Sutherlandia frutescens extracts can induce apoptosis in cultured carcinoma cells

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Abstract

Sutherlandia frutescens popularly known as cancer bush is endemic to Southern Africa. Whole plant parts have been used and traditional healers claim that it can treat cancer. In this study it is shown that a crude aqueous Sutherlandia frutescens whole plant extract induced cytotoxicity in neoplastic cells (cervical carcinoma) and CHO (Chinese Hamster Ovary cells) cell lines. Morphological observation and monitoring with other biological assays involving chromatin condensation as well as phosphotidyl serine externalisation point to apoptotic responses. Further biochemical assays showed similar DNA fragmentation patterns induced by Sutherlandia frutescens extracts compared to other inducers of apoptosis such as staurosporine and ceramide. Furthermore, Sutherlandia frutescens extracts induced apoptosis was confirmed by flow cytometric analysis. These findings warrant further research with a view to develop Sutherlandia frutescens extracts for use in anti-cancer therapy.

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1. Introduction

In South Africa traditional healers claimed that Sutherlandia frutescens commonly known as cancer bush or “kankerbos” treats cancer. The plant was first used by “khoi san” and the “Nama” people and is distributed mostly along the west coast of the Western Cape (Van Wyk, 1997). Extracts of this plant have been used to treat stomach cancer; decoctions consumed as blood tonic and used for other ailments like cough, uterine disease and eye infection (Moshe et al., 1998). Thomson (2002) described Sutherlandia frutescens as a poisonous herb and folklore on this plant describe that decoctions are curative against cancer. Based on these controversial claims this study was set out to analyse the cytotoxic effect of crude plant extract to induce apoptosis in cultured cells. In this study Sutherlandia frutescens (Fabaceae) extracts were used to evaluate its ability to induce apoptosis in CHO and neoplastic cells. Previous work done by Zhao et al. (2003) and Nile et al. (2003) showed that plant extracts are able to induce apoptosis in cancer cells. Previous studies have shown that medicinal plant extracts that induce apoptosis can be used for the purpose of generating therapeutic drug.

Induction of apoptosis, programmed cell death is one approach to cancer therapy (Los et al., 2003). Apoptotic cell death is a physiological mechanism that eliminates unwanted cells by triggering the cell’s intrinsic suicide program (Kerr et al., 1972). Impairment of the apoptotic mechanism ultimately generates a pathological condition that includes developmental defects like, autoimmune diseases, neurodegeneration or cancerous neoplasia (Reed et al., 2001). Apoptosis is characterized by morphological changes such as membrane blebbing, cell shrinkage, protein fragmentation, chromatin condensation and DNA degradation followed by rapid engulfment of cell debris by neighbouring cells (Chritop, 2003). It is therefore possible to take advantage of this intrinsic mechanism by manipulating the apoptotic process for therapeutic gains. The basis of this study was to broaden the under-
standing of the relationship between medicinal plants such as Sutherlandia frutescens and induced apoptosis, which could be beneficial in anti-cancer therapy.

2. Materials and methods

2.1. Materials

Hams F12 medium, Roswell Park Memorial Institute medium (RPMI) and Dulbecco’s Modified Eagle’s Medium (DMEM) and Foetal calf serum (FCS) were obtained from Gibco (Cape Town, South Africa), Staurosporine and Ceramide were supplied by Sigma (Cape Town, South Africa). ApoPercentageTM was supplied by Biocolor (Belfast, UK).

2.2. Cell culture

CHO cells were grown in Hams F12, Caski (cervical carcinoma) were grown in DMEM maintained as a monolayer while Jurkat T lymphoma cells were grown in RPMI suspension medium. Both Hams F12 and DMEM media were supplemented with 5% (v/v) foetal calf serum (FCS) and RPMI was supplemented with 10% (v/v) FCS and 0.1% (v/v) streptomycin was added in all media. The cultures were grown at 37°C in a humidified atmosphere of a 5% (v/v) CO2 in air.

