THE BIOCHEMICAL EFFECTS OF SUTHERLANDIA FRUTESCENS IN CULTURED H9 CANCEROUS T CELLS AND NORMAL HUMAN T LYMPHOCYTES

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2008

ABSTRACT

Indigenous plants have long been used by African populations in their cultural lives and health care. *Sutherlandia frutescens* (SF) is a popular traditional medicinal plant found in various parts of southern Africa and used for treatment or management of different diseases, including cancer and HIV/AIDS. In this study, the biochemical effects of various dilutions (1/50, 1/150, 1/200, and 1/300) of SF 70% ethanol (SFE) and deionised water (SFW) extracts in cancerous H9 and normal T cells were examined. Untreated, 70% ethanol-treated and camptothecin (CPT, 20μM) treated cells were used as reference samples for comparison. Cytotoxicity, apoptotic enzymes activity, oxidant scavenging and antioxidant promoting abilities, cellular morphology and cytokine signalling effects were assessed using the methylthiazol tetrazolium (MTT) assay, adenosine triphosphate (ATP) assay, caspase-3/-7 activity assay, thiobarbituric acid reactant substance (TBARS) and glutathione (GSH) assays, fluorescence microscopy and an ELISA-based cytokine analyses assay respectively.

Sutherlandia frutescens ethanol and water extract dilutions (1/50 and 1/200) were shown to be cytotoxic to H9 T cells in a dose- and time-dependent manner with the SFE extract having an average IC_{50} of 1/40 after 24 hours while SFW extract reached a similar IC_{50} only after 48 hours. In normal T cells, the SFE extract induced proliferation after 24 hours but this was reverse after 48 hours. The SFW extract dilutions did not significantly change cell viability after 24 hours but significantly increased cell viability after 48 hours. Both SFE and SFW extracts dilutions induced a dose- and time-dependent inhibition of caspase-3/-7 activity in both H9 and normal T cells. Both types of extracts were also shown to efficiently remove lipid peroxides from supernatants of treated cell lines, with SFW extract having a more lasting effect. In the GSH assay, the SFE and SFW extract dilutions reduced GSH levels in H9 T cells, with the SFW extract dilutions being more effective. In normal T cells, the higher dilutions (1/150 and 1/300) of SFW extract increased GSH levels significantly while lower dilutions (1/50) of both SFE and SFW extracts significantly inhibited GSH levels. Lower dilutions (1/50) of SFE and SFW extracts induced chromatin condensation in both H9 and normal T cells after 48 hours incubation. Using treated peripheral blood mononuclear cells (PBMCs) supernatants, SFE and SFW extract dilutions were shown to reduce the levels of pro-inflammatory cytokines IL 1β and TNF-α in a dose-dependent manner. These results further confirmed the anticancer abilities of SF and showed that higher concentrations of this medicinal plant can be toxic to normal T cells *in vitro* while lower concentrations can stimulate the immune cells. Therefore further studies should be conducted with regards to the effects of SF on the immune system in both *in vitro* and *in vivo* systems.

AUTHOR'S DECLARATION

The research reported in this dissertation, except where otherwise indicated, represents the original work done by the author. It has not been submitted for any degree or examination at any other university.

Experimental work for this research project was carried out in the Discipline of Medical Biochemistry in the School of Medical Sciences and Department of Medicine in the Faculty of Health Sciences at the Nelson R. Mandela School of Medicine, University of KwaZulu Natal, Durban, South Africa, under the supervision of Professor Anil A. Chuturgoon.

M. Ngcobo:..... Date: 15-04-2009

DEDICATION

Mr M S Ngcobo **Mrs N E Ngcobo**

27 November 2001 04 April 2008

"Zonke iziziba zogcwala inhlabathi".

Zulu expression

'Absence makes the heart grow fonder' (**William Shakespeare**). And the longer time passes the more we remember the good old days. Only if we could have it our own way!

ACKNOWLEDGEMENTS

'The aim of life is self-development. To realize one's nature perfectly - that is what each of us is here for'.

Oscar Wilde

And with those words I would like to express my gratitude and sincere thanks to the following people and organizations:

First and foremost, to my faithful departed mother Mrs Ngcobo, words won't really do justice to what I want to say about you. Thank you is the last word I would say to express my gratefulness and as the last one, they stand for all the other thousands which would come before it.

I would like to thank my supervisor, Professor A.A. Chuturgoon, for having allowed me to pursue my postgraduate studies in the Discipline of Medical Biochemistry. You have taught me the value of hard work.

To Professor Nceba Gqaleni, I hope you see the value of the money and time you invested into the construction of this thesis work. Thank you!

I am also appreciative to Miss Metse Serumula for the hours we spent in the laboratory together. I do not think I would have finished this early if it were not for you being around with your laughter. To Mrs Rene Myburg also, thank you for sharing your expertise with me.

I would also like to thank Professor Alain Assounga for allowing me to use his laboratory in the Department of Medicine to do some of my experiments and sharing some of your experiences with me.

Thank you to Devan Moodley, Leshern Karamchand and Kristina Naidoo of the Mycotoxin Research Unit for helping me with the use of the luminometer and other important work in the laboratory.

High fives to the following postgraduate students: Fortunate Ndlandla, Nhlanhla Jwara, Nkosana Jafta, Bongani Nkala and all the others I did not mention for their support and encouragement during the three years I have spent working towards completing this thesis.

Lastly but not least, I would like to thank the National Research Foundation (NRF) and LifeLab for their financial assistance.

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$\Delta\psi$ m	*mitochondrial proton gradient*
μg/ml	micrograms per millilitre
μl	microlitre
μm	micrometre
^{0}C	degrees Celsius
ABTS	2,2_-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid
AIDS	acquired immunodeficiency syndrome
AIF	apoptosis-inducing factor
AMP	adenosine monophosphate
ANOVA	one-way analysis of variance
APAF-1	apoptosis protease-activating factor-1
APCI	atmospheric pressure chemical ionisation
Apo-2L	apoptosis 2 ligand
ARV	antiretroviral
ATCC	American Type Culture Collection
ATP	adenosine triphosphate
ATPases	ATP synthase
B	bebbling nucleus
Bad	BclxL/Bcl-2 associated death promoter
Bax	Bcl-2 associated x protein
Bcl-2	B-cell lymphoma 2
BHT	butylated hydroxytoluene
BSA	bovine serum albumin
BSO	L-buthionine sulphonine

Ca^{2+}	calcium ion
CCM	complete culture media
cDNA	complementary DNA
CHO	Chinese Hamster Ovary
cm^3	cubic centimetre
CN	condensed nucleus
CO_2	carbon dioxide
COX-2	cyclooxygenase-2
CPT	camptothecin
CSI-MIS	co-ordination ion-spray mass spectrometry
CYP17	cytochrome P450 17
CYP21	cytochrome P450 21
CYP3A4	cytochrome P450 3A4
DC	dendritic cells
DMSO	dimethyl sulphoxide
DN	dividing nucleus
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DR	death receptor
DU-145	Human prostate carcinoma epithelial-like cell line
ECACC	European Collection of Cell Culture
ELISA	enzyme linked immunosorbent assay
ERK	extracellular signal-regulated protein kinase
ESI	electrospray ionization
EtOH	ethanol
FACS	fluorescence-activated cell sorting

FADD	Fas-associated death domain
Fc	fragment, crystallisable
FCS	foetal calf serum
FLICE	FADD-like interleukin 1β-converting enzyme
FLIP	FLICE-inhibitory protein
FMLP	L-formyl-L-methionyl-L-leucyl-phenylalanine
FN	fragmented nucleus
g	gravity force
GABA	gamma-aminobutyric acid
GCS	γ-glutamylcysteine synthetase
GM-CSF	granulocyte monocyte- colony stimulating factor
GSH	glutathione
GSSG	glutathione disulphide
GST	glutathione-S-transferase
h	hour
H_3PO_4	phosphoric acid
H9	Human T cell lymphoma cell line
HCl	hydrochloric acid
HCO_3	bicarbonate
HEPES	*N*-2-Hydroxyethylpiperazine-*N*'-2-ethane-sulfonic acid
HIV	Human immunodeficiency virus
HPLC	high pressure liquid chromatography
HUT-78	Human T cell lymphoma cell line
i.p.	intraperitoneal
IAP	inhibitor of apoptosis
ICAM	intracellular adhesion molecule

ICE	interleukin converting enzyme
IFN	interferon
IgE	immunoglobulin-E
IL	interleukin
kg	kilogram
LC	liquid chromatographic
LOOH	lipid hydroperoxide
LPS	liposaccharide
MALDI-TOF	matrix-assisted laser desorption and ionization time-of-flight
MAP	mitogen-activated protein
MCF-12A	Human non-tumorigenic mammary epithelial cell line
MCF-7	Human mammary adenocarcinoma cell line
MDA	malondialdehyde
MDA-MB-231	Human breast adenocarcinoma cell line
mg	milligram
MHC	major histocompatibility complex
mm	millimetre
mM^{-1}	millimolar
MOMP	mitochondrial outer membrane permeability
MRC	Medical Research Council
mRNA	messenger RNA
MS	mass spectrometer
MTT	3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide
N	normal nucleus
NAD^+	oxidised nicotinamide adenine dinucleotide
NADH	reduced nicotinamide adenine dinucleotide

NaOH	sodium hydroxide
NF- κB	nuclear factor-κB
NIH	National Institute of Health
NK	natural killer
nm	nanometre
NO	nitric oxide
$NO^{\cdot}$	nitric oxide ion
O_2	oxygen
$O_2^{\cdot-}$	superoxide
OH^{-}	hydroxyl
p53	phosphoprotein 53
PARP	poly (ADP-ribose) polymerase
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
PFA	paraformaldehyde
P-gp	P-gylcoprotein
pH	hydrogen-ion concentration
PHA	phytohaemaglutinin
PSF	Penicillin-Streptomycin-Fungizone
PUFA	polyunsaturated fatty acids
PXR	pregnane X receptor
RIP	receptor interacting protein
RLU	relative light units
RNA	ribonucleic acid
ROS	reactive oxygen species
rpm	revolutions per minute

RPMI	Roswell Park Memorial Institute
$RS^{\cdot}$	sulphur centred radical
RT	reverse transcriptase
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SF	*Sutherlandia frutescens*
SFE	*Sutherlandia frutescens* ethanol extract
SFW	*Sutherlandia frutescens* deionised water extract
SLE	systemic lupus erythematous syndrome
TB	tuberculosis
TBA	thiobarbituric acid
TBARS	thiobarbituric acid reactant substance
TCA	tricarboxylic acid cycle
TEAC	Trolox equivalent antioxidant capacity
T_h	T helper lymphocytes
TNF	tumour necrosis factor
TNFR	tumour necrosis factor receptor
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TRADD	TNF-receptor1-associated death domain
TRAIL	TNF-related apoptosis-inducing ligand
Trolox	6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid
T_s	T suppressor lymphocytes
UV	ultraviolet
VCAM	vascular adhesion molecule

CHAPTER 1

INTRODUCTION

Traditional medicines and plant-based remedies are widely used in Africa. Worldwide the trend has also shifted towards the use of traditional medicines due to concerns about the costs, invasiveness and potential for toxicity of conventional mainstream remedies (Cowan, 1999). Herbal medicines are often used as primary treatment for different types of ailments in Africa and in most cases they are the only source of treatment for many people (Harnett *et al.*, 2004). Most of these herbal and traditional medicines are not well-researched, poorly regulated, may contain adulterated products, and may produce adverse effects (Mills *et al.*, 2005[a]).

Despite the above stated flaws, the use of traditional medicines by Africans is widespread and is promoted by Ministries of Health of several African nations, including South Africa. Two herbal remedies (*Hypoxis hemerocallidea* and *Sutherlandia frutescens*) are currently recommended by the South Africa Ministry of Health for management of many ailments including cancer and human immunodeficiency virus (HIV) (Mills *et al.*, 2005[a]). Both of these remedies have been shown to significantly affect enzymes (cytochrome P450 enzymes *CYP3A4, CYP17* and *CYP21*) which are important for antiretroviral metabolism and therefore have the potential for drug interactions. Therefore efforts should be made by mainstream health professionals to provide validated information to traditional healers and patients on the careful use of herbal medicines (Mills *et al.*, 2005[b]).

South Africa has a rich diversity of medicinal plants (Bessong *et al.*, 2004). Very few studies have been conducted in South Africa to assess the value and efficacy of these herbal plants that are commonly used by traditional healers for treatment of different sicknesses. Of those that have been conducted, the medicinal plants have been shown to be safe and effective in improving the health status of patients and they have warranted further research (Tshibangu *et al.*, 2004). Plants are known to be a rich source of secondary metabolites. These secondary metabolites have been shown to have *in vitro* antimicrobial and antiviral activity. Twenty five to 50% of all current pharmaceutical products are derived from plants (Cowan, 1999).

Sutherlandia frutescens (SF), a member of the *Fabaceae* family, is a traditional herb widely used by South Africans to treat a variety of ailments including gastrointestinal problems, tuberculosis, diabetes, rheumatoid arthritis, osteoarthritis, HIV and internal cancers (Mills *et al.*, 2005[a]). The recommended therapeutic dose of SF in humans is 9 mg per kg body weight per day (Seier *et al.*, 2002). It is regarded as the most profound and multi-purpose of the medicinal plants in Southern Africa. *Sutherlandia frutescens* is known for its efficacy and safety for use in many different health conditions. The *Sutherlandia* plant grows mostly in the dry, stony grassland regions of Southern Africa, especially in the Cape and some parts of KwaZulu Natal (Figure 1.1).

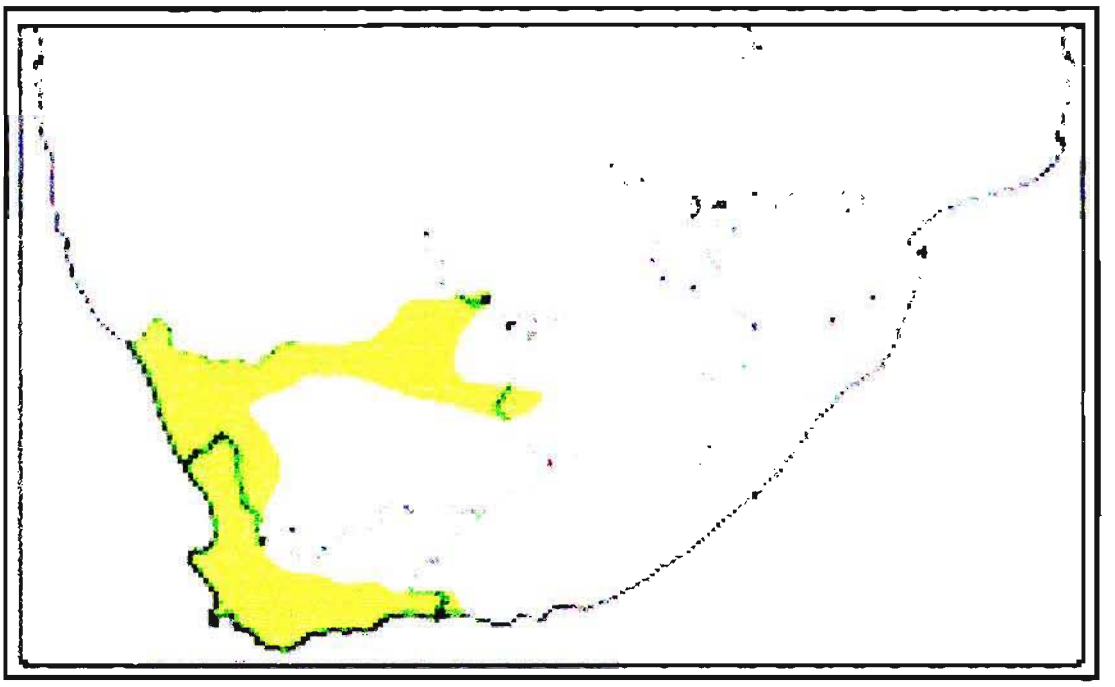


Figure 1.1: Geographical distribution of SF plant in South Africa. The plant mainly grows in the Cape region but it has also been shown to grow in other dry parts of the country like the midlands of KwaZulu Natal and the Free State (http://www.sahealthinfo.org/traditionalmeds/monographs/sutherlandia.htm).

Sutherlandia frutescens is mainly used as an immunotonic and a supplement which assists the body in mobilizing its own immune response to fight infections and cope with mental stress. It therefore acts as an adaptogenic tonic. An adaptogen refers to herbs that maintain health by increasing the body's ability to adapt to environmental and internal stress. They generally work by strengthening the immune system, nervous system and/or glandular systems (http://www.sutherlandia.org).

Strengthening of the immune response is very important in debilitating conditions such as cancer and HIV and acquired immune deficiency syndrome (AIDS). The immune system is responsible for the complex task of providing defence against a vast array of potential

pathogens, whilst ensuring that the same protective mechanisms are not turned against self. When their function is complete, the immune cells must be removed leaving only a small population of memory cells. Apoptotic mechanisms play an important role in the development, regulation and functioning of immune cells. Malfunctioning of apoptosis can cause autoimmune disease, immunodeficiencies, and lymphomas (Lowe and Lin, 2000).

Many studies have been undertaken to study the effects of this traditional herbal remedy on cancerous cell lines. To date, no study on the effects of SF extracts on the H9 T cell line as compared to normal T lymphocytes has been done. T lymphocytes are important immune cells involved in specific immune response. They are important in recognition and elimination of infected cells and are also involved in the removal of tumour cells. H9 cells are transformed cutaneous T lymphocytes which were clonally derived from a HUT 78 cell line. This cell line was acquired from a patient suffering from Sezary syndrome, a type of lymphoma. In this study tablets of SF from PhytoNova were used to determine their effects on these T cell lines. The tablets were made up of a mixture of different parts of the plant (stem, leaves, and fruits) which have all been shown to contain active ingredients. The aims of this study were:

- To determine the cytotoxicity of SF extracts in cultured H9 T-cells and isolated normal T cells.
- To determine apoptotic effects of SF extracts in cultured H9 T-cells and isolated normal T cells.
- To determine the morphological and nuclear effects of SF extracts in cultured H9 T-cells and isolated normal T cells.
- To determine the effects of SF on cytokine secretion in treated peripheral blood mononuclear cells (PBMCs).

CHAPTER 2

LITERATURE REVIEW

2.1 *Sutherlandia frutescens*

Sutherlandia frutescens (SF) (Insizwa/Unwele in Zulu, or Cancer bush) is a well known and widely used traditional medicinal plant in South Africa (Sia, 2004). *Sutherlandia frutescens* is regarded as the most profound and multi-purpose of all the medicinal plants in Southern Africa. Despite it broad spectrums of applications, not enough reports have been published about the efficacy of this plant in fighting diseases (Mills *et al*, 2005[a]).

Sutherlandia frutescens is a member of the *Fabaceae* family of pea and bean or pod-bearing plants. This family forms the second largest flowering plant family. The genus SF is closely related to *Lessertia* and some botanists consider them to form one genus. The *Lessertia* genus, which includes SF, is made up of approximately ±60 species in Africa and most of these are found in southern Africa. *Sutherlandia frutescens* has other closely related species that are often confused with it. These are *Sutherlandia montana* (mountain cancer bush), *Sutherlandia microphylla* (bitterblaar), and *Sutherlandia tomentosa* (eendjies or rooikeurtjie) (Xaba and Notten, 2003).

Sutherlandia frutescens is widely distributed throughout the dry parts of southern Africa, in the Western Cape and up the west coast towards Namibia and into Botswana. It is also found in arid regions of northern KwaZulu Natal (Xaba and Notten, 2003 and Goldblatt and Manning, 2000). *Sutherlandia frutescens* is a so-called pioneer plant in that it is able to become established in an environment where other plants do not thrive (Parceval

2.1.2 Preparation of *Sutherlandia frutescens* for medicinal use

Traditional healers usually prepare decoctions of SF according to the type of ailment pointed out by the patient. The preparation ranges from using each part of the plant alone (leaves, flowers, stems and roots) to using mixtures of these parts. Commercial preparations of SF have circumvented the need for preparing a mixture of the plant material with all the plants parts known to have active ingredients. These commercial preparations come in different forms ranging from tablets, powdered material used as a tea preparation, liquid extract preparations to gels for topical application.

2.1.3 Active constituents of *Sutherlandia frutescens*

Analyses of SF extracts have identified bioactive compounds which include L-canavanine, pinitol, GABA, flavanoids and unique triterpenoid glucosides (tentatively called SU1). The plant also contains amino acids like L-arginine, small amount of saponins but no alkaloids (van Wyk *et al.*, 1997). Gas chromatography/mass spectrometer profiling of tablet extracts of SF identified the presence of methyl paraben, propyl paraben, hexadecanoic acid, gamma sitosterol, and stigmast-4-en-3-one and other long chain alcohols. These were identified as normal compounds present in most plants (Figure 2.2) (Tai *et al.*, 2004).

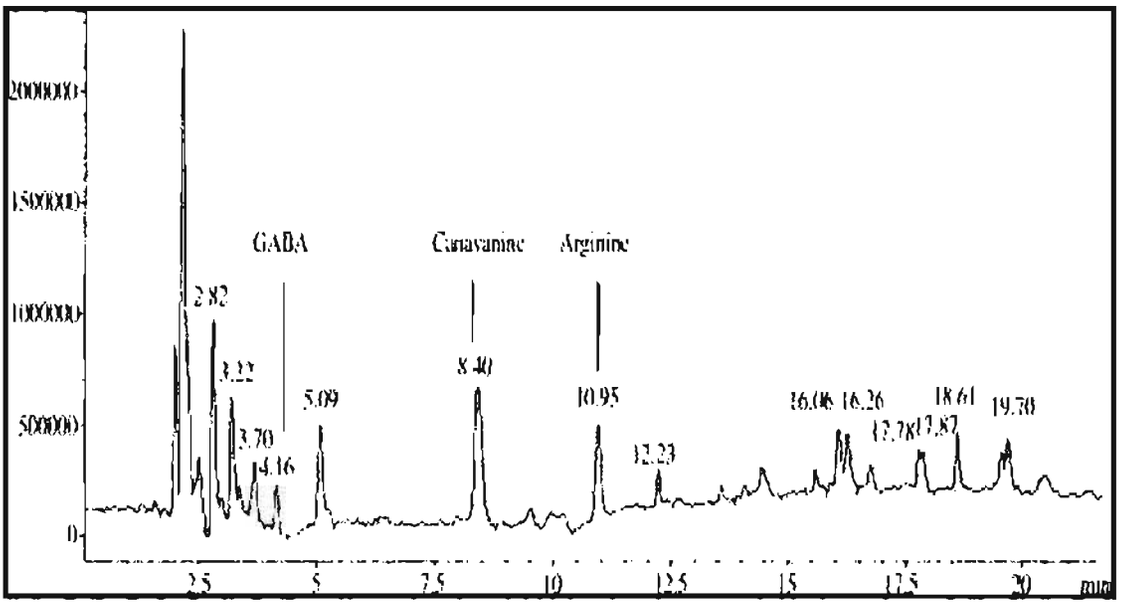


Figure 2.2: Liquid chromatographic/mass spectrometer (LC/MS) atmospheric pressure chemical ionisation (APCI) analysis chromatogram of amino acids in SF. GABA had a retention time of 4.16 minutes, canavanine- 8.40 minutes and arginine- 10.95 minutes (Tai *et al.*, 2004).

2.1.3.1 L-canavanine

L-canavanine is a non-protein amino acid that is the L-2-amino-guanidinooxy structural analogue of L-arginine (Figure 2.3 A). It is found in many leguminous plants and appears to play an important role in chemical defence of the plant. There is about 3 mg per dried gram of leaf material of SF (Tai *et al.*, 2004). L-canavanine has been shown to be toxic against microorganisms including retroviruses (including HIV), influenza, bacteria and yeast and also causes growth retardation in plant cells (Jang *et al.*, 2002; Green, 1988).

Several studies have demonstrated the anti-proliferative effects of L-canavanine on tumour cells in culture. When Jurkat T cells were treated with L-canavanine for 36 hours,

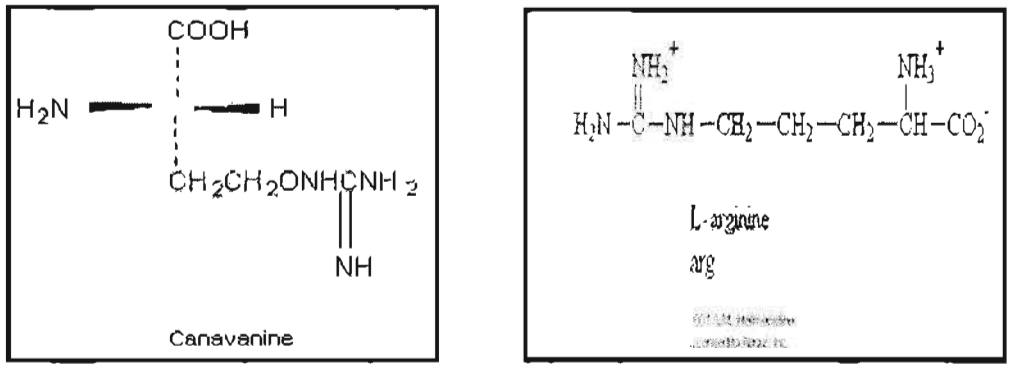
apoptotic cell death accompanying several biochemical events such as caspase-3 activation, degradation of poly (ADP-ribose) polymerase (PARP), and apoptotic deoxyribonucleic acid (DNA) fragmentation were induced in a dose-dependent manner (Jang *et al.*, 2002). A possible mechanism underlying the anti-tumour activity of L-canavanine is thought to be the incorporation of this compound into cellular proteins instead of L-arginine, and consequent formation of structurally aberrant proteins with impaired function. *In vivo*, the anti-proliferative effects of L-canavanine can result from its conversion to the arginase-mediated metabolite, L-canaline, which can disrupt biochemical pathways involving L-ornithine and the polyamines (Bence *et al.*, 2002). L-canavanine has also been shown to selectively inhibit inducible nitric oxide synthase and therefore can be used in the treatment of septic shock and chronic inflammation (Suzuki *et al.*, 2002).

2.1.3.2 L-arginine

L-arginine is a basic amino acid and because of structural similarity to L-canavanine (Figure 2.3 B), acts as antagonist to the anti-proliferative effects of this compound. There are approximately 3 mg per dried gram of leaves of L-arginine in SF (Tai *et al.*, 2004). L-arginine is particularly rich in nitrogen and is involved in a number of bodily functions. L-arginine is a precursor of nitrogen oxide which is required for the regulation of blood vessels and circulation and for the transmission of nerve impulses in neurons. It is also involved in the release of growth hormones from the pituitary gland, of insulin from the pancreas and noradrenalin from the adrenal gland (Parceval pharmaceuticals).

L-arginine has also important immune system functions. It is known to improve cellular immune responses, contributes to T lymphocyte formation and minimise their malfunction

and stimulates phagocytosis. L-arginine is also involved in the urea cycle which rids the body of excess nitrogen via urine excretion (Parceval pharmaceuticals).



A B

Figure 2.3: Structural similarities between canavanine (A) and arginine (B) make these compounds act as antagonist to each other (http//www.sutherlandia.org; www.chemistry.about.com/library/graphics/blargin.htm).

2.1.3.3 D-pinitol

D-3,O-methyl chiro inositol (D-pinitol) (Figure 2.4) is a glycan sugar found in many types of leguminous plants and is classified as a chiro-inositol. D-pinitol acts as a second messenger in a number of different metabolic processes including blood sugar metabolism (Lemar, 2002). Pinitol has also been described as being active against acute oedema in rat models (Ojewole, 2004). It is also thought to reduce the production of pro-inflammatory cytokines such as tumour necrosis factor (TNF)-α and interleukin (IL)-β (Tai *et al.*, 2004). It also has a positive effect in improving the ability of the muscles to store carbohydrates, allowing muscles to use growth whilst permitting burning of fat. This ability to increase muscle mass has strong applications to the treatment of wasting syndrome in cancer, HIV/AIDS and tuberculosis (TB) (http//www.sutherlandia.com/pinitol_frame/htm).

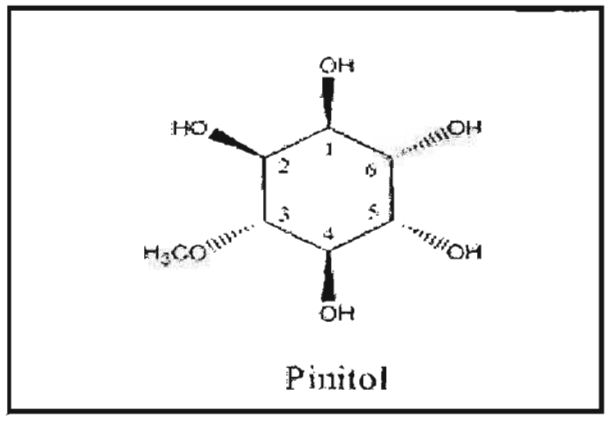


Figure 2.4: Structure of D-pinitol (Davis *et al.*, 2000).

2.1.3.4 γ Amino Butyric Acid (GABA)

Gamma amino butyric acid (GABA) is both an amino acid and an inhibitory neurotransmitter. There is about 0.4 mg per dried gram of leaves material in SF (Tai *et al.*, 2004). As an inhibitory neurotransmitter, GABA along with vitamin B_3 and inositol, acts outside the central nervous system to prevent anxiety and stress-related messages from reaching the motor centres of the brain. It has been used in the treatment of depression, manic depressive (bipolar) disorder and seizures (http//www.sutherlandia.com/GABA_frame/htm). GABA is supposed to have an anti-proliferative effect on tumour cells by inhibiting cell metastasis (Ortega, 2003). GABA, if present in a patient's circulation at sufficiently high levels, may potentially contribute to the improvement in mood and wellbeing experienced by many patients (Tai *et al.*, 2004).

2.1.3.5 Novel triterpenoid glucosides

Novel triterpenoid glucosides have been isolated and characterized in SF extracts, and are one of the key compounds used in the selection of raw material for propagation. These

compounds have promising biological activities, but this is still the subject of ongoing research (http://www.sutherlandia.org/chemistry.html). Moshe (1998) found a complex pattern of triterpenes in different species of SF, with limited variation within populations but large differences between populations. The major triterpene in commercial SF material is a cycloartane-type triterpene glycoside called SU1. Its structure has been determined as (24*R*)-25-_- d-glucopyranosyloxy-3_,7_,24-trihydroxycycloartane (Figure 2.5). At least 56 different triterpene glycosides have been detected in various species of SF and structural elucidation of the main compounds is ongoing (van Wyk and Albrecht, 2008).

The triterpenoid fraction of SF inhibited both the type 1 pregnenolone- and progesterone – induced difference spectra under LC/MS screening and elicited a type 11 difference spectrum in the absence of substrate. This therefore shows that the triterpenoid glucosides present in SF can influence the biosynthesis of glucocorticoids (Prevoo *et al.*, 2008).

Kikuchi *et al.*, (2007) studied the cancer chemo preventive activity of 48 natural and semi synthetic cycloartane-type triterpenoids. Many of the compounds (so-called astragalosides) are known from the genus *Astragalus L.*, a related species of SF. The most powerful inhibitory effects in an *in vivo* mouse skin carcinogenesis test were found in cycloartanes with hydroxylation at C-24 and with a 3-oxo group. This configuration is present in SU1 (Figure 2.5).

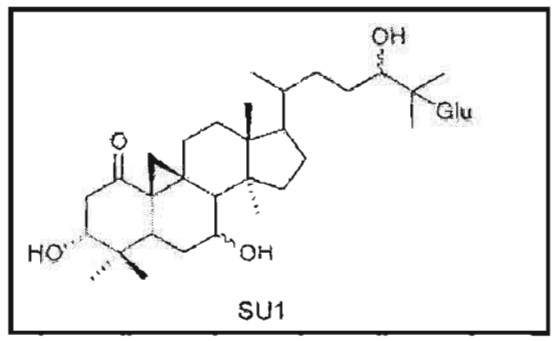


Figure 2.5: Chemical structure of triterpenoid glycosides found in *Sutherlandia frutescens* (van Wyk and Albrecht *et al.*, 2008).

2.1.4 Recommended dosage of *Sutherlandia frutescens*

The recommended therapeutic dose of SF in humans is 9 mg per kg body weight per day of the tablet preparation. That would be equal to about one 300 mg tablets taken twice a day after meals as recommended by the suppliers (Seier *et al.*, 2002).

2.1.5 Pharmacokinetics and pharmacology of *Sutherlandia frutescens*

There has been much work done to assess the pharmacokinetics of this medicinal plant. Mills *et al.*, (2005[b]) demonstrated *in vitro* effects of SF on cytochrome P450 3A4 (*CYP3A4*), pregnane X receptor (PXR), and P-gylcoprotein (P-gp). *Sutherlandia frutescens* produced near complete inhibition of *CYP* (96%) and activated the pregnane X receptor nearly twofold. P-glycoprotein expression was inhibited moderately in relative to the positive control (verapamil). Despite the use of relatively high doses, these results tentatively suggest that human consumption of SF could affect antiretroviral drug metabolism leading to bi-directional drug interactions and loss of therapeutic efficacy.

Prevoo *et al.*, (2008) investigated the interaction of SF with cytochrome P450 enzymes, *CYP17* and *CYP21*, which catalyse key reactions in glucocorticoid biosynthesis. They showed that that SF contains bioactive compounds capable of inhibiting the binding of natural substrates (pregnenolone and progesterone) and the catalytic activity of *CYP17* and *CYP21* enzymes involved in the biosynthesis of steroid hormones.

2.1.6 Toxicity of *Sutherlandia frutescens*

Sutherlandia frutescens has a relatively long history of safe usage in Africa. Minor side effects reported include occasional incidents of mild loose stool, mild constipation, dry mouth, and dizziness in cachetic patients (Seier *et al.*, 2002). A toxicology screening of SF in a primate model using dosages up to 9 times greater than the recommended dosage did not identify any clinical, haematological or physiological toxicity of this medicinal plant (MRC toxicity study, 2002). A more recent clinical trial of SF using healthy volunteers conducted by Johnson *et al.*, (2007) showed no adverse events on physical, vital, blood, and biomarker indices between the healthy treatment and placebo groups. However participants taking SF reported improved appetite compared to the placebo group. No traceable amounts of L-canavanine were found in participant plasma.

L-canavanine maybe associated with the autoimmune condition systemic lupus erythematous syndrome (SLE). Defective immune regulatory mechanisms, such as the clearance of apoptotic cells and immune complexes, are important contributors to the development of SLE (Mok and Lau, 2003). The non-protein amino acid L-canavanine can be incorporated in protein instead of arginine and may, if taken for a long time, result in autoimmunity (Prete, 1985).

2.1.7 Traditional uses of *Sutherlandia frutescens*

Sutherlandia frutescens has been and is still used in the treatment of many ailments in southern Africa. The Khoi San and Nama people were known to use the plant as a decoction for the washing of wounds and they also used it to treat internal fevers. The Cape settlers regarded it as giving successful results against chickenpox, stomach problems, and in the treatment of internal cancers (van Wyk *et al.*, 1997). It is also used to treat tuberculosis, diabetes, chronic fatigue syndrome, influenza, rheumatoid arthritis, osteoarthritis, peptic ulcers, gastritis, reflux oesophagitis, menopausal symptoms, anxiety, and clinical depression and HIV infection. It is said to be a useful bitter tonic and a little taken before meals will aid digestion and improve the appetite (Xaba and Notten, 2003; Mills *et al.*, 2005[b]; Gericke *et al.*, 2001; Dalvi, 2003).

There has not been conclusive scientific evidence that supports the use of SF for cure of these different ailments. *In vitro* studies have mainly focused on the anti-cancer properties of this plant on different tumour cell lines. There has also been no data on plant's immune stimulating abilities and the usefulness of that property in the fight against debilitating illnesses such tuberculosis, cancer and HIV/AIDS. Anecdotes have been provided from different clinical settings of how taking preparations of SF have greatly improved the well-being and $CD4^+$ counts of HIV/AIDS sufferers (Jenkins, 2005). To this end, no scientific data has supported this claim. A clinical study has been approved by the Medical Controls Councils of South Africa to look at the effects of SF on HIV/AIDS patients (Naidoo, 2007).

2.1.8 Scientific studies into the effects of *Sutherlandia frutescens*

A burgeoning volume of scientific data has been produced on the different effects of SF *in vitro* and *in vivo* settings. The *in vitro* studies have utilised cultured cell lines to ascertain the cellular and anti-proliferative effects of crude extracts of SF on tumour cells. Others have looked at the anti-inflammatory and anti-oxidant properties of the plant extracts in relation to what the plant is traditionally used for in herbal medicine. *In vivo* studies have also been conducted on rats and mice for similar reasons to the ones above. Recently a clinical trial of SF was completed in healthy adults to evaluate the safety of the plant (Johnson *et al.*, 2007)

2.1.8.1 Anti-proliferative properties of *Sutherlandia frutescens*

The effects of crude SF extracts which were prepared with 70% ethanol was investigated on cell numbers, morphology, and gene expression profiles on MCF-7 human breast adenocarcinoma cell line by Stander *et al.*, (2007). It was found that 1.5 mg/ml of the SF extract induced a statistically significant decrease in cell viability (50%) when compared to the vehicle-treated controls after 24 h exposure. Changes of cell morphology related to induction of apoptosis, i.e. cytoplasmic shrinking, membrane blebbing and apoptotic bodies, were observed after 24 h of exposure.

Aqueous extracts of SF inhibited growth of the oestrogen dependent cancer cell lines (DU-145 prostate cancer cell line, MCF-7 breast cancer cell line) and stimulated the growth of the MCF-12A non-malignant breast cell line and MDA-MB-231 breast cancer cell line (Steenkamp and Gouws, 2006). Chinkwo (2005) demonstrated that crude whole plant

extracts of SF induced cytotoxicity and apoptosis in three different cell lines (Chinese Hamster Ovary (CHO) cells, Caski cervical carcinoma cells, and Jurkat T lymphoma cells). Ethanolic extracts of commercially available SF tablets and powder preparations demonstrated a concentration dependent anti-proliferative effect of several tumour cell lines (Tai *et al.*, 2004).

2.1.8.2 Anti-oxidant and anti-inflammatory effects *Sutherlandia frutescens*

The study by Tai *et al.*, (2004) also demonstrated the anti-oxidant activity in reducing free radical cations with an estimated activity of 0.5 μl of SF extract equivalent to that of 10 μM of Trolox that is determined in a Trolox equivalent antioxidant capacity (TEAC) assay. *Sutherlandia frutescens* did not however significantly suppress lipopolysccharide stimulated nitric oxide production by murine macrophage/monocytes RAW 264.7 cells, nor did it significantly inhibit interleukin-1β and tumour necrosis factor-α expression in the same cell line.

Contrary to the finding by Tai *et al.*, (2004) in acute oedema rat models it was demonstrated that the extract of SF had a significant anti-inflammatory function (Ojewole, 2004). After an oral administration of 800 mg/ml of the extract, the animals were found to have reduced egg albumin-induced inflammation in their paws. *Sutherlandia frutescens* shoot aqueous extract (50-800 mg/kg i.p.) also produced significant ($p< 0.05$-0.001) analgesic effects against thermally- and chemically-induced pain stimuli in mice and caused significant ($p< 0.05$-0.001) hypoglycaemia in rats. Smith and Myburgh (2004) showed that warm water extract of SF is able attenuate an immobilisation stress-induced increase in plasma corticosteroid levels in rats.

Fernandes *et al.*, (2004) investigated the effects of the hot water extract of SF on both luminol and lucigenin enhanced chemiluminescence of neutrophils stimulated with L-formyl-L-methionyl-L-leucyl-phenylalanine (FMLP) as well as the superoxide and hydrogen peroxide scavenging properties in a cell free system. The results showed that *SF* exhibits significant superoxide and hydrogen peroxide scavenging activity at low concentrations. These antioxidant properties are also thought to account for some of the anti-inflammatory properties of this plant that have been described.

An investigation into the effects of methanolic extract of SF on 12-*O*-tetradecanoylphorbol-13-acetate (TPA)-induced COX-2 expression in mouse skin demonstrated that topical application of the extract inhibited TPA-induced COX-2 expression. This inhibition appeared to be mediated by blocking the catalytic activity of extracellular signal-regulated protein kinase (ERK) and inhibiting the activation of activator protein-1 (AP-1). This data supported the use of SF as an anti-tumour remedy (Kundu *et al.*, 2005).

These antioxidant and anti-inflammatory effects of SF in *in vitro* systems lends support to the use of this medicinal plant as an immune booster. The traditional use of this plant as an adaptogen also led to scientific investigations into its use in HIV/AIDS patients to boost $CD4^+$ counts. Accumulated evidence suggests that reactive oxygen species (ROS) can be scavenged through chemoprevention utilizing natural antioxidant compounds present in foods and medicinal plants.

2.2 The immune response

The immune system is an interactive network of lymphoid organs, cells, humoral factors, and cytokines. The essential function of the immune system in host defence is best illustrated when it goes wrong; underactivity resulting in the severe infections and tumours of immunodeficiency, overactivity in allergic and autoimmune disease. Immunity is divided into two parts determined by the speed and specificity of reaction, namely the innate and adaptive responses (Parkin and Cohen, 2001).

2.2.1 The innate response

The term innate is sometimes used to include physical, chemical, and microbiological barriers, but more usually encompasses neutrophils, monocytes, macrophages, complement, cytokines, and acute phase proteins of the immune system. These components provide the immediate host defence against the invading pathogen. Neutrophil recruitment forms the central feature of the early response to infection. The recruited neutrophils phagocytose the invading organisms by making pseudopodia which form a membrane-bound phagosome around the particle. Within this compartment the organism is killed either by a respiratory burst or by the oxygen-independent response which uses highly toxic proteins or enzymes (Parkin and Cohen, 2001).

Ingestion and killing of organisms is 100-fold more effective if the particle is first opsonised with specific antibody or complement. Complement binds to neutrophil Fc and C receptors, increasing adhesion between particle and phagocyte. This primes the cell for activation. Eosinophils play a major role in protection of the host from parasitic infections.

