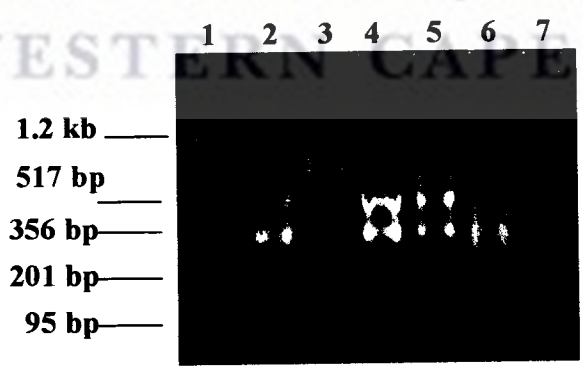


Figure 3.8: DNA fragmentation assay on CHO genomic DNA. Genomic DNA of untreated and treated CHO cells was extracted and size fractionated on a 1.8 % agarose gel and stained

with 0.5 µg / ml ethidium bromide. The CHO cells were treated with *S. frutescens* extracts from different geographical populations at a concentration of 3.5 mg / ml. Lane (1) Standard DNA molecular weight marker (Lamda DNA digested with restriction enzyme PstI). Lane (2) 1.3 µM staurosporine (Positive apoptotic control). Lane (3) Untreated (Negative control). Lane (4) *S. f.* extract 29, lane (5) *S. f.* extract 212, lane (6) *S. f.* 191 and lane (7) *S. f.* 206.

Staurosporine (see APPENDIX) is a compound that induces apoptosis and this in turn can be assayed by DNA fragmentation. As expected, lane 2 demonstrates that staurosporine induces DNA fragmentation of CHO genomic DNA when cells are treated with this compound. CHO cells that were not treated (Lane 3, control) had no fragmentation of the genomic DNA. CHO cells treated with extract 29 suggested apoptotic activity of the extract as indicated by DNA fragmentation of the genomic DNA (lane 4). There was slight fragmentation of genomic DNA with CHO cells treated with extract 212 (lane 5). There was no DNA fragmentation of genomic DNA (lane 6 and 7) from cells treated with extract 206 and 191 suggesting that these extracts were not apoptotic.

The high activity of extract 29 is in agreement with the results from the apoPercentage evaluation (section 3.2) and confirms the presence of high apoptotic compounds in this extract.

Based on the above result we wanted to evaluate apoptosis of extract 29 at different times to confirm early or late stages of apoptosis by observing DNA fragmentation.

CHO cells were exposed to 3.5 mg / ml of aqueous *S. frutescens* extract 29 at different times (Figure 3.9).

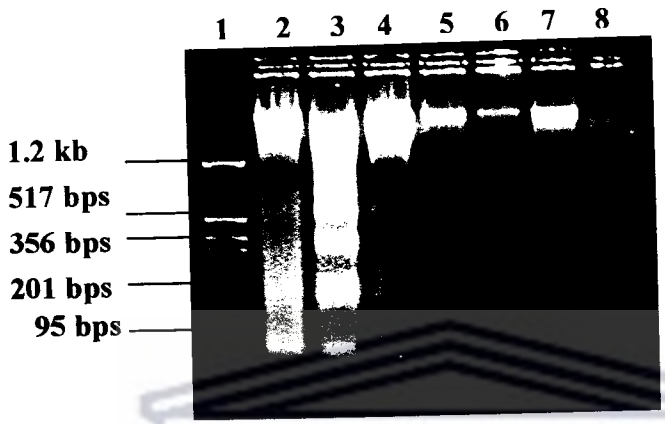


Figure 3.9: DNA fragmentation assay on CHO genomic DNA from cells treated for different time lengths with *S. frutescens* aqueous extract 29. CHO cells were treated with 3.5 mg / ml extract 29 for 2 h, 4 h, 8 h, 10 h and 24 h respectively. Genomic DNA of untreated and treated CHO cells was extracted and size fractionated on a 1.8 % agarose gel and stained with 0.5 µg / ml of ethidium bromide. Lane (1) Standard DNA molecular weight marker (Lamda DNA digested with restriction enzyme PstI). Lane (2) 24 h untreated (Negative control). Lane (3) 1.3 µM staurosporine (Positive apoptotic control). Lane (4) 2 h , lane (5) 4 h , lane (6) 8 h, lane (7) 10 h, and lane (8) 24 h treatments respectively.

There was slight fragmentation and a smear of the genomic DNA extracted from the 24 h untreated control (lane 2). This could be due to normal DNA degradation as result of cell age and lack of nutrients in the media after 24 h. As expected, lane 3 demonstrates staurosporine induced DNA fragmentation of genomic DNA of CHO after treatment. After 2 h, there was slight DNA fragmentation and smear of the

genomic DNA induced by extract 29 treatment on the CHO cells (lane 4). There was no DNA fragmentation of genomic DNA observed between 4 h and 8 h (lane 5 and 6). At 10 h there was slight fragmentation of genomic DNA (lane 7) and at 24 h there was complete DNA fragmentation (lane 8).

These results suggest that *S. frutescens* extract 29 can induce DNA fragmentation at late stages (10 h and 24 h) of treatment as a result of apoptosis. This result is in agreement with that observed by Ploski and Aplan, 2001.

From previous studies it has been shown that ceramide and staurosporine are known inducers of apoptosis (Haefena *et al.*, 2002; McKeague1 *et al.*, 2003). The DNA fragmentation pattern caused by *S. frutescens* extract 29 treatment was compared with other known inducers of apoptosis such as 1.3 µM staurosporine and 100 µM ceramide (Figure 3.10).

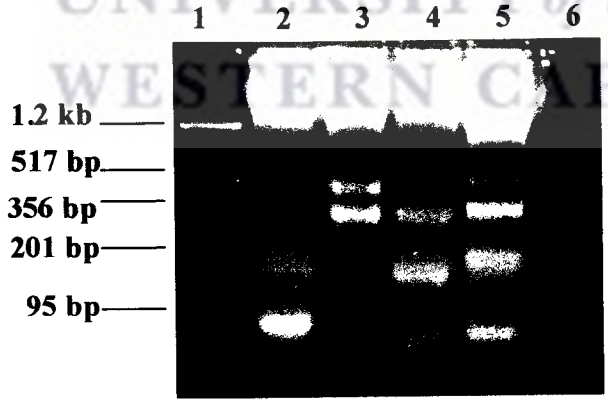


Figure 3.10: DNA fragmentation assay of CHO genomic DNA from cells treated with *S. frutescens* extract 29 and different known inducers of apoptosis. CHO cells were treated with 3.5 mg / ml extract, 1.3 µM staurosporine and 100 mM ceramide respectively. After ceramide treatment, two fractions of

genomic DNA were obtained, the supernatent and pellet. Genomic DNA of untreated and treated CHO cells was extracted and size fractionated on a 1.8 % agarose gel and stained with 0.5 µg / ml of ethidium bromide. Lane (1) Standard DNA molecular weight marker (Lamda DNA digested with restriction enzyme PstI). Lane (2) Positive control (staurosporine), lane (3) *S. f.* extract, lane (4) ceramide supernatant, lane (5) ceramide pellet fraction, and lane (6) untreated (negative control).

There was DNA fragmentation of genomic DNA extracted from staurosporine treated CHO cells (Figure 3.10, lane 2) and both fractions extracted from ceramide treated cells (Figure 3.10, lane 4 and 5). Genomic DNA extracted from cells treated with *S. frutescens* extract 29 also displayed fragmentation (Figure 3.10, lane 3) and as expected no fragmentation was observed with DNA extracted from untreated cells (Figure 3.10, lane 6). This result further suggests that *S. frutescens* extract 29 has apoptotic activity due to the fragmentation of genomic DNA when compared to known apoptotic inducers like staurospourine and ceramide.

3.4 Organic solvent fractionation and bioassay of *S. frutescens* extract 29

In order to isolate the active components present in *S. frutescens* extract 29, it was subjected to organic solvent extraction to isolate the secondary metabolites. The *S. frutescens* extract 29 was separated using organic solvents of different polarity and concentration as shown in Table 3.2.

Organic Solvents	Untreated Control	0.1% Extract 29	0.25% Extract 29	0.4% Extract 29	0.5% Extract 29
Petrol Ether	-	-	-	+	+
Chloroform	-	+	+	+	+
Ethyl acetate	-	-	-/+	+	+
n-Butanol	-	-	-	-	+
Water	-	-	-	-	-

Table 3.2: Separation and bioassay of *S. frutescens* extract 29 using different organic solvents of different polarities and concentrations. Activity was assayed based on morphological observation of cells. Cells were observed under a light microscope (Nikon) for cell death at 200X magnification. Cell death was indicated by cell shrinkage, cell disintegration and reduction in cell number.

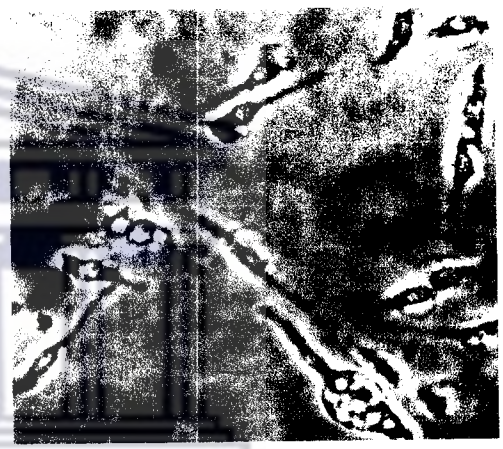
A bioassay of organic solvent fractionations was carried out in order to determine to which fraction the activity is confined. Confirmation of the most active fraction relied on minimum concentrations for cell death induction. The activity of petrol ether fraction was exhibited at a minimum concentration of 0.4 % (1 mg / ml), the activity of the chloroform fraction was exhibited at a minimum concentration of 0.1 % (0.25 mg / ml), the activity of ethyl acetate fraction was exhibited at a minimum concentration of 0.25 % (0.625 mg / ml), the activity of n-butanol was exhibited at a minimum concentration of 0.5 % (1.25 mg / ml) and the water fraction exhibited no activity.

CHO cells were exposed to the different organic fractions of *S. frutescens* extract 29 to observe morphological changes induced by plant metabolites that cause cell death (Figure 3.11).

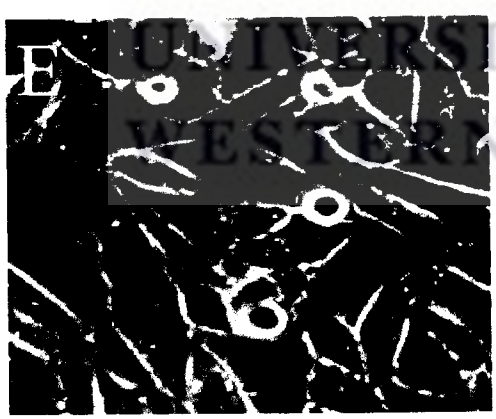
Untreated control

Petroleum ether fraction

Chloroform fraction



Ethyl actetate fraction



n-Butanol fraction

Water fraction

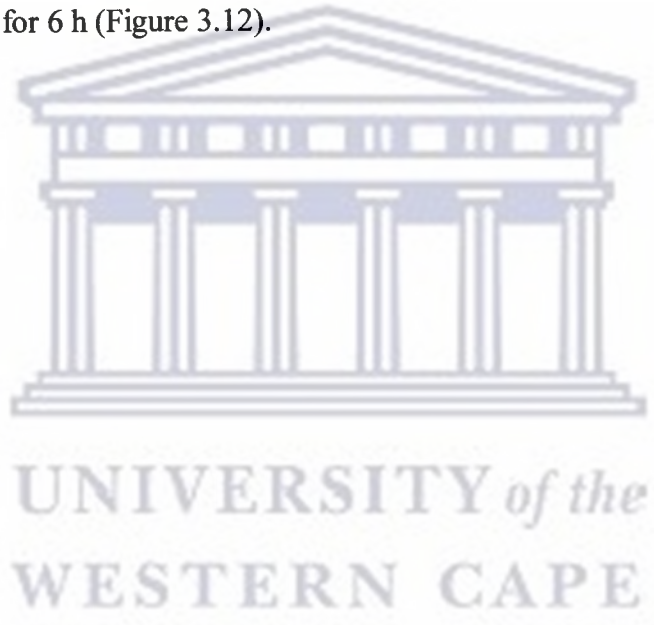
Figure 3.11: Morphological observation of CHO cells treated with organic solvent extracts of *S. frutescens*. CHO cells (0.25x 10^6) were seeded in six well tissue culture plates containing Hams F12 media and grown for 48 h. The different organic fractions were added to culture wells and incubated at 37^0C for 6 h. Cells were observed using an inverted light microscope (200X magnification). Treatments- (A) Untreated control, (B) petrol ether, (C) chloroform, (D) ethyl acetate, (E) n-butanol, (F) water.