2.3. Preparation of aqueous extract

Fresh and dried plant material was obtained from Kirstenbosch Botanical Gardens (Cape Town, South Africa), Bloemfontent and Free State. The plant specimens were authenticated by University of The Western Cape herbarium (Bellville, South Africa) where voucher specimens are kept. Leaves, stems and flowers of Sutherlandia frutescens were collected and washed with distilled water, dried in a ventilated oven for 72 h at 35°C and ground to a fine powder using Philips Cucina (HR173/37) domestic blender (Philips, IND/BRAS, Brazil). The plant material was passed through 850 μm pore size sieve and 10 g of powder was extracted in 1 L boiling water and allowed to cool prior to centrifugation at 1000 × g. The supernatant was freeze-dried for 72 h in the VIRTIS 5 L freeze drier (VIRTIS New York, USA) to obtain a dried powdered plant extract. Extracts were kept in desiccators until needed. Different doses were used to test apoptosis and were freshly prepared from extract stocks of 40 mg/mL stored at −20°C (449 mg of freeze dried plant material dissolved in 11.225 mL of distilled water to give a final concentration of 40 mg/mL).

2.4. ApoPercentageTM assays

CHO cells were seeded in a 96-well tissue culture plate. The cells were confluent for 24 h before treatment with the Sutherlandia frutescens extracts at concentrations between 1.5 and 10 mg/mL. All values were expressed as mean ± S.E.M. and this data was calculated from three independent experiments performed in triplicate and treated over a period of 5 min to 6 h. Experimental sets were gently washed with 2 × phosphate buffer saline (PBS) and replaced with 100 μL apoPercentageTM dye and incubated for 5 min to 6 h. The cell morphology was observed using an inverted microscope at 200× magnification (Nikon). Cell death was indicated by cell shrinkage, cell disintegration and reduction in cell number.

Fig. 1. Morphological observation of Sutherlandia frutescens WCP extract tested on CHO and cervical carcinoma (Caski) cells at different exposure times. CHO cells and Caski cells (2.5 × 10⁶) were grown in Hams F12 and RPMI media respectively. (A) Untreated control of CHO cells; (B and C) CHO cells induced with 3.5 mg/mL with Sutherlandia frutescens WCP extract and treated for 8 and 24 h respectively; (D) Untreated control of Caski cells; (E and F) are Caski cells treated with 3.5 mg/mL of Sutherlandia frutescens WCP extract and treated for 8 and 24 h, respectively. The cell morphology was observed using an inverted microscope at 200× magnification (Nikon). Cell death was indicated by cell shrinkage, cell disintegration and reduction in cell number.
a further 60 min at 37°C. After 60 min staining, the cells were washed twice with 100 µL PBS to remove un-trapped dye. The cells were visualised under a light microscope and photographs were taken using the 20× objective. Dye release agent (100 µL) was added and incubated for 10 min at room temperature. The cell bound dye recovered in solution was measured using a microtitre plate colorimeter. Absorbance was read at 550 nm (maximum dye absorbance) and 625 nm (minimum dye absorbance). The difference between these two values was taken as the real absorbance for the recovered dye that was trapped in the cells.

2.5. DNA fragmentation

Cells were grown in 25 cm² flasks and treated for the required time periods with respective apoptosis inducers before being transferred into 1.5 mL micro-centrifuge tubes. The cells were centrifuged at 16100 × g for 5 min. The pellet was resuspended in 0.5 mL TTE (Tris-EDTA (ethylene diamine tetracetic acid)) followed by vigorous vortexing. To the 0.5 mL solution already in the tube, 0.1 mL ice-cold 5 M NaCl and 0.7 mL ice-cold isopropanol were added. The tubes were vigorously vortexed and placed at −20 °C overnight to precipitate the DNA. Samples were centrifuged at 16,100 × g for 10 min at 4°C. The pellets were washed once with 0.5 mL 70% ice-cold ethanol and centrifuged at 16100 × g for 10 min at 4°C. The supernatant was removed and the tubes were dried at 37°C. The pellets were dissolved in 45 µL Tris-EDTA containing 5 µL loading buffer. The samples were analysed by electrophoresis on 1.8% agarose gels stained with ethidium bromide (0.5 µg/mL).