Such infections induce antigen-specific immunoglobulin-E (IgE) production, the antibodies coating the organism. Mast cells and basophils are involved in some of the most severe immunological reactions, such as angioedema and anaphylaxis. Natural killer cells have the morphology of lymphocytes but do not bear a specific antigen receptor (Parkin and Cohen, 2001).

The innate immune system basically functions as an immediate host defence mechanism which deploys a limited number of receptors with specificity for conserved microbial structures. Recognition of these structures induces co-stimulators, cytokines, and chemokines which recruit and activate antigen-specific lymphocytes and the adaptive immune response (Medzhitov and Janeway Jr, 2000).

2.2.2 Specific immunity

Specific or adaptive immunity is the hallmark of the immune system of the higher animals. This response consists of antigen-specific reactions through T lymphocytes and B lymphocytes (Figure 2.5). In contrast to the innate response which is rapid, adaptive immunity takes several days or weeks to develop and it is specific. The adaptive response has memory, so that subsequent exposure leads to a more vigorous and rapid response (Parkin and Cohen, 2001).

The characteristic of the specific immune response is the use of the antigen-specific receptors on T and B lymphocytes to drive targeted effector responses in two stages. Firstly within the lymphoid tissue, the antigen is presented to and recognised by the antigen specific T or B cell leading to cell priming, activation, and differentiation. This is

then followed by the effector response taking place, either due to the activated T cells leaving the lymphoid tissue and homing to the disease site, or due to the release of antibody from the activated B cells (plasma cells) into blood and tissues fluid, and thence to the infection area (Figure 2.6) (Parkin and Cohen, 2001).

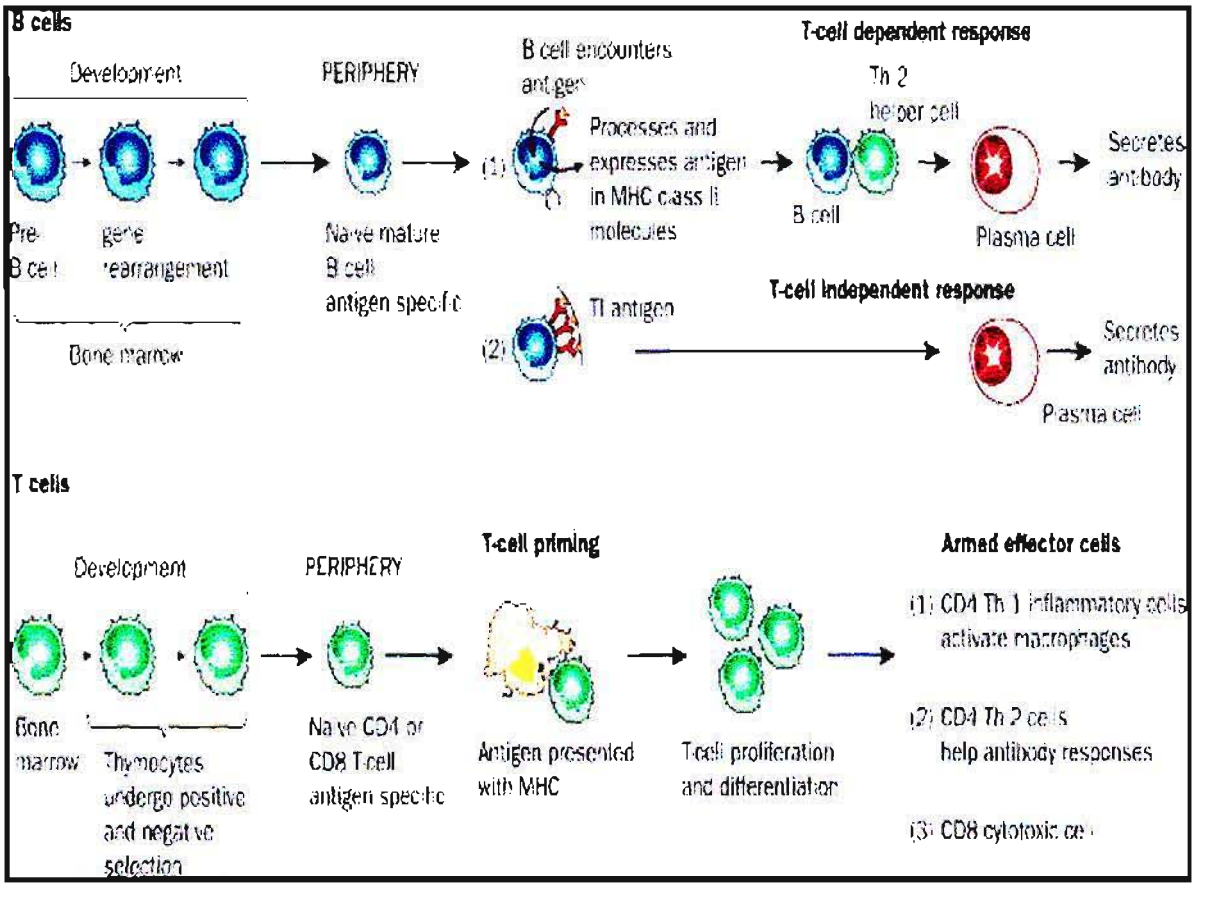


Figure 2.6: The role of T and B lymphocytes in the adaptive immune response (Parkin and Cohen, 2001).

Development of lymphocytes from progenitor cells takes place within the bone marrow. B cells remain within the marrow for the duration of their development while the T cells migrate to the thymus at an early stage as thymocytes. The cells that emerge from the thymus and bone marrow have undergone gene rearrangement are naïve- i.e. they have not

encountered their specific antigens. The lymphoid tissues provide the microenvironment for this activation. In addition to T and B lymphocytes, they contain efficient antigen-presenting cells and are able to produce the cytokines necessary to maintain T and B lymphocytes (Parkin and Cohen, 2001).

2.2.2.1 B lymphocytes

B cells produce antibody which serves to neutralise toxins, prevents organisms adhering to mucosal surfaces, activates complement, opsonises bacteria for phagocytosis, and sensitises tumour and infected cells for antibody dependent cytotoxic attack by killer cells. In a way, B cell antibodies act to enhance elements of the innate response (Parkin and Cohen, 2001).

2.2.2.2 T lymphocytes

In the development of T lymphocytes, the delicate process of positive selection of T cells that can react with self-MHC and peptide adequately to induce immune responses, but are not excessively MHC-reactive to cause self-tissue destruction occurs in the thymus (Figure 2.7).

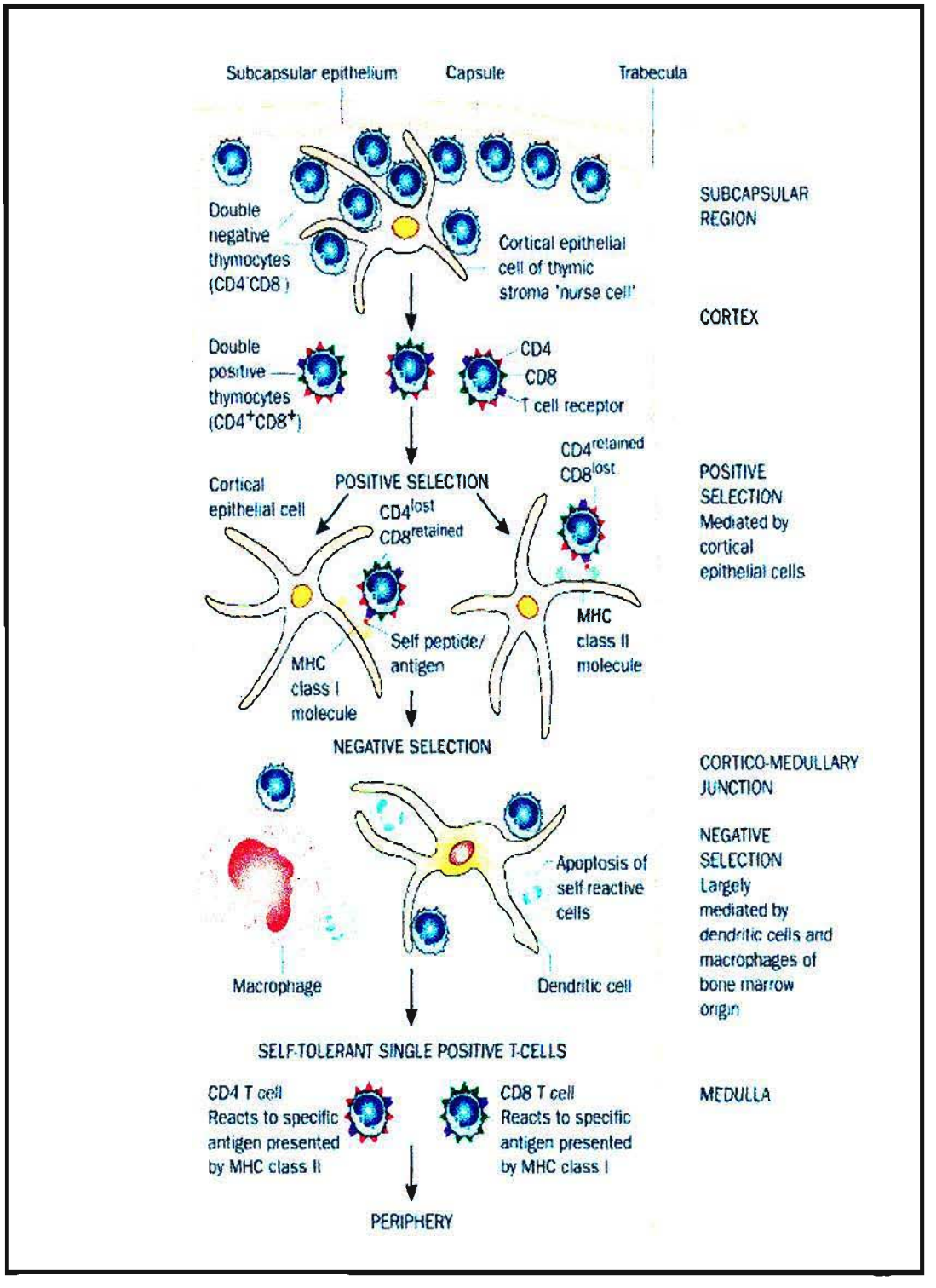


Figure 2.7: The development of T lymphocytes in the thymus (Parkin and Cohen, 2001).

Once activated, the T lymphocytes function to attack host cells that have been infected by viruses or microorganisms, cross-reactive transplanted tissues and tumour cells. They also produce cytokines which are chemical mediators that play a key regulatory role in the immune response. Since T lymphocytes are supposed to physically contact foreign or infected cells in order to destroy them, they are said to provide a cell-mediated immune response (Roitt *et al.*, 1993).

Antigen recognition by the T cell receptor is different for CD4+ and CD8+ cells. CD4 lymphocytes only recognise antigen presented with MHC class II and CD8 cells with MHC class I. T cells can also be differentiated into two populations depending on whether they use an α/β or γ/δ type of antigen receptor. Some of the activated T cells remain within the lymph nodes as central memory cells. Memory cells may live for up to ten years or more. They react more quickly on subsequent exposure because the log phase for their cell division is short (12 hours) and they have a longer lifespan due to decreased apoptosis (Parkin and Cohen, 2001). Different populations of effector T lymphocytes have different functions:

- T helper CD4+ cells
    - Th cells are subdivided functionally by the type of cytokines they produce. Precursor Th 0 lymphocytes become either Th 1 or Th 2 cells on stimulation. Th 1 and Th 2 cells are morphologically indistinguishable but only differ in the cytokines and response they produce. Th 1 cells produce IL 2, which induces T cell proliferation (including CD4+ cells in an autocrine response). Interleukin 2 stimulates CD8+ T cell division and cytotoxicity by decreasing the activation thresholds. Th 1 cells also produce

interferon γ which activates macrophages to kill intracellular pathogens and induces natural killer cells to cytotoxicity. Interleukin 12 secreted by the interferon-γ-(IFN γ)-stimulated macrophages further increases interferon γ production by T cells. A Th 1 response is essential to the host to control the replication of intracellular pathogens, but possibly contributes to the pathogenesis of autoimmune disease (Parkin and Cohen, 2001).

- T helper 2 cells in turn produce IL 4, 5, 6, and 10 that favour antibody production. IL 4 induces class-switching on B cells to IgE production and IL 5 promotes the growth of eosinophils. Interleukin 4 provides positive feedback to induce further Th 2 responses and suppress Th 1 differentiation. Thus the Th 2 response is associated with allergic reactions (Parkin and Cohen, 2001).

- T cytotoxic (CD8+) cells
  - These cells are directly cytotoxic to target cells bearing their specific antigen. They bind to the target cell and insert perforins into the cell membranes. They then release granules containing granzymes into the target cytoplasm. The granzymes activate caspase enzymes that induce DNA fragmentation and cell apoptosis. Some CD8+ T cells also have a suppressor (T_s) function in down regulating lymphocyte responses (Parkin and Cohen, 2001).

2.3 Apoptosis

Apoptosis, or programmed cell death, is a key event in biological homeostasis but is also involved in potentiation of human diseases (Ahr *et al.*, 2004). As important as cell division, programmed cell death allows the shaping of the organism during development and tightly controls cell number and tissue homeostasis in adult life (Ferraro-Peyret *et al.*, 2002) Apoptosis is a tightly controlled process in which cell death is executed through the activation of specific signalling pathways. Within cells, there are positive and negative regulatory pathways of apoptosis, and it is the balance between these pathways that determines cell fate (Pulido and Parrish, 2003).

The coordinated structural changes that make up the process of apoptosis are driven by a set of molecular interactions, sometimes known as terminal effector events. Cells that are undergoing apoptosis do so in a well-described pattern that is different from that of cells that are dying from necrosis due to physical injury, for example (DiBartolomies and Moné, 2003). That pattern includes cell shrinkage; development of bubble-like blebs on the surface; ROS formation, caspase activation; chromatin condensation and DNA fragmentation; mitochondrial depolarisation; phosphotidylserine externalisation; and finally phagocytosis of the cell fragments by macrophages and dendritic cells. Phagocytosis of apoptotic bodies is necessary to prevent induction of inflammation. Since apoptosis plays such an important role in processes of development, immune response, morphogenesis, and pathophysiology, its disruption can have severe consequences to the organism (Pulido and Parrish, 2003).

The importance of apoptosis is also underlined by the different regulatory mechanisms which are in place to safeguard its execution. These include death receptors, caspases, mitochondria, Bcl-2, and tumour-suppressor genes (Pulido and Parrish, 2003). The process of death is the result of an interaction between initiating stimuli, which can be physiological or the result of injury in various parts of the cell, and factors that determine the susceptibility of the cell to activation of the terminal effector events. These pathways can either be a response to receptor-mediated (e.g. Fas, TNF) or non-receptor-mediated (e.g. DNA damage, ultraviolet (UV) irradiation) signals (Alimonti *et al.*, 2003). Both the intracellular and extracellular pathways of apoptosis induction involve the transduction of signals which ultimately lead to activation of caspases but there are other pathways of apoptosis which do not involve activation of caspases that have been identified (Figure 2.8).

2.3.1 Extracellular activation of apoptosis

The extrinsic pathway involves the interaction of external signals or ligands with a receptor at the plasma membrane which initiates a cascade of events that leads to apoptosis. The "death receptors" of the TNFR family include TNFR1, Fas (CD95), DR3/WSL, and the TNF-related apoptosis-inducing ligand (TRAIL)/Apo-2L receptors (TRAIL-R1/DR4, TRAIL-R2/DR5). Members of this family are characterized by two to five copies of cysteine-rich extracellular repeats and an intracellular "death domain". Once a ligand binds to the receptor, an interaction occurs with death domain adaptor proteins such as FADD, RIP, Daxx, and TRADD (Figure 2.8) (Pulido and Parrish, 2003; Zimmerman *et al.*, 2001).

These proteins activate caspases and signalling pathways such mitogen-activated protein (MAP) kinase and nuclear factor (NF)-*k*B. Activation of kinase signalling pathways results in gene expression through (NF-*k*B) and/or activator protein-1 and inhibition of apoptosis. Activation of initiator caspases can lead to a caspase cascade which amplifies the apoptotic signal. The caspase cascade is regulated by a variety of proteins that acts as both cofactors (APAF-1) and inhibitors (FLIP and IAP) (Pulido and Parrish, 2003; Zimmerman *et al.*, 2001).

2.3.2 Intracellular activation of apoptosis

The intracellular pathway of apoptosis is initiated within the cell, most often through the disruption of cellular homeostasis. The mitochondria play a pivotal role in cell homeostasis and the disruption of the mitochondrial transmembrane potential is a vital initial event leading to apoptosis. Growth factors, cytokines and DNA damage appear to signal cell death through the mitochondria (Figure 2.8). The mitochondrial pathway of apoptosis is also a target of oncogenic mutations. These diverse signals affect the function of B-cell lymphoma (Bcl)-2 family members which, in turn, can modulate the mitochondrial transmembrane potential (Pulido and Parrish, 2003; Lowe and Lin, 2000).

The instability of the mitochondrial membrane results in the release of cytochrome *c* which binds to apoptotic activating factor-1 (APAF-1). Recently, a new protein with the dual name Smac/DIABLO has been discovered, which is released from the mitochondria along with cytochrome *c* during apoptosis. While cytochrome *c* forms an apoptosome with APAF-1 and procaspase 9 leading to caspase activation, Smac/DAIBLO functions to promote caspase activation by associating with the apoptosome and by inhibiting IAPs. It

has been shown that cytochrome *c* and Smac/DIABLO are released co-ordinately (Zimmerman *et al.*, 2001).

Mitochondrial-induced apoptosis may also involve a caspase-independent pathway. A change in the mitochondrial transmembrane potential may cause the release of apoptosis-inducing factor (AIF), which activates a deoxyribonuclease (DNase) leading to apoptosis (Pulido and Parrish, 2003). Endonuclease G has been identified as one of the proteins released along cytochrome *c* during apoptosis. It is a nuclease involved in mitochondrial DNA replication but when released from the mitochondria, it translocates to the nuclease where it induces DNA fragmentation independent of caspase activation (van Loo *et al.*, 2001).

Mitochondrial membrane permeability is regulated through the Bcl-2 family of proto-oncogenes. Within this family there are proto-oncogenes which are anti-apoptotic (Bcl-2) and those which are pro-apoptotic (Bad, Bax). Activated pro-apoptotic proto-oncogenes act to increase mitochondrial membrane permeability, thereby promoting apoptosis. The anti-apoptotic protein, Bcl-2, acts to inhibit the ability of Bax or Bad to increase membrane potential and the balance of these proteins normally determines cell fate (Figure 2.8) (Pulido and Parrish, 2003).

Other important factors involved in intracellular activation of apoptosis include excessive generation of reactive oxygen species (ROS) and tumour-suppressor genes. Excess ROS are involved in disruption of mitochondrial membrane potential leading to cytochrome *c* release and caspase cascade activation (see 2.3.4 below for detailed discussion). Activated tumour-suppressor genes like p53 respond to DNA damage by regulating gene expression.

This regulation of gene expression can suppress Bcl-2 activity and up-regulation of several genes that contribute to apoptosis, such as Bax or Bad (Pulido and Parrish, 2003).

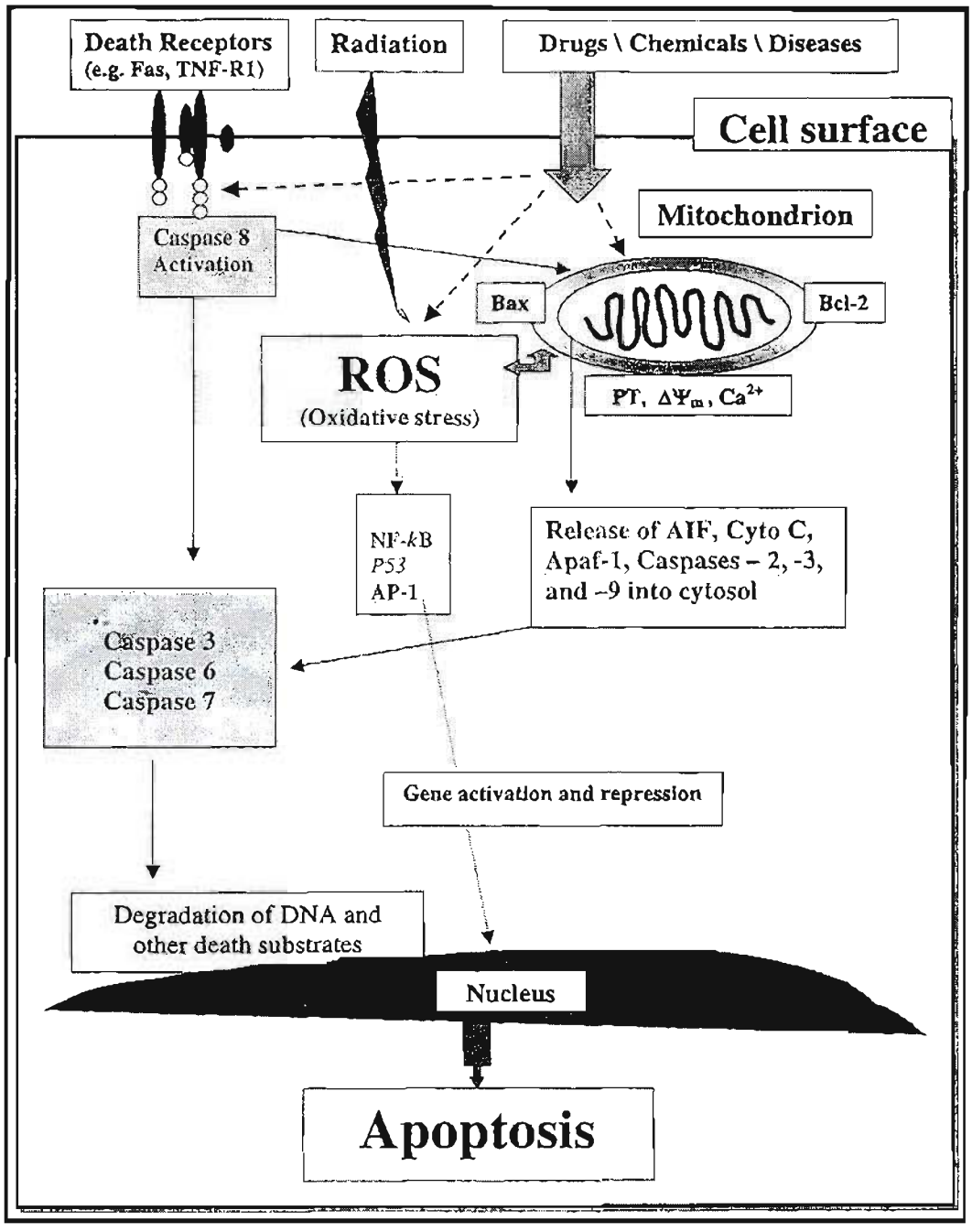


Figure 2.8: An overview of the apoptotic pathways starting from initiation of apoptosis externally or internally to activation of caspases and finally to cell degradation (Kannan and Jain, 2000).

2.3.3 Role of caspase activity in the apoptotic pathway

Caspases are aspartate-specific cysteine proteases which are found in the cytoplasm as proenzymes. The caspase gene family contains 14 mammalian members, of which, 11 human enzymes are known. The gene family is composed of two major subfamilies that are related to either ICE (caspase-1; inflammation group) or to the mammalian counterparts of ced-3 (apoptosis group). Caspases are activated to fully functional proteases by two cleavage events. The first proteolytic cleavage divides the chain into large and small caspase subunits, and a second cleavage removes the N-terminal prodomain. The active caspase is a tetramer of two large and two small subunits, with two active sites (Zimmerman *et al.*, 2001).

Based on their substrate specificity and function, caspases can be organised into initiator and executioner caspases. The self-activating caspases are generally known as initiators (i.e. caspases-8, -9, and -10), and they function to initiate and amplify a death signal. The other caspases are activated by other caspases and are known as effectors or executioner caspases (i.e. caspases-2, -3, -6, and -7). Activation of effector caspases causes the degradation of DNA, the nuclear lamina, the cytoskeleton, and other critical components of cellular integrity (Pulido and Parrish, 2003; DiBartolomies and Moné', 2003).

2.3.4 Oxidative stress and apoptosis

Under normal conditions, aerobic cells are enabled with extensive antioxidant defence mechanisms to counteract excessive ROS. When pro-oxidants overwhelm antioxidants, oxidative stress occurs. Reactive oxygen species have been shown to be both beneficial

and deleterious. At low concentration, ROS act as second messengers in signal transduction pathways. Excess amounts of ROS, however, can cause extensive oxidative damage to many vital components of the cell. The paradoxical role of ROS in physiological events can be illustrated by the role of superoxide radicals in the immune response. It is universally accepted that the production of superoxide radical by activated neutrophils and other phagocytes is an essential component of immunological defence mechanisms against bacteria. Many diseases results from unregulated changes in ROS levels (Table 2.1) (Kannan and Jain, 2000).

The mitochondria are a source of ROS and a target of excessive ROS generation. Excess ROS increases the mitochondrial membrane permeability and damages the respiratory chain resulting in increased ROS production (Pulido and Parrish, 2003). Damage to the mitochondrial membrane not only causes release of apoptosis-inducing cytochrome *c*, it also dramatically depletes the cellular energy supply. These effects potentiate occurrence of cell death either by necrosis or apoptosis (Kannan and Jain, 2000).

Reactive oxygen species, especially hydroxyl radicals, can react with all biological macromolecules, i.e. lipids, proteins, nucleic acids, and carbohydrates. The initial reaction generates a second radical, which reacts with a second macromolecule and this chain reaction is continued to amplify the ROS effect. Among the more susceptible targets of ROS are polyunsaturated fatty acids of membrane phospholipids. The lipid radicals generated during the early encounter with an oxidant add molecular oxygen to produce lipid dioxyl radical. This pro-oxidant reacts with an allelic hydrogen from another unsaturated side chain producing a lipid hydroperoxide (LOOH) and thus propagating the

chain reaction. Lipid peroxidation is an important biological consequence of oxidative cellular damage and aging (Kannan and Jain, 2000).

Table 2.1: Diseases that are influenced by oxidative stress or apoptosis (adapted from Kannan and Jain, 2000).

Neurodegenerative diseases (Alzheimer's, Parkinson's)
Autoimmune disease (e.g. rheumatoid arthritis)
Human immunodeficiency virus (AIDS)
Diabetes mellitus
Cancers of lung, colon, breast and others
Alcohol induced liver disease
Hepatitis-C induced liver disease
Ischemic reperfusion damage-heart, liver

2.3.5 Antioxidants, immune response and cell death

An antioxidant can be defined as a compound that inhibits or delays the oxidation of substrates even if the compound is present in a significantly lower concentration than the oxidized substrate. Mechanism of action may include the scavenging of ROS. Others include the prevention of ROS formation by metal binding or enzyme inhibition. Chain breaking antioxidants prevent damage by interfering with the free radical propagation cascades. The antioxidant compounds are usually recyclable in the cell or are irreversibly damaged, but their oxidation products are less harmful or can be further degraded to harmless substances in downstream processing. At the cellular and organism level the

antioxidant protection is provided by numerous enzymes and endogenous small molecular weight antioxidants such as ascorbic acid, uric acid glutathione, tocopherols and several others (Matkowski, 2008).

Over the past two decades, an expanding body of evidence from epidemiological and laboratory studies have demonstrated that some edible plants as a whole, or their identified ingredients with antioxidant properties have substantial protective effects on human carcinogenesis (Atawodi, 2004). The antioxidant activity of plant secondary metabolites has been widely established in *in vitro* systems. Medicinal compounds are of particular interest and much effort has been devoted to obtaining some of the most active and precious therapeutics. Numerous alkaloids, saponins, cardenolides, anthraquinones, polyphenols and terpenes have been reported from *in vitro* cultures (Matkowski, 2008).

Many of the protective functions of immune cells depend on the fluidity of the membranes of the cell. As the concentration of polyunsaturated fatty acids in the membranes is increased, the potential for membrane lipid peroxidation mediated by free radicals also is increased. Lipid peroxidation decreases membrane fluidity, which adversely affects immune responses. The degree of unsaturation of the lipids incorporated into membranes alters the exposure of membrane receptors and their activities (Bendich, 1993).

Oxidative stress may be detrimental in acquired immunity by activation of nuclear factor kappa B, which governs gene expression involving various cytokines, chemokines, and cell adhesion molecules, among others. However, antioxidant supplementation essentially reverses several immune deficiencies, resulting in increased levels of interleukin-2, elevated numbers of total lymphocytes and T-cell subsets, enhanced mitogen

responsiveness, increased killer cell activity, augmented antibody response to antigen stimulation, decreased lipid peroxidation, and decreased prostaglandin synthesis (Knight, 2000).

One of the most important antioxidants in the immune response is glutathione. Reduced glutathione (*y*-glutamyl-cysteinyl-glycine; GSH) is the most abundant low-molecular weight thiol in animal cells and is involved in many cellular processes including antioxidant defence, drug detoxification, cell signalling, and cell proliferation. Other novel roles of GSH include gene expression, protein glutathioylation, and nitric oxide (NO) metabolism (Table 2.2). The synthesis of GSH from glutamate, cysteine, and glycine is catalyzed sequentially by two cytosolic enzymes, *y*-glutamylcysteine synthetase and GSH synthetase. The synthesis of GSH is regulated primarily by *y*-glutamylcysteine synthetase activity, availability of cysteine, and GSH feedback inhibition. Glutathione can be transported out of cells via a carrier-dependent facilitated mechanism into plasma. Plasma GSH originates mainly from the liver but some of it can come from the diet. The interorgan metabolism of GSH functions to transport cysteine in a nontoxic form between tissues, and also helps maintain intracellular GSH concentrations and redox state (Wu *et al.*, 2004; Franco *et al.*, 2007; Armstrong *et al.*, 2002).

Adequate GSH concentrations are necessary for the proliferation of cells, including lymphocytes. Glutathione is also essential for activation of T-lymphocytes and polymorphonuclear leukocytes as well as for cytokine production, and therefore for mounting successful immune responses when the host is immunologically challenged. A shift in the GSH/glutathione disulphide (GSSG) redox state toward the oxidizing state therefore activates several signalling pathways which reduce cell proliferation and increase

apoptosis. Thus, oxidative stress plays a key role in the pathogenesis of many diseases, including cancer, inflammation, Alzheimer's and Parkinson diseases, HIV/AIDS, and diabetes (Wu *et al.*, 2004).

Table 2.2: Roles of glutathione in animals (adapted from Wu *et al.*, 2004)

Antioxidant defence
Scavenging free radicals and other reactive species
Removing hydrogen and lipid peroxides
Preventing oxidation of biomolecules
Metabolism
Synthesis of leukotrienes and prostaglandins
Conversion of formaldehyde to formate
Production of D-lactate from methylglyoxal
Formation of mercapturates from electrophiles
Formation of glutathione-NO adduct
Storage and transport of cysteine
Regulation
Intracellular redox status
Signal transduction and gene expression
DNA and protein synthesis, and proteolysis
Cell proliferation and apoptosis
Cytokine production and immune response
Protein glutathioylation
Mitochondrial function and integrity

2.3.6 Apoptosis and T lymphocytes regulation

Apoptosis has been shown to play a role in regulating the type of T lymphocytes released into circulation. In the thymus immature T lymphocytes are selected based on their ability to recognise self-antigens displayed by dendritic cells. Immature lymphocytes that lack the antigens receptors allowing then to respond to self-antigens escape the thymus, while self-reactive lymphocytes receive signals to undergo apoptosis. Several million precursor T-lymphocytes enter the thymus daily, with only 1 to 2% leaving to become function T-lymphocytes (DiBartolomies and Moné, 2003).

2.4 Apoptosis versus Necrosis

Apoptosis is characterised by chromatin condensation, nuclear fragmentation and formation of apoptotic bodies. These apoptotic bodies are eventually phagocytosed by macrophages. Apoptosis is tightly regulated by molecular mechanisms that appear to be evolutionary conserved and is therefore thought to be an active process in removing unnecessary cells. In most but not all forms of apoptosis, nuclear DNA is cleaved at internucleosomal sites. Apoptotic cells appear shrunken with membrane blebbing (Tsujimoto, 1997).

Necrosis (also referred to as accidental cell death) refers to the morphology most often seen when cells die from severe and sudden injury, such ischemia, sustained hyperthermia, or physical or chemical trauma. In necrosis there are early changes in mitochondrial shape and function, and the cell rapidly becomes unable to maintain homeostasis. The plasma membrane becomes the major site of damage, causing it to lose its ability to regulate

osmotic pressure which leads to cell swelling and rupture. Many of the substances released, such as prostaglandins and thromboplastin, are inducers of inflammation. Because cell injury of this type is often extensive, the inflammatory response is desirable, so that debris may efficiently be cleared away and the process of repair begins (Cohen, 1993; DiBartolomies and Moné, 2003).

In pathological regions or when cells are subjected to pathological reagents, it has often been observed that apoptotic cells also appear, indicating that accidental cell death can also progress by apoptosis. Furthermore, dying cells with non-apoptotic morphology are observed during naturally occurring cell death. Thus, apoptosis and some forms of necrosis must share common steps (Figure 2.9). Intracellular ATP levels have been implicated both *in vitro* and *in vivo* as a determinant of the cell's decision to die by apoptosis or necrosis. As apoptosis is an active process, it very much an ATP-dependent process while necrosis is ATP-independent (Tsujimoto, 1997).

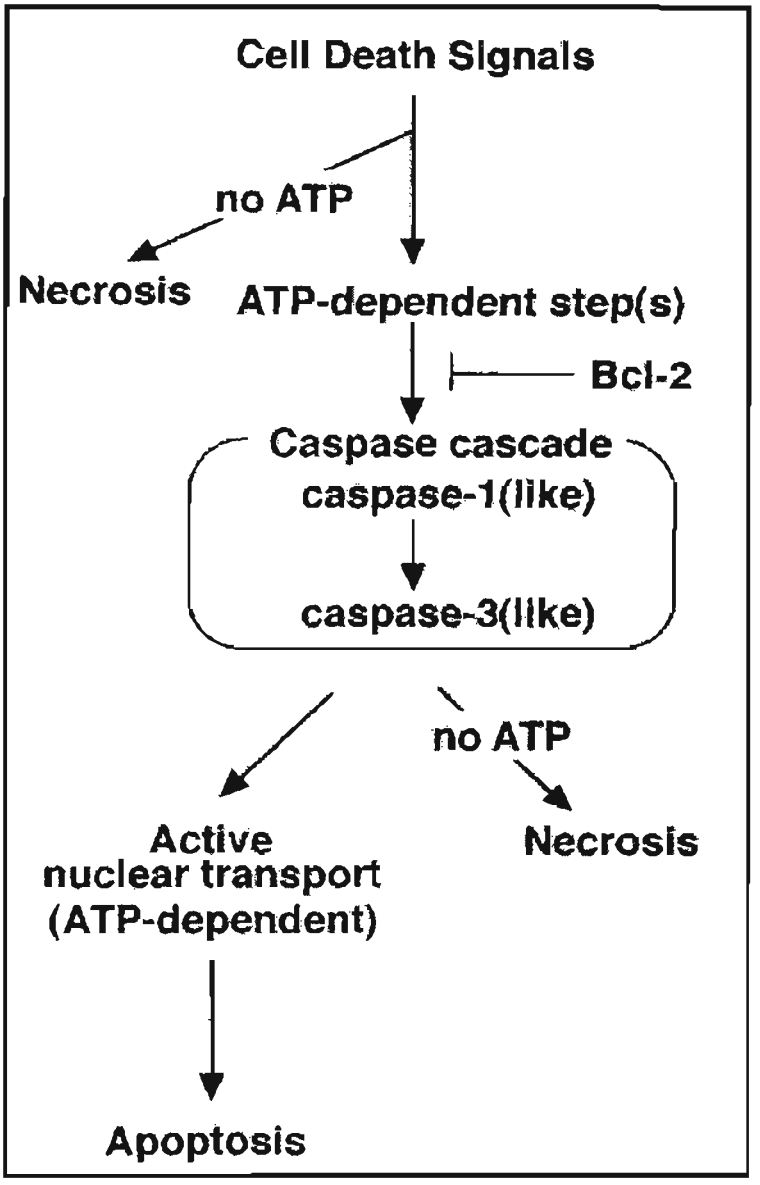


Figure 2.9: Diagram illustrating that apoptosis and some forms necrosis share a common signal transduction pathway (Tsujimoto, 1997).

2.5 Apoptosis and Cancer

The realization that apoptosis is a gene-directed program and maybe as important as mitosis has been significant for our understanding of developmental biology and tissue homeostasis. This genetic basis of apoptosis implies that cell death, like any other

metabolic or developmental program, can be disrupted by mutation (Lowe and Lin, 2000). In fact, defects in apoptotic pathways are now thought to contribute to a number of human diseases, ranging from neurodegenerative disorders to malignancy (Thompson, 1995). This has also led to the realization that apoptosis and the genes that control it have a reflective effect on the malignant phenotype. For one it is clear that some oncogenic mutations disrupt apoptosis leading to tumour initiation, migration and metastasis. Furthermore, compelling evidence points to other oncogenic mutations promoting apoptosis, thereby producing selective pressure to override apoptosis during multistage carcinogenesis (Lowe and Lin, 2000).

The evolution of the normal cell to a malignant one involves processes by which genes involved in normal homeostatic machineries that control proliferation and cell death suffer mutational damage which results in the activation of genes stimulating proliferation or protection against cell death, the oncogenes (e.g. Bcl-2), and the inactivation of genes which would normally inhibit proliferation, the tumour suppressor genes (e.g. phosphor-protein 53 (p53)) (Bertram, 2001). This formation of cancerous cells can result from sequential acquisition of mutations which arise as a consequence of damage to the genome. Mutations can be a result of errors in replication of DNA, the intrinsic chemical instability of certain DNA bases or from attack by free radicals generated during metabolism.

The immune response to a foreign invader involves the proliferation of lymphocytes (T and/or B cells). The immune system is responsible for the complex task of providing defence against a vast array of potential pathogens, whilst ensuring that the same protective mechanisms are not turned against self. When their job is complete, they must be removed leaving only a small population of memory cells. Apoptotic mechanisms play an important

role in the development, regulation and functioning of immune cells. Malfunctioning of apoptosis can cause autoimmune disease, immunodeficiencies, and lymphomas. Some B-cell leukaemia's and lymphomas express high levels of Bcl-2, thus blocking apoptotic signals they may receive. The high levels result from a translocation of the Bcl-2 gene into an enhancer region for antibody production. It is well documented that most cytotoxic anticancer agents induce apoptosis, raising the intriguing possibility that defects in apoptotic contribute to treatment failure (Lowe and Lin, 2000).

2.5.1 Apoptosis and cancer therapy

Most anticancer agents now in use were developed using empirical screens designed to identify agents that selectively kill tumour cells. It is now well established that anticancer agents induce apoptosis, and that disruption of apoptotic programs can reduce treatment sensitivity. An example of this disruption is seen in agents targeting p53 which become ineffective if p53 is lost or mutated. Other researchers have shown that p53 is not strictly required for drug-induced cell death. Indeed, at sufficient doses virtually all anticancer agents induce apoptosis and other forms of cell death independently of p53. The only thing unclear is the extent to which apoptosis contributes to treatment sensitivity in carcinomas (Lowe and Lin, 2000).

Anticancer agents have also been shown to induce apoptosis in normal tissues as well as in tumours. A link between apoptosis in tumours and that occurring in normal tissue has been suggested to contribute to the toxicity of chemotherapy (Lowe and Lin, 2000). This link has shown the need for an anticancer agent which will be selective for tumours while

sparing normal tissues, unlike chemotherapy and radiation which cause loss of normal cells.

2.5.2 Cancer, cytokines and the immune system

Cytokines are secreted or membrane-bound proteins that regulate the growth, differentiation and activation of immune cells. They also control immune cell trafficking and the cellular management of immune organs. Therefore, any disturbance in the regulation of cytokine production is thought to be one of the important factors in the development of diseases. Disorders such as HIV/AIDS, autoimmune disorders and cancer are known to be some of the diseases affected by cytokine dysregulation (Salazar-Onfray *et al.*, 2007; Borish and Steinke, 2003).

Tumour initiation and growth can be indirectly influenced by cytokines through their assorted immune activities. The role of cytokines in modulating the tumourogenesis is mediated by their ability to regulate antigen-specific anti-tumour responses and by the activation of non-specific mechanisms, including those involved in the processes of inflammation and innate responses. Cytokines can influence the growth of tumours by acting directly on tumour cells as growth promoters, growth inhibitors or indirectly by attracting inflammatory cell types and affecting angiogenesis (Salazar-Onfray *et al.*, 2007).

2.6 Conclusion

A search through the literature has demonstrated the lack of information with regards to the effects of SF on the immune system. This has given this study added importance as this plant is purported as an immune booster. A fully functional immune response is very important when dealing with diseases such as cancer, HIV/AIDS and other infections which are traditionally treated with SF. Cancer is a debilitating disease in which an individual's cells divide uncontrollable. Induction of apoptosis in cancerous cells has been shown to be an important process which is disrupted in carcinogenesis. Conventionally chemotherapy and radiation have been shown to induce cell death in both tumours and normal tissues. Therefore if SF is shown to be able to induce apoptosis only in transformed cancerous cells while sparing normal cells, it would have an important role in cancer treatment. Added to that, if SF can also be shown to protect normal T cells and cause their proliferation, it would also have a place in fighting many other infections in which the immune system is compromised.

CHAPTER 3

TISSUE CULTURE PROTOCOLS

3.1 Cell Culture

Tissue culture is a general term for the removal of cells, tissues, or organ from an animal or plant and their subsequent placement into an artificial environment conducive to growth (Ryan, 2008). Tissue culture can also be described as a method of keeping tissue alive and growing *in vitro* in a tissue culture media containing a rich mixture of essential amino acids, vitamins, and peptides, thus replicating conditions in which the tissue normally grows in an animal or human. The technique of culturing cells outside the organism may be used for a variety of cells provided the *in vitro* environment in which they are propagated replicates the *in vivo* system of the organism (Terse *et al.*, 1993).