The untreated control (Figure 3.11, A) shows morphologically intact cells while cells treated with the petrol ether fraction (Figure 3.11, B) shows a reduced number of cells. Cells treated with chloroform and ethyl acetate (Figure 3.11 C and D respectively) show morphologically distorted cells. Cells that were treated with the n-butanol fraction (Figure 3.11, E) are slightly swollen while cells treated with water (Figure 3.11, F) had no observable morphological change.

The observed morphological changes indicated successful bioactive fractionation of the *S. frutescens* extract 29 (Table 3.2 and Figure 3.11). These results also suggest that an active agent for cell death is confined to the chloroform and the ethyl acetate fraction both of which slightly differ in their polarity index. Since the solvents used to extract the activity are non-polar we speculated that the active compound might be a non-polar compound. Phytosterols, coumarins and organic acids are non-polar secondary metabolites that can be extracted with chloroform while coumarins, organic acids, flavoniods, hydrolysable tannins and lignins are non-polar and can be extracted with ethyl acetate (Tura *et al.*, 2002).

3.5 The activity of *S. frutescens* extract 29 on different cell lines

Achieving selective cytotoxicity in the destruction of cancer is crucial since it creates an opportunity for selectively targeting cancer cells with minimal damage to normal cells. Further fractionation of extract 29 shows that the chloroform fraction is the most active and hence contains the most active apoptotic compounds (see section 3.5). This fraction was subsequently used in an experiment to test different cell lines for susceptibility to cell death. CHO, Caski, HELA and NHF cells were grown and exposed to *S. frutescens* extract 29 at a concentration of 1.25 mg / ml (chloroform extract, 2.3.2) for 6 h (Figure 3.12).



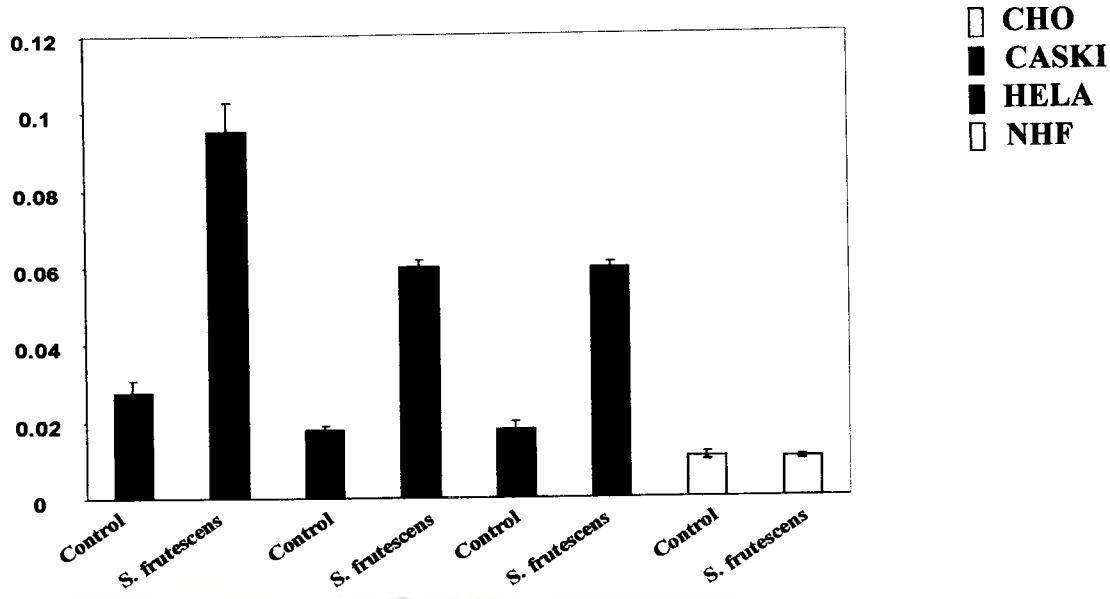


Figure 3.12: Investigation of apoptotic activity of *S. frutescens* extract 29 on different cell lines. CHO 22 (normal mouse cells), Caski (cancerous cells), Hela (cancerous cells) and Primary Human Fibroblasts (normal human cells) were cultured (2.2.1and 2.2.2) and treated with chloroform fraction of *S. frutescens* extract 29 (1.25 mg / ml). The percentage of cell death was determined using apoPercentage^{TM} assay. Absorbance was read at 550 nm (maximum dye absorbance) and 625 nm (minimum dye absorbance). The difference between these two values was taken as the real absorbance for the recovered dye that was trapped in the cells. Cells were considered non-apoptotic in the absorbance range of A550 ~ 0.0 - 0.04, and cells were considered apoptotic at an A550 > 0.04 compared to the untreated control.

The result shows that non-cancerous CHO cells became apoptotic when treated with the chloroform fraction of extract 29 (A550 ~ 0.1). The cancerous Caski and HELA cells became apoptotic (A550 ~ 0.06) when treated with the extract while the respective controls were unaffected. The non-cancerous primary normal human

fibroblast cells (NHF) showed no apoptosis (A550 < 0.02) when treated with the extract and was similar to the untreated control (A550 <0.02).

We demonstrated (Figure 3.12) that chloroform fraction of *S. frutescens* extract had the ability to induce apoptosis in Caski and HELA cells which are cancerous and not in the non-cancerous NHF cells. The extract also induced apoptosis in non-cancerous CHO cells, which are mouse fibroblast that are "functionally haploid". This result suggested that *S. frutescens* extract 29 may be inducing apoptotic cell death in specific cell lines with a selectivity for cancer cells. However, NHF resistance to apoptosis is not clear but may be as result of it having different apoptotic pathway.

The active compound in the extract may be specific for certain types of cancers. These cancers may have a single mutation, which confers susceptibility given that the activation of apoptosis involves targeting several pathways (Kerr *et al.*, 1972; Wyllie, 2000). A further test using staurosporine (known inducer of apoptosis) shows that it induces apoptosis on NHF cells (result not shown).

3.6 Preliminary separation and bioassay *S. frutescens* extract 29 using TLC

We further attempted to separate the above organic fractions into different secondary metabolites using TLC. Compounds were separated on the basis of their size, relative mobility and their affinity for the stationary phase (Cannell, 1998). TLC and preparative TLC were used to separate the secondary metabolites from the chloroform and ethyl acetate fraction (Figure 3.13).

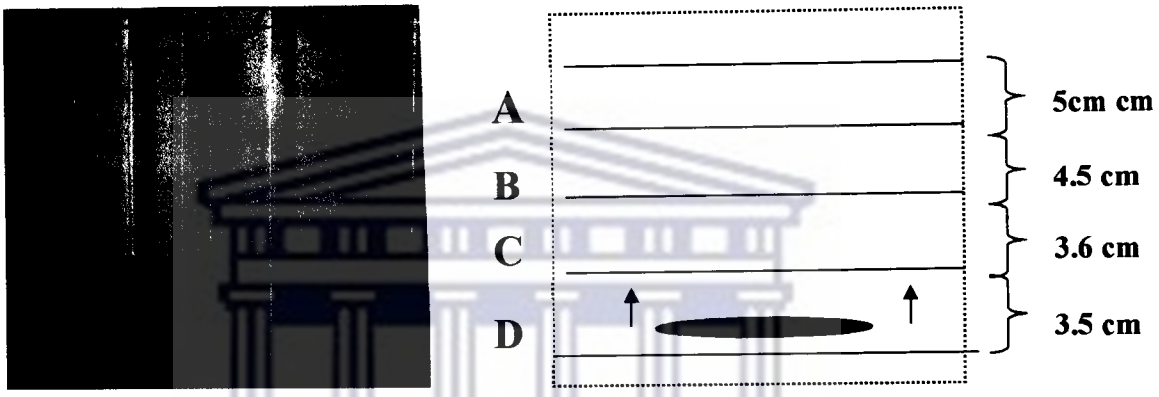


Figure 3.13: Separation of secondary metabolites in the chloroform and ethyl acetate fraction of *S. frutescens* extract 29 by Thin Layer Chromatography (TLC). TLC plates were divided into 4 quadrants. Active organic fractions (chloroform and ethyl acetate - 40 mg / ml) was applied at the origin of the TLC plates (2.5 x 10 cm; sample volume 1 ml). The solvent system contained toluene and acetone in a ratio of 4:1 (see section 2.4.1). Demarcations were made on the TLC plate, samples run and afterwards the respective areas (A) 5 cm, (B) 4.5 cm, (C) 3.6 cm, (D) 3.5 cm, scraped off and analysed further.

In order to test whether the separated fractions (secondary metabolites) obtained from TLC plates (Figure 3.13) had apoptotic activity, the fractions were tested on cultured cells. This will also enable us track regions eluted off the plate, which contain apoptotic compounds.

CHO cells were exposed to separate fractions eluted from TLC plates demarcated A – 5 cm, B - 4.5 cm, C - 3.6 cm and D - 3.5cm (concentration: 1.25 mg / ml), genomic DNA extracted and analyzed for DNA fragmentation (Figure 3.14).

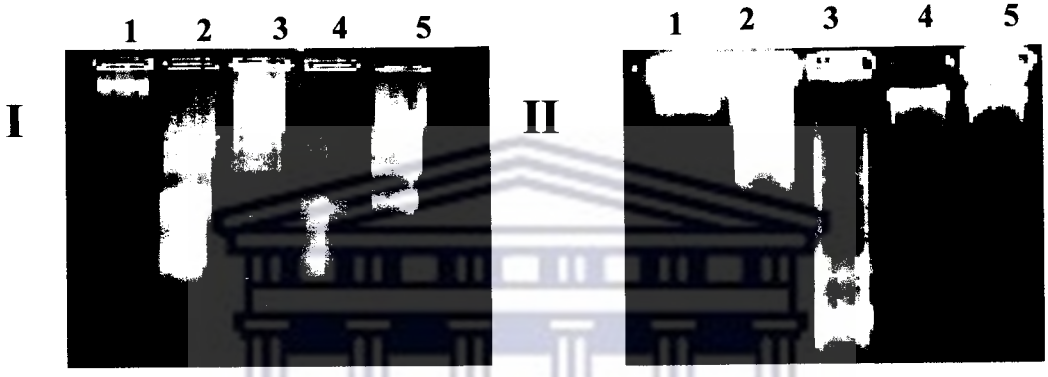


Figure 3.14: DNA fragmentation assay on CHO genomic DNA from cells treated with organic fractions eluted form the TLC plate. Genomic DNA of untreated and treated CHO cells was extracted and size fractionated on a 1.8 % agarose gel and stained with 0.5 µg / ml ethidium bromide. I (Chloroform fraction). Lane (1) untreated CHO cells (control), lane (2) TLC fraction A, lane (3) TLC fraction B, lane (4) TLC fraction C, lane (5) TLC fraction D. II (Ethyl acetate fractions): Lane (1) untreated CHO cells (control), lane (2) TLC fraction A, lane (3) TLC fraction B, lane (4) TLC fraction C, lane (5) TLC fraction D.

