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THE EFFECTS OF *Sutherlandia frutescens* IN CULTURED RENAL PROXIMAL AND DISTAL TUBULE EPITHELIAL CELLS

BY

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ABSTRACT

Sutherlandia frutescens (SF), an indigenous medicinal plant to South Africa (SA), is traditionally used to treat a diverse range of illnesses including cancer and viral infections. The biologically active compounds of SF are polar, thus renal elimination increases susceptibility to toxicity. This study investigated the antioxidant potential, lipid peroxidation, mitochondrial membrane potential and apoptotic induction by SF on proximal and distal tubule epithelial cells. Cell viability was determined using the MTT assay. Mitochondrial membrane potential was determined using a flow cytometric JC-1 Mitoscreen assay. Cellular glutathione and apoptosis were measured using the GSH-Glo^{TM} Glutathione assay and Caspase-Glo® 3/7 assay, respectively. The IC_{50} values from the cell viability results for LLC-PK$_1$ and MDBK was 15 mg/ml and 7 mg/ml, respectively. SF significantly decreased intracellular GSH in LLC-PK$_1$ ($p < 0.0001$) and MDBK ($p < 0.0001$) cells. Lipid peroxidation increased in LLC-PK$_1$ ($p < 0.0001$) and MDBK ($p < 0.0001$) cells. JC-1 analysis showed that SF promoted mitochondrial membrane depolarization in both LLC-PK$_1$ and MDBK cells up to 80% ($p < 0.0001$). The activity of caspase 3/7 increased both LLC-PK$_1$ (11.9-fold; $p < 0.0001$) and MDBK (2.2-fold; $p < 0.0001$) cells. SF at high concentrations plays a role in increased oxidative stress, altered mitochondrial membrane integrity and promoting apoptosis in renal tubule epithelia.

DECLARATION

This study represents the original work by the author and has not been submitted in any form to another University. The use of work by others has been duly acknowledged in the text.

The research described in this study was carried out in the Discipline of Medical Biochemistry, Faculty of Health Sciences, University of KwaZulu-Natal, Durban, under the supervision of Prof. A. A. Chuturgoon.

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Alisa Phulukdaree

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ABBREVIATIONS

$\Delta\Psi_m$	Mitochondrial membrane potential
AIDS	Acquired immunodeficiency syndrome
AIF	Apoptosis inducing factor
ALA	Alpha lipoic acid
Apaf-1	Apoptotic protease activating factor-1
ATM	Ataxia telangiectasia mutated protein
ATP	Adenosine triphosphate
ATR	ATM related
Bcl-2	B cell lymphoma-2
$CCl_3^{\cdot}$	Trichloromethyl
CCM	Complete culture media
$CD4^+$	Cluster differentiation
CHO	Chinese hamster ovary
Complex IV	Cytochrome oxidase
Complex V	Mitochondrial ATP synthase
COX	Cyclooxygenase
CYP	Cytochrome P450 enzymes
DCT	Distal convoluted tubule
DED	Death effector domain
DHLA	Dihydrolipoic acid
DISC	Death inducing signalling complex
DMSO	Dimethyl sulphoxide
DNA	Deoxyribose nucleic acid

DNA-PK	DNA-dependant protein kinase
ETC	Electron transport chain
$FADH_2$	Flavin-adenine dinucleotide dihydrogen
FADD	Fas associated death domain
FMN	Flavin mononucleotide
GABA	Gamma-amino-butyric-acid
GABA-T	GABA transaminase
GAD	Glutamate decarboxylate
GSH	Glutathione
GSSG	Oxidised glutathione
h	hour/s
H_2O_2	Hydrogen peroxide
HIV	Human immunodeficiency virus
HL60	Promyelocyte cell line
IAP's	Inhibitors of apoptosis proteins
IL-1β	Interleukin one beta
IC_{50}	Concentration of 50% cell growth inhibition
i.p	intraperitoneal
JNK	Jun NH_2-terminal kinase
MDA	Malondialdehyde
Mdm2	Mouse double minute 2
NADH	reduced nicotinamide adenine dinucleotide
NADPH	reduced nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear Factor kappa B
NO	Nitric oxide

NOS	Nitric oxide synthase
O_2	Oxygen
O_2^-	Superoxide
$OH^.$	Hydroxyl radical
$OONO^-$	Peroxynitrite
p53	Tumour suppressor protein p53
PCT	Proximal convoluted tubule
PG	Prostaglandin
PUFA	Polyunsaturated fatty acids
RIP	Receptor-interacting protein
ROS	Reactive oxygen species
$RS^.$	Thiyl
RT	Room temperature
SA	South Africa
s.e.	Standard deviation
SF	*Sutherlandia frutescens*
Smac	Second mitochondrial activator of caspases
SOD	Superoxide dismutase
STZ	streptozotocin
tBid	truncated bid
TNF-α	Tumour necrosis factor alpha
TNFR1	Tumour necrosis factor receptor 1
TRADD	TNFR1-associated death domain
TRAF2	TNF-associated factor 2
TRAIL	Tumour necrosis factor related apoptosis inducing ligand

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INTRODUCTION

Customarily, toxicology has been termed "the science of poisons" (Langman and Kapur, 2006). This science engages studying the properties of chemicals and the impact of these molecules on living organisms. Toxicological studies have provided a tool which considers the potential undesirable effects of chemicals in order to maintain and protect human health (Roberfroid, 1995).

Historically, toxicology dates as far back as 300 B.C where early man used animal venoms and plant extracts for hunting and as "bio-weapons" during war. Plant extracts formed the basis of therapeutics and experimental medicine during the early centuries. The discipline of toxicology integrates the understanding and applications of the biological sciences, chemistry, physics and mathematics to test its theories. Toxicology differs from other sciences with the absence of a single goal but its diversification has allowed for the interspersion of ideas and concepts from academe, industry as well as government. This gives the discipline of toxicology a unique but highly advantageous slant (Amdur *et al.*, 1991).

The majority of Africa's population reside in rural areas where there is a lack of basic health care facilities. These people are thus reliant on traditional remedies to treat a range of diseases. Traditional healers make use of herbal medicines despite the lack of evidence for its safety. It is therefore imperative that the identification, efficacy, therapeutic doses, toxicity, standardisation and regulation of these plants and their extracts be determined (Chattopadhyay, 2003).

The popular multi-purpose herbal remedy used by South Africans is *Sutherlandia frutescens* (SF). *Sutherlandia frutescens* is believed to have therapeutic potential against viral diseases, cancer, diabetes and a range of other conditions (Fernandes *et al.*, 2004). Herbal remedies are currently receiving much

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attention from the general public so much so, that these remedies are being made available in urban areas in the form of tablets. The spotlight, however, has shifted from the traditional conclusions of efficacy to the laboratories where experimentation will either prove or disprove the therapeutic potential to provide a cost effective and natural solution to health ailments.

CHAPTER 1

LITERATURE REVIEW

1.1   *Sutherlandia frutescens*

Traditional medicine is a common resort of majority of Africans that require healthcare as it is difficult and unaffordable to access healthcare facilities (Ojewole, 2008). In 2005 Mills *et al*. reported that approximately half of all pharmaceuticals were derived from plants. The diverse array of medicinal plants found in South Africa is estimated to be approximately 3 000 and is used regularly by the rural populace (van Wyk, 2008). A popular medicinal plant which is now commercially available due to its multi-purpose uses is SF. This plant has been used for a long time and it is assumed to be safe (Mills *et al*., 2005). The chemistry, pharmacology and toxicity of SF extracts are currently under critical analysis due to the increased usage and assumed safety of SF.

1.1.1   **Adaptogenic properties, Uses and Distribution of** *Sutherlandia frutescens*

Sutherlandia frutescens also referred to as the 'cancer bush' has been used for years by traditional healers to treat a variety of ailments. These include internal cancers, diabetes, uterine disease, influenza, human immunodeficiency virus (HIV) infection, depression, and arthritis (Gericke *et al.*, 2001).

Sutherlandia frutescens belongs to the family: Fabraceae/Leguminosa and is one of five currently recognised *Sutherlandia* species, all of which are indigenous to South Africa. This plant is distributed mainly in the Western Cape and Karoo regions (Fernandes *et al.*, 2004) in South Africa.

1.1.2   Toxicity

The recommended therapeutic dose of SF leaf powder for humans is 9mg/kg body weight/day with no side effects (Sia, 2004). This was determined during a toxicity study conducted on vervet monkeys (Medical Research Council and National Research Foundation of South Africa, 2002). During this study the dosage was increased nine times with no resulting haematological, clinical and physiological toxicity (Mills *et al.*, 2005).

A clinical trial conducted on healthy human volunteers showed no significant adverse changes in parameters following a daily dose of 800mg for 3 months (Ojewole, 2008). The commercially available SF tablet has a recommended dosage of 600mg/day.

1.1.3   Description and Biologically Significant Components of *Sutherlandia frutescens*

Sutherlandia frutescens is a perennial short-lived shrub that grows between 0.2-2.5m in height and has petiolate, stipulate and pinnate leaflets of 8-10 pairs with a terminal leaflet. Red and, seldomly white, flowers bear in axillary racemes and produces large and bladder-like pods containing numerous brown, laterally compressed, kidney shaped seeds. Leaves of SF are mainly used traditionally but all aerial parts are thought to have medicinal properties.

Biologically active compounds that have been isolated from SF include pinitol, flavonoids, saponins and amino acids such as gamma-amino-butyric-acid (GABA), L-canavanine (van Wyk, 2008), arginine, asparagine, proline, alanine, leucine, tryptophan and phenylalanine (Tai *et al.*, 2004). Other components of SF, along with commonly found plant-derived alcohols, include hexadecanoic acid, propyl parabens, methyl parabens, gamma sitosterol and sigmast-4-en-3-one (Sai, 2004).

1.1.4   L-Canavanine

L-canavanine is found at approximately 14.5mg per gram of dry SF leaves (van Wyk and Albrecht, 2008) and 3mg per gram in Phytonova SU1 SF tablets. Most leguminous plants in the subdivision Leguminosae synthesise L-canavanine (Bence and Crooks, 2003) which is a storage form of nitrogen in plant seeds and is used in the chemical defence mechanisms (Rosenthal, 1977).

L-canavanine is the L-2 amino-4-guanidinooxy structural analogue of L-arginine. The electron density of the guanidino group is reduced due to the destabilising effect of the oxygen atom (Bence and Crooks, 2003). As a result, the guanidino group of L-arginine has a pKa of 12.48 compared to 7.05 for the oxyguanidino group of L-canavanine (Swaffar *et al*., 1994). This makes L-canavanine less basic than L-arginine and exists in the amino, instead of the imino tautomeric form. These simple differences have huge implications with regard to their physiological roles. L-arginine is an important amino acid that is necessary for the normal development and growth of cells (Bence and Crooks, 2003) while L-canavanine is cytotoxic (Swaffar *et al*., 1994).

L-canavanine has been shown to be a potent inhibitor of inducible nitric oxide synthase (NOS) (Lincoln *et al.*, 1997), as well as other arginine-utilising enzymes (Hrabak *et al*., 1994). Nitric oxide synthase utilises L-arginine for the biosynthesis of nitric oxide (NO) and L-citrulline. The NOS reaction occurs in two steps, each using molecular oxygen (O_2) and reduced NADPH as co-substrates (Luzzi and Marletta, 2005). L-canavanine, a substrate in the NOS reaction, is converted to L-homoserine by the enzyme.

L-canavanine is responsible for structural changes induced in proteins when it is incorporated into a growing peptide chain in place of L-arginine. The outcome of the replacement is the initiation of

functional changes in the proteins containing L-canavanine. Thus the anti-metabolic properties of L-canavanine can be explained.

1.1.5　D-Pinitol
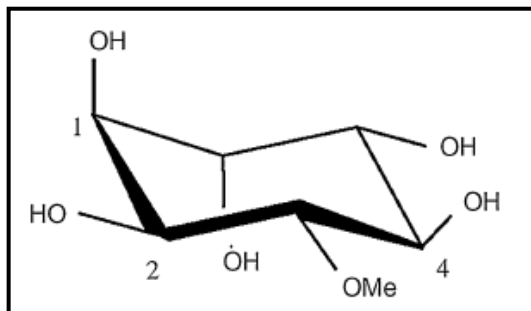
Figure 1.1　　Chemical structure of D-pinitol (Do *et al.*, 2008).

D-pinitol (Figure 1.1) is a legumous chiro-inositol sugar found at levels of 14mg per gram of dry SF leaf (Moshe, 1998; van Wyk and Albrecht, 2008). D-pinitol possesses anti-diabetic properties (Bates *et al.*, 2000) and has been implicated in treating wasting in cancer and acquired immunodeficiency syndrome (AIDS) (Ostlund and Sherman, 1996). This insulin-like effect makes glucose readily available to cells for increased metabolism, thus increasing intracellular ATP levels. Pro-inflammatory cytokines such as tumour necrosis factor alpha (TNF-α) and interleukin one beta (IL-1β) was reduced in rats with acute oedema treated with D-pinitol (Sia, 2004).