There are two main ways of obtaining cell cultures. When cells or cultures are derived directly from excised, normal animal tissue and cultured either as an explant culture or following dissociation into a single cell suspension by enzyme digestion, they are called primary cultures. The preparation of primary cultures is labour intensive and they can be maintained *in vitro* only for a limited period of time. Continuous cultures, on the other hand, are comprised of a single cell type that can be serially propagated in culture either for a limited number of cell divisions (approximately thirty) or otherwise indefinitely. They have this ability because they have been transformed into tumour cells or were obtained from clinical tumours. Transformed cell lines present the advantage of almost limitless availability, but the disadvantage of having retained very little of the original *in*

vivo characteristics (Freshney, 1987; European Collection of Cell Culture (ECACC) Handbook, 2006).

Cell lines derived from clinical human tumours have been crucial to building our understanding of the molecular pathophysiology of cancer and its treatment. They are also as important in forming an *in vitro* model system for rational drug discovery and development. This is because they are easy to maintain and manipulate *in vitro*. However, monolayers are generally poor at reflecting the *in vivo* sensitivity of the parent histology to classic chemotherapeutics (Greshock *et al.*, 2007). In this study, H9 lymphoma cells and normal T lymphocytes were used to study the effects of SF extract dilutions on cancerous and normal immune cells. H9 cells are transformed cutaneous T lymphocytes which were clonally derived from a HUT 78 cell line. This cell line was acquired from a patient suffering from Sezary syndrome, a type of lymphoma (Mann *et al.*, 1989).

Lymphocytes and the derived cell lines require specialised environments for their successful culture and growth. The essential requirements for an optimum cell culture environment are temperature, hydrogen-ion concentration (pH), gas phases and media containing the necessary nutrients. RPMI-1640 medium has been used for the culture of human normal and neoplastic leukocytes. RPMI-1640 was developed by Moore *et al.*, (1968) at Roswell Park Memorial Institute. RPMI-1640, when properly supplemented, has demonstrated wide applicability for supporting growth of many types of cultured cells, including fresh human lymphocytes (ECACC Handbook, 2006). For this study, RPMI-1640 was used for culturing the lymphocytes cell lines and was supplemented with foetal calf serum (FCS), *N*-2-Hydroxyethylpiperazine-*N'*-2-ethane-sulphonic acid (HEPES)

buffer, L-glutamine and Pen-Strep-Fungizone (PSF). A full discussion of how these cell lines were cultured follows below.

3.2 Materials and Methods

This study received full ethical approval on the 11th of March 2008 from the Biomedical Research Ethics Committee and Higher Degree Committee. This was after the initial ethics approval granted in 2007 was amended to remove the HIV part of the study. The reference number given to the first application was EXP057/06 and the same reference was kept for the amended protocol.

3.2.1 Materials

RPMI-1640 containing HEPES buffer (25mM), FCS, PSF, L-glutamine were purchased from *Adcock Ingram* (South Africa). Plastic tissue culture flasks and Histopaque 1077 were purchased from *Sigma Aldrich* (SA). BD IMagnet™ and BD IMag™ Human T lymphocytes Enrichment Set- DM for isolating T cells from peripheral blood mononuclear cells (PBMCs) were purchased from *BD BioSciences* (SA). Dimethyl sulphoxide (DMSO), Ethanol and all other organic solvents were purchased from *Merck Chemicals* (SA).

3.2.2 Methods

3.2.2.1 Aseptic techniques and good laboratory practices

Aseptic techniques were strictly adhered to at all times during culturing of cells in the tissue culture laboratory. Protective gear (i.e. laboratory coat, closed shoes and gloves) were used at all times. All cell culture work was carried out in a LabAire biological safety cabinet (Class 2) equipped with a vertical laminar flow hood and UV light. To minimise contamination, all work surfaces were swabbed with 70% ethanol before and after use. All materials used in the tissue culture were sterilised either by filtering with 0.45 μm filters or by autoclaving.

3.2.2.2 Growth of H9 cells

The T cell lymphoma cell line, H9, was a kind gift from Professor Raveen Parboosing (Department of Virology at Inkosi Albert Luthuli Central Hospital). The H9 suspension cells were cultured in RPMI-1640 medium supplemented with 10% FCS together with 1% PSF, and 1% L-glutamine. The cells were incubated at 37^0C in 5% carbon dioxide (CO_2). The CO_2 provides the natural buffering system where gaseous CO_2 balances with the CO_2 / HCO_3 content of the culture medium in order to maintain the pH at a range of 7.2 to 7.4.

FCS is a complex mixture of albumin, growth factors, growth inhibitors, hormones, amino acids, carbohydrates and lipids and is an important component of cell culture medium. This serum also provides buffering capacity to cultures and is important for slow growing cells or where the seeding density is low. Further, FCS has an advantage in that it can be used

for many different cell lines which have different growth factor requirements. The use of FCS in media also increases the risk of contamination. Heat inactivation of serum (incubation at 56^0C for 30 minutes) can help to reduce the risk of contamination since some viruses are inactivated by this process (ECACC Handbook, 2006).

Pen-Strep-Fungizone is an antibiotic combination of Penicillin, Streptomycin and Fungizone which are effective against Gram positive and Gram negative bacteria and fungi. L-glutamine is required in cell culture media formulations because it supports cell growth. Glutamine becomes essential cells that have high energy demands and synthesize large amounts of proteins and nucleic acids. It is an alternative energy source for rapidly dividing cells and cells that use glucose inefficiently. Although L-glutamine is synthesised by all normal cells, transformed cancerous cells are dependent on exogenous sources (ECACC Handbook, 2006).

The H9 cell line was maintained at $1 \times 10^5 - 1 \times 10^6$ cells/ml. Splitting was done twice weekly to maintain the flasks at 80%- 90% confluence. H9 cells grow as a single cell suspension with some clumping. Their morphology is mature lymphocytic (National Institute of Health (NIH), 2005).

3.2.2.3 Subculture of H9 cells

Cultures derived from blood (i.e. lymphocytes, B cells, etc) mostly grow in suspension as either single cells or clumps. For these cell types, subculturing by dilution of each flask is relatively easy. But cells that grow in clumps may necessitate that they are pelleted to a

single cell suspension by centrifugation and re-suspension by pipetting before they are counted and subcultured (ECACC Handbook, 2006).

H9 cells were subcultured while growing in exponential growth phase. Rapidly growing cells were bright, round and refractile when viewed under the microscope. Gently tapping on the side of the flasks allowed the removal of some clumps. Subculturing of each flask was dependent on the confluence of cells and their growth rate. The process of subculturing involved centrifugation to remove the old media and diluting the cells into two new flasks with new pre-conditioned RPMI-1640 media. A small quantity (100- 200 µl) of the cell suspension was used for cell quantification to determine the seeding density (Figure 3.1).

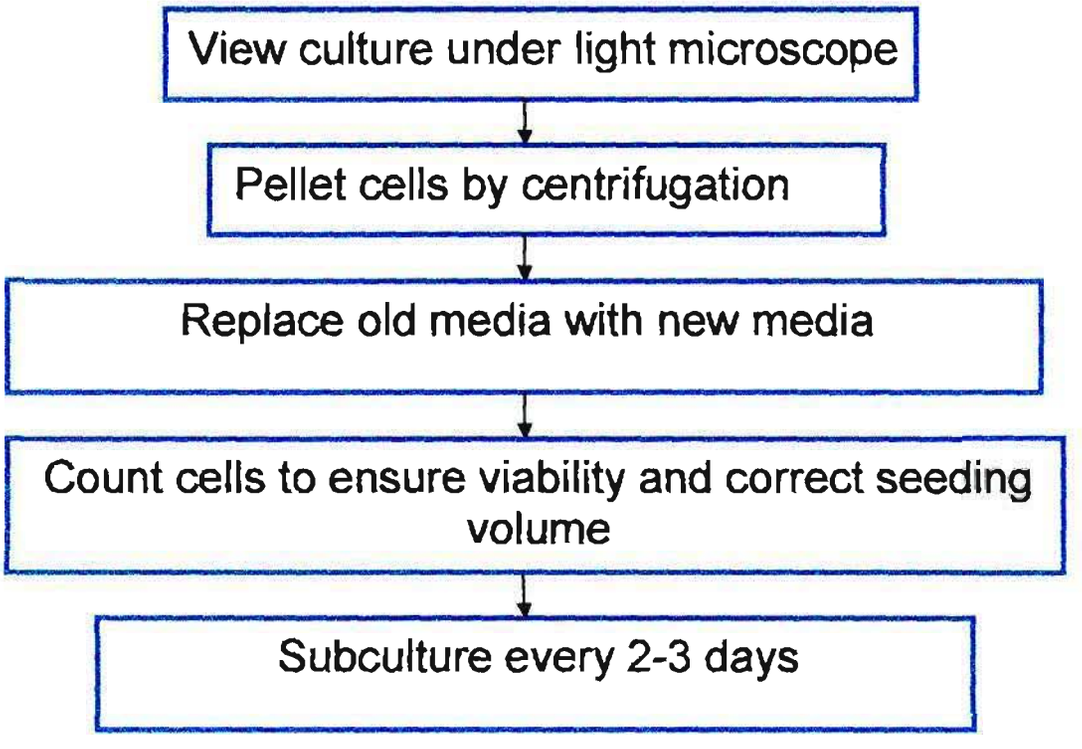


Figure 3.1: Schematic illustration of the subculture process in suspension cultures.

3.2.2.4 Cell quantification

Cell culture experiments require that the number of cells in an assay must be quantified to ensure maximum growth. This in turn gives results with better reproducibility (ECACC Handbook, 2006). Haemocytometers are counting chambers developed to count blood cells but are used for other cell types. It is a thick glass slide with two counting platforms in its centre. The grid is divided into 9 large squares, each 1mm x 1mm, by triple lines. Each large square is divided into 25 medium squares, each 0.23mm on a side, and each medium square is further divided into 16 small squares, each 0.05mm on a side (Figure 3.2). The haemocytometer can be used where cell densities are in the range 5×10^4 - 10^7 cells / ml.

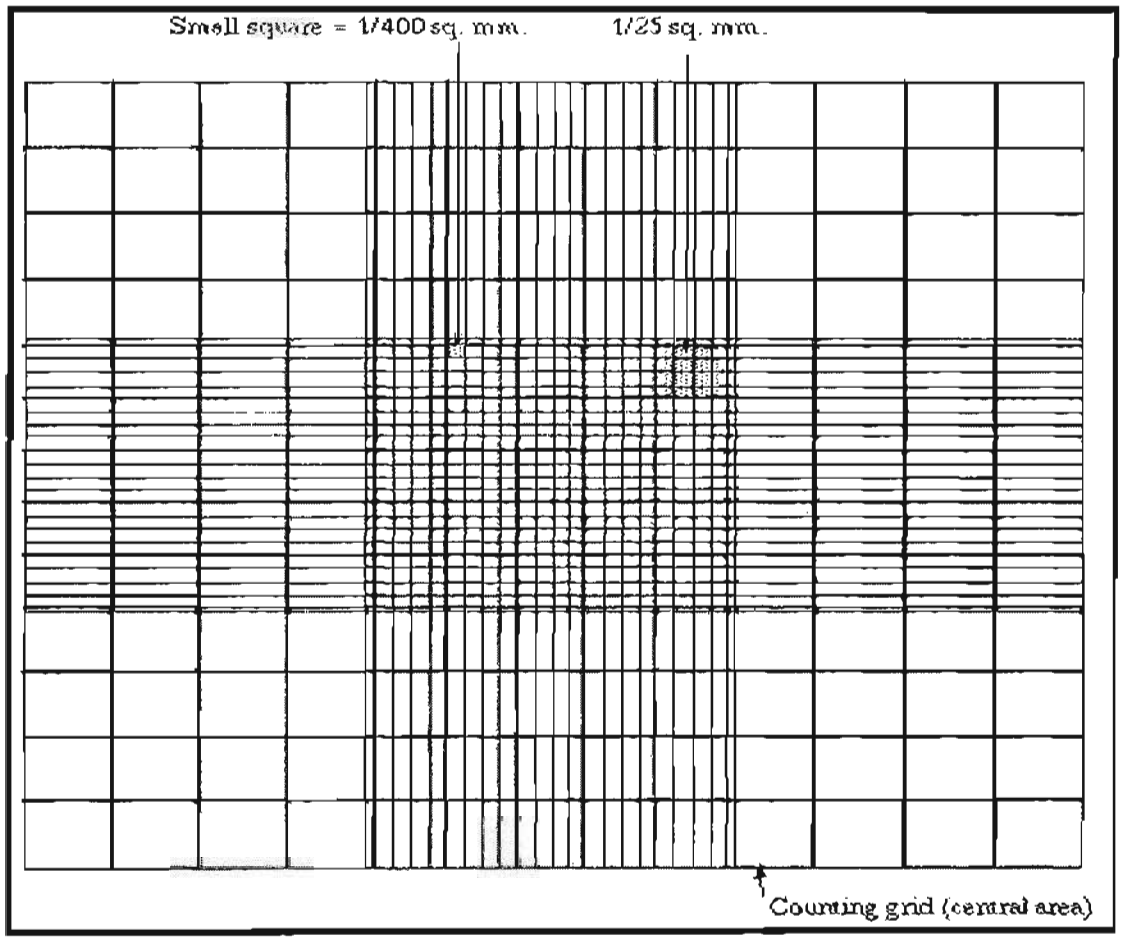


Figure 3.2: Diagram of the haemocytometer grid.

Trypan blue, a vital dye, is used to stain cells and viability is determined using a haemocytometer. The reactivity of trypan blue is based on the fact that the chromopore is negatively charged and does not interact with the cell unless the membrane is damaged. Therefore, all the cells which exclude the dye are viable (Freshney, 1987).

The H9 cells were suspended in RPMI-1640 and under sterile conditions 100-200uL of the cell suspension was placed into an equal volume of 0.4% Trypan blue (2× dilution) (appendix 1). This was mixed by pipetting up and down a few times and left at room temperature for 10 minutes. An equal amount (10 μl) was pipetted into each of the two haemocytometer chambers, covered with a clean coverslip and viewed under a light microscope (20 × magnification). The number of viable (appeared bright) and dead cells (stained blue) were counted. Ideally more than a hundred cells were counted in order to increase the accuracy of the cell count. The concentration of viable cells was calculated using the following formulae:

Concentration of viable cells = (Total unstained viable cells / Total number of cells [stained and unstained]) × 100

Total number of viable cells/ml = Average count per square × dilution factor × 10^4

Dilution factor = 2 (volume of cell suspension and trypan blue 1:1)

10^4 = correction factor (supplied by haemocytometer manufacturer)

The cell counts were repeated twice to ensure accuracy of the number of cells treated for each experiment.

3.2.2.5 Cryopreservation of cells

Cryopreservation enables stocks of cells to be stored preventing culture of many flasks at any given time. Other advantages of cryopreservation include reducing microbial contamination of cultures, reduced cross-contamination with other cell lines, reduced genetic drift and morphological changes and a reduction in cost. The basic principle of a successful cryopreservation is slow freeze and quick thaw. Although the precise requirement may vary with different cell lines as a general guide cells should be cooled at a rate of -1^0C to -3^0C per minute. Cells should be in a log phase of growth when they are stored. FCS and a cryoprotectant like dimethyl sulphoxide (DMSO) should also be used in the cryopreservation media. A cryoprotectant prevents the formation of ice crystals (ECACC Handbook, 2006).

The cryopreservation media for the H9 cells comprised RPMI-1640, DMSO and FCS (appendix 1). For cryopreservation, a confluent flask of H9 cells was identified and viewed under microscope to assess the degree of cell density and cell viability. The cells were then centrifuged at 400g for 10 minutes to remove culture which was then replaced with 2 ml of cryopreservation media. This suspension was aliquoted into cryovials and placed into a nalgene cooler (Figure 3.3). The nalgene cooler is a container which is filled with isopropanol and allows the slow freezing of preserved cells up to -70^0C without the necessity to monitor the freezing process. Cultures cryopreserved in this way were easily resuscitated when a new culture was needed.

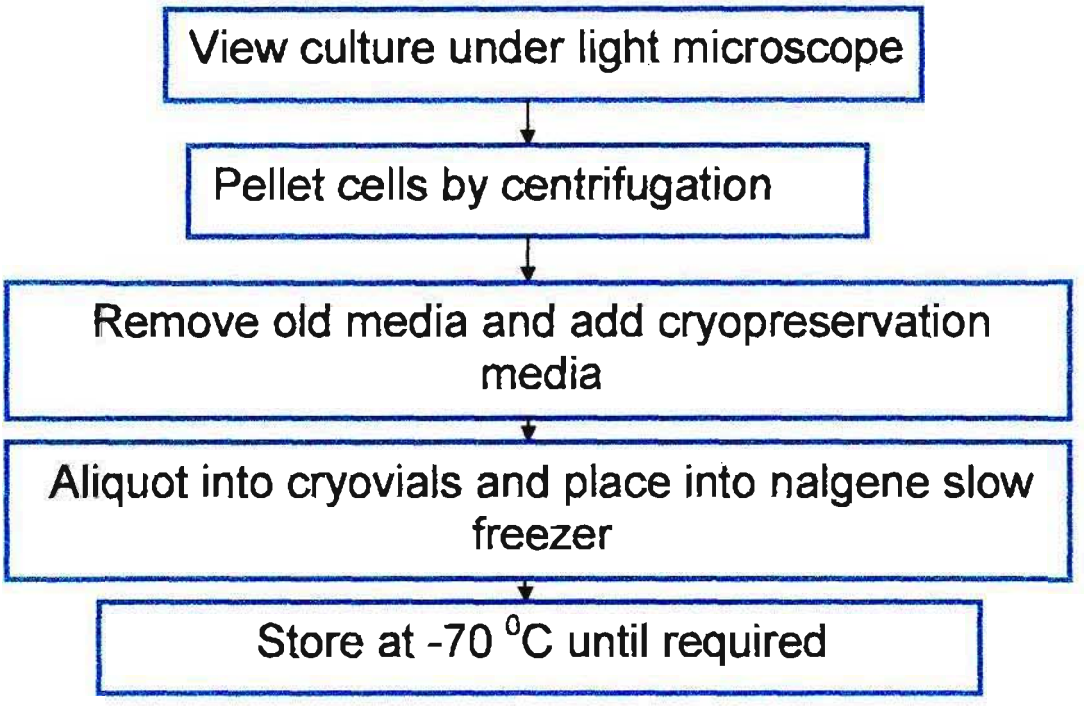


Figure 3.3: Schematic diagram representing the H9 cells cryopreservation process.

3.2.2.6 Resuscitation of Frozen Cells

It is vital to thaw cells correctly in order to maintain the viability of the culture and enable the culture to recover more quickly. Dimethyl sulphoxide is toxic above 4^0C therefore it is essential that cultures are thawed quickly and diluted in culture medium to minimize the toxic effects. A water bath at 37^0C is better to use for fast thawing than placing the cryovials in an incubator (ECACC Handbook, 2006).

For H9 cells resuscitation, the cryovials were removed from the -70^0C freezer and placed directly into the water bath at 37^0C. Water was prevented from entering the cryovials by sealing them completely. The cryovials were allowed to thaw for 1-2 minutes. The thawed

cells were then pipetted into flasks with pre-conditioned media. The flasks were placed into the incubator overnight to allow cells to recover. The media was removed the next day and replaced with new preconditioned media without any cryopreservation media.

3.2.3 Isolation of Human T cells from whole blood

3.2.3.1 Isolation of Peripheral Blood Mononuclear Cells (PBMCs) from whole blood

Blood from a healthy individual (myself) was drawn into lithium heparin vacutainer tubes (5ml). The blood was then carefully layered onto equal amounts of Histopaque 1077 in Sterilin tubes (15 ml). These tubes were then centrifuged at 1700 rpm for 30 minutes at 25^{0}C. After centrifugation, the tubes had four different layers (Figure 3.4) making up different components of blood and Histopaque 1077. These were the plasma layer at the top, the buffy coat, which had the PBMCs, was below the plasma, Histopaque 1077 made up the third layer and the red blood sunk to the bottom because of their higher density.

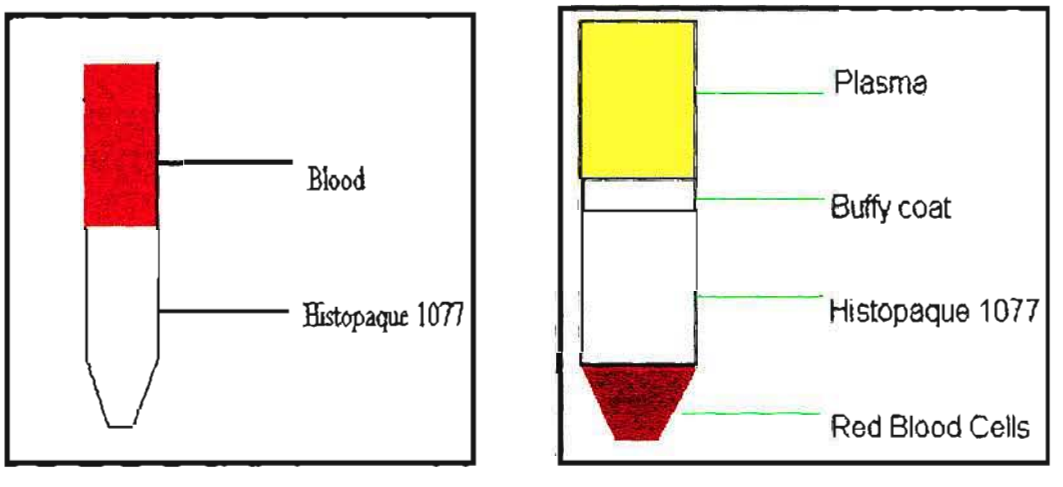


Fig 3.4: Separation of whole blood using sucrose density gradient yields 4 distinct layers following centrifugation.

The plasma layer was carefully aspirated and discarded. The buffy coat containing PBMCs was aspirated into new 15 ml centrifuge tubes and washed twice with phosphate buffered-saline (PBS, 5 ml), (2400 rpm for 20 minutes at 25^0C). The final pellet was re-suspended in BD IMag Human T lymphocytes isolation buffer.

3.2.3.2 Isolation of Human T cells from PBMCs

The BD IMag™ Human T Lymphocyte Enrichment Set – DM was used for negative selection of T lymphocytes from PBMCs. The Biotinylated Human T Lymphocyte Enrichment Cocktail contains monoclonal antibodies that recognize antigens expressed on erythrocytes, platelets, and peripheral leukocytes that are *not* T lymphocytes. The BD IMag™ Streptavidin Particles Plus– DM are magnetic nanoparticles that have streptavidin covalently conjugated to their surfaces. With these two components, the BD IMag™ Human T Lymphocyte Enrichment Set– DM avoids the inadvertent activation of the enriched T lymphocytes by using reagents that do not directly bind to those T cells (BD IMag™ Technical Data Sheet, 2006).

The Biotinylated Human T Lymphocyte Enrichment Cocktail simultaneously bound erythrocytes, platelets, and most leukocytes except the T lymphocytes. After washing away excess antibody, BD IMag™ Streptavidin Particles Plus – DM was added to the cell suspension and bound the cells bearing the biotinylated antibodies. The tube containing this labelled cell suspension was then placed within the magnetic field of the BD IMagnet™. Negative selection was then performed to enrich for the unlabelled T cells. Labelled cells migrated toward the magnet (positive fraction), leaving the unlabelled cells in suspension so they could be drawn off and retained (enriched fraction). The negative

selection was repeated twice to increase the yield of the enriched fraction (Figure 3.5). The combined enriched fraction contained T lymphocytes with no bound antibodies or magnetic particles. These cells were ready for downstream applications such as tissue culture (appendix 1).

3.3 Conclusion

The protocols used in tissue culture were according to published methods and were applied according to supplier's stringent instructions. The growth of H9 cells was optimal and they showed resilience to changes in their environment. The normal human T cells also showed optimal growth when incubated for up 48 hours despite the long processing procedures.

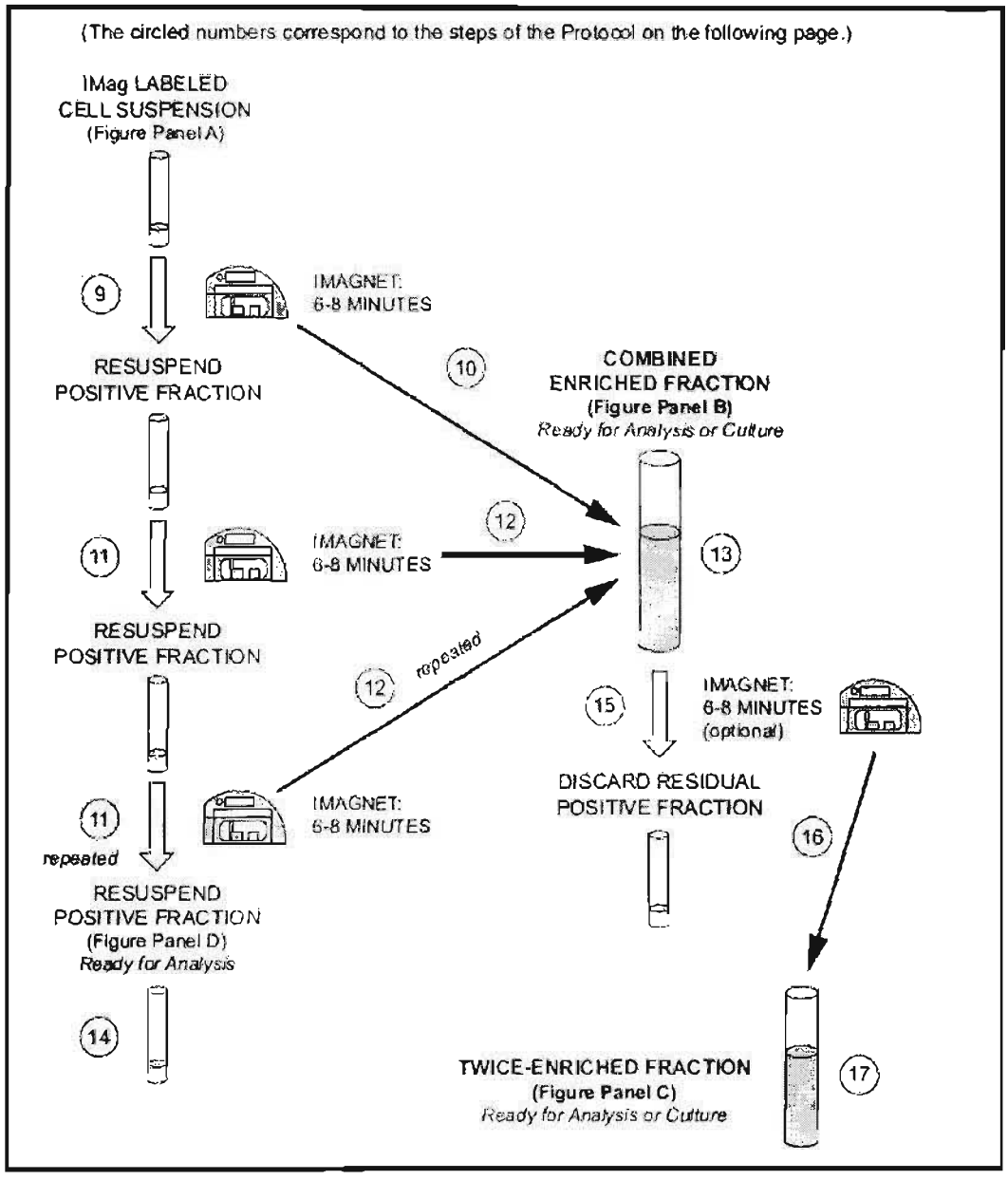


Figure 3.5: Human T lymphocytes enrichment flow chart using the BD IMag™ Human T Lymphocyte Enrichment Set – DM (BD IMag™ Technical Data Sheet, 2006).

CHAPTER 4

CYTOTOXICITY OF *SUTHERLANDIA FRUTESCENS* ON CULTURED H9 CELLS AND NORMAL T CELLS

4.1 Introduction

In vitro cytotoxicity assays are useful in that they measure the effects of toxic chemicals on the basic functions of cells, and that the toxicity can be measured by assessing cellular damage. The development of *in vitro* cytotoxicity assays has been driven by the need to rapidly evaluate the potential toxicity of large numbers of compounds, to limit animal experimentation whenever possible, and to carry out tests with small quantities of compound (www.noabbiodiscoveries.com).

There are three basic parameters upon which these measurements are based. The first assay type measures cellular metabolic activity. An early indicator of cellular damage is a decrease in the metabolic rate. Tests which can measure metabolic function measure cellular ATP levels or mitochondrial activity. A second parameter often tested is the measurement of membrane integrity. The cell membrane forms a functional barrier around the cell, and traffic into and out of the cell is highly regulated by transporters, receptors and secretion pathways. Damaged membranes lose this regulation and allow substances to migrate through the membrane unregulated. Changes in metabolic activity have been shown to be better indicators of early cell injury while effects on membrane integrity are indicative of more serious injury, leading to cell death. Another parameter often measured to indicate cytotoxicity is the direct quantification of cell numbers, since cytotoxicity

normally decreases cell numbers. Cell numbers can be measured by direct cell counting, or by the measurement of total protein or DNA, which are proportional to the number of cells (www.noabbiodiscoveries.com).

4.2 The MethylThiazol Tetrazolium (MTT) Assay

Chemosensitivity tests are commonly utilised in both research and clinical environments to determine the effects of compounds on tumour cells. A positively charged yellow tetrazolium salt, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), , is widely used as an indicator of cell viability and metabolism in cultured cells. This assay was first described by Mosmann (1983). The MTT assay offers a quantitative, convenient method for evaluating a cell population's response to external factors, whether it is an increase in cell growth, no effect, or a decrease in growth due to necrosis or apoptosis (Cooke and O'Kennedy, 1999; Newman *et al.*, 2000; ATCC, 2001).

The mechanism by which the tetrazolium salts are converted to coloured complexes appears to involve the reduction by the addition of electrons donated by several dehydrogenases, where this may be in cytosolic enzymes or mitochondrial enzymes of the respiratory chain. The reduction of the yellow MTT salt is followed by the formation of purple formazan crystals (Figure 4.1). MTT is taken up by cells by endocytosis, reduced in the cytosolic, endosomal or lysosomal compartments, and then transported to the extracellular space through exocytosis in the form of needle-like formazan. A solubilisation solution (DMSO) is added to dissolve the insoluble purple formazan product into a coloured solution. Different cell types have tremendous differences in their rate of MTT reduction (Cooke and O'Kennedy, 1999; Molinari *et al.*, 2005).

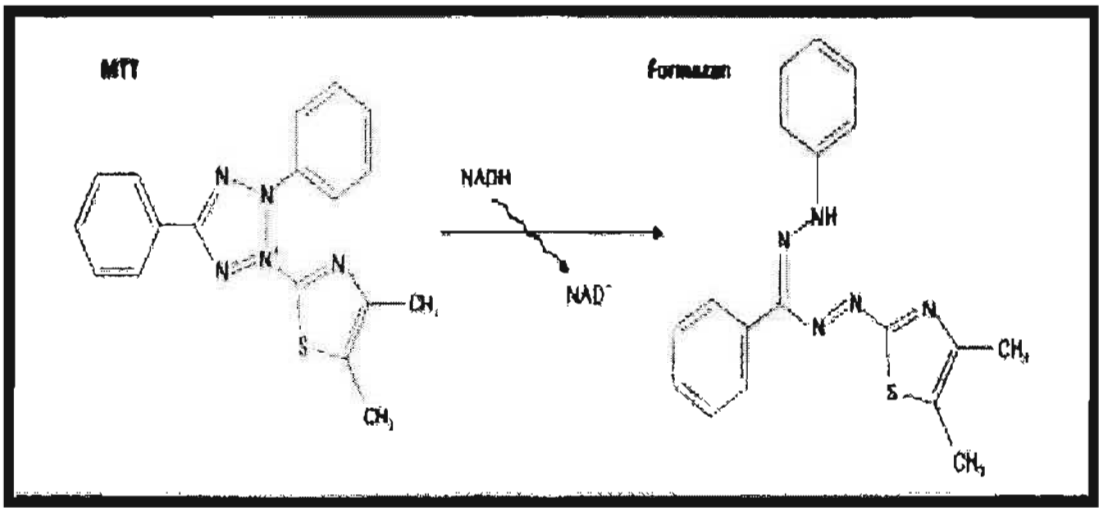


Figure 4.1: The reduction of the MTT salt to form formazan crystals in metabolically active cells (Rode *et al.*, 2004).

The absorbance of this coloured solution can be quantified by measuring at a certain wavelength (usually between 595 and 655 nm) by a spectrophotometer. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion is directly related to the number of viable cells. When the amount of purple formazan produced by cells treated with a compound of interest is compared with the amount of formazan produced by untreated control cells, the effectiveness of the compound in causing death of cells can be deduced, through the production of a dose-response curve (American Type Culture Collection (ATCC), 2001).

The advantages of tetrazolium salts in monitoring growth of cultures are apparent in the use of microplate assays where avoidance of radioisotopes replaces the use of [^{3}H]thymidine incorporation and related approaches. The assay has other advantages of being a rapid, semi-automated technique. The tetrazolium method also affords another advantage in that the yellow water soluble tetrazolium salt is converted upon reduction to a

lipid insoluble, purple formazan that becomes trapped within the cell (Newman *et al.*, 2000; Cooke and O'Kennedy, 1999).

The MTT assay also has various disadvantages when used to measure cell viability. These include the inability to distinguish between cytostatic and cytotoxic effects, as well as the observation that some test compounds may interfere with the reduction of the MTT salt. In addition, succinate dehydrogenase, localized in the mitochondria as part of the tricarboxylic acid cycle (TCA), is only one of a number of oxidative enzymes that can form reducing equivalents and therefore measuring its activity may not be a very good predictor of cytotoxicity (Cooke and O'Kennedy, 1999).

4.3 Luminescent Cell Viability ATP assay

One evident physiological difference in cells undergoing apoptosis versus necrosis is in intracellular levels of ATP. Cell death via the apoptotic pathway is an energy dependent process that requires ATP. Longer incubation of cells under ATP-depleting conditions results in necrotic cell death. Incubation of cells in glucose-free medium with an inhibitor of the mitochondrial F_0 F_1-ATPases reduces intracellular ATP levels and completely blocks the Fas/Apo-1-stimulated apoptosis. ATP supplied through glycolysis and oxidative phosphorylation restores the apoptotic cell death pathway in these cells. ATP depletion has also been shown to inhibit caspase-3 activation and when ATP is depleted after the activation of caspase-3, subsequent apoptosis is significantly blocked. Thus, ATP-dependent steps exist both upstream and downstream of caspase-3 activation in apoptotic signal transduction (Eguchi *et al.*, 1997).

Therefore, loss of ATP in cells has been used as an indicator of cell death. In contrast, cell proliferation has been recognised by increased levels of ATP. The advent of luminometers has allowed detection of changes in ATP levels in ultra-small amount of sample preparations. A luminometer is a photometer used for measuring very low light levels. For our experiments, we utilised a Modulus™ Microplate Luminometer (Turner BioSystems), a super sensitive luminometer. It also comes preloaded with luminescence and fluorescence protocols.

The luminometer in combination with the Promega CellTiter-Glo™ Luminescent Cell Viability Assay Kit provides a convenient, rapid, and sensitive procedure for determining the number of viable cells in a culture. Presence of metabolically active cells is signalled by ATP quantitation. The method utilises bioluminescent detection of the ATP levels for a rapid screening of cell death in cells. The assay uses luciferase as the detection enzyme because mammalian cells lack endogenous luciferase activity. Luciferase enzyme requires ATP in order to generate light. Metabolically active cells produce ATP as energy for respiration and other vital processes. After an equal volume of CellTiter-Glo™ Reagent is added to the cell culture, luminescence is measured. Light signal is proportional to the amount of ATP present which correlates with the number of viable cells present (Turner BioSystems Application Notes, 2008).

The cytotoxicity of SF was investigated on cancerous H9 T lymphocytes and normal T cells. Commercially available SF tablets (PhytoNova) were extracted with 70% ethanol and deionised water respectively and were diluted into 1/50 and 1/200 dilutions of each tablet extract. These dilutions were then used to treat the T cell lines over incubation periods of 24 and 48 hours. Tai *et al.* (2004) showed that SF had an IC_{50} of 1/150 (70%

SFE extract) using Jurkat T cells. We chose two concentrations which would cover this range as different cell lines have been shown to react differently to SF.

The use of an organic solvent extract like that of ethanol has been favoured as it has been shown to extract more of the plant's active ingredients. Because of the use of ethanol as an extracting solvent, this made it necessary that a vehicle (ethanol) control be included in the experimental setup. Camptothecin (CPT) at a concentration of 20 μM was included as positive control. The SFW extracts were included because commercial tablets which were ready for human consumption were used in this experiment. *Sutherlandia frutescens* tablet dosages are taken with water and the SFW extracts would better reflect what happens *in vivo* during absorption and metabolism. This type of experimental setup was utilised for both the MTT and ATP assays. A similar theme was followed for all the other assays that performed.

4.4 Materials and Methods

4.4.1 Materials

The tetrazolium salt (MTT) was purchased from *Sigma Chemical Company* (SA). DMSO and ethanol were purchased from *Merck Chemicals* (SA). Promega CellTiter-Glo™ Luminescent Cell Viability Assay Kit was purchased from *Promega* (SA). Modulus™ Microplate Luminometer was from *Turner BioSystems* (USA). SF tablets were purchased from *King Edward Pharmacy* (Durban).

4.4.2 Methods

4.4.2.1 Extraction of *Sutherlandia frutescens* tablets

Sutherlandia frutescens tablets (PhytoNova) were used in this study. Each tablet was extracted according to the method of Tai *et al.*, (2004). Briefly, SF tablets containing 300mg of raw herb powder (per tablet) compounded with inert excipients were extracted with 2.2 ml of 70% ethanol/triple distilled water at room temperature for 2 hours on an orbital shaker. The suspension was then centrifuged at 5 000 ×g for 10 minutes and the supernatant was removed. The extracts were filter sterilised with 0.45 μm filters. These extracts were further diluted to 1/50, 1/150, 1/200, and 1/300 (appendix 2) with complete culture medium (RPMI-1640 supplemented with 10% FCS, 1% L-glutamine, and 1% PSF). Each dilution was made up to 1 ml. The extracts could be stored at 4 ^{0}C for up to three months without the loss of efficacy.

4.4.2.2 Preparation of MTT Salt Solution

A 5mg/ml MTT salt solution was made by dissolving 5mg of the salt in 1 ml of Paste MTT salt solution was prepared just before it was used.

4.4.2.3 Preparation of Promega CellTiter-Glo™ Luminescent Cell Viability Assay Kit

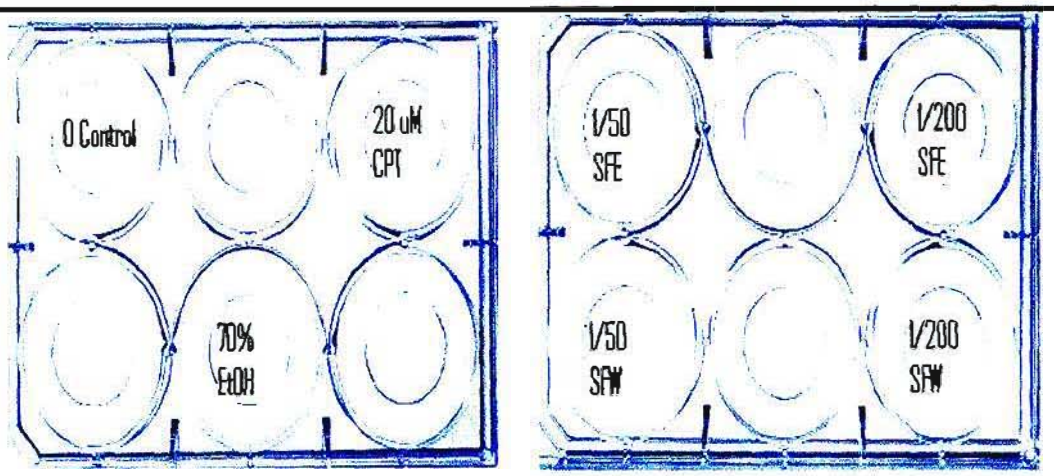
The cell viability kit for ATP was made up of a CellTiter-Glo™ Substrate and CellTiter-Glo™ Buffer. To make the CellTiter-Glo™ Reagent, the whole contents of one bottle of CellTiter-Glo™ Buffer (10ml) were transferred into one bottle of CellTiter-Glo™

substrate. The contents were mixed by inversion until the substrate was thoroughly dissolved. The reconstituted reagent was used when properly warmed up to room temperature and could be stored at 4 ^{0}C for two days with 5-20% loss of activity.

4.4.2.4 Treatment of H9 cells and normal T cells for both the ATP and MTT assays

A confluent flask (75 cm^3) with 30 ml of exponential growing H9 suspension cells was used for the MTT assay. A cell count was determined using trypan blue to give a cell number of 2.5 × 10^6 cells/ml. Aliquots of the cell suspension (2 ml) were transferred into each well of a 6-well plate. The 6-well plates were labelled according to the different treatment dilutions and types. Dilutions of SF (4 ml/well) were then added to each of the treatment wells. The well for the vehicle control (70% ethanol) had a similar amount of H9 cells and ethanol diluted into 4 ml. A CPT, 20 μM, positive control was also prepared. An untreated control was made in which 2 ml of cells in a well had 4 ml of CCM added to them (Figure 4.2). The 6 well plates were then incubated for 24 and 48 hours at 37 ^{0}C in an incubator.