As a negative control (TLC silica eluate control), CHO cells were treated with only silica eluate (blank silica plate was scraped and tested on cells). Genomic DNA extracted from these CHO cells that were not treated with the chloroform or ethyl acetate fractions from extract 29 had no fragmentation of the genomic DNA (Figure

3.14, lane I 1, lane II 1). Genomic DNA extracted from CHO cells treated with all the TLC chloroform fractions had DNA fragmentation (A-D, Figure 3.14, lane I 2-5). This indicates that all these four chloroform fractions eluted from the TLC plate induce apoptosis by DNA fragmentations. Genomic DNA extracted from CHO cells treated with the TLC ethyl acetate fractions had DNA fragmentation in only two fractions (A and B, Figure 3.14, lane II 2 and 3). Treatment with fractions C and D (Figure 3.14, lane I 4 and 5) resulted in no DNA fragmentation of genomic DNA. This suggests that some compounds from the ethyl acetate fraction has the ability to induce apoptosis when separated, while others are not able to induce fragmentation indicating the presence of non-apoptotic compounds.

The previous results (Table 3.2) showed the chloroform fraction of extract 29 having higher bioactivity than ethyl acetate fraction. The DNA fragmentation caused by the chloroform fractions (Figure 3.14) confirms the previous result.

The TLC plates were developed for identification of the secondary metabolites present in the two active organic fractions (chloroform and the ethyl acetate fraction) see Table 3.3.

Metabolite	Coumarin	Phytosterol	Flavoniod	Hydrolisable Tanins
Marker	Coumarin	B-Sistostero	Querceti	Gallic Acid
Spray	5% (Aq) KOH	Aris / H_2O_4	Naturstoffreage (A)	Iron 111
Fluorescen (UV)	365nM	Oven at °- 110°) 10mins Visible light	Yellow Band lilac (UV)	BlueBlack/ Green Visible light
Solvent	Acetone acid:	Toluene :	Acetate: acid:	Acetate: acid:
Solvent	18:1:1 (v/v)	4:1 (v/v)	18:1:1 (v/v)	18:1:1 (v/v)
Chlorofor	+	+	-	+
Ethyl	+	-	+	-

Table 3.3: Preliminary identification of secondary metabolites using TLC. Each plate was developed in a chromatographic tank through a saturated volatile mobile phase as monitored by the Whatman filter paper. Solvent compositions were chosen with relative mobility (R_f) of 0.5 and 0.2 for the solvent front and the retained spot respectively. Plates were examined under UV (254 nm), using various spray reagents. The presence and absence of secondary metabolites is indicated by the positive and the negative signs respectively Cannell , (1998).

The results in Table 3.3 suggests that coumarins, phytosterols and hydrolysable tannins are present in the chloroform fraction as indicated by the positive sign and

that flavaonoids are absent in this fraction. The ethyl acetate fraction shows the presence of coumarins and flavonoids and the absence of phytosterols and hydrolysable tannins. This suggests that the positive secondary metabolites identified may be some of the compounds that are responsible for the apoptosis activity observed in the previous experiments.

3.7 Cytotoxicity assay of *S. frutescens* extract 29

In this section we attempted to determine the cellular cytotoxicity of *S. frutescens* aqueous extract 29 using two different cell lines. When target cells are incubated with a cytotoxic agent, cytoplasmic LDH (lactate dehydrogenase) may be released into the culture supernatant due to plasma membrane damage. The LDH activity is measured by a substrate reaction and quantified with an ELISA plate reader (see section 2.5.4). CHO and Y10 cells were cultured in HAMS F12 media and exposed to *S. frutescens* aqueous extract 29 and assayed for cytoxicity (Figure 3.15).

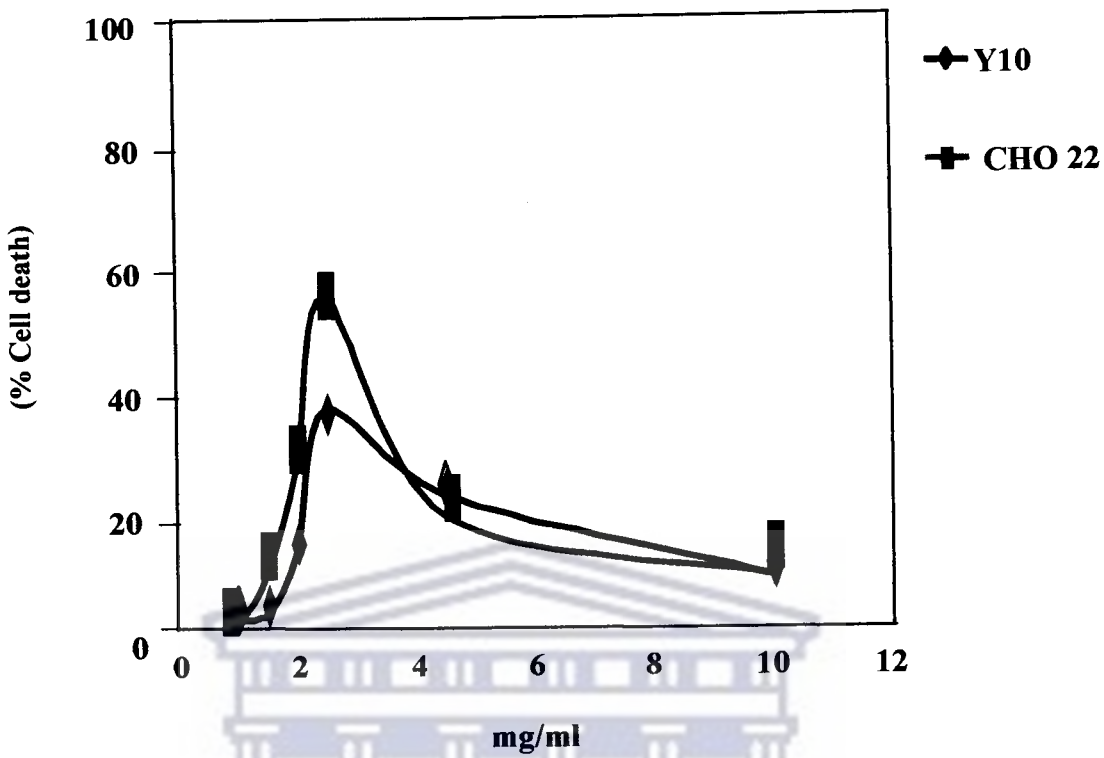


Figure 3.15: Cytotoxicity assay of *S. frutescens* extract 29 on CHO and Y10 cells. CHO 22 and Y10 cells were induced by *S. frutescens* extract 29 for 6 h and LDH release measured. as described in section 2.5.4. CHO 22 and Y10 cells were cultured in 25 cm^2 flasks until confluent. Cells were trypsinized and seeded (0.25 x 10^5 cells / ml) in a 96-well tissue culture plate. The cells was incubated for 24 hours at 37° C. The media was removed and 100 ml media containing *S. frutescens* extract 29 was added to the cells with concentrations ranging from 1 mg / ml to 10 mg / ml. To determine the percentage cell death, the average absorbance values (A492 nm) were calculated in triplicate and the resulting values were substituted in the following equation:

% cell death = Experimental value - Low control / High control - Low control

There was a sharp increase in the percentage cell death in both cell lines when the concentration of extract 29 was between 2 mg / ml and 4 mg /ml (Figure 3.15).

Cellular cytotoxicity was more marked in CHO 22 cells (cell death ≤ 60 %) than Y10 cells (cell death ≤ 40 %). There was a drop in cell death between 4 mg / ml to 10 mg / ml (<20 %) for both cell lines because at this stage it was too high dosage of extract 29 to the cells. The cells were necrotic and burst and could not retain the colour.

This result shows that 60 % of CHO cells was killed by the *S. frutescens* extract 29 as oppose to 40 % killed in Y10 cells. Cellular cytotoxicity does not differentiate between necrosis and apoptosis but it however quantifies the strength of killing by the extract and resistance to cell death of different cell lines.

It was shown previously in Figure 3.14 (I) that compounds eluted from the chloroform fraction of the TLC plate induce apoptosis resulting in genomic DNA fragmentation. We wanted to correlate the cellular cytotoxicity of compounds eluted from the chloroform fraction of the plate with LDH activity (Figure 3.16).

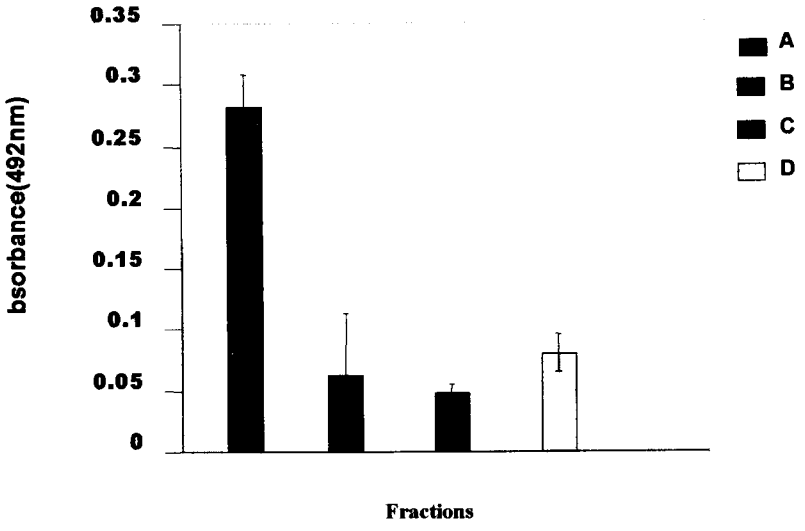


Figure 3.16: LDH assay to measure cytotoxicity on CHO 22 cells treated with the chloroform fraction of extract 29 eluted from TLC plates. CHO cells were grown on Hams F12, assayed for LDH with absorbance at 540 nm. The chloroform fraction from extract 29 eluted from the TLC was tested for activity by plotting the different fractions A, B, C and D against absorbance of 492 nm. Cells not affected by cytotoxicity had an absorbance range between 0.0 - 0.04, and cell death was considered when the absorbance was > 0.04

CHO cells were grown in HAMS F12 media and exposed to the different chloroform fractions from extract 29 eluted from the TLC plate at a concentration of 1.25 mg / ml. The control was CHO cells treated with a TLC plate containing no extract (silica only)- results not shown. Treated cells were analyzed for cytotoxicity using the LDH assay. The results show that fraction A caused a marked increase in cell death (A492 >0.25) while fraction B (A492 < 0.01) and C (A492 ≤ 0.05) caused little cell death. Fraction D (A492 < 0.1) caused slightly more cell death. The high activity in fraction

A suggest that it contains more of the cytotoxic compound than the other fractions B, C, and D.

The different secondary metabolites of the chloroform fraction of extract 29 had different cytotoxic effects. This supports the previous observation that different compounds have different cytotoxic effects (Paydas *et al.,* 2003).

3.8 Analysis of *S. frutescens* extracts using flowcytometry

In our previous analysis we have seen that *S. frutescens* extract 29 has the ability to cause apoptotic cell death in cultured cells as shown by morphological observation (microscopy), phosphotidylserine externalization (apoPercentage™ assay), chromatin condensation (Crossmon Trichrome) and nuclear fragmentation (agarose gel electrophoresis).

In this section flowcytometry was used to evaluate the behavior of cells treated with an apoptotic agent or *S. frutescens* extract 29 to determine apoptosis. Flowcytometry is a more accurate, qualitative and quantitative method to analyze apoptosis (Vermeulen *et al.,* 2002). In flowcytometry, fluorescent dye is added to cells treated with apoptotic agents (plant extract) and the cells passed through an argon laser (Figure 3.17). This causes the cells to emit fluorescence as well as expressing physical properties. Both the fluorescence and the physical properties can be analyzed by analog digital converter (ADC) and processed using integrated software (Becton

Dickinson-bioscience FACS Calibur™ soft ware San Jose, California, USA). The analyses of the fluorescence and physical properties indicate whether cells are apoptotic or not, shown by the addition of fluorescent dye.