1.1.6　Gamma amino butyric acid

In SF tablets there is more or less 0.4mg/g of GABA (Tai *et al*., 2004). Gamma amino butyric acid is an inhibitory neurotransmitter which mediates its effects outside the nervous system (van Wyk, 2008). This amino acid has been used and is thought to be responsible for relief of anxiety and stress (Sia, 2004).
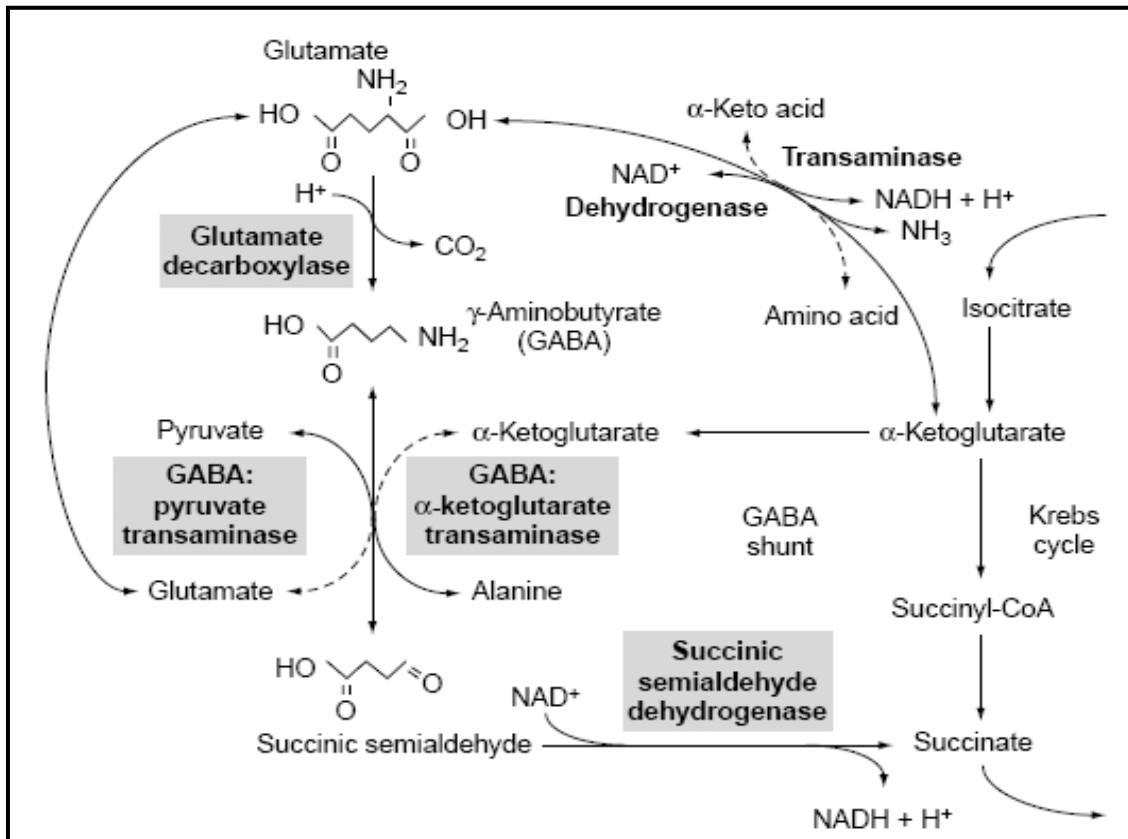
Figure 1.2   **The relationship of GABA to pathways in metabolism**

(**Shelp** *et al.***, 1999).**

In general, GABA was found to affect the absorption of ions in the renal tubules (Parducz *et al.*, 1992). Intracellularly, GABA is shunted along enzymes, glutamate decarboxylate (GAD), GABA transaminase (GABA-T) and succinic semialdehyde dehydrogenase to form intermediates that can enter the Kreb's cycle (Figure 1.2).

1.1.7   *Sutherlandia frutescens* **tablets**

Phyto Nova Sutherlandia[TM] Tablets are readily available as an over the counter complementary medicine. It is suggested that one tablet be taken twice a day after meals. Each tablet contains 300mg milled select SF subspecies microphylla chemotype. Side effects that have been documented include observed dizziness, loose stool, mild constipation and dry mouth (Mills *et al*., 2005).

1.1.8   Pharmacology of *Sutherlandia frutescens*

1.1.8.1   Stress

Stress is often associated with increases in circulating levels of steroid hormone. *Sutherlandia frutescens* is believed to have stress alleviating properties. This facet of SF was elucidated in a study by Prevoo *et al.* (2008) where experimental results indicated the attenuation of adrenal cytochrome P_{450} enzymes, *CYP17* and *CYP21*. These enzymes are actively involved in the biosynthesis of steroid hormones. The potential for SF to depress the activity of these enzymes provide insights to the mechanism behind the stress-relieving effects observed (Prevoo *et al.,* 2008).

1.1.8.2   Epilepsy

Many experiments are currently being conducted to determine the mechanism by which SF exerts its biological effect/s in cells. In an *in vivo* experiment, SF (50-400 mg/kg i.p) was found to possess significant anticonvulsant and antiseizure effects in *Balb/C* mice following epilepsy induction by pentylenetetrazole (Ojewole, 2008).

1.1.8.3   Antioxidant properties

Luminal and lucigenin enhanced chemiluminescence was used to investigate the hydroxyl free radical and superoxide scavenging properties of an aqueous extract of SF. This study revealed that SF displayed anti-oxidant potential with concentrations as low as 10µg/ml in cell free and stimulated neutrophil systems (Fernandes *et al*. 2004).

In another study by Tai *et al.* (2004) the antioxidant capacity of a 70% ethanolic extract of SF was determined. This study showed that 0.5µl of the ethanolic SF extract was comparable with 10µM of Trolox, an effective chemical with antioxidant activity.

1.1.8.5   Anti-inflammatory properties

During inflammation a vast array of factors are released and synthesised. One of these factors is the enzyme, cyclooxygenase (COX-2) which is involved in prostaglandin (PG) synthesis and inflammation. A study by Kundu *et al.* (2005) on the inflammatory properties of a methanolic extract of SF showed an inhibition of COX-2 induction *in vitro* and *in vivo*. In this study, tumour promoter 12-O-tetradecanoylphorbol-13-acetate was applied on mice skin followed by SF. The expression of cyclooxygenase-2 was found to be suppressed by 26% and 48% in mice treated with 100mg and 200mg of the SF extract, respectively.

1.1.8.6   Antiviral activity

The resort for primary health care for many HIV^+ individuals in South Africa is traditional herbal therapy as it is often difficult to access proper health care facilities (Mills *et al.*, 2005). In 2005 Harnett *et al.* reported that HIV^+ individuals who consumed SF showed an improvement in their mood, appetite, weight gain, cluster differentiation 4 positive ($CD4^+$) counts as well as decreased viral loads. It is thought that the antiretroviral activity of SF is through inhibition of HIV-1 target enzymes, such as HIV-1 reverse transcriptase (Harnett *et al.*, 2005).

1.1.8.7   Diabetes

A recent study showed that SF had hypoglycaemic properties and was useful in the treatment of type II diabetes. In this study crushed SF leaves in drinking water was administered to Wistar rats fed a high fat diet. The results showed that SF was an efficient hypoglycaemic mediator in comparison to metformin (the commonly used anti-diabetic drug) (Chadwick *et al.,* 2007).

The hypoglycaemic effect of SF was also demonstrated in streptozotocin (STZ)-induced diabetic rats (Type I diabetes) (Ojewole, 2004). This study found that SF caused significant hypoglycaemia in the STZ-treated rats when dosed with concentrations of 50-800mg/kg i.p. of the extract.

1.1.8.8 Apoptotic properties of *Sutherlandia frutescens*

The SF plant is commonly referred to as the 'cancer bush' and is used by traditional healers to treat cancer. To determine whether SF possessed anti-cancer properties, human breast adenocarcinoma cells were exposed to an ethanolic extract of SF. The results showed morphological characteristics of apoptosis and cell growth inhibition by affecting the mitogen activated protein kinase pathway (Stander *et al.*, 2007, Stander *et al.*, 2009).

The antiproliferative effect of SF was also demonstrated to be concentration dependant in breast cancer, the promyelocyte cell line HL60 and leukemia Jurkat cell line (Tai *et al.*, 2004).

A separate study by Chinkwo (2005) demonstrated the apoptotic properties of SF on Caski cells (cervical carcinoma), Chinese hamster ovary (CHO) and Jurkat T lymphoma cell line. The classical hallmarks of apoptosis such as cell shrinkage, breakdown and a decrease in cell numbers, was noted. This study used the apoPercentage^{TM} assay and Crossmon Trichrome stain which detects phosphatidylserine externalisation and chromatin condensation, respectively. Jurkat T cells treated with 3.5 mg/ml SF for 6 and 24 h were analysed by Annexin-V staining and flow cytometry showed approximately 84% apoptotic cells (Chinkwo, 2005).

Ethanolic extracts of SF was used to determine the apoptotic effects on the MCF-7 human breast carcinoma cell line (Stander *et al.*, 2007). Microscopical analysis showed that SF treated cells displayed altered morphology. The changes included cytoplasmic shrinking, membrane blebbing and

apoptotic bodies - representing the classical characteristics of apoptosis. Changes in the expression of 345 genes in cells treated with SF were found using cDNA microarray analysis. Changes have been noted in genes which code for proteins involved in apoptosis, cell cycle regulation and signal transduction. These include TNF superfamily members 10a and 10b, caspase recruitment domain family, member11 amongst others (Stander *et al.,* 2007).

1.2   The Nephron

1.2.1   Function of the Nephron

The route of elimination of polar compounds from the circulation occurs via the renal system. The kidney receives between 20% – 25% of the total cardiac output and therefore any substance in the systemic circulation reaches the kidney in large quantities. The functional unit of the kidney is the nephron. The kidney functions to filter blood, allowing substances to enter the Bowman's capsule and renal tubules. Filtered nutrients are actively reabsorbed at the proximal convoluted tubule (PCT) and ions are actively reabsorbed at the distal convoluted tubule (DCT) (Klaassen, 2000). The proximal and distal tubules, based on their functions, have different cell architectures (Young *et al.*, 2000).

1.2.2   Structure of the Nephron

The PCT epithelium (simple cuboidal epithelium) has a brush border of tall microvilli that extends into the lumen. This functions to increase the surface area 20-fold for the efficient reabsorption of molecules from the glomerular filtrate back into circulation.

Histologically, PCT cells stain intensely due to the high content of organelles and mitochondria. The PCT is responsible for the active reabsorption of approximately 100% of glucose and amino acids from the glomerular filtrate (Young *et al.*, 2000). The DCT cells are smaller simple cuboidal epithelial cells that stain less intensely due to the presence of fewer organelles. The DCT is involved in the active reabsorption of sodium from the tubular fluid and lacks a brush border (Young *et al.*, 2000).

The close proximity of filtered substances to the renal tubular epithelium and the ability of the kidney to concentrate toxicants increase the susceptibility of these cells to damage.

1.3 Biochemical Activity of PCT and DCT Epithelial Cells

1.3.1 Energy Generation

The mitochondrion is considered the energy generator of all aerobic eukaryotic cells. This organelle is responsible for production of most of the energy, signalisation, biosynthesis and apoptotic mechanisms (Saraste *et al*., 1999). The control of and the manner in which these cellular mechanisms occur plays a determinant role in cell physiology. The differences in energy utilisation lie not only between organs but are highly specific to the functionality of each cell type. Hence, the number and activity of organelles such as mitochondria in the proximal and distal convoluted tubular epithelial cells are different.

A distinctive feature of the mitochondria is the presence of a double membrane system, the inner and outer membranes. The outer membrane is not a significant permeability barrier as it contains transmembrane channel proteins (porins). The inner membrane is extremely convoluted forming cristae to increase the surface area and it presents a permeability barrier to most solutes. The inner mitochondrial membrane has transport proteins and protein complexes involved in electron transport and ATP synthesis. The high surface area provided by the cristae enhances the capacity of ATP production (Mathews *et al.*, 2000).

Energy generation by the mitochondria occurs primarily through oxidative phosphorylation. This is a process by which electrons are passed along a series of carrier molecules in the electron transport chain (ETC). These electrons are attained from the oxidation of reduced nicotinamide adenine dinucleotide (NADH) and flavin-adenine dinucleotide dihydrogen ($FADH_2$) which are produced during glycolysis,

Kreb's cycle and other oxidation reactions during the metabolism of nutrients. Five enzyme complexes make up the ETC, complexes I, II, III and IV transport electrons and complex V catalyses the synthesis of ATP. These complexes function to accept electrons from electron carriers and transfer them to the next carrier in the chain. The electrons, ultimately, combine with protons and oxygen to produce water (Mathews *et al.*, 2000).

Complex I (NADH: ubiquinone oxidoreductase) receives the free proton from NADH and transfers it to flavin mononucleotide (FMN) producing reduced FMNH. Succinate: ubiquinone reductase (Figure 1.3) is also known as complex II which transfers electrons from succinate to flavin-adenine dinucleotide (FAD). Electrons from complexes I and II are then accepted by coenzyme Q which transports these electrons to complex III.

Complex III is responsible for the deliverance of electrons from coenzyme Q to cytochrome C in a redox reaction coupled by the generation of a proton gradient across the membrane. Cytochrome oxidase (complex IV) also generates a proton gradient against the transmembrane by receiving electrons from cytochrome c in its active site with haem iron and copper.

