

## **The apoptosis inducing effects of *Sutherlandia* spp. extracts on an oesophageal cancer cell line**

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## **Abstract**

*Aim of study:* Oesophageal cancer is the ninth most common cancer in the world and the second most common cancer among South African men. It also has one of the lowest possibilities of cure, with the 5-year survival rate estimated to be only 10% overall. *Sutherlandia frutescens*, or the “cancer bush”, is a medicinal plant indigenous to southern Africa that is believed to have anti-cancer and anti-proliferative properties. The aim of this study was to investigate the potential apoptosis-inducing effects of two *S. frutescens* extracts and one *S. tomentosa* extract on the SNO oesophageal cancer cell line.

*Materials and Methods:* Cell viability and morphology of SNO cells were evaluated following exposure to the extracts. Apoptotic markers including cytochrome *c* translocation and phosphatidylserine externalisation was quantified by flow cytometry. The activity of caspase 3 and 7 was evaluated with spectrofluorometry. Apoptosis was evaluated in the presence of the pan-caspase inhibitor, Z-VAD-fmk. The effect of the extracts was compared to non-cancerous peripheral blood mononuclear cells (PBMCs).

*Results:* Time- and dose-response studies were conducted to establish treatment conditions of 2.5 and 5 mg/ml of crude plant extracts. Microscopy studies revealed that *S. frutescens*- and *S. tomentosa*-treated SNO cells had morphological features characteristic of apoptosis. Annexin V/propidium iodide flow cytometry confirmed that the extracts do, in fact, induce apoptosis in the SNO cells. Caspase inhibition studies seem to indicate that extracts A (*S. frutescens* (L.) R. Br. subsp. *microphylla* from Colesberg), B (*S. frutescens* (L.) R. Br. subsp. *microphylla* from Platvlei) and C (*S. tomentosa* Eckl. & Zeyh from Stil Bay) are able to induce caspase-dependent as well as –independent cell death. The *S. frutescens* and *S. tomentosa* extracts were found to be more cytotoxic to cancerous SNO cells when compared to the PBMCs.

*Conclusions:* *S. frutescens* and *S. tomentosa* extracts show promise as apoptosis-inducing anti-cancer agents.

## 1. Introduction

More than 80% of the world's population makes use of complementary and alternative medicines (Mainardi, 2009) which include herbalism and botanical medicines. Herbalism is the "medical use of preparations that contain exclusively plant material" (Ernst, 2003). Botanical medicines contain a number of active ingredients which have the same molecular targets as pharmaceutical drugs (Treasure, 2005). Thus herbal and botanical medicines represent an enormous medicinal resource at a fraction of the cost of conventional medicine. Many drugs used for the treatment of cancer have been discovered from medicinal plants (Balunas and Kinghorn, 2005). Vincristine, irinotecan, etoposide and paclitaxel are classic examples of plant-derived compounds that are used in cancer chemotherapy (Nobili *et al.*, 2009).

*Sutherlandia frutescens* (L.) R.Br. is a shrub indigenous to southern Africa. It has a long history of medicinal use by a number of cultural groups (including the Zulu, Xhosa, Sotho, Khoi-San and Cape Dutch) as a botanical medicine to treat ailments ranging from fevers, coughs and colds to peptic ulcers, dysentery and diabetes. It is locally referred to as the "cancer bush" because of its reported anti-cancer activity (reviewed by van Wyk and Albrecht, 2008). There are unpublished anecdotes of cancer patients who experienced an improved quality of life and survived for much longer than expected (and some that were apparently cured) after treatment with *Sutherlandia* (van Wyk and Albrecht, 2008). Furthermore, the use of *Sutherlandia tomentosa* as a medicinal plant is mentioned (van Wyk, 2008), who states that "both species of *Sutherlandia* have been used in traditional medicine but there are distinct local preferences for particular forms of *Sutherlandia frutescens*". However, these two plants belong to the same genus and are closely related. Because of this close taxonomical relationship (Moshe *et al.*, 1998), it seemed likely that *Sutherlandia tomentosa* (a plant also indigenous to South Africa, yet limited to the Cape coastal region) may also have anti-cancer activity and was included in this study.

The anti-tumour activity of *S. frutescens* was substantiated when it was found that methanolic extracts of *S. frutescens* inhibited 12-O-tetradecanoylphorbol-13-acetate-induced COX-2 expression in mouse skin (Kundu *et al.*, 2005). The toxicity of *Sutherlandia*

leaf powder has been studied in both adult vervet monkeys and humans and no toxic or other side effects were observed (Johnson *et al.*, 2007; Seieret *et al.*, 2002).

A number of studies have been performed to evaluate the effects of *S. frutescens* on different cell lines. Studies by Tai *et al.* (2004) and Stander *et al.* (2007) showed that ethanolic extracts of *S. frutescens* had concentration-dependent anti-proliferative effects on a variety of human tumour cell lines. Aqueous extracts of *S. frutescens* were found to inhibit the growth of MCF-7 cells (Steenkamp and Gouws, 2006) and induce cytotoxicity in cervical carcinoma (Caski) and Chinese hamster ovary (CHO) cells (Chinkwo, 2005).

It has been suggested that the vast catalogue of cancer cell genotypes is a manifestation of six essential alterations in cell physiology that collectively dictate malignant growth. One of these is the ability to evade apoptosis, a form of programmed cell death occurring in metazoans (Hanahan and Weinberg, 2000). This acquired resistance to apoptosis is a hallmark of most and perhaps all types of cancer. Therefore the search for compounds that can precisely activate or inhibit molecules that mediate the diverse forms of cell death, including apoptosis, is ongoing and ultimately aims to develop less toxic and more effective chemotherapeutic regimens. Apoptotic cells are typically characterised by morphological features such as cell shrinkage, chromatin condensation, blebbing of the cell membrane and the formation of apoptotic bodies (Kerr *et al.*, 1972), visible through microscopy. The biochemical features of apoptosis are considered to be the fragmentation of DNA in the cell (Cohen, 1997) and the externalisation of phosphatidylserine to the exterior of the cell membrane (Martin *et al.*, 1995).

A study by Chinkwo (2005) found that 3.5 mg/ml of aqueous *S. frutescens* extracts were able to induce apoptosis in CHO and Jurkat cells. CHO cells treated with *S. frutescens* extracts showed morphological features consistent with apoptosis. Phosphatidylserine externalisation and DNA fragmentation confirmed that *S. frutescens* extracts had induced apoptosis in the CHO cells. Flow cytometric analysis of *S. frutescens*-treated Jurkat cells revealed that more than 84% were apoptotic (Chinkwo, 2005). Stander *et al.* (2007) also observed morphological characteristics typical of apoptosis in MCF-7 cells treated with 1.5 mg/ml ethanolic *S. frutescens* extracts. A gene expression profile revealed that genes

thought to be involved in apoptosis, growth inhibition and NF- $\kappa$ B signalling were differentially expressed in *S. frutescens*-treated and vehicle control-treated cells. Further studies by Stander *et al.* (2009) showed that aqueous extracts of *S. frutescens* at 5 and 10 mg/ml were more cytotoxic to cancerous (MCF-7) than non-cancerous (MCF-12A) breast cell lines. These extracts activated both autophagic and apoptotic processes in MCF-7 cells.

Although previous studies have investigated the effects of *S. frutescens* extracts on various cell lines, no studies have involved an oesophageal cancer cell line. In South Africa, oesophageal cancer is the second most common cancer in men and one of the more challenging cancers to treat. The 5-year survival rate of oesophageal cancer sufferers is estimated to be only 10% (Johnstone, 2000). Conventional treatment of oesophageal cancer includes one, or a combination of, surgery, radiation therapy and chemotherapy. These treatments, however, have high rates of morbidity and mortality. In patients with resectable oesophageal cancer, surgery is the treatment of choice (Forshaw *et al.*, 2005; van Meerten and van der Gaast, 2005). However, patients tend to live only 12 to 18 months after the surgery and the 5-year survival rate seldom exceeds 25% (Johnstone, 2000). Furthermore it is estimated that, within a year of surgery, the disease will have recurred in almost half of the patients (Forshaw *et al.*, 2005). The 5 year survival rates of patients with oesophageal cancer treated with only radiation therapy has been found to be below 5% and there is a 77% local recurrence rate. A combination of chemotherapy and radiation therapy improves the 5 year survival rate to between 9 and 25% (Neuner, 2009). It is therefore important to determine what effects *Sutherlandia* spp. extracts have on oesophageal cancer cells. This study examined the potential apoptosis-inducing effects of three different *Sutherlandia* spp. extracts on a South African established oesophageal cancer cell line, the SNO cell line.

