EVALUATION OF *SUTHERLANDIA FRUTESCENS* FOR ANTI-CANCER ACTIVITY

BY

TANDEIH ALFRED GHOGOMU.

A THESIS SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN PHARMACEUTICAL SCIENCES, IN THE SCHOOL OF PHARMACY, FACULTY OF NATURAL SCIENCES, UNIVERSITY OF THE WESTERN CAPE.

SUPERVISORS: PROF. GEORGE J AMABEOKU
SCHOOL OF PHARMACY.

PROF. JASPER D. REES
DEPT. OF BIOCHEMISTRY.

MARCH, 2002.

EVALUATION OF *SUTHERLANDIA FRUTESCENS* FOR ANTI-CANCER ACTIVITY.

(TANDEIH ALFRED GHOGOMU)

KEY WORDS:

Anti-cancer

Activity

Apoptosis

Cholinesterase activity

Drug design

In-vitro

In-vivo

Medicinal plants

Sutherlandia frutescens

Treatment

ABSTRACT

EVALUATION OF *SUTHERLANDIA FRUTESCENS* FOR ANTI-CANCER ACTIVITY

TANDEIH ALFRED GHOGOMU.

MSc Thesis, School of Pharmacy, University of the Western Cape.

Purpose: The claim for anti-cancer activity of the plant *Sutherlandia frutescens* was investigated against some cancer cell lines in-vitro and against a dichlorvos induced chemical carcinogenesis in-vivo. A preliminary phytochemical analysis of the plant extract was also done.

Methods: In-vitro studies were done using a concentration of 10mg/ml aqueous plant extract to induce apoptosis in three cancer cell lines- human epidermoid cervical cancer, oesophageal cancer and lung cancer which were stained by both hematoxylin and eosin, and crossmon trichrome techniques. In the in-vivo study, phytosterols, coumarins, flavonoids and tannins resulting from organic solvent extraction were used in addition to the aqueous plant extract. The aqueous extract and isolates were injected intraperitoneally in varying test doses ranging from 12.5mg/kg to 1000mg/kg in mice chemically induced for carcinogenesis with dichlorvos (6mg/kg) and plasma activity levels of the used index butyryl cholinesterase determined.

Results: In-vitro results as seen on photomicrographs show cells treated with *Sutherlandia frutescens* extracts exhibiting features such as cell shrinkage, membrane blebbing, nuclear fragmentation and condensation, all consistent with apoptosis. While the in-vivo results reveal all test doses of both the aqueous extract and isolates significantly attenuating the effect of dichlorvos. $P<0.001$ for tests compared with controls in a paired Student's t-test.

Conclusion: Results suggest that *Sutherlandia frutescens*, has anti-cancer activity. This affirms claims of activity and conforms with reports of cancer risk reduction of phytosterols, coumarins, flavonoids and tannins, which are all isolates of the plant.

March, 2002.

DECLARATION

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

FULL NAME: TANDEIH ALFRED GHOGOMU.

DATE : March, 2002.

SIGNED: 

ACKNOWLEDGEMENTS

I wish to express my profound gratitude to the following;The Lord Almighty for His grace, protection and provision through out my study. My profound gratitude goes to my supervisors Prof George Jimboyeka Amabeoku and Prof Jasper D Rees for their friendly and fatherly care, in addition to the supervision of this project. I wish to express my thanks to Prof James Syce, coordinator postgraduate program, School of Pharmacy, for his guidance, advice and support during my study and to the whole staff of the school of Pharmacy for their support.

My appreciation also goes to Prof Richard Slaughter of the College of Pharmacy, Wayne State University, USA, for reviewing my thesis and for his advice and moral support. Many thanks to Jan Smit, Department of Anatomy, UWC, who has stood by me as an adoptive father and supported me through out my study. Also, the following in the Department of Anatomy, Dr Heyns, Prof C Leonard, Mrs Delport, Mr Arries and Mr Nkonjoana are worth mentioning for being a wonderful family.

My sincere appreciation also goes to the Department of Biochemistry, UWC and especially to Prof Jasper Rees' lab for the material and technical support during the in-vitro study. I want to particularly mention Mervin Meyer and Chinkwo Kenneth in Biochemistry and Evan Springfield in the School of Pharmacy for their technical assistance.

My family (the Mundingo Ghogomu family) and my fianceė Nduku Victorine are included in this prayer of thanks for all the moral and financial support, and for their prayers, as well as the United Crusade Team for Jesus Christ, especially Pastor Joshua Ghogomu for the prayers and support. I appreciate the following friends, Akoh Harry Asana, Egoh Benis, Vianney, Masha Tasha Roland, Chu Fidelis, Cham Gerald, Ticha Ignatius, Dr Weizhou wang and family, Kathy Hall, Pastor A. Volker and wife Joy, the Mowbray Baptist church, all my friends and colleagues at the school of Pharmacy for being there for me. Finally, I am thankful to the NRF for funding part of this work.

DEDICATION

This work is dedicated to the Lord Almighty, the Mundingo Ghogomu family and to my lovely fianceé Nduku M Victorine.

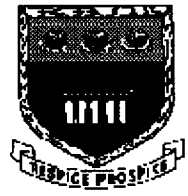
CONTENTS

List of Tables

List of figures

CHAPTER ONE

GENERAL INTRODUCTION



1.1 Introduction

The World Health Organization (WHO) defines health as " a state of complete physical, mental, and social well being and not merely the absence of disease or infirmity " (1,2,3). Though not a static condition, constant changes and adaptation to stress result in homeostasis (3), which when deregulated, results in disease.

According to the WHO, the world is in a health transition. Infection as a major cause of suffering and death is giving way to new epidemics of non-communicable disorders such as cardiovascular diseases, diabetes, and cancer (4,5).

The global incidence of cancer is soaring and physicians currently diagnose 10 million new cases of cancer each year. Statistical trends indicate that this number will double by 2020 (4,5). Given the fact that one in three people in the developed world will get cancer and one in four will die as a result, it is obvious that cancer is the condition most feared by the public. 70% of cancer patients will live in countries that between them will have less than 5% of the resources for cancer control (4). The flurry of the advances in the understanding of the disease at a molecular level has promised to reduce the fear and the world is now poised to see very significant advances in the diagnosis, detection, prevention, and treatment of cancers.

Techniques of molecular and cell biology, genetics and genomics have converged to reveal how the disease develops at the level of genes and molecules. However, all the approaches demonstrate that cancer will continue to become an increasingly important component in the global burden of diseases as this millenium continuously unfolds.

In the past three decades, there has been steady gains overall in the quality of care for cancer patients. Surgery has become more conservative due to technological improvements. Computers have made radiotherapy more precise with conformal therapy now routine for radical treatment, and the role of chemotherapy has become more defined. From the laboratory, we have seen an explosion in the understanding of cancer at

a molecular level. It is from a deeper understanding of the growth factor binding, signal transduction, transcription and cell cycle control, apoptosis and angiogenesis that novel systemic therapies will almost certainly emerge (4).
It is in this light of novel treatment and drug search that researches on medicinal plants have been vibrantly encouraged by the whole international community, as discovery of new drugs can emerge from plant screenings, and these plants are rooted in the traditional medicine practices of our societies.

The term " traditional medicine" refers to the ways of protecting and restoring health that existed before the arrival of modern medicine (6). As the term implies, the approaches to health belong to the traditions of each country, and have been handed down from generation to generation. A large proportion of the population in most developing countries, especially the rural dwellers, still relies on traditional practitioners, including traditional birth attendants, herbalists and bone-setters and on local medicinal plants to satisfy their primary health care needs (6).

Medicinal plants are the oldest known health care products. Their importance is still growing although it varies depending on ethnological, medical and historical background of each country. Medicinal plants are also important for pharmacological research and drug development, not only when plant constituents are used directly as therapeutic agents, but also when they are used as basic materials for the synthesis of drugs or as sources for pharmacologically active compounds.

In prospecting for new medicines, researches in the identification, collection and analysis of medicinal plants have increased tremendously between 1960 to date (5). Between 1960 and 1980, the United States National Cancer Institute collected samples of almost 35,000 plants species and now receives almost 10,000 plant, marine, invertebrate, fungal, and algal samples each year (5). The importance of medicinal plants in medicine today can not be over emphasized as they are key ingredients in everything from aspirin to contraceptives pills and are the foundation of health care systems all over the world. For example, up to 80% of the people in Africa go to traditional medicine doctors, who often administer extracts of local plants (5).

Plant active ingredients are discovered daily for the treatment of diseases. Since cancer research currently focuses strongly on biochemical pathways operating in normal and cancer cells, and the way in which they control cell division and programmed cell death (apoptosis); many plants are used in these researches for their active influences on these pathways. So researchers are not only identifying new molecules and new pathways for cell regulation and cell growth, but are also identifying potent anti-cancer plants and their active ingredients. The combination of these two aspects of research will certainly culminate in the development of new targets for therapeutic intervention in cancer and new drug discovery.

Identifiable "anti-cancer" plants particularly in South Africa as gathered from literature and also from local traditional practitioners include; *Tulbaghia violacea, Solanum tomemtosum, Leonotis spp, Helichrysum excisum, Elytropappus spp, Dicoma capensis, Catella asiatica, Combretum caffrum and Sutherlandia frutescens* (cancer bush), (7), which is the plant used in this study.

Sutherlandia frutescens is the plant of choice in this work, due to the fact that it is highly acclaimed by local traditional practitioners for excellent anti-cancer activity. Further more, the Department of Biochemistry at the University of the Western Cape, has been working extensively, evaluating the apoptosis inducing properties of the plant on Chinese Hamster Ovary (CHO) cells, thus also trying to authenticate activity claims, and there after isolating possible active compounds.

1.2 Research Problem

The investigation explores the question " Is *Sutherlandia frutescens* able to induce programmed cell death (apoptosis) of cancer cells in-vitro, and is it able to beneficially influence or interfer with a dichlorvos initiated chemical carcinogenesis in-vivo?"

Standard chemical tests identified secondary metabolites, which were squentially extracted using petroleum ether, chloroform, and ethyl acetate. The secondary metabolites were then used in the in-vivo investigations to verify their influence on a dichlorvos initiated carcinogenesis.

1.3 Hypothesis

The hypothesis here is stated as *Sutherlandia frutescens* is able to cause programmed cell death (apoptosis) of cancer cells in-vitro and in-vivo, able to attenuate a chemical carcinogenesis initiated by dichlorvos.

1.4 Aim and Objectives

The aim of this study is to affirm the claims by traditional medicine practitioners that *Sutherlandia frutescens* has anticancer activity.

The following objectives were set for this study;

1) The identification and collection of the plant.

2) The extraction and preparation of the aqueous plant extract.

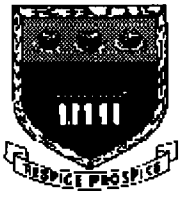
3) The treatment and evaluation of some cultured cancer cell lines with the aqueous plant extract in-vitro.

4) The treatment and evaluation of cholinestarase using the aqueous plant extract against the process of dichlorvos induced chemical carcinogenesis in-vivo.

5) The treatment and cholinesterase activity evaluation of organic solvent extracted fractions of secondary metabolites against the process of dichlorvos induced chemical carcinogenesis.

CHAPTER TWO

LITERATURE SURVEY



2.1 CANCER REVIEW

2.1.1 Introduction.

Cancer is a disease process that can be well traced as far back as 500-400 B C in Greece, and 1600 -1890 in ancient Egypt (8). Putting together different theories, and coupled with the understanding of the disease process, cancer can be defined as a neoplastic disease, with a fatal natural course, and unlike benign tumors (which are often well circumscribed and confined), exhibit the properties of invasion, immortality and metastasis and are highly anaplastic (8-11). Cancer includes two broad categories known as carcinoma (malignant lesions of epithelial origin) and sarcoma (malignant lesions of supportive tissue origin), both often used synonymously with carcinoma (2).

2.1.2 Cell differentiation and cancer

For a normal cell development, orderly sequences of cell division, cell specialization, and correct spatial positioning of cells in tissue are needed. The disruption of these processes essentially defines cancer. In this process of development, the cell makes a stable commitment to one of several alternative developmental pathways usually before real cellular differentiation (8).

Differentiation implies the cell switches on a particular set of genes responsible for the control of a physiological task in a tissue. This is recognized when the tissue architecture shows cells with the correct shape, polarity and orientation with respect to their neighbours, and also appropriate arrangement of organelles, and synthesize proteins with functions in specialized metabolism, signaling, transport, contraction and other functions. Some molecules involved in the process of differentiation and other mechanisms in cell development are similar to those that appear to regulate cancer cell behaviour. The checks in the cycle during cell division are an example of the growth regulation. Three known control points in the cell cycle at which a decision may be taken on whether to proceed are at: the G1 phase (commitment to chromosome replication), the G2 phase (commitment to mitotic division), and the spindle assembly check point (12). Developmental control genes broadly classify as nuclear and cytoplasmic genes. The

nuclear developmental regulatory genes include the tissue specific transcription factor and the nuclear hormone receptors. Some nuclear hormone receptors, including receptors for steroid hormones, thyroid hormone, and retinoic acid are involved in a range of normal growth control mechanisms. Cytoplasmic developmental control genes are often exercised at the cell surface. Examples are the genes with epidermal growth factors repeat motifs, c-ras proto-oncogene expressed in human glioblastomas and thought to function in epithelial development (8).

In malignancy, invasion and metastasis involve changes in the spatial relationship of cells to one another and to the extracellular matrix. Though extracellular matrix modules, their receptors, and cell-cell adhesion molecules may not be direct targets for transformation, their expression may be severely altered by the process (8).

2.1.3 Oncogenes and cancer

Oncogenes may be derived from proto-oncogenes by mutations that affect their function or level of expresssion. They are identified by genetic changes that represent gain-of-function associated with the acquisition of immortality, morphological transformation, and matastasis. Oncogenes are present in both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) tumor viruses. The DNA oncogenes inhibit the activities of cellular tumor suppressors, while some RNA oncogenes like the v-oncogenes and c-oncogenes mostly express as fusion proteins (8).

Cellular oncoproteins derived from several types of genes, produce gene products that will likely affect pathways that regulate growth. Such oncoproteins may lack regulatory functions or increased activity. Examples are cytoplasmic tyrosine kinases, Ras proteins and G-proteins. Nuclear oncoproteins may be involved directly in regulating gene expression. Examples are JUN, and FOS (8).

2.1.4 Carcinogenesis

The neoplastic transformation of cells is called carcinogenesis. This can result from a variety of agents, principally chemical carcinogens, oncogenic viruses, and radiant energy.

Chemical Carcinogens

Chemical carcinogens are extremely diverse and include both natural and synthetic products. Some are direct-reacting and require no chemical transformation to induce carcinogenicity, but others are indirect-reacting and become active only after metabolic conversion. Such agents are referred to as procarcinogens and their active end products are called ultimate carcinogens. All chemical carcinogens, both the direct-reacting and the ultimate carcinogens, are highly reactive electrophiles (have electron-deficient atoms) that react with the electron-rich atoms in RNA, cellular proteins, and mainly DNA (13).