The active site of complex IV has a haem iron and a copper molecule that is uses to reduce oxygen into two water molecules utilising two protons from the mitochondrial matrix simultaneously pumping a proton across the membrane (Figure 1.3) (Mathews *et al.*, 2000).
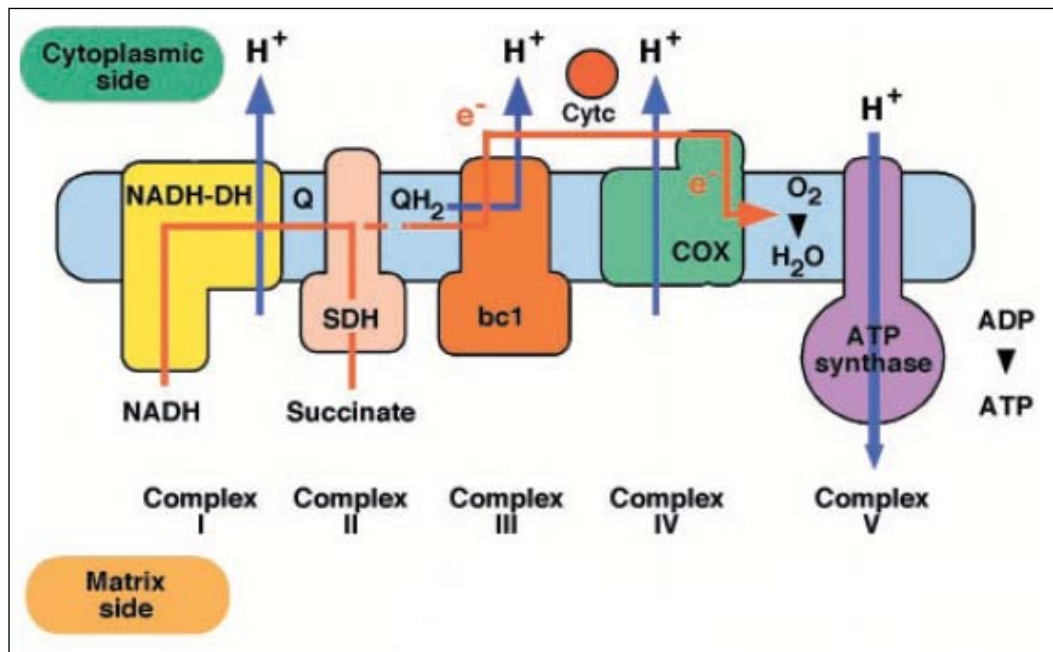
Figure 1.3 **The transmembrane proteins of the inner mitochondrial matrix that are involved in the electron transport chain and oxidative phosphorylation. Complex I (NADH-DH), Complex II (SDH), Complex III (bc1), Complex IV (COX) transfers electrons to oxygen. Complex I, III IV translocate protons across the membrane creating a proton gradient that Complex V(ATP synthase) uses to synthesise ATP (adapted from Mathews, 2000).**

Mitochondrial ATP synthase (Complex V) synthesises ATP from ADP and Pi, using a proton motive force across the membrane and it can hydrolyse ATP to pump protons against an electrochemical gradient. It is made up of two major complexes (F_1 and F_0). The F_0 complex is embedded within the inner membrane while the F_1 complex protrudes into the matrix.

1.3.2   Free Radicals

Any molecule that is capable of independent existence when it contains one or more unpaired electrons is regarded as a 'free radical'. This class of molecules include superoxide ($O_2^{.-}$), hydroxyl ($OH^.$), thiyl ($RS^.$), trichloromethyl ($CCl_3^.$) and nitric oxide ($NO^.$) (Halliwell and Chirico, 1993; Jones, 2006).

The oxygen-centred radicals are considered the most important *in vivo* generated free radicals. These reactive oxygen species (ROS) arise as a result of normal metabolic processes as well as physical irradiation. NADPH oxidase is responsible for the production of ROS in inflammatory cells during cell-mediated immunity and antimicrobial activity. The production of ROS by reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase in non-inflammatory cells is used for cell signalling. Superoxide generation in eukaryotic aerobic cells occurs in abundance at the mitochondrial ETC during the synthesis of ATP (Cadenas and Sies, 1998).

Cytochrome oxidase, complex IV in the ETC, contains haem iron and copper which transfers one electron at a time to oxygen. This process is not efficient and therefore allows for the generation of incompletely reduced oxygen species (Jones, 2006). Depending on the number of electron reductions of oxygen different ROS are formed. A single electron reduction produces superoxide, two electron reductions produces hydrogen peroxide (H_2O_2) and three-electron reduction produces the hydroxyl radical ($OH^.$) (Halliwell and Chirico, 1993).

The $OH^.$, most reactive of the ROS, has a half life of 10^{-9}s and is responsible for the damage of biological molecules such as cellular lipids, proteins, and DNA (Pastor *et al.*, 2000). Polynucleotide strand breakage, double stranded DNA breaks, base alterations and nucleotide base lesions occur as a result of $OH^.$ damage (Mathews *et al.*, 2000). Superoxide radicals and H_2O_2 may not be as detrimental as $OH^.$, however $O_2^{.-}$ readily reacts with NO to form peroxynitrite ($OONO^-$) (Klatt & Lamas, 2000;

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Ridnour *et al.*, 2004). This reactive nitrogen species causes lipid peroxidation and protein damage by nitration of tyrosyl hydroxyl groups (Mathews *et al*., 2000).

The cell attempts to dissipate the superoxide radical by converting it to H_2O_2, a reaction catalysed by superoxide dismutase. Hydrogen peroxide, however, reacts with transition metals, iron and copper, during the Fenton reaction giving rise to highly reactive $OH^.$. The $OH^.$ is then free to react with polyunsaturated fatty acids (PUFA) to produce a carbon-centred lipid radical (Halliwell and Chirico, 1993). The lipid radical can then react with iron resulting in lipid alkoxyl radicals or with $O_2^{.-}$ forming lipid peroxyl radicals.

Lipid peroxidation is initiated when these reactive lipid peroxyl radicals are not detoxified by cellular antioxidants. The lipid alkoxyl radical undergoes cyclisation forming an intermediate product which can degrade into malondialdehyde (MDA). Nucleotide bases, cytosine, adenine and guanine, of DNA are targets of MDA which result in the formation of DNA adducts (Halliwell and Chirico, 1993).

1.3.3   Antioxidants

Oxidative stress is dealt with *in vivo* by both enzymatic and non-enzymatic antioxidant systems. The enzymatic antioxidant mechanisms include superoxide dismutase (SOD) and peroxidases. The non-enzymatic antioxidant system includes molecules such as vitamin C, vitamin E, beta-carotene, flavonoids, glutathione (GSH) and uric acid (Mathews *et al*., 2000).

Superoxide dismutase is a metalloenzyme that catalyses the dismutation of two $O_2^{.-}$ molecules to H_2O_2 and O_2. Peroxidases include catalase and GSH peroxidase. Catalase reduces H_2O_2 to water and O_2, and GSH peroxidase reduces H_2O_2 to water along with the oxidation of GSH (Mathews *et al*., 2000).

Vitamin C (ascorbic acid) is a major extracellular antioxidant but is also capable of scavenging radicals in the aqueous phases of the cytoplasm. Vitamin E and beta-carotene act as antioxidants in hydrophobic environments (Mathews *et al.*, 2000). Both vitamin C and vitamin E detoxify intermediates formed during lipid peroxidation. The lipid peroxyl radical is acted upon in the membrane by the reduced form of vitamin E forming lipid hydro-peroxide and a vitamin E radical. Vitamin C then regenerates vitamin E by accepting the free radical and forming an ascorbyl radical. The vitamin E radical can also be reduced by GSH. The oxidised GSH and ascorbyl radical are subsequently reduced to GSH and ascorbate monoanion by dihydrolipoic acid (DHLA) which is then converted to alpha-lipoic acid (ALA). Dihydrolipoic acid is regenerated from ALA using NADPH (Mathews *et al.*, 2000).

Glutathione (Figure 1.4), also called gamma-glutamylcysteinylglycine, is a tripeptide that is present in most cells (Sies, 1999). This molecule is responsible for protecting the cell against oxidative stress. This is accomplished by acting as a cofactor for detoxification enzymes, scavenging hydroxyl radicals and singlet O_2, detoxifying H_2O_2 and lipid peroxides. GSH also plays a role in the regeneration of vitamin C and vitamin E as mentioned previously.
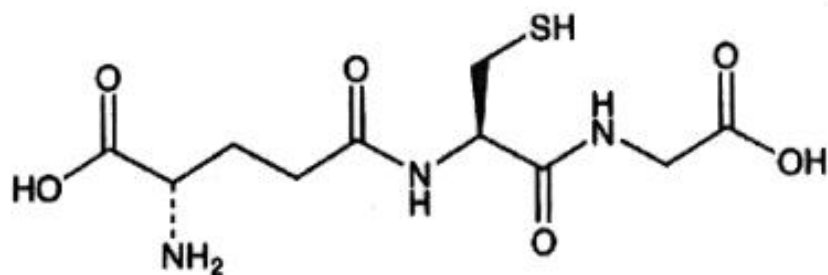

Figure 1.4   **Structure of glutathione (Sies, 1999).**

An important feature of GSH is the presence of a free thiol group with which it forms conjugates with a range of electrophilic compounds nonenzymatically, or through the action of GSH–*S*-transferase (Sies, 1999). The resulting GSH-conjugates are removed by the biliary system. They may also be metabolised further by hydrolysis and N-acetylation reactions resulting in mercapturic acid which is excreted by the renal system (Dickinson and Forman, 2002).

The reduction of peroxides by GSH peroxidase produces oxidised glutathione (GSSG). The depletion of GSH is circumvented by GSH reductase catalysing the reduction of GSSG to GSH using NADPH. During oxidative stress the level of transcription of GSH peroxidase is up-regulated and the rate of post-translational modification is increased. Aberrant expression of this enzyme and its activity has been associated with many pathologies including hepatitis (Downey *et al.*, 1998), HIV (Banki *et al.*, 1998) and cancers of various tissues including the kidney (Okamoto *et al.*, 1994).

1.3.4   Cells Response to Stress

Cell signalling and signal transduction are the two means by which cells communicate with each other (Poli *et al.*, 2004). Extracellular signals such as hormones, cytokines, growth factors and neurotransmitters trigger signal transduction and allow information to be transferred from the outside to the intracellular elements (Thannickal and Fanburg, 2000). When a cell is placed under stress due to heat shock or xenobiotics, for example, several signalling cascades are activated to enhance cell repair mechanisms and promote cell survival.

1.4   The Fate of the Cell

Cells that are unable to recover from oxidative stress and chemical damage are signalled to undergo cell death. Cell death may occur either through necrosis or apoptosis. Necrosis usually occurs in cells following acute tissue injury and results in the release of intracellular molecules into the extracellular

matrix, initiating an inflammatory response. Tissue homeostasis and the demise of individually affected cells occur via programmed cell death/ apoptosis.

1.4.1 Apoptosis

A variety of mechanisms can induce apoptosis in cells. Intracellular pro- and anti-apoptotic factors, the severity of the stimulus and the stage of the cell cycle determine whether the signalled cell will undergo apoptosis or not. Apoptosis can be stimulated by extracellular or intracellular signals.

Extrinsic signals include binding of death inducing ligands (from the surface of other cells or soluble factors) to death receptors. Intrinsic apoptotic signals are produced following exposure to radiation, viral infection, growth factor deprivation or oxidative stress (Figure 1.5) (Hengartner, 2000).

1.4.1.1   Extrinsic Apoptotic Activation

Fas, tumour necrosis factor receptor 1 (TNFR1) and tumour necrosis factor related apoptosis inducing ligand (TRAIL), death receptor 4 and death receptor 5 are the best characterised and understood extrinsic death receptors which belong to the TNF-α superfamily.