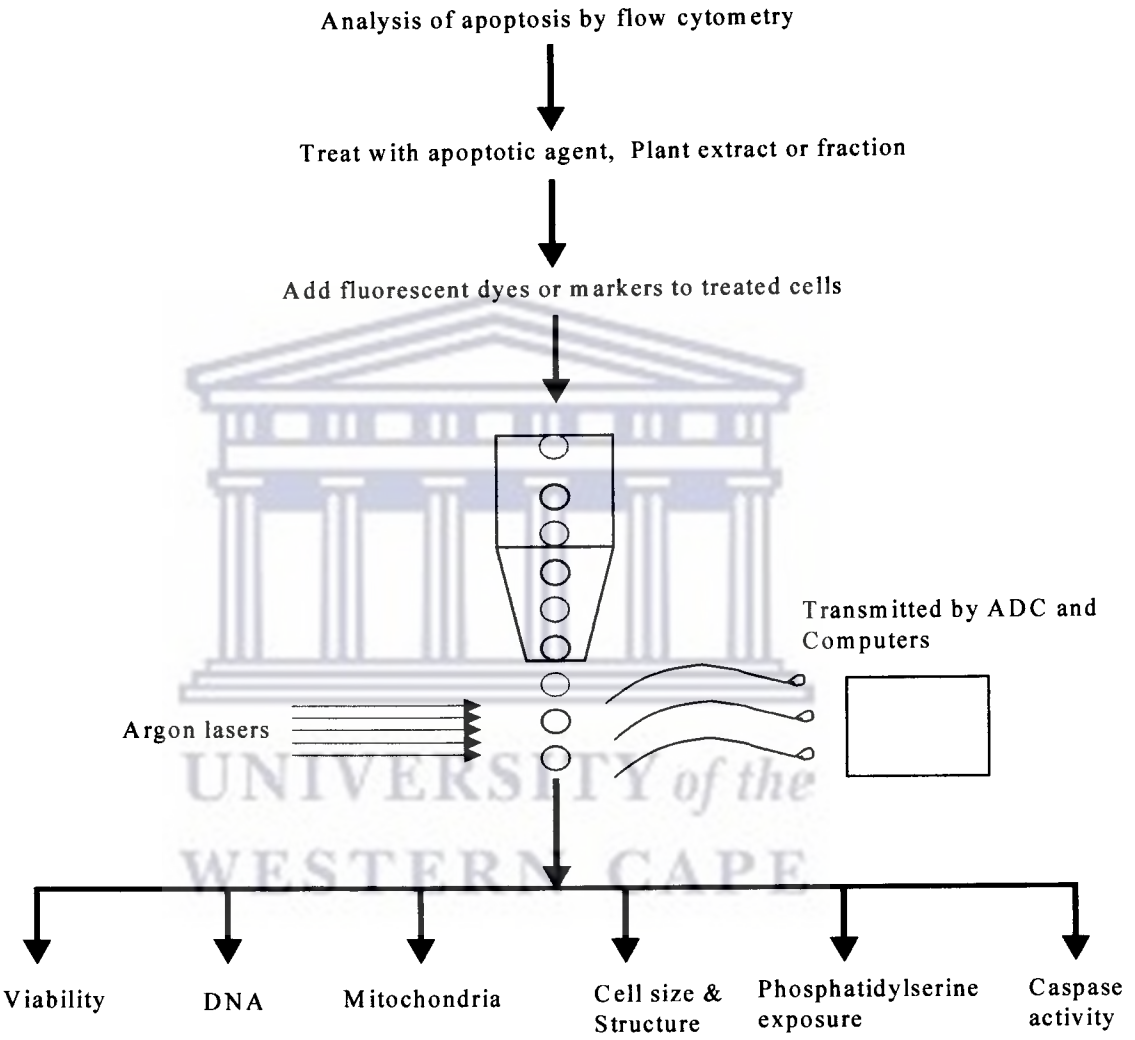


Figure 3.17: Flow-chart of the analysis of *S. frutescens* extract 29 for the induction of apoptosis using flowcytometry.

In order to validate the use of flowcytometry analyses control experiments were first done. Cancerous cells (2.2.1) were starved and then subjected to fluorescent dyes, annexin-V PE and 7-AAD for flowcytometry analyses (figure 3.18). Annexin- V PE is a cell surface binding dye that fluoresces green and reports phosphotidylserine at early stages of apoptosis. 7-AAD is a nuclear DNA accessibility dye that fluoresces red because of cell permeability due to progressive cellular membrane and nuclear membrane damage and measures late apoptosis and necrosis.

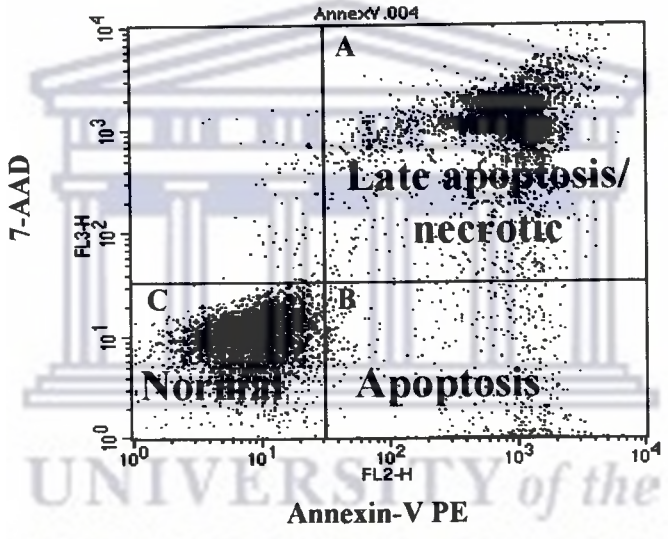


Figure 3.18: Dot plot of cells, stained with dyes for nuclear DNA and cell surface phosphotidylserine and then subjected to flowcytometric analyses for apoptosis. Jurkat T Lymphoma (10^5) cells were seeded (section 2.2.1) and starved without fresh media for 96 h and stained with annexin-V PE (stains phosphotidylserine) and 7-AAD (stains nuclear DNA) according to the manufacturers instructions (2.6.1). The data generated was analysed by BD-bioscience FACS calibur™ software (San Jose, California, USA). (A) Late apoptotic cells have a mixed population of apoptotic and necrotic cells. (B) Population of apoptotic cells. (C) Normal cells.

JURKAT-T lymphoma cells grown first in RPMI media and then starved of media for 96 h to differentiate between normal, apoptotic and necrotic cells within the same cell population using flowcytometry (Figure 3.18). The cells in quadrant A (Figure 3.18) show that the cell population is a mix of apoptotic cells (positive for annexin-V PE) and necrotic cells (positive for 7-AAD). The cells in quadrant B were positive for annexin only indicating apoptosis. The cells in quadrant C (Figure 3.18) were negative for both dyes indicating normal cells.

Cell viability was also analyzed via the flowcytometry software (Figure 3.19).

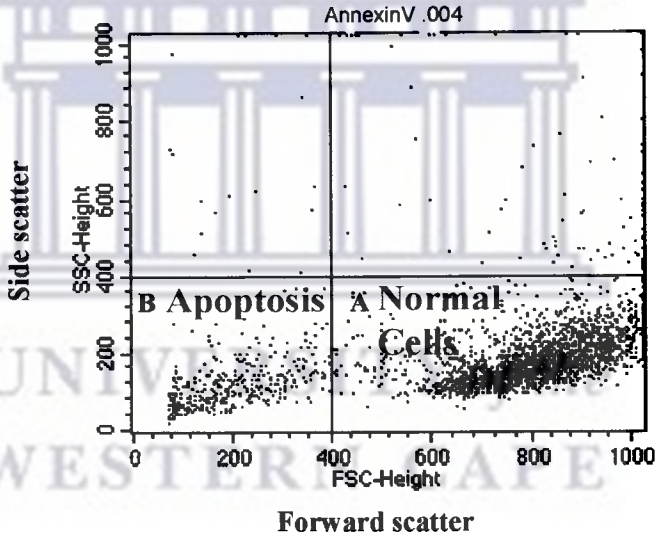


Figure 3.19: Dot plot of cells subjected to flowcytometric analyses for physical properties. Jurkat T Lymphoma (10^5) cells were seeded (section 2.2.1) and starved without fresh media for 96 h. The data generated was analysed by BD-bioscience FACS calibur ^{TM} software (San Jose, California, USA). (A) Normal cells. (B) Apoptotic cells.

A decrease in forward scatter with no change in side scatter indicates cell shrinkage and is a characteristic of apoptosis. An increase in side scatter with no change in forward scatter is indicative of necrosis. JURKAT-T cells were grown RPMI and starved for 96 h prior to analysis by flowcytometry. Cells in quadrant A (Figure 3.19) are normal (normal cell size) based on their forward scatter (FSC) pattern whereas cell in quadrant B are apoptotic (cell shrinkage) based on the side scatter (SSC) pattern. These scatter plots are helpful as it indicates the parameters to be used for the study of apoptosis.

With these findings we proceeded to evaluate staurosporine, which is a known inducers of apoptosis as our positive control to study apoptosis. JURKAT-T cells were grown on RPMI media and the treated cells exposed to staurosporine. Both the treated and untreated were stained simultaneously with 7–AAD and annexin- V PE and subjected to flowcytometric analyses. The result was obtained as a histogram where the relative fluorescent shift (X-axis) was measured against the cell count (Y-axis). Apoptosis was determined by a shift in cell population (along the X-axis) from the first decade to the second decade from 10^1 to 10^2 demarcated as M1 and M2 (Figure 3.20).

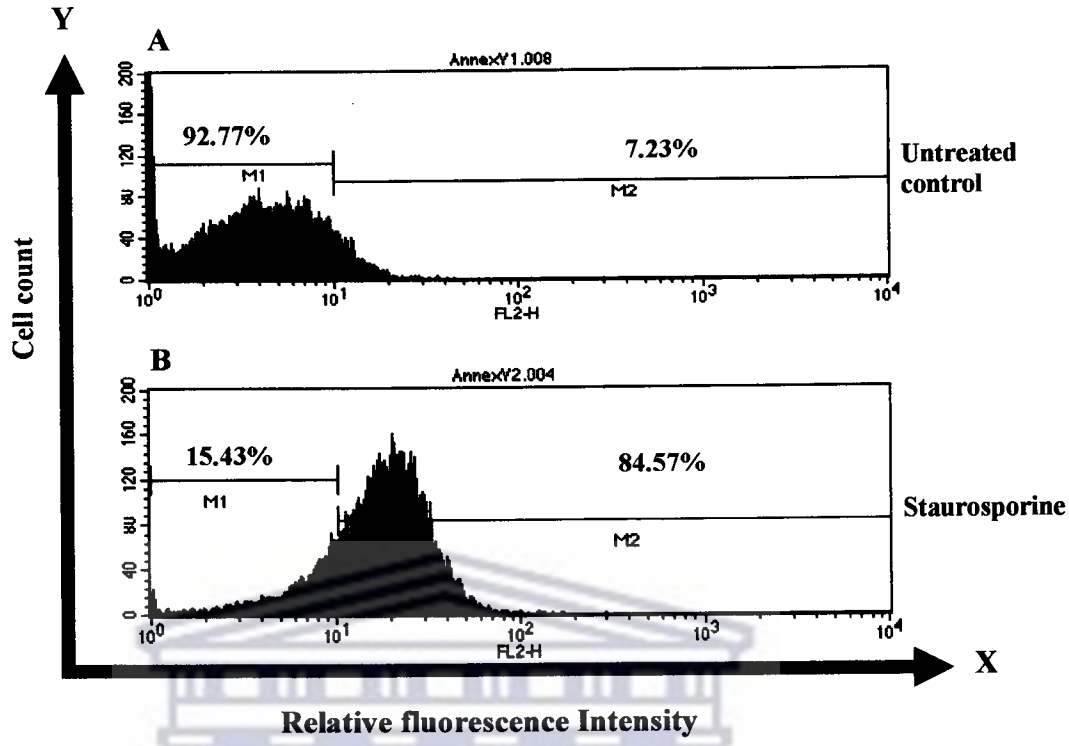


Figure 3.20: Histogram of Jurkat T cells stained with annexin-V PE. Jurkat T (1X 10^5) cells were cultured in a 5 ml culture flask (section 2.6.1) and treated where appropriate with staruosporine (1.3μM), stained with annexin-V PE and subject to flowcytometry. (A) Untreated cells, control. (B) Cells treated with staurosporine, positive control. The X and Y axis represent the relative fluorescence intensity and cell counts respectively. M1 and M2 represent the percentages of viable cells and non-viable (apoptotic) cells respectively.