The carcinogenicity of many chemicals, particularly weak carcinogens, is augmented by agents that by themselves have little or no cancerous activity. Such augmenting agents are referred to as promoters. However, strong carcinogens have no need for promoting agents. The transforming effect of carcinongens on DNA are noted to be irreversible. However, the effect of promoters are transient, reversible, and appear to involve epigenetic mechanisms. Several chemical carcinogens may act in concert or with other types of carcinogenic influences (e.g viruses or radiation) to induce neoplasia-cocarcinogenesis (13).

Some major chemical carcinogens include;

A) Direct- Acting carcinogens.

These substances do not require metabolic conversion to become carcinogenic. They are generally weak carcinogens, depending on time-dosage considerations, and may not

produce tumors. However, they have importance because some of them are cancer chemotherapeutic drugs (13).

1) Alkylating agents.

Beta-propiolactone, Dimethyl sulfate, Dioxybutane, anticancer drugs (cyclophosphamide, chlorambucil, nitrosureas, etc).

2) Acylating agents.

1-acetyl-imidazole, Dimethylcarbamyl chloride.

B) Indirect-Acting Agents.

These chemicals require metabolic conversion to become active. Examples include polycyclic hydrocarbons in fossil fuel, benz(a)anthracene, benzo(a)pyrene, and other carcinogens produced in cigarette smoke, polycyclic hydrocarbons that may be produced from animal fats in the process of broiling meat, and are present in smoked meats and fish. The principal active products in many hydrocarbons are epoxides, which form covalent bonds with molecules in the cell, principally DNA, and also with RNA and proteins (13).

Another class of indirect agents, are the aromatic amines and azodyes. Beta-naphthylamine is implicated in bladder cancers. Some azo-dyes used to color food for example, "butter yellow" to make margerine enticing, and scarlet red for maraschino cherries are dangerous. Most of the aromatic amines and azodyes are converted into ultimate carcinogens in the liver by the cytochrome P-450 oxygenase systems, and therefore may induce hepatocellular carcinomas (13).

C) Procarcinogenes that require metabolic activation.

1) Polycyclic and heterocyclic-aromatic hydrocarbons.

Benz(a)anthracene, Benzo(a)pyrene, Dibenz(a,h)anthracene, 3-Methylcholanthrene, 7,12-Dimethylbenz(a)anthracene.

2) Aromatic amines, amides, azo-dyes

2-Naphthylamine(beta-naphthylamine),Benzidine,2-Acetyllaminofluorene, Dimethylaminoazobenzene.

D)-Natural plants or microbial products

Aflatoxin B1, Griseofulvin, Cycasin, Safrole, Betel nuts.

E)-Others

Nitrosamine and amides, vinyl chloride, nickel, chromium, insecticides with organophospates like methlion and dichlorvos, fungicides and polychlorinated biphenyls (13).

Mechanisms of action of chemical carcinogens

A summary of the insights into the fundamental nature of carcinogenesis;

1) The bulk of chemical carcinogens are mutagens. They bind directly to DNA and to specific sites within the molecule, inducing mis-coding errors during transcription and replication. However, it should be noted that binding to RNA or cytoplasmic proteins may also take place and be carcinogenic. Normal cells can be converted to cancer cells by transfection (transfer of genes by recombinant DNA) of DNA from chemically transfromed cells.

2) The carcinogenicity of chemical agents is dose dependent, and multiple fractional doses over time have same oncogenicity as a simple comparable dose. The time interval between fractional doses can be considerably extended (within limits) and thus the critical carcinogenic effect, which has been termed initiation, is virtually irreversible.

3) The carcinogenicity of chemical agents can be significantly enhanced by the subsequent administration of promoters such as phorbol esters, which themselves are non-tumorigenic. Strong carcinogens or sufficiently large doses of weak initiators may not require the action of promoters, which appear to interact with membrane receptors to stimulate cell replication.

4) To be effective, the promoter must come after the initiator. A reversed order, may not yield any tumor. Fractional doses of promoters when widely spaced are without effect,

indicating that their action is reversible. On the other hand, when the intervals are not too prolonged, the promoting effect is additive.

5) The initiation-promotion two-stage sequence has given rise to the recognition that carcinogenesis involves more than a single event.

6) Two or more initiators, be they chemical agents, oncogenic viruses, or radiant energy may act in concert to induce malignant transformation referred to as cocarcinogenesis. Thus, sub-effective doses of one may be synergized by the actions of one or more additional influences.

Oncogenic Viruses

Many viruses, are suspected to be involved in human carcinogenesis and are divided into two classes - single-stranded RNA viruses and double stranded DNA viruses.

Selected RNA carcinogenic viruses in humans include human T-cell leukemia virus (HTLV-1) for T-cell leukemia/lymphoma and mammary tumor virus for breast carcinoma. DNA carcinogenic viruses in humans include, human papillomavirus (HPV) for common warts and closely related condylomata, squamous cell carcinoma, vulvar and cervical carcinoma; herpes viruses (HSV-2) for vulvar and cervical carcinoma; Epstein-Barr virus (EBV) for Burkitt's lymphoma nad nasopharyngeal carcinoma; cytomegalovirus (CMV) for Kaposi's sarcoma, and hepatitis B virus (HBV) for hepatocellular carcinoma.

Radiation Carcinogenesis

Radiation, whatever its source, sunlight, X-rays, nuclear fission radionuclides, is an established carcinogen (13). Though its carcinogenicity is has been proven, the ultimate mechanism is not understood. Some of the proposals about the mechanism include;

1) Radiation-induced mutations may activate proto-oncogenes, or act by damaging control regions, or permit over expression of proto-oncogenes;

2) Radiation mutations may render cells vulnerable to other carcinogenic influences, for example viruses;

3) Radiation might cause cell death, permitting survivors to proliferate and thereby become vulnerable to oncogenic influences; and

4) Amplification over time of radiation-induced mutations might ultimately lead to the neoformation of cellular oncogenes.

Cholinesterase and Carcinogenesis

Cholinesterases are enzymes that show high activity with certain esters of choline. They are of two types, namely;

1) True cholinesterase (acetyl choline hydrolase, acetylcholinesterase) present in nerve tissue and erythrocytes, and responsible for the hydrolysis of acetylcholine at synapses and the neuromuscular junction (14,15).
2) Pseudocholinesterase (acetylcholine acyl-hydrolase cholinesterase, butyryl cholinesterase), present in liver, heart muscle, intestine and other non-nervous tissue and which can be determined in blood serum or plasma (14,15).

Determination of cholinesterase levels in serum or plasma is of great significance in detecting possible insecticide poisoning and the detection of patients with atypical forms of the enzyme. Measurements of serum/plasma cholinesterase activity can also serve as sensitive measures of liver function if the patient's normal or baseline level is known. In the absence of known inhibitors, any decrease in activity in serum/plasma levels reflects impaired synthesis of the enzyme by the liver. A 30 to 50 per cent decrease in level is obtained in acute hepatitis, and chronic hepatitis of long duration and decreases of 50 to 70 per cent in advanced cirrhosis and carcinomas (15,16,17).

2.1.5 Apoptosis (Programmed cell death)

Apoptosis or programmed cell death is a process where by extraneous, dangerous or effete cells are eliminated from tissue (18,19). It occurs in both physiological and pathological conditions, and effected by the cysteine proteases called caspases. This form of cell death comes as a result of cell being provoked into suicide. It is also an active process characterized by morphological and biochemical changes. The changes include cellular shrinkage, chromatin condensation and margination and formation of apoptotic bodies. During this process, DNA is fragmented into 180-200 base pairs due to the action of endonucleases that cleave the nucleosomes. The DNA fragments are often

detected as a ladder on agarose gel electrophoresis (18). In pathology, differential staining can be used to identify morphological changes in apoptosis.

Caspases are the main executioners in apoptosis, with initiators identified as caspases 1,2,4,5,8,9,10,11, and 13; and effectors as caspase 3,6, 7, and 14. The effectors are usually activated proteolitically by upstream caspase, whereas initiator caspases are thought to be activated through regulated protein-protein interactions. Some caspases either have death effector domains (DED), for example caspase 8, or an activation and recruitment domain (CARD), for example caspase-2 and caspase-9, which allows binding to and association with upstream modules (18,20,21).

A second set of apoptosis regulators are the Bcl-2 family, which are divided into three groups. Group 1 members include Bcl-2 and Bcl-XL, and the key feature of this group is that they all posses anti-apoptotic activity, thus protecting the cell from death. Group 2 consists of Bcl-2 family members with proapoptotic activity, and include Bax and Bad. Group 3 consists of a large and diverse collection of proteins, which include Bid and other divergent homologues of Bcl-2 and Bax (18-23).

The mitochondria, are the cell's armory in addition to being the power house. They house and influence apoptotic proteins, the most prominent being cytochrome-C. In addition to its involvement in mitochondrial oxidative phosphorylation, cytochrome-C is one of the components required for the activation of caspase-9 in the cytosol. It is not clearly understood how cytochrome-C manages to cross the mitichondrial membrane, but it is clear that the Bcl-2 family (Bax and Bad) is intimately involved in the regulation of this process (21,24). Other pro-death denizens also present in the mitochondria and released upon induction of apoptosis are AIF (a flavo-protein), Smac/DIABLO, and several pro-caspases including pro-caspase-2,-3 and –9 (21,24).

Death receptor-cell surface receptors transmit apoptosis signals initiated by specific death ligands and play an important role in apoptosis. This is common and important with the immune system. Death receptors belong to the tumor necrosis factor (TNF) receptor gene superfamily. They contain a death domain, which enables them to engage the cell's

apoptotic machinary, but in some instances they mediate functions that are distinct from or even counteract apoptosis. Some molecules that transmit signals from death receptors contain death domain themselves. The best characterized death receptors are the CD95 (Fas or Apo1) and the TNFR-1 (p55 or CD120a). Others are the avian CAR-1, death receptor 3 (DR3, APO3, WSL-1, TRAMP, LARD), and DR5 (Apo2, TRAIL-R2, TRICK2, or KILLER) (18). The p75 nerve growth factor (NGF) receptors also contain a death domain. The ligands that activate these receptors with the exception of NGF, are structurally related molecules that belong to the TNF gene superfamily. CD95 ligand (CD95L) binds to CD95; TNF and lymphotoxin alpha bind to TNFR-1; Apo3 ligand binds to DR4 and DR5. The ligand for CAR is unknown (22,25,26).

CD95 and CD95L play an important role mainly in three types of physiological apoptosis.

1) Peripheral deletion of activated mature T-cells at the end of an immune response.
2) Killing of targets such as virus infected cells or cancer cells by cytotoxic T-cells and by natural killer cells, and
3) Killing of inflammatory cells at "immune-privileged" sites such as the eye.

During signal transmission, an adapter protein called FADD (Fas-associated death domain or Mort1) binds to CD95 through its own death domain. FADD also contains a death effector domain that binds to an analogous domain of caspase-8. The death effector domain is a specific example of a broader homophilic interaction domain termed CARD (caspase recruitment domain), which is found in several caspases including caspases-2, -8, -9, and –10. Upon recruitment by FADD, there is caspase-8 oligomerization and its activation through self-cleavage. Caspase-8 then activates down stream effector caspases such as caspase-9 committing the cell to apoptosis (25, 26,27).

P53 also known as the cellular gate keeper is very important in apoptosis. It was originally classified as an oncogene due to oncogenic mutations, but now known as a tumor suppressor (28, 29). In response to DNA damage, p53 activity increases and triggers either of two pathways, depending upon the stage of the cycle and the cell phenotype (30). Early in the cycle, p53 checks DNA and allows it to be repaired before

replication. If damaged DNA can not be repaired, p53 causes apoptosis, so that the cell does not perpetuate itself. Loss of p53 function is common in some cancers and may contribute to drug resistance and the progression of a wide variety of tumors (31, 32).

p53 has a sequence-specific DNA binding domain that recognizes a particular sequence in proteins that are activated by p53. They include the cdk inhibitor p21 and the protein GADD45 activated to respond to DNA damage. Activation of these and other genes is thought to be the key by which p53 causes cell cycle arrest (33). The ability of p53 to activate these target genes is enhanced by the DNA damage. Mutant p53 lacks this pro-apoptotic ability and will therefore allow the perpetuation of cells with damaged DNA.

2.1.6 Anti-neoplastic drugs

Antineoplastic drugs are usually classified according to their mechanisms of action. The major groups include alkylating agents, anti-metabolites, plant alkaloids, and anti-tumor antibiotics, and miscellaneous groups of hormones, enzymes, monoclonal antibodies (34-36).

A) Alkylating Agents

These are the largest class of anti-cancer agents, comprising five subgroups: nitorgen mustards, alkyl sulfonates, nitrosoureas, ethylenimines and triazenes.

Alkyalting agents are compounds that are capable of introducing alkyl groups into nucleophilic sites on other molecules through the formation of covalent bonds. These nucleophilic targets for alkylation include the sulfhydryl, amino, phosphate, hydroxyl, carboxyl, and imidazole groups, and are present in macromolecules and low-molecular-weight compounds within cells. The mechanism of reaction between the alkylating agent and the nucleophilic target involves one of the two mechanisms.

1) A first order nucleophilic substitution (Sn1) with the initial formation of a solvated, highly reactive, electrophilic carbonium ion, followed by a reaction with a nucleophile; or

2) A second order nucleophilic substitution (Sn2) in which a transition complex is immediately formed between the drug and its target molecule (34-36).

Macromolecular sites of alkylation damage include DNA, RNA and various enzymes. In alkylation, the 7-nitrogen (N7) and the 6-oxygen (O-6) of guanine are particularly susceptible to attack by electrophilic compounds. The possible consequences of N7 guanine alkylation would be:

1) Cross linkage, where bi-functional alkylating agents, such as the nitrogen mustards, may form covalent bonds with each of two adjacent guanine residues, with the 2-chloroethyl group functioning as a covalent bridge between strands of DNA. Such cross linkages will inhibit DNA replication and transcription. Also, cross links may be produced between DNA and a near by protein.

2) Mis-pairing of bases, where alkylating at N7 changes the O6 of guanine to its enol tantomer, which can then form base pairs with thymine. This occurrence may lead to gene miscoding, with adenine-thymine pairs replacing guanine-cytosine. The result is the production of defective proteins.

3) Depurination, whereby N7 alkylation may cause cleavage of the imidazole ring and excision of the guanine residue, this leading to DNA strand breakage (34-36).

1)Nitrogen Mustards

a) Mechlorethamine is a derivative of the gas sulfur mustard. Its principal disadvantage is the instability of the 2-chloroethyl group, which readily reacts with water molecules and hydroxyl ions. In aqueous solution, mechloethamine loses a chloride atom and forms a cyclic ethylnimonium ion. This carbonium ion interacts with nucleophilic groups such as the N7 and O6 of guanine, and leads to an interstrand cross-linking of DNA (34-36).

b) Cyclophospahmide

This is the most common nitrogen mustards. The parent drug is inactive and must be enzymatically converted to cytotoxic metabolites. As with the other nitrogen mustards, cyclophosphamide administration results in the formation of cross-linkages within the DNA due to a reaction of the two chloroethyl moieties of the cyclophosphamide with adjacent nucleotide bases. Cyclophospahmide is activated metabolically by cytochrome P-450 enzyme system before the formation of the cyclic ethylnimonium ion can occur. The metabolites, phosphoramide mustards and acrolein, are thought to be the ultimate active cytotoxic moieties derived here (34-36).