The interaction of TNF-α with TNFR1 causes receptor trimerisation and the clustering of intracellular death domains (Van Antwerp *et al*., 1998). This allows intracellular TNFR1-associated death domain (TRADD) to bind to the death domain followed by the recruitment of TNF-associated factor 2 (TRAF2) to the death receptor. The NF-kB and the Jun NH_2-terminal kinase (JNK)/Ap-1 pathway are then activated by TRAF2.
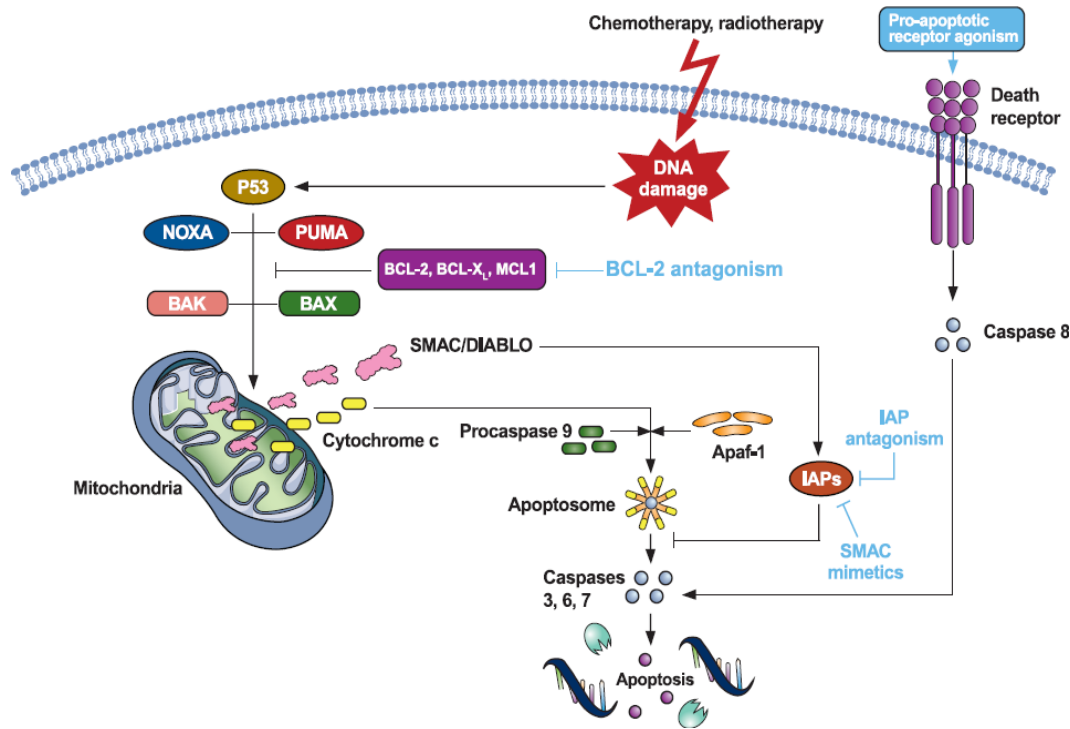
Figure 1.5 **Programmed cell death – extrinsic and intrinsic activation**

(Hengartner, 2000).

The TNFR1 also recruits receptor-interacting protein (RIP)-associated ICH-1 homologous protein with death domain which uses caspase recruitment domain to recruit caspase 2. Fas associated death domain (FADD) interaction with TRADD also induces apoptosis by the recruitment and cleavage of procaspase 8 using its death effector domain (DED) (Ashe and Berry, 2003).

The death inducing signalling complex (DISC), formed by the interaction of procaspase 8 with the DED of FADD, is a multi-protein complex and is responsible for the fate of the cell as its composition activates either apoptotic or survival pathways. The apoptotic pathway can occur rapidly by the recruitment of a high number of caspase 8 to DISC, or slowly with few caspase 8 molecules recruited and slow initiation of apoptosis following the involvement of the mitochondrial apoptotic pathway.

Caspase 8 cleaves downstream caspase 3 as well as pro-apoptotic molecule Bid to truncated Bid (tBid). Second mitochondrial activator of caspases (Smac)/Diablo, apoptosis inducing factor (AIF), cytochrome c, and endonuclease G are mitochondrial factors whose release is stimulated by truncated bid (tBid) (Ashe and Berry, 2003).

1.4.1.2 Intrinsic pathway of activation for apoptosis

As described by Chowdhury *et al.* (2008), a range of factors including cell stress, oxidative stress, heat shock and DNA damage can lead to the intrinsic activation of apoptosis. The mitochondrion is an important organelle in the process of apoptosis as the mitochondrial membrane is the site at which anti-apoptotic proteins B cell CLL/lymphoma 2 (Bcl-2) and Bcl-X are located.

Pro-apoptotic molecules such as cytochrome c is located within the mitochondria and pro-apoptotic protein Bax and Bad mediate apoptosis by interacting directly with the mitochondrial membrane or with mitochondrial associates Bcl-2 and Bcl-X (Gross *et al.,* 1998, Lalier *et al.*, 2007). Bax is thought to have the ability of forming pores in the mitochondrial membrane thereby promoting the release of cytochrome C and AIF (Lalier *et al.*, 2007). The release of cytochrome c, which may also be caused by cell stress, involved in apoptotic activation makes it available to form an apoptosome with apoptotic protease activating factor-1 (Apaf-1) and caspase 9 (Yang *et al.,* 1997).

Damage to cellular DNA and protein leads to the generation of pro-apoptotic molecules such as Bax following the activation of p53 (Figure 1.5). The p53 protein, also known as the tumour suppressor protein, has been associated with key responses of the cell to DNA damage (Haffner and Oren, 1995). The p53 protein plays an integral role in monitoring cellular stress and inducing apoptosis, when required (Hosfseth e*t al.*, 2004). The maintenance of appropriate levels of p53 is accomplished by mouse double minute 2 (Mdm2). This enzyme binds to the p53 protein and promotes its

polyubiquinitation and degradation, therefore maintaining low intracellular levels of this protein (Brooks and Gu, 2003).

Changes in chromatin structure are a consequence of DNA damage. This phenomenon leads to activation of the chromatin-bound protein kinase, ataxia telangiectasia mutated protein (ATM), ATM related (ATR) and DNA-dependant protein kinase (DNA-PK) proteins which then phosphorylates p53 (Hickman *et al.*, 2002; Kubbutat and Vousden, 1998). Activated p53 accumulates in the nucleus and binds to DNA sequences and induces transcription of proapoptotic proteins. p53 acts as a promoter of apoptosis by suppressing anti-apoptotic protein gene expression, Bcl-2, Bcl-xL (Yu and Zhang, 2005) and cFLIP (Ashe and Berry, 2003).

1.4.1.3   Caspases – Effectors of Apoptosis

The caspases, a group of cysteine proteases, are effectors of apoptosis that are present in cells in an inactive form. Stimuli that promote apoptosis initiate the cleavage of the procaspases thereby initiating the caspase cascade for the execution of apoptosis. The binding of appropriate ligands to death receptors activates the initiator caspases 10 and 8. These activate downstream caspases in the caspase cascade until the effector caspases 3, 6 and 7 are activated. The targets of effector caspases include nuclear lamins, ICAD/DNA fragmentation factor 45, PARP-1 and P 21- activated kinase 2 that result in morphological features of apoptotic cells (Kerr *et al*., 1972).

The intrinsic pathway of apoptosis ultimately results in the activation of executioner caspase 3 as well. Release of cytochrome c from the mitochondria occurs in concert with Smac/DIABLO. The formation of the apoptosome with cytochrome c, apaf-1 and procaspase 9 renders caspase 9 active. The presence of inhibitors of apoptosis proteins (IAP's) is able to react with caspase 9 and prevent further activation

of the caspase cascade. The release of Smac/DIABLO allows it to bind to IAP's thereby allowing caspase 9 to cleave and activate caspase 3 (Ekert *et al*., 2001).

The features of apoptosis are DNA fragmentation by endogenous nucleases that are activated by effector caspases, cytoplasmic shrinkage due to the cleavage of lamins and actin filaments, chromatin condensation due to the degradation of nuclear structural proteins, externalisation of phosphatidylserine on the membrane of cells to promote phagocytosis, membrane blebbing and the formation of apoptotic bodies. This process is intricate and involves several protein families, sub cellular compartments and signal transduction cascades (Hengartner, 2000).

The availability, utilisation and popularity of medicinal plants are increasing amongst the South African communities. Scientific evidence that supports the effectiveness of herbal therapies such as SF is emerging, proving its antiviral, antioxidant, antiproliferative and hypoglycaemic properties. Naturally, traditional therapeutics is accepted by the populous; however, continued research by *in vitro*, *in vivo* (animal) experiments and clinical trials is imperative to validate the efficacy of SF.

CHAPTER 2

SCIENTIFIC PAPER PUBLICATION

PREFACE

The following paper has been published in the South African Journal of Science, Volume 106, Article #10, Pages 54 - 58, documents the effect of *Sutherlandia frutescens* on cultured renal epithelial cells. This study was undertaken in light of the lack of literature regarding the therapeutic value and potential toxicity of the above medicinal plant.

THE EFFECTS OF *SUTHERLANDIA FRUTESCENS* EXTRACTS IN CULTURED RENAL PROXIMAL AND DISTAL TUBULE EPITHELIAL CELLS

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ABSTRACT

Sutherlandia frutescens (SF), a medicinal plant indigenous to South Africa, is traditionally used to treat a diverse range of illnesses, including cancer and viral infections. The biologically active compounds of SF are polar, thus renal elimination increases susceptibility to toxicity in that organ. This study investigated the antioxidant potential, lipid peroxidation, mitochondrial membrane potential and apoptotic induction by SF extracts on proximal and distal tubule epithelial cells. Cell viability was determined using the MTT assay. Mitochondrial membrane potential was determined using a flow cytometric JC-1 Mitoscreen assay. Cellular glutathione and apoptosis were measured using the GSH-Glo™ Glutathione assay and Caspase-Glo® 3/7 assay, respectively. The IC_{50} values from the cell viability results for LLC-PK$_1$ and MDBK were 15 mg/mL and 7 mg/mL, respectively. SF extracts significantly decreased intracellular glutathione in LLC-PK$_1$ ($p < 0.0001$) and MDBK ($p < 0.0001$) cells, while lipid peroxidation increased in treated LLC-PK$_1$ ($p < 0.0001$) and MDBK ($p < 0.0001$) cells. JC-1 analysis showed that SF extracts promoted mitochondrial membrane depolarization in both LLC-PK$_1$ and MDBK cells by up to 80% ($p < 0.0001$). The activity of caspase 3/7 increased in both LLC-PK$_1$ (11.9-fold; $p < 0.0001$) and MDBK (2.2-fold; $p < 0.0001$) cells. SF extracts at high concentrations appear to increase oxidative stress, to alter mitochondrial membrane integrity, and to promote apoptosis in renal tubule epithelia.

INTRODUCTION

Sutherlandia frutescens (SF), a member of the Leguminosae family, is a multipurpose medicinal plant endemic to South Africa.[1] Commonly known as 'cancer bush', it has been used in crude form for years by traditional healers to treat a variety of ailments including internal cancers, diabetes, uterine disease, influenza, HIV, depression, and arthritis.[1] Various doses of SF leaf powder have been administered to humans, but have produced no known side effects.

Leaves of SF contain the biologically active compounds L-canavanine, D-pinitol, gamma amino butyric acid (GABA), parabens, saponins, cycloartane glycosides and triterpenoid diglucoside.[2,3,4] L-canavanine stores nitrogen in seeds and is used in plant chemical defense mechanisms.[5,6] Its production by SF is dependent on the availability of abiotic factors.[7] L-canavinine, a non-protein amino acid, is a structural analogue of L-arginine. It can be recognised by arginine-utilising enzymes such as arginyl-tRNA synthetase, and consequently can be incorporated into newly synthesised peptides.[5] Canaline, produced by arginase-mediated hydrolytic cleavage of L-canavanine, has been shown to have anti-tumour properties.[8] D-pinitol, a chiro-inositol sugar, possesses anti-diabetic properties and is used in the treatment of wasting in cancer and HIV/AIDS patients.[9,10]

GABA, an inhibitory neurotransmitter, mediates most of its effects inside the nervous system.[11] It has been used as a drug for the relief of anxiety and stress.[12] Interestingly, GABA has been shown to affect the absorption of ions in the renal tubules.[13] Intracellularly, GABA is metabolised through the action of the enzymes glutamate decarboxylase, GABA transaminase and succinic semialdehyde dehydrogenase, and is transformed into citric acid cycle intermediates.[14]

Many experiments have been done to determine the mechanism(s) of action of SF extracts. In an in vivo experiment it was concluded that SF extracts possessed anticonvulsant effects in mice subjected to the induction of epilepsy.[15] SF-treated human breast adenocarcinoma cells in culture showed morphological characteristics of apoptosis and cell growth inhibition.[16,17] An antiproliferative effect of SF was demonstrated to be concentration-dependent in breast cancer and leukaemia cell lines, with no significant antioxidant effects.[2] Another study, by contrast, showed that SF extracts displayed antioxidant potential (hydroxyl free radical and superoxide scavenging properties) in cell-free and stimulated neutrophil systems.[18] An apparent antiretroviral activity of SF has been thought to be mediated by the inhibition of HIV-1 target enzymes, such as HIV-1 reverse transcriptase.[19]

The route of elimination of polar compounds from the circulation occurs via the renal system. The kidney functions to filter blood, allowing substances to enter Bowman's capsule and the renal tubules. Filtered nutrients are actively reabsorbed at the proximal convoluted tubule (PCT), while ions are actively reabsorbed at the distal convoluted tubule (DCT).[20] Based on their contrasting functions, the PCT and DCT epithelia have different cell architectures.[21]

The PCT epithelium has a brush border of tall microvilli that extends into the lumen to increase the surface area 20-fold for the efficient reabsorption of molecules from the glomerular filtrate back into circulation. Histologically, PCT cells stain intensely with eosin due to their high content of organelles and mitochondria. The PCT is responsible for the active reabsorption of 99% of glucose and amino acids from the glomerular filtrate.[21] The DCT cells are smaller, simple cuboidal epithelial cells (stain less intensely due to fewer organelles) that actively reabsorb sodium from the tubular fluid.[21] The close proximity of filtered substances to this kind of tubular epithelium increases the susceptibility of these cells to damage.

To date, limited scientific evidence has been available on the mechanism by which SF extracts affect cellular processes and the side effects related to their use. This medicinal plant, however, continues to be recommended as a traditional remedy and is used by a large portion of the South African community. In this study, the nephrotoxic and apoptotic effects of SF extracts on two kidney cell lines, LLC-PK$_1$ (PCT epithelium) and MDBK (DCT epithelium), were investigated and compared.