Normal cells remain in the first decade and the percentage indicates the number of normal cells in the population. The second decade indicates cell death and the percentage indicates the number of cells that die naturally within the population. A shift along the X-axis (relative fluorescence) therefore indicates apoptosis and shift to

the third decade indicates necrosis. The percentage of cells in the untreated sample that are normal is 92.77 % (Figure 3.20). Cells treated with staurosporine showed 84.57 % of apoptotic cell death.

The analyses of the cells for apoptosis based on the use of dyes, physical properties and positive apoptotic inducers validate the use of flowcytometry to study apoptosis induced by *S. frutescens* extract 29.

Firstly the chloroform fraction of *S. frutescens* extract 29 was analyzed using HPLC chromatogram and the chloroform fraction was also analyzed for apoptosis using flowcytometry (Figure 3.21).

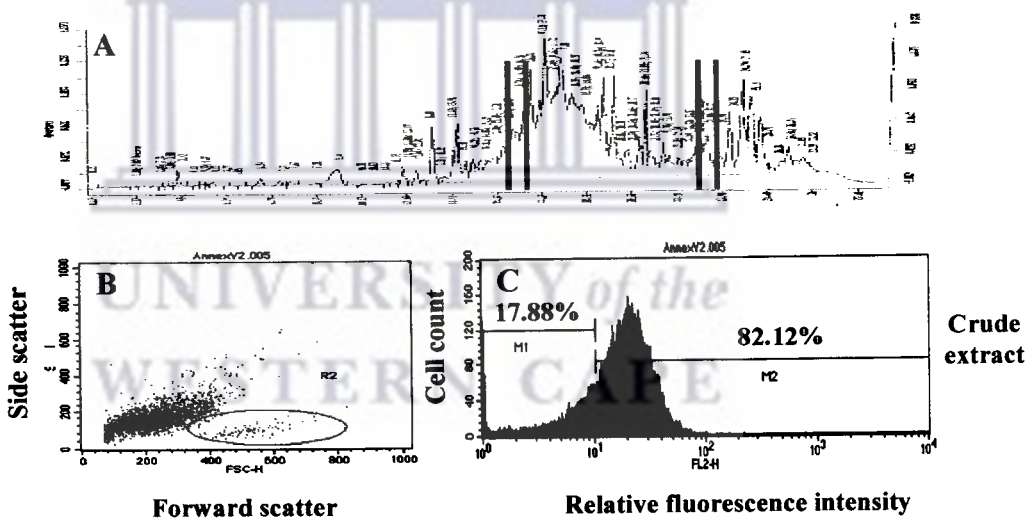


Figure 3.21: HPLC and flowcytometry of the chloroform fraction of *S. frutescens* extract 29.

The HPLC method is described in section 2.4.2. Jukat T (1 x 10^5) cells were treated with the chloroform fraction of extract 29 and the cells subject to flowcytometry as described in section 2.6.1. (A) HPLC Chromatogram. (B) Dot plot of cells subjected to flowcytometric analyses for

physical properties where the X and Y axis represent the forward and side scatter respectively. R2 represents the normal cell population in the graph and the region outside R2 represents apoptotic cells. (C) Histogram of Jurkat T cells treated with the chloroform fraction of extract 29 stained with annexin-V PE where the X and Y axis represent relative fluorescence intensity and cell count respectively. M1 and M2 represent the percentages of viable cells and non-viable (apoptotic) cells respectively.

The HPLC chromatogram showed the peak profile of the chloroform fraction of the *S. frutescens* extract 29 (Figure 3.21 A). This profile is unique for the particular extract and will help identify peaks of interest for later analyses. The flowcytometric results indicate that the chloroform fraction of extract 29 had apoptotic activity. The dot plot (Figure 3.21 B) indicates that cells in the R2 region are normal (FSC pattern) while cells outside the R2 region (SSC pattern) are apoptotic. This is further indicated by the histogram result where 82.12 % of the population of the cells were apoptotic, decade M2 (Figure 3.21 C).

3.9 Bioactivity of HPLC fractions of *S. frutescens* extract 29 by flowcytometry

After the above analysis the peaks were fractionated into separate active peaks, demarcated by black lines (Figure 3.21 A), and each peak analyzed using flow cytometry (Figure 3.22 and 3.23). Only two peaks, fraction 46 (F46) and fraction 49 (F49), are discussed below.

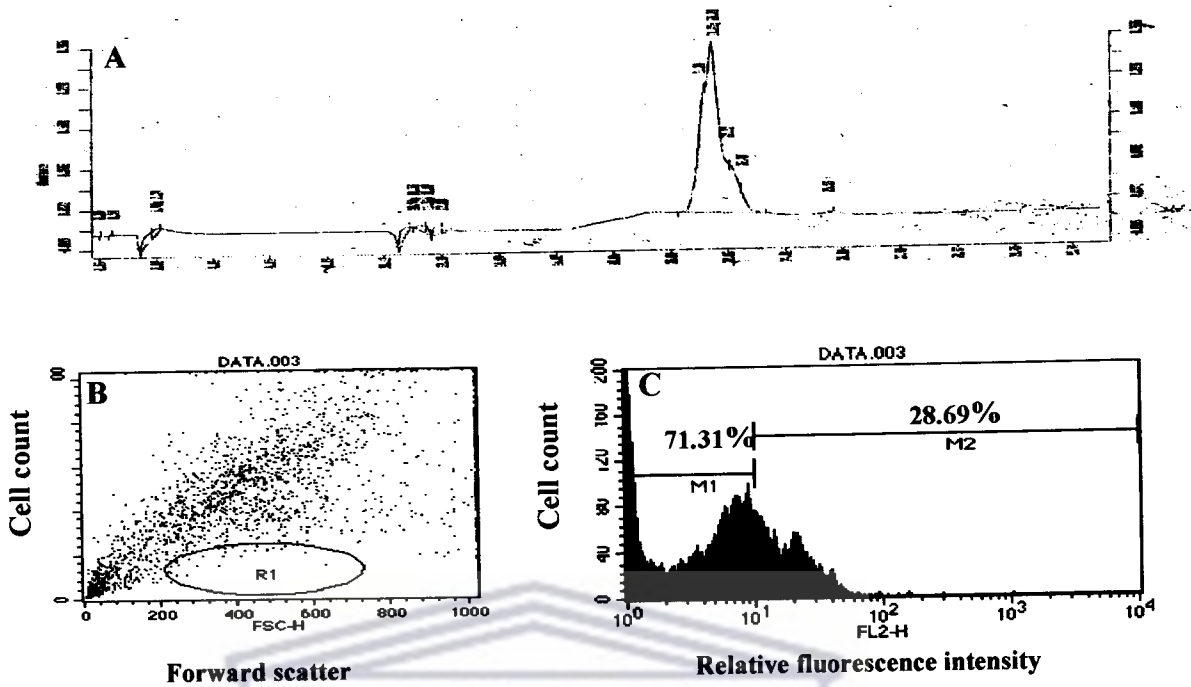


Figure 3.22: HPLC and flow cytometry of the HPLC fraction 46 of *S. frutescens* extract 29. The HPLC method is described in section 2.4.2. Jukat T (1 x 10^5) cells were treated with HPLC fraction 46 of extract 29 and the cells subject to flowcytometry as described in section 2.6.1. (A) HPLC Chromatogram. (B) Dot plot of cells subjected to flowcytometric analyses for physical properties where the X and Y axes represent the forward and side scatter respectively. R1 represents the normal cell population and the region outside R1 represents apoptotic and necrotic cells. (C) Histogram of Jurkat T cells treated with HPLC fraction 46 of extract 29 stained with annexin-V PE where the X and Y axes represent relative fluorescence intensity and cell count respectively. M1 and M2 represent the percentages of viable cells and non-viable (apoptotic) cells respectively.

JURKAT-T cells were treated with fraction 46 of extract 29 obtained from HPLC (Figure 3.22 A) and subject to flowcytometry. The flowcytometric results indicate that fraction 46 of extract 29 had apoptotic and necrotic activity. The dot plot (Figure 3.22 B) indicates that cells in the R1 region are normal (FSC pattern) while cells outside the R2 region (SSC pattern) are apoptotic and necrotic. Apoptosis however is further indicated by the histogram result where 71.31 % of the population of the cells was viable (M1) while 28.69 % of the cells were apoptotic M2 (Figure 3.22 C).

JURKAT-T cells were also treated with fraction 49 of extract 29 obtained from HPLC (Figure 3.23 A) and subject to flowcytometry.

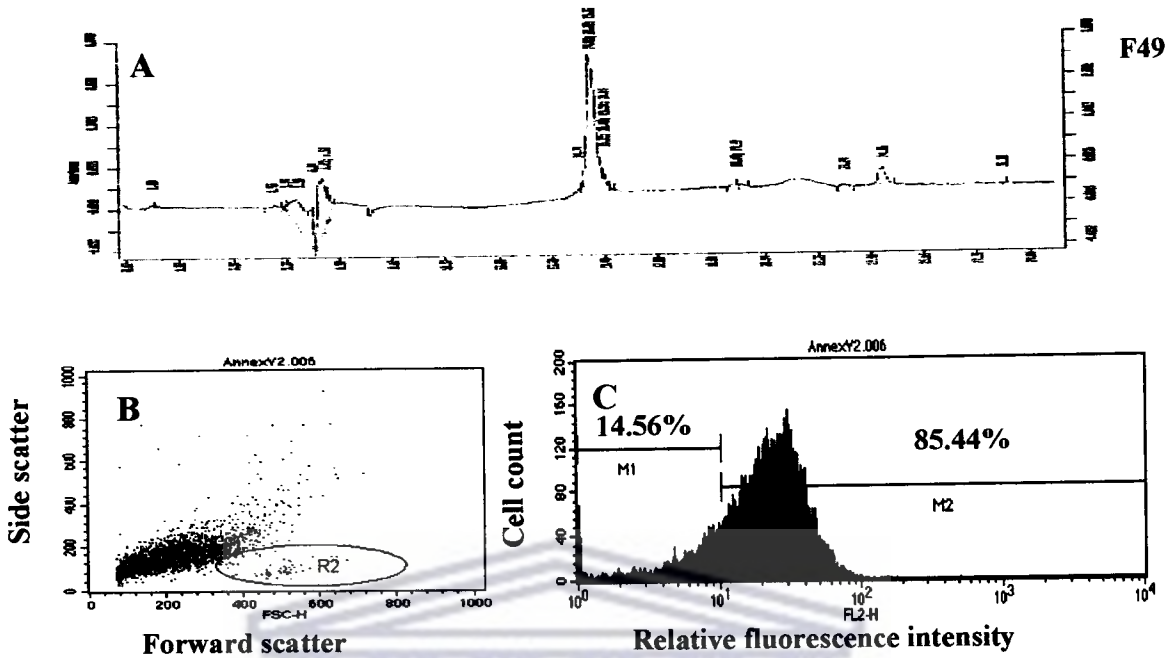


Figure 3.23: HPLC and flow cytometry of the HPLC fraction 49 of *S. frutescens* extract 29. The HPLC method is described in section 2.4.2. Jukat T (1 x 10^5) cells were treated with HPLC fraction 46 of extract 29 and the cells subject to flow cytometry as described in section 2.6.1. (A) HPLC Chromatogram. (B) Dot plot of cells subjected to flowcytometric analyses for physical properties where the X and Yaxes represent the forward and side scatter respectively. R2 represents the normal cell population and the region outside R2 represents apoptotic cells. (C) Histogram of Jurkat T cells treated with HPLC fraction 46 of extract 29 stained with annexin-V PE where the X and Y axis represent relative fluorescence intensity and cell count respectively. M1 and M2 represent the percentages of viable cells and non-viable (apoptotic) cells respectively.