## 2. Materials and Methods

### 2.1 Materials

Hank's balanced salt solution (HBSS), Dulbecco's modified Eagle's medium (DMEM), foetal bovine serum (FBS), trypsin/versene, penicillin/streptomycin/fungizone cocktail and gentamycin were supplied by Highveld Biologicals (Lyndhurst, South Africa). Z-VAD-fmk, dimethyl sulfoxide (DMSO), cycloheximide (CHX), hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), Histopaque® and 70% ethanol were supplied by Sigma Aldrich (Steinheim, Germany).

### 2.2 Cell culture

SNO (ATCC, cat no. CCL-185) oesophageal cancer cells, an adherent cell line, were cultured in DMEM supplemented with 10% foetal bovine serum, 1% penicillin/streptomycin/fungizone cocktail and 0.5% gentamycin. The cells were maintained in a humidified atmosphere at 37°C with 5% CO<sub>2</sub>.

### 2.3 Preparation of crude extracts of *Sutherlandia frutescens* and *S. tomentosa*

The *Sutherlandia* plants used in this study (summarised in Table 1) were kindly donated by Professor Ben-Erik van Wyk from the Department of Botany and Plant Biotechnology at the University of Johannesburg. Extracts were annotated as follows: extract A (*S. frutescens* (L.) R. Br. subsp. *microphylla* from Colesberg, South Africa); B (*S. frutescens* (L.) R. Br. subsp. *microphylla* from Platvlei, South Africa) and C (*S. tomentosa* Eckl. & Zeyh from Stil Bay, South Africa). Due to the apparent chemical, genetic and geographical variation, *Sutherlandia frutescens* is divided into three subspecies and several regional forms (van Wyk and Albrecht, 2008), and plants from different geographical localities were thus included in this study.

The available plant material was dried in an oven at 30°C overnight, finely ground to a powder and autoclaved. Solutions of 100 mg/ml in 70% ethanol were prepared and allowed to extract for 24 h. The supernatant was then removed and filtered through a 0.2 µm filter. The ethanol was then evaporated off and the sticky residue was re-suspended in sterile

water to make stock solutions of 100 mg/ml which were again filtered through a 0.2 µm filter.

#### *2.4 Treatment of SNO cells with Sutherlandia spp. extracts*

SNO cells were seeded at a concentration of  $6 \times 10^5$  cells per cell culture dish (3 cm diameter; Corning Inc, NY), (final volume of 1 ml) and treated with 2.5 or 5 mg/ml of extract A, B or C (see Table 1) for 24 h. In each case vehicle controls, necrotic control (10% H<sub>2</sub>O<sub>2</sub>) and/or apoptotic control (0.5 mM CHX) was included. The cells were then removed from the plates by trypsinisation, re-suspended in 1 ml of fresh media, and used for various assays.

#### *2.5 AlamarBlue® viability assay*

From each of the harvested cell suspensions 100 µl was transferred to the wells of a 96-well plate containing 10 µl of alamarBlue® (AbDSerotec, Kidlington, UK). The plate was incubated in the dark for 2 h at 37°C. The fluorescence was measured at an excitation wavelength of 544 nm and emission wavelength of 590 nm on the Synergy™ HT multi-detection microplate reader (Biotek®, Vermont, USA).

#### *2.6 ATP levels as an indication of cell viability*

The CellTiter-Glo® viability assay (Promega, Madison, USA) was used to determine the amount of adenosine triphosphate (ATP) present in the cell sample, which is indicative of the mitochondrial activity. Fifty µl of the harvested cell suspensions were placed in the wells of a 96-well plate and 50 µl of the reconstituted CellTiter-Glo® substrate in buffer was added. The plate was agitated for 2 min and incubated at room temperature for 10 min in the dark. The ATP levels of the cells were then quantified via luminescence detection on the Synergy™ HT multi-detection microplate reader (Biotek®, Vermont, USA).

#### *2.7 Morphological studies*

Treated cells, still attached to their plates, were examined under the AxioCam MR™ colour light microscope (Carl Zeiss, Jena, Germany) at a 400 X magnification to study the morphology of the cells. Micrographs of the cells were taken using AxioVison™ software.

### *2.8 AnnexinV-FITC/propidium iodide flow cytometric analysis*

This assay is based on the fact that apoptotic cells have exposed phosphatidylserine molecules (Fadok *et al.*, 1992) and thus bind annexin V, while necrotic cells have compromised membranes and thus take up propidium iodide (Vermes *et al.*, 1995). Four different populations of cells are easily distinguished: those that are unlabelled (viable cells), those that have bound annexin V-FITC only (early apoptotic), those that have been stained with propidium iodide (necrotic) and those that have both bound annexin V and been labelled with propidium iodide (late apoptotic/necrotic cells).

After the treated cells were harvested, they were washed with 1 ml cold PBS and re-suspended in 100 µl of 1x'binding' buffer and placed into flow tubes. The cells were labelled with FITC-conjugated annexin V and propidium iodide (PI) according to the manufacturer's instructions of the Annexin V-FITC Apoptosis Detection Kit (BD Biosciences, San Diego, USA). The contents of the tubes were gently mixed and incubated in the dark for 15 min before 400 µl of the binding buffer was added.

The cell samples were then analysed on the FACS Aria™ Flow Cytometer (BD Biosciences, San Diego, USA). Propidium iodide signal (emitted at 617 nm) was read on the PE-A channel while the annexin V-FITC signal (emitted at 530 nm) was read on the FITC-A channel. The data was obtained and analysed using BD FACSDiva™ 6.0 software.

### *2.9 Cytochrome c release assay*

The InnoCyte™ Flow Cytometric Cytochrome C Release Kit (Calbiochem®, San Diego, USA) was used to assess the release of cytochrome c from the mitochondria into the cytoplasm, which would suggest that the intrinsic apoptotic pathway had been activated. The cell samples were analysed on the FACS Aria™ Flow Cytometer. The data was obtained and analysed using FACSDiva™ 6.0 software.

### *2.10 Caspase 3/7 Assay*

The Caspase-Glo® 3/7 (Promega, Madison, USA) assay was used to determine the levels of active caspases 3 and/or 7 in order to ascertain whether the mode of cell death induced by the plant extracts was caspase-dependent. Caspases 3 and 7 are both executioner caspases



and their activation is considered a biochemical hallmark of apoptosis (Denault and Salvesen, 2002; Kroemer *et al.*, 2009; Zimmermann *et al.*, 2001).

In a 96 well plate, 50  $\mu$ l of the Caspase-Glo<sup>®</sup> solution was added to 50 $\mu$ l of cells and agitated for 30 seconds. The plate was then incubated for 1 h at 37°C, in the dark. The luminescence was then read on the Synergy™ HT multi-detection microplate reader.

### *2.11 Caspase inhibition studies*

To further verify whether the treatments with plant extracts induce caspase-dependent apoptosis, Z-VAD-fmk, a pan-caspase inhibitor, was added to the cells prior to the different treatments. SNO cells were pre-treated with 20  $\mu$ M Z-VAD-fmk (in DMSO) to inhibit caspases. After 1 h the cells were treated with the extracts for 24 h as previously described. The cells were then studied by light microscopy and their viability and caspase 3/7 levels were determined as described above. A DMSO vehicle control was also included for these experiments.

### *2.12 Isolation and treatment of peripheral blood mononucleocyte cells (PBMCs)*

After the effects of the various extracts had been studied on a cancerous cell line, it was important to establish what effects they have on non-cancerous cells. For this study peripheral blood mononuclear cells (PBMCs), isolated from whole blood, were used as a control non-cancerous cell line. Whole blood, from a volunteer, was collected into three 3 ml anticoagulant tubes containing EDTA. The whole blood was diluted four times with PBS, poured onto 20 ml of Histopaque and centrifuged at 400  $xg$  for 30 min. The upper serum layer was removed and discarded. The opaque PBMC layer was transferred to a new 50 ml tube. The tube was filled to 45 ml with DMEM, supplemented with 10% FBS and 1% penicillin/streptomycin/fungizone cocktail, and gently inverted. The solution was then centrifuged at 600  $xg$  for 10 min. The supernatant was washed with 5 ml of supplemented DMEM and centrifuged at 300  $xg$  for 10 min. The pellet was then re-suspended in 2 – 3 ml of supplemented DMEM. The PBMCs were plated in cell culture dishes at a concentration of  $1 \times 10^6$  cells per plate and allowed to adhere for 1 h. The spent media was discarded and fresh, supplemented media was added to the cells. The cells were then treated with extracts

A, B or C (at 2.5 or 5.0 mg/ml) or 10% H<sub>2</sub>O<sub>2</sub> (necrotic control) or 0.5 mM CHX (apoptotic control) for 24 h.

### *2.13 Cell viability of treated PBMCs*

Following the 24 h treatment, the PBMCs were removed from their plates by trypsinisation. Into the wells of a 96-well plate 100 µl of the harvested cell suspension was added, followed by 10µl of alamarBlue®. The plate was incubated in the dark for 2 h at 37°C. The fluorescence of each well was read at an excitation wavelength of 544 nm and emission wavelength of 590nm on the Synergy™HT multi-detection microplate reader.

