EFFECTS OF *SUTHERLANDIA FRUTESCENS* EXTRACTS ON NORMAL T-LYMPHOCYTES *IN VITRO*

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Abstract

Sutherlandia frutescens (SF), a popular traditional medicinal plant found in various parts of southern Africa, is used for treatment or management of HIV/AIDS and other diseases including cancer. However, its toxicity profile has not been fully established. The aims of this study were to examine the effects of 70% ethanol (SFE) and deionised water (SFW) extracts on normal isolated human T cells. An experimental study on normal human lymphocytes treated with doses SF extract doses ranging from 0.25 to 2.5 mg/ml. Untreated, vehicle-treated (Ethanol) and camptothecin (CPT) treated normal T cells were used as controls. Induction of cell death, changes in intracellular ATP, caspase-3/-7 activity and nuclear changes were analysed using flow cytometry, luminometry and nuclear staining (Hoechst) respectively. The highest concentration (2.5 mg/ml) of SFE extract induced significant necrosis (95%), depletion of ATP (76%), and inhibition of caspase-3/-7 activity (11%) following a 24 hour incubation period ($p< 0.001$). The 2.5 mg/ml concentration of SFW showed the same trend but were less effective (necrosis- 26%, ATP- 91%, & caspase-3/-7- 15%). These effects showed a time-dependence over 48 hours of incubation, with high doses of SFE extracts eliminating viable cells by necrosis, depleting ATP levels and decreasing caspase-3/-7 activity ($p< 0.001$). The activity of SFE extract was independent of ethanol. The SFW extract dilutions were less toxic than the SFE extracts. Significant DNA fragmentation as demonstrated by Hoechst staining was also seen over 48-hour incubation for high doses of both types of SF extracts. These results showed that although high concentrations of SF extracts can be toxic to normal T cells *in vitro,* SFW fractions were relatively safe for use.

Keywords: *Sutherlandia frutescens*, T lymphocytes, flow cytometry, luminometry, necrosis, apoptosis

Introduction

Sutherlandia frutescens (SF) (a member of the *Fabaceae/Lugemniosa* family of plants) is one of the herbal remedies currently recommended by the South African Health Department for management of a variety of ailments. These include treatment of cancer and as an immune booster/modulator in human immunodeficiency syndrome (HIV) infection (Mills et al., 2005). *Sutherlandia frutescens* is an old South African herbal remedy traditionally used for the treatment of stomach and internal cancers (Chinkwo, 2005; Steenkamp et al., 2006). SF is regarded as the most profound and multi-purpose medicinal plant in southern Africa (Mills et al., 2005; Sia, 2004) as its extracts are mainly used as an immune tonic and a supplement which boosts the immune response, fight infections and relieve mental stress. Strengthening the immune response is very important in debilitating conditions such as cancer and HIV/AIDS. For example, anecdotal evidence has demonstrated improved CD^{+}_{4} cells and decreased viral loads in patients taking SF tablets (Morris, 2001; Jenkins, 2005; Harnett et al., 2005).

Most scientific data on the effects of SF on immune cells were obtained from transformed lymphocyte cell lines. For example, the cytotoxicity of SF ethanol extracts on Jurkat and HL60 leukaemia cells at 1/150 and 1/200 dilutions respectively, has been demonstrated (Tai et al., 2004). SF extracts can also induce apoptosis in Jurkat T lymphocytes (Chinkwo, 2005). The elemental composition of SF extracts, following chemical analysis, comprise several biological active compounds such as L-canavanine, pinitol, gamma-aminobutyric acid, methyl- and propyl parabens and saponins, but no alkaloids(Tai et al., 2004; Stander et al., 2007). Recently, similar to our work, Korb et al., (2010) described the apoptosis inducing effects ethanol extracts of SF on normal lymphocytes *in vitro*. L-canavanine, a natural L-arginine analogue and its metabolite canaline, are two of the many factors contributing to *in vitro* antiproliferative and apoptotic activity (Chinkwo, 2005). High doses of L-canavinine block DNA synthases *in vitro* and affect B-cell function in autoimmune mice at low doses. L-canaline is cytotoxic to human peripheral blood mononuclear cells (PBMCs) *in vitro* through the disruption of polyamine biosynthesis (Bence et al., 2002). Additionally, a newly discovered tritephene SU1, has been demonstrated to be a major component of commercial SF preparations (Stander et al., 2009; Van Wyk and Albrecht, 2008). Several of triterpenoid compounds, believed to be responsible for pharmacological activity in medicinal plants, are implicated in the resolution of inflammatory and immune diseases, although their role has not been clearly defined (Rios, 2010).