Other nitrogen mustards include, melphalan (L-phenylalamine mustard) and cholrambucil, all with the same general principle of action.

B) Alkyl Sulfonates

Busulfan is a bifunctional methane-sulfonic ester that acts by cleavge of the alkyl-oxygen bond, producing an electrophilic butyl compound that forms intrastrand cross-linkages with DNA.

Other alkylating agents of interest include the ethylenimines for example thiotepa, and the triazenes for example Decarbazine.

C) Antimetabolites

Antimetabolites used in cancer chemotherapy are drugs that are structurally related to the naturally occurring compounds such as vitamins, amino acids or nucleotides. These drugs can compete for binding sites on enzymes or can themselves become incorporated into DNA or RNA and thus, interfere with cell growth and proliferation. The antimetabolites in clinical use include the folic acid analogue methotraxate, the pyrimidines (fluorouracil and cytarabine), and the purines (thioguanine and mercaptomine) (34-36).

Methotrexate competitively inhibits the binding of folic acid (FH2) to the enzyme dihydrofolate reductase. This enzyme catalyzes the formation of tetrahydrofolate (FH4), which is in turn converted to N5, N10- methylenetetrahydrofolate, an essential cofactor for the synthesis of thymidylate, purines, methionine and glycine. The major mechanism by which methotrexate brings about cell death appears to involve inhibition of DNA synthesis through a blockade of the biosynthesis of thymidylate and purines (34-36).

Thioguanine (6-Thioguanine) is an analogue of the natural purines, guanine, in which a hydroxyl group has been replaced by a sulfhydryl group in position 6. The proposed mechanisms of cytotoxicity are;

1) In corporation of the thionucleotide analogue into DNA or RNA, and

2) Feed back inhibition of purine nucleotide synthesis.

Both actions require initial activation of the drug by the enzyme, hypoxanthineguanine-phosphoribosyl- transferase (HGPRTase). The product of this reaction, 6-thioguanine-5-monophosphate (6-TGMP), can eventually be converted to deoxy-6-thioguanine-triphosphate (dTGTP), which is incorporated into DNA.

Cytarabine (cytosine arabinoside; 1-B-D-arabinofuranosylcytosine) is an analogue of the pyrimidine nucleosides, cytidine and deoxycytidine, with an arabinose sugar moiety replacing ribose or deoxyribose. Cytarabine competitively inhibits DNA polymerase.

Fluorouracil (5-fluorouracil, 5-FU) is a halogeneted pyrimidine analogue, which must be activated metabolically. The active metabolite which inhibits DNA synthesis is the deoxyribonucleotide 5-fluoro-2' deoxyuridine-5'- phosphate (Fd UMP).

D) Anti-tumour Antibiotics

Anthracyclines (doxorubicin and daunorubicin) are products of *Streptomyces pencetins*. Their chemical structures consist of a planar anthracycline ring system, which is attached by a gylcosidic linkage to the sugar, daunosamine. Doxorubicin binds tightly to DNA by its ability to intercalate between base pairs and thus concentrating in the nuclear structures. Intercalation results in inhibition by steric hindrance of DNA synthesis and DNA-dependent RNA synthesis, and the production of single-strand breaks in DNA. Anthracycline-DNA strand breaks are generated by the enzyme, topoisomerase II. In addition to the intercalation mechanism described, the anthracycline ring of doxorubicin can undergo a one-electron reduction to form free radicals and participate in further electron transfer. These active substances can then react with tissue macromolecules. This interaction suggest an alternative mechanism of cytotoxicity for anthracyclines. Bleomycins are a group of glycopeptides isolated from *Streptomyces verticullus*. Bleomycin binds to DNA, partly by an intercalation mechanism without severely altering the nucleic acid secondary structure. It results in a single and double-strand splits and DNA fragmentation. Bleomycins form a bleomycin-Fe2+ complex, which donates electrons to molecular oxygen, thus forming superoxides and hydroxyl free radicals. These highly reactive intermediates then attack DNA. Mitomycin (mitomycin C), also a *streptomyces* derived antibiotic, inhibits DNA synthesis through its ability to alkylate

double-strand DNA and bring about interstrand cross-linking. Dactinomycin (actinomycin D) yet another *streptomyces* product, binds non-covalently to double-strand DNA by partial intercalation, inhibiting DNA-directed RNA synthesis. Plicamycin (mithramycin) produced by *Streptomyces tranashiensis*, binds to DNA and inhibits transcription (34-36).

E) Vinca alkaloids

Vincristine and Vinblastine are derived from *Canthatranthus roseus* (*Vinca rosea L*) and bind avidly to tubulin, a class of proteins, that forms the mitotic spindle during cell division.

F) Enzymes

L-asparaginase derived from the bacteria *Escherichia coli* and *Erwinia carotovora,* is a good antitumor agent for acute lymphocytic leukemias and certain lymphomas. It catalyzes the hydrolysis of L-asparagine to aspartic acid and ammonia. L-glutamine can also undergo hydrolysis by this enzyme. Tumor cells sensitive to L-asparaginase are deficient in the enzyme, asparagine synthetase and therefore cannot synthesize asparagine. Depletion of exogenous asparagine and glutamine inhibits protein synthesis in cells lacking asparagine synthetase, with subsequent inhibition of nucleic acid synthesis and cell death.

G) Hormonal agents

Some hormones inhibit cancer growth while others stimulate their growth. Prednisone is a glucocorticoid used in many combination chemotherapies. Progestins are useful in endometrial and renal carcinomas and also like anti-estrogens, glucocorticoids and androgens, they cause breast cancer regressions. Estrogens act by binding to a cytoplasmic protein receptor. The hormone-receptor complex is translocated into the nucleus and induces the synthesis of ribosomal RNA (rRNA) and messenger RNA (mRNA) at specific sites on the DNA of the target cell. Other examples of hormones used to treat cancer include, Estramustine phosphate sodium (Emcyt), which is a hybrid structure combining estradiol and nornitrogen mustard in a single molecule and used for prostatic carcinomas, and flutamide, a non steriodal antiandrogen compound, which

competes with testosterone for binding to androgen receptors and also used in prostsatic cancer. Buserelin and leuprolide are peptide analogues of the hypothalmic hormone luteinizing hormone-releasing hormone (LH-RH) and are both active agents in prostatic carcinomas and breast cancer. Octreotide acetate is a synthetic peptide analogue of the hormone somastostatin, which inhibits the pituitary secretion of growth hormone and the secretion of insulin and glucagon by the pancreatic islet cells. Another hormone is tamoxifen, a synthetic anti-estrogen used in breast cancer.

H) Miscellaneous agents

Hydroxyurea is a simple derivative of urea used to reduce high circulating granulocytes in chronic myelocytic leukemia. It inhibits the enzyme, ribonucleotide reductase and thus, depletes intracellular pools of deoxyribonucleotides, resulting in the impairment of DNA synthesis.

Procarbazine is useful against lymphomas and small-cell anaplastic (oat cell) lung cancers. It may auto-oxidize spontaneously to form azoprocarbazine and during this reaction, hydrogen peroxide and free radicals are generated. These reactive products may degrade DNA. Also cell toxicity may result from a transmethylation reaction that can occur between the N-methyl group of procarbazine and the N-7 position of guanine.

Mitoptane, a derivative of the insecticide DDT, can cause reduction in both tumor size and adreno-cortical hormone secretion. Hexamethylmelamine inhibits both DNA and RNA synthesis but the molecular mechanisms are not understood. Cisplastin has broad antitumor activity and is especially useful in the treatment of testicular and ovarian cancers. It binds to DNA at nucleophilic sites such as the N-7 and O-6 of guanine, producing alterations in DNA structure and inhibition of DNA synthesis. Carboplastin, an analogue of cisplastin, has a similar mechanism with DNA cross-linking. Etoposide a semi-synthetic derivative of podophyllotoxin, is another anti-tumor agent that affects DNA but not well understood. Mitoxanthrone is a synthetic anthraquinone that has similar structure and mechanism to the anthracyclines. It intercalates with DNA and produces a single-strand DNA breakage. Interferon alpha-2b is a recombinant DNA product derived from the interferon alpha-2b gene of human white blood cells. It binds to

a plasma membrane receptor but the mechanism is poorly understood (34-36). It is useful in the treatment of hairy cell leukemia.

I) Monoclonal antibodies

These are mostly murine proteins produced by immunizing mice with an antigen of interest (for example, human cancer cells). The sensitized splenic B lymphocytes of the mouse are then fused with a murine myeloma cell line. The resulting hybrid cells are screened for the production of the desired antibodies, which can be purified and produced in large quantities. They can be used in various diagnostic applications such as radioimmunoassays, histochemical analysis and tumor imaging as well as providing therapeutic reagents for serotherapy. Trials have been done in lymphomas, leukaemias, melanoma and colorectal carcinomas with tumor remissions in a few patients (34-36).

2.2 MEDICINAL PLANTS

2.2.1 Medicinal plants: A source of natural pharmacological products

Modern chemical methods have led to a dramatic increase in the number of natural and synthetic molecules available for pharmacological research, of which medicinal plants are a major source (37), as many have been demonstrated to contain biologically active ingredients (38).

Plants are considered to be medicinal if they possess pharmacological activities of possible therapeutic use (39). Clues on the pharmacological activities of such plants are given to researchers by the communities to which the plants belong. But they have to be carefully investigated if drugs that meet the criteria of modern health standards are to be developed.

The goals of medicinal plant researches for drug discovery and development would include;

i) The identification of the active molecules or compounds of the plants and investigation of the extract in order to ensure they are safe, effective and of constant activity

ii) The isolation of these active compounds and the determination of their structure in order that they may be synthesized, structurally modified or simply extracted more efficiently (39).

The series of procedures involved in the selection, identification and screening of medicinal plants fall into seven stages:

1) The investigation of usage in traditional medicine,
2) Identification of the plants,
3) Harvesting the correct plant family,
4) Investigation of activity of whole plant and of the active ingredients as well,
5) Classical pharmacological investigation,
6) Determination of structure, and,
7) Synthesis and structural modification.

Criteria for the selection of medicinal plants.

A set of criteria are used for the selection of plants for investigation (40). These include;

1) Selection based on traditional usage

Traditional medicine forms a major aspect of health care in many countries and thus give a clear objective to be pursued as to what plants to be investigated, on determined models. However, variety model investigations are advisable as efficacy for other disease situations other than those treated by such local communities are sometimes discovered.

2) Poisonous plants

Scientists do not often investigate poisonous plants because they are shorn by the local populations; mean while these plants can be useful sources for drugs or useful guides for the investigations in elucidating some disease conditions. Many important drugs of plant origin used in medicine today come from poisonous plants, for example tubocurarine (arrow poison); atropine (poison) and picrotoxin (fish poison). A beneficial approach will be to screen such plant extracts on many biological models as possible. Where

therapeutic benefit does not result directly, the explanations for the mechanisms of poisoning can be possible ways to counter similar physiological or pathological situations (40).

3) Selection based on chemical composition

In certain instances, scientists may decide to extract a class of compounds such as alkaloids for investigation. Plants with such determined compounds are collected, extracted and screened on a wide range of models possible. This approach is greatly helped by chemo-taxonomic information relating different classes of compounds of different plant species.

4) Screening for a specific biological activity

Specific biological activities can be screened for in plants by the use of specific models for such diseases.

5) Combination of criteria

Several criteria can be put together to make plant selection and screening even more meaningful. For example, plants used in traditional medicine and which are also known to contain particular types of compounds, for example alkaloids or glycosides, may be investigated. The merging of such criteria will depend on available expertise and facilities. However, the extract should still be screened for as wide a range of biological activities as possible.

2.2.2 Technology in the separation, isolation and structural analysis of plant products

The separation of large or small quantities of complex mixtures efficiently, rapidly and inexpensively is seldom achieved by the use of one ideal chromatographic technique. the long route from a crude plant extract containing hundreds of constituents to a pure compound requires several separation steps and techniques. Within the last 15 years, new separation techniques such as centrifugal TLC, flash chromatography, low-, medium-, and high-pressure liquid chromatography (HPLC), droplet counter-current chromatography (DCCC) and rotation locular counter-current chromatography (RLCC),

reverse phase liquid chromatography and so on, became commercially available (37). The techniques when used in combination give better results.

Analytical screening of plant extracts for biologically-active natural products

Photodiode assay detectors for high-performance liquid chromatography (HPLC) enables a marked improvement in peak identification (37). It provides a simultaneous record of chromatograms obtained at different wavelengths. The measurements of the UV spectrum of each eluted compound, makes this a very useful tool for the analysis of complex mixtures, during plant screenings and for chemotaxonomic studies. For the elucidation of the structure, mass spectrometry using soft ionization techniques such as fast atom bombardment (FAB-MS), field desorption (FD-MS) or desorption chemical ionization (D/CI-MS) (37) are helpful. Information on the molecular weight, the nature and sequences of sugars and other molecules, positions of attached groups are easily obtained.

2.2.3 Anti-cancer medicinal plants

Anti-cancer medicinal plants are wide spread across the globe and vary from region to region. However, very little scientific data is often available on these different geographical anti-cancer medicinal plants. Most information gathered for any research are often based on information stemming from traditional usage and traditional medicine practitioners from local communities to which the plants belong.

In South Africa, a Pharmacopia on traditional medicinal plants is still under preparation, so researchers get information mostly from the local medicine practitioners. However, some literature exist that has been compiled by eminent scientists. This literature generally give exhaustive lists of plants known to the communities, and some pharmacological properties based on the reports of the traditional medicine practitioners, following long periods of usage. These plants in most cases have not been scientifically investigated or at times just minimally researched into.

A few South African plants claimed to treat cancer include; *Tulbaghia violacea*, *Solanum tomentosum*, *Leonotis species*, *Helichrysum excisum*, *Elytropappus species*, *Dicoma*

capensis, *Catella asiatica*, *Combretum caffrum* and *Sutherlandia frutescens*, the plant of choice in this research.

2.2.4 *Sutherlandia frutescens*

This plant belongs to the group *Fabaceae* and is commonly called "Cancer bush". It grows to about 1 meter in height (7), with slightly hairy leaves and red flowers. There are six known species of *Sutherlandia* which are difficult to tell apart and are likely to be combined (7). The genus particularly found in Southern Africa (South Africa, Botswana and Namibia). The plant is multi-purpose and its is one of the most extensively used medicinal plants in South Africa. The Zulu people call it "Unwele or Insiswa", the Tswana people call it Phetola and the Afrikaans manes are "Kankerbos, Kalkoenbos, Bitterblaar and Gansies. Traditional medicine practitioners use the plant as decoction in the treatment of cancer, HIV/AIDS, TB, Stress, fatigue, gastritis, urogenital tract infections, diabetes and respiratory tract ailments. Pinitol, GABA and Canavanine are some metabolites that have been identified in *Sutherlandia frutescens.* However, more scientific investigations are still needed to authenticate the different therapeutic claims and evaluate its full toxicity. In addition to the anti-viral activity of L-Canavanine, some scientists also attribute the plant's anti-tumorigenic properties to this metabolite (7). However, on going studies on this plant in the Department of Biochemistry at the University of the Western Cape, have so far not establish L-Canavanine to induce apoptosis (42).