2.6. Crossmon trichrome stain

Hydrated smears were stained in a 0.1% acid fuchsin/orange G stain for 5 s, rinsed in water and transferred in 1% light green for 30 min. After staining the smears were briefly rinsed in water and dehydrated through ascending grades of ethyl alcohol, cleared in xylene and mounted in Canada balsam.

2.7. Flow cytometry (immunofluorescence) analysis

CHO cells (or Jurkat T cells were appropriate) were plated in a 5 mL culture flask and induced with the appropriate Sutherlandia frutescens extract or staurosporine. Cells were washed twice with cold PBS and then resuspended with 1x binding buffer. The resuspended cells (100 µL) were gently vortexed and incubated for 15 min at room temperature (25°C) in the dark. The binding buffer (400 µL) was added in each tube and analysed by flowcytometry using 488 nm wavelengths within 1 h (Vermeulen et al., 2002). Both the fluorescence and the physical properties of the cells were analysed by analog digital converter (ADC) and processed using integrated software (Becton Dickinson-Bioscience FACS Calibur™ software San Jose, California, USA).

2.8. Statistical analysis

Results are expressed as mean ± standard errors of the means for each series of experiments. Levels of statistical significance were calculated using Wilcoxon sign rank sum test.

3. Results

3.1. Morphological changes in cultured cells induced by Sutherlandia frutescens extracts

Based on the controversial claims of the effect of Sutherlandia frutescens, morphological screens (bioactivity testing) of collections of the Sutherlandia frutescens microphylla obtained from different selected populations in South Africa were undertaken. It was observed that a particular extract from the Western Cape Province (WCP) was more

![Fig. 2. Apoptotic effects of Sutherlandia frutescens extracts on CHO cells.](image)
active (cytotoxic) compared to the other two from Northern Province (NP) and Orange Free State (OFT) (data not shown). The morphological changes indicated that cell death occurred in both CHO and Caski cells (Fig. 1). In untreated controls (Fig. 1A and D) the cells appear morphologically normal while cells B and E treated at 6h with WCP extract were vacuolated with condensed nuclei and showing membrane damage. At 24h the treated cells (Fig. 1C and F) show morphological disintegration. This experiment suggests that there are active constituents in the plant extract that causes morphological changes and cell death. This morphological assay was not enough to confirm apoptosis.

3.2. The investigation of Sutherlandia frutescens induced apoptosis on CHO cells using apoPercentage
tm
assay and cromson trichrome stain

In order to confirm that the morphological cell death observed in Fig. 1 was due to apoptosis an assay that takes advantage of the ‘flip-flop’ mechanism in apoptotic cells was first utilized. During the apoptotic process (Martin et al., 1994; Savill and Fadok, 2000) a flip-flop occurs resulting in externalisation of a phospholipid in the plasma membrane, phosphotidyl serine. The apoPercentage™ dye gains entrance into the cells, and when it is trapped, it is a diagnostic tool for apoptosis. The apoPercentage™ assay (Fig. 2) indicated that CHO cells not treated with WCP extract (control) had no dye and therefore is morphologically intact (Fig. 2A). An apoPercentage™ assay was carried out on CHO cells treated with Sutherlandia frutescens aqueous extracts from WCP at a concentration of 3.5 mg/mL (Fig. 2B). CHO cells treated with WCP extract showed cellular shrinkage and contained pink dye when observed under the microscope (Fig. 2B). This trapped dye indicates that the cells were positive for apoptosis and that Sutherlandia frutescens extract from the WCP activated apoptosis. Cells dying of apoptosis displayed condensed chromatin (Anton, 1999). It was confirmed that chromatin condensation was induced by Sutherlandia frutescens extracts (3.5 mg/mL) from WCP using cromson trichrome stain (Fig. 2C and D). Since Sutherlandia frutescens extract from WCP activated apoptosis in

![Figure 3](image_url)