### *2.14 Statistical analyses*

Three biological repeats and two technical repeats of the various assays were performed. To determine whether there were significant differences between the treated and untreated cells, the two-tailed Student's *t*-test was performed in Microsoft Excel. If the probability value (*P*-value) was less than 0.05, it was concluded that the values for the treated cells were significantly different with respect to the untreated control cells. The error bars on the bar graphs were used to represent the standard error of the mean (SEM) for each condition.

### **3. Results**

#### *3.1 Concentration and time trials*

In order to determine the optimal treatment conditions, concentration- and time-response studies were initially conducted in a preliminary study. SNO cells were treated with extracts A, B and C at concentrations of between 0.5 and 10 mg/ml for 3, 24 or 48 h. Vehicle, necrotic (10% H<sub>2</sub>O<sub>2</sub>) and apoptotic (0.5 mM CHX) controls were included. From these dose- and time-response studies (results not shown), the 24 h treatment with extracts A, B and C at concentrations of 2.5 and 5 mg/ml was elected for further analysis, based on the significant differences observed in the results when compared to each other as well as the untreated and vehicle controls.

Treatment with extracts B and C resulted in a large reduction in cell viability, similar to the result seen for those cells treated with CHX (Fig 1). Extract A, on the other hand, did not significantly affect the cell viability at 2.5 mg/ml, while at 5 mg/ml the viability was only slightly reduced. These conditions were used in subsequent experiments since almost all the extracts were able to significantly reduce the SNO cell viability. In all cases, the vehicle controls had no statistically significant effect on the cells when compared to the untreated controls, and are henceforth not included in the results. In order to affirm the data obtained with the alamarBlue® assay, the ATP assay was performed.

#### *3.2 ATP assay*

The amount of ATP present in the differentially treated-cells was measured, which is an indicator of cellular metabolic activity (Los et al., 2001) and thus an indirect measure of cell death. The results of this assay (Fig. 2) revealed that the levels of ATP in SNO cells treated with any extract, at either 2.5 or 5 mg/ml, were extremely low relative to the untreated control cells. Of the extracts, extract B resulted in the largest reduction in ATP and extract C, the smallest reduction in ATP levels. H<sub>2</sub>O<sub>2</sub>-treated cells had the lowest ATP levels overall.

#### *3.3 Changes in morphology following treatment with extracts*

The treated cells were examined by inverted light microscopy at 400 X magnification in order to observe the effects the various treatments had on their morphology. The

morphology of normal SNO cells is irregular and spindly-shaped. These cells were quite closely packed and adhered strongly to the dishes in which they were cultured (Fig. 3 A).

Cells treated with a necrotic inducer, H<sub>2</sub>O<sub>2</sub>, showed morphological features consistent with necrosis, including swelling and bursting of the cell as well as leakage of cell debris (Fig. 3 B). SNO cells treated with CHX, an apoptotic inducer, displayed morphological features indicative of apoptosis (Fig. 3 C). They had detached from the culture dish and become spherical. Their cell membranes showed signs of blebbing and apoptotic bodies were also evident.

Many of the SNO cells treated with extracts A, B and C (Fig. 3 D – I) had morphological features similar to those cells treated with CHX. Extract A-treated cells had detached from their culture dish and become condensed. Membrane blebbing was also seen in these cells and they resembled the CHX-treated cells. Cells treated with 2.5 mg/ml of extract B were also morphologically similar to the CHX-treated cells. When cells were treated with 5 mg/ml of extract B, the cells appeared quite swollen and were comparable to the H<sub>2</sub>O<sub>2</sub>-treated cells. Extract C-treated cells detached from their culture dishes and appeared condensed although no clear membrane blebbing was seen. At the higher concentration of extract C much cell debris was evident and some swollen necrotic-looking cells were seen.

#### *3.4 The externalisation of phosphatidylserine*

Since the morphological studies indicated that the extracts appeared to induce apoptosis in the SNO cells, the annexin V-FITC/propidium iodide flow cytometric assay was performed to confirm this and the results are summarised in Table 2. As expected, the majority (94%) of the untreated control cells were found to be viable. Treatment of the SNO cells with the various extracts tended to shift the cell population from viable to apoptotic. Approximately 50% of extract A-treated cells were viable; at 2.5 mg/ml 36% and 13% were in the early and late stages of apoptosis respectively. When the concentration of extract A was doubled there was a shift from early apoptosis into the late stage of apoptosis (21% each). The majority of cells (66 and 74%) treated with extract B, at 2.5 and 5 mg/ml respectively, were found to be in the late stages of apoptosis. Extract C-treated cells were more or less evenly distributed between the viable, early apoptotic and late apoptotic quadrants. This

confirmed that the extract-treated cells were in fact undergoing apoptosis as they showed similar results to the CHX-treated cells, which were 39% early apoptotic and 46% late apoptotic/necrotic. Cells treated with H<sub>2</sub>O<sub>2</sub> were either necrotic (32%) or late apoptotic/necrotic (41%).

### *3.5 The release of cytochrome c from the mitochondria*

The cytochrome *c* levels in the mitochondria were measured to determine whether or not the intrinsic apoptotic pathway could have been induced by the extracts. Cells treated with 2.5 and 5 mg/ml of extract B and 5 mg/ml of extract C had significantly lower levels of cytochrome *c* compared to the untreated control cells, as did those cells treated with H<sub>2</sub>O<sub>2</sub> (Fig. 4). SNO cells treated with the apoptotic inducer (CHX) did not have significantly lower levels of cytochrome *c* in the mitochondria. This was also true of cells treated with extract A (at 2.5 and 5 mg/ml) and extract C (at 2.5 mg/ml).

### *3.6 Caspase 3/7 levels*

To further confirm the cell death pathway induced by the extracts, the levels of active caspase 3/7 were measured. The SNO cells treated with CHX had significantly higher levels of caspase 3/7. SNO cells treated with extract A, at both concentrations, also had significantly increased caspase 3/7 levels (Fig. 5). In contrast, extract B reduced the caspase 3/7 levels significantly at 5 mg/ml. At both concentrations of extract C the SNO cells showed increased levels of active caspase 3/7 compared to the untreated control but these levels were statistically insignificant.

### *3.7 Caspase inhibition studies*

To verify whether cell death was in fact caspase-dependent, the well-known pan-caspase inhibitor, Z-VAD-fmk, was added to the SNO cells prior to the 24 h treatment with the extracts. The caspase 3/7 levels were then measured to confirm the caspase inhibition. The cell viability was determined via alamarBlue® assay and the cell morphology was visualised.

Overall, the caspase inhibitor was able to significantly reduce the amount of caspase 3/7 present in the SNO cells irrespective of treatment (Fig. 6). In spite of the decreased levels of executioner caspases, the alamarBlue® results (Fig. 7) show that the cell viability was

significantly decreased following treatment with the extracts with the exception of 2.5 mg/ml extract C and CHX. Thus the extracts were still able to induce cell death despite the caspase inhibition.

The morphology of the SNO cells following treatment with the extracts in the presence of the pan caspase inhibitor, Z-VAD-fmk, was also studied by light microscopy. The cells seen in Fig 8, pre-treated with Z-VAD-fmk, were dissimilar to those cells depicted in Fig. 3. The untreated control cells, and the DMSO control cells, have typical SNO cell morphology. They are closely-packed and spindly-shaped (Fig. 8 A). SNO cells treated with extracts A, B or C in the presence of Z-VAD-fmk (fig 8 D – I), especially at 5 mg/ml, resemble the Z-VAD-fmk pre-treated CHX-treated SNO cells (fig 8 C). Growth appears inhibited, although many of the cells were still attached to their culture dishes. A number of cells have irregular cell membranes and signs of blebbing were apparent.

### *3.8 Effect of extracts on PBMCs*

When non-cancerous PBMCs were treated with the extracts, significant reductions in cell viability were only observed in those cells treated with 5 mg/ml of extract B or C. Although 2.5 mg/ml of extract B also caused quite a large reduction in cell viability, it was statistically insignificant. Treatment of PBMCs with H<sub>2</sub>O<sub>2</sub> and CHX, however, resulted in significantly decreased cell viability as expected. When comparing the viability of SNO cells and PBMCs treated with the various extracts, it was found that the PBMCs all had higher viabilities than the SNO cells, with the exception of the 2.5 mg/ml extract A treatment, where the relative levels of viability were similar.

Typical PBMCs are rounded, spherical and adherent. Such morphology was observed in the microscopic examination of the untreated control cells (not shown). PBMCs treated with extracts A and C were morphologically similar to the untreated control cells while those treated with extract B morphologically resembled the PBMCs treated with H<sub>2</sub>O<sub>2</sub>. The PBMCs treated with CHX did not have typical apoptotic morphology and instead looked similar to the untreated control cells.