Fig 1 : Picture of *Sutherlandia frutescens*.

2.3 DRUG DISCOVERY, DESIGN, AND DEVELOPMENT

This encompasses many areas/specialties which include protein and enzyme studies, molecular biology/genetics and bioinformatics, drug design proper, combinatorial libraries/chemoinformatics, structural biology and analytical chemistry, pharmacology and toxicology.

2.3.1 Protein/enzymology

With apoptosis as indicator, anti-cancer drug design will definitely center around proteins, enzymes and genes traced or thought to be involved in apoptosis.

Structural information has been used with some success to improve protein and enzyme functions. By using direct evolution, protein engineers have not only caused mutagenesis but also attempted to mimic the natural processes by which protein variants can arise and are tested for "fitness" within living systems (43). Incorporation of recombination into a method for direct evolution of single genes has been developed. A population of mutant genes can be selected on the basis of their containing mutations, thus making it a suitable proposal for their mechanisms.

On the aspect of mechanisms and properties, the sequence-to-function approach is the most commonly used function prediction method and an alternative approach will be the sequence-to-structure-to-function paradigm, where the aim is to determine the structure of the protein of interest, for example, p53, CD95, Bax, Bcl, and caspases as with apoptosis. Thereafter, the functionally important residues in the structure can be identified (44).

The sources of proteins which will be broadly divided as exogenous and endogenous, also play an important role in elucidating structure-function relationships. In this area, protein mimicry can be done. Though the mimics might not necessarily be proteins themselves, the structure of some unknown receptors, enzymes or targets could be unraveled using them (45).

2.3.2 Molecular biology/genetics/bio-informatics

The knowledge of bio-informatics is today a key aspect of drug discovery in the genomic revolution, contributing to both target discovery and target validation. Genome- wide data sources are available including expressed sequence tags, microbial genome sequences, model organism sequences, polymorphisms, gene expression data and proteomics (46).

Genomics on the other hand has caused an increase in large molecule targets; however, this has shifted the focus of bioinformatics from target identification to target validation. The challenges to bioinformatics is evolving from the creating of long lists of genes and proteins to that of creating short lists of the targets most likely to be crucial in disease and least likely to fail for developmental reasons (46).

The ideal role of bioinformatics here would be primarily to provide as many clues as possible to function and role of genes, proteins and targets that may be involved with apoptosis.

Another attempt of interest would be to intergrate bioinformatics more intimately into the discovery process itself by establishing "wet-dry cycles". Such cycles occur whenever a computational model can be linked to a biological one, such that predictions from the former can be immediately tested at the bench with the results being fed back for producing refinement of the model. Avoiding to just hand down candidate genes to an independent bench validation process can thus be achieved by using bioinformatics function to follow targets through the pipeline by for instance, modeling biological systems, suggesting experiments and using the results for the bench to refine the model further. In this way, bioinformatics can add more value by shortening cycle times. Value can also be added to targets already in development by continuing to search for homologues, both orthologues and paralogues that can provide additional models in other species and follow on targets that can use existing assays and compound libraries. Useful tools in bioinformatics include the gene databases: EMBL, DDJ, GenBank, and the protein databases like SWISSPROT, TrEMBL, MIPS and PIR.

2.3.3 Combinatorial libraries/chemo-informatics

Combinatorial chemistry allows rapid increases in the size of compound collections to support high throughput screening (HTS) programmes, and rapid exploration of the structure-activity relationships (SAR) around chemotypes of interest in anti-cancer drug design (47,48).

To ensure that the products of a combinatorial library possess the desired characteristics, substantial effort is required in the library design process. In view of the large number of potential products that can be synthesized from combinations of available reagents, computational methods able to rapidly evaluate molecular similarity and diversity, calculate physicochemical properties and select the most suitable reagents based on the structures of either combinatorial reagents or their products are essential. Computer tools used for these purposes include QANTA, Cerius2 4.5, Catalyst 4.6, HypoGen, C2.MFA, C2.QSAR, C2 GA, LUDI, Search/Compare amongst many others (49).

Combinatorial libraries can be divided into two classes: diversity libraries and focused libraries. Diversity libraries are intended to yield products that interact with a single biological receptor or possibly a family of related receptor. These libraries might be designated based on information obtained from the structures of target receptors or both. Focused libraries are useful for both lead optimization and lead discovery purposes (47,48).

The end goal of combinatorial libraries and chemoinformatics is to be able to simulate, model and predict general " drug-likeness" of molecules (50).

2.3.4 Analytical chemistry and structural biology

Drug design sometimes requires the structural elucidation of both receptors/targets and ligands. These receptors and targets are mostly proteins and in apoptosis would include, CD95, TNFR-1, caspases, p53 and cyclins. Other tragets would include inhibitors such as p21, Rb, cdc, p16 and so on. Ligands could either be proteins too or inorganic molecules.

Tools frequently used for detailed structural analysis include, X-ray crystallography, NMR, mass spectrometry and so on. A good area for ligand search is the area of medicinal plants, where by medicinal plants undergo crude extraction and the active compounds or molecules in the crude extracts are thereafter separated and isolated by using tools like TLC and HPLC in addition to the above named structure analysis methods.

2.3.5 Pharmacology and toxicology

Any identifiable ligands of apoptosis must undergo a therapeutic evaluation before such can be proclaimed a good anti-cancer drug. Therapeutic evaluations will determine the efficacy of the compound as well as the selective toxicity. Most available anti-neoplastic drugs are known to be highly toxic to normal and transformed cells. However, it is hoped that through the induction of apoptosis in cancer cells, a compound with lesser toxicity will be identified.

Two types of therapeutic evaluations are obtainable for apoptosis inducing compounds. These are the in-vitro and the in-vivo analysis. In-vitro evaluations are done on cultured cancer cells upon which the potential anti-cancer compounds are tested for the induction of apoptosis. While in-vivo evaluations require the induction of cancer in laboratory animals and thereafter, treating with the compounds. This may need a thorough pathological analysis.

2.3.6 Drug design proper

This aspect involves the identification and selection, the characterization, validation and optimization of targets. Possible known targets that can be exploited in anti-cancer drug design include, CD95, TNFR-1, p53, caspases and cyclins. Whatever the situation at hand, four pertinent aspects are taken into account and are together known as rational drug design (51-59).

Rational drug design

1- Receptor structure unknown and ligand structure unknown:

Generate 3D structures, perform chemical similarity and diversity, apply combinatorial Chemistry (51-59).

2- Receptor structure unknown and ligand structure known (Analogue-based drug design) :

Develop pharmacophore models and hypotheses, perform database searching, similarity searching, 2D and 3D quantitative structure activity relationships (QSAR) (51-59).

3- Receptor structure known and ligand structure unknown. (De Novo Design):

Find ligands or ligand fragments to fill the receptor sites, de novo design, receptor-based 3D seraching (51-59).

4- Receptor structure known and ligand structure known. (Structure based drug design):

Receptor-ligand interaction and dynamics, docking (51-59).

2.3.7 Two types of drug design

Anti-cancer drug discovery can either be direct or indirect. Direct discovery will mean the identification and optimization of a molecule or compound that has potency and would induce apoptosis without all the screenings, separations and chemical structure alterations. Such a discovery, not necessitating any major works in finding and identifying such compounds, is most often a question of chance.

Indirect discovery will require HTS through separations, isolations and possible optimization. All the tools and techniques previously mentioned viz; TLC, HPLC, mass spectrometry and NMR will definitely come into play here.

Taking for instance the extraction and separation of plant material, possible outcomes can be:

1) No active compounds identified.

2) Partially active or active but highly toxic compounds identified.

3) Active and efficacious compounds identified.

Were a compound is identified to be active and efficacious, such a compound is developed for dosage forms that can be administered to humans and thereafter clinical trials can be done upon approval of the Medicines Control Council.

The active but toxic and the partially active compounds can be structurally modified through diversity focus and combinatorial chemistry. Another option would be to return to the crude extract and trace possible pathways, receptor, genes and other targets involved in apoptosis, and then search the databases for molecules with drug-likeness to such receptors and targets. These could also be mimics of the partially active or the active but toxic compounds. The biological evaluations then follow after such samplings.

2.3.8 Evaluation of apoptosis in anti-cancer drug testing

In the discovery, design and development of anti-cancer drugs with apoptosis as indicator, some assays would be done to authenticate or prove for apoptosis. Biochemical assays include DNA fragmentation analysis, caspase assays, PARP, immuno-flourescence and flow cytometry. Pathological/cytological evaluations would involve determining morphographic changes by simple differential staining of apoptosing cells and observing cellular alterations.

Typical cellular changes in apoptosis include DNA fragmentation, membrane blebbing, cell shrinkage, chromatin condensation, and disassembly into membrane-enclosed vesicles known as apoptotic bodies (13-16).

Much progress has occurred in the unraveling of the mysteries of cancer using molecular biology and it is hopeful that cancer research will no longer represent a collection of complex, apparently chaotic phenomena. Instead, it will soon become a logical discipline able to explain the hidden details of genes and proteins driving malignant cell proliferation. Apoptosis is one of such highly promising areas through which understanding cancer-causing processes and relating them with treatment targets and novel drug screenings will definitely bear fruit.

2.3.9 The Problem

As already previously noted, medicinal plants today play an important role in health care delivery. Traditional medicine practice has remained less organized, still to a greater part based on cultural beliefs. Moreover, the claims of therapeutic successes by traditional medicine practitioners are hardly backed by scientific evidence. As a consequence, proper pharmacological interpretation of efficacy is far from being a reality. *Sutherlandia frutescens* is used to treat many conditions amongst which is cancer (7) and a major problem is that the mechanisms through which it produces its actions are still just speculations, as well as its full toxicity potential. This is supported by the fact that very little scientific data exist about *Sutherlandia frutescens*.

This project attempts to investigate the possible mechanisms of anti-carcinogenecity of *Sutherlandia frutescens* extracts based on apoptosis and its influence on the enzyme butyryl cholinesterase.

CHAPTER THREE

MATERIALS AND METHODS

METHODS

3.1 Plant Material.

3.1.1 Selection, collection and Identification of *Sutherlandia frutescens*.

The criteria for selecting plant species is a key step in a phytotherapeutic study, and are based on many factors, the most common being information about the history and applications as it pertains to the community to which the plant belongs (60,61). *Sutherlandia frutescens* was selected due to its common and popular use in the South African traditional medicine circles for the management of conditions like depression, anxiety, wasting in HIV/AIDS patients and cancer, such that a herbal product of the plant is alleged to be sold in alternative health shops. However, no concrete scientific findings yet exist.

The plant material was collected from the Kristenbosch Botanic Garden, Cape Town, South Africa, and authenticated by Botanists of the Kristenbosch Botanic Garden and Dr. Gillian Scott, taxonomist of the of the South African Traditional Medicine Research Group, School of Pharmacy, University of the Western cape. A voucher specimen (number TRAD 25) was deposited in the Herbarium of the Department of Botany, University of the Western Cape.

3.1.2 Preparation of aqueous extract of *Sutherlandia frutescens*.

The leaves, young stem and flowers of the plant were collected and washed with distilled water, dried in a ventilated oven for 72 hours at 30° C and thereafter ground to a fine powder using the Waring laboratory grinder (KENWOOD P09 2NH). An extract was made from 10g of fine powder in 1 liter of boiled water and allowed to cool and centrifuged at 2500 rpm. The supernatant was freeze-dried for 72 hours in the LSL SECFROID SR, Model 3021, Switzerland, freeze dryer to obtain the dried plant extract, which was kept in a dessicator. The *Sutherlandia frutescens* test/working solutions of different doses, used for both the in-vitro and in-vivo experiments were freshly prepared daily just before administration, by dissolving a given quantity of the dried extract in an appropriate volume of phosphate buffered saline (PBS).

3.2Phytochemical characterization of *Sutherlandia frutescens* and preparation of organic solvent extracts.

3.2.1 Phytochemical characterization of *Sutherlandia frutescens.*

Objectives:

The aim was to detect the groups of compounds (secondary metabolites) present in the whole aqueous extract of the plant and to produce a fingerprint chromatogram characteristic of the plant. The constituents were detected chemically, while the chromatogram was by a High Performance Liquid Chromatography (HPLC).

Detection of chemical constituents (secondary metabolites).

Using standard methods, coumarins, phytosterols, flavovoids and tannins were identified to be bioactive substances in the aqueous extract of *Sutherlandia frutescens* (62,63).

1) Detection of coumarins.

60 µl of aliquots of ethyl acetate and chloroform extracts were applied in the form of narrow bands (15mm by 2mm) at the base of two 5 by 10 TLC plates pre-coated with Kieselgel 60 F250 (Merck). The solvent system used was ethyl acetate:formic acid:water (18:1:1 v/v) and detection by fluorescence in UV light of wave length 365nm. The applied marker was 6-Methoxy-7-(3', 3'-dimethylallyloxy)-coumarin, and the spray a 5% aqueous solution of potassium hydroxide (KOH).

2) Detection of flavonoids.

The same procedure and solvent system as for coumarins were used. In this case, detection was depicted by yellow and pale lilac bands under UV light, using quercetin as marker and 1% methanolic solution of 2-aminoethyl diphenylborinate (Naturtoff reagenz A), followed by a 5% solution of polyethylene glycol 400 in methanol as sprays.

3) Detection of hydrolyzable tannins.

The same procedure and solvent system as for coumarins and flavonoids were used and blue-black/ green-brown colors were detected using Garlic acid marker and a 10% solution of iron-III-chloride in methanol as spray.

4)Detection of phytosterols.

60µl of petroleum ether extract of the plant was applied at the base of a TLC plate pre-coated with Kieselgel 60 F250 and the solvent medium used was toluene:acetone (4:1). The finished chromatogram was sprayed with anisaldehyde/sulfuric acid reagent, followed by heating in an oven at 100-110° C for 10 minutes. The marker was ß-sitosterol.

After identification of the different secondary metabolites present in the plant, bulk isolation of the different metabolites was done, following an extraction sequence, using petroleum ether first to extract phytosterols, followed by chloroform for coumarins and ethyl acetate for flavonoids (extraction by exclusion). The remaining water portion after the three solvent extractions contained the tannins.

