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 IC50 values from the cell viability results for LLC-PK, and MDBK were 15 mg/mL and 7 mg/mL, respectively. SF extracts significantly decreased intracellular glutathione in LLC-PK, (p < 0.0001) and MDBK (p < 0.0001) cells, while lipid peroxidation increased in treated LLC-PK (p < 0.0001) and MDBK (p < 0.0001) cells. JC-1 analysis showed that SF extracts promoted mitochondrial membrane depolarization in both LLC-PK, and MDBK cells by up to 80% (p < 0.0001). The activity of caspase 3/7 increased in both LLC-PK, (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 triterpene diglucoside.2–4 L-canavanine stores nitrogen in seeds and is used in plant chemical defense mechanisms.4 Its production by SF is dependent on the availability of abiotic factors.2 L-canavanine, 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 arginine-mediated hydrolytic cleavage of L-canavanine, has been shown to have anti-tumour properties.6 D-pinitol, a chiro-inositol sugar, possesses anti-diabetic properties and is used in the treatment of wasting in cancer and HIV/AIDS patients.7,8,9

GABA, an inhibitory neurotransmitter, mediates most of its effects inside the nervous system.10 It has been used as a drug for the relief of anxiety and stress.10 Interestingly, GABA has been shown to affect the absorption of ions in the renal tubules.11 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.12

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.13 SF-treated human breast adenocarcinoma cells in culture showed morphological characteristics of apoptosis and cell growth inhibition.14,15 An antiproliferative effect of SF was demonstrated to be concentration-dependent in breast cancer and leukaemia cell lines, with no significant antioxidant effects.7 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.16 An apparent antitumoral activity of SF has been thought to be mediated by the inhibition of HIV-1 target enzymes, such as HIV-1 reverse transcriptase.17

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.22 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-PK1 (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-PK1 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 frutescens* 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 sterile tubes and centrifuged (5 645 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% penstreplusigibzone.

**Cell culture and cytotoxicity assay**

LLC-PK1 and MDBK cells were cultured (37 °C, 5% CO₂) to confluency in 75 cm² flasks in CCM. The cytotoxicity of SF in LLC-PK1 and MDBK cells was measured using the MTT assay.²² LLC-PK1 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 sulfoxide (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 IC₅₀ (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 IC₅₀ values of the SF extracts.

**Lipid peroxidation assay**

Oxidative damage of both cell lines was assessed by 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% H₂PO₄ (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 Modulus²³ microplate luminoimeter (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 Modulus²³ microplate luminoimeter (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 (ΔΨₘ) of LLC-PK₁ and MDBK cells (both those treated with SF extract and their untreated controls; 48 h) was assessed using fluorescein-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% CO₂, 10 min). Cells were then washed with JC-1 Mitoscreen wash buffer (400 µL, 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 v4.0.2 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 was determined using one-way ANOVA followed by Tukey’s multiple comparison test. The student’s t-test (paired and unpaired) was used to determine significance between two groups. The significance level was defined as p < 0.05.

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**TABLE 1**

<table>
<thead>
<tr>
<th>SF (mg/mL)</th>
<th>LLC-PK1 CELL VIABILITY (%)</th>
<th>MDBK CELL VIABILITY (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>24</td>
<td>25 ± 0.0*</td>
<td>15 ± 0.3*</td>
</tr>
<tr>
<td>12</td>
<td>72 ± 2.1</td>
<td>24 ± 14.0</td>
</tr>
<tr>
<td>6</td>
<td>89 ± 33.0</td>
<td>103 ± 12.7</td>
</tr>
<tr>
<td>2.4</td>
<td>107 ± 10.9</td>
<td>118 ± 3.7</td>
</tr>
<tr>
<td>1.2</td>
<td>103 ± 2.9</td>
<td>122 ± 9.3</td>
</tr>
<tr>
<td>0.6</td>
<td>122 ± 14.0</td>
<td>96 ± 7.9</td>
</tr>
<tr>
<td>0.3</td>
<td>133 ± 11.0</td>
<td>101 ± 10.3</td>
</tr>
</tbody>
</table>

* p < 0.001
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 \( p < 0.0001 \)% 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 \( p < 0.0001 \)% 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.26

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.27 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,27 and increased availability of reducing equivalents such as NADH,

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 \( p < 0.0001 \) in the case of LLC-PK1 cells and to 15% and 24% \( p < 0.0001 \) 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 \( \Delta \Psi_m \) 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.

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Lipid peroxidation assays

In order to determine whether mitochondrial damage and cytotoxicity were related to oxidative stress, we measured the

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

FIGURE 2

Levels of MDA in SF-treated cells
GSH concentration in the LLC-PK$_1$ cells proliferated rapidly and possessed high metabolic activity similar to those of metformin, the common diabetes type-2 drug. SF extracts increased the uptake of glucose by the kidney cells and resulted in increased glycolysis, which increased the amount of ATP generated and the ultimate fate of the cell. The number of mitochondria that are affected determines the 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 ∆m$_{m}$ within all the mitochondria that are present.

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 ∆m$_{m}$. GABA was shown to penetrate up to 50% 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. A change in ∆m$_{m}$ 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. 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-ketoglutarate transaminase and succinic semialdehyde dehydrogenase to form succinate which can enter the Krebs cycle and produce substrates for the ETC.

The oxidative ability of L-canavanine to induce ROS and subsequent peroxidation was determined previously in a mouse glial cell line (N11)\textsuperscript{27}. 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 mitochondrial membranes are depolarised, pro-apoptotic signals are released. It was demonstrated that changes in ∆m$_{m}$ 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. The release of cytochrome c, Apaf-1 and calcium ions into the cytosol results in the activation of downstream caspases that execute apoptosis. 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 ∆m$_{m}$ 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$_1$ cells, 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 LD₅₀ of 280 mg/kg body weight in rats. 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. 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). 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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