A similar treatment procedure was followed for the normal T cells. T cells were isolated from PBMCs using the Human T Lymphocyte Enrichment Set – DM (BD IMag™), washed with PBS and then diluted in RPMI-1640 media. A cell count (as described previously) of 1.5- 2 × 10^6 cells/ml was determined. Separate 6 well plates were used for the treatment of normal T cells. The treatment with SF and the three controls were performed similarly to the H9 cells.



CPT = Camptothecin

EtOH = Ethanol

SFE = 70% ethanol extract of *Sutherlandia frutescens*

SFW = deionised water extract of *Sutherlandia frutescens*

Figure 4.2: A schematic illustration of the treatment procedure in 6 well plates for both the H9 cells and normal T cells in performing the MTT assay.

4.4.2.5 The MTT assay

At the end of each incubation period (24 and 48 hours), a 100 µl from each treatment concentration was pippetted into three different wells of a 96-well plate (Figure 4.3). A freshly prepared MTT salt solution (10 µl of 5 mg/ml working solution) was added into each of the 96 wells. The untreated control, 70% ethanol treated cells and the CPT treated cells were also treated in the same way. This procedure was the same for both the H9 cells and the normal T cells. The 96-well plate with treated cells and the MTT salt were then incubated for 4 hours at 37 ^{0}C.

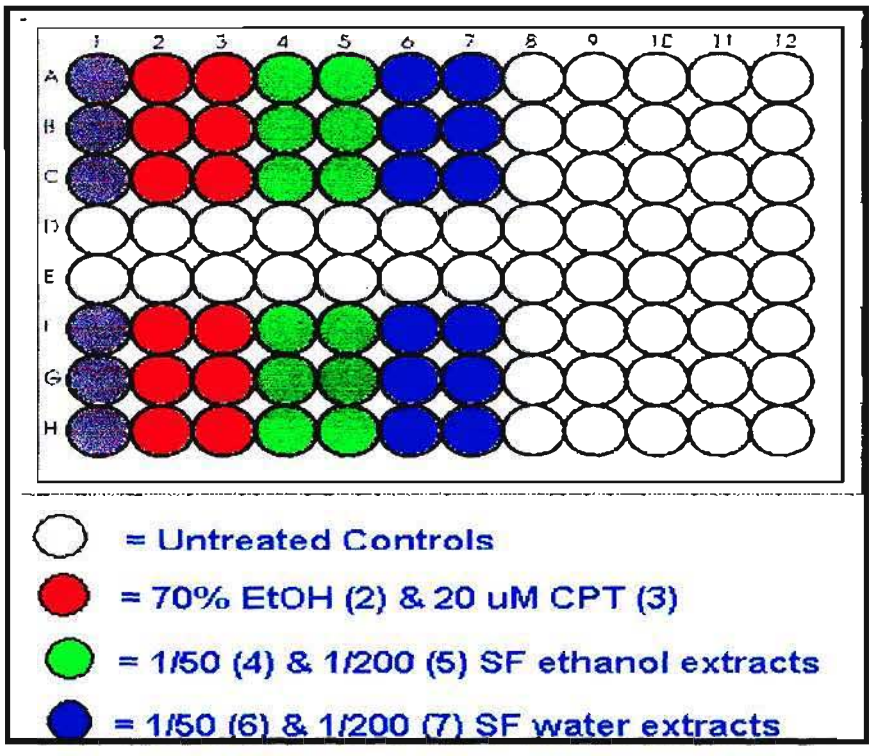


Figure 4.3: An illustration outlining the set up of the MTT assay in the 96-well plate. The wells from column A to C were for the treatment of H9 cells and columns F to H were for normal T cells.

After incubation with the MTT salt, the 96-well plate was centrifuged at 500 ×g for 10 minutes to pellet the suspension cell lines. The culture media and excess MTT salt were aspirated and DMSO (100 µl/well) was added to each well. The plate was incubated for an hour at 37 ^{0}C to allow solubilisation of the purple formazan. The absorbance of the treated wells and controls was then spectrophotometrically determined at a wavelength of 595 nm with a reference wavelength of 655 nm using a Bio-Tek microplate reader. The assay was repeated three times and cell viabilities of the different treatments were the average of the triplicate readings. The cell viabilities of the treated cells as compared to the untreated control were used to generate a dose-response curve for the SF extracts.

4..2.5.1 Statistical analyses of the MTT results

The percentage cell viability for the average absorbance of each treatment was calculated using the following formula:

$$\text{Cell Viability} = \frac{\text{Average absorbance of treated cells}}{\text{Average absorbance of untreated control cells}} \times 100$$

Data analyses were done on *Microsoft Excel* to obtain the averages, cell viability percentages and the standard deviations. The different levels of significances within the separate treated groups were analysed using one-way analysis of variance (*ANOVA*) and the differences between the treated cells and the control cells were analysed using the *Tukey* and *Bonferroni* methods for multiple comparisons. Differences with $p < 0.05$ were considered statistically significant.

4.4.2.6 Luminescent Cell Viability ATP assay

Luminescent Cell Viability ATP assay was only done on H9 T cells in order to confirm the anti-proliferative effects of SF on cancerous cells. After the end of each incubation period (24 and 48 hours), a 100 μl from each treatment concentration was pipetted into two different wells of a white opaque 96 well plate. It is crucial to use white opaque plates when doing a luminescence assay as these plates are able to reduce the cross-reactions and background luminescence due to the specially formulated composition of the white resin. This increases assay sensitivity by reflecting emitted light into the detector.

The ATP assay was done in duplicate, due to the high sensitivity of the assay. Cells were aliquoted into designated wells of a 96-well plate as in the MTT assay and allowed to come

to room temperature. The CellTiter-Glo™ Reagent was mixed immediately before use and was added to the wells with treated cells at 25 μl per well. The plate was shaken on a plate shaker for 2 minutes at 300 rpm. This plate was then incubated out of direct light for 10 minutes at room temperature after shaking.

The Modulus™ Microplate Luminometer from Turner BioSystems comes with a preinstalled protocol for analysis of samples for the Promega CellTiter-Glo™ Luminescent Cell Viability Assay Kit. The instrument was turned on 20 minutes before use to pre-warm and pre-condition it. At the end of the incubation period, the plate was put into the luminometer and the relative light units (RLU) of the samples were measured. A dose response curve was also generated for the ATP levels using the RLU and the dilutions of SF and different control samples. Basically the RLU, which are equivalent to luminescence, reflect the amount of ATP in a given sample.

4.4.2.6.1 Statistical analysis of ATP assay results

As mentioned above, the amount of luminescence obtained for each sample represents the amount of ATP detected by the luciferase enzyme. Therefore a comparison of the average reading of each sample to the next sample gives the differences in ATP levels between samples. Data analyses were done on *Microsoft Excel* to obtain the averages, percentage ATP levels relative to the untreated control and the standard deviations of each treatment. The different levels of significances within the separate treated groups were analysed using one-way analysis of variance (*ANOVA*) and the differences between the treated cells and the control cells were analysed using the *Tukey* and *Bonferroni* methods for multiple comparisons. Differences with $p< 0.05$ were considered statistically significant.

4.5 Results and Discussion

4.5.1 The MTT assay

Sutherlandia frutescens 70% ethanol (SFE) and deionised water (SFW) extracts dilutions were incubated with H9 T cells and normal T cells for 24 and 48 hours (appendix 5 for all spectrophotometer readings). In the H9 T cells after 24 hours, the cell viability decreased significantly (p= 0.001) in both dilutions of the SFE extract as compared to the untreated control cells (Figure 4.4). The 50% inhibition (IC_{50}) of cell viability for the SFE extract dilution was estimated to be 1/40 after 24 hours incubation. The number of viable cells in the SFE extract dilutions was significantly (p< 0.05) lower than that of the vehicle control cells (70% ethanol-treated), which indicated that the effect of the extract was independent of ethanol. The SFE extract vehicle control was made by using the equivalent amount of 70% ethanol as used in making up the lowest dilution (1/50) of the SFE extract.

The positive control, camptothecin, induced the higher levels of cell death when compared to both the SFE and SFW extracts but this effect was not statistically significant (p> 0.05). Camptothecin is a secondary metabolite of *Camptotheca* plant and is known to induce apoptosis in cancer cells but is also known to induce apoptosis cell death in normal cells. Camptothecin acts by interrupting the breakage/reunion cycle of mammalian DNA topoisomerase 1 by trapping a reversible topoisomerase 1-DNA cleavable complex (Chen *et al.*, 1993). The 1/50 SFW extract dilution significantly (p= 0.049) decrease H9 T cell viability when compared to the untreated control cells. The cytotoxicity of the SFW extracts to H9 T cells showed a dose-dependent trend after 24 hours of incubation, with the lowest dilution (1/50 SFW) inducing the higher cytotoxicity (Figure 4.4).

The effects of the SFE and SFW extracts on normal T cells after 24 hours showed a different effect as observed on H9 T cells. Overall both the SFE and SFW extracts dilutions significantly (p= 0.003) increased cell proliferation when compared to the controls. The SFE extract dilutions were more potent at increasing cell proliferation, with the highest dilution (1/200) being more effective as compared to the untreated control cells. Both SFE extract dilutions were not significantly (p= 0.122 and p=0.232) different from the vehicle control, which meant the observed proliferative effects were related to the use of ethanol for extracting the tablets. The same dose-dependent cell proliferative effects were observed with the SFW extract dilutions but this was not significant (p= 0.391 and p= 0.139). Camptothecin did not induce significant normal T cell cytotoxicity as in H9 T cells. The effects of CPT on normal T cells were significantly (p= 0.005) different to the proliferative effects of the SFE and SFW extracts (Figure 4.4).

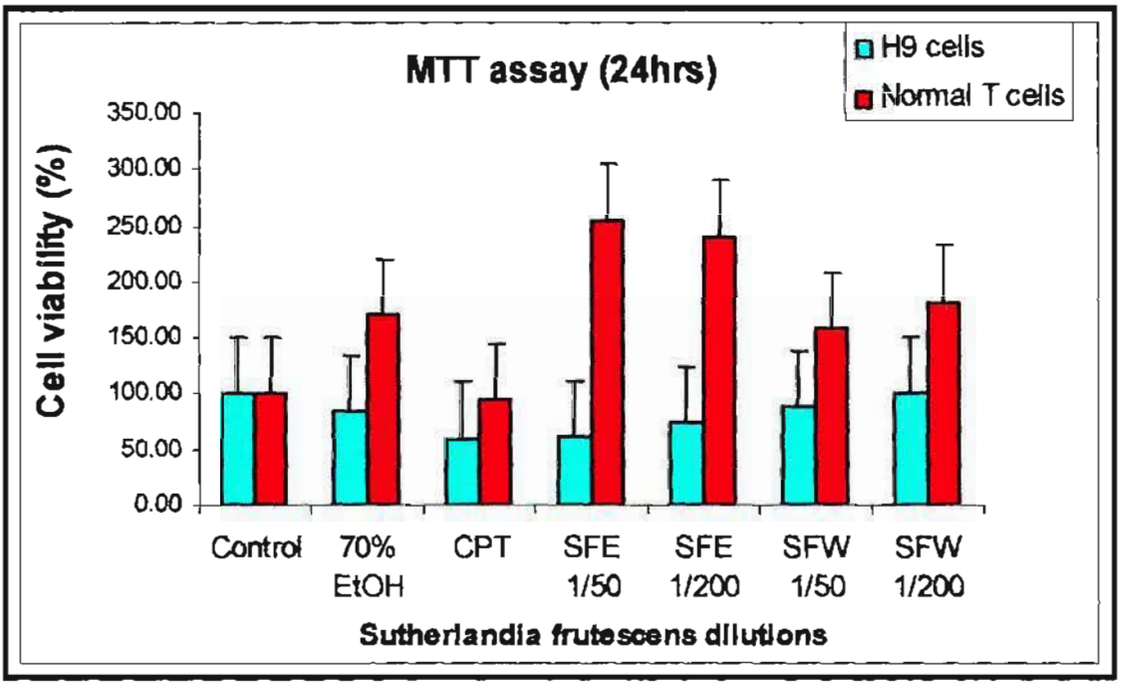


Figure 4.4: A dose response curve of different extract dilutions of *Sutherlandia frutescens* on the H9 T cells and normal T cells over a period of 24 hours.

The dose-dependent decrease in H9 T cell viability was still evident after incubation of these cells with the extracts of SF for 48 hours (Figure 4.5). In the SFE extract dilutions, this trend also became time-dependent as the lowest dilution (1/50) significantly (p= 0.001) decreased cell viability to 27% of the untreated control cells. This was well below the IC_{50} of the extract (1/105) after 48 hours. There was significant (p= 0.001) difference between the vehicle control and the SFE extract dilutions treated cells. In the SFW extract dilutions, the lowest dilution (1/50) was shown to have significantly (p= 0.014) decrease the number of viable cells as compared to the untreated control cells (appendix 5).

The effects of the SF extracts on normal T cells change after 48 hours incubation as compared to the 24 hours incubation (Figure 4.5). The proliferative effects of SFE extract dilutions were significantly reversed as compared to levels of viability at 24 hours, i.e. 254% cell viability at 24 hours versus 109% viability after 48 hours in the 1/50 SFE extract dilution. The vehicle control was not statistically significant (p= 0.611) from the SFE extract dilutions, meaning that the effects of the extract was not independent of ethanol. In contrast to the reversal of cell proliferation in the SFE extract dilutions, the SFW extract dilutions showed a time-dependent increase in cell viability after 48 hours incubation. This proliferation was significantly higher than the untreated cells (p= 0.004), the SFE extract dilutions (p= 0.008) and positive control (p= 0.001).

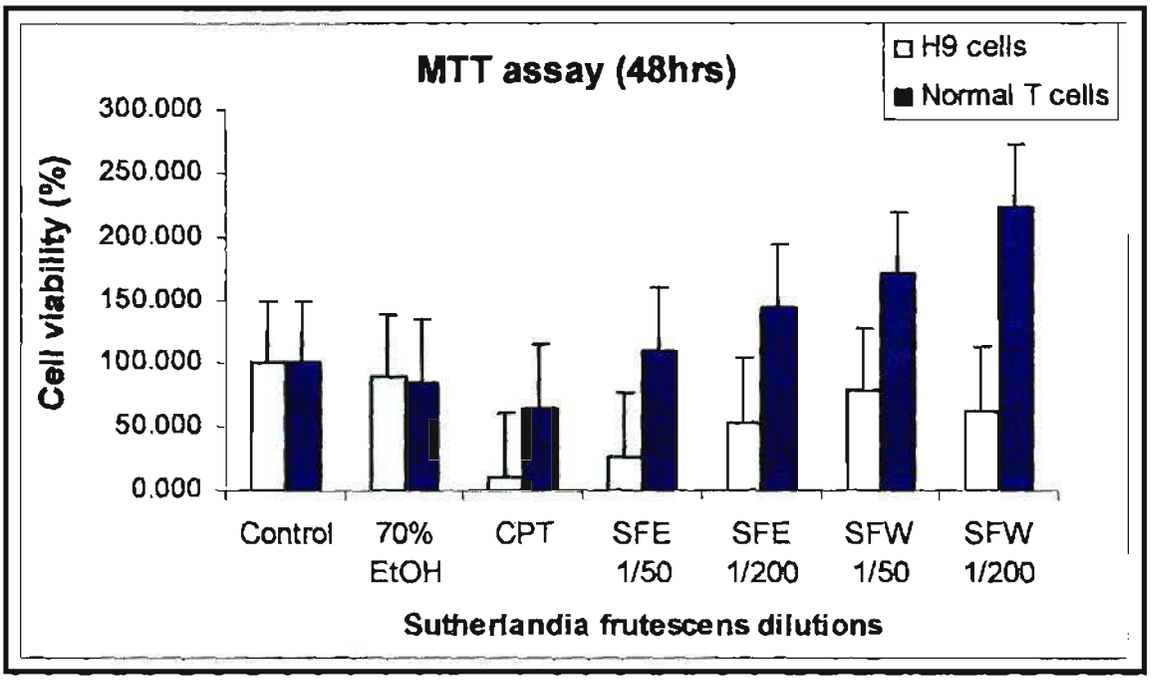


Figure 4.5: A dose response curve of different extract dilutions of *Sutherlandia frutescens* on the H9 T cells and normal T cells after 48 hours incubation.

Sutherlandia frutescens is a traditional remedy that has been purported as having anticancer properties. Some of the anticancer theories have been proven in *in vitro* assays with transformed malignant cell lines. *Sutherlandia frutescens* is also said to be a potent immune booster and an adaptogen. The immune boosting effects of this plant have not been conclusively tested *in vitro* while a few studies have looked at the effects of SF on blood parameters *in vivo* on healthy subjects. No study has shown that SF can selectively induce cytotoxicity in malignant cells while sparing the normal cells.

Tai *et al.*, (2004) looked at the effects of the SF on different malignant cell lines. Three of these cell lines were related to the immune system, i.e. human leukaemia Jurkat cell line, human pre-myelocyte HL60 cells and murine RAW 264.7 macrophage/monocyte cells. Using SFE extract dilutions of each SF tablet, they showed that SF extracts induced 50%

inhibition of proliferation of Jurkat cells at 1/150 and HL60 cells at 1/200 dilutions. These were higher dilutions than we found in our experiments (1/40 after 24 hours and 1/105 after 48 hours) but different cell lines have been shown to react differently to this medicinal plant. Suspension cell lines like Jurkat and H9 T cells, which are normally found in blood, have been shown to respond to higher concentrations of SF than attachment cultures.

Sutherlandia frutescens ethanol extracts from plant material of the SF plant were tested for their influence on cell numbers, morphology and gene expression profiles in a MCF-7 human breast adenocarcinoma cell line. A statistically significant decrease to 50% viability of malignant cell numbers was observed after 24 hours of exposure to 1.5 mg/ml SF extract when compared to the vehicle-treated controls (Stander *et al.*, 2007). The results of this were consistent with these findings as they showed that the 70% ethanol used for extraction of tablets did not play a significant role inducing cytotoxicity in H9 T cells.

Aqueous extracts of SF at a concentration of 50 μg/ml were tested for their cytotoxic ability *in vitro* against three human cancer cell lines: DU-145 prostate cancer cells, MDA-MB-231 and MCF-7 breast cancer cells and a non-malignant breast cell line MCF-12A. The aqueous extracts inhibited growth of the oestrogen-dependent cancer cell lines and stimulated the growth of the MCF-12A and MDA-MB-231 cells (Steenkamp and Gouws, 2006). Chinkwo (2005) found the crude aqueous whole plant extracts of SF to induce cytotoxicity in Jurkat T cells among the cell lines he used. The results obtained in study demonstrated comparable results in H9 T cells treated with aqueous extracts of SF over 24 and 48 hours (Figures 4.4 and 4.5).

Sutherlandia frutescens has been shown to contain active ingredients like L-canavanine, L-arginine, D-pinitol, GABA, and unique triterpenoid glucosides. Flow cytometric cell cycle analyses of different cell lines treated with SFE extracts of SF showed that the different cell lines were arrested at different phases of the cell cycle. This suggested that different active ingredients contained in the extracts may be involved in the inducing cell death in different cell lines (Tai *et al.*, 2004). Of all the active ingredients contained in SF, L-canavanine has been the only compound shown to induce cytotoxicity in tumour cell lines *in vitro*. L-canavanine is able to contribute to the production of canavanine-containing the proteins that can disturb protein synthesis as well as RNA and DNA metabolic pathways (Sia, 2004).

There has not been much information with regards to the anti-proliferative effects of the other active ingredients of SF. L-arginine, an antagonist of L-canavanine, is a precursor of nitric oxide (NO) which is synthesised by the nitric oxide synthase enzyme. Increased production of NO can lead to NO-induced cytotoxicity by oxidative injury within cells (Sia, 2004). The cytotoxic effects of the unique triterpenoid glucosides have not been tested. Haridas *et al.*, (2004) associated triterpenoids with anti-tumour activity. These observations suggest that there may be many bioactive compounds present in SF, possibly acting synergistically to induce the anti-proliferative effects seen in H9 T cells in this study and in other tumour cell lines.

According to the MTT assay results, the extracts of SF exhibited selectivity in inducing cytotoxicity in H9 T cells while causing proliferation in normal T cells. No published study has looked directly at the effects of SF extracts on normal T cells *in vitro*. This is in direct

contrast to the reported use of this traditional herbal remedy as an immune tonic. There have been suggestions that this plant be used in clinical trials on HIV/AIDS patients.

A safety study of SF on vervet monkeys was conducted by the Medical Research Council (MRC) of South Africa. Monkeys given a dose nine times the recommend dose of SF (81 mg/kg body weight per day for 3 months) did not show significant changes to relevant haematological, biochemical, and physiological parameters (MRC, 2001). A more recent clinical trial on healthy human adults given SF showed that there were no significant differences in general adverse events or physical, vital, blood, and biomarker indices between the treatment and placebo groups ($p > 0.05$) (Johnson *et al.*, 2007).

Fernandes *et al.*, (2004) looked at the antioxidant potential of SF on normal neutrophils isolated from whole blood. Although the main purpose was to assess the oxidant scavenging properties, they also looked at cell viability of the neutrophils treated with hot SFW extracts of SF using an acridine orange/propidium iodide staining method. The SF hot SFW extracts, up to concentrations of 40μg/ml, had no adverse effect on cell viability at 30 minutes of incubation with viability maintained at $\geq$ 95%. The deionised SFW extracts used in our experiments with normal T cells showed a time-dependent increase in cell viability of the treated cells. The difference with the results of Fernandes *et al.* (2004) may be as a result of the longer incubation periods utilised in our experiments (24 and 48 hours versus 30 minutes).

Anecdotes from field clinics using SF to treat wasted HIV/AIDS patients have reported improvements in appetite and body weight gains in patients. D-pinitol has been suggested to contribute to the positive effects of SF (Tai *et al.*, 2004). D-pinitol is a glycan sugar

which an isomer of D-chiro inositol, meaning they can work interchangeably. D-chiro inositol has been identified in putative insulin mediator fractions that have hypoglycaemic activity and appears to act downstream in insulin-signalling pathways to mimic the effects of insulin. The two isomers have been shown to lower blood glucose concentration in rats and also increased the rate of glucose disappearance in insulin-resistant and hyper-insulinemic monkeys (Davis *et al.*, 2000).

Therefore there is a possibility that D-pinitol might contribute to the cell proliferation seen in normal T cells (Figures 4.4 and 4.5). This effect might occur via the increased shuttling of glucose or nutrients into cells caused by the insulin like activity of D-pinitol. Increase cellular glucose would be supportive of the metabolic pathways needed for cell proliferation by providing an energy source. Increased metabolism would also increase activity of dehydrogenase and this translates to increased formation of formazan crystals. Such an effect would also require the suppression of the anti-proliferative activity of compounds such as L-canavanine. Cell signalling mechanisms would need to play an important role in such response machineries. Cytokines play an important role in such a process; whether a cell would divides and replicates or undergoes senescence. Although the mechanism is unknown, SF active compounds may affect cell signalling coordination in different ways according to the metabolism of the specific cell line.

Another important observation in the MTT assay results was the fact that there was no statistically significant difference between the SFE extract treated isolated normal T cells and the vehicle treated isolated normal T cells (Figures 4.4 and 4.5). The initial increase in cell viability of SFE extract treated cells was mirrored by an increase in viability of vehicle treated cells. The reverse was observed after 48 hours as both sets of treatment type

decrease cell viability. *Sutherlandia frutescens* ethanol extracts have been used successfully in malignant cell lines without the ethanol influencing the effects of the plant (Tai *et al.*, 2004; Stander *et al.*, 2007 as examples). Even in malignant H9 T cells used in parallel with normal T cells in this study there was a statistically significant difference between the SFE extract treated cells and vehicle treated cells.

The effects of ethanol on the SFE extract treated normal T cells were to initially enhance the proliferative effects of SF after 24 hours incubation (Figure 4.4). The composition of ethanol makes this organic solvent a good source of carbon chains for the glycolytic pathway in energy generation. Human lymphocytes cultured with ethanol and subsequently assayed for natural killer (NK) activity to K562 cells have enhanced NK activity compared to lymphocytes cultured without exposure to ethanol. Lymphocytes retained their enhanced cytolytic ability for several hours after removal from the ethanol-containing medium (Rice *et al.*, 1983).

Chronic intake of alcohol is associated with increased risk of hepatoxicity. The ingestion of alcohol produces oxidative stress generating free radicals of oxygen and ethanol. Oxidative stress has been shown to be a precursor of mitochondrial depolarisation leading to apoptosis or necrosis. These processes play an important role in production of injury which appears in the liver and in other organs and tissues (Colomé *et al.*, 2003).

Organic solvents have been used in extracting active ingredients of plant material because of their efficiency in binding these active compounds. If it is assumed that the same effect occurred when SF tablets were extracted with 70% ethanol, then more active compounds were found in the SFE extracts than the deionised SFW extracts. Compounds like L-

canavanine have been shown to be toxic at high concentrations. L-canavanine may be associated with serious toxicities including a systemic lupus erythematous syndrome (Mills *et al.*, 2005[b]). At high doses it blocks any DNA synthesis *in vitro* and affects B cell function in autoimmune mice at low doses (Sia, 2004).

L-canavanine has also been shown to be cytotoxic to PBMCs in culture. The suggested mechanism of L-canavanine toxicity is due it antagonist effects to L-arginine and the metabolic conversion of L-canavanine to L-canaline, which is toxic to human PBMCs by disrupting polyamine biosynthesis (Bence *et al.*, 2002). Therefore a combination of ethanol and anti-proliferative compounds such as L-canavanine acting synergistically could have resulted in drastic reduction in cell proliferation after 48 hours incubation with the SFE extract dilutions of SF.

4.5.2 Luminescent Cell Viability ATP assay

The MTT assay has been previously shown to underestimate the cytotoxicity of various compounds, e.g. the growth inhibitory effects of interferons (IFNs). Cells have also been observed to metabolise the tetrazolium salt when lethally damaged and have lost their ability to exclude vital dyes. Formazan production can also be induced by drugs that cause perturbations of the cell cycle (Cooke and Kennedy, 1999). An ATP based assay was therefore used to confirm the cytotoxicity of SF on cancerous H9 T cells.

The luminescent cell viability ATP assay uses recombinant luciferase to catalyse the following reaction: ATP + d-Luciferan + O_2 → Oxyluciferan + AMP + PPi + CO_2 + Light (560 nm). When ATP is the limiting component in the reaction, the intensity of the emitted

light is proportional to the concentration of ATP. Based on this reaction, the amount of ATP in SF extract dilutions treated H9 T cells was quantified using a luminometer. In H9 T cells incubated for 24 hours, the change in ATP levels was significant for all the samples analysed (p= 0.001) (Figure 4.6, appendix 5 for luminometer readings). Comparing between the samples, only the positive control (CPT) had significantly reduced levels of ATP when compared to the untreated control (p= 0.001). There was no significant difference between the SF treated cells and the untreated control cells (p> 0.05). ATP levels were significantly reduced in the lowest dilution of the SFE extract (1/50) than in the in the vehicle treated cells (p= 0.001). The SFE extract dilutions reduced ATP levels more than the deionised SFW extract dilutions, but this was not statistically significant (p> 0.05).

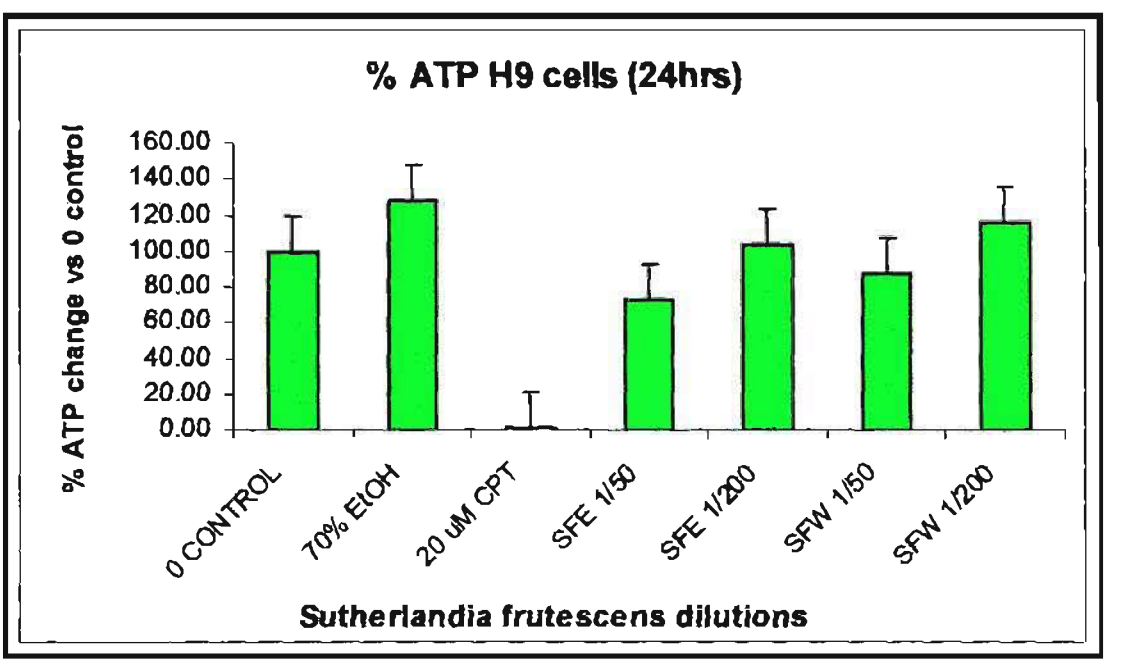


Figure 4.6: Schematic illustration of changes in ATP levels in SF treated H9 cells after 24 hours incubation.

After 48 hours incubation, the concentration of ATP in all the samples was reduced according to the luminescence readings (Figure 4.7). CPT inhibited production of ATP in H9 T cells when compared to the untreated cells (p= 0.001). H9 T cells treated with SFE extract dilutions had significantly (p= 0.001) reduced levels of ATP when compared with the untreated control cells. There was a significant difference (p= 0.001) in ATP levels of the SFE extract dilution treated cells and the vehicle treated cells. The lowest dilution of the deionised SFW extract dilution (1/50 SFW) also reduced the ATP levels significantly (p= 0.001).

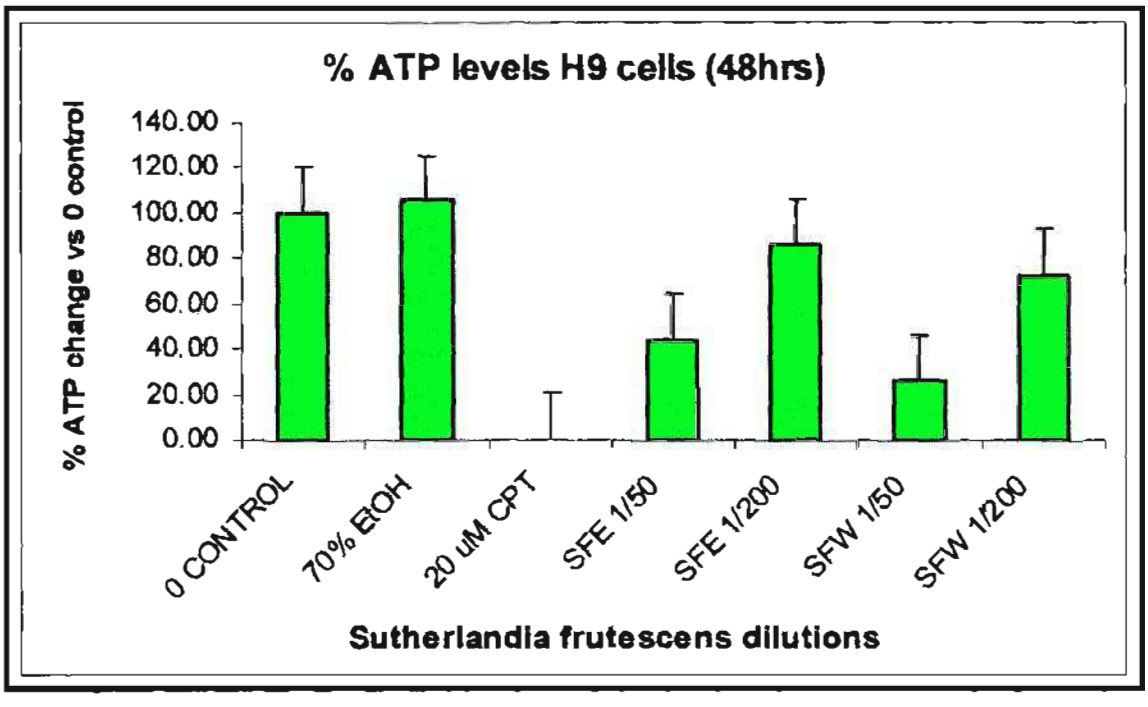


Figure 4.7: A bar graph illustrating the reduced levels of ATP in SF treated H9 cells after 48 hours incubation.

Mitochondria are the sites of oxidative phosphorylation during which substrate oxidation by the electron transport chain builds a proton gradient ($\Delta\psi$ m) and fuels ATP synthesis. Loss of $\Delta\psi$ m is a common event following exposure to toxins leading to cellular necrosis

or apoptosis (Lim *et al.*, 2007). In this study, SF has been shown to have growth inhibitory and cytotoxic effects induced by reduction in levels of ATP in a dose and time-dependent manner. Therefore active ingredients in SF, like most other plant secondary metabolites, target the mitochondria for induction of toxicity leading to cell death.

Mitochondria are not only important in ATP generation but are also a major source of ROS production and the earliest target of drug-induced cellular injury. Excess ROS is capable of reacting with NADH dehydrogenase, aconitase, and other enzymes that may account for inhibition of ATP production (Lim *et al.*, 2007). ATP levels have been shown to determine the mode of cell death. Intracellular ATP levels were measured during cell death and shown to remain unchanged until the very end of the apoptotic process such as disruption of plasma membrane. Since it has been reported that loss of mitochondrial membrane potential which halts mitochondrial ATP production is an early step in apoptosis, intracellular ATP required for the rest of apoptotic processes must be provided from glycolysis (Tsujimoto, 1997). Leist *et al.*, (1997) have shown that intracellular energy levels are dissipated in necrosis but not in apoptosis of neuronal cells. They also report demise of human Jurkat T cells caused by apoptotic triggers (staurosporin and CD95 stimulation) changed from apoptosis to necrosis when cells were pre-emptied of ATP. Therefore, it is possible for apoptosis and necrosis to occur simultaneously in cell cultures exposed to the same insult.

Another determinant of cell death mode might be the concentrations of the toxic reagents or drugs used. The same reagent might induce apoptosis at lower concentrations but induce cell death by necrosis at higher concentrations. This is probably due to the rapid collapse of cellular integrity including plasma membrane disruption, a large calcium influx, as well as

intracellular ATP depletion (Tsujimoto, 1997). That might be the case with treatment of cell cultures with SF. The lowest dilutions for both the SFE and SFW extracts caused the highest depletion of ATP after 48 ours incubations. The high concentrations of the extracts might induce apoptosis during early incubation, but the mode of cell death changes later when ATP reserves are depleted.

4.5.3 Comparison of the effects of *Sutherlandia frutescens* extracts between MTT and ATP depletion assays on H9 cells.

Sutherlandia frutescens reduced cell viability in a time-dependent and dose-dependent mode in the MTT assay. The SFE extract dilutions were more effective in reducing cell viability in the cancerous cell line and the effects were statistically independent of ethanol. The SFW extract dilutions did not significantly affect cell viability after 24 hours incubation but were more effective after 48 hours.

When compared to MTT assay results, the ATP assay produced a very similar trend (Table 4.1). The SFE extract dilutions were more effective in reducing ATP levels in H9 cells than the SFW extract dilutions and the effect was also time and dose-dependent. The SFW extracts also showed a time and dose-dependent reduction in ATP levels, with the effect more pronounced after 48 hours incubation. The similarity in the results obtained with both the MTT and ATP assays gives proof that SF is effective in inducing cell death in malignant H9 cells.

Table 4.1: Comparison of changes in cell viability assayed by the MTT assay and the percentage ATP concentrations measured by luminometry in H9 T cells treated with different dilutions of SF.

**= p value significantly different from the untreated control cells*

Treatment types and Concentrations	**MTT assay (% cell viability vs Control)**		**Luminescent ATP assay (% ATP levels vs Control)**	
	24 hrs	48 hrs	24 hrs	48 hrs
0 Control	100	100	100	100
70% Ethanol	83.44	89.168	128.8	105.65
20 μM CPT	60.68*	10.594*	1.44*	0.17*
1/50 SFE	61.48*	27.345*	72.01*	26.44*
1/200 SFE	73.12*	54.769*	103.37	72.75*
1/50 SFW	88.87	63.567*	86.90	44.30*
1/200 SFW	100.59	78.388	115.43	86.12

4.6 Conclusion

Cytotoxicity and cell viability assays are very important in evaluating the efficacy of purported medicinal plants against cancer models. The tetrazolium (MTT) assay and the luminescent cell viability assay are important assays in measuring metabolic function of cell cultures treated with anticancer agents. These assays were utilised in measuring the cytotoxic effects of SFE and SFW extract dilutions in cancerous H9 T cells and normal T cells.

In the MTT assay, the extracts showed a time and dose dependent cytotoxic effects in H9 T cells. The SFE extract was more effective in reducing H9 cell viability and this effect was independent of ethanol. The effect of the extracts in normal T cells was the reverse of what was observed in cancerous H9 cells, with cell proliferation evident after 24 hours incubation with both types of extracts. The SFE extract was more potent in causing proliferation after 24 hours but this proliferation was shown to be dependent on ethanol when statistically analysis was done. Cell proliferation was reversed in SFE extract treated cells and this was also shown to be dependent on ethanol when compared with vehicle treated cells. The SFW extracts treated normal T cells showed a time and dose dependent increase in cell proliferation.

The luminescent ATP assay was utilised to confirm the cytotoxicity (as shown by the MTT assay) of SF in cancerous H9 T cells. A time and dose dependent decrease in ATP metabolism was observed with both SFE and SFW extracts. The ATP reducing diminishing effects of the SFE extracts were shown to be statistically independent of ethanol. Reduction in ATP metabolism has been shown to correspond with a decrease in cell viability. The luminescent ATP assay results combined with the MTT assay results confirmed that SF is cytotoxic to cancerous H9 T cells and causes cell proliferation in normal T cells in a dose dependent manner.

CHAPTER 5

CASPASE 3/7 ACTIVATY IN H9 T CELLS AND ISOLATED NORMAL T CELLS AFTER TREATMENT WITH *SUTHERLANDIA FRUTESCENS*

5.1 The role of caspase enzymes

Early biochemical events normally dictate the mode of cell death. Cell death can follow two different pathways, i.e. apoptosis or necrosis. Necrosis is associated with acute cellular dysfunction in response to severe stress conditions or exposure to a toxicant. It is a passive process and is associated with a rapid depletion of ATP. Necrosis has been associated with some proteases, mainly calcium-activated or lysosomal proteases. Apoptosis, on the other hand, is a physiological process that occurs during development of multi-cellular organisms, during the immune response, or exposure to stress and xenobiotic agents. Several protease families are implicated in apoptosis, the most prominent being caspases (Samali *et al.*, 1999).

Caspases are cysteine-containing aspartic acid-specific proteases involved in the initiation and execution of apoptosis. Initiator caspases (caspases-6, -8, -9 or -10) are able to activate effector caspases or amplify the caspase cascade by increased activation of effector caspases (caspase-3 and -7). Upon activation, effector caspases act on a variety of cellular proteins to cause arrest of the cell cycle and inactivate DNA repair, inactivate inhibitors of apoptosis, and dismantle the cytoskeleton of cells. During apoptosis, several important proteins such as Bcl-2 family members, cytochrome *c*, and apoptosis protease activation

factor-1 (APAF-1) are closely related to caspases. Pathways to activation of caspases have been shown to involve cellular organelles such as the mitochondria (cytochrome *c* release), endoplasmic reticulum (caspase-12 initiation), death receptors (Fas- or TNF-mediated), and granzyme B (Wang *et al.*, 2005; Samali *et al.*, 1999).

The tumour cells of highly curable malignancies such as leukaemia's, testicular and Wilms cancer easily undergo caspase-dependent apoptosis. Most chemotherapeutic agents have been shown to be potent inducers of apoptosis. However, chemotherapeutic drugs also induce apoptosis in normal cells, limiting their use in cancer therapy (Samali *et al.*, 1999). Recently, it has become clear that inhibition of caspases does not always prevent irreversible loss of cellular function, although it does prevent the acquisition of apoptotic morphology. Alterations in mitochondrial membrane structure and function can occur in a caspase-independent fashion and have a higher predictive value for cell death than caspase activation (Green and Kroemer, 1998).

Detection of caspase activity provides a useful assay for analyzing one of the earliest known biochemical events associated with apoptosis. A variety of fluorogenic caspase substrates have been developed for the detection of functional caspase activity using flow cytometry. Immunocyto-, -histochemistry, and blotting methods have also been utilised to study caspase activation in tissues and cell lines. Enzyme linked immunosorbent assay (ELISA) based methods have also been developed to achieve high throughput in a short space of time. In this study, we utilised a luminometer based method to analyse for caspase-3/-7 activation after treatment of suspension cell lines with SF.

5.2 Caspase-Glo® 3/7 Assay

The Caspase-Glo® 3/7 Assay is a homogenous, luminescent assay that measures caspase-3 and -7 activities. The assay provides a luminogenic caspase-3/-7 substrate, which contains the tetra peptide sequence DEVD, in a reagent optimised for caspase activity, luciferase activity and cell lysis. Addition of Caspase-Glo® 3/7 Reagent in an "add-mix-measure" format results in cell lysis, followed by caspase cleavage of the substrate and generation of a "glow-type" luminescent signal produced by luciferase (Figure 5.1). Luminescence is proportional to the amount of caspase activity present. The Caspase-Glo® 3/7 Reagent relies on the properties thermostable luciferase (Ultra-Glo™ Recombinant Luciferase), which is formulated to generate a stable "glow-type" luminescent signal and improve performance across a wide range of assay conditions (Promega, 2005).