3.2.2 Preparation of organic solvent extracts of *Sutherlandia frutescens.*

Using the above mentioned sequence (petroleum ether, chloroform, ethyl acetate) of extraction, the different extracts were obtained. 50ml of the aqueous extract of the plant was transferred into a separating funnel and extracted with 3 times 50ml aliquots of petroleum ether (B.P 40-60° C). The aliquots were combined and reduced to near dryness on a rotary evaporator (<40° C). The residue weighed and taken up with 1ml of methanol and an additional 1ml of dimethyl sulfoxide (DMSO) for petroleum ether extract, to give a stock solution (concentration 100mg/ml). After the petroleum ether extraction, chloroform extraction was next using the same aqueous solution of the plant as above, using the same procedure. The residue was taken up with DMSO and again a stock solution of 100mg/ml obtained. This was followed by the third solvent extraction with ethyl acetate using the same procedure and the residue taken up with DMSO to a give solution of concentration 100mg/ml. After the three solvent extractions, the remaining water portion was evaporated in the same way and the residue taken up with DMSO, and a stock solution of 100mg/ml obtained.

3.2.3 HPLC analysis of the aqueous extract of *Sutherlandia frutescens.*

The chromatographic system included the Beckman HPLC system consisting of a double pump programmable solvent model 126; Diode Array Detector, Module model 168; Samsung computer 386 with management system Gold (Gold V 601) software supplied by Beckman; a column, C18 Bondapak 5µm dimensions 250 by 4.6mm). The chromatographic conditions included the mobile phase; solvent A:Methanol; solvent B:Acetic acid; mode;gradient:flow rate 1ml/min; injection volume 10µl; detector UV at 270 and 360nm. The HPLC operating conditions were programmed to give the following; at 0min, solvent A:45%, solvent B55%; 1min, solvent A:80%, solvent B:20% and at 8min, solvent A:45%, solvent B:55%. The run rate was for 13min.

3.2.4 HPLC data analysis of the aqueous plant extract.

The characteristic feature of each compound present in the *Sutherlandia frutescens* extract was the retention time, which is the time from injection of the sample to emergence of the peak.

3.3 IN-VITRO ASSESSMENT METHODS (for Apoptosis).

3.3.1 Cancer cell culture and the induction of apoptosis.

Cancer cell lines of human cervical epidermoid carcinoma (CASKI), human esophageal cancer ($WHCO_3$) and human lung cancer (NH157) obtained from the Departments of Biochemistry and Physiology at the University of the Western Cape, were used. The cells were cultured for 3 days at 30° C, in RPMI medium with 10% fetal calf serum, supplemented with penicillin-streptomycin. The cells were thereafter treated with a 10% aqueous plant extract of *Sutherlandia frutescens* for 4 hours at 37° C (as established from preliminary studies), along side two controls (a negative control using RPMI as "inducer" and a positive control using 1.5µM Staurosporine, a known inducer of apoptosis, time also 4h). After treatment, the cells were scraped off and smeared on clean glass slides.

Smears were then fixed in 95% ethyl alcohol for 15min and stained using the Hematoxylin and Eosin method (64) and a Modified trichrome stain-Crossmon trichrome stain (65). The stained cells were examined and photographed under a computerized

Zeiss binocular microscope system consisting, of a Zeiss microscope, a photographic unit and camera, and a processing computer using a graphic converter-microscope image software.

3.3.2 Hematoxylin and Eosin staining technique.

Smears were hydrated through descending grades of ethyl alcohol (95%, 70%, 50%) to water for 2min each and stained for 15min in 1% Mayer's acid hematoxylin. The slides were thereafter washed in water, differentiated in 1% acid alcohol briefly, blued in scotts alkaline tap water for 1min , rinsed in water and counter stained in 1% eosin for 1min. After counterstaining, the smears were dehydrated through ascending grades of ethyl alcohol, cleared in xylene and mounted with Canada balsam.

3.3.3 Modified (Crossmon) trichrome technique.

Hydrated smears were stained in a 0.1% acid fuchsin/orange G stain for 5 seconds, rinsed in water and transferred in 5% phosphotungstic acid for 1min, rinsed briefly in water and stained in 1% light green for 30min. 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.

3.3.4 Data analysis for in-vitro studies.

Features consistent with apoptosis were depicted by cellular and nuclear chromatin changes seen in the treated cells and brown-pinkish color changes on Crossmon stained smears, all illustrated in photo-micrographs.

3.4 IN-VIVO ASSESSMENT METHODS- Anti-carcinogenesis.

3.4.1 Experimental animals.

Male albino mice bought from the University of Cape Town, Cape Town, South Africa weighing 20-30g each, were used in 24 groups consisting of 10 per group. Each group represented a particular treatment dose of either the aqueous extract or the different solvent extracts. The mice were housed in perpex cages, given unlimited supply of water and food. They were handled according to the University of the Western Cape's Ethics Committee recommendations on the use and care of laboratory animals.

3.4.2 Drugs / Chemicals and other materials.

Dichlorvos (Riedel-de Haen; Germany), Butyryl Cholinesterase kit (Sigma), Dimethyl Sulfoxide (DMSO) (Merck), Methanol (Merck), Chloroform and Ethyl Acetate (Saarchem), Petroleum Ether (BDH), Lithium Heparin Vacutainers (BD vacutainer systems,Pre-analytical solutions, UK).

Dichlorvos was diluted in PBS to the required concentration, and each vial of butyryl cholinesterase reagent was dissolved in 10ml of de-ionized water as recommended by the manufacturer.

Preliminary studies were done to establish threshold but non lethal acute doses of both dichlorvos and the extracts, before the study proper. The following established doses were used for the study; aqueous extract-12.5mg/kg, 25mg/kg, 50mg/kg, 100mg/kg, 200mg/kg, 500mg/kg, and 1000mg/kg; coumarins from the chloroform extract- 50mg/kg, 100mg/kg, 200mg/kg, and 500mg/kg; flavonoids from the ethyl acetate extract-50mg/kg, 100mg/kg, 200mg/kg; phytosterols from the petroleum ether extract- 50mg/kg, 100mg/kg, 200mg/kg, 500mg/kg, 1000mg/kg; tannins from the remaining water fraction-50mg/kg, 100mg/kg, 200mg/kg. The duration of treatment was 4 days, with daily intra-peritoneal (i.p) administration of both the carcinogen and the plant extracts. The control groups received PBS (for negative control) and dichlorvos alone (for positive control) during the same period. Fresh solutions were prepared on the days of the experiments.

3.4.3 Procedure.

Mice were pre-treated daily with the appropriate plant extracts 1hour prior to the administration of the carcinogen. Carcinogenesis was initiated in the animals using dichlorvos, a known and proven carcinogen (66,67), in a daily dose of 6mg/kg. After four days of treatment, blood samples were collected in Lithium heparin " vacutainers", the plasma separated by centrifuging at 300g and immediately assayed for butyryl cholinesterase activity.

Principle of analysis for carcinogeneis.

Dichlorvos like all organophosphates and other carcinogens, significantly reduces plasma/serum activity levels of the enzyme butyryl cholinesterase by 50% or more in cancer or carcinogenesis (15,68). The ability of the plant extracts/secondary metabolites to revert this trend was taken as an indication or index of an anti-carcinogenic activity. Control groups were also treated concurrently.

The method used for the evaluation of cholinesterase activity was as per the Cholinesterase BTC kit No 421 -Sigma diagnostics. (69).

3.4.4 Data analysis for in-vivo studies (plasma/serum cholinesterase activity).

The absorbances of samples were measured using the Spectronic Unicam (Heyios) spectrophotometer at a wavelength of 405nm and the cholinesterase activity was determined using the formula;

$$\text{Cholinesterase activity (U/L)} = \frac{\Delta A \text{ per min} \times TV \times 1000}{13.6 \times LP \times SV}$$

where: ΔA per min = change in absorbance per minute at 405nm

TV = Total volume= Volume of cholinesterase reagent (1.0ml) + sample volume (0.01ml) = (1.01 ml).

SV = Sample volume (0.01 ml).

13.6 = Millimolar absorptivity of 5-thio-nitrobenzoic acid.

LP = Lightpath (1-cm).

1000 = Conversion of units per mL to units per liter.

$$\text{Cholinesterase Activity (U/L)} = \frac{\Delta A \text{ per min} \times 1.01 \times 1000}{13.6 \times 1.0 \times 0.01}$$

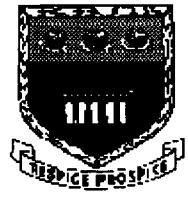
$$= \Delta A \text{ per min} \times 7426$$

3.4.5 Statistical analysis.

The results of the cholinesterase activities (U/L) were analyzed using the paired Student's t-test.

CHAPTER FOUR

RESULTS



RESULTS

4.1 Phytochemical characterization of *Sutherlandia frutescens* .

The chemical analysis of the plant identified the presence of phytosterols, coumarins, flavonoids and tannins as the secondary metabolites (Table 1).

Table 1. Phytochemical characterization of *Sutherlandia frutescnens.*

	Secondary metabolites detected			
Solvents	**Coumarins**	**Phytosterols**	**Flavonoids**	**Tannins**
1-Water	+	+	+	+
2-Petroleum ether	-	+	-	-
3-Chloroform	+	+	-	-
4-Ethyl acetate	+	-	+	-

4.2 HPLC Chromatograms

The HPLC spectra for the aqueous extract of *Sutherlandia frutescens* at 270nm and 360nm showed major peaks representing significant retention times. The following peaks with retention times (min) were obtained; at 270nm: 2.35, 2.38, 2.48, 3.23, 3.32, 5.29, and 6.31 (figure 2); and at 360nm: 0.47, 2.73, 5.29, 5.90, 6.11, 6.43, 6.71, and 7.24 (figure 3). No further identifications were done on the peaks.

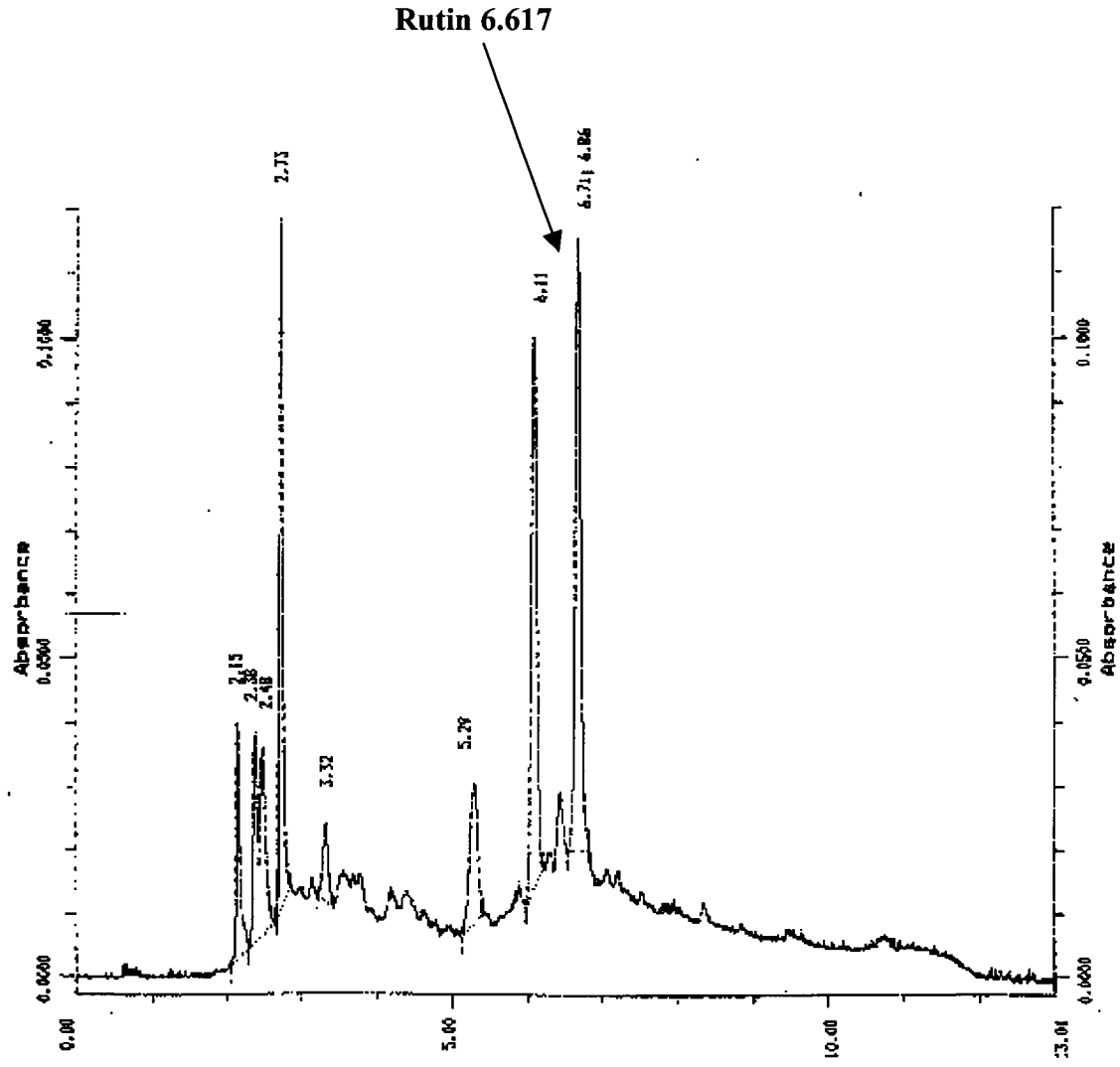


Fig 2: HPLC chromatogram of the aqueous extract of *Sutherlandia frutescens* at 270nm.