In this study, we aimed to determine the effects of SF ethanol and aqueous tablet extract dilutions on isolated normal human T lymphocytes. Data obtained from this study will give insight into the toxic side effects or *in vitro* immunomodulatory effects of SF extracts.

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Materials and Methods

Reagents and equipment

RPMI-1640 with HEPES buffer (25 mM), foetal calf serum (FCS), penstrep-fungizone (PSF), & L-glutamine were purchased from *Adcock Ingram* (SA). Normal whole blood was obtained from the South African National Blood Supply (SANBS). Histopaque 1077, dimethyl sulphoxide (DMSO), bisbenzimide were purchased from *Sigma Aldrich* (SA). Ethanol was bought from *Merck Chemicals* (SA). Promega CellTiter-Glo™ Luminescent Cell Viability and Promega Caspase 3/7™ assay kits were products of *Promega* (SA). *Sutherlandia frutescens* tablets (*PhytoNova*) were available from local Chemist shop. BD IMagnet™ and BD IMag™ Human T-lymphocytes Enrichment Set- DM were purchased from *BD BioSciences* (USA). Modulus™ Microplate Luminometer was from *Turner BioSystems* (USA). The inverted fluorescent microscope was from *Olympus Microscopes* (USA). The FACSCalibur flow cytometer used for flow cytometric analysis and annexin V-FITC apoptosis detection kit were purchased from *BD Biosciences* (USA).

Extraction of active ingredients from *Sutherlandia frutescens* tablets

Each of the SF tablets was extracted according to the method by Tai *et al.* 2004 with a slight variation. Briefly, SF tablets containing 300 mg of raw herb powder (per tablet) compounded with inert excipients were extracted in 2.2 ml of 70% ethanol/triple distilled water at room temperature for 2 hours on an orbital shaker. The suspension was then centrifuged at 2 000 *g* for 10 minutes and the supernatant was removed. The extracts were filter sterilised twice using 0.45 µm filters to give a stock solution of 136.4 mg/ml of each extract type. These extracts were further diluted to 2.5, and 0.5 mg/ml with complete culture medium (RPMI-1640 supplemented with 10% FCS, 1% L-glutamine, and 1% PSF). Different concentration ranges were used to measure enzyme activity (caspase-3/-7) (2.5, 1.5, and 0.25 mg/ml). Both types of extracts were prepared and used on the same day.

Isolation of T-lymphocytes and treatment with SF extracts

Normal whole blood (acquired from the SANBS, ethics approval number EXP057/06) was carefully layered onto equal amounts of Histopaque 1077 then centrifuged at 600 *g* for 30 minutes at 25 °C. After centrifugation, the buffy coat layer containing PBMCs was isolated and washed twice in phosphate buffered-saline (PBS, 5 ml) (1150*g* for 20 minutes at 25 °C). The final pellet was re-suspended in BD IMag Human T lymphocytes isolation buffer.

The BD IMag™ Human T Lymphocyte Enrichment Set – DM (cat number: 557874) was used for negative selection of T lymphocytes from PBMCs. The Biotinylated Human T Lymphocyte Enrichment Cocktail contains monoclonal antibodies that recognize antigens expressed on erythrocytes, platelets, and peripheral leukocytes that are not T lymphocytes. Isolation of lymphocytes from PBMCs was done according to manufacturer instructions. Following isolation, the T-cells were washed with PBS and then diluted in RPMI-1640 media to make a suspension of 1.5- 2 × 10^6 cells/ml and their viability determined using trypan blue. Aliquots of the cell suspension (2 ml) were transferred into each well of a 6-well plate. Doses of SF extracts (also 2 ml) were then added to each of the treatment wells. Vehicle control (70% ethanol), camptothecin (CPT-20 µM) and untreated control wells were also included in the treatment setup. The 6-well plates were then incubated for 24 and 48 hours at 37 °C in an incubator (5% CO_2, 95% humidity).

Annexin V-FITC staining by flow cytometry

A sample (2 ml) of 24/48-hour treated/control cell suspension from each well was aliquoted into labelled sterilin tubes. The tubes were centrifuged (500 *g*, 10 minutes) to remove the incubation media. The cell pellets were then washed thrice in PBS and incubated with annexin V-FITC staining solution (20 µl) for 20 minutes. The staining solution was removed and access stain washed away with assay buffer. The resultant cell suspensions in the assay buffer were analysed by flow cytometry for levels of live, apoptotic and necrotic cells.