The flowcytometric results indicate that fraction 49 of extract 29 had apoptotic activity. The dot plot (Figure 3.23 B) indicates that cells in the R2 region are normal

(FSC pattern) while cells outside the R2 region (SSC pattern) are apoptotic. Apoptosis is further indicated by the histogram result where 14.56 % of the population of the cells was viable (M1) while 85.44 % of the cells were apoptotic (M2) (Figure 3.23 C).

Previously the apoPercentage™ assay was used to determine apoptosis in a colometric analyses (section 3.2). We wanted to investigate whether an apoPercentage™ dye could also report apoptosis in flowcytometry. A control experiment was designed where CHO cells were grown and treated with 1.25 mg / ml of *S. frutescens* chloroform extract or 1.3 μM staurosporine for 6 h prior to flowcytometry using the apoPercentage™ dye (Figure 3.24).

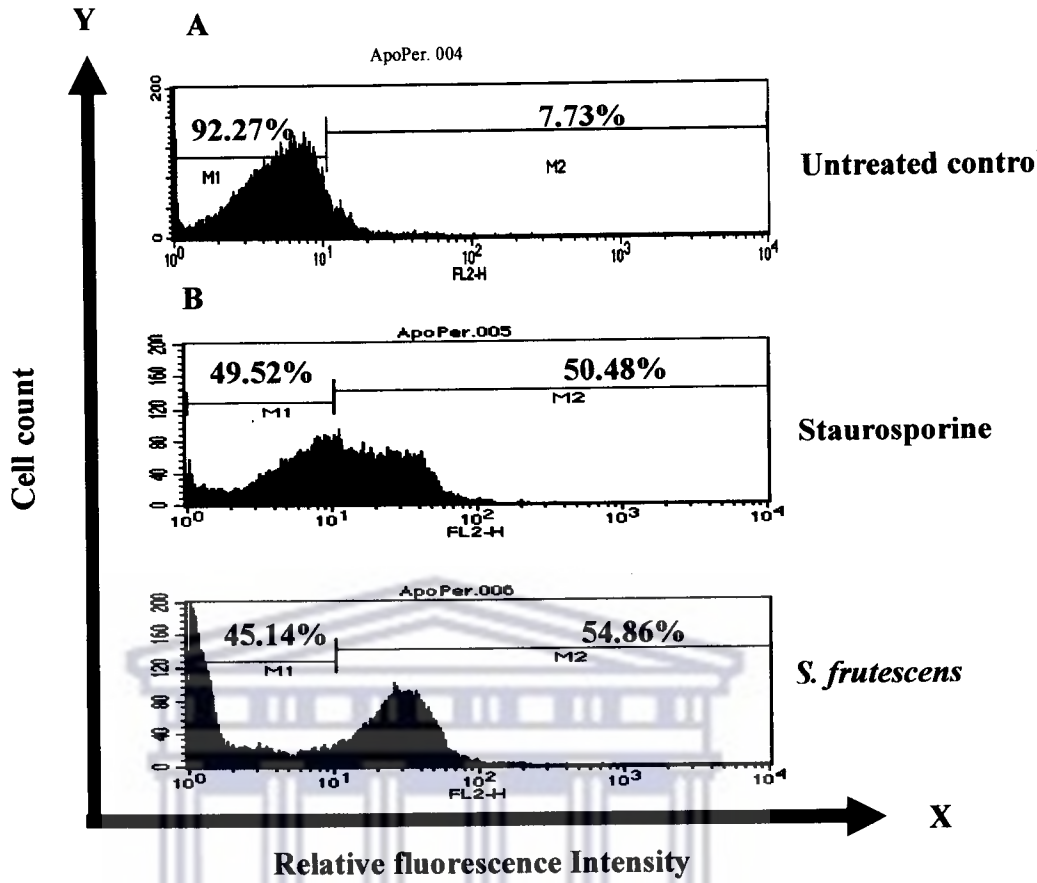


Figure 3.24: Histogram analysis of CHO cells stained with apoPercentage™ dye. CHO (1X 10^5) cells were cultured in a 5 ml culture flask (section 2.6.1) and treated where appropriate with *S. frutescens* extract 29 (1.25 mg / ml) or staurosporine (1.3 µM), stained with apoPercentage™ dye and subject to flowcytometry. (A) Untreated cells, control. (B) Cell treated with staurosporine, positive control. (C) Cells treated with *S. frutescens* extract 29. The X and Y axes represent the relative fluorescence intensity and cell counts respectively. M1 and M2 represent the percentages of viable cells and non-viable (apoptotic) cells respectively.

The percentage of cells in the untreated sample is primarily normal with 92.27 % (decade M1) of the cells viable (Figure 3.24 A). The positive control in which cells were treated with staurosporine had 50.48 % (decade M2) of the cells being apoptotic (Figure 3.24 B). Cells treated with extract 29 of *S. frutescens* had 54.86 % (decade M2) of the cells being apoptotic (Figure 3.24 C).

The ability of the apoPecentage™ dye to stain CHO cells and to emit fluorescence in flowcytometric analysis (section 3.10, Figure 3.24) suggests that the apoPercentage™ dye can be use as a fluorescent reagent for the study of apoptosis using flowcytometry.

The results in section 3.9 and this section (3.10) show that peaks fractionated from the chloroform fraction of *S. frutescens* extract 29 via HPLC induce apoptosis as analyzed by flowcytometry. The detection and analyses of apoptosis using flowcytometry have been used previously (Theodoridis *et al*, 2002; Vermeulen *et al.*, 2002; Herzig *et al.*, 2003; Nile, 2003).

We demonstrated that 82.2 % of the cells undergo apoptosis within 6 h when exposed to total crude extract 29 after chloroform fractionation (section 3.9, Figure 3.21). In a subsequent analysis two active peaks, F46 and F49, (section 3.10, Figure 3.22 and Figure 3.23) were isolated by bioactivity-guided fractionation. Fraction 46 induced slight apoptosis with 28.69 % cells being apoptotic and 71.31 % non apoptotic. Fraction 49 induced apoptosis significantly with 85.44 % of the cells showing

apoptosis. We thus concluded that both F46 and F49 might be different compounds that cause necrosis and apoptosis respectively. With our interest on apoptosis, F49 will be more valuable to investigate as a source of compounds that may have anti-cancer activity.

3.10 Aqueous extracts from various *S. frutescens* subspecies and the ability of these extracts to induce apoptosis

In the previous sections *S. frutescens* extract 29 were shown to induce apoptosis in cultured cells. In this section experiments were carried out utilizing extracts from different subspecies of *S. frutescens* to evaluate the strength of apoptosis inducing agents present in these extracts. CHO cells were treated with the various extracts from the *S. frutescens* subspecies and tested for apoptosis using the apoPercentage™ assay (section 2.5.1). Plants were collected as batches from the field, fresh frozen in liquid nitrogen and taken to the laboratory for extraction.

CHO cells were grown in a 96 well plate and treated with aqueous *S. fructescens* extracts (2.3.1) from *S. frutescens* roboster, *S. frutescens* tomantosa, *S. frutescens* white, and *S. frutescens* microphylla at concentration of 1.25 mg / ml (Figure 3.25).

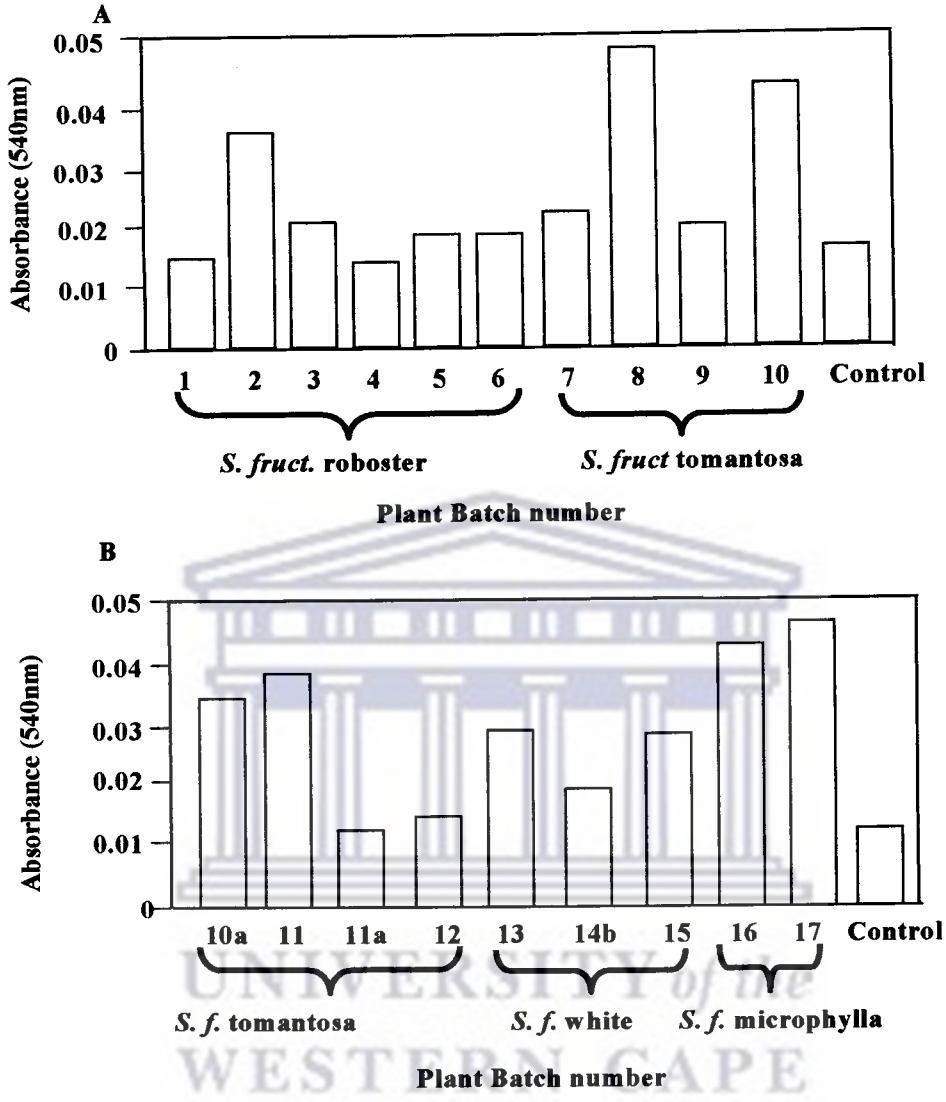


Figure 3.25: Graph indicating the ability of extracts from different subspecies of *S. frutescens* to induce apoptosis on CHO cells. CHO cells (0.25 x10^6) were seeded in a 96 well plate and allowed to grow for 24 h as described (section 2.2.1). The cells were treated with extracts from different batches of *S. frutescens* subspecies at a concentration of 1.25 mg / ml. The percentage of cell death was determined using apoPercentage^{TM} assay. Absorbance was read at 550 nm (maximum dye absorbance) and 625 nm (minimum dye absorbance). The difference between these two values was taken as the real

absorbance for the recovered dye that was trapped in the cells. An absorbance reading above 0.04 at 550nm indicated cell death. The extracts from the following *S. frutescens* subspecies were tested for inducing apoptosis, *S. f. roboster*, *S. f.* tomantosa, *S. f. white* and *S. f.* microphylla.

The results obtained from the apoPercentage™ assay indicate that the extract from *S. frutescens* roboster batch 2 had a higher apoptotic activity (A550 >0.03) than the other batches (1, 3, 4, 5, 6) of the same subspecies (A550 <0.03) and the control (A 550 <0.02) (Figure 3.25 A).