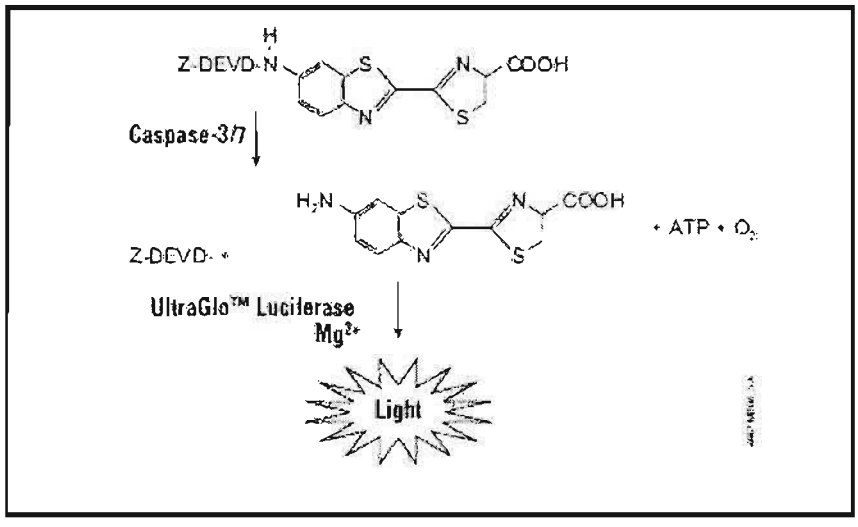


Figure 5.1: Caspase-3/7 cleavage of the luminogenic substrate containing the DEVD sequence which results in luciferase reaction and generation of light (Promega, 2005).

The Caspase-Glo® 3/7 Assay is designed for use with multi-well plate formats, making it ideal for automated high-throughput screening of caspase activity or apoptosis. Cell washing, removing medium and multiple pipetting steps are not required. The caspase and luciferase enzyme activities reach a steady state so that the luminescent signal peaks in approximately one hour and is maintained for several hours with minimal loss of signal. This results in a rapid, sensitive and flexible caspase-3/-7 activity assay (Promega, 2005).

5.3 Materials and Methods

5.3.1 Materials

Caspase-Glo® 3/7 Assay kit was purchased from *Promega* (SA). Modulus™ Microplate Luminometer was purchased from *Turner BioSystems* (USA).

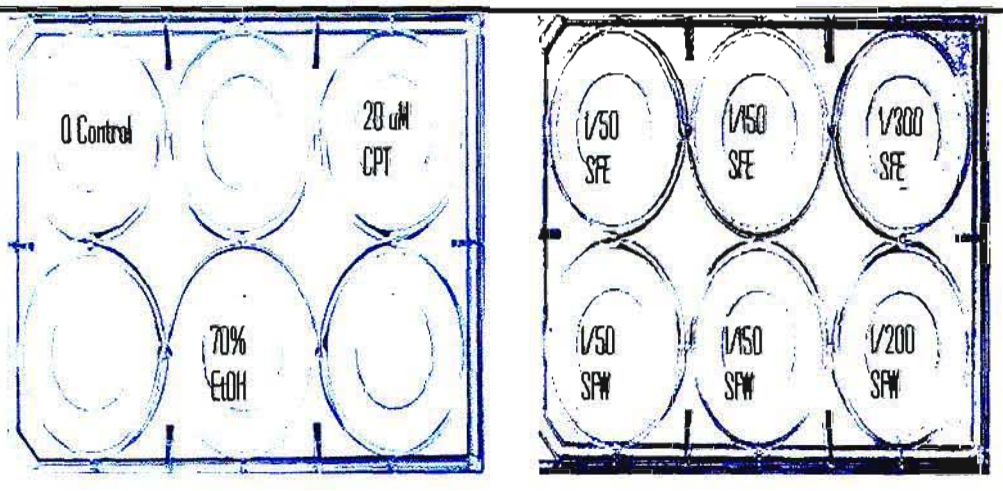
5.3.2 Methods

5.3.2.1 Preparation of the Caspase-Glo® 3/7 Reagent

The Caspase-Glo® 3/7 Assay kit was made up of Caspase-Glo® 3/7 buffer and lyophilised Caspase-Glo® 3/7 substrate. The buffer and substrate were equilibrated to room temperature before use. To make up the reagent, the contents of the Caspase-Glo® 3/7 buffer were transferred into the bottle of Caspase-Glo® 3/7 substrate. This solution was mixed by swirling the contents until the substrate was thoroughly dissolved to form the working reagent.

5.3.2.2 Treatment of H9 cells and isolated normal T cells for Caspase-Glo® 3/7 Assay

H9 T cells and isolated normal T lymphocytes were treated in a similar manner to what was described for the MTT and ATP assays (paragraph 4.4.2.4, chapter 4). The difference was that three different dilutions of each of the SFE and SFW extracts (1/50, 1/150, and 1/300) were utilised to increase the range of concentrations. The vehicle control (70% ethanol) and positive control (20 µM CPT) were also included as before. The incubation periods were kept at 24 and 48 hours. The full illustration of the treatment setup for the Caspase-Glo® 3/7 Assay is in Figure 5.2 below.



CPT = Camptothecin

EtOH = Ethanol

SFE = 70% ethanol extract of *Sutherlandia frutescens*

SFW = deionised water extract of *Sutherlandia frutescens*

Figure 5.2: A schematic illustration of the treatment procedure in 6 well plates for both the H9 cells and isolated T cells in performing the Caspase-Glo® 3/7 Assay.

5.3.2.3 Caspase-Glo® 3/7 Assay

At the end of each incubation period (24 and 48 hours), a 100 μl of each treatment type was pipetted into duplicate wells of a white-walled 96-well plate compatible with the luminometer. This was done for the H9 T cells and isolated normal T cells. A 100 μl of RPMI-1640 was added into separate duplicate wells to serve as a blank for the reaction (Figure 5.3). Preconditioned Caspase-Glo® 3/7 reagent was added into each well at a ratio of 1:1 to the amount of cells in each well. The treated cells and the Caspase-Glo® 3/7 reagent were allowed to equilibrate to room temperature before they were mixed. After mixing the two components, the plate was covered and gently shaken on a plate shaker at 300-500rpm for 30 seconds. The plate was then incubated at room temperature in the dark for an hour. Luminescence for each sample was measured in a plate-reading Modulus™ Microplate Luminometer as directed by the manufacturer (Figure 5.4). The assay was repeated three times to confirm the observed results.

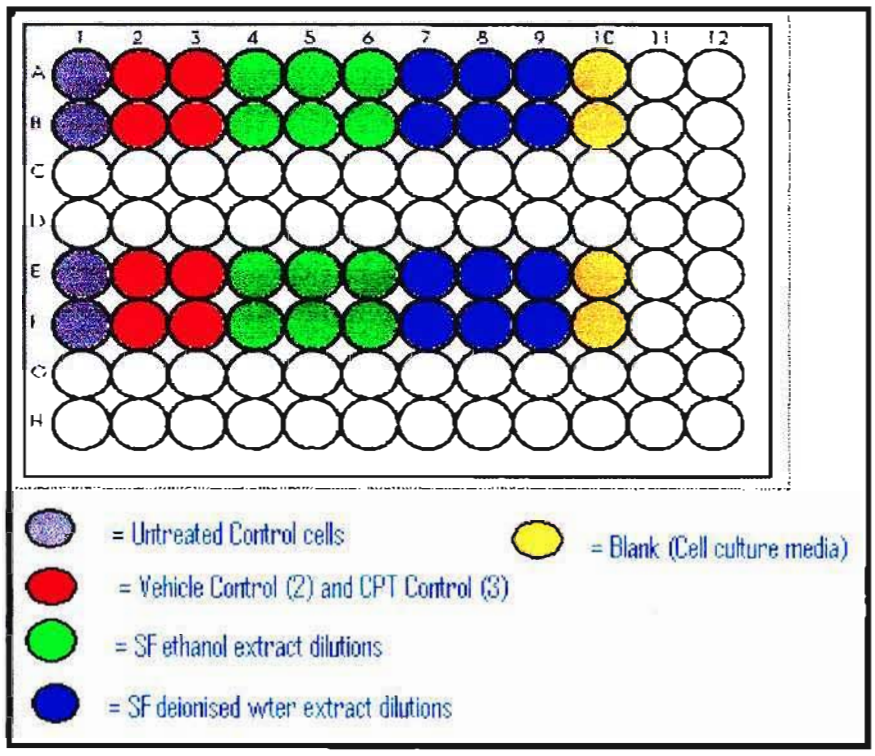


Figure 5.3: An illustration outlining the setup of the caspase assay in the white-walled 96 well plate. The wells from column A to B were for the treatment of H9 cells and columns E to F were for normal T cells.

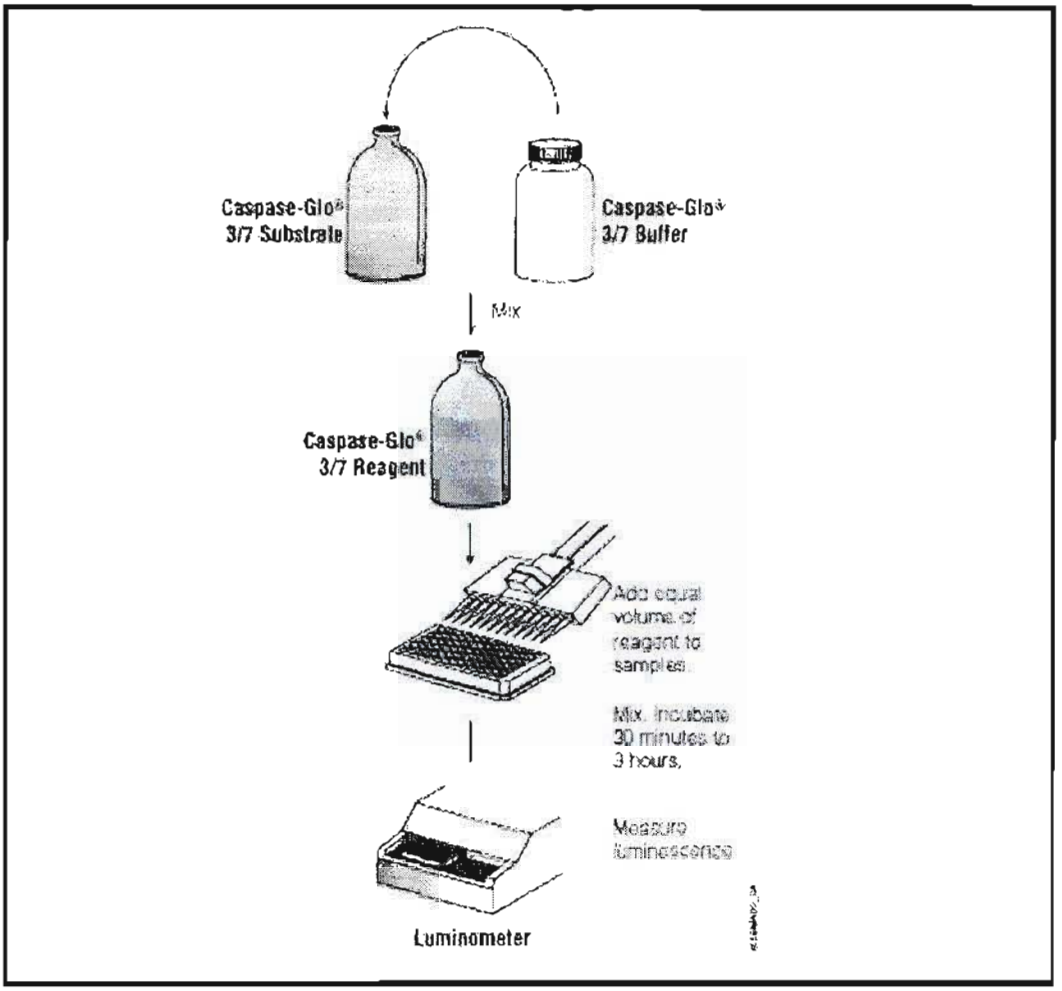


Figure 5.4: A schematic diagram of the Caspase-Glo® 3/7 Assay protocol (Promega, 2005).

5.3.2.4 Statistical analysis

The amount of luminescence obtained for each sample represented the amount of caspase activity detected by the luciferase enzyme. All the obtained luminescence readings were subtracted from the average reading of the blank. Therefore a comparison of the average reading of each sample to the next sample gives the differences in caspase activity between samples. Data analyses were done on *Microsoft Excel* to obtain the averages, percentage caspase activity relative to the untreated control and the standard deviations of each

treatment. The percentage caspase activity was calculated using the formula similar to the one used to calculate cell viability in the MTT assay (paragraph 4.3.2.5.1). The different levels of significances within the separate treated groups were analysed using one-way analysis of variance (*ANOVA*) and the differences between the treated cells and the control cells were analysed using the *Tukey-Kramer* multiple comparison methods. Differences with $p< 0.05$ were considered statistically significant.

5.4 Results and Discussion

H9 T cells exhibited high caspase-3/-7 activity in the absence of an apoptosis inducing agent. This activity did not seem to lead to induction of apoptosis and cell death. This observation was confirmed by repeating the caspase-3/-7 assay with different cultures of H9 T cells which were grown at different times within a space of three months. Incubation of this cell line with a known inducer of apoptosis, CPT, did not increase caspase-3/-7 activity but seemed to decrease it significantly. This decrease correlated with a decrease in cell viability as confirmed by the MTT assay in chapter 4.

One-way analysis of variance in treated H9 T cells found that there was a statistically significant difference ($p< 0.0001$) between the samples after 24 hours incubation (Figure 5.5). The SFE and SFW extract dilutions showed a concentration dependent decrease in caspase-3/-7 activity, with the lowest dilutions exhibiting the larger decrease in enzyme activity. The lowest dilution of the SFE extract significantly ($p< 0.001$) decreased caspase-3/-7 activity compared to the untreated control. There was no significant difference ($p> 0.05$) between the lowest SFE extract dilution and both the positive control (CPT) and vehicle control (70% ethanol). The highest SFE extract dilution (1/300) increased caspase-

3/-7 activity (107%) when compared to the untreated control but this was not significant (p> 0.05). The lowest dilution of the deionised SFW showed no significant (p> 0.05) decrease in caspase-3/-7 activity. The highest SFW extract dilution (1/300) induced the highest caspase-3/-7 activity (121%) but this was not significantly (p> 0.05) different from the untreated control.

In isolated normal T cells the SF extracts also showed a concentration dependent decreased in caspase-3/-7 activity as seen with the H9 T cells (Figure 5.5). The inhibition was significant at higher concentrations for both extracts (p< 0.001). The vehicle control did not change caspase-3/-7 activity when compared to untreated normal T cells but was significantly (p< 0.001) different from SFE extract dilutions. The highest SFW extract dilution significantly (p< 0.01) increased caspase-3/-7 activity (140%) when compared to the untreated control. The positive inducer of apoptosis, CPT, slightly increased caspase-3/-7 activity but this was not significant.

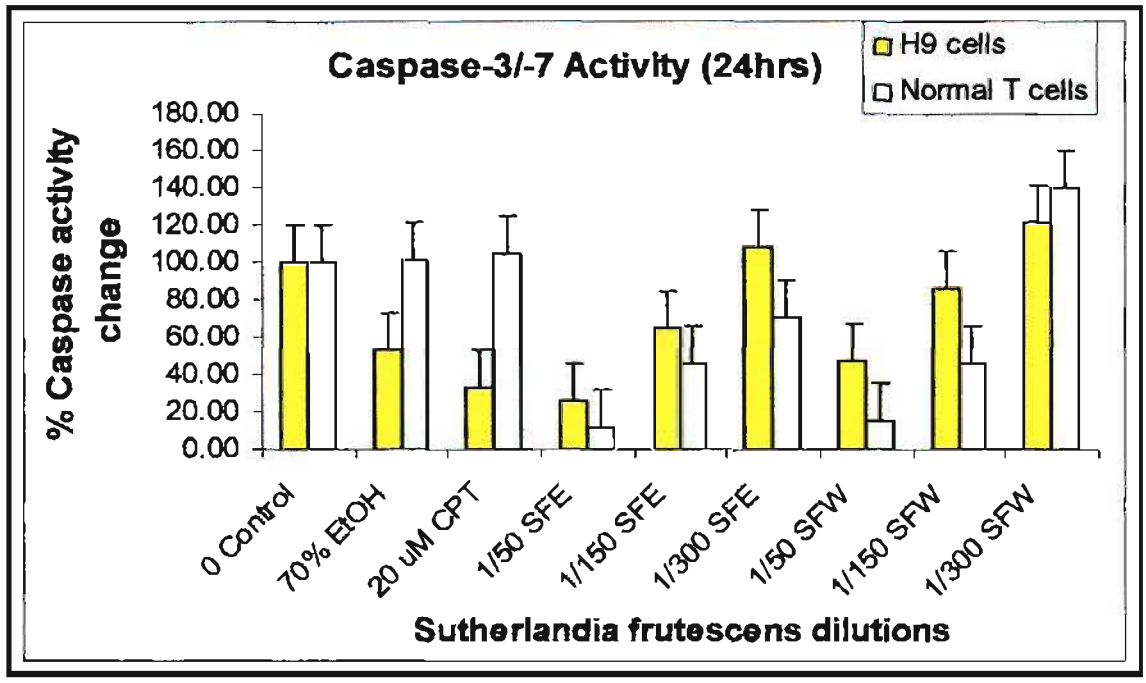


Figure 5.5: A graphical illustration of changes in caspase-3/-7 activity relative to the untreated control cells in H9 T cells and normal T cells after 24 hours incubation with *Sutherlandia frutescens* extract dilutions.

The decrease in caspase-3/-7 activity seen in both H9 T cells and isolated normal T cells after 24 hours incubation with SF extracts was either maintained or increased after 48 hours (Figure 5.6). This trend was concentration dependent with highest concentrations of the SFE extract completely inhibiting caspase-3/-7 activity in both cell lines ($p< 0.001$). The vehicle control was significantly different ($p< 0.001$) from the SFE extract dilutions, meaning the extracts activity was independent of ethanol. In H9 T cells CPT decreased caspase-3/-7 activity in a concentration and time-dependent manner while in normal T cells enzyme activity remained similar to the untreated control cells ($p> 0.05$).

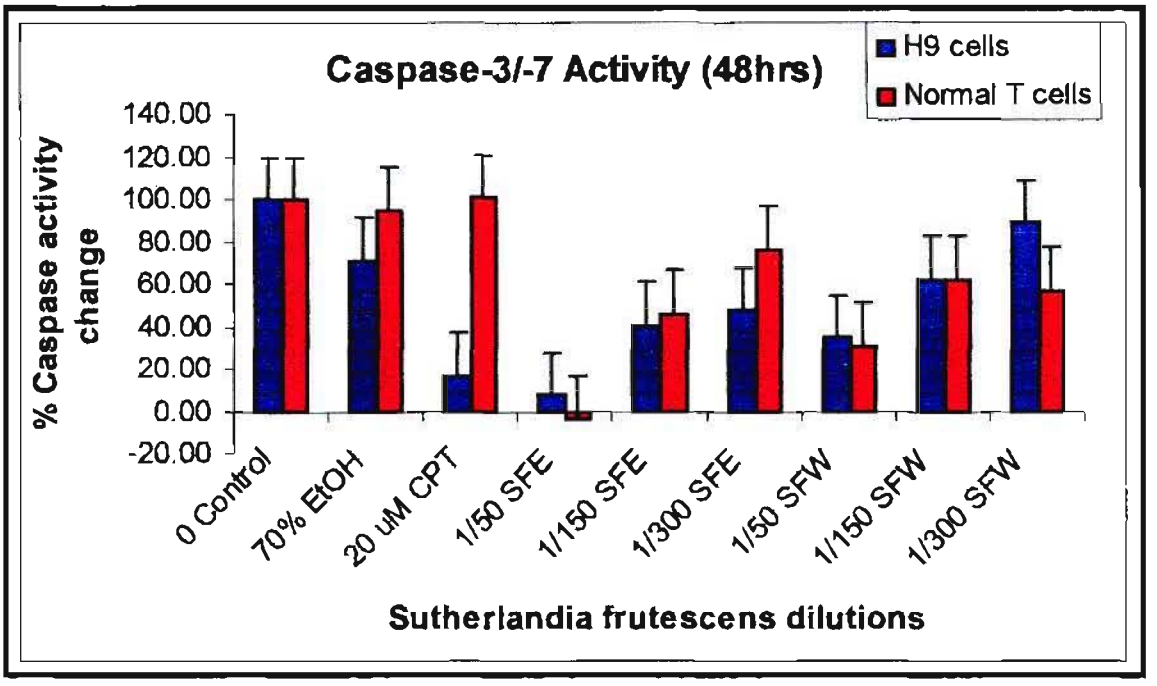


Figure 5.6: A graphical illustration of changes in caspase-3/-7 activity relative to the untreated control cells in H9 T cells and normal T cells after 48 hours incubation with *Sutherlandia frutescens* extract dilutions.

A comparison of the effects of SF extracts on both H9 and normal T cells showed that higher concentrations of both extracts decreased the caspase-3/-7 activity in both cell lines. This was regardless of the effects of the extracts on cell viability (as seen in the MTT assay and ATP assays in chapter four). The MTT assay showed that SF decreased cell viability in H9 T cells while it induced cell proliferation in normal T cells. Therefore the decreased caspase-3/-7 activity had little to do with changes in cell viability but more to do with the effects of SF on the caspase-3/-7 enzymes directly. *Sutherlandia frutescens* extracts inhibited activation of caspase-3/-7 enzymes in concentration and time-dependent manner.

The pharmacokinetics of SF have not been fully studied. Harnett *et al.*, (2004) looked at the anti-HIV activities of organic and aqueous extracts of SF. Their results showed that SF

extracts contained active ingredients against the reverse transcriptase (RT) enzyme of HIV. The aqueous extracts retained ±30% inhibitory activity of RT in the presence of bovine serum albumin (BSA). The study did not reveal the active ingredients of SF that were responsible for inhibition of RT. Inhibition of the RT enzyme was assumed to be a potential mechanism for the clinically seen improvements o AIDS sufferers when administered with SF.

The effects of SF on antiretroviral (ARVs) metabolism were studied on microsome-based *in vitro* fluorometric microtitre assay. Extracts from SF showed significant inhibition of cytochrome P450 3A4 (*CYP 3A4*) metabolism. The extracts activated the modulator of *CYP3A4* expression, pregnane X receptor, approximately twofold and also inhibited the activity of the P-glycoprotein enzyme. These results identified the potential for clinically significant drug interactions. They suggested that co-administration of ARVs and SF may result in early inhibition of drug metabolism and transport followed by the induction of drug exposure with more prolonged therapy. These bidirectional drug interactions may put patients at risk of treatment failure, viral resistance or drug toxicity (Mills *et al.*, 2005[b]).

The effect of SF extracts on caspase-3/-7 activity and expression has not been studied. Activation of effector caspases like caspase-3/-7 has been purported as one of the 'points of no return' in the apoptotic process. Using cell cycle analyses in various cell lines, Tai *et al.*, (2004) showed that SFE extract dilutions of SF tablets arrested the cell cycle at various phases in different cell lines. It was suggested that various "actives", not a single compound, in the extracts induced cell cycle arrest in these cell lines. Chinkwo (2005) demonstrated that SF extracts induced apoptosis in three different cell lines (CHO, Caski and Jurkat T cells). This apoptosis was indicated by the flip-flop translocation of

phosphotidylserine to the outer membrane. The above mentioned studies suggest that SF can induce apoptosis but whether this involved activation of caspse-3/-7 was not indicated.

The use of caspase inhibitors has revealed that other pathways for apoptosis may be initiated. Effector caspases are activated at late stages of the apoptotic process, after mitochondrial outer membrane permeability (MOMP) disruption. In this case caspase activation constitutes a sign rather than a mechanism of cell death and the decisive event has occurred upstream or at the level of MOMP, which frequently marks the 'point of no return' of the lethal process. When inhibition of caspases does not restrain cell death, this often leads to a shift in the morphology of cell death from the aspects of classical apoptosis to the occurrence of apoptosis-like autophagic cell death or even necrosis (Kroemer and Martin, 2005).

Although caspase-independent cell death is not well studied compared to caspase-dependent apoptosis, several mechanisms have been suggested. ROS production is required for caspase-independent cell death in many different types of cells. The poly (ADP-ribose) polymerase-1 (PARP-1) hyperactivation-mediated depletion of cellular NAD^+ and its precursor ATP is responsible for DNA damage-reagent-induced caspase-independent cell death. It has been reported that PARP-1 hyperactivation prompts mitochondrial dysfunction, which in turn releases apoptosis-inducing factor (AIF) from the mitochondria to the nucleus. AIF induces DNA and chromatin condensation in the absence of DNA fragmentation (Xu *et al.*, 2006; LeBlanc, 2003).

The mechanisms of caspase-independent cell death described above would apply to the higher concentrations of SF (1/50, 1/150) which were seen to significantly inhibit caspase-

3/-7 activity. Higher dilutions of the extracts were shown to either not change the caspase-3/-7 activity or increase the activity of the enzymes when compared to the untreated control samples. As was the case with the effects of SF on ATP metabolism in H9 T cells, the modes of cell death might be affected by the concentration of the cytotoxic compound used (Tsujimoto, 1997). *Sutherlandia frutescens* might induce apoptosis at lower concentrations while higher concentrations might induce cell death by some form of necrosis due to the rapid MOMP disruption, ROS generation, ATP depletion, and inhibition of caspase-3/-7.

Sutherlandia frutescens is supposed to be an efficient anticancer medicinal herb. Inhibition of caspase-3/-7 activity by higher concentrations of SF extracts is against the notion of induction of the more favourable apoptotic pathway of cell death in cancerous cells. Use of SF along with apoptosis-inducing chemotherapeutic agents might lead to drug interactions. Chemotherapeutics targeting pathways leading to effector caspase activation might be inhibited, which ultimately cause treatment failure. On the other hand, caspase inhibition might lead to induction of other forms of cell death which are not only specific for cancerous cells but also target normal cells. Therefore, there is a need to find concentrations or dosages of SF which will induce enough cell death in cancerous cells without affecting other therapeutic agents or inducing cell death in normal cells.

Sutherlandia frutescens has been shown to interact with other enzymes and other cell components. Most of the studies on the pharmacokinetics of SF have not specified the active ingredient(s) responsible for inhibiting cellular enzymes. L-canavanine might be considered a candidate for inhibiting these enzymes because of it mechanism of action. Its ability to antagonise L-arginine in protein synthesis might lead to formation of defective

enzymes/proteins and therefore malfunction. A study by Jang *et al.*, (2002) showed that L-canavanine induced apoptotic cell death in Jurkat T cells. This apoptosis was accompanied by several biochemical events such as caspase-3 activation, degradation of PARP, and DNA fragmentation. The results of this study effectively exclude L-canavanine as an inhibitor of caspase-3/-7 activity as seen in the current study.

Prevoo *et al.*, (2008) recently demonstrated on the influence of SF on adrenal steroidogenic cytochrome P450 enzymes. Using differential fraction to study the influence of each active ingredient on substrate binding, they showed that SU1 triterpenoid glucosides fraction was more effective in inhibiting enzyme activity while L-canavanine and the other compounds did not affect substrate binding to *CYP17* and *CYP21*. Therefore, it is possible that high concentrations of the SU1 triterpenoid glucosides may be responsible for the inhibition of caspase-3/-7 activity demonstrated by this study.

5.5 Conclusion

In this experimental setup, the results obtained have shown that higher concentrations of SF have an inhibitory effect on caspase-3/-7 activity *in vitro*. Cell viability assays in the previous chapter have indicated that inhibition of effector caspases does not prevent cell death in cancerous H9 T cells. Therefore, inhibition of these caspases may have activated alternative pathways of cell death.

CHAPTER 6

OXIDANT SCAVENGING PROPERTIES AND ANTIOXIDANT PROMOTING ABILITIES OF *SUTHERLANDIA FRUTESCENS* EXTRACTS ON H9 T CELLS AND NORMAL T CELLS

6.1 Introduction

Oxidative stress can damage many biological molecules like proteins and DNA, which are often more significant targets of injury than are lipids, and lipid peroxidation often occurs late in the injury process. Free radicals are the main source of oxidative stress. A free radical can be defined as any species capable of independent existence that contain one or more impaired electrons, an impaired electron being the one that is alone in an orbital. Examples of free radicals include superoxide ($O_2^{\cdot-}$) and hydroxyl ($OH^{\cdot}$) - both which are oxygen centred radicals ($RS^{\cdot}$, a sulphur centred radical), nitric oxide ($NO^{\cdot}$, in which the unpaired electron is delocalised between both atoms), etc. A feature of the reaction of free radicals with non-radicals is that they usually proceed via chain reactions: one radical begets another. Lipid peroxidation is the most studied biologically relevant free radical chain reaction (Halliwell and Chirico, 1993).

Lipids are oxidised by three distinct mechanisms; enzymatic oxidation, non-enzymatic, free radical mediated oxidation, and non-enzymatic, non-radical oxidation. The relative susceptibilities of lipids to oxidation depend on the reaction milieu as well as their structure. Both isolated polyunsaturated fatty acids (PUFAs) and those incorporated into lipids are readily attacked by free radicals, becoming oxidised into lipid peroxides. By

contrast, both monosaturated and saturated fatty acids are more resistant to free radical attack. Lipid hydroperoxides are formed as the major primary products of oxidation, however, they are substrates for enzymes and they also undergo various secondary reactions. The occurrence of lipid peroxidation in biological membranes cause impairment of membrane functioning, changes in fluidity, inactivation of membrane-bound receptors and enzymes, and increased non-specific permeability to ions such as calcium (Ca^{2+}) (Niki *et al.*, 2005; Halliwell and Chirico, 1993).

Direct exposure of cells to oxidants such as hydrogen peroxide or redox-cycling quinones causes multiple intracellular alterations, including elevation of Ca^{2+}, depletion of ATP, and oxidation of NADH, reduced glutathione (GSH) and lipids. Necrosis is the characteristic end point of such a dramatic disturbance in cell homeostasis. Lower concentrations of oxidants are known to promote the alternative apoptotic death pathway. However, recent experiments demonstrating apparently normal apoptosis even when cells are cultured at low oxygen tensions show that ROS cannot be essential mediators of apoptosis. Depletion of antioxidants plays a more important role in cell death (Slater *et al.*, 1995).

It is now well established that ROS are continuously produced *in vivo*. Consequently, organisms have evolved not only antioxidant defence systems to protect against them but also repair systems that prevent the accumulation of oxidatively damaged molecules. The human body has a multiplicity of different antioxidant defences. However, probably because some free radicals and other ROS play vital biological roles, antioxidant defences are not 100% effective. Depletion of antioxidant defences and/or rises in ROS production can tip the ROS-antioxidant balance and cause oxidative stress, which may result in tissue injury (Halliwell and Chirico, 1993).

The extent of lipid peroxidation can be determined by measuring 1) losses of unsaturated fatty acids, 2) amounts of primary peroxidation products, and 3) the amount of secondary products, such as carbonyls and hydrogen gases. The detection and identification of lipid peroxidation products has been performed by using various techniques such as co-ordination ion-spray mass spectrometry (CSI-MIS) and electrospray ionization (ESI) or matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry. Their detection has been successfully accomplished in several *in vitro* systems, including cell culture and in *in vivo* systems (Halliwell and Chirico, 1993).

To learn as much as possible about the occurrence of lipid peroxidation in biological materials, it is important to use techniques that give specific chemical information about what is present. High pressure liquid chromatography (HPLC) is the most preferred method for doing this but it is quite a tedious assay (Halliwell and Chirico, 1993; Niki *et al.*, 2005). The thiobarbituric acid reactant substance (TBARS) content, indicative of lipid peroxidation, was studied in T cell lines treated with extracts of SF for our experiments.

Antioxidant defences may be divided into four categories: prevention of the formation of active oxidants, scavenging, quenching and removal of active oxidants, repair of damage and excretion of toxic oxidant products, and adaptive responses. The inhibition of enzymatic lipid oxidation may be achieved by either the activation or reaction of the enzyme. Free radical mediated lipid peroxidation may be inhibited by the inhibition of chain initiation and chain propagation and/or acceleration of chain termination. Many natural and synthetic supplements and drugs with radical-scavenging capacity have been explored. The antioxidant activity *in vivo* is determined by several factors, such as reactivity towards radicals, absorption, distribution, localization and mobility of oxidant,

and interaction with other antioxidants. Functions that are independent of antioxidant action, such as induction of phase 11 biotransformation enzymes, may also be important in the total defence network against oxidative stress (Niki *et al.*, 2005).

Evidence has been presented that oxidative stress may influence the function of the immune system of humans and experimental animals and modify the pro-oxidant capacity of macrophages and neutrophils. These effects may cause a decrease in the function of these cells and modify the immune response to viruses and bacteria. Chemical antioxidants such as β-carotene, vitamin E, vitamin C and reduced glutathione (GSH) improve the proliferative capacity of lymphocytes and increase host defence and immunoglobulin synthesis (Periera *et al.*, 1998). Antioxidant enzymes activities can be studied using various spectrophotometric, ELISA or molecular expression methods. We utilised a luminometer based assay to study changes in GSH levels in T cell lines treated with extracts of SF.

6.2 Thiobarbituric acid reactant substances (TBARS) assay

The sensitivity of measuring TBARS has made this assay the method of choice for screening and monitoring lipid peroxidation. It is popular because of it simplicity and cheapness. Although much controversy has appeared in the literature regarding the specificity of TBARS toward compounds other than malondialdehyde (MDA), it remains one of the most widely employed assays to determine lipid peroxidation. If lipoprotein fractions are first acid precipitated from the sample, interfering soluble TBARS are minimized (Oxi-Tek, 2002; Halliwell and Chirico, 1993).

The principle of the assay is that lipid hydroperoxides can degrade into, among other things, malondialdehyde, which forms 1:2 adduct with thiobarbituric acid (TBA) (Figure 6.1). The result is a coloured product with a maximum absorbance at 532 nm. Butylated hydroxytoluene (BHT) is added to the TBA solution to prevent oxidation in the reaction during heating. The pH for the reaction is 1.5 and heat is added to stimulate the reaction. Butanol is used to separate the lipid-TBA products from the unreacted TBA. Finally, spectrophotometric measurements are made at both 532 nm (maximum absorbance for TBARS) and 600 nm. The absorbance at 600 nm is due to the solution and will be subtracted from the absorbance at 532 nm. Absorbances from the blank reaction are measured to account for substances that may also result in a signal at 532 nm (Oxi-Tek, 2002).

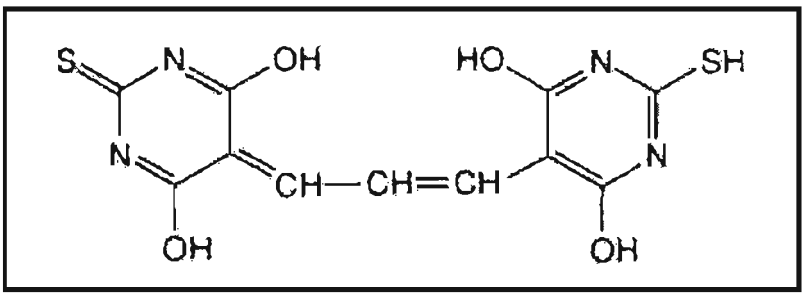


Figure 6.1: The MDA-TBA adduct (Oxi-Tek, 2002).

6.3 GSH-Glo™ Glutathione Assay

Reduced glutathione, the most abundant non-protein thiol, is an antioxidant found in eukaryotic cells. The presence of glutathione is required to maintain the normal function of the immune system and is also critical for the proliferation of lymphocytes. Cells of the immune system are also known to produce free radicals as part of their normal function, resulting in a need for higher concentrations of GSH than other cells. Oxidative stress and

free radicals can cause a decrease in GSH levels either by oxidation or reaction with thiol group. A change in GSH levels is important for assessing toxicological responses and can promote oxidative stress, potentially leading to apoptosis and cell death (Promega, 2007).

The GSH-Glo™ Glutathione Assay is a luminescence-based assay for the detection and quantification of glutathione. The assay is based on the conversion of a luciferin derivative into luciferin in the presence of GSH, catalysed glutathione-S-transferase (GST). The signal generated in a coupled reaction with firefly luciferase is proportional to the amount of GSH present in the sample. The assay generates a stable luminescent signal and is simple, fast and easily adaptable to multiwall formats such as 96-well plates. This assay can be used for detection and quantification of GSH in cultured cells or various biological samples. The GSH-Glo™ Glutathione Assay can be applied for measuring glutathione levels as an indicator of cell viability or oxidative stress. It can also be applied for screening drugs and new chemical entities for their capacity to modulate glutathione levels in cells (Promega, 2007).

Detection of GSH using the GSH-Glo™ Glutathione Assay is the result of the combination of two chemical reactions (Figure 6.2). The first reaction involves the generation of luciferin from a luminogenic substrate, catalysed by GST in the presence of GSH. The luciferin produced in the first reaction is detected as a luminescent signal generated by the luciferase enzyme. The second reaction is initiated by the addition of an equal volume of Luciferin Detection Reagent, which simultaneously stops the GSH-Glo™ reaction and initiates a luminescent signal that is directly proportional to the amount of luciferin formed in reaction 1 (Promega, 2007).

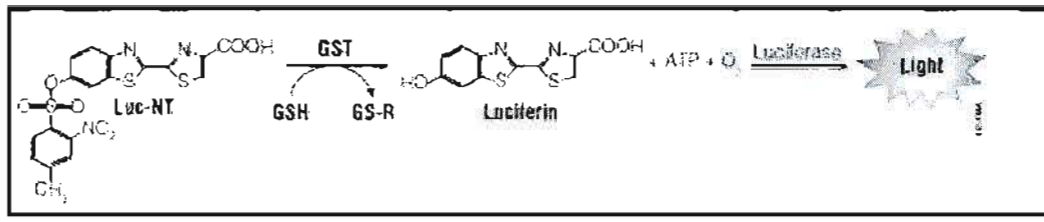


Figure 6.2: The two reactions of GSH-Glo™ Glutathione Assay (Promega, 2007)

We therefore aimed to analyse for the oxidant scavenging properties of SF in supernatants of isolated normal T cells and H9 T cells. The second objective was to analyse for the ability of SF to change GSH levels in the intracellular environment of both the normal and cancerous T cells.

6.4 Materials and Methods

6.4.1 Materials

Butylated hydroxytoluene (BHT), thiobarbituric acid (TBA), phosphoric acid (H_3PO_4), hydrochloric acid (HCl) and sodium hydroxide (NaOH) were purchased from *Sigma Aldrich* (SA). GSH-Glo™ Glutathione Assay Kit was purchased from *Promega* (USA).

6.4.2 Methods

6.4.2.1 TBARS assay

Supernatants collected from treated cells used for the MTT assay and stored at -70^{0}C were used for the TBARS assay. These supernatant samples were thawed and then kept on ice

until the beginning of the assay. The rational behind the use of supernatant is based on the fact that when lipid peroxides are formed in cell membrane, they are released into the surrounding environment of the cells. The use of supernatants in the *in vitro* system is similar to the use of serum and plasma for analysis of lipid peroxidation *in vivo*.

For each treatment concentration a sample and blank test tube was prepared by addition of 200 µl 7% H_3PO_4 into each tube. In the sample test tube 400 µl TBA/BHT solutions was added and in the blank test tube 400 µl 3 mM HCl was added instead. 400 µl of the supernatant was added into both sample and blank test tubes and they were vortex vigorously. Both sets of test tubes were pH to 1,5 using 1 M HCl and 1 M NaOH. The solutions were heated at 100^0C for 15 minutes and then left to cool to room temperature.

Butanol (1.5 ml) was added into each tube and the solutions were vortexed for 40-60 seconds. The solutions separated into two phases after vortexing and butanol (top) phase was transferred into eppendorf centrifuge tubes. The tubes were centrifuged at 13 200 rpm for 6 minutes. Absorbance of the butanol phase was measured at 532 nm and 600 nm on a spectrophotometer. The final absorbance of the sample was calculated by subtracting the absorbance reading of the blank from that of the corresponding sample. The concentration of lipid peroxides was calculated using the following formula:

Concentration = Absorbance/absorptivity coefficient

The absorptivity coefficient for TBARS given by Dr Marcelo is 156 mM^{-1} (Ramos-Vasconcelos and Hermes-Lima, 2002).

6.4.2.2 GSH-Glo™ Glutathione Assay

6.4.2.2.1 Preparation of GSH-Glo™ and Luciferin reagents

The GSH-Glo™ reagent for suspension cultures was prepared by diluting the Luciferin-NT substrate and Glutathione-S-Transferase 1:50 in GSH-Glo™ reaction buffer. Each reaction (well) of a 96-well plate required 50 μl of the GSH-Glo™ reagent; the total volume of the reagent prepared was adjusted according to the number of assay wells. The GSH-Glo™ reagent needed to be prepared immediately before use and could not be stored for future use.

The luciferin detection reagent was prepared by transferring the contents of one bottle of luciferin detection buffer to the bottle of lyophilised luciferin detection reagent. The contents were mixed by inversion several times until the substrate was thoroughly dissolved. Each reaction (well) of a 96-well plate required 100 μl of the luciferin detection reagent meaning the luciferin detection reagent needed to be 2:1 with the GSH-Glo™ reagent for reaction to work properly. The prepared luciferin detection reagent could be stored for up to 4 weeks at -20^{0}C.

6.4.2.2.2 Treatment of cell lines and layout of the GSH-Glo™ Glutathione Assay

H9 and isolated normal T cells were treated in a similar manner as in the caspase-3/-7 assay but without the CPT control samples (paragraph 5.3.2.2). Three different dilutions of each extract of SF (1/50, 1/150, and 1/300) was used to treat the cell lines. The vehicle

control (70% ethanol) was also included. The incubation periods were kept at 24 and 48 hours.