ATP assay by luminometry

The luminescent cell viability ATP assay kit from Promega uses recombinant luciferase to catalyse the following reaction: ATP + d-Luciferan + O_2 → Oxyluciferan + AMP + PPi + CO_2 + Light (560 nm). When ATP is the limiting component in the reaction, the intensity of the emitted light is proportional to the concentration of ATP. Based on these principles, the levels of ATP in SF extract treated normal T cells were analysed according to manufacturer's instruction. Briefly, a sample (100 µl) of 24/48-hour treated/control cell suspension from each treatment concentration was pipetted into two different wells of a white opaque 96-well plate. The working CellTiter-Glo™ Reagent (cat number: G7570) was prepared immediately before use and was added to the wells with treated cells at 100 µl per well. The plate was shaken on a plate shaker for 2 minutes at 30 *g*. This plate was then incubated in darkness for 10 minutes at room temperature after shaking. At the end of the incubation period, the plate was put into the luminometer and the relative light units (RLU) of the samples were measured. A dose response curve was also generated for the ATP levels using RLU and the dilutions of SF extracts and different control samples.

Caspase-3/-7 assay by luminometry

A sample (100 µl) of 24/48-hour treated/control cell suspension of each treatment type was pipetted into duplicate wells of a white-walled 96-well plate compatible with the luminometer. A 100 µl of RPMI-1640 was added into separate duplicate wells to serve as a blank for the reaction. Preconditioned Caspase-Glo® 3/7 reagent (cat number: G8091) was added

into each well at a ratio of 1:1 to the amount of cells in each well. The treated cells and the Caspase-Glo® 3/7 reagent were allowed to equilibrate to room temperature before they were mixed. After mixing the two components, the plate was covered and gently shaken on a plate shaker at 30 *g* for 30 s. The plate was then incubated at room temperature in the dark for 1 hour. Luminescence of each sample was measured in a plate-reading Modulus™ Microplate Luminometer, as directed by the manufacturer.

Nuclear staining protocol

Samples of 24/48-hour treated/control cell suspensions were washed in PBS and stained with a Hoechst working solution (100 µl, 15 minutes at 37 °C). Unbound Hoechst stain was removed by washing in PBS. The cells were then fixed in 10% paraformaldehyde (PFA;5 minutes). After another washing step in PBS, the cells pellets were re-suspended in 100 µl PBS. This suspension was pipetted into glass slides then covered with coverslips. The prepared slides were then viewed under an Olympus TH4-200 inverted fluorescent microscope (excitation filter 350 nm and a barrier filter of 450 nm) Kukuruga, 1997.

Statistical analysis

Data analysis was done on Microsoft Excel, Microsoft Corporation, USA, to obtain descriptive statistics. The different levels of significances within the separate treated groups were analysed using one-way analysis of variance (*ANOVA*) and the differences between the treated cells and the control cells were analysed in GraphPad Instat software (version 3) using the *Tukey-Kramer* multiple comparison test. Differences with $p< 0.05$ were considered statistically significant.

Results

Flow cytometry

The different extraction methods used to extract the SF tablets were shown to have slightly varying abilities in their effects on normal T cells. For example, the 70% ethanol extract doses of SF (SFE) were more effective in inducing cell death than the aqueous extract doses (SFW). This effect maybe observed as a result of the active compound being mainly soluble in organic solvents. Over 24 hours of incubation, the 2.5 mg/ml SFE extract dose induced significant levels of necrosis ($p< 0.001$) while apoptosis was minimal. Cell viability at this concentration was also significantly reduced ($p< 0.001$) than in the untreated controls. This 2.5 mg/ml SFE extract was also more effective in reducing the percentage of live cells the positive control (20 µM CPT) but CPT induced more apoptosis ($p< 0.001$). The effects of the SFE extract doses were found to be dependent on the 70% ethanol used for tablet extraction (Figure 1A).

The aqueous extract dilution of SF at similar dilutions as the SFE extract dilutions had a reduced death-inducing effect in the treated normal T cells over 24 hours incubation. The 2.5 mg/ml SFE dose significantly reduced ($p< 0.001$) live cells and this cell death was induced more by necrosis than apoptosis. The low 0.5 mg/ml SFW extract dose induced more apoptosis than necrosis while the percentage of live cells was similar to that of untreated control cells. The aqueous extract doses had significantly higher live cells ($p< 0.001$) than CPT while this positive control induced significantly higher ($p< 0.001$) more apoptosis than the SFW extract doses (Table 1). A similar trend of effects was observed over the 48 hours incubation period as compared to 24 hours (Table 1).

Table 1: Apoptosis, necrosis and live T cells in samples treated with *Sutherlandia frutescens* (ethanol and water extract doses) over 24 (A) and 48 (B) hours incubation. SFE and SFW extracts induced necrotic cell death on normal T cells at low doses while higher doses had more viable cells. High doses of both extracts induced more apoptosis than necrosis over 48 hours of incubation. The effects of the SFE extract were independent of ethanol while CPT decreased cell viability more potently than both SFE and SFW extracts.