## DISCUSSION

*S. frutescens* has been used as a traditional medicine by a number of different cultural groups to treat a wide variety of diseases. *S. frutescens* has become known as the “cancer bush” due to its reported use by the Khoi-San and Cape Dutch people for the treatment and prevention of internal cancers (van Wyk and Albrecht, 2008). However, little is known about how the plant exerts its anti-cancer activity. The aim of this study was to investigate the potential apoptosis-inducing effects of three different *Sutherlandia* spp. extracts on the SNO oesophageal cancer cell line.

The first phase of this study involved the selection of treatment conditions for the SNO cell line. In order to determine these, time and dose-response trials were initially conducted. Based on these studies it was decided that extracts A, B and C at concentrations of 2.5 and 5 mg/ml would be used for the remainder of the study. Under these conditions almost all the extracts are able to produce a significant decrease in cell viability. These concentrations are similar to the concentrations of *S. frutescens* used in previous studies (Tai *et al.*, 2004; Chinkwo, 2005; Stander *et al.*, 2007; Stander *et al.*, 2009; Korb *et al.*, 2010 and Phulkkdarre, *et al.* 2010). The ATP assay supported that the extracts do in fact significantly decrease the cell viability as detected by alamarBlue® at concentrations of 2.5 and 5 mg/ml (Fig. 1 and 2).

Once the treatment conditions were established the effects of these treatments on the morphology of SNO cells was investigated. The results showed that the morphology of the extract-treated SNO cells was markedly different from that of the untreated cells (Fig. 3) and was most obvious in cells treated with extracts B and C and the high concentration of extract A. Based on the morphological features observed, including cell shrinkage, spherical cell shape and plasma membrane blebbing, the treated cells can be classified as apoptotic (Kroemer *et al.*, 2009). These findings are consistent with previous studies which have also found morphological characteristics of apoptosis following *S. frutescens* treatments of various cell lines (Chinkwo, 2005 and Stander *et al.*, 2007).

Since the morphological studies indicated that the extracts may induce apoptosis in the SNO cells, a number of other apoptotic markers were also examined in order to confirm this

supposition. The exposure of phosphatidylserine on the external surface of the plasma membrane was one such marker (Fadok *et al.*, 1992). The annexin V- propidium iodide flow cytometric assay showed that apoptosis was, in fact, the dominant mode of cell death induced by extracts A, B and C (Table 2).

Apoptosis can follow either the intrinsic or extrinsic pathway. To investigate whether the intrinsic, or mitochondrial, apoptotic pathway was triggered by the extracts the mitochondrial cytochrome *c* levels were studied. It was found that the cytochrome *c* levels in the mitochondria were only significantly reduced for only a few treatments (Fig. 4). CHX, an apoptotic inducer, treatment did not cause a decrease in the mitochondrial cytochrome *c* levels of the SNO cells. This is because CHX-mediated apoptosis occurs through the extrinsic pathway which involves a FADD-dependent mechanism (Tang *et al.*, 1999). Thus extract A and lower concentrations of extract C may also induce apoptosis via the extrinsic pathway. Alternatively according to Hengartner (2000), the release of cytochrome *c* from the mitochondria is sometimes a very late apoptotic event. The cells treated with extract B were mostly late apoptotic (see Table 2) while the cells treated with extracts A or C were mostly early apoptotic, which may account for the reduction in cytochrome *c* in cells treated with extract B and not with extracts A and C.

The levels of active caspase 3/7 were determined to ascertain whether the mode of cell death induced by the extracts was caspase-dependent. Caspases 3 and 7 are both executioner caspases and their activation is considered a biochemical hallmark of apoptosis (Denault and Salvesen, 2002; Kroemer *et al.*, 2009; Zimmermann *et al.*, 2001). Extract A was able to increase the levels of active caspase 3/7, which indicates that caspase-dependent cell death is induced in the SNO cells by extract A. Extract B caused the caspase levels to decrease significantly and extract C caused an increase in caspase 3/7 levels which was not found to be statistically significant (Fig. 5). The decrease in caspase 3/7 levels in SNO cells treated with extract B may be explained by the fact that many of these cells are in the late stages of apoptosis, also known as secondary necrosis (Table 2). In secondary necrosis many of the cells' proteins, including caspases, become degraded. Thus it may well be that extract B increased caspase 3/7 activity during the early stages of apoptosis and the caspases were subsequently degraded during the later stages of apoptosis. An alternative explanation may



be that extract B induces a caspase-independent form of cell death. The caspase 3/7 levels of the SNO cells treated with extract C were not significantly increased which may indicate that extract C also induces a caspase-independent form of cell death.

In order to verify whether the mode of cell death induced by the extracts was caspase-dependent, the treatments were carried out in the presence of a caspase inhibitor. The pan-caspase inhibitor Z-VAD-fmk was added to the cells before treatment with the extracts and was able to inhibit caspase 3/7 activity (Fig. 6). Despite this inhibition, the extracts were still able to significantly reduce the SNO cell viability (Fig. 7). The viability of the SNO cells treated with CHX or *Sutherlandia* spp. extracts in the presence of Z-VAD-fmk tended to be lower than in the absence of the pan-caspase inhibitor (Fig. 7 vs. Fig. 1). The morphology of the SNO cells treated with the extracts in the presence and absence of the caspase inhibitor was also quite different (Fig. 8 vs. Fig. 3). This indicates that the type of cell death seen in Fig. 8 is different from the type of cell death seen in Fig. 3.

Taken together, these results show that even if the caspases are inhibited, the extracts are still able to induce cell death. This observation is by no means a novel one. According to Chipuk and Green (2005) caspase inhibition tends to hinder cell death but does not prevent it. Lemaire *et al.* (1998) suggested that the inhibition of caspases causes the mode of cell death to switch from apoptosis to necrosis. According to Leist and Jäätelä (2001), the inhibition of caspases may cause caspase-independent forms of cell death to be revealed. Caspase-independent cell death is defined by Chipuk and Green (2005) as a form of cell suicide induced by pro-apoptotic conditions that occurs in the absence of caspase activity and is morphologically different from apoptosis. Studies by Cummings and Schnellmann (2002; 2004) involving caspase inhibition by Z-VAD-fmk found that significant fractions of apoptosis induced by diverse toxicants in renal epithelial cells and in four different cancer cell lines (Caki-1, A549, A172 and L1210) is actually caspase-independent. In all probability, caspase-independent cell death pathways have the same function as apoptosis and are important defence mechanisms should the apoptotic pathway be evaded (Brökeret *al.*, 2005; Leist and Jäätelä, 2001). Thus the *Sutherlandia* spp. extracts seem to induce caspase-independent cell death.

Once the effect of the extracts on the SNO oesophageal cancer cell line was established, it was important to determine what effects the extracts had on non-cancerous cells. It was found that the extracts were less cytotoxic to the non-cancerous PBMCs than to the cancerous SNO cells. Most of the PBMCs cells treated with extracts A and C had normal, healthy morphology (results not shown). These extracts did not significantly reduce the cell viability (Fig. 9). The PBMCs that were treated with extract B, however, showed morphological features consistent with apoptosis as well as necrosis. Although extract B caused significant cell death of the PBMCs, it was still less cytotoxic to the PBMCs than to the SNO cells. Interestingly, the differential effects of the *S. frutescens* extracts on normal and cancerous cells were also observed by Stander *et al.* (2009). This group found that 10 mg/ml of ethanolic *S. frutescens* extract was more lethal to tumorigenic MCF-7 cells than to non-tumorigenic MCF-12A cells.

In conclusion *S. frutescens* and *S. tomentosa* extracts were found to have greater cytotoxic effects in cancerous SNO cells than in non-cancerous PBMCs. The mode of cell death induced by extracts A, B and C in SNO cells appears to have many features associated with apoptosis. These include apoptotic morphology, externalisation of phosphatidylserine, cytochrome *c* release (for extracts B and C) and caspase 3/7 activation (for extract A). However, since the extracts are still able to induce cell death when in the presence of a caspase inhibitor, it suggests that the extracts may also induce a caspase-independent programmed cell death in SNO cells. This study shows that *Sutherlandia* spp. extracts may be a promising alternative chemotherapeutic cancer treatment which induces apoptosis in cancer cells while exerting minimal cytotoxic effects in normal cells. Future studies would include the investigation of biochemical and molecular mechanisms induced by the individual active compounds of *S. frutescens* and *S. tomentosa* on the SNO cells in comparison to their synergist properties.

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## **Declaration of Interest**

The authors of this study have no conflicts of interest or any financial disclosures to make.

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## Tables

**Table 1**

The species of *Sutherlandia* (tribe Galegeae, family Fabaceae) used in the study and the dates and locations of collection in the Western Cape region of South Africa.