MATERIALS AND METHODS

Materials

Sutherlandia frutescens tablets (Phyto Nova™, Cape Town, South Africa) were purchased from a local pharmacy. The LLC-PK$_1$ and MDBK cell lines were purchased from Highveld Biologicals (Johannesburg, South Africa). All tissue culture reagents, the GSH-Glo™ Glutathione Assay and the Caspase-Glo® 3/7 Assay were obtained from Whitehead Scientific (Johannesburg, South Africa). The JC-1 dye was purchased from BD Biosciences (South Africa). All other reagents were purchased from Merck (South Africa) unless otherwise stated.

Preparation of *Sutherlandia frutescens* extracts

Phyto Nova Sutherlandia™ tablets were used to prepare an aqueous extract of the active ingredients of the plant. Sixty tablets were crushed to a fine powder in a pestle and mortar, weighed and suspended in deionised water (1.2 g per 10 mL). The mixture was continuously stirred at room temperature for 1.5 h, and thereafter transferred to 50 mL sterilin tubes and centrifuged (3 645 g, 10 min) at room temperature. The upper aqueous layer (SF extract) was removed, vacuum filtered and stored at 4 °C. SF extract dilutions (24 mg/mL, 12 mg/mL, 6 mg/mL, 2.4 mg/mL, 1.2 mg/mL, 0.6 mg/mL, and 0.3 mg/mL) were prepared using complete culture media (CCM), comprising Eagle's minimum essential medium, 10% foetal calf serum, 1% L-glutamine and 1% penstrepfungizone.

Cell culture and cytotoxicity assay

LLC-PK$_1$ and MDBK cells were cultured (37 °C, 5% CO$_2$) to confluency in 75 cm³ flasks in CCM. The cytotoxicity of SF in LLC-PK$_1$ and MDBK cells was measured using the MTT assay.[22] LLC-PK$_1$ and MDBK cells (10 000/well) were incubated with varying SF extract dilutions for 48 h in triplicate in microtitre plates, together with the respective controls (cells incubated with CCM only). The cells were then incubated with the MTT substrate (5 mg/mL) for 4 h. Thereafter all supernatants were aspirated, and dimethyl sulphoxide (DMSO) (100 µL/well) was added to the wells. Finally, the optical density was measured at 570 nm, with a reference wavelength of 690 nm, by an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Tek µQuant). The data were translated to 'percentage cell viability' versus 'concentration of extract', from which the IC$_{50}$ (half the maximal inhibitory concentration) values for each cell line and for the combination of treatments were determined. For all subsequent biochemical assays, both cell lines were grown to confluency and treated with the determined IC$_{50}$ values of the SF extracts.

Lipid peroxidation assay

Oxidative damage of both cell lines was assessed using the thiobarbituric acid assay, because lipid peroxidation is commonly quantified by levels of malondialdehyde (MDA). After the 48 h incubation with SF extract, the culture fluid from each SF-treated flask and from the untreated controls (500 µL) was dispensed into duplicate glass tubes (one representing the sample, one representing a negative control), followed by addition of 7% H$_3$PO$_4$ (200 µL). A positive control of 1% MDA was prepared. Thiobarbituric acid (1%, w/v)/ 0.1 mM butylated hydroxytoluene solution (400 µL) was added to sample tubes. To the tubes with the negative controls, 400 µL of 3 mM HCl was added. The solution was adjusted to pH 1.5 and heated at 100 °C for 15 min. Once cooled, butanol (1.5 mL) was added and the sample then centrifuged (8 400 g, 6 min). Following centrifugation, the butanol phase (200 µL) from each sample and

from the blanks was aliquoted into a microtitre plate. The optical density was measured at 532 nm, with a reference wavelength of 600 nm, by an ELISA plate reader. The sample means of ten replicates were calculated and divided by the absorption coefficient, 156 mM^{-1}.

Glutathione assay

The GSH-Glo™ Glutathione Assay (Promega, Madison, USA) was used to measure glutathione (GSH) levels. Cells (those treated with SF extract and the untreated controls after 48 h incubation) were transferred to an opaque microtitre plate (50 µL of 10 000 cells/well, 10 replicates). GSH standards (0 µM – 5 µM) were prepared from a 5 mM stock solution diluted in water. Five two-fold dilutions of the GSH stock were prepared and transferred into wells (50 µL) of the microtitre plate. The 2X GSH-Glo™ Reagent was prepared according to the manufacturer's instructions, added to the experimental wells (50 µL/well), and incubated at room temperature. Reconstituted Luciferin Detection Reagent (50 µL) was added to each well and incubated. The luminescence was measured on a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA). A standard curve was derived using the GSH standards (0 µM – 5 µM) and the GSH concentration in each sample was extrapolated from the equation.

Caspase-3/7 assay

The apoptotic potential of SF extracts on both cell lines was determined using the Caspase-Glo®3/7 assay (Promega). Caspase-Glo®3/7 Reagent was reconstituted according to the manufacturer's instructions and added to both the SF-treated and control cells (following 48 h incubation) in the wells of a microtitre plate (10 µL reagent per 50 µL of 10 000 cells/well, 10 replicates) and incubated in the dark (30 min). The luminescence was measured on a Modulus™ microplate luminometer (Turner BioSystems). The caspase-3/7 activity of the SF-treated samples was represented as X-fold change compared to the control (cells incubated with CCM only).

Mitochondrial membrane potential

The mitochondrial membrane potential ($\Delta\Psi_m$) of LLC-PK$_1$ and MDBK cells (both those treated with SF extract and their untreated controls; 48 h) was assessed using fluorescence-activated cell sorting (FACS) and the JC-1 Mitoscreen assay (BD Biosciences) according to the manufacturer's instructions. Cells (approximately 100 000) were transferred to polystyrene cytometry tubes. The JC-1 dye (150 µL) was added to the cells and allowed to incubate (37 °C, 5% CO$_2$, 10 min). Cells were then washed with JC-1 Mitoscreen wash buffer (400 g, 5 min) and resuspended in 300 µL flow cytometry sheath fluid. Flow cytometry data from stained cells (15 000 events) was obtained using a FACSCalibur (BD Biosciences) flow cytometer with CellQuest PRO v4.02 software (BD Biosciences). Cells were gated to exclude debris using FlowJo v7.1 software (Tree Star Inc., Ashland, USA)

Statistical analysis

Results are expressed as the means, with error bars representing the standard deviations (s.d.) of the means. Statistical significance

TABLE 1
Viability of PCT and DCT cells incubated with SF for 48 h using the MTT assay

SF (mg/mL)	LLC-PK$_1$ CELL VIABILITY (%)	MDBK CELL VIABILITY (%)
24	25 ± 0.0*	15 ± 0.3*
12	72 ± 2.1	24 ± 14.0*
6	89 ± 33.0	103 ± 12.7
2.4	107 ± 10.9	118 ± 3.7
1.2	103 ± 2.9	122 ± 9.3
0.6	122 ± 14.0	96 ± 7.9
0.3	133 ± 11.0	101 ± 10.3

*$p < 0.001$

The levels of GSH were decreased significantly in LLC-PK₁ cells treated with SF extracts ($p < 0.0001$) compared with the relevant controls. The MDBK cells treated with SF also showed a decrease in GSH levels ($p < 0.0001$). Results are presented as the mean ± s.d. and n = 10. (***$p < 0.0001$).

FIGURE 1
Basal GSH concentrations in LLC-PK₁ and MDBK were different from each other

between samples for the MTT assay was determined using the one-way ANOVA Tukey Kramer Multiple Comparisons test. The statistical significances of the lipid peroxidation assay, GSH assay, caspase-3/7 assay and mitochondrial depolarisation assay were determined by the Mann-Whitney test for non-parametric data using GraphPad InStat Software, (GraphPad Software Inc., La Jolla, USA).

RESULTS

Cytotoxicity
The cytotoxic effects of SF extracts (MTT assay) were determined in both LLC-PK₁ and MDBK cells treated with a range of extract dilutions for 48 h (see Table 1). The IC₅₀ for SF extract was determined as 15 mg/mL and 7 mg/mL dilutions for the LLC-PK₁ and MDBK cells, respectively. The cell viability of both cell lines treated with concentrations between 6 mg/mL and 0.3 mg/mL was more than 89%. However, at higher SF extract concentrations of 24 mg/mL and 12 mg/mL, respectively, cell viability was decreased to 25% and 72% of controls ($p < 0.001$) in the case of LLC-PK₁ cells and to 15% and 24% ($p < 0.001$) of controls in the case of MDBK cells.

GSH assays
The intracellular concentrations of GSH in both SF-extract-treated renal epithelial cell lines were determined. They decreased significantly in SF-treated LLC-PK₁ cells as compared with controls ($p < 0.0001$), whilst there also was a significant decrease in GSH in SF-treated MDBK cells as compared with controls ($p < 0.0001$) (see Figure 1).

Lipid peroxidation assays
In order to determine whether mitochondrial damage and cytotoxicity were related to oxidative stress, we measured the levels of lipid oxidation products in both SF-treated cell lines. There were significantly higher levels ($p < 0.0001$) of MDA in both the SF-treated cell lines as compared with the respective control cells (see Figures 2A and 2B).

Mitochondrial membrane potential analyses
To determine whether LLC-PK₁ and MDBK cells were metabolically viable after SF-extract treatment, we investigated changes in $\Delta\Psi_m$. The SF-treated LLC-PK₁ cells had a significantly higher ($p < 0.0001$) percentage of depolarised mitochondria as compared with the untreated cells (80.2% vs 54.6%) (see Figure 3). Similarly, the MDBK cells had a significantly higher ($p < 0.0001$) percentage of depolarised mitochondria as compared with controls (81.7% vs 58.2%) (see Figure 3).
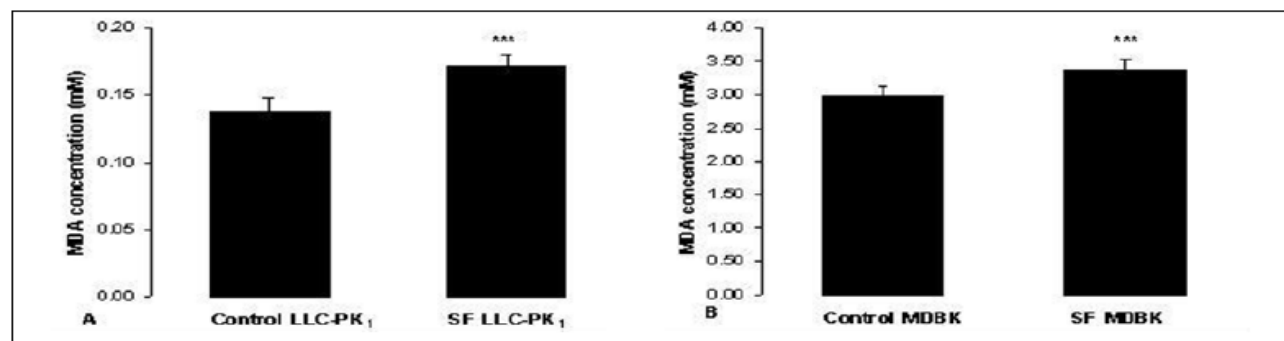
Caspase-3/7 assay
A significant change in $\Delta\Psi_m$ is a good indicator of apoptosis. The intracellular activities of caspase 3/7 in both SF-treated cell lines were measured (as a fold-change in comparison with the relevant controls). The activity of caspase 3/7 in SF-treated LLC-PK₁ cells showed a highly significant 11.9-fold increase ($p < 0.0001$) compared with the controls. The SF-treated MDBK cells also showed a significant 2.2-fold increase ($p < 0.0001$) over controls (see Table 2). These results indicated that PCT cells were more susceptible to apoptotic induction by SF than were DCT cells.

DISCUSSION

SF is used by many South Africans as a traditional remedy or ameliorant for many diseases, including HIV infection. The crude plant concoction is normally taken orally and the main route of elimination of the absorbed constituent polar compounds is via the renal system. The potential for injury by noxious compounds in the PCT region is high due to the ability of the PCT cells to concentrate substances that have been filtered by the glomerulus.[20]
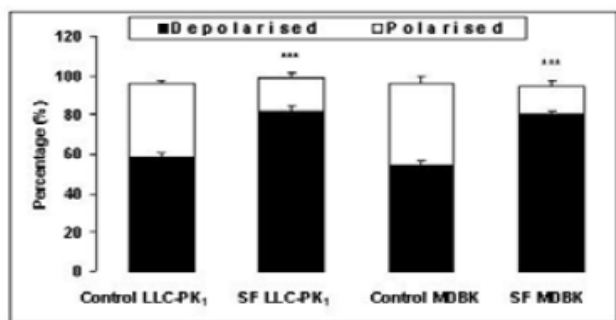
The results of this investigation have shown that both kinds of renal tubular cells, LLC-PK₁ and MDBK cells, are affected by SF extracts *in vitro*. This sensitivity may be attributed to the location and functional capacities of these cells. The intact PCT is susceptible to injury, as it is at this site of the nephron where toxicants accumulate and where there is an abundance of mitochondria for active re-absorption and transportation of ions, low molecular weight proteins, glutathione conjugates and heavy metals.[20] Cells treated with SF extracts *in vitro*, at concentrations of 6 mg/mL and lower, displayed greater than 89% viability. At such low concentrations, SF extracts appeared to promote cell metabolism as evidenced by the increased conversion of the MTT salt. This might have been due to both increased mitochondrial reductase enzyme activity,[22] and increased availability of reducing equivalents such as NADH,

The concentrations of MDA in LLC-PK₁ cells ($p < 0.0001$) [A] and MDBK cells ($p < 0.0001$) [B] treated with SF were significantly higher than in their respective controls. Results are presented as the mean ± s.d. and n = 10. (***$p < 0.0001$).