	24hrs			48hrs		
Sample type	**Apoptotic T cells (%+ stdev)**	**Necrotic T cells (%+ stdev)**	**Live T cells (%+ stdev)**	**Apoptotic T cells (%+ stdev)**	**Necrotic T cells (%+ stdev)**	**Live T cells (%+ stdev)**
0 Control	8.385±0.728	11.365±2.171	80.235±1.464	13.2±1.838	11.55±1.909	75.2±0.141
70% ethanol	7.445±1.732	9.06±2.036	83.405±0.431	11.65±2.051	12.605±0.997	75.695±1.124
20 µM CPT	16.8±3.960*	14.245±1.619	68.455±4.872	37.6±4.384**	19.85±1.061**	42.5±3.253
SFE 2.5 mg/ml	2.02±0.764	95.355±2.750**	2.61±2.008**	2.795±2.185	93.87±6.406**	3.275±4.306**
SFE 0.5 mg/ml	7.115±2.355	8.2±0.948	84.645±1.351	17.565±2.638	11.3±1.273	71.135±3.910
SFW 2.5 mg/ml	11.245±0.502	25.77±2.588*	62.985±3.090*	17.9±0.849	63.4±4.101**	18.7±3.252**
SFW 0.5 mg/ml	3.725±0.389	14.57±1.881	81.555±2.482	20.5±1.273	8.875±1.266	70.565±0.092

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(**- significant difference ($p< 0.001$) & *-slightly significant ($p< 0.01$) when compared to untreated control cells).

Both doses of the SFE extract significantly reduced the percentage of live cells when compared to the untreated control ($p< 0.001$). The high SFE extract dose (2.5 mg/ml) induced cell death by necrosis while more apoptosis occurred in the low dose (0.5 mg/ml) extract dilution. The 2.5 mg/ml SFW extract also induced more necrosis while the lower concentration (0.5 mg/ml) did not reduced cell viability significantly ($p> 0.05$) over 48 hours incubation when compared to the untreated control (Table 1). Overall, both types of SF extracts lowered the cell viability of normal T cells. Higher concentrations induced significant levels of necrosis while the lower doses induced cell death by apoptosis.

ATP assay

Following the first 24 hours of incubation, the SFE extract doses were shown to significantly reduce the ATP concentrations in treated normal T cells in comparison to untreated control T cells ($p< 0.001$) (Figure 1A). The effects of SFE extract doses were dependent on increasing ethanol doses ($p> 0.05$). The positive control, CPT, had a similar effect as the 2.5 mg/ml SFE extract while it was more effective in reducing ATP levels than the SFW extract doses (Figure 1A).