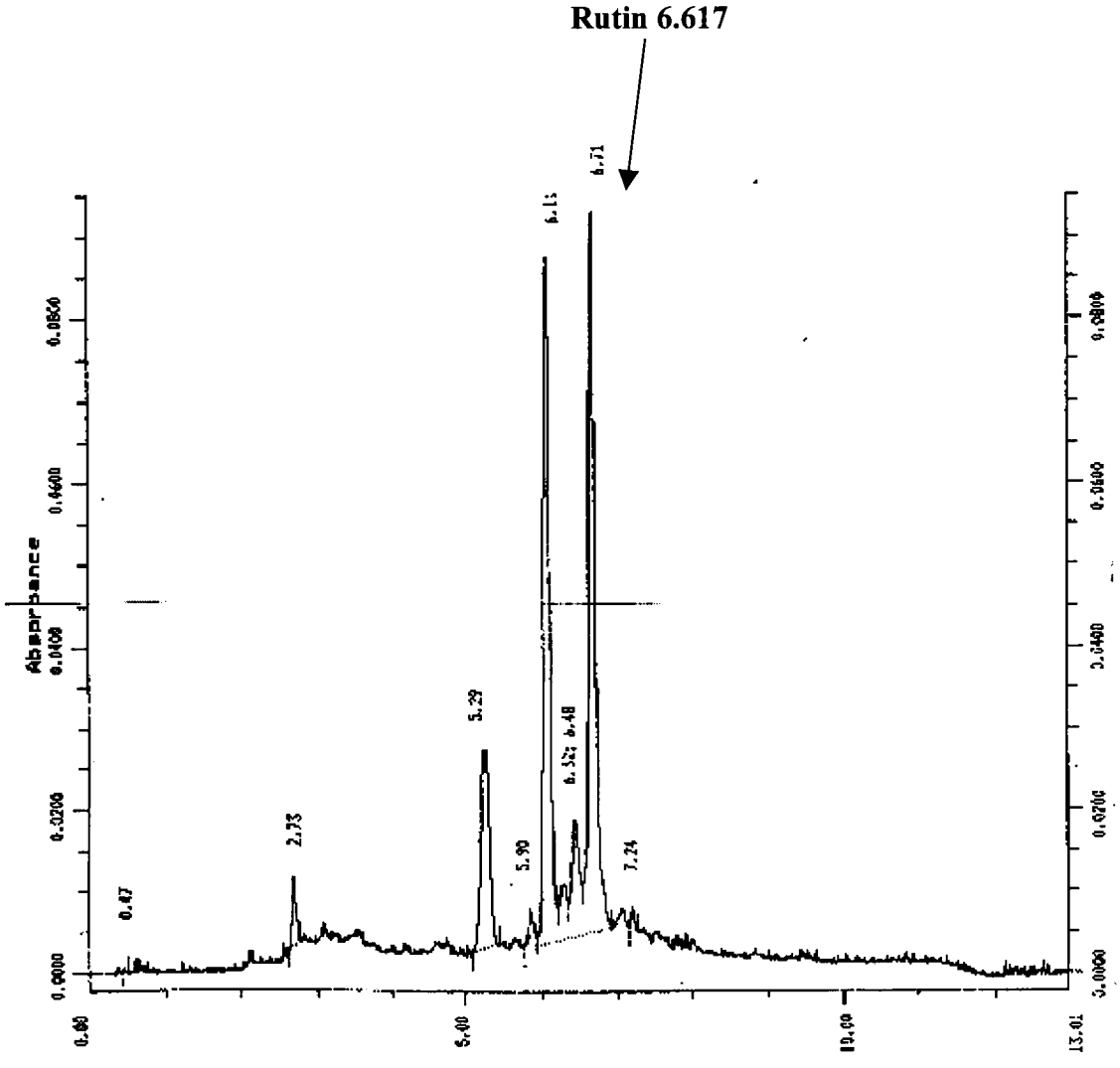


Fig 3: HPLC chromatogram of the aqueous extract of *Sutherlandia frutescens* at 360nm.

4.3 IN-VITRO ASSAY.

4.3.1 Results of cultured cancer cell lines treated with the aqueous extract and stained using the hematoxylin and eosin method.

The hematoxylin and eosin microphraphs of both the positive controls and tests for the three cell lines used: human epidermoid cervical cancer (CASKI), human oesophageal cancer (WHCO3) and lung cancer (NH157), showed features such as cell shrinkage, nuclear fragmentation and membrane blebbing, all consistent with apoptosis. While the negative controls show regular malignant cells (figures 4 to 12). The cells per slide were not quantified as the evaluation was based on morphological appearance only.

4.3.2 Results of cultured cancer cell lines treated with aqueous extract of *Sutherlandia frutescens* and stained using Crossmon trichrome technique.

Micrographs showed positive controls and tests positive for apoptosis, stained dark brown- pink; while the negative control, negative for apoptosis stained blue-green (figures 13 to 21). Evaluation here was based on the colour changes only.

4.4 IN-VIVO ASSAY.

Effect of crude aqueous extract of *Sutherlandia frutescens* and its isolates on dichlorvos-induced reduction in butyryl cholinesterase activity.

The normal plasma butyryl cholinesterase activity was 895.00 ± 41.22U/l. Dichlorvos (6mg/kg) was shown to significantly reduce the butyryl cholinesterase activity to 412.00 ± 13.89U/l, which is over 50% reduction. The aqueous extract (12.5-1000mg/kg) significantly attenuated the effect of dichlorvos (6mg/kg) against the butyryl cholinesterase activity. The highest aqueous extract effect against dichlorvos effect was obtained with 50mg/kg (aqueous extract). The enzyme activity was 1033.70 ± 24.69U/l. The isolates, coumarins (50-500mg/kg), flavonoids (50-200mg/kg), phytosterols (50-1000mg/kg) and tannins (50-200mg/kg), all significantly increased the enzyme activity by antagonising dichlorvos (6mg/kg) effect (Table 2).

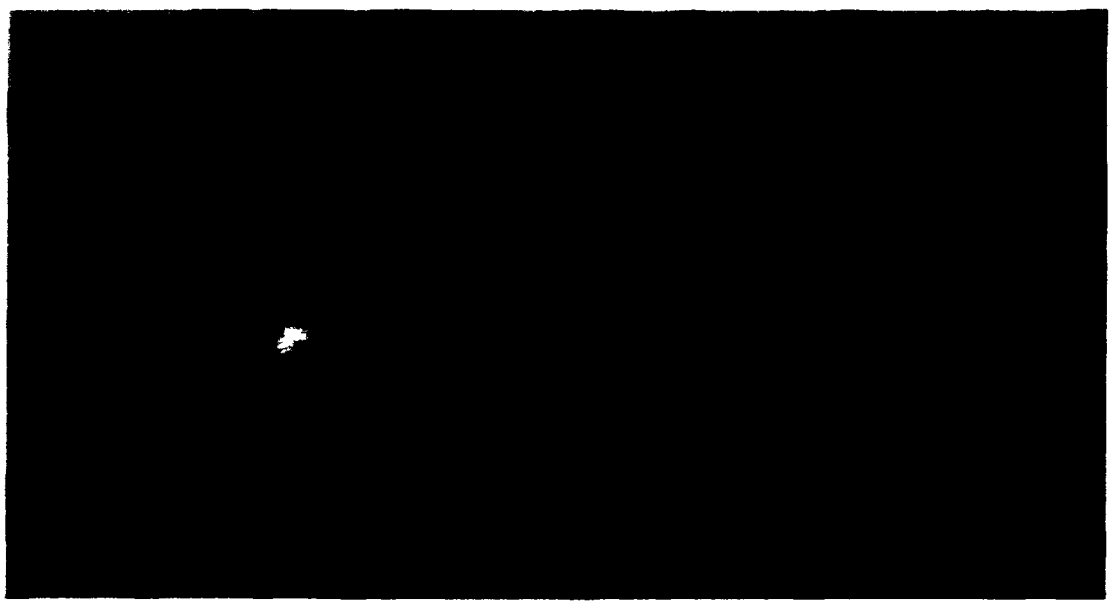


Fig 1: Human epidermoid cervical cancer cells (CASKI) treated with RPMI (negative control). Original magnification x200.

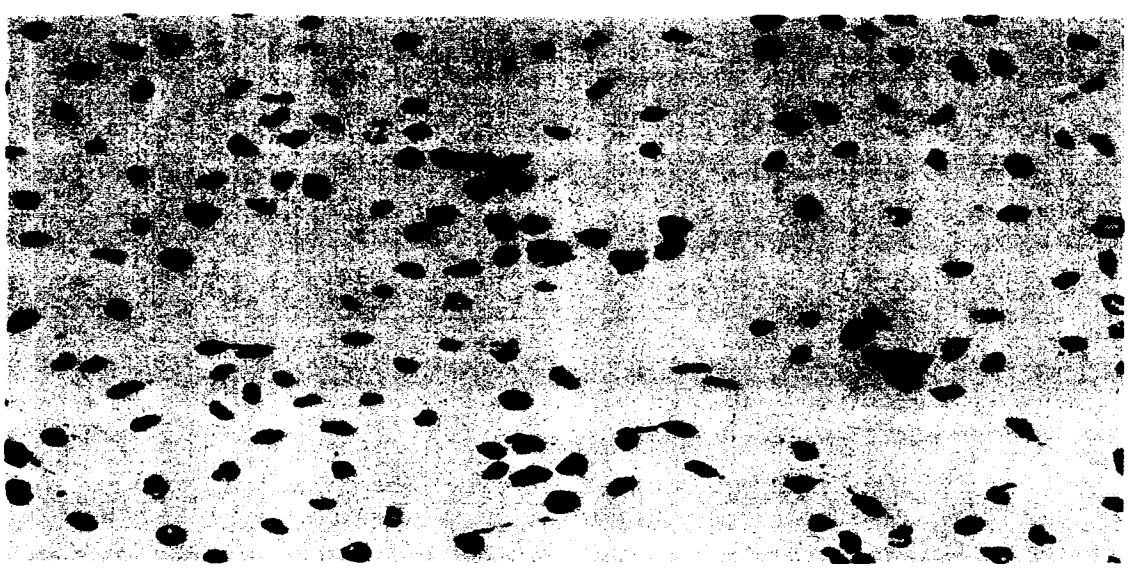


Fig 2: Human epidermoid cervical cancer cells (CASKI) treated with Staurosporine (positive control). Original magnification x200.

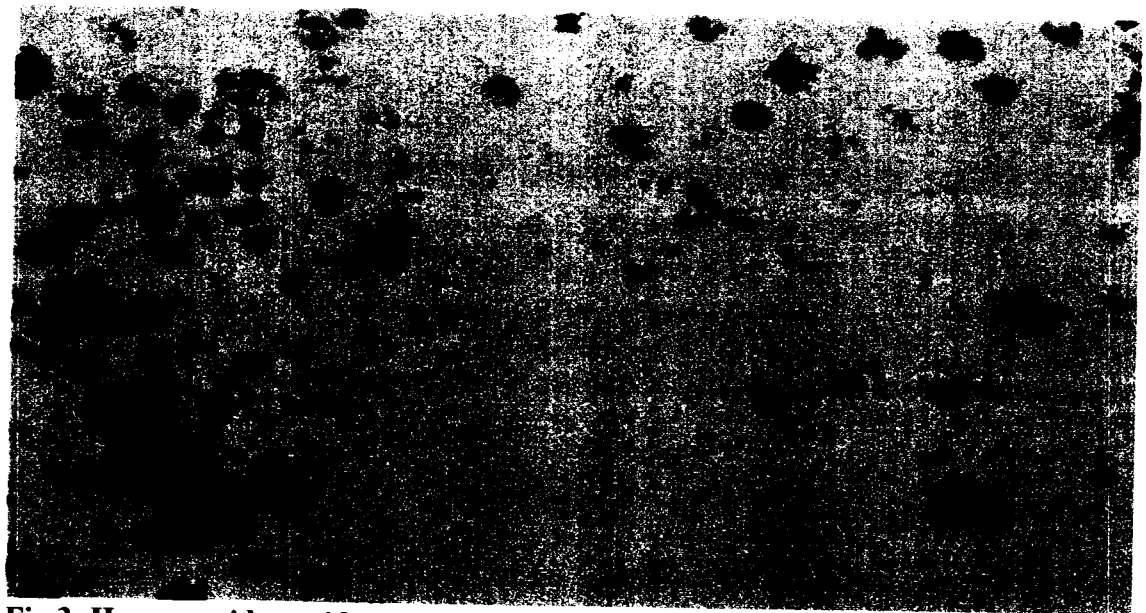


Fig 3: Human epidermoid cervical cancer cells (CASKI) treated with 10% aqueous extract of *Sutherlandia frutescens* (test). Original magnification x200.

Fig 4: Human oesophageal cancer cells (WHCO3) treated with RPMI (negative control). Original magnification x200.

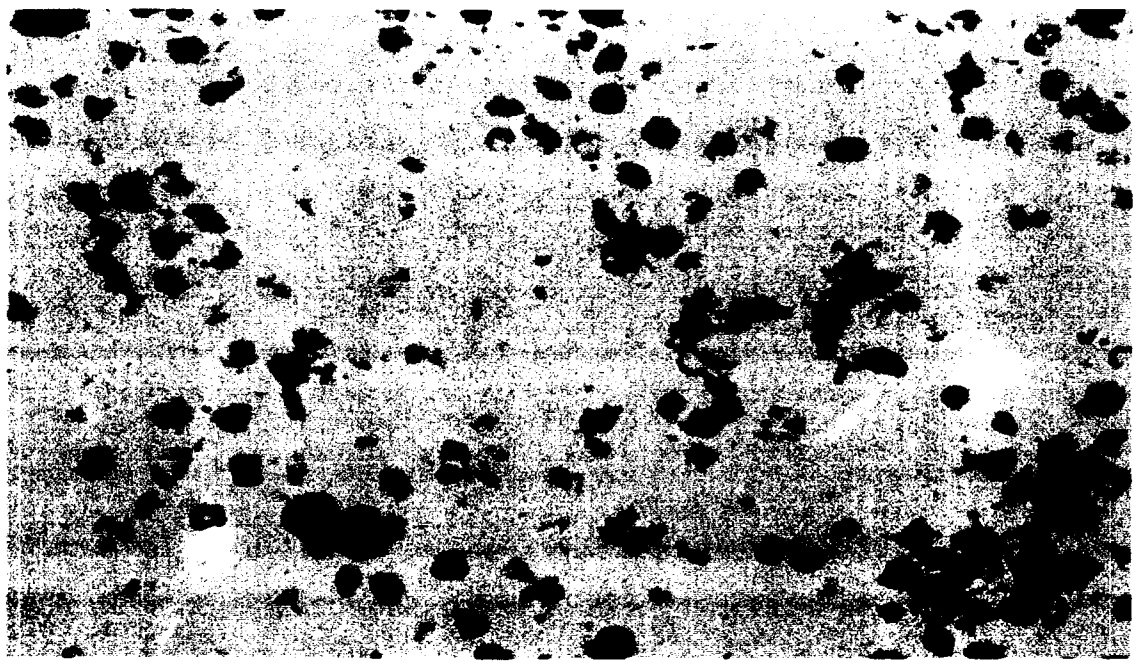


Fig 5: Human oesophageal cancer cells (WHCO3) treated with Staurosporine (positive control). Original magnification x200.

Fig 6: Human oesophageal cancer cells (WHCO3) treated with 10% aqueous extract of *Sutherlandia frutescens* (test). Original magnification x200.