After the end of each incubation period, 1 ml of each treatment type was transferred into labelled eppendorfs and centrifuged to pellet the cells. The supernatants were removed and stored while the treated cells were washed 2× by centrifugation using PBS. The media was removed because cell culture media has been shown to interfere with the GSH-Glo™ Glutathione Assay kit reagents and decrease their sensitivity. After the last PBS wash was removed, 100 µl of PBS was added into each eppendorf and vortexed thoroughly. 50 µl of each treatment was then transferred into duplicate wells of a white-walled 96-well plate (Figure 6.3). 50 µl/well of prepared GSH-Glo™ reagent was added into the treated cells. The solution was mixed by shaking on a plate shaker and incubated for 30 minutes at room temperature in the dark.

At the end of this incubation period, prepared luciferin detection reagent was added (100 µl/well) to stop the GSH-Glo™ reaction and initiate the luminescence to be detected by the luminometer. The plate was shaken again and incubated for 15 minutes at room temperature in the dark. Luminescence was read using the Modulus™ Microplate Luminometer from Turner BioSystems (Figure 6.4). The assay was repeated three times to show repeatability of results.

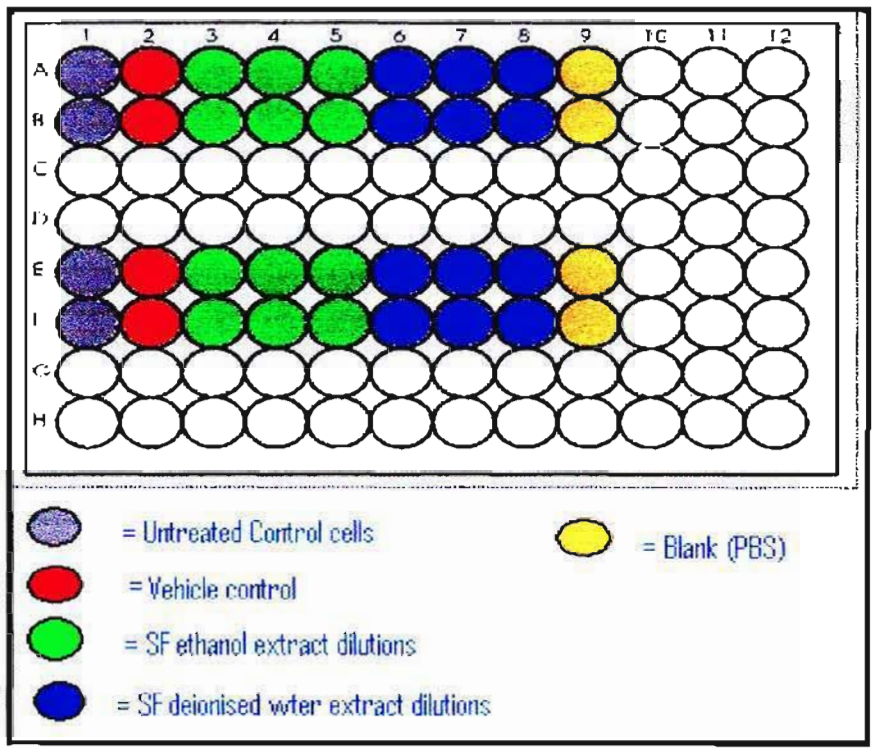


Figure 6.3: *An illustration outlining the set up of the GSH assay in the white-walled* 96 well plate. The wells from column A to B were for the treatment of H9 cells and columns E to F were for isolated normal T cells.

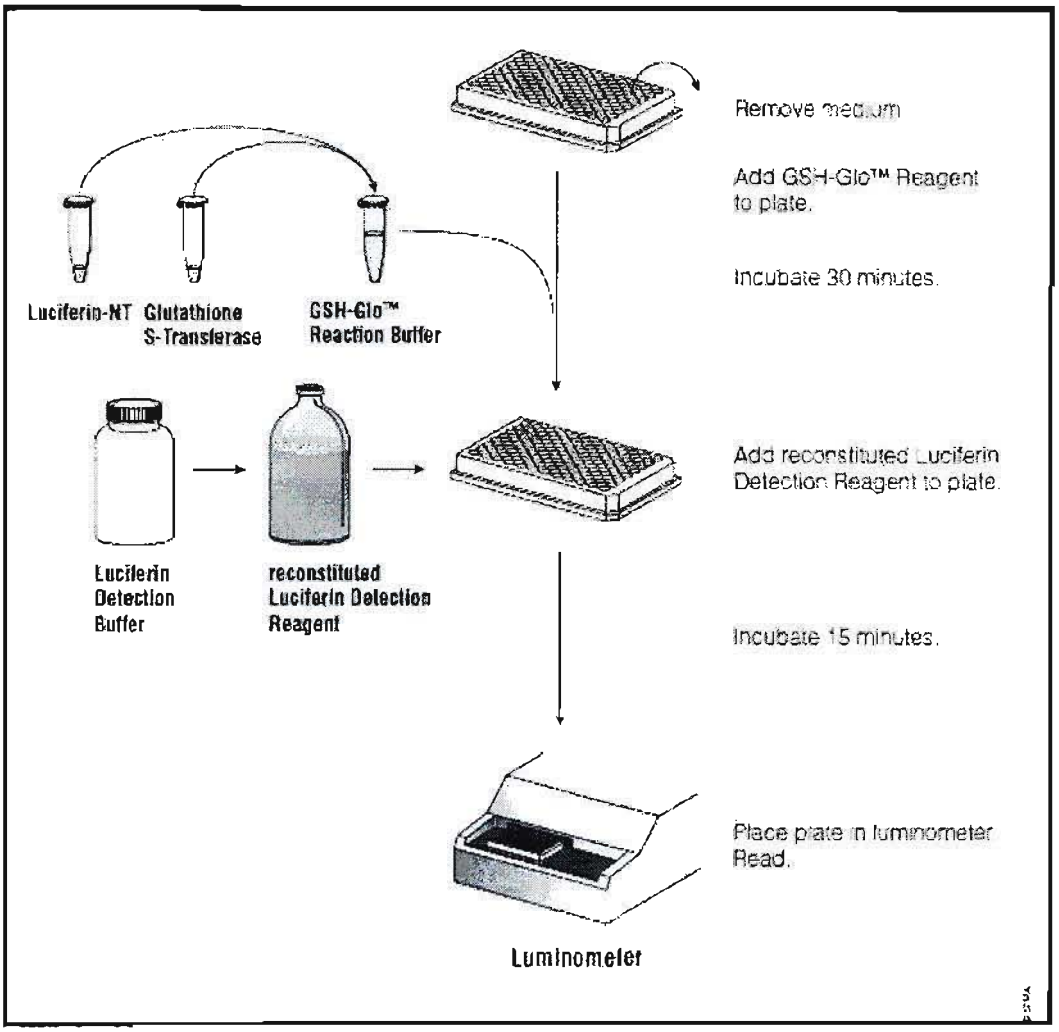


Figure 6.4: An outline of the steps involved in the GSH-Glo™ Glutathione Assay (Promega, 2007).

6.5 Results and Discussion

6.5.1 TBARS assay

The lipid peroxide concentrations in the SF extracts treated samples were compared to the untreated control samples and the SFE extracts was also compared to the vehicle control (70% ethanol). After 24 hours incubation, there was an extremely significant difference ($p< 0.0001$) between all the samples grouped together as calculated by *ANOVA* (Figure 6.5). There was a very significant difference ($p< 0.001$) between the lipid peroxide concentrations in the treated samples and the untreated control samples. This was regardless of the SF extraction method and the type of cell line under analysis. Sutherlandia frutescens ethanol extract dilutions exhibited lipid peroxides scavenging properties which were significantly enhanced ($p< 0.001$) than the vehicle control, again showing the independent action of the extracts.

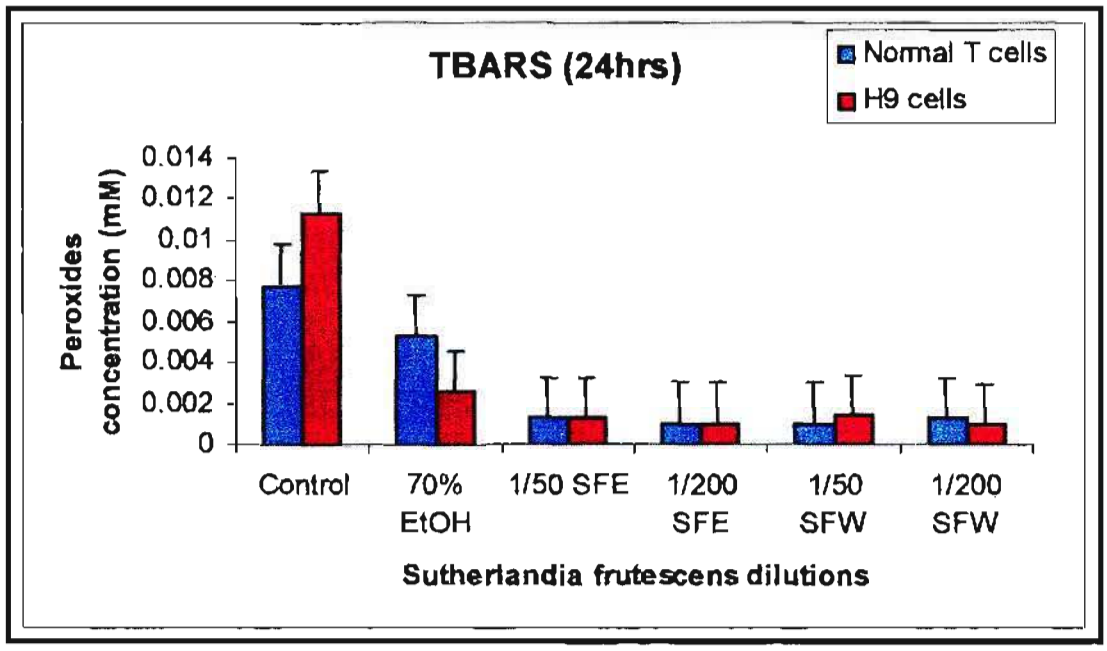


Figure 6.5: A bar graph illustrating the changes in supernatant lipid peroxides after 24 hours incubation of H9 and normal T cells with extract dilutions of *Sutherlandia frutescens*.

After 48 hours incubation with both H9 and normal T cells, the SF extracts still showed a significant ($p< 0.001$) lipid peroxide scavenging properties when compared to the untreated control (Figure 6.6). The extracts were more effective in removing the lipid peroxides in the normal T cells supernatants than in H9 cells but this difference was not significant ($p> 0.05$). The SFW extracts was able to maintain lipid peroxide concentration at similar levels as there were after 24 hours incubation. The SFE extracts lost some of the lipid peroxides scavenging properties after 48 hours but they were still significantly lower than the untreated control. The loss of lipid peroxide scavenging properties in the SFE extracts coincided with a significant drop in peroxides in the vehicle control of the H9 cell supernatants. This significant difference ($p< 0.01$) showed the SFE extracts had lost their scavenging properties.

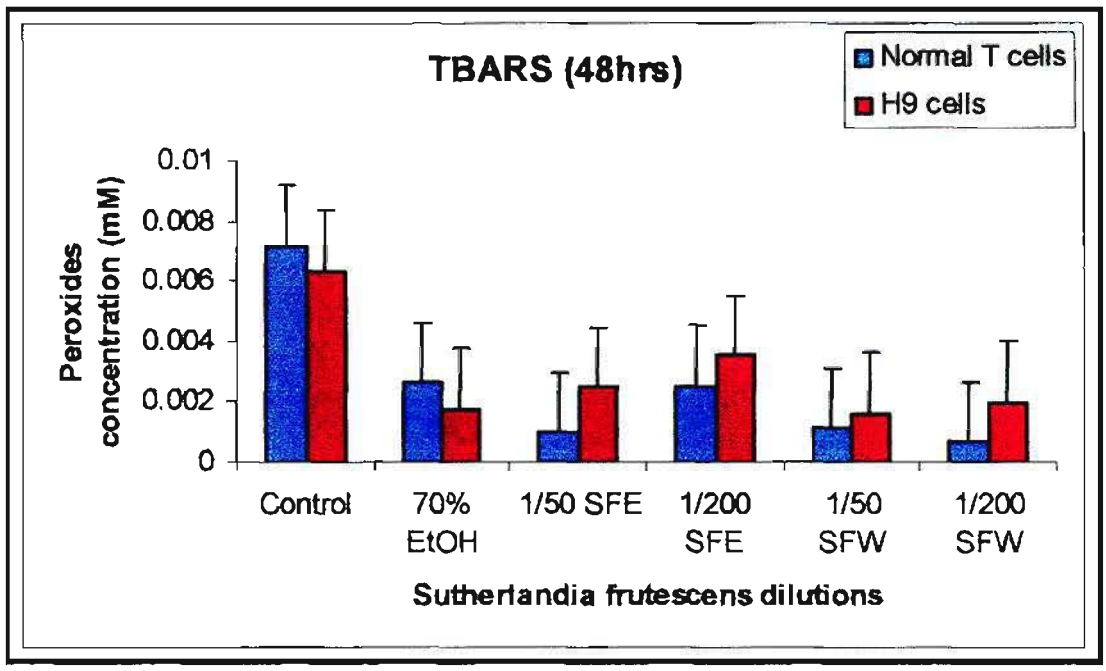


Figure 6.6: A bar graph illustrating the changes in supernatant lipid peroxides after 48 hours incubation of H9 and normal T cells with extracts of *Sutherlandia frutescens*.

Molecular oxygen and its reaction products can cause injury to biological organisms in different ways. ROS products induce point mutations, deletions and gene amplification and rearrangement in mammalian cells, which may induce proto-oncogene activation and/or tumour suppressor gene inactivation. They are thought to play a role in the multiple steps leading to carcinogenesis. Free radical alterations of unsaturated lipids in cell membranes may result in loss of membrane fluidity and eventually cell lysis. TBARS is one of the aldehyde products of ROS degradation of membrane lipids and these aldehydes can cause cross links in nucleic acids, proteins and lipids (Tuzgen *et al.*, 2003).

These results have shown that treatment of H9 and normal T cells with SF extracts for up to 48 hours reduced the level of lipid peroxides in supernatants. These findings are in agreement with the traditional use of this plant as an antioxidant. They are also in

agreement with some of the *in vitro* and *in vivo* studies which used SF in studying different aspects of oxidative stress reduction. Fernandes *et al.*, (2004) looked at the hydrogen peroxide and superoxide radical scavenging properties of SF plant-extracts in phagocytes in a cell free system. The results indicated that hot SFW extracts possessed ROS scavenging properties at very low concentrations. It was concluded that these scavenging activities could account for some of the anti-inflammatory properties of this medicinal plant.

Using another cell free system, Tai *et al.*, (2004) demonstrated the antioxidant activity of SF in reducing free radical activity. The reduction of 2,2_-azinobis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) radical by the 0.5 μl SFE extracts was found to be equivalent to 10 μM of Trolox, a chemical with potent antioxidant activity. These results are strongly in agreement with our results with respect to the SFW extract in which the highest dilution (1/200) was more effective in decreasing the generation of lipid peroxides. The compounds directly responsible for the free radical scavenging properties in SF have not been identified. Fernandes *et al.*, (2004) suggested that the antioxidant activity of SF extracts could be due to phenolic compounds such as tannins and flavanoids.

Cells exhibit a broad spectrum of responses to oxidative stress, depending on the stress type and level encountered. Oxidative stress exceeding the antioxidant capacity level leads to oxidative damage, but low-level stress may enhance the defence capacity. The differential response of a single cell line to SF was demonstrated by Tai *et al.*, (2004). Extracts of SF were shown to be toxic to RAW 264.7 monocytes/macrophages while it totally blocked nitrite production at higher concentrations. A similar mechanism may be proposed for our results since a reduction in lipid peroxides in cancerous H9 cells did not

result in a decrease in cytotoxicity (MTT and ATP, chapter 4). This suggests that the two mechanisms, lipid peroxides scavenging and cell cytotoxicity, occurred independently of each other in the cell system used.

6.5.2 GSH-Glo™ Glutathione Assay

Changes in GSH levels in SF extract-treated cells were compared to those in the untreated control cells (appendix 5). The use of ethanol for extraction was evaluated by comparing the SFE extracts effects to those of the vehicle control. After 24 hours incubation, there was a significant difference ($p< 0.0001$) between the samples analysed (treated and control samples) in both the H9 cells and normal T cells (Figure 6.7). In H9 cells samples the SFE extracts treated samples showed a concentration dependent change in GSH concentration with lower dilutions significantly ($p< 0.001$ and $p< 0.05$ respectively) decreasing GSH levels when compared to the control. The effect of the SFE extracts in reducing GSH levels in these cancerous cells was independent of the vehicle ($p< 0.001$). The SFW extracts did not show concentration dependence in affecting GSH levels with the highest dilution (1/300) reducing GSH levels more significantly ($p< 0.001$) than the lowest dilution.

In normal T cells, the effects of SF extracts on GSH levels were concentration dependent for both types of extracts after 24 hours (Figure 6.7). The lowest dilution of the SFE extracts (1/50) significantly ($p< 0.001$) inhibited GSH synthesis to such an extent that GSH was eliminated from the treated cells when compared to the untreated cells. This elimination of GSH levels was in contrast to the significant ($p< 0.001$) increase in GSH levels in the highest dilutions (1/300 SFE). The inhibition/elimination of GSH synthesis in the higher concentrations of SFE extracts was also significantly ($p< 0.001$) to the high

GSH levels in the vehicle control (70% ethanol), implying that the extract inhibited GSH independently of ethanol. The SFW extracts increased the levels of GSH in a concentration-dependent manner, with the lower concentrations of the extract inducing the higher levels of GSH.

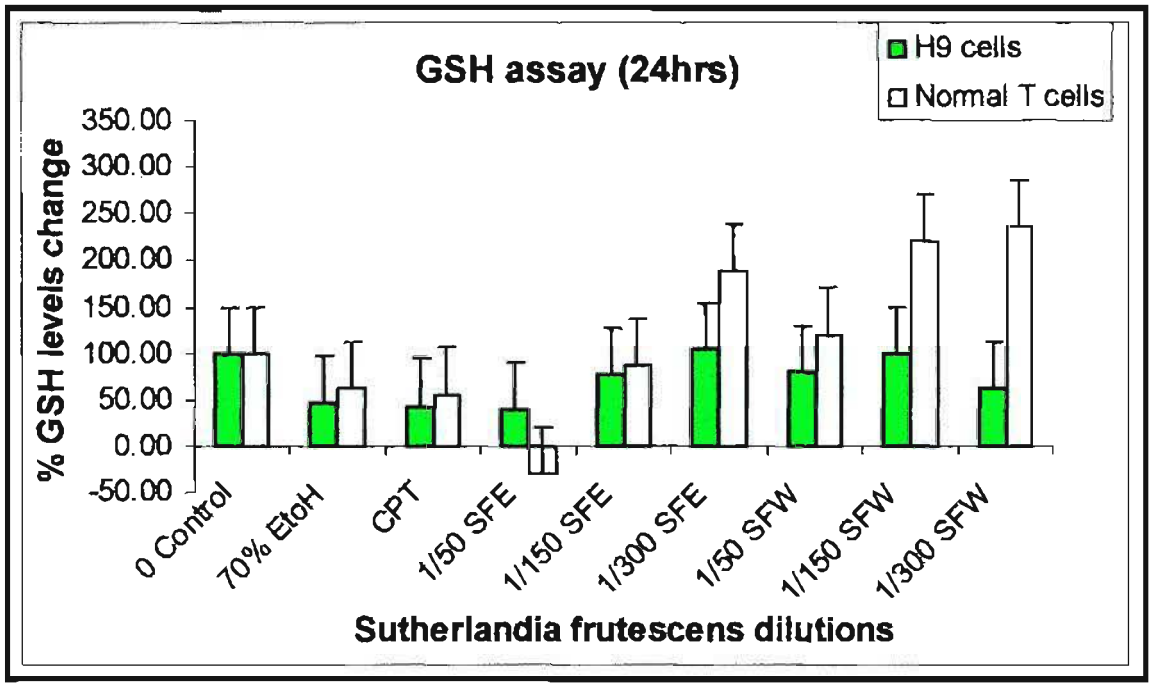


Figure 6.7: A graphical illustration of the effects of extract dilutions of *Sutherlandia frutescens* on GSH levels in H9 and normal T cells after 24 hours incubation.

A time- and concentration-dependent trend in GSH level changes was seen after 48 hours incubation with SF in both H9 and normal T cells (Figure 6.8). The extracts reduced GSH levels in H9 cells while the lower concentrations of the extracts significantly ($p< 0.001$) increased GSH levels in normal T cells. The SFW extract dilutions were more effective in reducing GSH levels in the cancerous H9 cells and were also more effective in increasing GSH levels in normal T cells. Higher concentrations of the SFE extracts continued to inhibit GSH synthesis significantly ($p< 0.001$) in normal T cells when compared to the

untreated normal T cells or the H9 cells treated with similar concentrations of SF extracts. The normal T cell vehicle control had a significantly ($p< 0.001$) increased level of GSH after 48 hours than the highest concentration of the SFE extract (appendix 5).

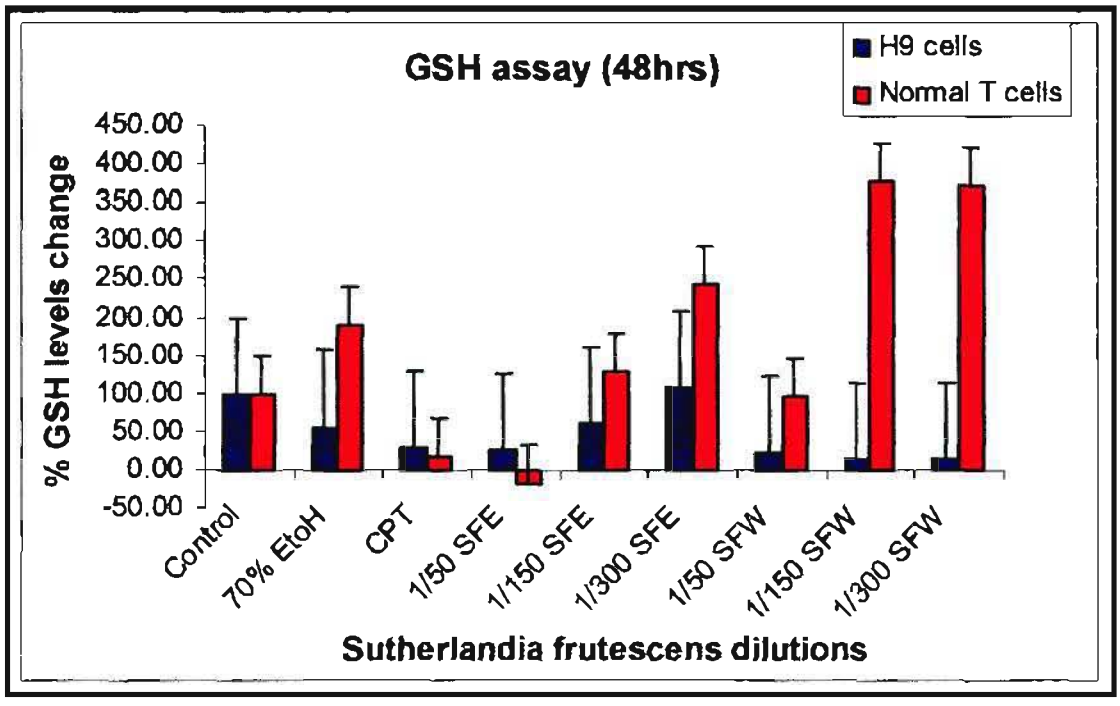


Figure 6.8: A graphical illustration of the effects of extract dilutions of *Sutherlandia frutescens* on GSH levels in H9 and normal T cells after 48 hours incubation.

Antioxidative defence mechanisms which protect against oxidation include enzymes such as glutathione peroxidise, superoxide dismutase, catalase, a selenium-dependent free radical scavenger and smaller molecules such as the carotenoids, ascorbic acid, glutathione, bilirubin, urate and the tocopherols. These antioxidants are proposed to protect cells and their molecular components from oxidative stress by scavenging free radicals and quenching lipid peroxidation chain reactions. Intracellular glutathione plays an important role in the protection of cells against damage from free radicals and also influences cytotoxicity to some chemotherapeutic agents (Tuzgen *et al.*, 2003).

Many chemotherapeutics agents have profound effects on the cellular redox status, and alteration of redox status plays an important role in the induction of cell death. Cellular redox potential is largely determined by GSH which is mainly found in the cytosol and a small, but significant percentage located in the mitochondria. Mitochondrial GSH is important in protecting the organelle from ROS produced during coupled mitochondrial electron transport and oxidative phosphorylation (Armstrong *et al.*, 2002). We showed in chapter 4 that SF reduced ATP levels in treated H9 cells, implying an inhibition/disruption in the electron transport chain. Disruption of ATP synthesis would have consequently led to an increase in ROS production. As a response to increased oxidative stress, mobilisation of the GSH content of cells could lead to depletion of the antioxidant levels.

Another mechanism contributing to depletion of intracellular GSH as seen in H9 cells might be a result of GSH efflux from the cell. GSH is negatively charged at physiological pH, and the large intracellular negative potential of -30 to -60 mV facilitates its extrusion (Franco *et al.*, 2007). By exacerbating the loss of GSH in the extracellular environment, SF could cause the gradient diffusion of GSH out of the cells and thereby decrease cell survival abilities.

Alterations in GSH concentration have been demonstrated as a common feature of many pathological conditions such as AIDS, neurodegenerative disorders, and cancer (Franco *et al.*, 2007). Carcinogenesis induces uncontrollable cell proliferation and increasing the GSH content of cells would strength their non-response to senescence. Ideally, chemotherapeutic agents should therefore reduce the GSH content of cancerous cells. *Sutherlandia frutescens* extracts reduced GSH levels in H9 cells over a period of 48 hours (Figures 6.7 and 6.8).

Intracellular GSH loss is an early hallmark in the progression of cell death (Franco *et al.*, 2007).

Changes in GSH levels can be used as an indicator of cell viability (Promega, 2007). Although there was no direct correlation between the results of the cell viability assays (chapter 4) and the GSH assay results, the general trend pointed towards a relationship between cell viability and GSH content. The SF extracts decreased both cell viability and GSH levels in H9 cancerous cells while the same extracts significantly increased cell viability and GSH levels in normal T dells.

The ability of high concentrations of SFE extract dilutions to inhibit or eliminate GSH in normal T cells might possibly be due to the effects of this medicinal plant on the synthesis pathway of GSH. The availability of the essential amino acid, cysteine is generally accepted as the rate limiting amino acid for GSH synthesis. The cysteine generated from transsulphuration of methionine serves as a substrate for γ-glutamylcysteine synthase (GCS) in the GSH synthesis pathway. The GCS enzyme might also be inhibited by SF extracts and consequently depleting the GSH content. L-canavanine and the SU1 triterpenoid glucosides found in SF have been reported to affect enzymes activities.

In a recent clinical trial of SF on healthy adults, it was shown that the treatment group exhibited higher total protein and albumin than the placebo group (Johnson *et al.*, 2007). As noted in the above paragraph, the protein content plays an important role in determining the GSH content of the cells. Lower concentrations of the SF extracts were shown to significantly increase the GSH content of normal T cells. This ability is relative to the capacity to increase cell proliferation but might also be caused by an increased protein

content of the cells. An extract which can increase GSH levels in normal cells while significantly decreasing GSH levels in cancerous cells is of interest. An ideal trait of a chemotherapeutic agent is to target cancerous cells while sparing the normal tissue.

Certain lymphocyte functions, such as a DNA synthetic response, are favoured by relatively high levels of GSH. Interleukin-2 dependent functions, including cytotoxic T-cell activity, lymphokine-activated killer cells and natural killer cells, are particularly sensitive to GSH depletion. Certain lymphocyte signal pathways, on the other hand, are enhanced by oxidative stress and favoured by low intracellular glutathione levels (Dröge and Breitkruetz, 2000). Therefore the ability of SF to discriminate between normal and cancerous tissue might lie in its capacity to enhance certain cell signalling pathways under specific conditions while suppressing others.

The available evidence suggests that the normal lymphocytes from healthy human subjects have, on average, an optimal GSH level. There is no evidence that immunological functions such as resistance to infection or the response to vaccination may be enhanced in healthy subjects by administration of GSH. However, immunological functions in disease may be greatly enhanced and potentially restored by supplements which increase GSH levels. The best studied case of GSH deficiency is HIV infection. HIV-positive individuals have, in general, abnormally low GSH levels and several groups have also reported a significant decrease in intracellular GSH levels in PBMCs from HIV-infected patients (Dröge and Breitkruetz, 2000). Increased GSH levels in normal T cells induced by low concentrations of SF extracts shows that this medicinal plant can have a positive influence on immunological functions.

6.5.3 Correlation between the TBARS and GSH-Glo™ Glutathione assay results

Analysis of lipid peroxide concentrations in the supernatants of SF extract treated cells showed that this medicinal plant scavenged oxidative stress products. The removal of peroxides did not depend on the concentration of the extracts or the type of cell line treated with the extracts. Higher dilutions of the extracts were as effective as lower dilutions in both the H9 and normal T cells. Targeting of ROS products in all supernatants was therefore as a result of specific activity compounds in SF extracts which scavenged for these products. Tannins and flavanoids have been suggested as possible contributors to these oxidant scavenging mechanisms (Fernandes *et al.*, 2004).

Sutherlandia frutescens extracts induced different effects on the GSH concentration of H9 cells and normal T cells. The extracts caused a time-dependent decrease in GSH content in H9 cells with the SFW extract dilutions being more effective than the ethanol extracts. In normal T cells, the extracts negatively affected the levels of GSH at higher concentrations but enhanced the GSH content at lower concentrations. The SFW extract dilutions were also more effective in increasing the GSH content of normal T cells than the ethanol extracts. The GSH content is closely related to cell viability in a specific sample, and therefore the effects of SF extracts on cell proliferation also determined the GSH levels. Another distinction in affecting GSH levels was the ability to discriminate between normal and cancerous cells.

After treatment of a human B lymphoma (PW) with L-buthionine sulphonine (BSO), an inhibitor of γ-glutamylcysteine synthetase, there was an early decline in cellular GSH, followed by an increase in ROS production. This increase in ROS induced a variety of

apoptotic signals in the absence of any external apoptotic stimuli (Armstrong *et al.*, 2002). Franco *et al.*, (2007) recently demonstrated that loss of intracellular GSH was paralleled by generation of ROS including superoxide anions, lipid peroxides, etc. However, inhibition of ROS formation by a variety of antioxidants showed that GSH loss was independent from the generation of ROS. Furthermore, GSH depletion was observed to be necessary for ROS formation. High extracellular content of thiols like GSH inhibited apoptosis while inhibition of ROS generation was ineffective in preventing cell death.

The above statements give credibility to the observed differential effects of SF extracts on TBARS and GSH content in H9 and normal T cells. *Sutherlandia frutescens* was shown to be an excellent scavenger of lipid peroxides in extracellular environment of both cell lines. While removal of ROS might be expected to improve cell survival, this was not the case with treated H9 cells. Decrease in GSH levels of the H9 cancerous cells reflected this cell death more accurately than ROS. From these results it can be concluded that the GSH concentration in cells determined cell fate.

6.6 Conclusion

In this chapter we analysed for the oxidant scavenging and antioxidant promoting properties of different extracts of SF. The TBARS assay showed that extracts SF are excellent scavengers of lipid peroxides in supernatants in treated cell lines. This scavenging ability did not depend on the concentration of the extracts but rather on the type of extraction method used, with the SFW extract dilutions more effective in this regard. The ROS scavenging properties of SF have been hypothesised as dependent on the presence of tannins and flavanoids in the extracts.

The results of the GSH assay did not correlate with the effects on lipid peroxide removal. SF extracts decreased GSH levels in H9 cancerous cells while increasing the GSH levels in normal T cells depending on the type and concentration of the extracts. High concentrations of the SFE extracts negatively affected the GSH content in normal T cells independent of ethanol and this again showed that high concentrations of SF can seriously suppress T cell proliferation. Lower concentrations of the SFW extracts significantly increased GSH levels in normal T cells and this in turn showed their ability to increase T cell proliferation. These results show that cell fate is mostly dependent on the concentration of antioxidants, not on the levels of oxidative stress.

CHAPTER 7

MORPHOLOGICAL EFFECTS OF *SUTHERLANDIA FRUTESCENS* EXTRACTS ON H9 T CELLS AND NORMAL T CELLS

7.1 Introduction

Apoptosis and necrosis are two forms of cell death with clearly distinguishing morphological features. These cell death mechanisms can occur simultaneously in cell cultures exposed to the same stimulus, the intensity of the same initial insult determining the prevalence of one over the other. This suggests that there are common early events in both apoptosis and necrosis, while a downstream controller may be required to direct cells towards apoptosis. Levels of intracellular ATP have been shown to be one of the important determinants of occurrence of morphological features of apoptosis like nuclear condensation and DNA fragmentation (Leist *et al.*, 1997).

Apoptosis is characterised by chromatin condensation, nuclear fragmentation and formation of apoptotic bodies which are phagocytosed by other cells. In most but not all forms apoptosis, nuclear DNA is cleaved at internucleosomal sites. By contrast, cellular necrosis is defined by electron-lucent cytoplasm, mitochondrial swelling loss of plasma membrane integrity without drastic morphological changes in the nuclei. Nuclear DNA is randomly cleaved as a consequence of cellular degeneration in necrosis (Tsujimoto, 1997).

The morphological definition of cell death modes makes electron microscopy the best way to assess apoptosis or necrosis. Electron microscopy application can be inconvenient in

cases where apoptosis and necrosis occur in the same cell culture. A versatile tool like the confocal laser scanning microscope, powerful for both morphological analysis and macromolecular localisation, can also be utilised. One of the convenient ways is to use fluorescence microscopy in conjunction with different fluorescent dyes for cell staining (Sgonc and Gruber, 1998; Tsujimoto, 1997).

Fluorescence microscopy technique is the most rapidly expanding technique employed today in medical and biological sciences. Fluorescence is a member of the ubiquitous luminescence family of processes in which the susceptible molecules from electronically excited states created by either a physical, mechanical, or chemical mechanism. Fluorescence differs from other forms of photoluminescence in that it is a property of some atoms and molecules to absorb light at a particular wavelength and subsequently emit light of longer wavelength after a brief interval, termed fluorescence lifetime (Olympus microscopy manuscript).

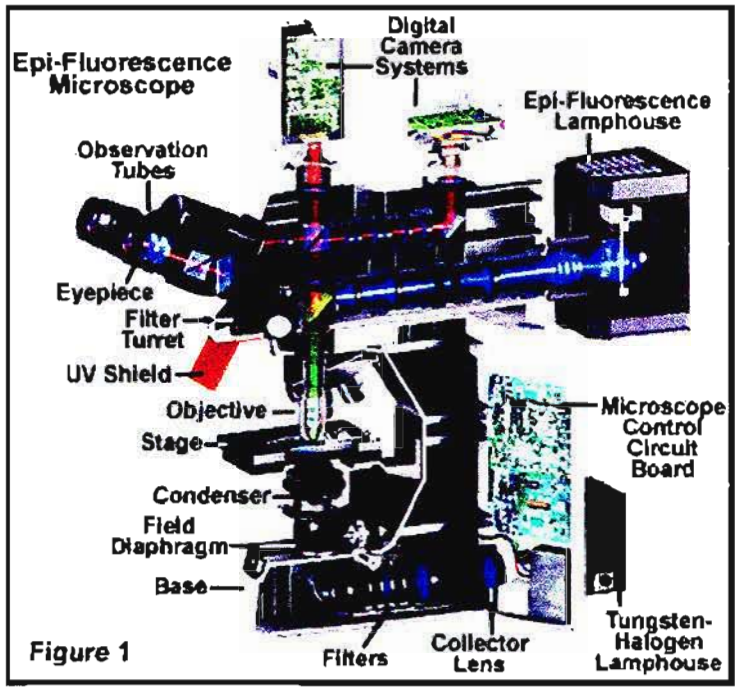


Figure 7.1 Illustration of a typical fluorescence microscope (Nikon microscopy: http://www.microscopyu.com/articles/fluorescence/fluorescenceintro.html).

Fluorescence microscopes can be used with a variety of fluorescent dyes to distinguish between viable, apoptotic and necrotic cells. Propidium iodide (PI) and the Hoechst stains are some of the most widely used fluorescent dyes. Hoechst dyes stain nuclei of all cells (varying intensity of blue) whereas PI stains only cells with disrupted membrane integrity (red staining). Propidium iodide is therefore a cell viability dye marker as only compromised cells have leaky membranes. Microscopy techniques like the use of fluorescent dyes are primarily qualitative determinants of cell death (Tsujimoto, 1997).

7.2 The Hoechst stains

The Hoechst or bisbenzimidazole dyes, Hoechst 33342 and 33258, are potent DNA minor groove binding ligands. Like many other DNA minor groove-binding ligands, these

Hoechst dyes have strong A+T base pair specificity. They induce highly specific protein-linked, single-stranded DNA breaks on DNA in the presence of mammalian DNA topoisomerase I (Chen *et al.*, 1997).

The Hoechst dyes are highly positively charged and their passive diffusion across the cellular membrane is expectedly slow. However, Hoechst 33342 due to the ethoxy substitution on the 4-phenyl ring of its parent compound Hoechst 33258, has enhanced membrane permeability and is about 2 orders of magnitude more cytotoxic than Hoechst 33258 (Figure 7.2). The higher cytotoxicity of Hoechst 33342 can be explained by its higher lipophilicity which is one of the crucial determinants for passive diffusion across the cellular membrane (Chen *et al.*, 1997).

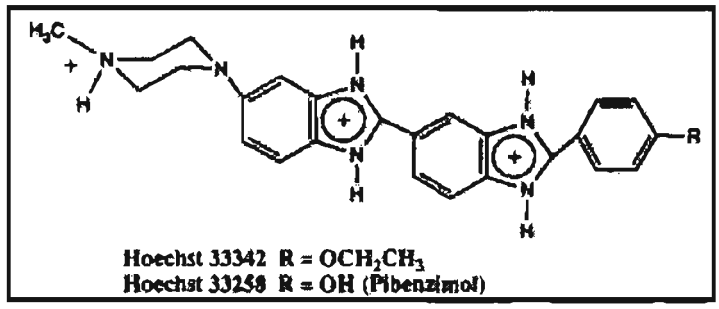
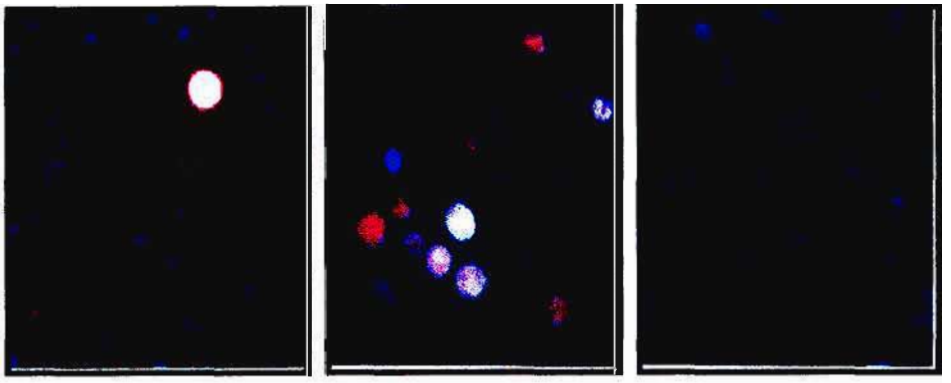


Figure 7.2: Chemical structures of Hoechst 33342 and 33258 (Chen *et al.*, 1997).

Hoechst 33342 has been widely used, along with fluorescent microscopes, to stain morphological changes in the nuclei of cells undergoing processes leading to cell death. Cells to be stained with Hoechst 33342 do not require permeabilisation but do require physiological conditions for active transportation of the dye. The staining protocol is very sensitive to cell concentration and pH of the media when using cell suspensions. Cells should be approximately 1-2 × 10^6/ml in buffered media (pH 7.2). Incubation of cells at 37 ^{0}C for an hour is usually enough for the dye to be transported into cells while the staining

signal has a short half-life. Hoechst 33342 has a broad fluorescence range, allowing for co-staining with fluorescent dyes. It also works with permeabilized paraformaldehyde- (PFA) and ethanol-fixed cells (Kukuruga, 1997).

Apoptotic or necrotic cells have defined morphologies when stained with Hoechst 33342 and analysed under a fluorescent microscope. The nuclei of viable and necrotic cells appear round in shape, with those on necrotic cells more blue-green. The apoptotic cells appear to have fragmented nuclei and stain with an intensely blue-green fluorescence (Figure 7.3 (a), (b) and (c)). It is important to distinguish between intensely stained cells with nuclear condensation and those cells appearing blue-green as a result of undergoing mitotic cell division (Tsujimoto, 1997).



A B C

Figure 7.3: Photomicrographs of Hoechst 33342 stained Jurkat T cells grown under ATP-depleting and ATP-supplying conditions and subsequently induced to die by oligomycin. A) Shows apoptotic cells (indicated fragmented nuclei- FN), B) necrotic cells and C) untreated normal cells (Eguchi *et al.*, 1997).

The aim of the work reported in this Chapter was to analyse the morphological effects of SF extract dilutions on cultured H9 T cells and normal T cells using the Hoechst 33342 dye and fluorescent microscopy.