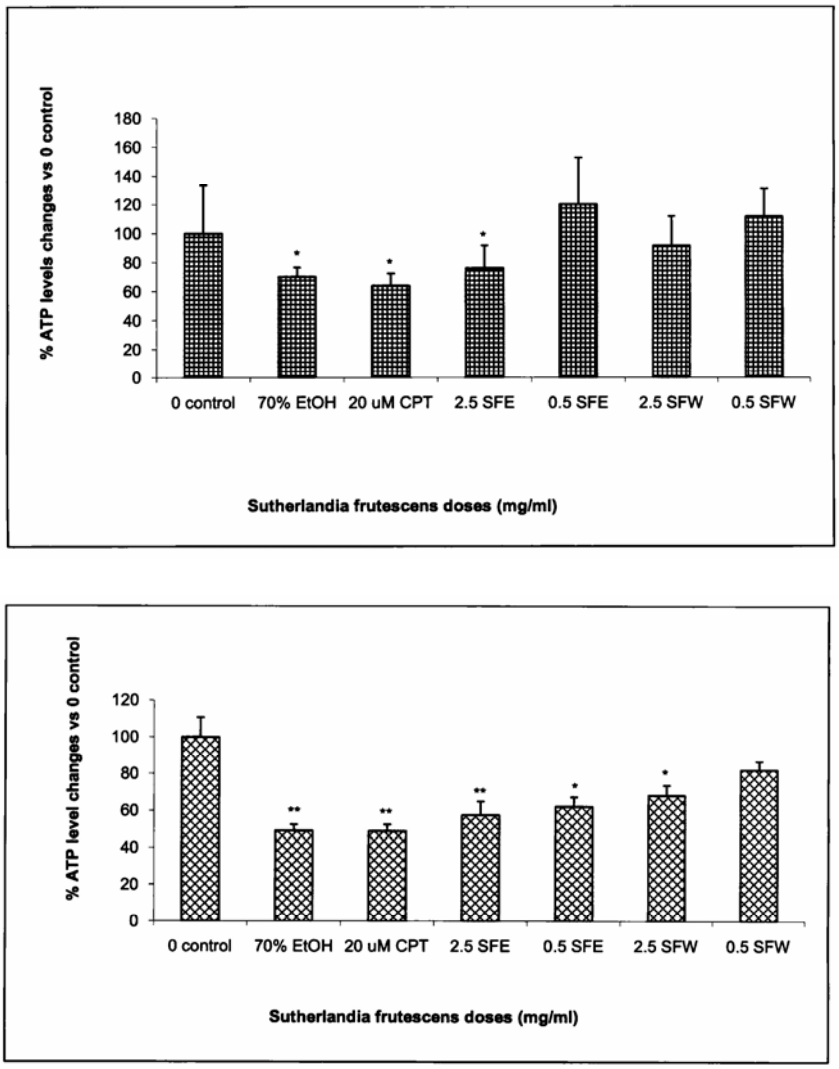

Figure 1: Illustration of the changes in ATP levels in *Sutherlandia frutescens* extract doses (SFE and SFW) treated samples after 24 (A) and 48 (B) hours incubations. The untreated control sample was standardised as a reference point (100%) for all the samples analysed. The high dose of SFE (2.5 mg/ml) significantly reduced ATP after 24 hours while there was no change in the low dose of SFE (0.5 mg/ml). SFW doses (2.5 & 0.5 mg/ml, respectively) did not change ATP levels after 24 hours (A). SFE doses further reduced ATP after 48 hours and this was dependent on ethanol at high doses. SFW doses decreased ATP levels after 48 hours but this effect was not as significant ($p> 0.05$) as the positive control, CPT (B). (**- significant difference ($p< 0.001$) & *-slightly significant ($p<0.01$) when compared to untreated control cells).

Over the 48-hour incubation period, all the extract doses (SFE and SFW) significantly reduced ATP levels in treated normal T cells when compared to the untreated control T cells ($p< 0.001$) (Figure 1B). A concentration dependent

trend was seen in the SFE extract doses, with the high dose (2.5 mg/ml) more effective in reducing ATP levels. In the SFW extract, both doses had comparable effectiveness with the 2.5 mg/ml dose slightly but not significantly more effective. The trend in the reduction of ATP levels in treated normal T cells over the 48 hours was consistent with the reduction in the number of live T cells measured by flow cytometry (Table 1).

Caspase-3/-7 assay

Since caspase-3/-7 activity assay targets enzyme activity, the range of extract dilutions was increased to assess the rate of activity with increasing concentrations. In isolated normal T-cells, the SF extracts showed a dose dependent decrease in caspase-3/-7 activity over 24 hours incubation. The inhibition was significant at higher doses for both extracts ($p< 0.001$). The vehicle control did not change caspase-3/-7 activity when compared to untreated normal T cells but was significantly different from SFE extract doses ($p< 0.001$). The 2.5 mg/ml deionised SFW extract doses significantly ($p< 0.01$) increased caspase-3/-7 activity (140%) in comparison to the untreated control. The positive inducer of apoptosis, CPT, slightly increased caspase-3/-7 activity but this was not significant (Figure 2A).