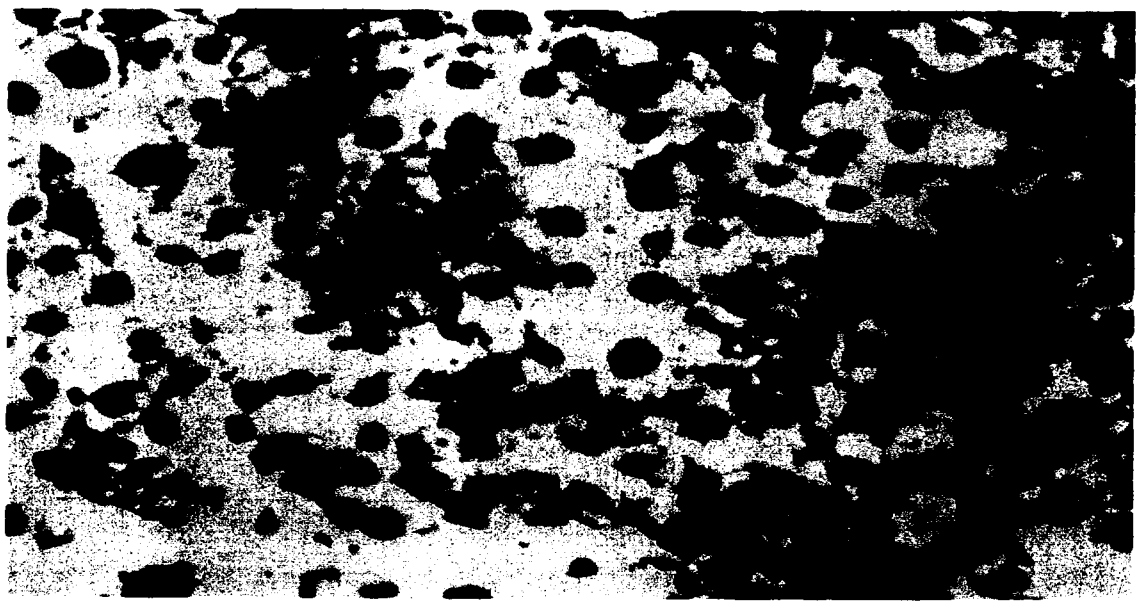


Fig 7: Human lung cancer cells (NH157) treated with RPMI (negative control). Original magnification x200.

Fig 8: Human lung cancer cells (NH157) treated with Staurosporine (positive control). Original magnification x200.

Fig 9: Human lung cancer cells (NH157) treated with 10% aqueous extract of *Sutherlandia frutescens* (test). Original magnification x200.

Fig 10: Human epidermoid cervical cancer cell (CASKI) treated with RPMI- negative for apoptosis (negative control). Original magnification x100.

Fig 11: Human epidermoid cervical cancer cells (CASKI) treated with Staurosporine –positive for apoptosis (positive control). Original magnification x100.

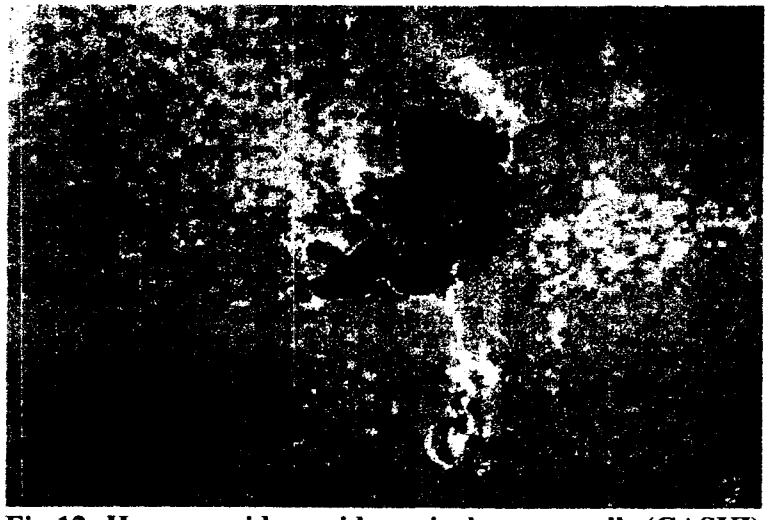


Fig 12: Human epidermoid cervical cancer cells (CASKI) treated with aqueous extract of *Sutherlandia frutescens* –test smear positive for apoptosis. Original magnification x100.

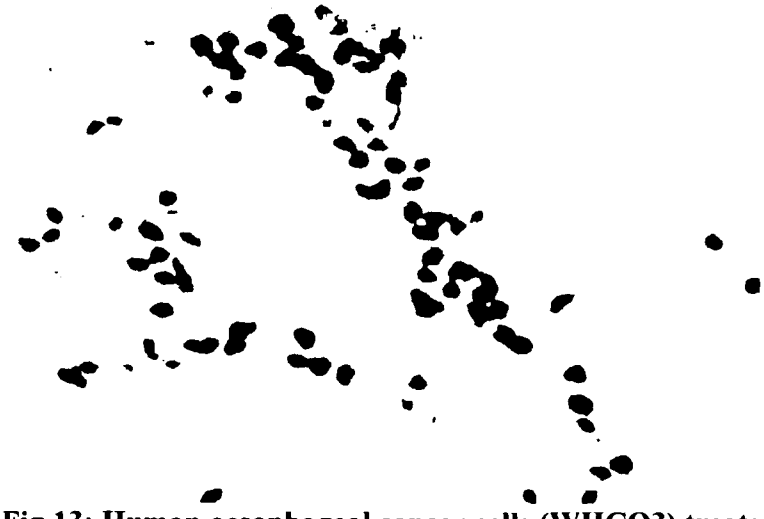


Fig 13: Human oesophageal cancer cells (WHCO3) treated with RPMI-negative for apoptosis (negative control). Original magnification x100.

Fig 14:Human oesophageal cancer cells (WHCO3) treated with Staurosporine - positive for apoptosis (positive control). Original magnification x100.

Fig 15: Human oesophageal cancer cells (WHCO3) treated with 10% aqueous extract of *Sutherlandia frutescens* –test, positive for apoptosis. Original magnification x100.

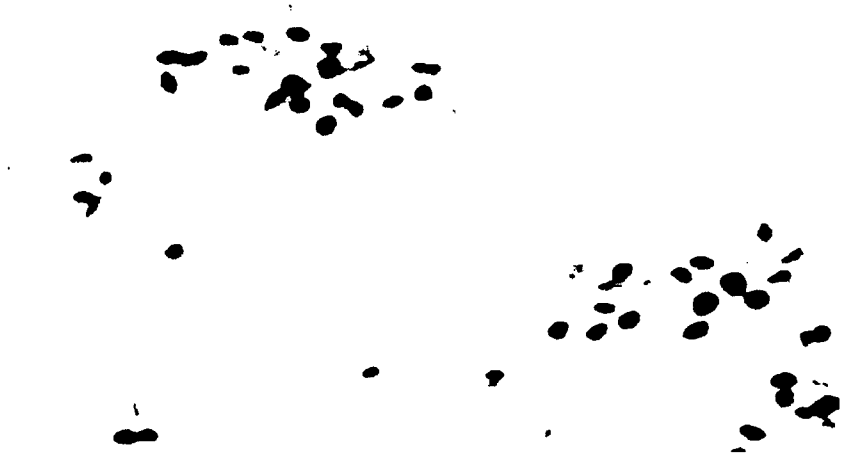


Fig 16: Human lung cancer cells (NH157) treated with RPMI - negative for apoptosis (negative control). Original magnification x100.

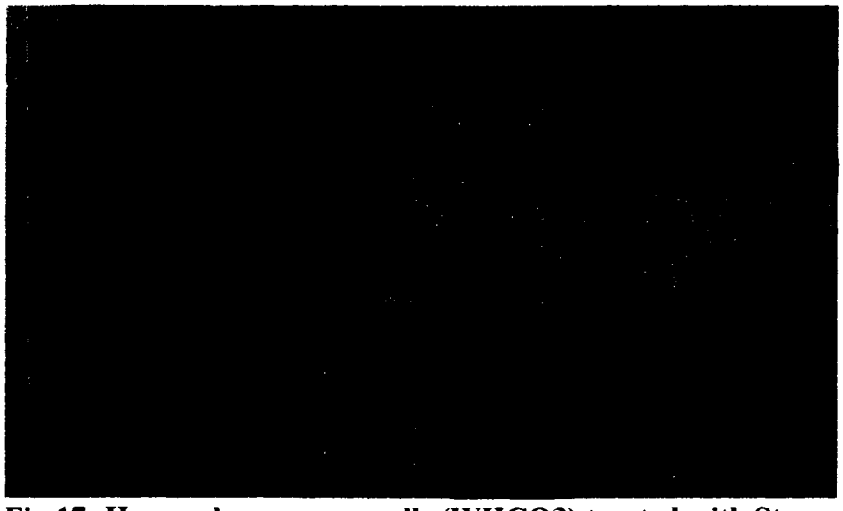


Fig 17: Human lung cancer cells (WHCO3) treated with Staurosporine – positive for apoptosis (positive control). Original magnification x100.

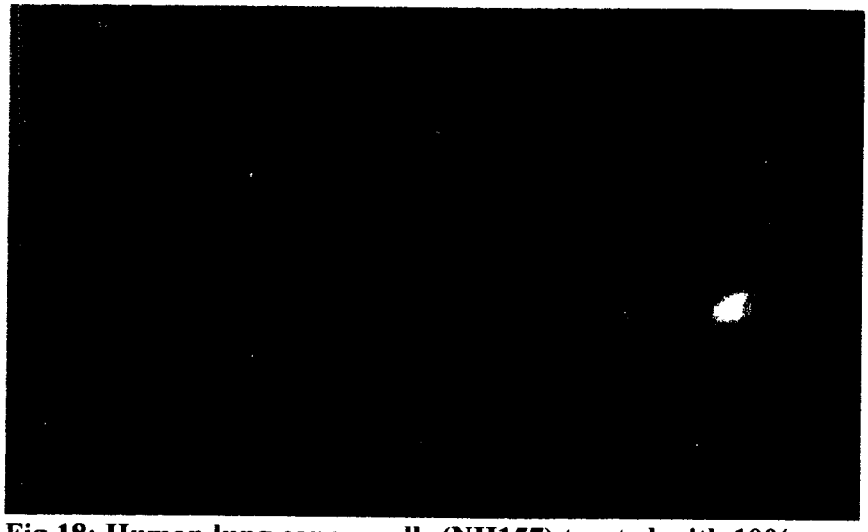


Fig 18: Human lung cancer cells (NH157) treated with 10% aqueous extract of *Sutherlandia frutescens* –test, positive for apoptosis. Original magnification x100.

Table 2 :
The effect of the crude aqueous extract of *Sutherlandia frutescens* and its isolates on dichlorvos induced reduction in butyryl cholinesterase activity.

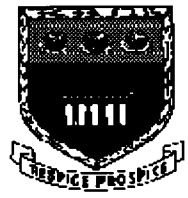
Treatment Group	Treatment doses (mg/kg) A	B	C	D	E	F	Plasma cholinesterase activity (U/l). Mean ±SEM
Control 1	6.0	-	-	-	-	-	412.00 ± 13.89
Control 2	-	-	-	-	-	-	895.00* ± 41.22
1	6.0	12.5	-	-	-	-	726.26* ± 24.91
2	6.0	25.0	-	-	-	-	739.63* ± 21.23
3	6.0	50.0	-	-	-	-	1033.70* ± 24.69
4	6.0	100.0	-	-	-	-	779.73* ± 28.40
5	6.0	200.0	-	-	-	-	742.28* ± 29.28
6	6.0	500.0	-	-	-	-	721.81* ± 29.58
7	6.0	1000.0	-	-	-	-	699.53* ± 36.44
8	6.0	-	50.0	-	-	-	815.38* ± 17.65
9	6.0	-	100.0	-	-	-	712.90* ± 23.97
10	6.0	-	200.0	-	-	-	761.91* ± 20.43
11	6.0	-	500.0	-	-	-	574.77* ± 20.43
12	6.0	-	-	50.0	-	-	726.26* ± 24.90
13	6.0	-	-	100.0	-	-	717.35* ± 24.38
14	6.0	-	-	200.0	-	-	1033.70* ± 31.03
15	6.0	-	-	-	50.0	-	748.54* ± 22.38
16	6.0	-	-	-	100.0	-	802.02* ± 31.18
17	6.0	-	-	-	200.0	-	818.78* ± 16.39
18	6.0	-	-	-	500.0	-	864.39* ± 19.03
19	6.0	-	-	-	1000.0	-	1029.24* ± 25.27
20	6.0	-	-	-	-	50.0	646.06* ± 44.21
21	6.0	-	-	-	-	100.0	632.70* ± 17.33
22	6.0	-	-	-	-	200.0	797.55* ± 48.14

***P < 0.001 compared with dichlorvos control (control 1), in student's t-test. n=10/group.**

A: Dichlorvos
B: Aqueous extract
C: Coumarins
D: Flavonoids
E: Phytosterols
F: Tanins

CHAPTER FIVE

DISCUSSION & CONCLUSIONS.



5.1 DISCUSSION

In this study, the anti-cancer activity of the plant *Sutherlandia frutescens* was investigated so as to serve as a prima facie evidence that it is beneficial or not in its current use in traditional medicine for anti-cancer activity and that it can be further designed and developed into a novel anti-cancer drug. The experimental design was such that the initial in-vitro work was done on cancer cell lines in culture, as a solid based standard screening for any anti-cancer activity. The second phase then necessitated the authentication of the in-vitro results. This is mindful of the fact that in-vivo evaluations may sometimes not be mirror images of in-vitro results.

The pytochemical analysis of interest identified coumarins, flavonoids, phytosteroles and tannins in the plant. However, some papers have also documented the presence of L-canavanine, GABA and pinitol in the plant (70), in relation to the purposes for which the plant was used.

HPLC analyses showed numerous peaks representing the presence of many compounds, thus proving how chemically complex the plant is. This means therefore that only further fractionation and chromatography, followed by NMR and mass spectrometry can identify specific leads for further elaboration in an anti-cancer drug design and development plan.

In the in-vitro assessment, the induction of apoptosis was used as the indicator for anti-carcinogenecity. The results shown on hematoxylin and eosin (H&E) stained and crossmon stained photo- micrographs, show features consistent with apoptosis. These features on the H&E micrographs are cell shrinkage, chromatin condensation, dissolution of nuclear membrane and nuclear fragmentation, and membrane bebbing. While the positive dark brown to pink-red colouration of some crossmon stained smears indicates that the cells did undergo apoptosis. This result suggest that *Sutherlandia frutescens* has anticancer properties as claimed.

Simply defined as programmed cell death or activation-induced cell death (19,25,71), apoptosis is a genetically controlled response for cells to commit suicide. These cells can

undergo apoptosis spontaneously or when induced with a chemotherapeutic agent or substance as is the case in the first phase of this study. Other stimuli to which cells can respond to in addition to chemotherapy, include irradiation, some hormones and the tumor necrosis factor (TNF). Despite the wide variety of stimuli that can activate apoptosis, it is worthy to note that response can only occur in effete or abnormal cells, characterized by damaged DNA (beyond repair) or a dysregulated cell cycle as in the case with cancer cells. The signaling pathways induced by the wide variety of stimuli converge into a common death pathway either at a mitochondrial step, culminating in the release of cytpchrome C and apoptosis inducing factors from the mitochondria into the cytosol or at the following step at which caspase-3-like cysteine proteases, also called the executioners of apoptosis, are activated (72). Finally, active caspases cleave limited set of cellular proteins whose activation/inactivation results in the typical apoptotic morphological changes including membrane bebbing, cell shrinkage, nuclear condensation and or fragmentation (19,25,71).

The chain of processes and pathways culminating in this programmed cell death, have been traced and elucidated in-vitro, such that in addition to the characteristic morphological changes, the "behind-the-scene" biochemical processes can be evaluated, using indices such as caspases, p53 and kinases/cyclins, with biochemical assay kits.