FIGURE 2
Levels of MDA in SF-treated cells

Both cell lines contained approximately 50% depolarised mitochondria without SF treatment. Results are presented as the mean ± s.d. and n = 15 000. (***$p < 0.0001$).

FIGURE 3
Depolarised mitochondria determined by the JC-1 Mitoscreen assay of control and SF-treated LLC-PK₁ and MDBK cells

TABLE 2
Caspase-3/7 activity of PCT and DCT cells incubated with SF for 48 h.
RLU – relative light units

	Control – Caspase-3/7 activity (RLU)	SF – Caspase-3/7 activity (RLU)	Fold Change	p value
LLC-PK₁	16 127 ± 346	191 706 ± 13455***	11.9	$p < 0.0001$
MDBK	5 137 ± 576	11 365 ± 488***	2.2	$p < 0.0001$

***$p < 0.0001$

a byproduct of metabolic pathways such as glycolysis and the Krebs cycle.[23] The anti-diabetic and favourable cellular metabolic properties of SF were previously demonstrated in male Wistar rats.[24]

The hypoglycaemic potential of SF was shown in diabetogenic rats, where it exhibited properties similar to those of the extracts similar to those of metformin, the common diabetes type-2 drug.[24] SF extracts increased the uptake of glucose by the kidney and resulted in increased glycolysis, which increased the rate of the Krebs cycle and produced more reducing equivalents (NADH and FADH₂). The high concentration of reducing equivalents donate electrons to the electron transport chain (ETC), resulting in increased ATP production via oxidative phosphorylation. This increased ATP concentration, is utilised for the active reabsorption of substances by the PCT and DCT. The continuous flux of electrons along the ETC and pumping of protons out of the matrix results in a negative mitochondrial membrane potential, during which process reactive oxygen species (ROS) are generated as a byproduct, rendering the mitochondria susceptible to oxidative damage.[25]

Free radicals attack polyunsaturated fatty acids, a major component of cell membranes, thereby forming lipid peroxyl radicals. Lipid peroxidation is initiated when the reactive lipid peroxyl radicals are not detoxified by antioxidants. The lipid alkoxyl radical undergoes cyclisation forming an intermediate product which can degrade into MDA.[26] Lipid peroxides can be detoxified by conjugation to antioxidant molecules such as ascorbic acid, α-tocopherol, uric acid and GSH.[27] Glutathione concentrations were significantly decreased in SF-treated LLC-PK₁ and MDBK cells with a corresponding increase in lipid peroxidation compared to the controls ($p < 0.0001$).

Both the LLC-PK₁ and MDBK cells were transformed cell lines which proliferated rapidly and possessed high metabolic activity which resulted in increased production of ROS, which, when not sequestered rapidly, increased lipid peroxidation. The MDA concentrations in the control LLC-PK₁ and MDBK cells were 0.14 mM and 3.0 mM, respectively (see Figures 2A and 2B), whereas the concentrations of GSH in LLC-PK₁ and MDBK cells were 2.85 µM and 2.1 µM. respectively (see Figure 1). The increased basal GSH concentration in the LLC-PK₁ cells suggested that these

cells were better protected from increased ROS production and subsequent peroxidation than the MDBK cells were.

The oxidative ability of L-canavanine to induce ROS and ensuing lipid peroxidation was determined previously in a mouse glial cell line (N11)[27]. In this study, a luminol-amplified chemiluminescence assay showed that 1 mM of L-canavanine significantly ($p < 0.05$) increased ROS production and lipid peroxidation in N11 cells.[27] L-canavanine, a structural analogue of L-arginine, is a competitive inhibitor of arginine-utilising enzymes such as nitric oxide (NO) synthase, which synthesises NO from L-arginine.[28] Nitric oxide is involved in many biological processes and has the ability to spontaneously react with superoxide radicals to produce peroxynitrite and subsequently, nitrate.[27] Nitric oxide, therefore, serves as a reactive radical scavenger that ultimately prevents oxidative damage to cellular components.[29] The possible decreased activity of NO synthase and inhibition of NO synthesis by SF may explain the significant lipid peroxidation in these renal cell lines.

Another possibility for the increased lipid peroxidation in both the SF-treated LLC-PK₁ and MDBK cells may be due to a decreased $\Delta\Psi_m$. GABA was shown to penetrate up to 60% of the mitochondrial matrix volume in rat brain and liver cells. The weak acidic nature of GABA may contribute to the proton gradient disruption within mitochondria.[30] A change in $\Delta\Psi_m$ promotes formation of ROS as it offsets the normal functioning of the ETC.

In addition to its acidic nature, high concentrations of GABA cause the reduction of intra-mitochondrial NAD⁺ stimulated by glutamate.[30] Excessive intra-mitochondrial NADH is dissipated by donating its electrons to the ETC. The metabolism of GABA involves the enzymes GABA: pyruvate transaminase, GABA: α-ketogluterate transaminase and succinic semialdehyde dehydrogenase to form succinate which can enter the Krebs cycle and produce substrates for the ETC.[14]

ROS production in the mitochondria increases susceptibility for membrane damage, altering the mitochondrial permeability transition. This may account for the high percentage of depolarised mitochondria noted after treatment of cells with SF extracts. The alteration of the $\Delta\Psi_m$ within a cell will not, however, occur simultaneously to all the mitochondria that are present.[31] The number of mitochondria that are affected determines the amount of ATP generated and the ultimate fate of the cell. The amount of ATP available will determine the type of cell death that occurs within the cell.[20]

When mitochondrial membranes are depolarised, pro-apoptotic signals are released. It was demonstrated that changes in $\Delta\Psi_m$ favoured the transition of a tightly bound form of cytochrome c (to cardiolipin in the inner mitochondrial membrane) into its loosely bound form, followed by the release of cytochrome c into the extra-mitochondrial environment.[32] The release of cytochrome c, Apaf-1 and calcium ions into the cytosol results in the activation of downstream caspases that execute apoptosis.[25,33,34] This study showed that SF-extract treatments increased caspase-3/7 activity in both cell lines (by 11.9-fold in LLC-PK₁ cells and by 2.2-fold in MDBK cells).

When comparing the change in $\Delta\Psi_m$ of LLC-PK₁ and MDBK cells with caspase-3/7 activity, it became evident that SF extracts are potent apoptotic inducers. The percentage mitochondrial membrane depolarisation in both cell lines appears to be equal at basal levels; following SF-extract treatments, an almost similar increase in depolarisation occurs in both LLC-PK₁ and MDBK cells.

The 11.9-fold increase of caspase activity in LLC-PK₁, as compared to a 2.2-fold increase in MDBK cells, may be due to the difference in mitochondrial numbers and cellular architecture of both cell lines. It is known that PCT epithelium contains more mitochondria than do DCT cells. The depolarisation of mitochondria allows for the increased release of more pro-apoptotic signals, thereby resulting in increased apoptosis.

There exists a real concern about the unregulated use of the crude plant derivatives of SF. Evidence that the recommended dosages are in such concentrations that they may be potentially harmful to the renal tubular cells, could be attained *in vivo*. Ojewole determined that SF extract had an LD_{50} of 1 280 mg/kg body weight in rats.[35] In a clinical trial on ten healthy adults receiving a dose of 800 mg/day of SF extract, no significant changes in haematological, physiological and biochemical markers were noted after three months of treatment.[36] The only changes observed in this *in vivo* study were a decrease in respiratory rate ($p < 0.04$), and increases in platelet count ($p < 0.05$), mean cell haemoglobin ($p = 0.01$), protein ($p < 0.03$) and albumin levels ($p < 0.03$).[36] Chronic doses of SF extract, however, may eventually lead to renal toxicity.

CONCLUSION

The aqueous SF extracts studied here were not cytotoxic at low concentrations, but had the potential to increase oxidative stress, alter the integrity of mitochondrial membranes, and promote apoptosis in renal tubule epithelia at high concentrations in vitro.

ACKNOWLEDGEMENTS

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CHAPTER 3

CONCLUSION

Sutherlandia frutescens is one of approximately 3 000 medicinal plants that is utilised daily to treat a wide range of illnesses in our population. These plants are now commercially available and their therapeutic potential remains to be elucidated. Continuous research into the safety and efficacy of the medicinal plant, SF, for human ailments need to be intensified.

The conclusions drawn from the current study indicate that SF is not cytotoxic to renal tubule epithelial cells at low concentrations. The plant extract at higher concentrations, however, induces cell death via apoptosis in renal tubule epithelia. Also, high concentrations of SF water extracts increased oxidative stress and altered the integrity of the mitochondrial membrane.

This *in vitro* experiment provides evidence that SF, if consumed moderately, may in essence; prove to be a safe therapeutic. Further research such as clinical trials are required to prove the safety and efficacy of this plant extract.

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APPENDIX 1
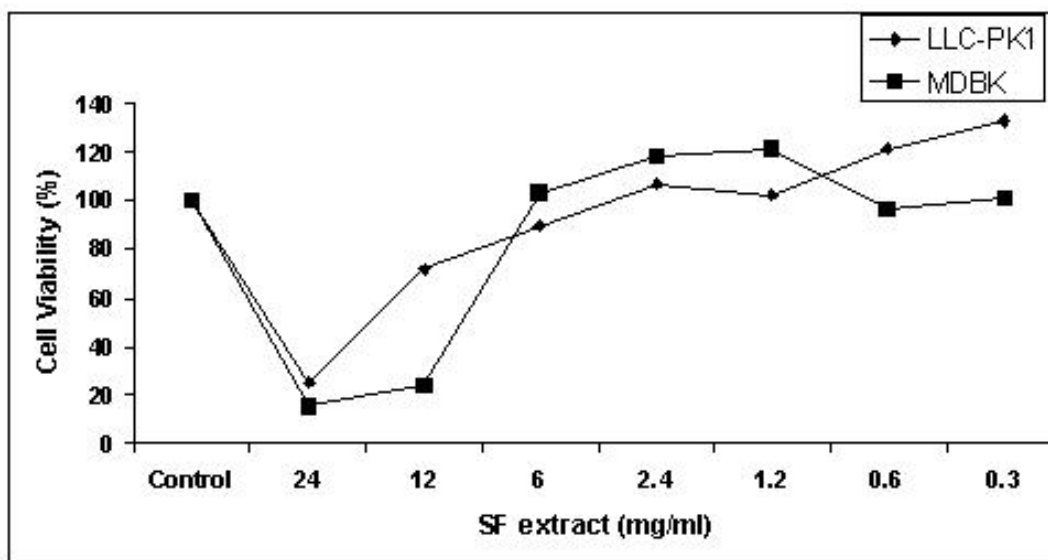
Graphical representation of cell viability (%) of cells exposed to SF for 48h.

THE EFFECTS OF *SUTHERLANDIA FRUTESCENS* EXTRACTS IN CULTURED RENAL PROXIMAL AND DISTAL TUBULE EPITHELIAL CELLS

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ABSTRACT

Sutherlandia frutescens (SF), a medicinal plant indigenous to South Africa, is traditionally used to treat a diverse range of illnesses, including cancer and viral infections. The biologically active compounds of SF are polar, thus renal elimination increases susceptibility to toxicity in that organ. This study investigated the antioxidant potential, lipid peroxidation, mitochondrial membrane potential and apoptotic induction by SF extracts on proximal and distal tubule epithelial cells. Cell viability was determined using the MTT assay. Mitochondrial membrane potential was determined using a flow cytometric JC-1 Mitoscreen assay. Cellular glutathione and apoptosis were measured using the GSH-Glo™ Glutathione assay and Caspase-Glo® 3/7 assay, respectively. The IC_{50} values from the cell viability results for LLC-PK$_1$ and MDBK were 15 mg/mL and 7 mg/mL, respectively. SF extracts significantly decreased intracellular glutathione in LLC-PK$_1$ ($p < 0.0001$) and MDBK ($p < 0.0001$) cells, while lipid peroxidation increased in treated LLC-PK$_1$ ($p < 0.0001$) and MDBK ($p < 0.0001$) cells. JC-1 analysis showed that SF extracts promoted mitochondrial membrane depolarization in both LLC-PK$_1$ and MDBK cells by up to 80% ($p < 0.0001$). The activity of caspase 3/7 increased in both LLC-PK$_1$ (11.9-fold; $p < 0.0001$) and MDBK (2.2-fold; $p < 0.0001$) cells. SF extracts at high concentrations appear to increase oxidative stress, to alter mitochondrial membrane integrity, and to promote apoptosis in renal tubule epithelia.