7.3 Materials and Methods

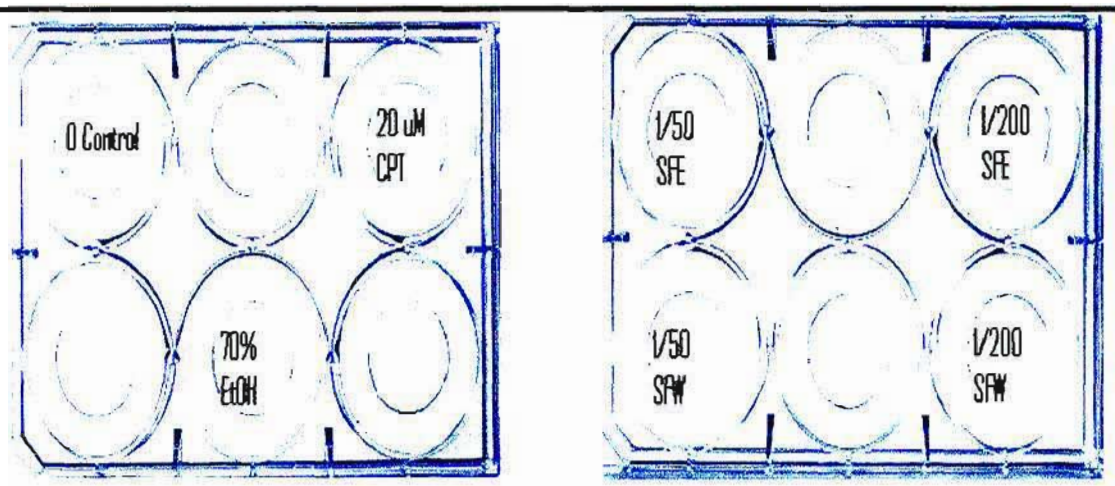
7.3.1 Materials

Bisbenzimide and DMSO were purchased from *Sigma Aldrich* (SA). PFA was purchased from *Merck Chemicals* (SA). Glass slides and coverslips were purchased from *Corning* (Netherlands). Inverted fluorescent microscope was supplied by *Olympus* (USA).

7.3.2 Methods

7.3.2.1 Treatment of cell lines and outline of incubation procedure

Normal T cells and H9 T cells were treated in a similar procedure to that outlined in paragraph 4.4.2.4 (chapter 4). Briefly, the cell lines were treated with various dilutions of SFE and SFW extracts and incubated over a period of 48 hours. A positive control (20 μM CPT) and vehicle control (70% ethanol) were also included for both cell lines (Figure 7.4). The 48 hours incubation period was chosen based on the levels of cytotoxicity as shown by MTT and ATP assays. Both cell lines incubated over this period could be expected to show distinct patterns of cell death related to SF treatment.



CPT = Camptothecin

EtOH = Ethanol

SFE = 70% ethanol extract of *Sutherlandia frutescens*

SFW = deionised water extract of *Sutherlandia frutescens*

Figure 7.4: A schematic illustration of the treatment procedure in 6 well plates for both the H9 cells and isolated T lymphocytes in performing the Hoechst staining assay.

7.3.2.2 The Hoechst staining protocol

At the end of the 48 hours incubation period, the treated cells and controls were removed from their allocated wells and dispensed into labelled sterilin tubes. The tubes were centrifuged at 1500 rpm to pellet the cells. The resultant supernatant was removed and stored at -70 ^{0}C for future use. The pelleted cells were washed by vortexing (PBS, 500 µl) and transferred to labelled eppendorf tubes. They were pelleted by centrifugation again and the pellets were stained with a Hoechst working solution (100 µl). The stained cells were incubated for 15 minutes at 37 ^{0}C. The Hoechst stain was washed away with PBS and the cells were fixed in 10% PFA for 5 minutes. After another wash step with PBS (500 µl,

vortex), the cells pellets were re-suspended in 100 μl PBS. This suspension was pipetted into glass slides which were covered with coverslips. The prepared slides were then viewed under an Olympus TH4-200 inverted fluorescent microscope (excitation filter 350 nm and barrier filter of 450 nm).

7.4 Results and Discussion

Staining suspension cultures on glass slides for viewing under a microscope presents with several challenges. Because the cultures do not attach to the slides or coverslips, they remain in constant motion in freshly prepared slides. The repeated movement of these cells makes focusing and adjustment of the fluorescent microscope very difficult. Another challenge involves the multiple centrifugation steps that are involved in the staining protocol and this may result in loss of cell numbers.

Untreated H9 cells stained with Hoechst (Figure 7.5 A) generally displayed nuclei with homogenous chromatin staining characteristic of viable cells. Some of the cells showed signs of early condensation while a few were already fragmented. The normal T cells (Figure 7.5 B) displayed nuclei with a similar pattern to the H9 cells but with some cells showing dividing nuclei.

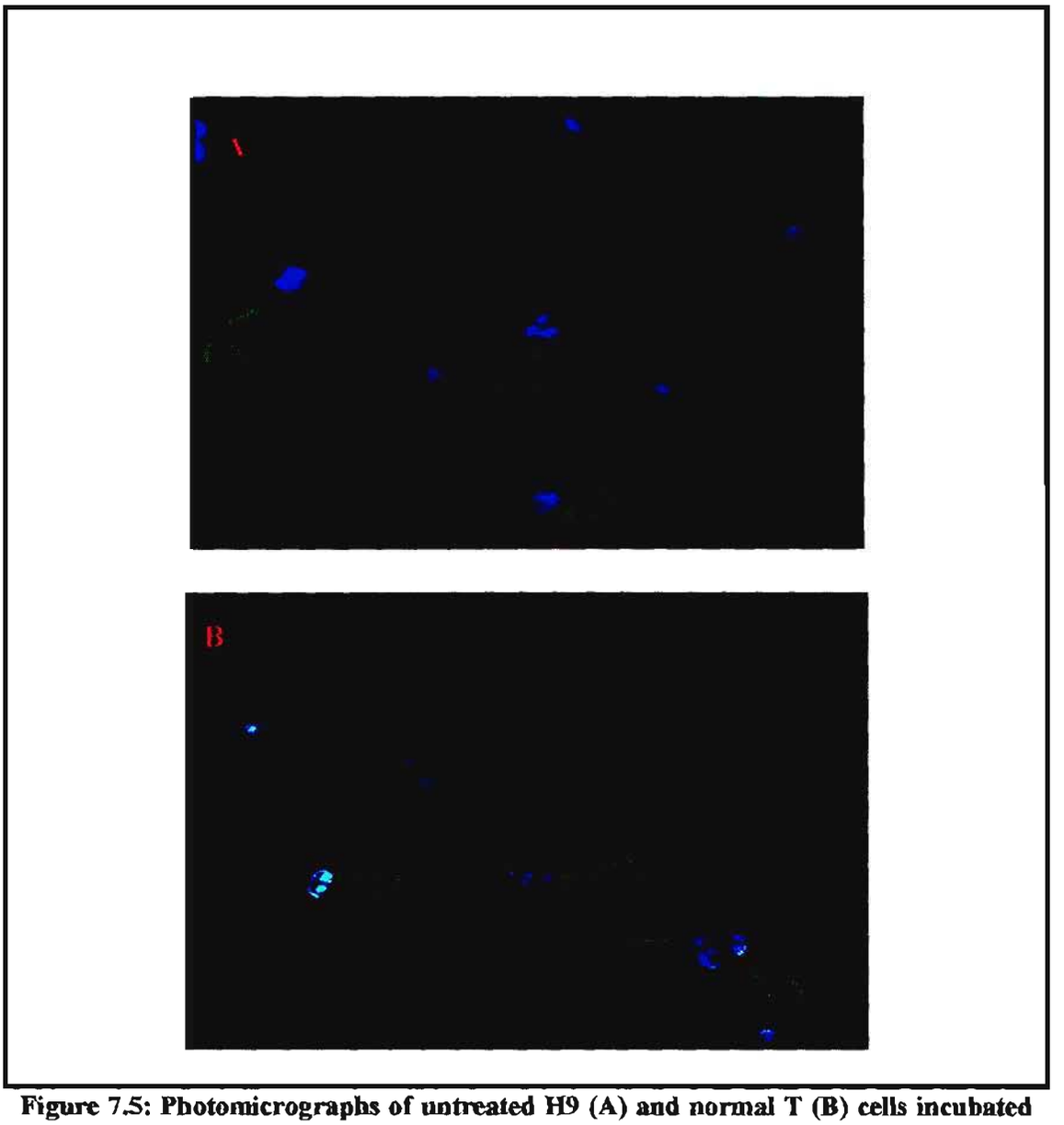


Figure 7.5: Photomicrographs of untreated H9 (A) and normal T (B) cells incubated for 48 hours and stained with Hoechst. Cells can be seen displaying normal (N), fragmented (FN), condensed (CN) and dividing (DN) nuclei (A & B- 600×).

H9 and normal T cells treated with 20 μM CPT predominantly displayed condensed nuclei with bright blue-green staining indicative of apoptotic (Figure 7.6 A & B). The levels of condensed nuclei were similar in both cell lines and this was consistent with the known effects of CPT in suspension cells.

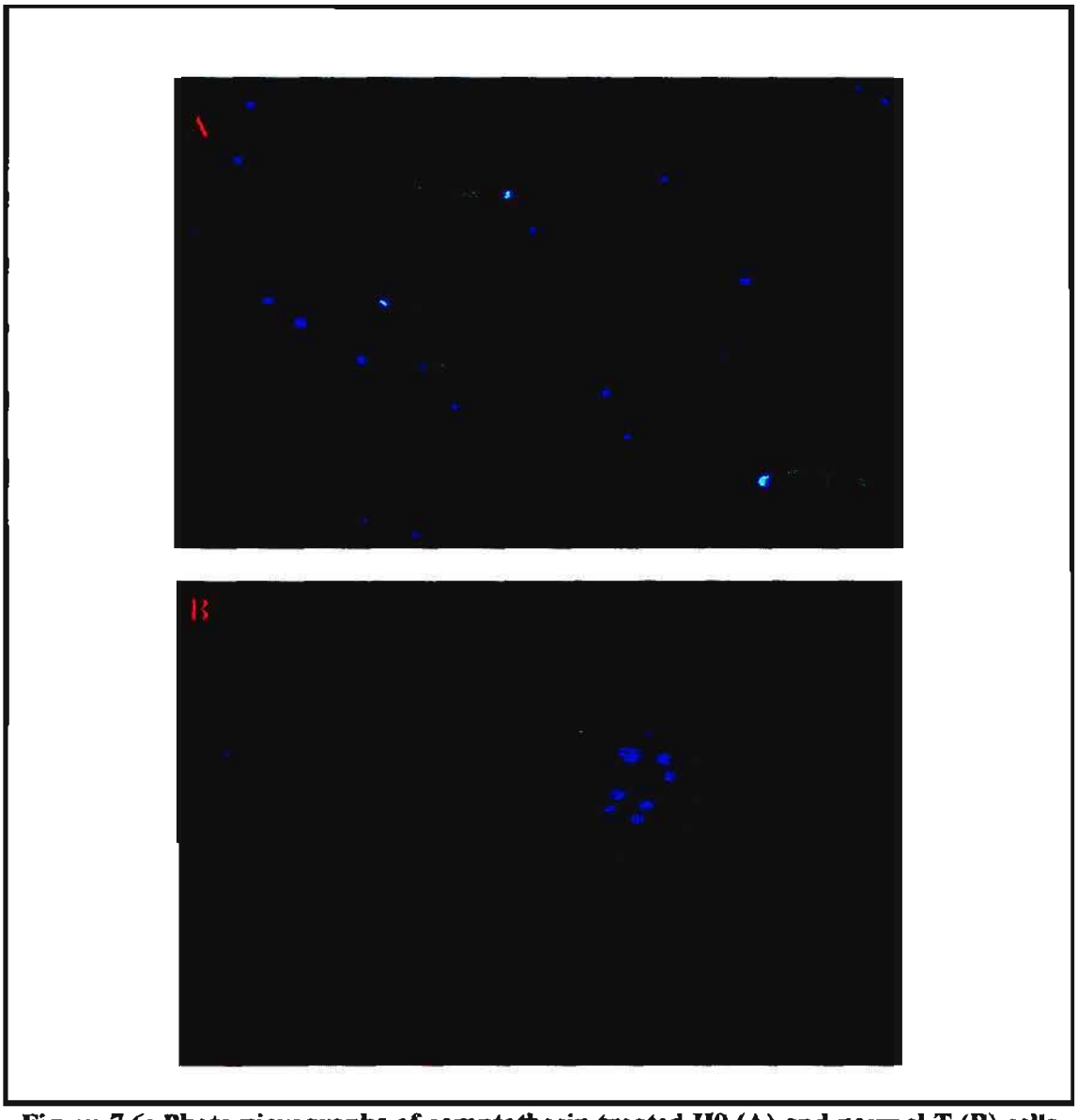


Figure 7.6: Photomicrographs of camptothecin treated H9 (A) and normal T (B) cells stained Hoechst. Both cell lines show extensive nuclei condensation (CN) after 48 hours incubation (A- 200×, B- 600×).

The 70% ethanol treated H9 cells (Figure 7.7 A) stained with Hoechst 33342 exhibited similar nuclei nomenclature to those of untreated H9 cells. Most cells showed normal nuclei while condensed nuclei were also observed in the stained cultures. Treatment of normal T cells with 70% ethanol (Figure 7.7 B) induced more condensed nuclei than the untreated cells.

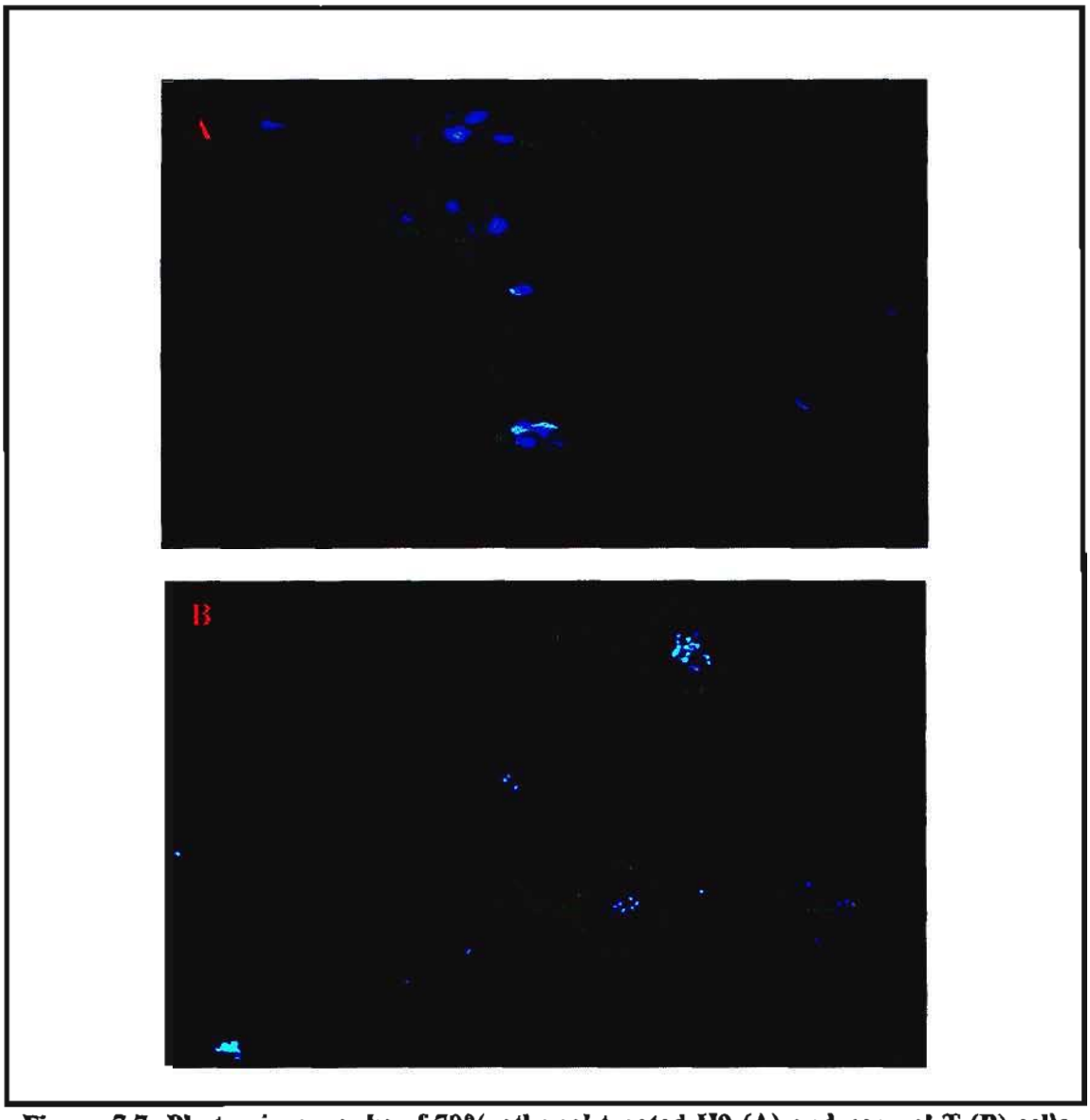


Figure 7.7: Photomicrographs of 70% ethanol-treated H9 (A) and normal T (B) cells stained with Hoechst and displaying condensed (CN) and some normal (N) nuclei (A- 600×, B- 200×).

H9 cells treated with the SFW extract dilutions (Figure 7.8 A) displayed a significant number of cells with condensed and fragmented nuclei indicative of induction of cell death. The condensed nuclei were an indication of early stages of apoptosis while fragmentation was indicative of the execution stage of cell death. This is consistent with results of the cytotoxicity assays which showed that the SFW extracts reduced cell viability

in a concentration dependent manner. The effects of the SFW extract on normal T cells results in some nuclei showing bright blue-green staining, indicative of nuclei condensation (Figure 7.8 B). This staining pattern suggests that cells may be undergoing mitosis which causes the nucleus to fragment into two parts. This is supported by the cell proliferation shown in MTT assays in chapter 4.

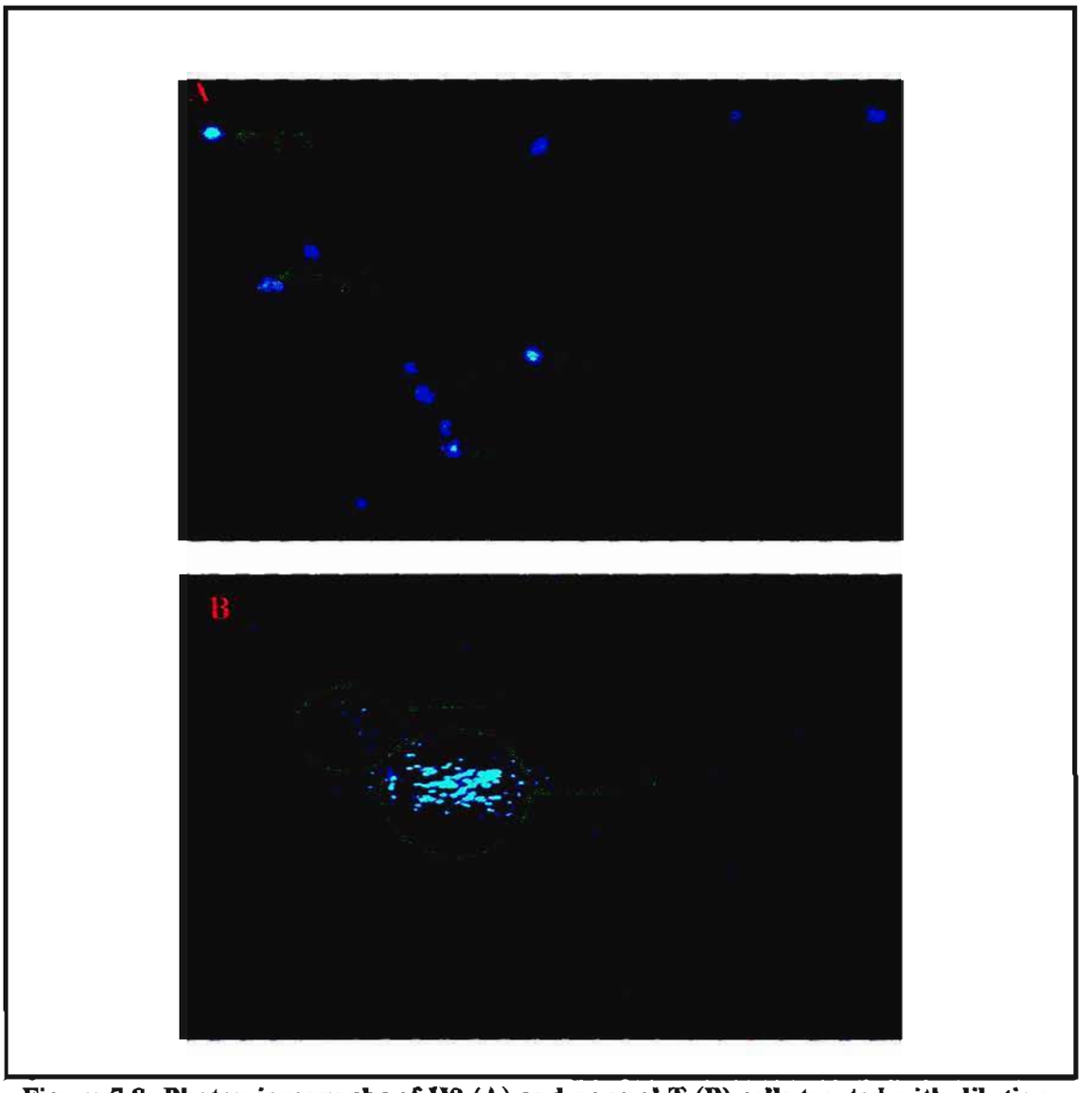


Figure 7.8: Photomicrographs of H9 (A) and normal T (B) cells treated with dilutions of SFW extract dilutions and stained with Hoechst. Cells with fragmented (FN) and condensed (CN) nuclei were seen in H9 cells while condensed (CN) and normal (N) nuclei appeared in normal T cells (A- 600×, B- 200×).

Similar to the effects of SFW extracts on H9 cells, the SFE extracts induced different levels of condensation, fragmentation and blebbing in the nuclei of treated H9 cells (Figure 7.9 A). The effects of SFE extracts on nuclei were more pronounced than those of the vehicle control. While less pronounced than in H9 cells, the SFE extracts also induced condensation and fragmentation of chromatin in normal T cells (Figure 7.9 B). The observed effects were expected as the lower dilutions of the SFE extract were previously shown to reduced cell viability below those of untreated controls in cytotoxicity assays (chapter 4).

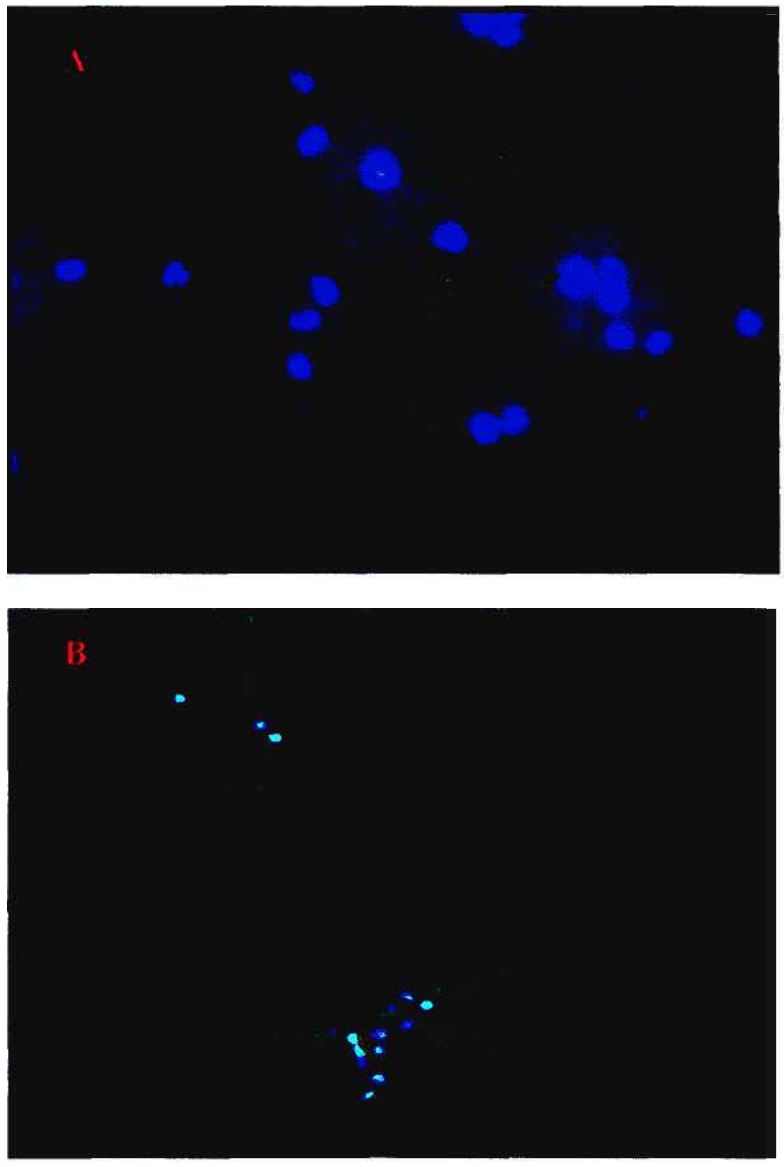


Figure 7.9: Photomicrographs of H9 (A) and normal T (B) cells treated with dilutions of SFE extracts over 48 hours stained with Hoechst. Treated H9 cells displayed condensed (CN), blebbing (B) and normal (N) nuclei while normal T cells had condensed (CN) and normal (N) nuclei (A- 600×, B- 400×).

Several studies have presented evidence of cytotoxic effects of various SF extracts on a number of cancerous cell lines (Tai *et al.*, 2004; Steenkamp and Gouws, 2006; Stander *et*

al., 2007; Chinkwo, 2005). The experiments on malignant H9 T cells also showed similar findings (MTT and ATP cytotoxicity assays). Cytotoxicity assays suffer the disadvantage of not being able to specify the type of cell death occurring. Only a few studies which have sort to ascertain the type of cell death induced by these SF extracts.

Tai *et al.*, (2004) predicted the occurrence of apoptosis in several cell lines treated with ethanol extracts of SF using flow cytometric cell cycle analysis. Chinkwo (2004) gave a clearer picture of the occurrence of apoptosis in SF treated carcinoma cells using annexin V staining, DNA fragmentation and morphological cell staining assays. DNA fragmentation as indicated by the formation of a DNA ladder in an agarose gel was observed in CHO cells treated with aqueous extracts of SF. Treated CHO cells stained with the crossmon trichome dye showed chromatin condensation indicative of apoptosis.

In this experimental setup, H9 and normal T cells treated with aqueous and ethanol extracts over 48 hours and stained with Hoechst 33342 showed chromatin condensation, fragmentation of the nuclei and some blebbing of nuclei (indicators of apoptosis). Important triggers of apoptosis included reduced GSH levels, increased ROS, lipid peroxidation, effector caspases activation and levels of ATP high enough to drive the apoptotic process. We have shown in chapters 4, 5, and 6 that extracts of SF decrease ATP levels, reduce GSH levels and inhibit caspase-3/-7 activation in a concentration and time dependent manner in treated H9 cells. Inhibition of caspase-3/-7 was also observed in treated isolated normal T cells.

Chromatin condensation and DNA fragmentation in apoptosis are known to be dependent on activated effector caspases (caspase-3 and -7). These caspases, when activated, are

7.5 Conclusion

Treatment of cancerous H9 T cells with SFE and SFW extracts over 48 hours induced chromatin condensation and fragmentation of the nuclei. Normal T cells treated in a similar approach were seen to have normal nuclei while some cells had condensed chromatin but there was no fragmentation or blebbing of the nuclei. These results show that SF can induce apoptosis in malignant cell lines and also have similar effects on normal cells. The condensed chromatins of normal T cells may be another indication that higher concentrations of SF can be harmful to normal tissues.

CHAPTER 8

THE EFFECTS OF *SUTHERLANDIA FRUTESCENS* ON CYTOKINE SIGNALLING IN NORMAL PERIPHERAL BLOOD MONONUCLEAR CELLS

8.1 Introduction

Cytokine and chemokines are secreted proteins with growth, differentiation, and activation functions that regulate and determine the nature of the immune responses. They also control immune cell trafficking and the cellular arrangement of immune organs. Cytokines produced in response to an immune insult determine initially whether an immune response develops and subsequently whether that response is cytotoxic, humoral, cell-mediated, or allergic. Depending on underlining conditions, cytokines can be grouped according to those that are predominantly mononuclear phagocytic-derived or T-lymphocyte derived; mediate cytotoxic (anti-viral and anti-cancer), humoral, cell-mediated, or allergic immunity; and those that are immunosuppressive (Borish and Steinke, 2003).

A cascade of responses can be seen after secretion of cytokines and often several cytokines are required to synergise their expression in order to produce optimal function. Cytokines and chemokines can promote angiogenesis, metastasis, subversion of adaptive immunity and changing the response to hormones and drug treatment. Another factor in analysing cytokine function is that each cytokine may have a completely different function, depending on the cellular source, target and most importantly, specific phase of the

immune response during which it is presented (Borish and Steinke, 2003; Germano *et al.*, 2008).

The measurement of soluble cytokines in serum and plasma has become very important in the study and management of many diseases. Therefore it has become important to develop rapid, precise and cost-effective measurements of these cytokines in both clinical and research laboratories. Techniques such as cDNA microarrays enabled analysis of global gene expression but evaluation of DNA and RNA alone is limiting. Disparity between the relative expression levels of mRNA and their corresponding proteins have been shown. Protein-based analyses are more useful in the analysis of cytokines. Two dimensional SDS-PAGE in combination with mass spectrometry has been used in cytokine levels analyses but has mass limitations. Surfaced-enhanced laser desorption and ionisation coupled with mass spectroscopy have also been used (Elshal and McCoy Jr, 2006; Huang *et al.*, 2001).

Antibody-based protein array systems have been developed for a simple, flexible, cost effective, highly sensitive, and high-throughput approach. Protein array technology has been further developed to examine protein-protein interactions (Huang *et al.*, 2001). Enzyme linked immunosorbent assays (ELISAs) have long been the standard for quantitative analysis of cytokines. Although they are not suited for high throughput multiplex analyses, ELISAs have other advantages. ELISAs, in general, use immobilised antibodies to capture a soluble ligand, with subsequent detection of the captured ligand by a 2nd reporter antibody. They use enzyme amplification of a colorimetric substrate on flat surfaces in 96-well plates. ELISA methodologies generally study one analyte at a time, and thus avoid cross-reactivities (Elshal and McCoy Jr, 2006; Tam *et al.*, 2002).

8.2 Multi-Analyte Profiler ELISArray Kit for Human Inflammatory Cytokines

The human inflammatory cytokines and chemokines multi-analyte ELISArray kit analyses a panel of 12 pro-inflammatory cytokines using a conventional ELISA protocol under uniform conditions. The cytokines and chemokines represented by this array are IL 1α, IL 1β, IL 2, IL 4, IL 6, IL 8, IL 10, IL 12, IL 17α, IFN-γ, TNF-α, and GM-CSF (IL- interleukin; IFN- interferon; TNF- tumour necrosis factor; GM-CSF- granulocyte monocyte- colony stimulating factor). The pro-inflammatory cytokines (IL 1, IL 6, and TNF-α) are also endogenous pyrogens. IL 4 and IL 10 can also act as anti-inflammatory cytokines depending on their concentration (SABiosciences manual, 2008).

The complete kit features the best possible combination of capture and detection antibodies for each protein in the panel and all the needed colorimetric detection reagents. Using the same ELISA protocol and development or incubation times, it allows for easy of profile for the levels of a focused panel of 12 proteins related to inflammation with this array (SABiosciences manual, 2008).

In the study reported in this Chapter the effects of SF extracts on cell signalling were analysed by a cytokine ELISA method. PBMCs were utilised because they are characterised by the presence of all the cells of the immune system. They also represent an environment closer to the *in vivo* system than the use of a single cell line which may only produce a limited range of cytokines.

8.3 Materials and Methods

8.3.1 Materials

The Multi-Analyte Profiler ELISArray^{TM} Kit was purchased from *SABiosciences* (USA).

8.3.2 Methods

8.3.2.1 Treatment of isolated peripheral blood mononuclear cells (PBMCs)

Peripheral blood mononuclear cells (PBMCs) (whole blood from a healthy individual) were isolated according to a method outlined in paragraph 3.2.3.1 of chapter three. The PBMCs were then treated with various dilutions of SF ethanol and deionised water extracts in RPMI-640 media over a 24 hours incubation period. Untreated control cells, CPT (20 μM) and PHA (10 μg/ml) treated PBMCs were included in the experiment as controls.

At the end of the incubation period, the cytotoxicity of SF extract dilution and control samples were assessed by the ATP assay as outlined in paragraph 4.4.2.6 of chapter 4. 2 ml of each treatment was then dispensed into sterilin tubes and centrifuged at 1000 g for 10 minutes to pellet the cells. The supernatants were stored at -20 ^{0}C and later used for the cytokine analysis and the pelleted cells were stored in storage media (80% RPMI-1640, 10% FCS and 10% DMSO) at 70^{0}C for future use.

8.3.2.2 Multi-Analyte Profiler ELISArray assay

The ELISArray kit uses a standard ELISA technique and for our purposes two kits were purchased to accommodate the number of samples in our experiments. Each kit included 96-well plate coated with antibodies for the various cytokines included in the microarray. Each row of the plate from 1 to 12 represented a single cytokines in the following order: IL 1α, IL 1β, IL 2, IL 4, IL 6, IL 8, IL 10, IL 12, IL 17α, IFN-γ, TNF-α, and GM-CSF. The kit had a negative controls and positive controls to show that assay worked. The negative and positive control for each cytokine to be analysed was done in single wells and experimental samples and controls were assayed in duplicates for statistical purposes (Figures 8.1 and 8.2).

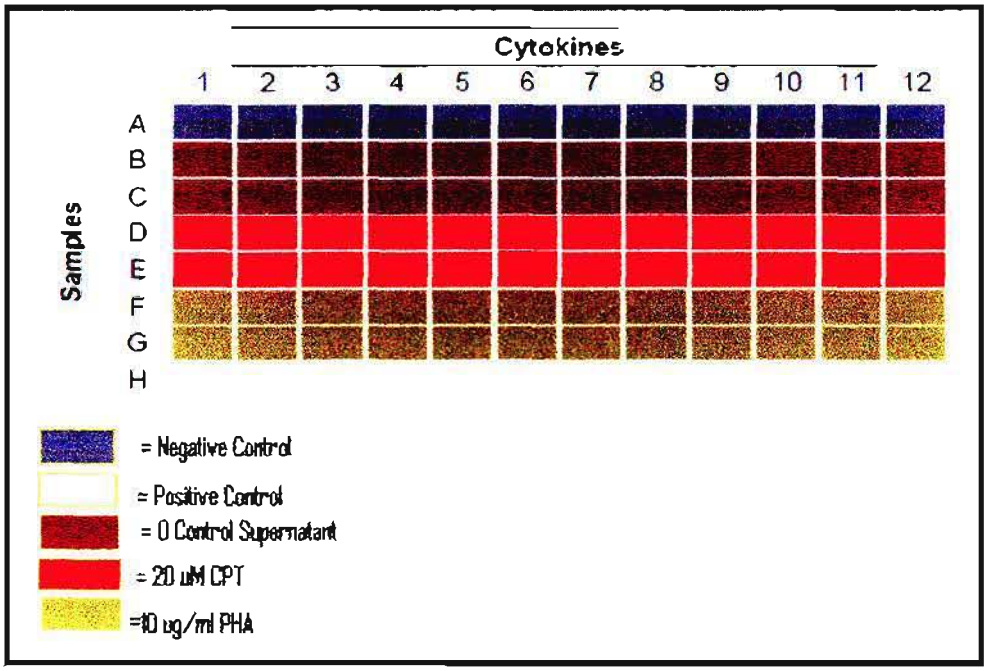


Figure 8.1: Diagram illustrating the setup of the control samples in the antibodies coated wells of the 96-well plate.

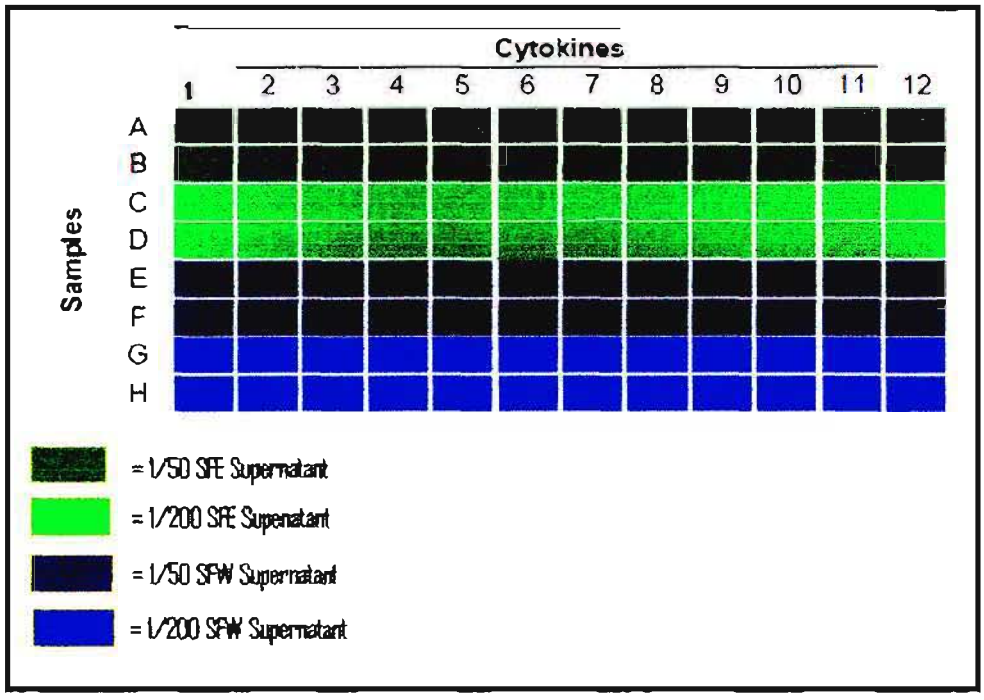


Figure 8.2: Diagram illustrating the setup of the *Sutherlandia frutescens* extract dilutions treated samples in the antibodies coated wells of the 96-well plate.

Incubation of the samples in the 96-well plates allowed the capture antibodies to bind their specific protein of interest. After washing away unbound protein, biotinylated detection antibodies were added to the wells to also bind the captured analyte. After washing again to remove unbound material, an avidin-horseradish peroxidise conjugate was added. The wells were again washed and the colorimetric substrate solution was added, which produced a blue colour in direct proportion to the amount of protein analyte present in the initial sample. The colour development was stopped by adding the stop solution, and the absorbance at 450 nm with reference at 570 nm was read in a Bio-Tek plate reader and compared across all samples (Figure 8.3).

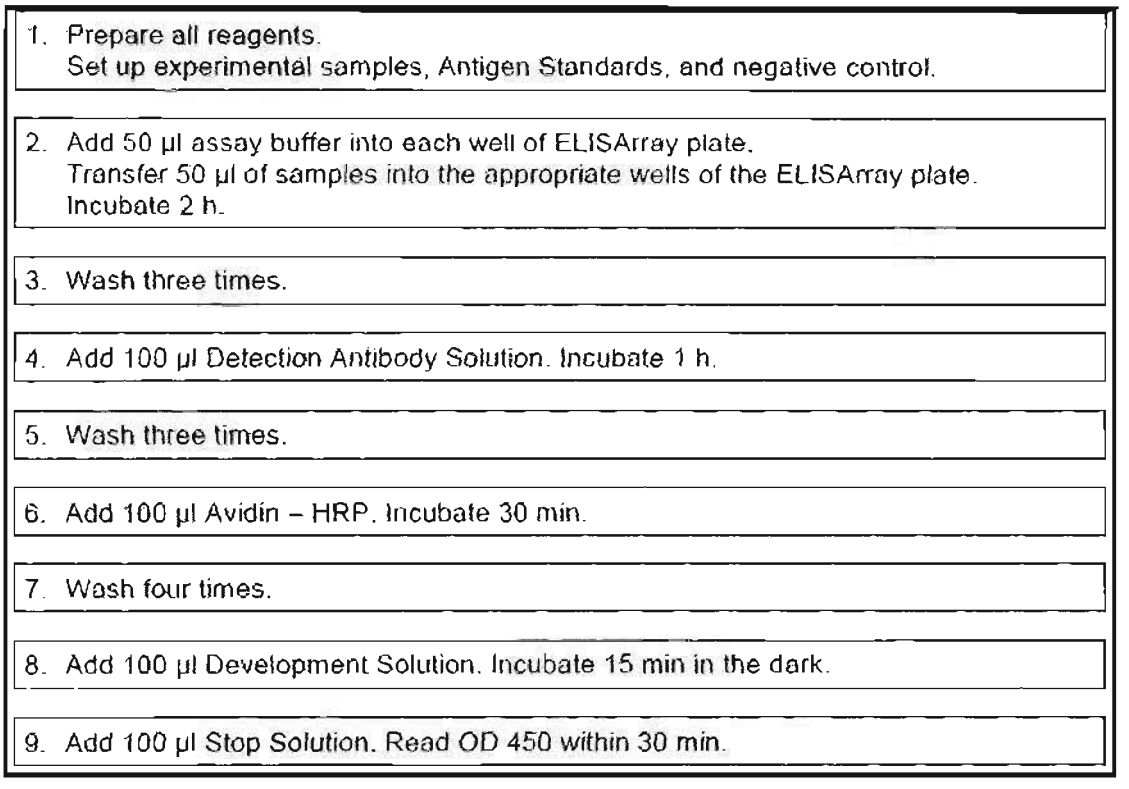


Fig 8.3: A flow diagram of the Multi-Analyte Profiler ELISArray assay procedure (SABiosciences manual, 2008).

8.3.2.3 Statistical analysis

The absorbance obtained for each sample represented the amount of cytokine present in the sample. All the absorbance readings were subtracted from the negative control. Therefore a comparison of the average absorbance of each sample to the next sample gives the differences in cytokine levels (e.g. IL 1α levels in the untreated control and 1/50 SFE samples) between samples. Data analyses were done on *Microsoft Excel* to obtain the averages and the standard deviations of each treatment. The different levels of significances within the separate treated groups were analysed using one-way analysis of variance (*ANOVA*) and the differences between the treated cells and the control cells were

analysed using the *Tukey-Kramer* multiple comparison methods. Differences with $p< 0.05$ were considered statistically significant.