Extract	Voucher Specimen	Species	Plant parts	Date collected	Location collected
A	RAU 11	<i>S. frutescens</i> (L.) R.Br. subsp. <i>microphylla</i>	Leaves Stems	03/02/2009	Colesberg
B	RAU 7	<i>S. frutescens</i> (L.) R.Br. subsp. <i>microphylla</i>	Leaves Stems	15/11/2000	Platvlei
C	Van Wyk 3669	<i>S. tomentosa</i> Eckl.&Zeyh.	Leaves *	1996	Stil Bay

\* Stems not available



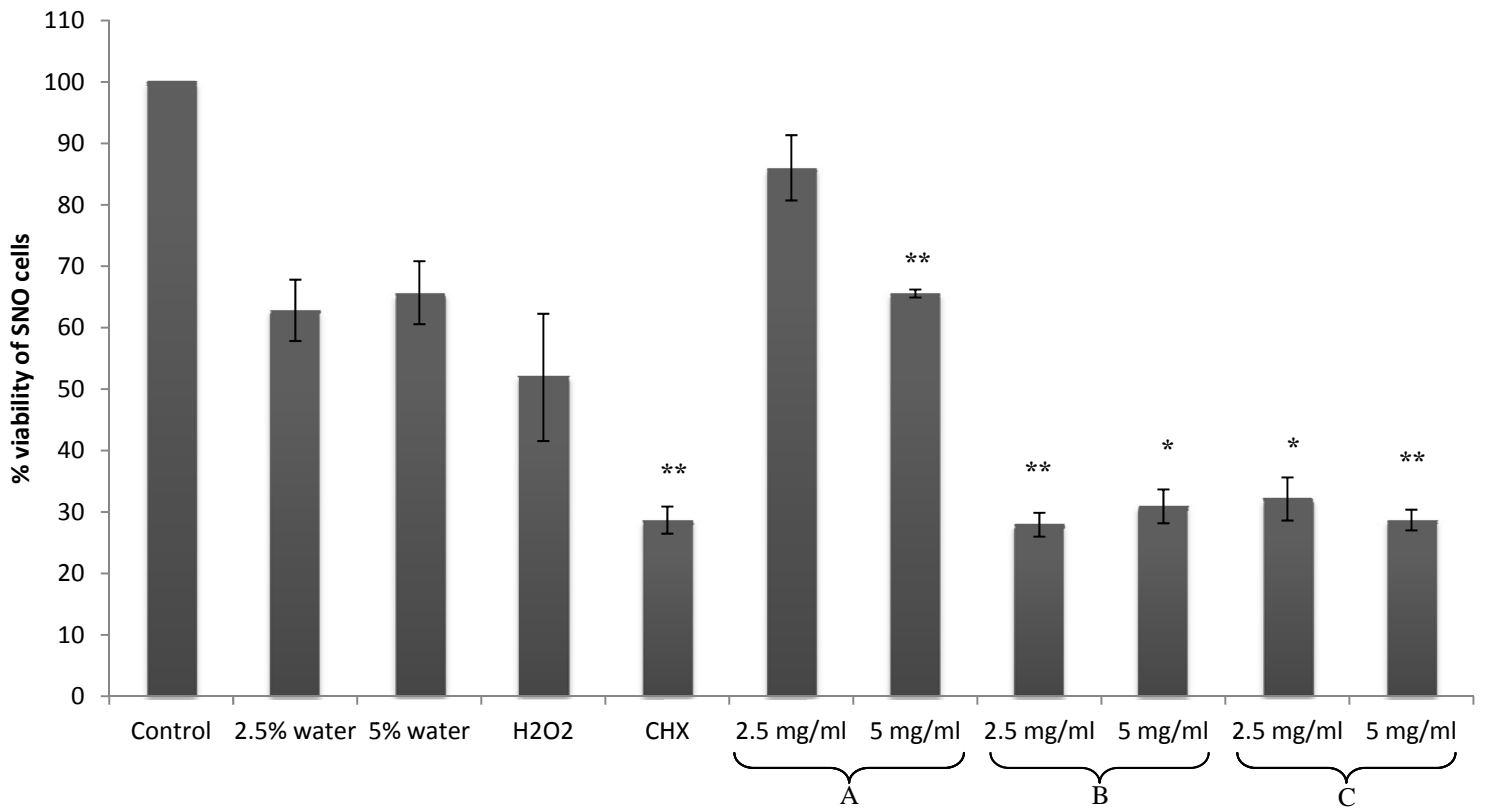
**Table 2**

The percentage of the SNO cell population  $\pm$  the SEM (n= 6; asterisks indicate significant differences (\*\*  $P < 0.005$  and \*\*\*  $P < 0.001$ ) when compared to untreated control) that is alive, apoptotic or necrotic, following various treatments, as determined by the AnnexinV-FITC/propidium iodide flow cytometric assay<sup>1</sup>.

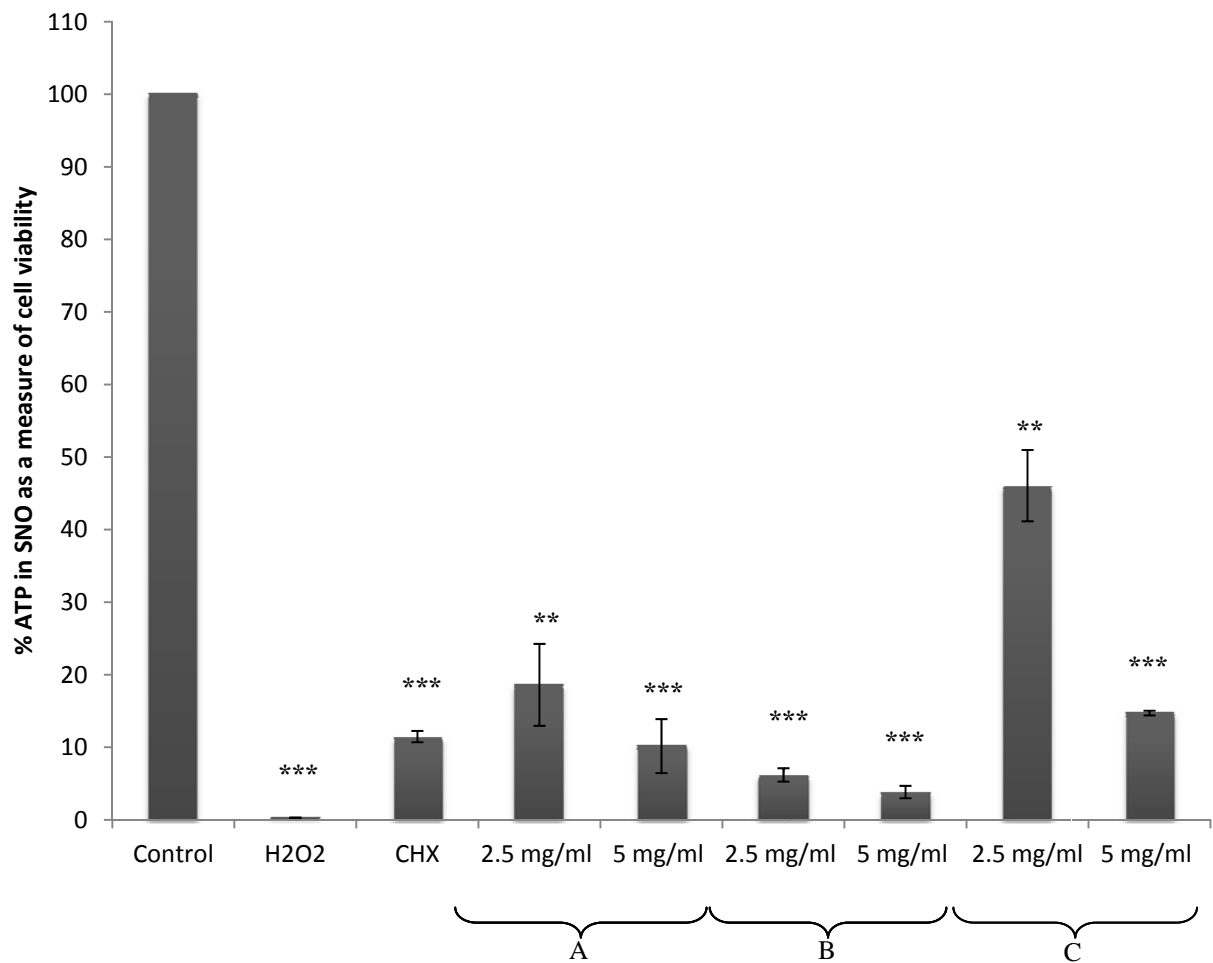
	Q1 PI+ FITC -	Q2 PI+ FITC +	Q3 PI- FITC-	Q4 PI- FITC +
	Necrotic	Late apoptotic or necrotic	Live	Early apoptotic
Control	3.4% $\pm$ 0.10	2.6% $\pm$ 0.16	93.7% $\pm$ 0.61	3.8% $\pm$ 0.36
H <sub>2</sub> O <sub>2</sub>	32.1%** $\pm$ 2.78	41.1%*** $\pm$ 1.25	23.3%*** $\pm$ 0.85	6.7% $\pm$ 1.28
CHX	3.6% $\pm$ 0.85	46%*** $\pm$ 0.56	13.8%*** $\pm$ 0.51	38.5%*** $\pm$ 0.56
2.5 mg/ml A	0.7%*** $\pm$ 0.09	12.7%*** $\pm$ 0.73	50.6%** $\pm$ 3.75	35.8%** $\pm$ 3.15
5 mg/ml A	2.1%** $\pm$ 0.09	21.0%*** $\pm$ 0.90	50.4%*** $\pm$ 1.71	21.8%*** $\pm$ 1.08
2.5 mg/ml B	0.2%*** $\pm$ 0.03	65.6%*** $\pm$ 4.72	12.9%*** $\pm$ 2.03	24.8%* $\pm$ 2.34
5 mg/ml B	0.8%* $\pm$ 0.29	73.9%*** $\pm$ 3.51	7.2%*** $\pm$ 0.57	13.5%*** $\pm$ 0.39
2.5 mg/ml C	0.8%*** $\pm$ 0.10	30.8%*** $\pm$ 1.79	36%*** $\pm$ 2.06	29.6%*** $\pm$ 1.00
5 mg/ml C	2.3% $\pm$ 0.21	31.7%*** $\pm$ 1.36	30%*** $\pm$ 2.62	31.8%*** $\pm$ 1.92