Extracts from batches 8 and 10 of *S. fructescens* tomantosa had high apoptotic activity (A550 >0.04, two folds higher than the control) and were higher than other batches (7, 9, 10a, 11, 11a and 12) (Figure 3.25). *S. frutescens* tomantosa batch 10a and 11 also induced high apoptotic activity (A550 > 0.03) as compared to batches 7, 9, 11a and 12 (A550 < 0.02).

Extracts from batch 13 and 15 of *S. frutescens* white* had higher apoptotic activity (A550 ~ 0.03) than batch 14.

* Indigenous name of *S. frutescens*

Two extracts from the *S. frutescens* microphylla batches showed high apoptotic activity (16 and 17) (A550 > 0.04) compared to the control and to the other subspecies extracts (*S. f.* roboster, *S. f.* tomantosa, *S. f.* white*).

Following these results we investigated the apoptotic activity of extracts from different parts of the plant. Aqueous extracts were obtained from both the white and pink flowers of *S. frutescens* white* and from the seeds and pods of *S. frutescens* white* and *S. frutescens* tomantosa. CHO cells were grown and treated with the crude extracts from the flowers, seeds and pods (2.3.1) and tested for apoptosis using the apoPercentage™ colorimetric assay (Figure 3.26).

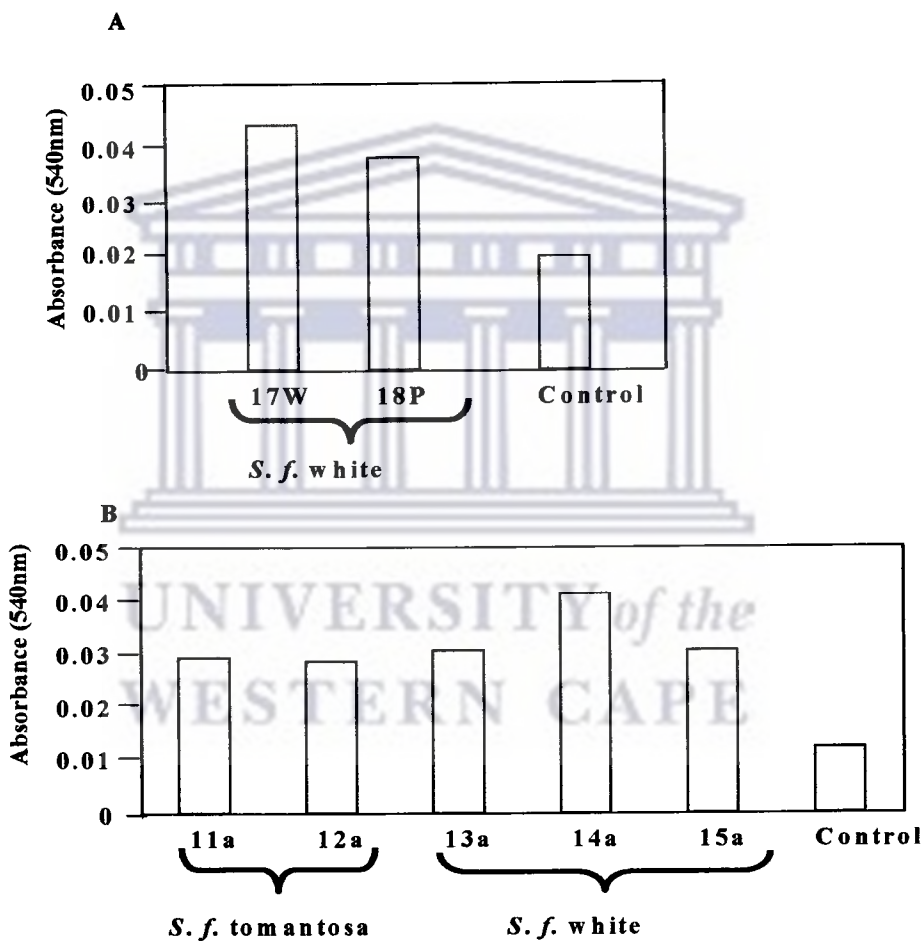


Figure 3.26: Graph indicating the ability of extracts from different parts of the *S. frutescens* subspecies to induce apoptosis on CHO cells. CHO cells (0.25 x10^6) were seeded in a 96 well plate and allowed to grow for 24 h as described (section 2.2.1).

The cells were treated with extracts from flowers, seeds and pods obtained from *S. frutescens* subspecies at a concentration of 1.25 mg / ml. The percentage of cell death was determined using apoPercentage™ assay. Absorbance was read at 550 nm (maximum dye absorbance) and 625 nm (minimum dye absorbance). The difference between these two values was taken as the real absorbance for the recovered dye that was trapped in the cells. An absorbance reading above 0.06 at 550nm indicated cell death. The extracts from the following *S. frutescens* subspecies were tested for inducing apoptosis,*S. f.* tomantosa and *S. f. white*

The results indicate that extracts from the white flowers of *S. frutescens* white* (batch 17W) have higher apoptotic activity (A550 >0.04) than the extracts obtained from pink flowers (batch 18P, A550 <0.04) (Figure 3.26 A). Extracts obtained from the seeds and pods from *S. frutescens* white* (batch 14a) has higher apoptotic activity (A550 ~0.04) than extracts of batch 13a and 15a (A550 ~0.03) (Figure 3.26 B). Seed and pod extracts from of *S. frutescens* tomantosa batches show similar apoptotic activity (A550 ~0.03) (Figure 3.26 B).

We found that two batches of the *S. frutescens* microphylla extracts (16 and 17, Figure 3.25) had the highest apoptotic active and *S. frutescens* white* and *S. frutescens* roboster were the least active. These differences in activity could be as a result of genetic variation within the subspecies studied leading to a difference in the level of apoptotic compound production. It could also mean that the age of the plant

was different or environmental stress responses could be responsible for the presence of different apoptotic compounds in the different subspecies.

The analysis comparing the white flowers and pink flowers shows that the white flowers are slightly higher in apoptotic activity compared to the pink flowers seen in Figure 3.26 A. The reason of this difference in activity is not understood. We speculated that the active ingredients *S. frutescens* might be more concentrated in the in the white flowers than in the pink flowers of the plants. The seeds and pods of *S. frutescens* white* did not show any significant difference in activity compared to extracts of *S. frutescens* tomantosa. Seeds and pods of *S. frutescens* microphylla and *S. frutescens* roboster were not available at the time of collection and could therefore not be analyzed.

3.11 Chromatogram of extracts obtained from different *S. frutescens* subspecies

The HPLC chromatographic profiles of the extracts from the different *S. frutescens* subspecies were obtained to determine the variation in peak patterns between the subspecies. Chloroform extracts from the different subspecies were subjected to HPLC analysis (Figure 3.27).

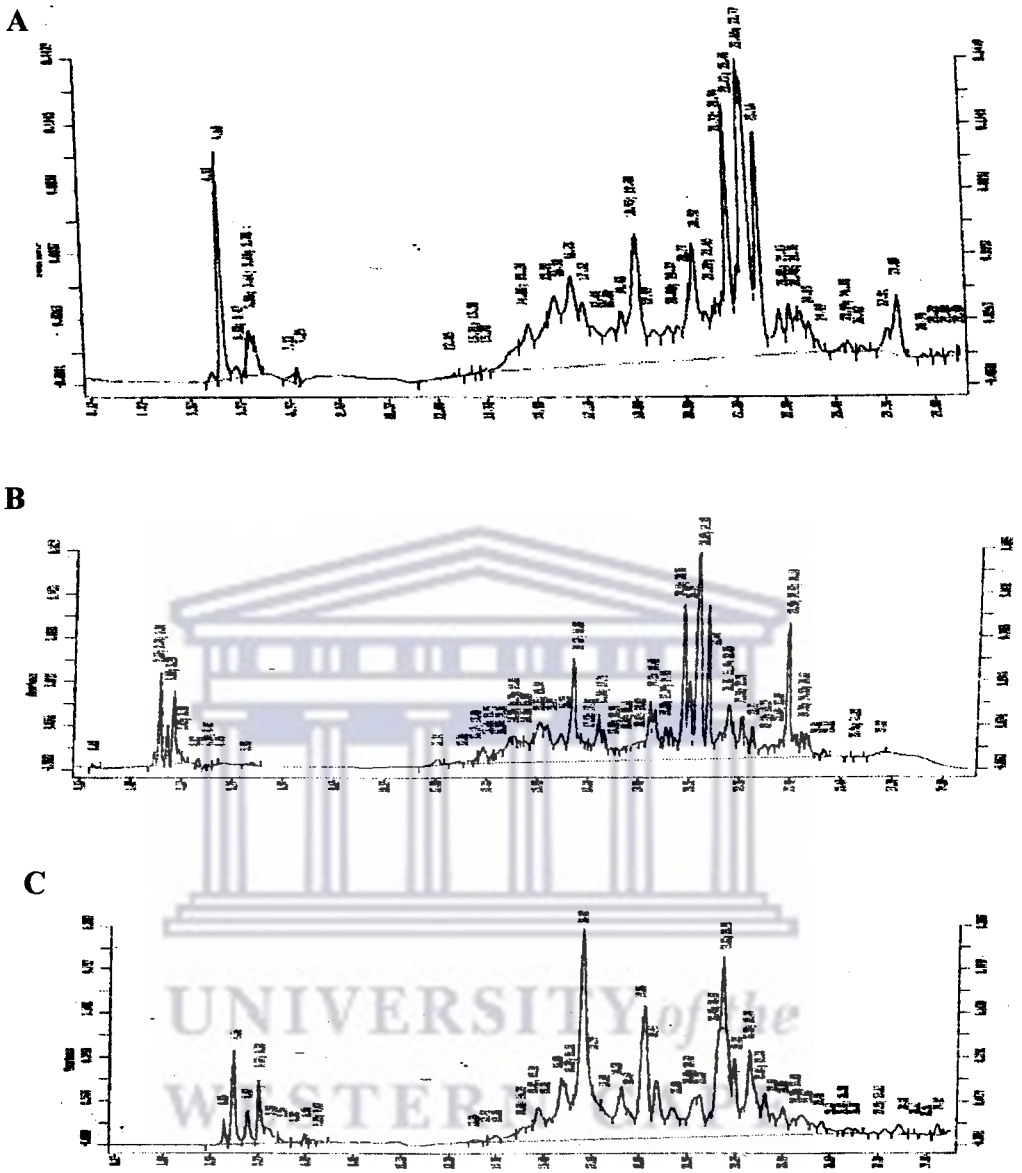


Figure 3.27: HPLC chromatogram of chloroform extracts from different subspecies of *S. frutescens.* The HPLC method is described in section 2.4.2. (A) *S. frutescens* white* batch 13. (B) *S .frutescens* tomantosa batch 8. (C) *S. frutescens* roboster batch 16. X and Y axis represent the time and absorbance. Each peak is indicated at the top of the peak by the time of elution.

The HPLC profile of the extract obtained from *S. frutescens* white* (batch 13), *S. frutescens* tomantosa (batch 8) and *S. frutescens* roboster (batch 16) had different peak profiles (Figure 3.27). The different HPLC profiles could indicate different compounds and/or quantities of compounds in the plant extracts.

HPLC was also used to study the chromatographic profile of extracts from seeds and pods obtained from the two subspecies *S. frutescens* tomantosa and *S. frutescens* white* (Figure 3.28).