INTRODUCTION

Sutherlandia frutescens (SF), a member of the Leguminosae family, is a multipurpose medicinal plant endemic to South Africa.[1] Commonly known as 'cancer bush', it has been used in crude form for years by traditional healers to treat a variety of ailments including internal cancers, diabetes, uterine disease, influenza, HIV, depression, and arthritis. [1] Various doses of SF leaf powder have been administered to humans, but have produced no known side effects.

Leaves of SF contain the biologically active compounds L-canavanine, D-pinitol, gamma amino butyric acid (GABA), parabens, saponins, cycloartane glycosides and triterpenoid diglucoside.[2,3,4] L-canavanine stores nitrogen in seeds and is used in plant chemical defense mechanisms.[5,6] Its production by SF is dependent on the availability of abiotic factors.[7] L-canavinine, a non-protein amino acid, is a structural analogue of L-arginine. It can be recognised by arginine-utilising enzymes such as arginyl-tRNA synthetase, and consequently can be incorporated into newly synthesised peptides.[5] Canaline, produced by arginase-mediated hydrolytic cleavage of L-canavanine, has been shown to have anti-tumour properties.[8] D-pinitol, a chiro-inositol sugar, possesses anti-diabetic properties and is used in the treatment of wasting in cancer and HIV/AIDS patients.[9,10]

GABA, an inhibitory neurotransmitter, mediates most of its effects inside the nervous system.[11] It has been used as a drug for the relief of anxiety and stress.[12] Interestingly, GABA has been shown to affect the absorption of ions in the renal tubules.[13] Intracellularly, GABA is metabolised through the action of the enzymes glutamate decarboxylase, GABA transaminase and succinic semialdehyde dehydrogenase, and is transformed into citric acid cycle intermediates.[14]

Many experiments have been done to determine the mechanism(s) of action of SF extracts. In an in vivo experiment it was concluded that SF extracts possessed anticonvulsant effects in mice subjected to the induction of epilepsy.[15] SF-treated human breast adenocarcinoma cells in culture showed morphological characteristics of apoptosis and cell growth inhibition.[16,17] An antiproliferative effect of SF was demonstrated to be concentration-dependent in breast cancer and leukaemia cell lines, with no significant antioxidant effects.[2] Another study, by contrast, showed that SF extracts displayed antioxidant potential (hydroxyl free radical and superoxide scavenging properties) in cell-free and stimulated neutrophil systems.[18] An apparent antiretroviral activity of SF has been thought to be mediated by the inhibition of HIV-1 target enzymes, such as HIV-1 reverse transcriptase.[19]

The route of elimination of polar compounds from the circulation occurs via the renal system. The kidney functions to filter blood, allowing substances to enter Bowman's capsule and the renal tubules. Filtered nutrients are actively reabsorbed at the proximal convoluted tubule (PCT), while ions are actively reabsorbed at the distal convoluted tubule (DCT).[20] Based on their contrasting functions, the PCT and DCT epithelia have different cell architectures.[21]

The PCT epithelium has a brush border of tall microvilli that extends into the lumen to increase the surface area 20-fold for the efficient reabsorption of molecules from the glomerular filtrate back into circulation. Histologically, PCT cells stain intensely with eosin due to their high content of organelles and mitochondria. The PCT is responsible for the active reabsorption of 99% of glucose and amino acids from the glomerular filtrate.[21] The DCT cells are smaller, simple cuboidal epithelial cells (stain less intensely due to fewer organelles) that actively reabsorb sodium from the tubular fluid.[21] The close proximity of filtered substances to this kind of tubular epithelium increases the susceptibility of these cells to damage.

To date, limited scientific evidence has been available on the mechanism by which SF extracts affect cellular processes and the side effects related to their use. This medicinal plant, however, continues to be recommended as a traditional remedy and is used by a large portion of the South African community. In this study, the nephrotoxic and apoptotic effects of SF extracts on two kidney cell lines, LLC-PK$_1$ (PCT epithelium) and MDBK (DCT epithelium), were investigated and compared.

MATERIALS AND METHODS

Materials

Sutherlandia frutescens tablets (Phyto Nova™, Cape Town, South Africa) were purchased from a local pharmacy. The LLC-PK$_1$ and MDBK cell lines were purchased from Highveld Biologicals (Johannesburg, South Africa). All tissue culture reagents, the GSH-Glo™ Glutathione Assay and the Caspase-Glo® 3/7 Assay were obtained from Whitehead Scientific (Johannesburg, South Africa). The JC-1 dye was purchased from BD Biosciences (South Africa). All other reagents were purchased from Merck (South Africa) unless otherwise stated.

Preparation of *Sutherlandia frutescens* extracts

Phyto Nova Sutherlandia™ tablets were used to prepare an aqueous extract of the active ingredients of the plant. Sixty tablets were crushed to a fine powder in a pestle and mortar, weighed and suspended in deionised water (1.2 g per 10 mL). The mixture was continuously stirred at room temperature for 1.5 h, and thereafter transferred to 50 mL sterilin tubes and centrifuged (3 645 *g*, 10 min) at room temperature. The upper aqueous layer (SF extract) was removed, vacuum filtered and stored at 4 °C. SF extract dilutions (24 mg/mL, 12 mg/mL, 6 mg/mL, 2.4 mg/mL, 1.2 mg/mL, 0.6 mg/mL, and 0.3 mg/mL) were prepared using complete culture media (CCM), comprising Eagle's minimum essential medium, 10% foetal calf serum, 1% L-glutamine and 1% penstrepfungizone.

Cell culture and cytotoxicity assay

LLC-PK$_1$ and MDBK cells were cultured (37 °C, 5% CO$_2$) to confluency in 75 cm³ flasks in CCM. The cytotoxicity of SF in LLC-PK$_1$ and MDBK cells was measured using the MTT assay.[22] LLC-PK$_1$ and MDBK cells (10 000/well) were incubated with varying SF extract dilutions for 48 h in triplicate in microtitre plates, together with the respective controls (cells incubated with CCM only). The cells were then incubated with the MTT substrate (5 mg/mL) for 4 h. Thereafter all supernatants were aspirated, and dimethyl sulphoxide (DMSO) (100 µL/well) was added to the wells. Finally, the optical density was measured at 570 nm, with a reference wavelength of 690 nm, by an enzyme-linked immunosorbent assay (ELISA) plate reader (Bio-Tek µQuant). The data were translated to 'percentage cell viability' versus 'concentration of extract', from which the IC$_{50}$ (half the maximal inhibitory concentration) values for each cell line and for the combination of treatments were determined. For all subsequent biochemical assays, both cell lines were grown to confluency and treated with the determined IC$_{50}$ values of the SF extracts.

Lipid peroxidation assay

Oxidative damage of both cell lines was assessed using the thiobarbituric acid assay, because lipid peroxidation is commonly quantified by levels of malondialdehyde (MDA). After the 48 h incubation with SF extract, the culture fluid from each SF-treated flask and from the untreated controls (500 µL) was dispensed into duplicate glass tubes (one representing the sample, one representing a negative control), followed by addition of 7% H$_2$PO$_3$ (200 µL). A positive control of 1% MDA was prepared. Thiobarbituric acid (1%, w/v)/ 0.1 mM butylated hydroxytoluene solution (400 µL) was added to sample tubes. To the tubes with the negative controls, 400 µL of 3 mM HCl was added. The solution was adjusted to pH 1.5 and heated at 100 °C for 15 min. Once cooled, butanol (1.5 mL) was added and the sample then centrifuged (8 400 *g*, 6 min). Following centrifugation, the butanol phase (200 µL) from each sample and

from the blanks was aliquoted into a microtitre plate. The optical density was measured at 532 nm, with a reference wavelength of 600 nm, by an ELISA plate reader. The sample means of ten replicates were calculated and divided by the absorption coefficient, 156 mM^{-1}.

Glutathione assay

The GSH-Glo™ Glutathione Assay (Promega, Madison, USA) was used to measure glutathione (GSH) levels. Cells (those treated with SF extract and the untreated controls after 48 h incubation) were transferred to an opaque microtitre plate (50 µL of 10 000 cells/well, 10 replicates). GSH standards (0 µM – 5 µM) were prepared from a 5 mM stock solution diluted in water. Five two-fold dilutions of the GSH stock were prepared and transferred into wells (50 µL) of the microtitre plate. The 2X GSH-Glo™ Reagent was prepared according to the manufacturer's instructions, added to the experimental wells (50 µL/well), and incubated at room temperature. Reconstituted Luciferin Detection Reagent (50 µL) was added to each well and incubated. The luminescence was measured on a Modulus™ microplate luminometer (Turner Biosystems, Sunnyvale, USA). A standard curve was derived using the GSH standards (0 µM – 5 µM) and the GSH concentration in each sample was extrapolated from the equation.

Caspase-3/7 assay

The apoptotic potential of SF extracts on both cell lines was determined using the Caspase-Glo®3/7 assay (Promega). Caspase-Glo®3/7 Reagent was reconstituted according to the manufacturer's instructions and added to both the SF-treated and control cells (following 48 h incubation) in the wells of a microtitre plate (10 µL reagent per 50 µL of 10 000 cells/well, 10 replicates) and incubated in the dark (30 min). The luminescence was measured on a Modulus™ microplate luminometer (Turner BioSystems). The caspase-3/7 activity of the SF-treated samples was represented as X-fold change compared to the control (cells incubated with CCM only).

Mitochondrial membrane potential

The mitochondrial membrane potential ($\Delta\Psi_m$) of LLC-PK$_1$ and MDBK cells (both those treated with SF extract and their untreated controls; 48 h) was assessed using fluorescence-activated cell sorting (FACS) and the JC-1 Mitoscreen assay (BD Biosciences) according to the manufacturer's instructions. Cells (approximately 100 000) were transferred to polystyrene cytometry tubes. The JC-1 dye (150 µL) was added to the cells and allowed to incubate (37 °C, 5% CO$_2$, 10 min). Cells were then washed with JC-1 Mitoscreen wash buffer (400 *g*, 5 min) and resuspended in 300 µL flow cytometry sheath fluid. Flow cytometry data from stained cells (15 000 events) was obtained using a FACSCalibur (BD Biosciences) flow cytometer with CellQuest PRO v4.02 software (BD Biosciences). Cells were gated to exclude debris using FlowJo v7.1 software (Tree Star Inc., Ashland, USA)
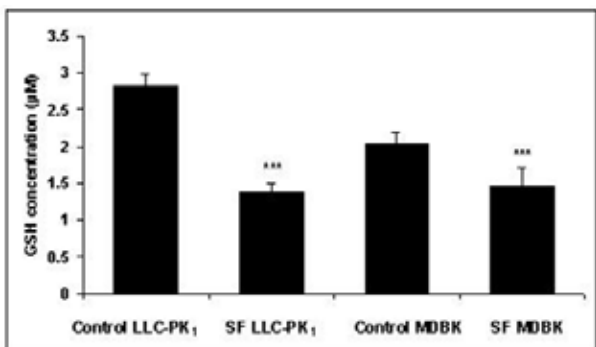
Statistical analysis

Results are expressed as the means, with error bars representing the standard deviations (s.d.) of the means. Statistical significance

TABLE 1
Viability of PCT and DCT cells incubated with SF for 48 h using the MTT assay

SF (mg/mL)	LLC-PK$_1$ CELL VIABILITY (%)	MDBK CELL VIABILITY (%)
24	25 ± 0.0*	15 ± 0.3*
12	72 ± 2.1	24 ± 14.0*
6	89 ± 33.0	103 ± 12.7
2.4	107 ± 10.9	118 ± 3.7
1.2	103 ± 2.9	122 ± 9.3
0.6	122 ± 14.0	96 ± 7.9
0.3	133 ± 11.0	101 ± 10.3

*p < 0.001

The levels of GSH were decreased significantly in LLC-PK$_1$ cells treated with SF extracts (p < 0.0001) compared with the relevant controls. The MDBK cells treated with SF also showed a decrease in GSH levels (p < 0.0001). Results are presented as the mean ± s.d. and n = 10. (***p < 0.0001).

FIGURE 1
Basal GSH concentrations in LLC-PK$_1$ and MDBK were different from each other

between samples for the MTT assay was determined using the one-way ANOVA Tukey Kramer Multiple Comparisons test. The statistical significances of the lipid peroxidation assay, GSH assay, caspase-3/7 assay and mitochondrial depolarisation assay were determined by the Mann-Whitney test for non-parametric data using GraphPad InStat Software, (GraphPad Software Inc., La Jolla, USA).

RESULTS

Cytotoxicity

The cytotoxic effects of SF extracts (MTT assay) were determined in both LLC-PK$_1$ and MDBK cells treated with a range of extract dilutions for 48 h (see Table 1). The IC$_{50}$ for SF extract was determined as 15 mg/mL and 7 mg/mL dilutions for the LLC-PK$_1$ and MDBK cells, respectively. The cell viability of both cell lines treated with concentrations between 6 mg/mL and 0.3 mg/mL was more than 89%. However, at higher SF extract concentrations of 24 mg/mL and 12 mg/mL, respectively, cell viability was decreased to 25% and 72% of controls (p < 0.001) in the case of LLC-PK$_1$ cells and to 15% and 24% (p < 0.001) of controls in the case of MDBK cells.