<sup>1</sup>This assay counts the number of cells that are viable, apoptotic or necrotic based on the exposure of phosphatidylserine (PS) molecules on apoptotic cells and compromised cell membranes in necrotic cells. Viable cells, with intact membranes and unexposed PS molecules will remain unlabelled. Propidium iodide enters the compromised membranes of necrotic cells and stains the DNA. Early apoptotic cells, with exposed PS molecules, bind Annexin V-FITC and late apoptotic cells, with compromised cell membranes and exposed PS molecules are stained by both propidium iodide and annexin V-FITC.

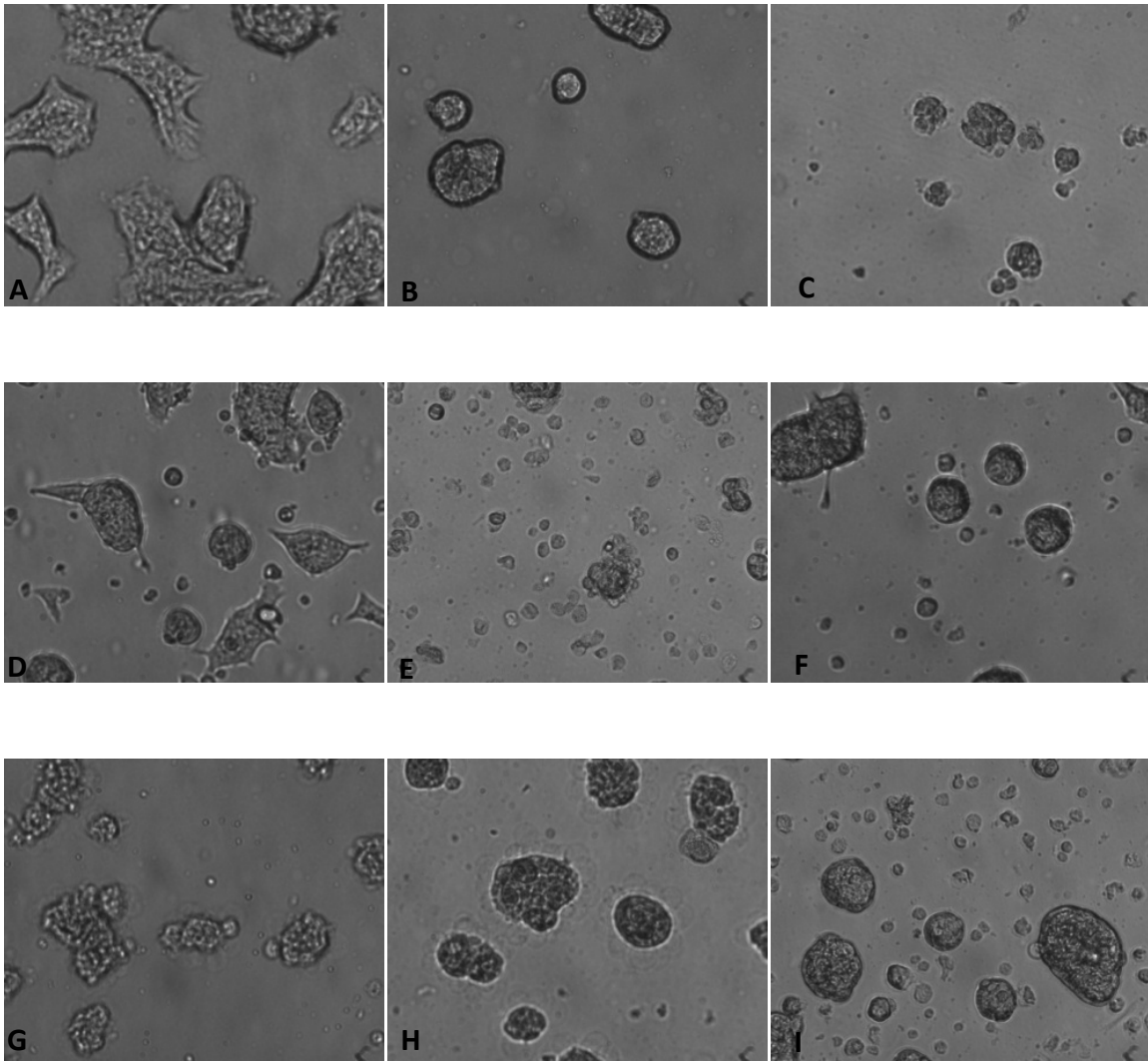
## Figures



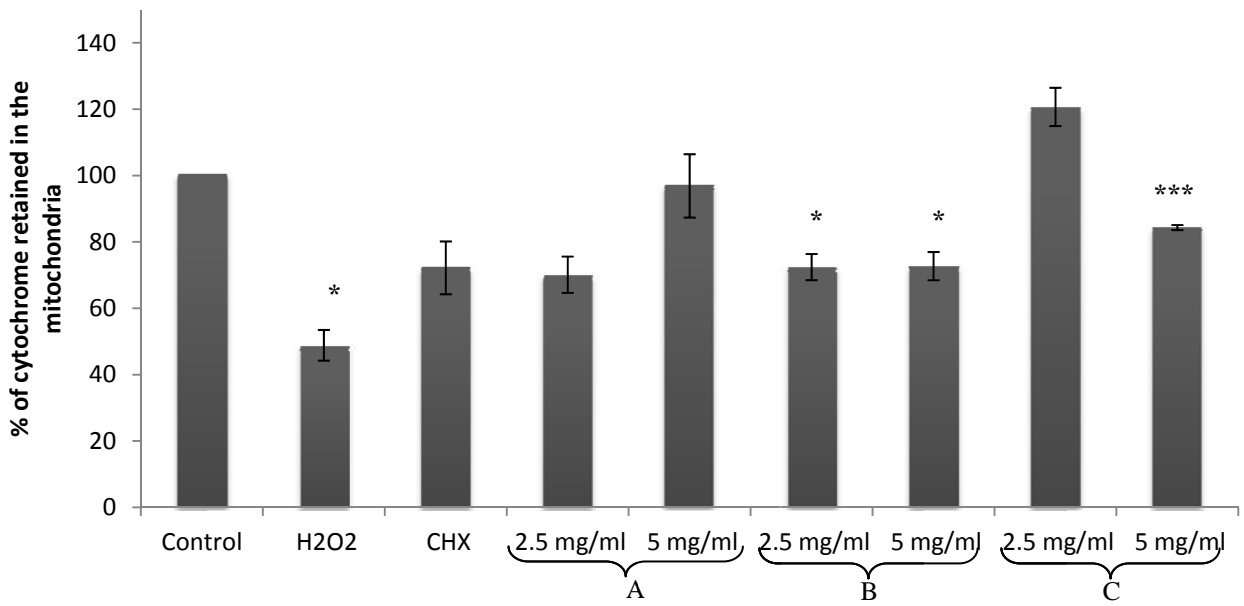
**Fig. 1.** The cell viability of SNO cells as determined by the alamarBlue® assay following treatment with the vehicle control (2.5 and 5% water), necrotic (H<sub>2</sub>O<sub>2</sub>) or apoptotic (CHX) inducers or *Sutherlandia* spp. extracts A, B or C (at 2.5 and 5 mg/ml). The cell viability is expressed as a percentage relative to the untreated control cells. Error bars are representative of the SEM (n = 6) and asterisks indicate significant differences (\*  $P < 0.05$ ; \*\*  $P < 0.005$  and \*\*\*  $P < 0.001$ ) when compared to untreated control.