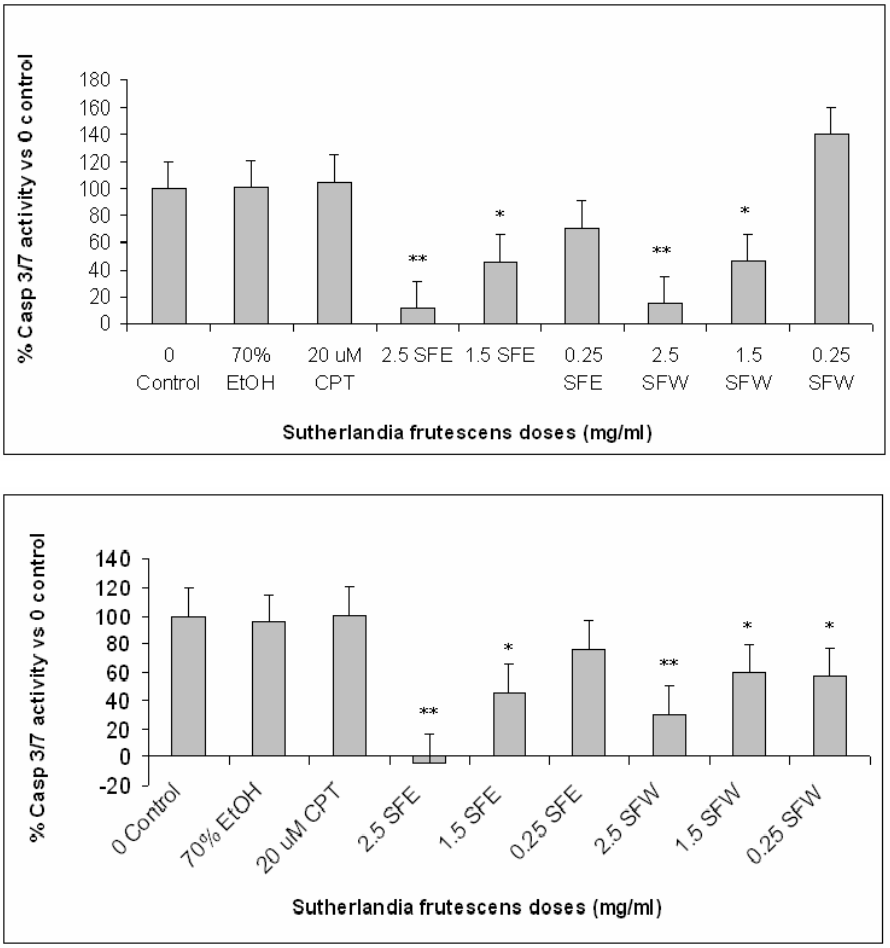

Figure 2: Graphical comparisons of changes in caspase-3/-7 activity of normal T cells after 24 (A) and 48 (B) hours incubation with *Sutherlandia frutescens* extract doses (SFE and SFW). The untreated control was again used as a reference (100%) for all the samples. SFE and SFW extract doses showed a concentration- and time-dependent decrease in decreasing caspase-3/-7 activity, with high doses being more effective. High doses of the SFE extract were more potent in reducing caspase-3/-7 activity over this period and their effect was independent of ethanol ($p< 0.001$). High doses of the SFW extract significantly reduced caspase-3/-7 activity while the positive control, CPT, did not change enzyme activity significantly over this period. (**- significant difference ($p<0.001$) & *-slightly significant ($p<0.01$) when compared to untreated control cells).

The decrease in caspase-3/-7 activity seen in normal T cells after 24 hours incubation with SF extract doses was either maintained or increased after 48 hours (Figure 2B). This trend was dose-dependent with high doses of the SFE extracts, which completely inhibited caspase-3/-7 activity ($p< 0.001$). The SFW extract doses also showed a dose-dependent trend in decreasing caspase-3/-7 activity, with the high dose (2.5 mg/ml) more effective (Figure 2B). Caspase-3/-7 activity was significantly decreased in T cells with decreased cell viability (necrotic) than those which were apoptotic and had decreased ATP levels (Table 1 and Figure 1A & B).

Nuclear staining

Untreated normal T cells (Figure 3A) stained with Hoechst generally displayed nuclei with homogenous chromatin staining, characteristic of viable cells. Some of the cells showed signs of early condensation while a few were already fragmented. Some normal T cells, which displayed dividing nuclei, could also be observed. Normal T cells treated with 20 µM CPT (Figure 3B) predominantly displayed condensed nuclei with bright blue-green staining, characterising an apoptotic process. This was consistent with the known effects of CPT: inducing apoptosis in suspension cells. Treatment of normal T cells with 70% ethanol induced more condensed nuclei than the untreated cells (Figure 3C).