GSH assays

The intracellular concentrations of GSH in both SF-extract-treated renal epithelial cell lines were determined. They decreased significantly in SF-treated LLC-PK$_1$ cells as compared with controls (p < 0.0001), whilst there also was a significant decrease in GSH in SF-treated MDBK cells as compared with controls (p < 0.0001) (see Figure 1).

Lipid peroxidation assays

In order to determine whether mitochondrial damage and cytotoxicity were related to oxidative stress, we measured the levels of lipid oxidation products in both SF-treated cell lines. There were significantly higher levels (p < 0.0001) of MDA in both the SF-treated cell lines as compared with the respective control cells (see Figures 2A and 2B).

Mitochondrial membrane potential analyses

To determine whether LLC-PK$_1$ and MDBK cells were metabolically viable after SF-extract treatment, we investigated changes in $\Delta\Psi_m$. The SF-treated LLC-PK$_1$ cells had a significantly higher (p < 0.0001) percentage of depolarised mitochondria as compared with the untreated cells (80.2% vs 54.6%) (see Figure 3). Similarly, the MDBK cells had a significantly higher (p < 0.0001) percentage of depolarised mitochondria as compared with controls (81.7% vs 58.2%) (see Figure 3).
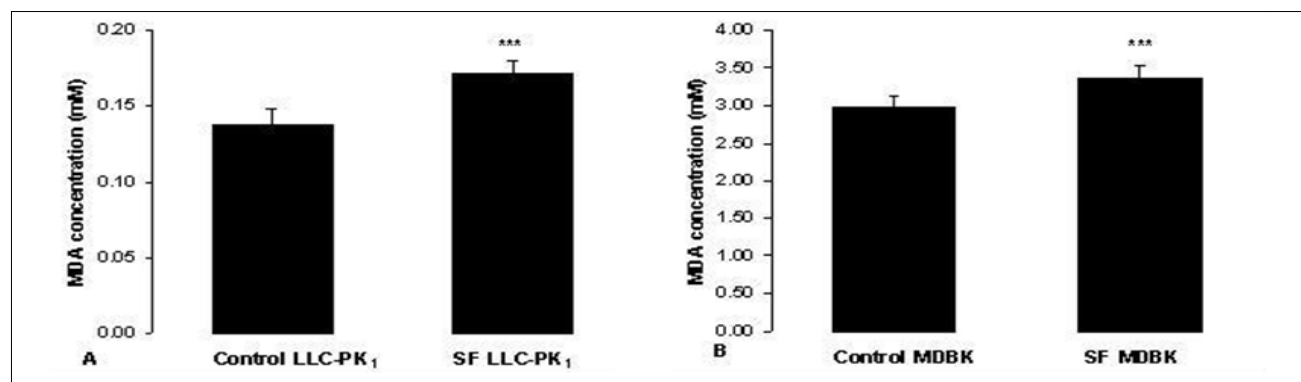
Caspase-3/7 assay

A significant change in $\Delta\Psi_m$ is a good indicator of apoptosis. The intracellular activities of caspase 3/7 in both SF-treated cell lines were measured (as a fold-change in comparison with the relevant controls). The activity of caspase 3/7 in SF-treated LLC-PK$_1$ cells showed a highly significant 11.9-fold increase (p < 0.0001) compared with the controls. The SF-treated MDBK cells also showed a significant 2.2-fold increase (p < 0.0001) over controls (see Table 2). These results indicated that PCT cells were more susceptible to apoptotic induction by SF than were DCT cells.
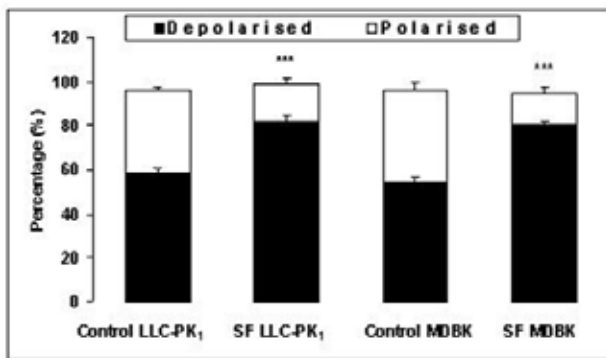
DISCUSSION

SF is used by many South Africans as a traditional remedy or ameliorant for many diseases, including HIV infection. The crude plant concoction is normally taken orally and the main route of elimination of the absorbed constituent polar compounds is via the renal system. The potential for injury by noxious compounds in the PCT region is high due to the ability of the PCT cells to concentrate substances that have been filtered by the glomerulus.[20]

The results of this investigation have shown that both kinds of renal tubular cells, LLC-PK$_1$ and MDBK cells, are affected by SF extracts *in vitro*. This sensitivity may be attributed to the location and functional capacities of these cells. The intact PCT is susceptible to injury, as it is at this site of the nephron where toxicants accumulate and where there is an abundance of mitochondria for active re-absorption and transportation of ions, low molecular weight proteins, glutathione conjugates and heavy metals.[20] Cells treated with SF extracts *in vitro*, at concentrations of 6 mg/mL and lower, displayed greater than 89% viability. At such low concentrations, SF extracts appeared to promote cell metabolism as evidenced by the increased conversion of the MTT salt. This might have been due to both increased mitochondrial reductase enzyme activity,[22] and increased availability of reducing equivalents such as NADH,

The concentrations of MDA in LLC-PK$_1$ cells (p < 0.0001) [A] and MDBK cells (p < 0.0001) [B] treated with SF were significantly higher than in their respective controls. Results are presented as the mean ± s.d. and n = 10. (***p < 0.0001).

FIGURE 2
Levels of MDA in SF-treated cells

Both cell lines contained approximately 50% depolarised mitochondria without SF treatment. Results are presented as the mean ± s.d. and n = 15 000. ($^{***}p < 0.0001$).

FIGURE 3
Depolarised mitochondria determined by the JC-1 Mitoscreen assay of control and
SF-treated LLC-PK$_1$ and MDBK cells

TABLE 2
Caspase-3/7 activity of PCT and DCT cells incubated with SF for 48 h.
RLU – relative light units

	Control – Caspase-3/7 activity (RLU)	SF – Caspase-3/7 activity (RLU)	Fold Change	p value
LLC-PK$_1$	16 127 ± 346	191 706 ± 13455***	11.9	$p < 0.0001$
MDBK	5 137 ± 576	11 365 ± 486***	2.2	$p < 0.0001$

$^{***}p < 0.0001$

a byproduct of metabolic pathways such as glycolysis and the Krebs cycle.[23] The anti-diabetic and favourable cellular metabolic properties of SF were previously demonstrated in male Wistar rats.[24]

The hypoglycaemic potential of SF was shown in diabetogenic rats, where it exhibited properties similar to those of the extracts similar to those of metformin, the common diabetes type-2 drug.[24] SF extracts increased the uptake of glucose by the kidney and resulted in increased glycolysis, which increased the rate of the Krebs cycle and produced more reducing equivalents (NADH and FADH$_2$). The high concentration of reducing equivalents donate electrons to the electron transport chain (ETC), resulting in increased ATP production via oxidative phosphorylation. This increased ATP concentration, is utilised for the active reabsorption of substances by the PCT and DCT. The continuous flux of electrons along the ETC and pumping of protons out of the matrix results in a negative mitochondrial membrane potential, during which process reactive oxygen species (ROS) are generated as a byproduct, rendering the mitochondria susceptible to oxidative damage.[25]

Free radicals attack polyunsaturated fatty acids, a major component of cell membranes, thereby forming lipid peroxyl radicals. Lipid peroxidation is initiated when the reactive lipid peroxyl radicals are not detoxified by antioxidants. The lipid alkoxyl radical undergoes cyclisation forming an intermediate product which can degrade into MDA.[26] Lipid peroxides can be detoxified by conjugation to antioxidant molecules such as ascorbic acid, α-tocopherol, uric acid and GSH.[27] Glutathione concentrations were significantly decreased in SF-treated LLC-PK$_1$ and MDBK cells with a corresponding increase in lipid peroxidation compared to the controls ($p < 0.0001$).

Both the LLC-PK$_1$ and MDBK cells were transformed cell lines which proliferated rapidly and possessed high metabolic activity which resulted in increased production of ROS, which, when not sequestered rapidly, increased lipid peroxidation. The MDA concentrations in the control LLC-PK$_1$ and MDBK cells were 0.14 mM and 3.0 mM, respectively (see Figures 2A and 2B), whereas the concentrations of GSH in LLC-PK$_1$ and MDBK cells were 2.85 µM and 2.1 µM. respectively (see Figure 1). The increased basal GSH concentration in the LLC-PK$_1$ cells suggested that these

cells were better protected from increased ROS production and subsequent peroxidation than the MDBK cells were.

The oxidative ability of L-canavanine to induce ROS and ensuing lipid peroxidation was determined previously in a mouse glial cell line (N11)[27]. In this study, a luminol-amplified chemiluminescence assay showed that 1 mM of L-canavanine significantly ($p < 0.05$) increased ROS production and lipid peroxidation in N11 cells.[27] L-canavanine, a structural analogue of L-arginine, is a competitive inhibitor of arginine-utilising enzymes such as nitric oxide (NO) synthase, which synthesises NO from L-arginine.[28] Nitric oxide is involved in many biological processes and has the ability to spontaneously react with superoxide radicals to produce peroxynitrite and subsequently, nitrate.[27] Nitric oxide, therefore, serves as a reactive radical scavenger that ultimately prevents oxidative damage to cellular components.[29] The possible decreased activity of NO synthase and inhibition of NO synthesis by SF may explain the significant lipid peroxidation in these renal cell lines.

Another possibility for the increased lipid peroxidation in both the SF-treated LLC-PK$_1$ and MDBK cells may be due to a decreased $\Delta\Psi_m$. GABA was shown to penetrate up to 60% of the mitochondrial matrix volume in rat brain and liver cells. The weak acidic nature of GABA may contribute to the proton gradient disruption within mitochondria.[30] A change in $\Delta\Psi_m$ promotes formation of ROS as it offsets the normal functioning of the ETC.

In addition to its acidic nature, high concentrations of GABA cause the reduction of intra-mitochondrial NAD$^+$ stimulated by glutamate.[30] Excessive intra-mitochondrial NADH is dissipated by donating its electrons to the ETC. The metabolism of GABA involves the enzymes GABA: pyruvate transaminase, GABA: α-ketogluterate transaminase and succinic semialdehyde dehydrogenase to form succinate which can enter the Krebs cycle and produce substrates for the ETC.[14]

ROS production in the mitochondria increases susceptibility for membrane damage, altering the mitochondrial permeability transition. This may account for the high percentage of depolarised mitochondria noted after treatment of cells with SF extracts. The alteration of the $\Delta\Psi_m$ within a cell will not, however, occur simultaneously to all the mitochondria that are present.[31] The number of mitochondria that are affected determines the amount of ATP generated and the ultimate fate of the cell. The amount of ATP available will determine the type of cell death that occurs within the cell.[20]

When mitochondrial membranes are depolarised, pro-apoptotic signals are released. It was demonstrated that changes in $\Delta\Psi_m$ favoured the transition of a tightly bound form of cytochrome c (to cardiolipin in the inner mitochondrial membrane) into its loosely bound form, followed by the release of cytochrome c into the extra-mitochondrial environment.[32] The release of cytochrome c, Apaf-1 and calcium ions into the cytosol results in the activation of downstream caspases that execute apoptosis.[25,33,34] This study showed that SF-extract treatments increased caspase-3/7 activity in both cell lines (by 11.9-fold in LLC-PK$_1$ cells and by 2.2-fold in MDBK cells).

When comparing the change in $\Delta\Psi_m$ of LLC-PK$_1$ and MDBK cells with caspase-3/7 activity, it became evident that SF extracts are potent apoptotic inducers. The percentage mitochondrial membrane depolarisation in both cell lines appears to be equal at basal levels; following SF-extract treatments, an almost similar increase in depolarisation occurs in both LLC-PK$_1$ and MDBK cells.

The 11.9-fold increase of caspase activity in LLC-PK$_1$, as compared to a 2.2-fold increase in MDBK cells, may be due to the difference in mitochondrial numbers and cellular architecture of both cell lines. It is known that PCT epithelium contains more mitochondria than do DCT cells. The depolarisation of mitochondria allows for the increased release of more pro-apoptotic signals, thereby resulting in increased apoptosis.

There exists a real concern about the unregulated use of the crude plant derivatives of SF. Evidence that the recommended dosages are in such concentrations that they may be potentially harmful to the renal tubular cells, could be attained *in vivo*. Ojewole determined that SF extract had an LD_{50} of 1 280 mg/kg body weight in rats.[35] In a clinical trial on ten healthy adults receiving a dose of 800 mg/day of SF extract, no significant changes in haematological, physiological and biochemical markers were noted after three months of treatment.[36] The only changes observed in this *in vivo* study were a decrease in respiratory rate ($p < 0.04$), and increases in platelet count ($p < 0.05$), mean cell haemoglobin ($p = 0.01$), protein ($p < 0.03$) and albumin levels ($p < 0.03$).[36] Chronic doses of SF extract, however, may eventually lead to renal toxicity.

CONCLUSION

The aqueous SF extracts studied here were not cytotoxic at low concentrations, but had the potential to increase oxidative stress, alter the integrity of mitochondrial membranes, and promote apoptosis in renal tubule epithelia at high concentrations in vitro.

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