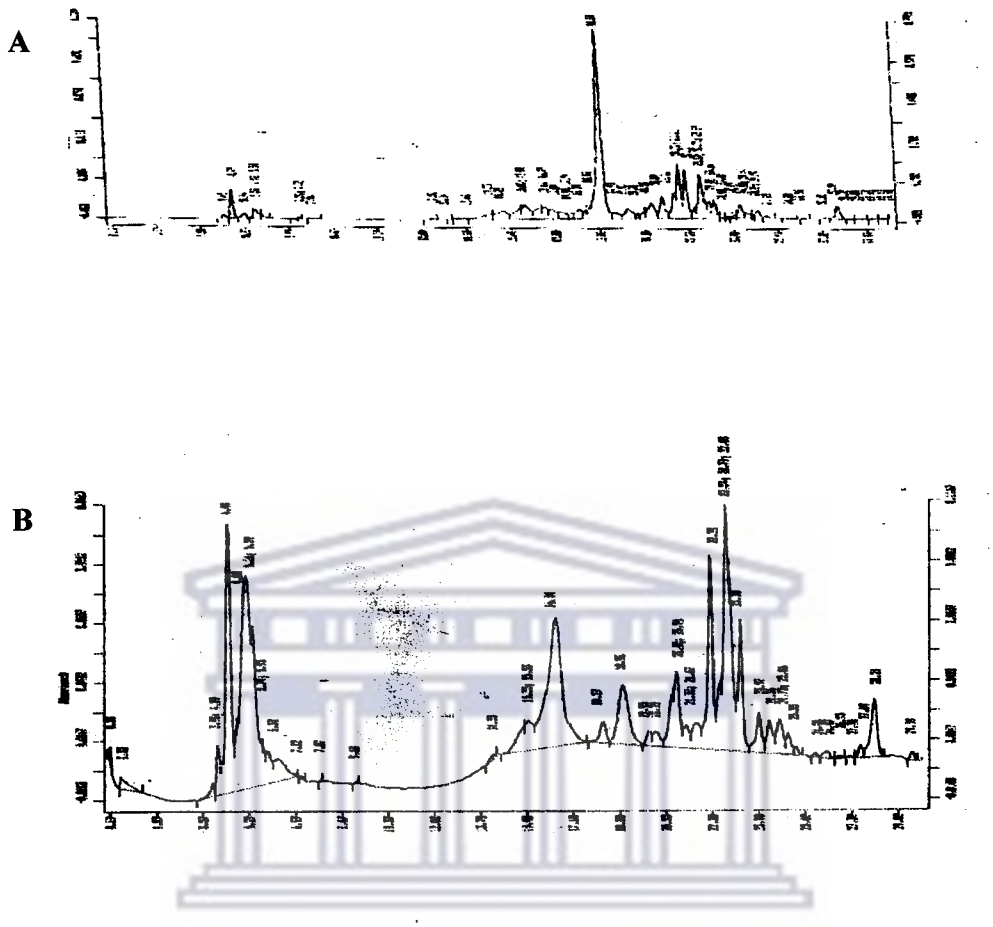


Figure 3.28: HPLC chromatogram of chloroform extracts from the seeds and pods of different subspecies of *S. frutescens.* The HPLC method is described in section 2.4.2. (A) *S. frutescens* tomantosa seeds and pods, batch 11A. (B) *S .frutescens* white* seeds and pods, batch 14A. X and Y axis represent the time and absorbance. Each peak is indicated at the top of the peak by the time of elution.

The HPLC profile of the extract obtained from *S. frutescens* tomantosa seeds/pods

white (batch 14A) (Figure 3.28). The latter extract also had a higher peak density which could indicate more compounds.

Lastly, HPLC was also used to study the chromatographic profile of extracts from the white and pink flowers from the *S. frutescens* white* subspecies (Figure 3.29).

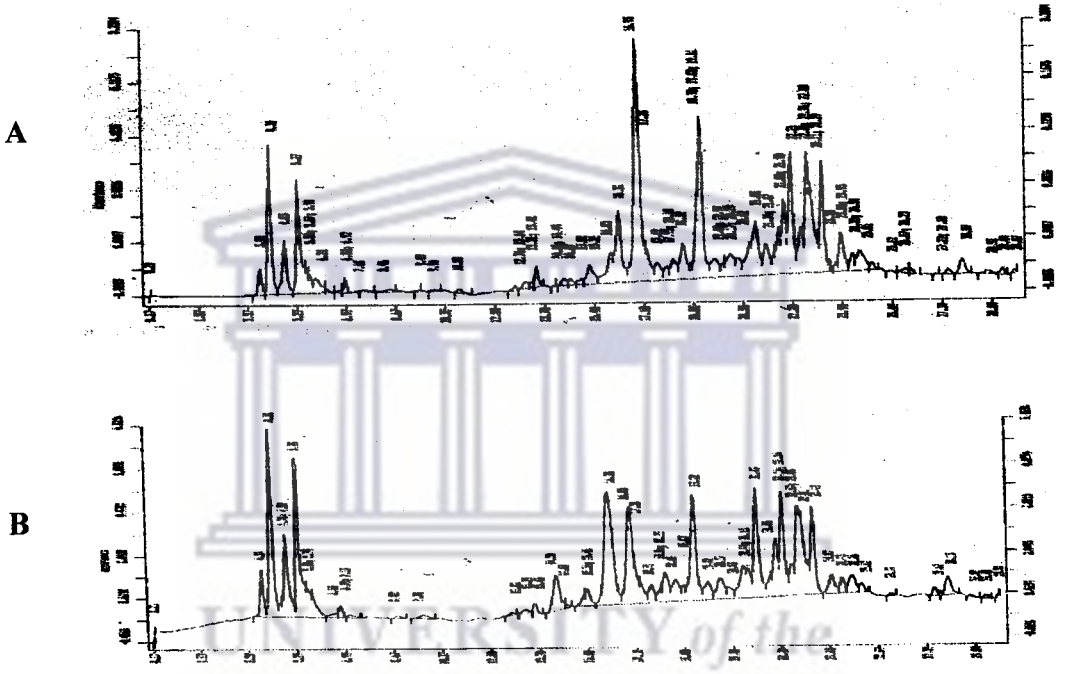


Figure 3.29: HPLC chromatogram of chloroform extracts from white and pink flowers of subspecies *S. frutescens* white*. The HPLC method is described in section 2.4.2. (A) *S. frutescens* white* white flowers, batch 17. (B) *S .frutescens* white* pink flowers, batch 18. X and Y axis represent the time and absorbance. Each peak is indicated at the top of the peak by the time of elution.

The HPLC profile of the extract obtained from *S. frutescens* white* pink and white flowers had slight differences in peak profile and density (Figure 3.28). Previously it

was shown that extract from the white flowers had slightly more apoptotic activity than the pink flowers (section 3.11, Figure 3.26). Comparing the difference in their peak density it may be that the increase in activity of the white flowers could be due to the concentration of the active compounds present in that extract being greater.

A HPLC was also done on the total chloroform and total ethyl acetate fraction of *S. frutescens* extract 29 (result not shown). The HPLC profile of the chloroform fraction has more complex peaks than those of the ethyl acetate fraction. This suggests that the chloroform fraction of *S. frutescens* extract 29 has a larger number of complex compounds than the ethyl acetate fractionation. Even though the complexity and number does not necessarily suggest more apoptotic activity we could speculate that the chloroform fraction of *S. frutescens* extract 29 may have a higher concentration of the bio-active compounds (secondary metabolites) than the ethyl acetate fraction (Table 3.2).

The chromatographic profile and peak prominence of subspecies of *S. frutescens* extracts (*S. frutescens.* white*, *S. frutescens.* tomantosa, *S. frutescens.* roboster) and the different parts (white flowers, pink flowers, seeds and pods) were examined. The HPLC profile shows that the peak profiles are different from each other for the different subspecies and different parts of the plant. Since the peak profile does not correlate with the bioactivity of the various extracts (whole plant part, seeds and pods,

white and pink flowers) we infer that the activity of the extracts is not reflected in the HPLC profile.

However, if a fraction from a subspecies or part of the plant is found that gives reproducible apoptotic activity, the HPLC profile of this fraction could be used as a template for HPLC identification of promising extracts from other *S. frutescens* subspecies or plants.

CHAPTER FOUR

General Conclusion

Apoptosis is a form of programmed cell death that occurs naturally in cells (Zimmermann *et al.,* 2001; Kimball, 2003). The activation of apoptosis can be beneficial to cancer therapy because it eliminates potentially cancerous cells from the body. *S. fructescens*, commonly known as "kankerbos" is claimed by traditional healers to have beneficial effects against cancer. We sought validation of this claim by sampling *S. frutescens* plants from different geographical locations in South Africa, obtaining the aqueous extracts and testing the ability of the extracts to induce apoptosis in CHO, (Chinese Hamster Ovary), CASKI (cervical carcinoma), JURKAT (T lymphoma), HELA (variant form of cervical cancer cell) and NHF (normal human fibroblast) cells.

One particular extract from *S. frutescens*, extract 29, caused the most apoptosis on treated cells. Extract 29 was separated based on polarity, using organic solvents yielding partially fractionated secondary metabolites. We assayed and quantified the induction of apoptosis based on morphological changes; various biochemical assays and flowcytometric analysis. Active fractions were further separated and purified using TLC and HPLC. We have demonstrated that extract 29 from *S. frutescens* has the ability to activate apoptosis in cells and that this bioactivity can be traced during the fractionation of the crude extract. Based on morphological observations of cells,

we also cast doubt on the suggestion that canavanine is the active compound present in *S. frutescens* responsible for the beneficial effect against cancer (Van Wyk, 1997).

Our data also demonstrates that *S. frutescens* extract bioactivity, varies between different geographical collections of the same species and different subspecies studied. In order to extract more of the apoptotic compounds and to know which part of the plant contains a higher concentration of these compounds, it will be vital to evaluate extracts from different parts of *S. frutescens* (seeds, roots, stems, leaves and flowers).

In summary, we have partially validated the claim by traditional healers that *S. frutescens* has anti-cancer properties by identifying an extract in the plant that has marked apoptotic activity. However this apoptotic activity seems not to be universally present in all *S. frutescens* species tested.

We anticipate that some of these compounds in these *S.frutescens* extracts, if structurally identified and characterized (MS, ^{1}HNMR) may be candidates for anti-cancer drug development.

A characterized compound from *S. frutescens* can be used to study apoptotic pathways based on the expression of certain genes like p53 and the activation of caspases and the cleavage of PARP during chromosomal DNA damage. This will pave the way for the identification of other novel candidate compounds that will be

therapeutic targets for cancer. The study of additional cell lines both normal cells as well as cancer cells will also be vital to evaluate any resistance to these plant extracts.

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APPENDIX

Preparation of apopercentage™ dye

The dye was added to complete medium at a concentration of one in twenty dilution 0.5 ml of dye in 9.5 ml medium 10 mls [10 µm] of this working stock dye solution was used for 96 well plate format. The final concentration becomes 10 µm. To each of the well is added 100 ul / well of the prepared dye.

Preparation of canavanine

solubility of canavanine = 100 mg/ml

1 molar concentration of canavanine = mw / 1 = 274.2 g / 1

0.274 g in 1 ml = 1 molar

100 g in 1 ml = .365 m

at solubility of 50mg / ml concentration = $\frac{.365\ M}{2}$

working concentration = 0.182 m

$c_1v_1 = c_2v_2$

182 mm x v_1 = 10 mm x 10 mls

$v_1 = \frac{10x10}{182}$

=. 549 mls or 549 µl

549 µl of 1ml (0.182 m) was diluted with 549 µl media(100 mm)

Preparation of staurosporine

DMSO supplied at 100 % concentration

Solubility of staurosporine = 5 mg / ml which is 0.5 mg /100 µl

500 µg staurosporine was weighed

500 µg was dissolve in 100 µl of 100 % DMSO

Dissolve 0.5g in 200 µl of 100 % DMSO

=2.5 mg / ml

Molecular weight (mw) = 466.5 g

466.5 g mol^{-1} = 1 m

.466.5 mg / l = 1m

2.5 mg / ml = 5400 µm

to prepare 10 ml of 54 µm working concentration

$= c_1v_1 = c_2v_2$

$v_1 \times 5400\ \mu m = 54\ \mu m \times 10\ ml$

$$v_1 = \frac{540}{5400}$$

0.1 ml = 100 µl

dilute 100 ul of stock in to 9.9mls of hams f12 to get a.

final concentration 54 µm

concentration of dmso in the stock $c_1v_1 = c_2v_2$

$$100\ \% \times 0.1\ \text{ml} = c_2 \times 10\ \text{mls}$$

$$c_2 = \frac{100 \times .0.1}{10}$$

$$= 1\ \%$$