8.4 Results and Discussion

8.4.1 Interleukins 1α, 1β, 2, and 4

Incubation of PBMCs with extracts of SF produced different changes in the levels of IL 1α, IL 1B, IL 2, and IL 4. Although these cytokines were present in low concentrations in supernatants of treated cells, the changes between the treated samples and the different controls were significant (Figure 8.4). IL 1α levels decreased significantly in CPT treated supernatants ($p< 0.001$) and were unchanged in PHA treated samples ($p> 0.05$). IL 1α levels were unchanged in SFE extract dilutions treated samples while it was significantly increased in the 1/50 SFW extract dilution ($p< 0.001$) as compared to the untreated control. The SFW extract dilutions generally increased IL 1α levels relative to the SFE extract dilutions which either decreased or maintained levels to that of the untreated control. A similar trend to that of IL 1α was seen with IL 1β concentrations but this cytokine was found in higher concentrations relative to the others in this group (Figure 8.4).

The IL 1 family of cytokines represents four peptides and IL 1α and 1β have similar biological activities and interact with similar affinities to the two IL 1 receptors. IL 1 is primarily produced by cells of the mononuclear phagocytic lineage but is also produced by oestoblasts, neutrophils, glial cells, and numerous other cells. One of the most important biological activities of IL 1 is its ability to activate T lymphocytes by enhancing the production of IL 2 and expression of IL 2 receptors. IL 1 augments B cell proliferation and

increases immunoglobulin synthesis (Borish and Steinke, 2003). Therefore higher concentrations of SF extracts, which tend to reduce expression of IL 1 cytokines, can suppress the immune response. This is in contrast to lower SF concentrations which enhanced the IL 1 cytokine levels and therefore can stimulate the immune response.

Concentrations of IL 2 in all control samples and SF extract treated samples did not show any significant changes ($p > 0.05$) (Figure 8.4). IL 2, which is produced mainly by T helper cells, is a central regulator of T cell function. IL 2 induces proliferation and activation of both $CD4^+$ and $CD8^+$ T cell, potentiates the cytotoxicity of $CD8^+$ T lymphocytes and NK cells and stimulates B cell function (Alfano and Poli, 2005). As stated above, increased production of pro-inflammatory IL 2 is dependent on the actions of another pro-inflammatory cytokine, IL 1. *Sutherlandia frutescens* extract dilutions increased IL 1 levels while maintaining IL 2 to those of normal untreated cells. This might be a means to limit the pro-inflammatory actions of both cytokines.

Interleukin 4 concentrations were unchanged in SF extract treated samples when compared with the untreated control samples. The SFE extracts decrease IL 4 levels while this cytokine was either maintained (1/200) or increased (1/50) in the SFW extracts but all these changes were not statistically significant ($p > 0.05$). Phytohaemaglutin significantly increased ($p < 0.001$) IL 4 levels compared to all the various samples and controls (Figure 8.4). Interleukin 4 is an anti-inflammatory cytokine that down-regulates both the innate and the adaptive immune responses. It is mainly produced by T cells, NK cells and mast cells and targets other T cells, eosinophils and endothelial cells for its regulatory actions (Alfano and Poli, 2005).

The effects of the SF extracts on this group of cytokines (IL 1α, IL 1β, IL 2, and IL 4) can be useful in predicting the effects of this medicinal plant in diseases such as cancer and HIV/AIDS. The increased production of pro-inflammatory IL 1 can be useful in stimulating the immune response against cancer cells while the up-regulatory effect of this cytokine on IL 2 can be useful in HIV infection. Interleukin 2 production has been shown to be deficient in HIV infected individuals as well as after *in vitro* infection of PBMCs. Due to the short half-life IL 2, changes in the levels of this cytokine are not easy to assay directly (Alfano and Poli, 2005).

The reduction or maintenance to normal levels of IL 1β by the SFE extract dilutions and increased secretion caused by SFW extract dilutions contradict the results obtained by Tai *et al.*, (2004). They found that there was no significant change or inhibition of IL 1β mRNA expression in RAW 264.7 macrophage cells treated with dilutions of an ethanol extract of SF. This pro-inflammatory cytokine, along with TNF-α, has been implicated in the cachexia seen in patients with HIV/AIDS. Agents that can reduce their production are thought be clinically useful. This study has also demonstrated that high concentrations of SF extracted by 70% ethanol can reduce the levels of IL 1β and therefore can alleviate the wasting diseases seen in cancer and HIV/AIDS patients. Lower concentrations may need to be taken chronically to have a similar effect.

Maintenance of IL 4 levels at those of normal untreated cells allows the process of immune stimulation to remain unimpeded. By not decreasing an anti-inflammatory cytokines like IL 4, our data supports the traditional use and scientific data showing that SF is a useful anti-inflammatory herbal plant (Fernandes *et al.*, 2004 and Kundu *et al.*, 2005).

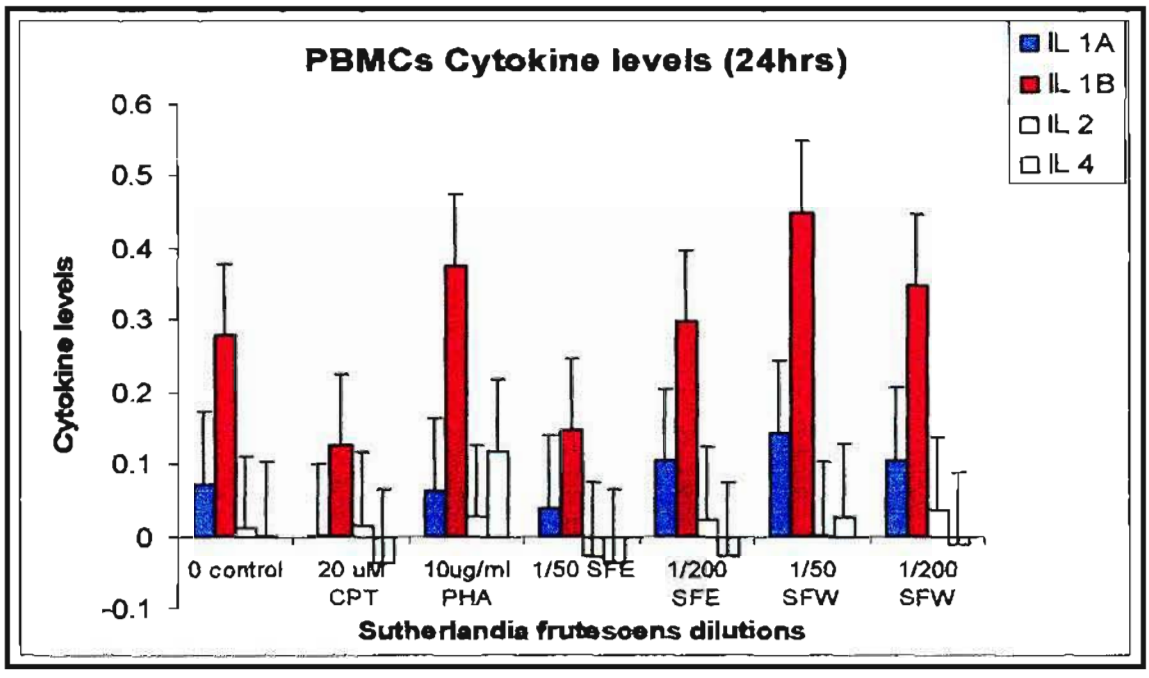


Figure 8.4: Graphical illustration of changes in concentrations of IL 1A, IL 1B, IL 2 and IL 4 in controls and *Sutherlandia frutescens* extract dilutions treated samples.

8.4.2 Interleukins 6, 8, 10, and 12

The presence of IL -6 and -8 was relatively high in all the analysed supernatants when compared to the other cytokines. Interleukin 6 concentrations in supernatants of SF extract treated PBMCs were significantly decreased when compared to the untreated cells, with the SFE extract dilutions being more effective ($p< 0.001$) than SFW extract dilutions ($p< 0.01$) (Figure 8.5). Interleukin 6 expression was virtually eliminated in CPT treated supernatants ($p< 0.001$) while this cytokine was maintained at similar levels in PHA treated supernatants to that of the untreated control ($p> 0.05$). Interleukin 8 concentrations were significantly increased in SF extract dilutions treated samples ($p< 0.001$) when compared to the control samples. Phytohaemaglutin maintained IL 8 levels to that of the

untreated control while this cytokine was significantly decreased ($p< 0.001$) in CPT treated supernatants (Figure 8.5).

The ability of SF extracts to significantly decrease the levels of IL 6 is very important in the use of this herbal medicine in the treatment of various forms of cancer. Interleukin 6 is a key growth-promoting and anti-apoptotic inflammatory cytokine and is one of the effector signals of activated NF-κB in the promotion of neoplasia (Germano *et al.*, 2008). Depending on the type of cancer model, IL 6 activates different major pathways of cell proliferation, inducing tumour growth, metastasis, and resistance to chemotherapy (Salazar-Onfray *et al.*, 2007). Decreased levels of IL 6 might also be useful in HIV infections as this would reduce the ability of infected T lymphocytes to resist induction of apoptosis.

Interleukin 8 (CXCL8) is the most important chemo-attractant of polymorph nuclear cells and is derived mostly from mononuclear phagocytes, endothelial, and epithelial cell but also from T cells, eosinophils, neutrophils, and fibroblasts. Interleukin 8 is one of the most important chemo-attractants of neutrophils and its synthesis is enhanced by IL 1 (Borish and Steinke, 2003). The ability of SF to up-regulate both IL 1 and IL 8 while the pro-inflammatory IL 2 is left stable may be important in maintaining a balance between immune stimulation and resisting excessive inflammation which might damage normal tissues.

Sutherlandia frutescens ethanol extract dilutions significantly reduced ($p< 0.001$) and SFW extracts slightly ($p< 0.01$) decreased IL 10 concentrations when compared to the untreated control. PHA significantly increased ($p< 0.001$) while CPT significantly decreased ($p<$

0.001) the levels of IL 10. Concentrations of IL 12 did not change in either control samples or SF extract dilutions treated samples ($p > 0.05$) (Figure 8.5).

Interleukin 10 is a cytokine mostly secreted by dendritic cells (DC), Th2 cells, B cells, monocytes, and macrophages. This cytokine inhibits the production of all pro-inflammatory cytokines and chemokines and the expression of DC-co-stimulatory molecules, therefore shutting-off T-cell activation. Interleukin 10 levels have been found elevated in lymph nodes of HIV-infected individuals (Alfano and Poli, 2005). Therefore the ability of SF extracts to suppress the secretion of this cytokine, as shown in the results obtained in this study, might be useful in allowing activation of T-cells and reduction of HIV replication. Interleukin 10 has also been shown to down-regulate VEGF synthesis and inhibit angiogenic cytokine production (Minozzo *et al.*, 2007). Such effects can be useful in the treatment of cancer and inhibition of the production of IL 10 by SF extracts might limit the anticancer abilities of this herbal plant.

Tumour necrosis factor-α is produced by neutrophils, activated lymphocytes, monocytes, macrophages, NK, DC, and mast cells. Tumour necrosis factor-α interacts with endothelial cells to induce adhesion molecules known as intracellular adhesion molecule (ICAM)-1, vascular adhesion molecule (VCAM)-1, and E-selectin, thus permitting accumulation of granulocytes into inflammatory sites. Its pro-inflammatory actions allow for tumour regression by host immune reaction and inhibition of tumour angiogenesis (Minuzzo *et al.*, 2007). These important actions are tempered by severe side effects of this cytokine. Tumour necrosis factor is responsible for the severe cachexia (more so than IL 1β) seen in chronic infections such as cancer and HIV/AIDS. Other side effects include induction of vascular leakage, negative inotropic effects, and it is the primary endogenous mediator of toxic shock and sepsis (Borish and Steinke, 2003).

Tai *et al.*, (2004) demonstrated that SF did not significantly inhibit the expression of liposaccharide (LPS) induced TNF-α mRNA expression in RAW 264.7 cells. Our results are in contrast to Tai *et al.*, (2004), as we have demonstrated that higher concentrations of both SFE and SFW extracts significantly reduced the levels of TNF-α in SF treated PBMCs. Even the lower concentrations reduced this cytokine but this was not statistically significant. These preliminary results provide evidence for the anti-wasting abilities of SF as noted by traditional healers and community workers. D-pinitol, one of active ingredients found in SF, has been suggested as a contributor of the positive effects against wasting.

Levels of IL 17A did not show any significant change in either the controls or SF treated samples. The levels of IFN-γ in SF and CPT treated samples were also unchanged while PHA induced a significant increase ($p< 0.001$) in the concentration of this cytokine (Figure

8.6). The lower dilutions of the SF extracts reduced GM-CSF levels, but not significantly ($p > 0.05$) while the higher dilutions increased this cytokine (not significant $p > 0.05$).

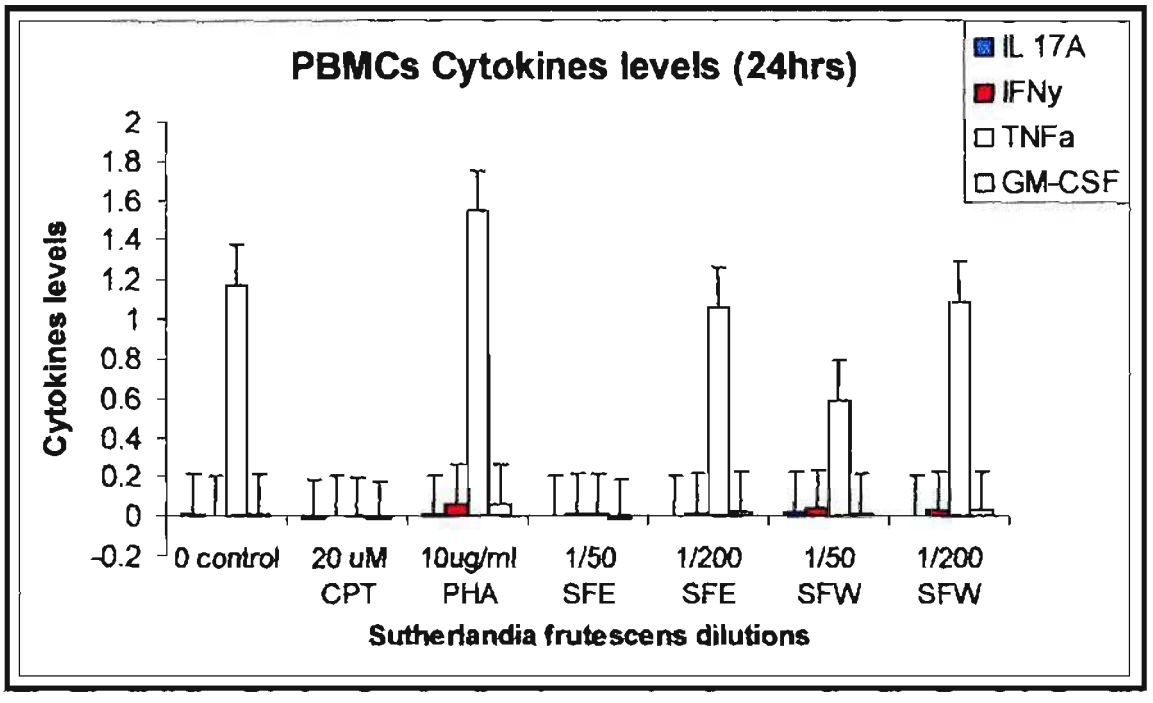


Figure 8.6: Graphical illustration of changes in the concentrations of IL 17A, IFN-γ, TNF-α, and GM-CSF in controls and *Sutherlandia frutescens* extract dilutions treated samples.

8.4.4 Contribution of changes in cell viability in the production of the cytokines analysed

As mentioned in the paragraph 8.3.2.1 above, before the supernatants of treated PBMCs were analysed for cytokines analyses, an ATP assay was performed to determine the cytotoxicity of SF extracts on these treated cells. Peripheral blood mononuclear cells treated with the negative control (20 µM CPT) had significantly reduced ATP levels (51%) when compared to the untreated control cells ($p < 0.001$). PHA (10 µg/ml) significantly

increased ATP levels (161%) over the 24 hours incubation period ($p< 0.001$). The 70% ethanol control was not included in the cytokines analyses setup because it was shown to have not changed ATP levels (97%) when compared to the untreated PBMCs ($p> 0.05$) (Figure 8.7). 70% ethanol was also shown to have not played a statistically significant role in the effects of SFE extracts on ATP levels in treated PBMCs (appendix 5).

The lower dilutions of SF extracts (1/50 SFE and 1/50 SFW) significantly ($p< 0.001$) reduced ATP levels (25% and 60%, respectively) when compared to the untreated control. The 1/50 SFE extract dilution was more potent in reducing ATP levels than CPT ($p< 0.001$). The dilutions (1/200 SFE and 1/200 SFW) did not change ATP levels to a significant when compared to the untreated control (Figure 8.7). These results further confirmed the cytotoxicity of higher concentrations of SF on cultured normal white blood cells *in vitro* (appendix 5).

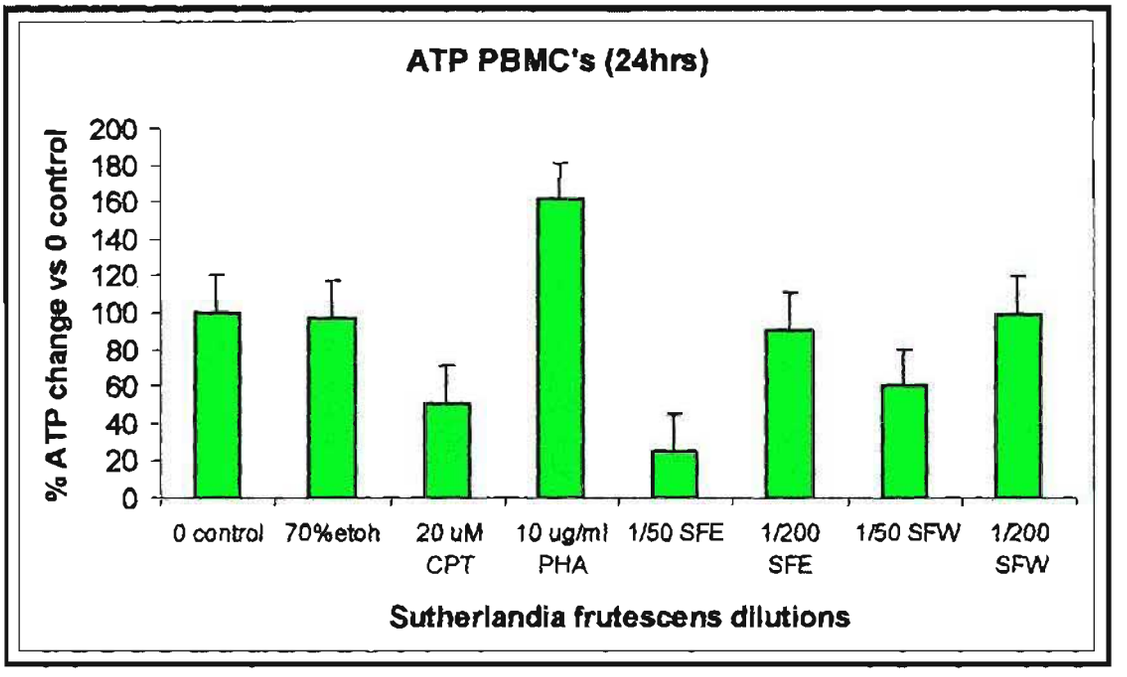


Figure 8.7: Graphical illustration of changes in PBMCs ATP concentrations after treatment with various dilutions of *Sutherlandia frutescens* extract dilutions over 24 hours incubation period.

The changes in ATP levels in the treated and control samples related to the effects of SF extract dilutions in cytokine concentrations in different ways. In the negative (CPT) and positive (PHA) controls, the changes in the cytokines were directly related to the number of viable cells (equivalent to % ATP levels) in each sample. In the CPT-treated PBMCs, all the cytokines decreased significantly as the viability of the cells decreased. PHA stimulated the immune cells to proliferate and this was reflected by the concomitant increase in most of the cytokines when compared to the untreated control cells.

The level of cytokines in supernatants of SF extracts-treated PBMCs related differently to cell viability as represented by ATP concentrations. Some of the cytokines were up-regulated (increased production) above or maintained to the levels of the untreated controls

while ATP levels were shown to be depleted by dilutions of the SF extracts (Figure 8.7). These observations give an impression that the cytotoxicity (changes in ATP concentrations) of the SF extracts was not related to the amount of cytokines secreted by the treated PBMCs. A possible exception to this would be the cells treated with the 1/50 SFE extract dilution, which produced the levels of cytokines generally consistent viable cell.

8.5 Conclusion

Peripheral blood mononuclear cells were shown to secrete high concentrations of pro-inflammatory cytokines than their anti-anti-inflammatory counterparts. It was the effect of SF extracts on these pro-inflammatory cytokines, as shown in the preliminary screening, which therefore can determine the outcome in disease conditions. The ELISA method used in this study has shown that high concentrations of SF extract dilutions can reduce the production of IL 1β and TNF-α and this effect is regarded as being helpful in fighting muscle wasting in cancer and HIV/AIDS patients. The changes cytokine levels which occurred as a result of SF extracts-treatment were largely not related to changes in ATP content (and therefore cell viability) but the cytokines themselves might be one of the effectors of these changes in viability. Changes in the other cytokines, as effected by SF extract dilutions, can also be helpful in fighting many other diseases.

CHAPTER 9

CONCLUSION

The past decade has seen a renewed interest in traditional medicine by scientists in search of new pharmaceuticals. This has resulted in a tremendous increase in publications concerning traditional medicinal plants and in particular ethnopharmacology (Editorial (*Journal of Ethnopharmacology)*, 2008). *Sutherlandia frutescens* has a long history of medicinal use in southern Africa. It has been used for a wide variety of ailments including diabetes, stress, fever, internal cancer and more recently, HIV/AIDS (van Wyk and Albrecht, 2008; van Wyk, 2008; Ojewole, 2008). The aims of this research thesis were to investigate the anticancer abilities of SF using a T lymphocytes cell line, H9, and to ascertain whether this traditional medicinal plant can stimulate normal human T cells to proliferate.

Using the MTT and ATP cytotoxicity assays, the effects of SFE and SFW extract dilutions in H9 and normal T lymphocytes were studied over 24 and 48 hours incubation. The SFE extracts were shown to be more potent in inducing cytotoxicity in H9 T cells with a calculated IC_{50} of 1/40 after 24 hours. Similar dilutions of the SFW extracts did not inducing significant cytotoxicity over 24 hours but were more effective after 48 hours incubation. Using the more sensitive ATP assay on treated H9 T cells, it showed similar trends to that of the MTT assay but with varying cytotoxicity levels. In the treated isolated normal T cells after 24 hours, the SFE extract dilutions induced an ethanol dependent cell proliferation while SFW extract did not significantly change cell viability. After 48 hours, SFE extracts-induced proliferation was reversed while the SFW extracts a very significant

increase in cell viability over the similar period. Reversal of proliferation by SFE extract dilutions was indicative of a possible cytotoxicity of high concentrations of the SF plant on normal cells *in vitro*.

The mode of cell death induced by SF extract dilutions in treated cell lines was assessed using a luminometer based caspase-3/-7 activity assay. Dilutions of both SFE and SFW extracts were shown to inhibit caspase-3/-7 activity in both H9 and normal T cells in a concentration and time-dependent manner, with higher concentrations more effective in this action. Lower concentrations of the extracts increased activity of caspases-3/-7 activity above the levels of untreated cells, raising the possibility of induction of apoptosis by these concentrations. Inhibition of caspase-3/-7 activity by higher concentrations of SF extracts did not impact on induction of cytotoxicity as shown by the MTT and ATP assays. Therefore it is possible to conclude that higher concentrations of SF extracts induce more necrosis than apoptosis.

The ability of SF extract dilutions to scavenge for lipid peroxides in supernatants of treated cell lines was also investigated. Both SFE and SFW extract dilutions were shown to significantly reduce lipid peroxides in the extracellular environment of treated cells when compared to all the controls used. This was regardless of the concentration of SF extracts used to treat the cells or the type of cell line (normal T cells or H9 T cells). With regards to the effects of SF extracts on the intracellular GSH content, SF extracts decreased GSH levels in H9 cancerous cells while increasing the GSH levels in normal T cells depending on the type and concentration of the extracts. High concentrations of the SFE extracts negatively affected the GSH content in normal T cells independent of ethanol and this again showed that high concentrations of SF can seriously suppress T cell proliferation.

Lower concentrations of the SFW extracts significantly increased GSH levels in normal T cells over 48 hrs.

Treatment of cancerous H9 T cells with SFE and SFW extract dilutions over 48 hours induced chromatin condensation and fragmentation of the nuclei. Normal T cells treated in a similar approach were seen to have normal nuclei while some cells had condensed chromatin but there was no fragmentation or blebbing of the nuclei. These results show that SF can induce apoptosis in malignant cell lines and also have similar effects on normal cells. The condensed chromatins of normal T cells may be another indication that higher concentrations of SF can be harmful to normal tissues.

In the cytokines analyses of supernatants from treated PBMCs, high concentrations of SF extracts were shown to reduce the production of IL 1β and TNF-α, and that this effect can be helpful in fighting wasting in cancer and HIV/AIDS patients. The cytokine changes which occurred as a result of SF extracts-treatment were largely not related to changes in cell viability but the cytokines themselves might be one of the effectors of these changes in viability. Changes in the other cytokines as affected by these extracts can also be helpful fighting many other diseases.

This study has therefore shown for the first time that while high concentrations of SF extracts were more effective in inducing cytotoxicity to cancerous H9 T cells, they can be harmful to *in vitro* normal T cells by reducing cell viability, GSH levels and inducing chromatin condensation. Similar harmful effects on cell viability were also shown in treated PBMCs. Lower concentrations of the SF extracts were shown to increase cell viability, GSH content and being effective scavenging for lipid peroxides in normal T

cells. These results suggest that SF may be useful in cancer patients if taken in low doses. Induction of cytotoxicity in cancer cells and immune stimulation and tolerance by these doses can improve the diseases outcome. Further studies should be undertaken on the immune stimulating abilities of this medicinal plant both *in vitro* and *in vivo*.

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APPENDICES

Appendix 1: Reagents for cell culture

1.1 Preparation of complete culture media (CCM)

RPMI-1640 was supplemented with heat inactivated 10% foetal calf serum (FCS), 1% L-glutamine and 1% Pen-Strep-Fungizone (PSF).

1.2 Preparation of cryopreservation media

H9 and normal T lymphocytes were cryopreserved in 80% RPMI-1640 supplemented with 10% FCS and 10% DMSO.

1.3 Preparation of 0.4% trypan blue stain

Trypan blue stain was prepared by dissolving 0.4g of trypan blue dye in 0.85% sodium chloride (NACl) and this solution was stored at room temperature.

1.4 Preparation of buffer for labeling and enrichment of T lymphocytes (BD IMag™ Buffer)

Phosphate buffered saline (PBS) was supplemented with 0.5% BSA, 2 mM EDTA, and 0.1% sodium azide.

Appendix 2: Preparation of *Sutherlandia frutescens* extracts dilutions

SF 70% ethanol and deionised water extracts were diluted into working concentrations in prepared RPMI-1640. These dilutions were normally made up to 1 ml as illustrated below,

Dilution (1000 μl)	Extract stock (μl)	RPMI-1640 (μL)
1/50	20	980
1/150	6.67	992.33
1/200	5	995
1/300	3.3	996.7

Appendix 3: Reagents for TBARS assay

3.1 20 mM butylatedhydroxytoluene (BHT) in ethanol

0.1102g of BHT was dissolved in 25 ml ethanol and stored at room temperature.

3.2 50 mM sodium hydroxide (NaOH)

50mg of NaOH was dissolved in 2 ml deionised water and stored at room temperature.

3.3 TBA/BHT solution

125μl of 20 mM BHT was added in 25 ml deionised water. 0.25 g of TBA was then dissolved in 25 ml of deionised water. To make the TBA/BHT solution, 0.5g of TBA and 125μl of 20mM BHT were added into 25 ml of the 50mM NaOH and stored at room temperature.

3.4 3 mM HCl in H_2O

An initial concentration of 32 % HCl stock was diluted to a 1M solution using molarity. The 1 M HCl was then diluted to a 3 mM working solution and stored at room temperature.

3.5 0.1% H_3PO_4

85% H_3PO_4 stock was diluted to 0.1% working solution in deionised water and stored at room temperature.

3.6 2% H_3PO_4

85% H_3PO_4 stock was diluted to the 2% working solution in deionised water and stored at room temperature.

3.7 7% H_3PO_4

85% H_3PO_4 stock was again diluted to the 7% working solution in deionised water and stored at room temperature.

Appendix 4: Reagents for Hoechst 33342 staining

4.1 Hoechst 33342

Hoechst stain stock solution was prepared by dissolving 4 mg of bisbenzimide in 2 ml of DMSO. A working solution was then prepared by adding 1.4 ml of the stock solution into 1 ml of PBS.

4.2 Paraformaldehyde (10%)

1 gram of paraformaldehyde was dissolved in PBS (10 ml) and heated for 30-40 minutes in 60 ^{0}C water bath. The pH was then adjusted to 7.2 with 1 M HCl.

Appendix 5: Raw data from plots used in the main script

1.1 MTT assay spectrophotometer readings

		Spectrophotometer readings					
	Sample type	1	2	3	Average	Cell viability (%)	STD DEV
	Control	1.603	1.64	1.492	1.578	100.00	0.077
	SFE 1/50	0.854	1.07	0.987	0.970	61.48	0.109
	SFE 1/200	1.122	1.236	1.104	1.154	73.12	0.072
	SFW 1/50	1.437	1.394	1.377	1.403	88.87	0.031
	SFW 1/200	1.583	1.633	1.547	1.588	100.59	0.043

Repeat							
		1	2	3	Average	Cell viability (%)	
	Control	0.167	0.163	0.135	0.155	100	0.017
	Vehicle treated	0.097	0.172	0.119	0.129	83.44	0.039
	Camptothecin	0.118	0.077	0.088	0.094	60.86	0.021

		Spectrophotometer readings					
	Sample type	1	2	3	Average	Cell Viability (%)	STD DEV
	Control	0.134	0.123	0.112	0.123	100.00	0.011
	70% EtOH	0.218	0.209	0.2	0.209	169.92	0.009
	CPT	0.116	0.112	0.119	0.116	94.04	0.004
	1/50 SFE	0.313	0.342	0.284	0.313	254.47	0.029
	1/200 SFE	0.259	0.296	0.332	0.296	240.3794	0.037
	1/50 SFW	0.198	0.195	0.191	0.195	158.27	0.004
	1/200 SFW	0.188	0.224	0.259	0.224	181.8428	0.036

H9 cells 48 hrs		Spectrophotometer readings					
	Sample type	1	2	3	Average	Cell viability (%)	STD DEV
	Control	1.106	1.441	1.238	1.262	100.000	0.169
	70% EtOH	0.85	1.412	1.113	1.125	89.168	0.281
	20 μM CPT	0.112	0.153	0.136	0.134	10.594	0.021
	SFE 1/50	0.313	0.33	0.392	0.345	27.345	0.042
	SFE 1/200	0.741	0.615	0.717	0.691	54.769	0.067
	SFW 1/50	0.86	1.094	1.013	0.989	78.388	0.119

	SFW 1/200	0.755	0.797	0.854	0.802	63.567	0.050

Norm T cells 48 hrs		Spectrophotometer readings					
	Sample type	1	2	3	Average	Cell Viability (%)	STD DEV
	Control	0.22	0.277	0.191	0.229	100.00	0.036
	70% EtOH	0.2	0.147	0.24	0.196	85.32	0.038
	20 μM CPT	0.178	0.114	0.156	0.149	65.12	0.027
	1/50 SFE	0.211	0.251	0.29	0.251	109.30	0.032
	1/200 SFE	0.207	0.33	0.452	0.330	143.75	0.100
	1/50 SFW	0.381	0.398	0.39	0.390	169.91	0.007
	1/200 SFW	0.392	0.632	0.512	0.512	223.26	0.098

5.2 Luminometer readings-ATP assay on normal T cells

ATP levels in normal T cells (24 hrs)	Luminometer readings (RLU)				
Sample types	1	2	Average	STD DEV	% ATP
CONTROL	4821270	5842430	5331850	722069.2	100.00
70% EtOH	7088020	6580800	6834410	358658.7	128.1808
20 μM CPT	77084.2	76963.3	77023.75	85.48921	1.44
SFE 1/50	3849430	3829950	3839690	13774.44	72.01
SFE 1/200	5089080	5933930	5511505	597399.2	103.37
SFW 1/50	4625390	4641270	4633330	11228.86	86.90
SFW 1/200	5697430	6611850	6154640	646592.6	115.43

ATP levels in normal T cells (48 hrs)		Luminometer readings (RLU)				
	Sample types	1	2	Average	STD DEVIATION	% ATP
	CONTROL	5388560.0	4711580.0	5050070.0	478697.1	100.00
	70% EtOH	5266200	5405240	5335720.0	98316.1	105.6564
	20 μM CPT	8223.2	8813.7	8518.5	417.5	0.17
	SFE 1/50	2248540.0	2225480.0	2237010.0	16305.9	44.30
	SFE 1/200	4380720.0	4317140.0	4348930.0	44957.8	86.12
	SFW 1/50	1576370.0	1094320.0	1335345.0	340860.8	26.44
	SFW 1/200	4209190.0	3138220.0	3673705.0	757290.1	72.75

5.3 Luminometer readings- Caspase-3/-7 assay

Caspase-3/-7 activity in normal T cells (24 hrs)		Luminometer readings (RLU)					
	Samples	1	2	Average	minus blank	% Caspase activity	STD DEV
	Control	64409.5	62339	63374.25	34944.4	100.00	1464.065
	70% EtoH	58958.4	68493.4	63725.9	35296.05	101.01	6742.263
	20 μM CPT	57160.4	72939.5	65049.95	36620.1	104.80	11157.51
	1/50 SFE	29949	35172.3	32560.65	4130.8	11.82	3693.431
	1/150 SFE	42429.2	46227.4	44328.3	15898.45	45.50	2685.733
	1/300 SFE	49077.3	57382.6	53229.95	24800.1	70.97	5872.734
	1/50 SFW	26034.5	41593.9	33814.2	5384.35	15.41	11002.16
	1/150 SFW	35067.9	54020.7	44544.3	16114.45	46.11	13401.65
	1/300 SFW	77064	77684.6	77374.3	48944.45	140.06	438.8305
	BLANK	27083.2	29776.5	28429.85			

Caspase-3/-7 activity in H9 cells (24 hrs)		Luminometer readings (RLU)					
	Samples	1	2	Average	minus blank	% activity	STD DEV
	0 Control	13323.5	13001.1	13162.3	6959.455	100.00	227.9712
	70% EtOH	10073.9	9732.55	9903.225	3700.38	53.17	241.3709
	20 μM CPT	8411.4	8584.54	8497.97	2295.125	32.98	122.4285
	1/50 SFE	8894.71	7121.43	8008.07	1805.225	25.94	1253.898
	1/150 SFE	12456.5	10567.4	11511.95	5309.105	76.29	1335.795
	1/300 SFE	16439.6	10997.8	13718.7	7515.855	107.99	3847.934
	1/50 SFW	9681.5	9214.07	9447.785	3244.94	46.63	330.5229
	1/150 SFW	10503.3	13883.2	12193.25	5990.405	86.08	2389.95
	1/300 SFW	10835.6	18486.4	14661	8458.155	121.53	5409.933
	BLANK	6212.85	6192.84	6202.845			

		Luminometer readings (RLU)					
	Samples	1	2	Average	minus blank	% Caspase activity	STD DEV
	Control	210235	198147	204191	158458.4	100.00	8547.507
	70% EtoH	187194	205418	196306	150573.4	95.02	12886.31
	CPT	211085	199391	205238	159505.4	100.66	8268.907
	1/50 SFE	55876.5	24071.8	39974.15	-5758.45	-3.63	22489.32
	1/150 SFE	145244	92848	119046	73313.4	46.27	37049.57
	1/300 SFE	192092	141749	166920.5	121187.9	76.48	35597.88
	1/50 SFW	89655.2	100017	94836.1	49103.5	30.99	7326.899
	1/150 SFW	144627	139698	142162.5	96429.9	60.86	0
	1/300 SFW	141681	129681	135681	89948.4	56.76	8485.281
	BLANK	42226.1	49239.1	45732.6			

		Luminometer readings (RLU)					
	Samples	1	2	Average	minus blank	% activity	STD DEV
	0 Control	223964	164564	194264	188061.2	100.00	42002.14
	70% EtOH	190094	90618.4	140356.2	134153.4	71.33	70339.87
	20 μM CPT	39619.2	37645.9	38632.55	32429.71	17.24	1395.334
	1/50 SFE	21315.8	19557.3	20436.55	14233.71	7.57	1243.447
	1/150 SFE	107086	58452.6	82769.3	76566.46	40.71	34389.01
	1/300 SFE	71946.6	119863	95904.8	89701.96	47.70	33882.01
	1/50 SFW	69359.1	74134.9	71747	65544.16	34.85	3377.001
	1/150 SFW	118283	129279	123781	117578.2	62.52	7775.346
	1/300 SFW	188656	158237	173446.5	167243.7	88.93	21509.48
	BLANK	6212.85	6192.84	6202.845			

5.4 Luminometer readings- GSH assay

			Luminometer readings (RLU)					
GSH content in H9 cells (24 hrs)	Sample type		1	2	Average	minus blank	% GSH change	Std dev
	0 Control	1	265154	290217	277685.5	137331	100.00	17722.22
	70% EtoH	2	210477	197792	204134.5	63780	46.44	8969.65
	CPT	3	192073	206933	199503	59148.5	43.07	10507.61
	1/50 SFE	4	191026	199160	195093	54738.5	39.86	5751.607
	1/150 SFE	5	240515	253141	246828	106473.5	77.53	8927.93
	1/300 SFE	6	288957	274446	281701.5	141347	102.92	10260.83
	1/50 SFW	7	258141	239872	249006.5	108652	79.12	12918.13
	1/150 SFW	8	269165	280889	275027	134672.5	98.06	8290.12
	1/300 SFW	9	233351	214220	223785.5	83431	60.75	13527.66
	BLANK	10	139792	140917	140354.5			

			Luminometer readings (RLU)					
GSH content in normal T cells (24 hrs)	Sample type		1	2	Average	minus blank	% GSH change	Std dev
	0 CONTROL	1	190176	215125	202650.5	53074	100.00	17641.61
	70% EtOH	2	179422	185874	182648	33071.5	62.31	4562.253
	20 μM CPT	3	178386	179670	179028	29451.5	55.49	907.9251
	1/50 SFE	4	123903	142312	133107.5	-16469	-31.03	13017.13
	1/150 SFE	5	195434	195842	195638	46061.5	86.79	288.4996
	1/300 SFE	6	250136	247875	249005.5	99429	187.34	1598.768
	1/50 SFW	7	210216	216239	213227.5	63651	119.93	4258.904
	1/150 SFW	8	262736	269567	266151.5	116575	219.65	4830.246
	1/300 SFW	9	269559	279913	274736	125159.5	235.82	7321.384
	BLANK	10	144530	154623	149576.5			

			Luminometer readings (RLU)					
	Sample type		1	2	Average	minus blank	% GSH change	Std dev
	Control	1	1887520	1543190	1715355	1575001	100.00	243478.08
	70% EtoH	2	1073380	991640	1032510	892155.5	56.64	57798.91
	20 μM CPT	3	405452	756697	581074.5	440720	27.98	248367.72
	1/50 SFE	4	538086	566469	552277.5	411923	26.15	20069.81
	1/150 SFE	5	1049280	1175520	1112400	972045.5	61.72	89265.16
	1/300 SFE	6	1852100	1841870	1846985	1706631	108.36	7233.70
	1/50 SFW	7	587427	437173	512300	371945.5	23.62	106245.62
	1/150 SFW	8	309832	383755	346793.5	206439	13.11	52271.45
	1/300 SFW	9	395702	319582	357642	217287.5	13.80	53824.97
	BLANK	10	139792	140917	140354.5			

			Luminometer readings (RLU)					
	Sample type		1	2	Average	minus blank	% GSH change	Std dev
	CONTROL	1	200401	216431	208416	31999	100.0	11334.92
	70% EtOH	2	215560	259134	237347	60930	190.4	30811.47
	20 μM CPT	3	176539	187826	182182.5	5765.5	18.0	7981.114
	1/50 SFE	4	162400	178445	170422.5	-5994.5	-18.7	11345.53
	1/150 SFE	5	212093	222806	217449.5	41032.5	128.2	7575.235
	1/300 SFE	6	254754	253004	253879	77462	242.1	1237.437
	1/50 SFW	7	206048	208977	207512.5	31095.5	97.2	2071.116
	1/150 SFW	8	294061	299532	296796.5	120379.5	376.2	3868.581
	1/300 SFW	9	297552	292864	295208	118791	371.2	3314.917
	BLANK	10	162136	190698	176417			

5.5 PBMCs ATP assay- luminometer readings (RLU)

ATP levels in PBMCs (24 hrs)		Luminometer readings (RLU)					
	Sample types	1	2	Average	minus blank	% ATP change vs. control	STD DEV
	0 control	25959	26518	26238.5	26187.5	100	395.2727
	70% EtoH	27013	24031	25522	25471	97.26396	2108.592
	20 μM CPT	14711	11903	13307	13256	50.61957	1985.556
	10 μg/ml PHA	43671	40786	42228.5	42177.5	161.0597	2040.003
	1/50 SFE	6405	6931	6668	6617	25.26778	371.9382
	1/200 SFE	23685	23785	23735	23684	90.4401	70.71068
	1/50 SFW	16113	15265	15689	15638	59.71551	599.6266
	1/200 SFW	26247	25778	26012.5	25961.5	99.13699	331.6331