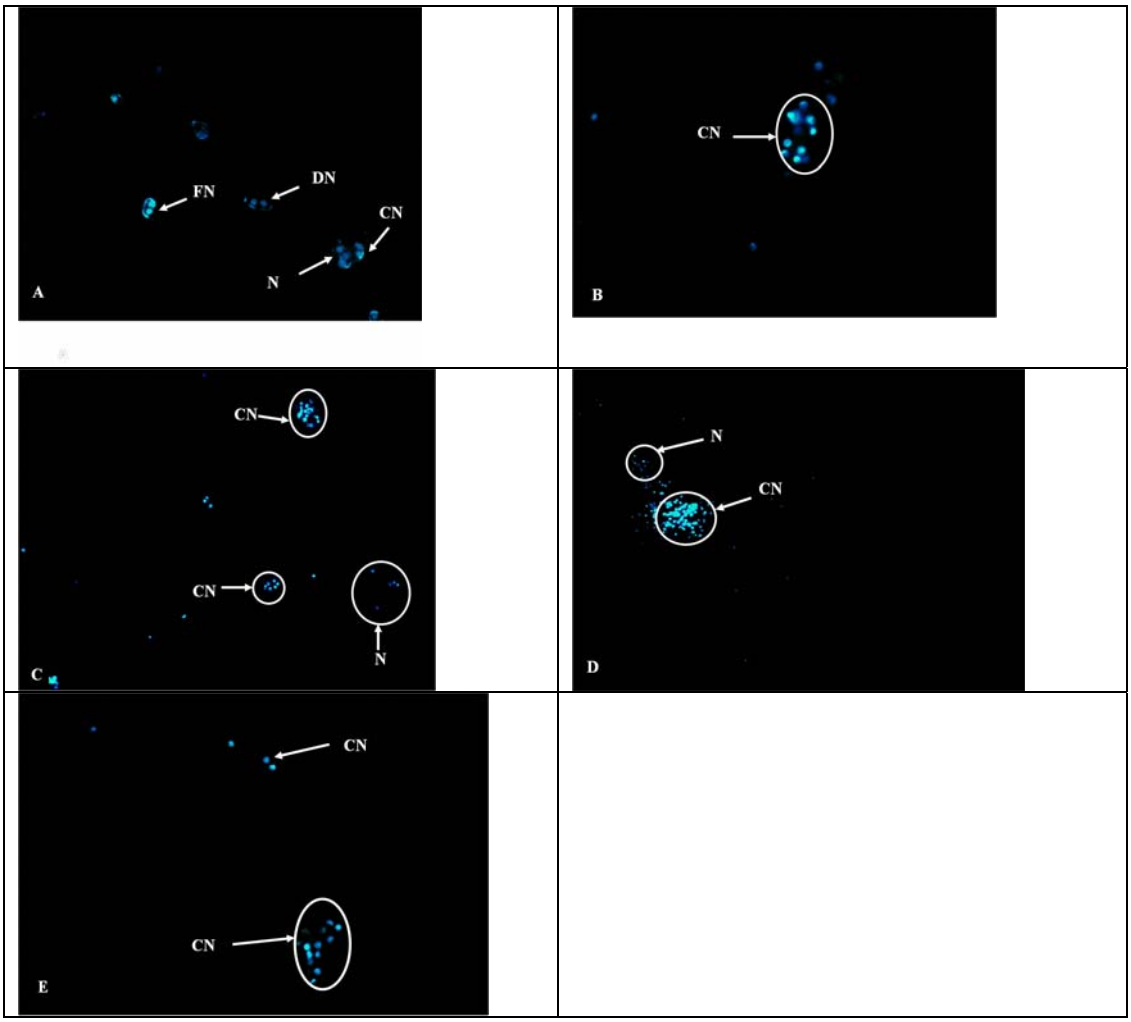

Figure 3. (A) Untreated control lymphocytes stained with Hoechst over 48 hours (600× magnification). Intact nucleus (N), dividing nucleus (DN) can be seen along some fragmented and condensed nuclei (FN and CN respectively). (B) Lymphocytes treated with 20 µM CPT and stained with Hoechst (600× magnification). Condensed nuclei (CN) can be seen indicating induction of cell death. (C) Micrographs of lymphocytes treated with 70% ethanol over 48 hours (200× magnification). Induction of cell cytotoxicity was indicated by appearance of condensed nuclei (N). (D) Micrographs of lymphocytes treated with SFW (1.5 mg/ml) over 48 hours and stained with Hoechst (200× magnification). The SFW extract induced some cytotoxicity as represented by condensed nuclei (CN) but normal nuclei (N) were present. (E) Micrographs of lymphocytes treated with SFE extract (1.5 mg/ml) over 48 hours and stained with Hoechst (400× magnification). The cytotoxicity of the SFE extract was shown by appearance of condensed nuclei (CN) after staining.

The effects of the SFW extract on normal T cells results in some nuclei showing bright blue-green staining, indicative of nuclei condensation (Figure 3D). This staining pattern suggests that cells may be undergoing some form of cell death, which is consistent with the flow cytometry results. These results demonstrated that SFW extract dilutions can induce apoptosis. The SFE extract also induced condensation and fragmentation of chromatin in normal T cells (Figure 3E). The observed effects are consistent with previous findings where 2.5 mg/ml SFE extract was shown to reduce cell viability below that of untreated controls in the flow cytometry assay.