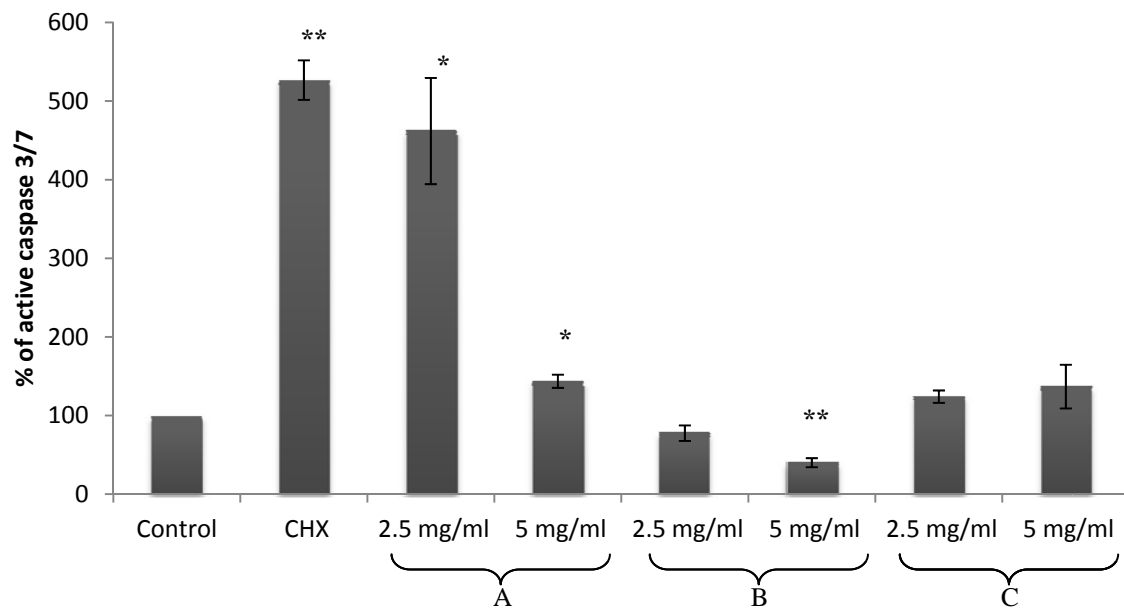
**Fig. 2.** The ATP levels of SNO cells treated with extracts A, B and C, necrotic (H<sub>2</sub>O<sub>2</sub>) or apoptotic (CHX) inducers. The ATP levels were determined by the CellTiter-Glo™ assay and expressed as a percentage relative to untreated control cells. Error bars are representative of the SEM (n = 6) and asterisks indicate significant differences (\* *P* < 0.05; \*\* *P* < 0.005 and \*\*\* *P* < 0.001) relative to the untreated control.



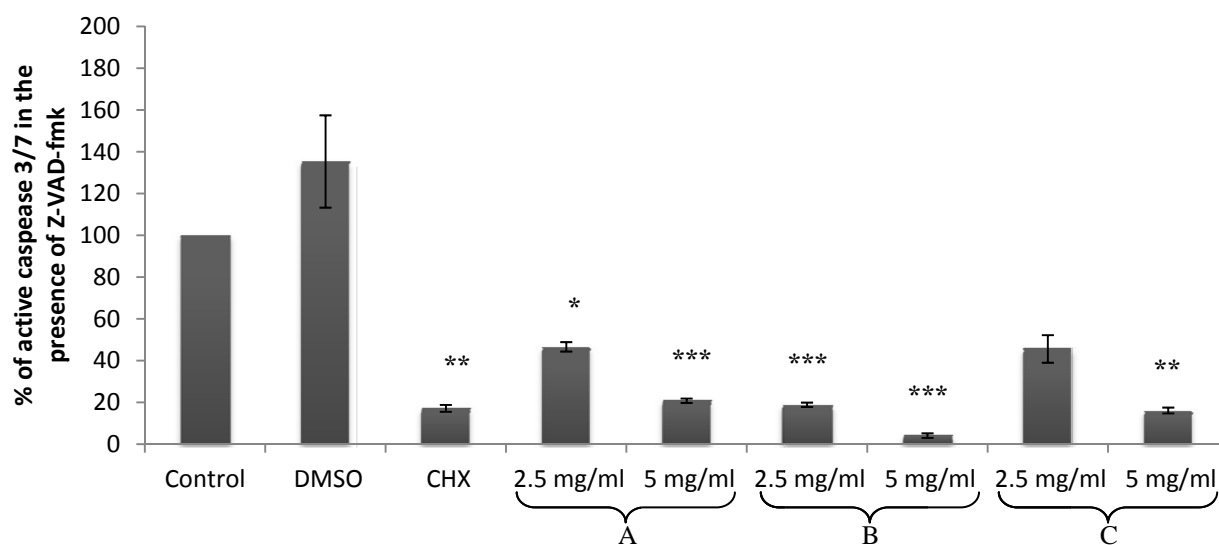
**Fig. 3.** The morphology of SNO cells, at 400X magnification, compared to the untreated control cells (A) which were treated with the following: H<sub>2</sub>O<sub>2</sub>, a necrotic inducer(B), CHX an apoptotic inducer (C) 2.5 and 5 mg/ml of *S. frutescens* extract A (D and G respectively), 2.5 and 5 mg/ml of *S. Frutescens* extract B (E and H respectively) and 2.5 and 5 mg/ml of *S. tomentosa* extract C (F and I respectively). Apoptotic morphological features include spherical cell shape, plasma membrane blebbing and small apoptotic bodies. Swelling of the cells and its organelles are morphological features associated with necrosis.



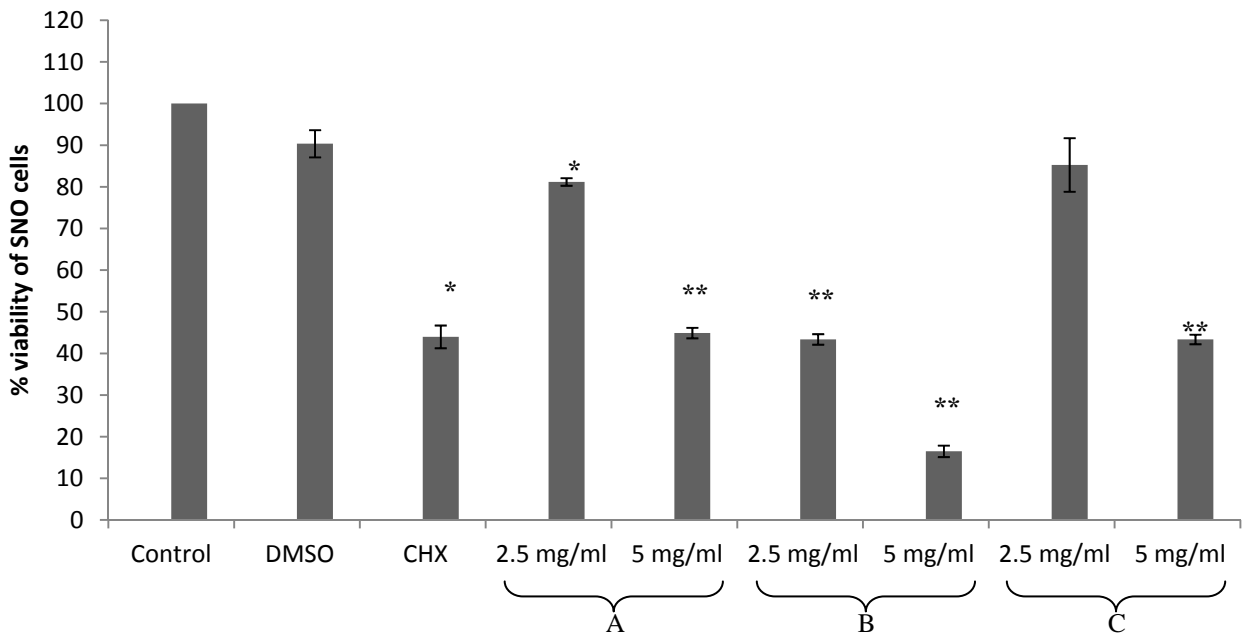
**Fig. 4.** The levels of cytochrome *c* present in the mitochondria of the cells treated with extracts A, B and C, necrotic (H<sub>2</sub>O<sub>2</sub>) or apoptotic (CHX) inducers, expressed as a percentage relative to the untreated control cells. Error bars are representative of the SEM (n = 6) and asterisks indicate significant differences (\*  $P < 0.05$ ; \*\*  $P < 0.005$  and \*\*\*  $P < 0.001$ ).