Proteolysis of cellular proteins (cyto-skeletal protein lysis) results from the activation of Ca/Mg-dependent nucleases, especially DNAse I, which degrade the nuclear lamins, vimentin and other cyto-skeletal proteins and chromosomal DNA. This leads to multiple nicks and breaks within the DNA and the cytoplasm and finally results in the generation of DNA oligomers, their sizes being multiples of nucleosome-associated DNA (DNA-fragmentation)- the hallmark of apoptosis. The cytoplasmic alterations result in membrane blebbing and cell shrinkage.

The "behind-the-scene" biochemical changes from activations/inactivations of cleaved cellular proteins giving rise to both nuclear and cytoplasmic protein degradations culminate into visible morphological changes in the nuclei and cytoplasm of affected cells. These changes were subsequently well demonstrated by differential H & E staining

as seen in the photo-micrographs. The principle for H & E staining being such that all relatively more acidic cellular components like the nuclei or its fragments have more affinity for the basic dye hematoxylin and all less acidic cell components like the cytoplasm, having affinity for the acid dye eosin thus, differential staining. The principle of the modified (crossmon) trichrome stain is such that apoptotic cells and fragments stain dark brown to pink-red. It seems that condensed nuclear material is stained by fuchsin in a way that subsequent phosphotungstic acid treatment is not able to remove the dye (65). The described color change as it is in the presence *Sutherlandia frutescens* indicates apoptosis, while non-apoptotic cells or smears pick up the secondary stain of light green after being de-stained by phosphotungstic acid treatment.

In-vitro cancer cell transformations are sometimes not true representations of in-vivo spontaneous cancers, due to deletions and/or alterations in some factors or properties in the process of mimicking in-vivo carcinogenesis in the test tube (8). This sometimes presents discrepancies in the results of in-vitro and in-vivo experiments meant to be reflective of one another. Mindful of the possible discrepancies, it was worth while authenticating the impressive in-vitro results with the animal study. The benefits of this second phase of study include the fact that the induction of carcinogenesis in the mice was ab initio in a biogenetic process of spontaneous initiation of chemical carcinogenesis. In addition, pharmacological processes such as drug absorption, transportation, bioavailability, biotransformation and the excretion of active plant metabolites of *Sutherlandia frutescens* could invariably be attested to be therapeutically enhancing viz-a-viz, the biological system of choice- mice, as the results were also impressive. These factors that would otherwise determine the possible "drug" efficacy, could not be ensured in the in-vitro experimental model used in this study.

In the in-vivo study, dichlorvos (dichloro- dimethyl phosphoric acid ester – DDP) was used as the carcinogen. This chemical is a cholinesterase inhibitor and like most chemical inducers of cancer, in relative high non-lethal doses cause mutagenesis. Increase of mutation frequency as much as 3-fold have been observed in liver cells after giving multiple doses in mice (66,67). Neoplasms induced by dichlorvos include adenomas of the exocrine pancreas, mononuclear cell leukemia, squamous cell papilloma of the

forestomach and fibroadenomas of the mammary gland in rats and mice (66). The index of measure in the in-vivo study was the level of activity of the enzyme butyryl cholinesterase (pseudo-, plasma cholinesterase).

The enzyme cholinesterase exist in two forms: acetyl cholinesterase found in erythrocytes and nervous tissue, and buytryl cholinesterase in non-nervous tissue. In carcinogenesis induced by organophosphates, of which dichlorvos is one and in many carcimomas in general, there is a drastic reduction of butyryl cholinesterase levels in plasma/serum by 50% or more (66-68).

The results obtained after treatment with both the aqueous extract of *Sutherlandia frutescens* and its isolates, showed that they increase the level of the enzyme butyryl cholinesterase which suggest that, the extract and isolates counteract the process of dichlorvos induced carcinogenesis. All the test were analyzed in comparism to a dichlorvos control, and all were significant with $P<0.001$. It not only re-affirms the claims for anti-cancer activity of *Sutherlandia frutescens*, but also authenticates the in-vitro results. In addition, the results conform with reports of cancer risk reduction by phytosterols, coumarins, flavonoids, and tannins (73), which are all components of *Sutherlandia frutescens.*

5.2 CONCLUSION

On the basis of this study, *Sutherlandia frutescens* extracts induce apoptosis and counteract dichlorvos induced carcinogenesis; two parameters consistent with anti-cancer activity. The study was based on evaluating the anti-cancer activity only and no toxicity evaluations were done as a part of this work. However, it is probable that the plant and its ingredients may present some side effects and/or toxicity especially in high doses or in long term use, as in the case with many therapeutic substances. This does not in any case rule out its activity against cancer as observed in this study and would only add to the profile of the plant, mindful of the fact that the ultimate end point of any plant study would be for the discovery of leads and hits towards novel drug design and development.

My recommendation would be that subsequent studies be carried out to develop true animal tumour models for the anti-cancer activity evaluation of the plant and its metabolites. Moreso, extensive pharmacological, toxicological and phytochemical screening of this plant, would be very valuable in understanding the different machanisms of action and enhancing the safety and efficacy of the plant.

REFRENCES:

1) Read, A.E. Barrit, D. W. Henver, R. L. (1984). Introduction. *Modern Medicine, A Text book for students.* 3rd ed. Pitman Ltd.: 1
2) Dorland, I. Newman, W.A. (2000). Dorland's Illustrated Medical Dictionary. 29th ed. W.B Saunders: 789.
3) Anderson, K.N. Anderson, L.E. Glanze, W.D. (1998). Mosby's Medical, Nursing and Allied Health Dictionary. 5th Ed. Mosby: 732.
4) Sikora K. (1999). *Cancer Strategy* 1:2-4.
5) Developing a global strategy for cancer. *WHO : Geneva*: March 2001.
6) Traditional Medicine fact sheet # 134. *WHO: Geneva*: Sept 1996.
7) Van Wyk B.E, Van Oudtshoorn B, Gerick N (1997). Medicinal plants of South Africa (Ed). Briza pub:246-247.
8) Peckman M, Pirech H, Veronesi U (1995).Cancer. *Oxford text book of Oncology.* Oxford Univ press. Vol1:1-5.
9) Walter J.B, Isreal U.S, (1987). Tumors: *General Pathology.* 6th Ed . Churchill livingstone:335-341.
10) Robbin S.L, Kumar V, (1980). Neoplasia. *Basic Pathology.* 4th Ed. W.B Saunders: 182-212.
11) Robbin S.L, Cotron R.S, Kumar V (1984). Neoplasia. *Pathologic Basis of disease.* 3rd Ed. W.B Saunders: 214-253.
12) Lewin B (1997). Oncology and Cancer. *Gene VI.* 6th Ed . Oxford Univ press:1087-1170.
13) Nicolin C (1982). Chemicals as carcinogens. *Chemical carcinogenesis.* NATO: 1-71.
14) Varley H, Gowenlock A.H, Bell M (1980). Enzymes. *Practical Clinical Biochemistry.General Topics and commoner tests.* 5th Ed. William Heinman Med Books Ltd. Vol 1: 753-761.
15) Tietz N.W (1982). Enzymes. *Fundamentals of Clinical Chemistry.* 2nd Ed. W.B Saunders & Co:643-649.
16) Wallach J (2000). Interpretation of Diagnostic Tests. 7Th Ed. Lippincott Williams & Wilkins: 934.
17) Acetyl-cholinesterase (1997). Calzyme Lab. [Online]. Available http://www.calzyme.com/catalog/actlchol.html

18) Hengartner M. (1998). Death by crowd control. *Science* 281: 1298-1299.

19) Miller L.J, Marx J (1998). Apoptosis. *Science* 281: 1301.

20) Thornberry N.A, Lazebnik Y (1998). Enemies within. *Science* 281:1312-1316.

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

22) Ashkenazi A, Dixit V.M (1998). Death receptors: Signaling and modulation. *Science* 281:1305-1308.

23) Even G, Littlewood T (1998).A matter of life and cell death. *Science* 281: 1317-1321.

24) Adams J.M, Cory S (1998). The Bcl-2 protein family: Arbiters of cell survival. *Science* 281:1322-1325.

25) Hengartner O.M (2000). The Biochemistry of apoptosis. *Nature* 407:770-775.

26) Nicholson D. W (2000). From bench to the clinic with apoptosis-based therapeutic agents. *Nature* 407:810-816.

27) Krammer P.H (2000). CD95's deadly mission in the immune system. *Nature* 407:789-794.

28) Kinzler K.W, Vogelstein B (1996). Life and death in a malignant tumor. *Nature* 379:19-20.

29) Chang F, Syrjanen S, Syrjanen K (1995). Implications of the p53 tumor-suppressor gene in clinical oncology. *J. of Clinical Oncology*: 13(4): 1009-1022.

30) Rich T, Allen R.L, Wyllie A.H (2000). Defying death after DNA change. *Nature* 407:777-782.

31) Jones P.A (2000). Death and methylation. *Nature* 409: 141-143.

32) Hann B.C, Lane D.P (1995). The dominating effect of mutant p53. *Nature Genetics* (9): 221-222.

33) Churman B, Groudine M (1997). Killer in search of a motive. *Nature* 389:122-123.

34) Craiz C.R, Stitzel R.E (1990). Antineoplastic agents: *Modern Pharmacology* 3rd. Little Brown & co: 787-820.

35) Mycet M.J, Harvey A.R, Champe C.P (1997). Anticancer drugs:*Lippincott's illustrated reviews: Pharmacology.*2nd Ed. Lippincott-Raven pub: 373-398.

36) Calabresi P, Chabner B.A (1996). Chemotherapy of neoplastic disease. *Goodman & Gilman's- The Pharmacological Basis of Therapeutics.* 9th Ed. The Mc Graw-Hill Co. Inc: 1225-1233.

37) Rasolonjanahary R, Sevenet T, Kordon C (1986). New Screening strategies for natural products active on the brain. *Advances in medicinal phytochemistry.* John Libbey: 81-87.

38) Luu B (1986). Use of in-vitro cell cultures to study cytotoxic properties of natural products. *Advances in medicinal phytochemistry.* John Libbey: 97-101.

39) Cave A (1986). Methodology of research on medicinal plants. *Advances in medicinal phytochemistry.* John Libbey: 47-61.

40) Williamson E.M et al (1999). *Pharmacological methods in phytotherapy research.* John Wiley & Son: Vol 1.

41) Hostettman K (1998). Current level of technology in separation, isolstion and structural analysis of natural products. *Advances in medicinal phytochemistry.* John Libbey:67-76.

42) Chinkwo K, et al (2001). Isolation of Apoptosis inducing agents. *Poster/Conference Unpublished.* Bellville : University of the Western Cape.

43) Minshul, J Stemmer, W.P.C.(1999). Protein evolution by molecular breeding. *Current opinion in Chemical Biology.* 3: 284-290.

44) Skolinck, J. Fetrow, J.S. (2000). From genes to protein structure and function: novel applications of computational approaches in the genomic era. *TIBTECH.* Vol. 18: 34-39.

45) Beeley, N.R.A. (2000). Can peptides be mimicked? *DDT* Vol. 5. # 8 August 2000: 354-363.

46) Searl, D.B. (2000). Using bioinformatics in gene and drug discovery. *DDT* Vol. 5. # 4:135-142.

47) Beno, B.R. Mason, J.S.(2001). The design of combinatorial libraries using properties and 3D pharmacophore fingerprints. *DDT* Vol. 6. # 5: 251-258.

48) Bohrn, H.J. Stall, M. (2000). Structure-based library design: molecular modelling merges with combinatorial chemistry. *Current opinion in Chemical Biology.* 4: 283-286.

49) Rational Drug Design. (1999). Molecular simulations Inc. [Online] Available. http://www.msi.com/life/rational/index.html

50) Clark, D.E. Pickett, S.D. (2001). Computational methods for the prediction of "drug-likeness". *DDT* Vol. 5, # 2: 49-58.

51) Understand activity data. (1999). Molecular simulation Inc. [Online]. Available http://www.msi.com/life/rational/activity.html

52) Suggest new active molecules. (1999) Molecular simulation Inc. [Online]. Available http://www.msi.com/life/rational/new_active.html

53) General modeling. (1999). Molecular simulation Inc. [Online]. Available http://www.msi.com/life/rational/gen_model.html

54) Analog-based drug design. (1999). Molecular simulation Inc. [Online]. Available http://www.msi.com/life/rational/rulk.html

55) De novo drug design. (1999). Molecular simulation Inc. [Online]. Available http://www.msi.com/life/rational/rklu.html

56) Structure based drug design. (1999) Molecular simulation Inc. [Online]. Available http://www.msi.com/life/rational/rklk.html

57) Modeling tutorials. (1999). Center for molecular modelling. [Online]. Availble http://cmm.info.nih.gov/modeling/tutorials.html

58) Drug design lectures.(1999). UNIMI Italy. [Online]. Available http://users.unimi.it/~ddl/

59) Anti-cancer drug.(1999). OUP UK. [Online]. Available http://www3.oup.co.uk/antican/

60) Lipp, F.J (1989). Methods for Ethnopharmacological field work. *J. of Ethnopharmacology, 25: 139-150.*

61) Williamson, E.M . Okpako, D.T and Evans, F.J. (1996). Pharmacological Methods in phytotherapy research. Vol 1: selection, preparation and pharmacological evaluation of plant material. Enlgand: Wiley: 2-190.

62) Harborne, J.B (1984). Phytochemical Methods: A guide to morden techniques of plant analysis, 2nd ed. London: Chapman & Hall: 84-274.

63) Moffat, A.C. Jackson, J.V. Moss, M.S. Widdop, B & Greenfield, E.S (1986). Clarke's isolation and identification of drugs, 2nd ed. London: *The Pharmaceutical Press:* 201-221.

64) Drury, R.A.B. Wallington, E.A. (1980). General Staining Procedures. Carlton's Histological Techniques. 5th ed. Oxford University Press: 125-149.

65) Anton, E. (1999). Detection of Apoptosis by a Modified Trichrome Technique. *J. of Histechnol. Vol. 22 (4):* 301-304.

66) Chan et al (1991). Carcinogenesis Studies of Dichlorvos in Fischer rats & B6C3F1 mice. *Jpn J can Res. 82(2)*: 157-164.

67) Pletsa, V et al (1999). Induction of somatic Mutations but not Methylated DNA adducts in Lambdalac Z transgenic mice by Dichlorvos. *Cancer Lett.* 15: 146(2): 155-160.

68) Clinical toxicology review.(1995). [Online]. Available http://www.maripoisoncenter.com

69) Cholinesterase BTC enzyme analysis. Sigma diagnostics. Dec. 1995.

70) *Sutherlandia.(2000).* [Online]. Available http://www.sutherlandia.org

71) Ken Ohta (2001). Apoptosis & its Induction in Eosinophils Activated with IL-5. *ACI International.* 13/6: 262-267.

72) Apoptosis(2000)[Online]Available http://www.cancer.dk/english/biological_research/apoptosis/apoptosis.htm

73) Phytochemical (2002). [Online]. Available http://www.mckinley.uiuc.edu/health-info/nutrit/phytochemicals.htm