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Discussion

The main purpose of this study was to assess the toxicity and immunomodulatory effects of SF extracts on normal T cells. Very few studies have associated the active ingredients of SF with cytotoxicity to normal, non-transformed cell lines. *Sutherlandia frutescens* extracts contain active ingredients such as L-canavanine, L-arginine, D-pinitol, GABA, and unique triterpenoid glucosides. L-canavanine is the most toxic component of SF extracts. Bence et al. (2002) associated L-canavanine and it metabolite L-canaline to cytotoxicity against immune cells *in vitro*. However, we did not isolate or measure this in our study. *Sutherlandia* extracts are used as is without separating into its components. Although Tai et al. (2004) confirmed the presence of 3 mg/g L-canavanine in commercially available tablets of SF, it is not clear how active these compounds are in presence of other components in the *Sutherlandia* extract. Accumulation of misfolded proteins in the endoplasmic reticulum (ER) contributes to ER stress and may activate both apoptosis and autophagy (Stander et al., 2009; Ding and Yin, 2008).

The data presented in this research study suggest that high doses of SF extracts are cytotoxic to normal T cells. This cytotoxicity occurs due to the induction of necrotic cell death, which results in depletion of cellular ATP, inhibition of caspase-3/-7 activity and induction of DNA fragmentation. These observations are consistent with the pathophysiology of cell death, demonstrable in the course of determining the cytotoxicity of a test compound *in vitro* (Tsujimoto, 1997). The type of cell death occurring in a particular cell line is dependent on the intracellular ATP content. Intracellular ATP levels measured during cell death were shown to remain constant until the very end of the apoptotic process. Intracellular energy levels are dissipated during necrosis but not in apoptotic cells (Leist et al., 1997). Therefore, the dose of the SF extract used, determined the extent and the effect response in cellular components. While the same test compound could induce apoptosis at lower concentrations, it could induce cell death by necrosis at higher concentrations. This is probably due to the collapse of cellular integrity, including plasma membrane disruption, a large calcium influx, as well as intracellular ATP depletion (Tsujimoto, 1997). This observation is true for the different doses of SF extracts, with higher doses inducing necrosis, while lower doses induced significant levels of apoptosis.

Effector caspases are activated at late stages of the apoptotic process, after mitochondrial outer membrane permeability (MOMP) disruption. In this case, caspase activation constitutes a sign, rather than a mechanism of cell death and the decisive event has occurred upstream or at the level of MOMP, which frequently marks the 'point of no return' of the lethal process. When inhibition of caspases does not restrain cell death, this often leads to a shift in the morphology of cell death from the aspects of classical apoptosis to the occurrence of apoptosis-like autophagic cell death or even necrosis (Kroemer and Martin, 2005). Therefore high concentrations of SF extracts dissipated ATP levels and inhibited caspase-3/-7, which did not restrain the cell death process but shifted it to necrosis. This is also the reason why there were T cells with fragmented and condensed nuclei under Hoechst 33342 staining. A recent study showed that morphological hallmarks of apoptosis were less prominent in human non-tumorigenic epithelial mammary gland cells (MCF-12A) after exposure to water extracts of SF (Stander et al., 2009). They concluded that SF extracts exerted a differential action mechanism in normal cells when compared to cancer cells. Normal T cells treated with high doses of SF extracts had increased necrotic activity than apoptosis.

Although a study on the SF effects on PBMCs has recently been published, the results were based on the effects of a single acute dose of SF extract on PBMCs over a shorter time of incubation (Korb et al., 2010). Korb et al., (2010) studied the effects of a SF extract over 12 hours on PBMCs while our study was over 48 hours on T lymphocytes alone. While their study concluded that the single high dose (7.5 mg/ml) induced apoptotic pathophysiology on PBMCs over 12 hours, our results showed that over a longer period high doses (2.5 mg/ml) of SF extract induced more necrotic cell death, depletion of ATP, inhibition of caspase-3/-7 activity and DNA fragmentation. Our longer time of incubation results are supported by those of Fernandes et al., (2004), demonstrating that the viability of human neutrophils did not change much after treatment with SF extract doses over an hour. All these studies, including our study, show that the observed results are dependent on the incubation periods over which the results are taken. We used longer incubation periods because *Sutherlandia frutescens* and other alternatives medicines are usually recommended for chronic use by patients with immune system deficiencies.

Based on the results of this study, the water extracts of SF may be safer to use than the 70% ethanol extracts. SF extracts are usually taken with water in its raw formulation. It is important to note the fundamental differences in the constituents of water (polar) and ethanol (polar and non-polar) extracts, as they may likely affect efficacy of the extract itself (Tai et al., 2004). Considerable caution should therefore be extended to traditional health practitioners who prescribe how doses of SF to patients for long term use.

Conclusion

Toxicity of SF extracts was dose and time-dependent, with SFE fractions being more potent in inducing cell death over 48 hours. Aqueous extracts are much safer to use as they were relatively less toxic *in vitro*. Further studies should be conducted on the effects of the SF tablets *in vivo*.

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