**Fig. 5.** The levels of active caspase 3/7 in SNO cells treated with extracts A, B and C or CHX (an apoptotic inducer), relative to the untreated control cells, as determined by the Caspase-Glo™ assay. Error bars are representative of the SEM (n = 6) and asterisks indicate significant differences. (\*  $P < 0.05$ ; \*\*  $P < 0.005$  and \*\*\*  $P < 0.001$ ).

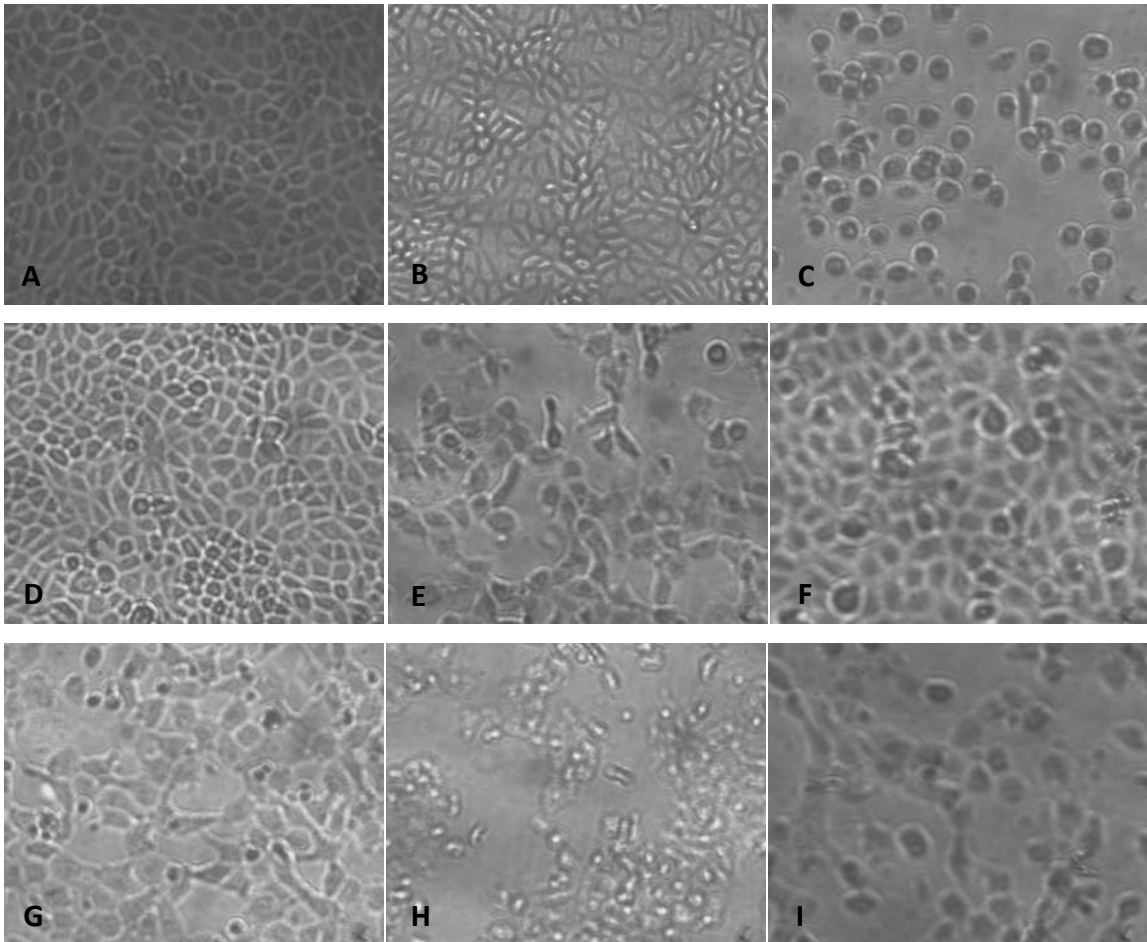


**Fig. 6.** The levels of active caspase 3/7 present in the SNO cells treated with extracts A, B and C, DMSO (a vehicle control) or CHX (an apoptotic inducer) in the presence of the pan-caspase inhibitor Z-VAD-fmk. The caspase 3/7 levels are expressed as a percentage, relative to the untreated, uninhibited control cells. Error bars are representative of the SEM (n = 6) and asterisks indicate significant differences (\*  $P < 0.05$ ; \*\*  $P < 0.005$  and \*\*\*  $P < 0.001$ ).

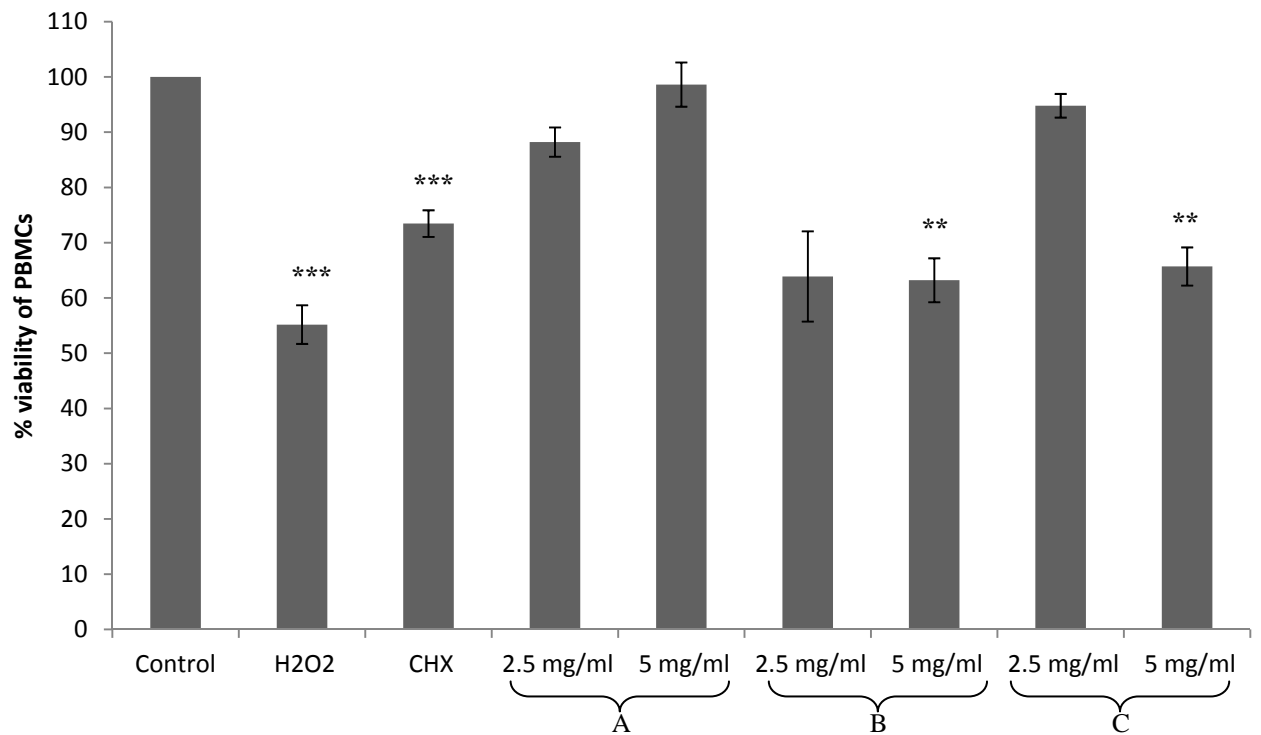


**Fig. 7.** The cell viability of the SNO cells, pre-treated with Z-VAD-fmk or DMSO (vehicle control) followed by treatment with extracts A, B and C, or CHX (an apoptotic inducer). Cell viability was determined by the alamarBlue® assay and expressed as a percentage of the untreated control cells. Error bars are representative of the SEM and asterisks indicate significant differences (\*  $P < 0.05$ ; \*\*  $P < 0.005$  and \*\*\*  $P < 0.001$ ).





**Fig. 8.** The morphology of the SNO cells at 200X magnification compared to the untreated control cells (A) and DMSO-treated control cells (B) following treatment in the presence of the pan caspase inhibitor, Z-VAD-fmk. Treatments were as follows: CHX (C) 2.5 and 5 mg/ml of extract A (D and G respectively), extract B (E and H respectively) and extract C (F and I respectively).



**Fig. 9.** The cell viability of peripheral blood mononuclear cells (PBMCs) treated with extracts A, B or C, necrotic (H<sub>2</sub>O<sub>2</sub>) or apoptotic (CHX) inducers. Cell viability was determined by the alamarBlue<sup>®</sup> assay and expressed as a percentage relative to the untreated control. Error bars are representative of the SEM (n = 6) and asterisks indicate significant differences (\* P< 0.05; \*\* P< 0.005 and \*\*\* P< 0.001).