**Fig. 3.** (A) Effect of Sutherlandia frutescens extracts (1 WCP, 2 NP, 3 OFT) for 6h from different selected plant population in South Africa. The percentage cell death was determined using apoPercentage™ assay. Absorbance was read at 540nm (maximum dye absorbance) and 625 nm (minimum dye absorbance). The difference between these two values was taken as the real absorbance for the recovered dye that was trapped in the cells. An absorbance reading above 0.05 at 540 nm indicated cell death. Cells not affected by cytotoxicity had an absorbance range between 0.0 and 0.05. Where error bars are not shown, the standard error of triplicates is smaller than the size of the symbol. Results are expressed as absorbance (peak heights) for triplicate experiments ± S.E.M. Values that differed significantly from untreated control are indicated as follows: extract 1 = P < 0.05 (at concentrations 1.5–5 mg/mL). Extract 2 = P < 0.05 (at concentrations 4.5–5 mg/mL). Extract 3 = P < 0.05 (at concentration 5 mg/mL) (B) The effect of different Sutherlandia frutescens extracts (1 WCP, 2 NP, 3 OFT) on CHO cells (cell death) at specified time-intervals and at a concentration of 3.5 mg/mL. Cell death was determined using the apoPercentage™ assay. Absorbance was read at 540 nm (maximum dye absorbance) and 625 nm (minimum dye absorbance). The difference between these two values was taken as the real absorbance for the recovered dye that was trapped in the cells. An absorbance reading above 0.05 at 540 nm indicated cell death. Cells not affected by cytotoxicity had an absorbance range between 0.0 and 0.05. Where error bars are not shown, the standard error of triplicates is smaller than the size of the symbol. Results are expressed as absorbance (peak heights) for triplicate experiments ± S.E.M. Values that differed significantly from untreated control are indicated as follows: extract 1 = P < 0.05 (at times 45–75 min).
CHO cells, it was necessary to investigate the dose response and time of activation of apoptosis by the different *Sutherlandia frutescens* extracts collected from the various selected populations.

### 3.3. Dose response and time course experiment on CHO cells by *Sutherlandia frutescens* induced apoptosis using apoPercentage™ assay

In this section, *Sutherlandia frutescens* extracts from different selected populations were tested to establish which population was more active by evaluating dose response and the time course experiment. The different collections of *Sutherlandia frutescens* aqueous extracts from separate selected collection were again tested using apoPercentage™ assay on CHO cells to establish a dose response (Fig. 3). The result shows a rise in absorbance ($A_{550} > 0.05$) when the dose of extract from WCP is increased from 1 to 4 mg/mL ($A_{550} > 0.1$) for 6 h. There was no increase in absorbance observed with extracts from OIT at all concentrations. Extract from NP showed a slight increase ($A_{550} < 0.08$) at 3.5 and 4.5 mg/mL. The dose response indicates different activities in the different populations tested. The time course experiment was intended to evaluate the shortest possible time for activation and detection of apoptosis using the different collections of *Sutherlandia frutescens* extracts with the apoPercentage™ assay (Fig. 3B). CHO cells were exposed to different *Sutherlandia frutescens* aqueous extracts at concentrations of 3.5 mg/mL at specified time interval of 0–75 min to all treated and untreated cells and subsequently 5 min interval for the different triplicate samples. Extract from WCP showed an exponential increase in activity between 0 and 65 min ($A_{550} > 0.01$) and a drop in activity between 65 and 75 min ($A_{550} ≥ 0.10$). Extracts from NP and

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**Fig. 5.** Histogram analysis of Jurkat T cells stained with annexin-V PE dye. Jurkat T ($1 \times 10^5$) cells were cultured in a 5 mL culture flask (Section 2.1) and treated where appropriate for 6 h with *Sutherlandia frutescens* WCP extract (3.5 mg/mL) or staurosporine (1.3 μM) stained with annexin-V dye and subject to flowcytometry. (A) Untreated cells (control). (B) Cell treated with staurosporine, positive control. (C) Cells treated with *Sutherlandia frutescens* WCP extract. The X- and Y-axis represent the relative fluorescence intensity and cell counts, respectively. M1 and M2 represent the percentages of viable cells and non-viable (apoptotic) cells, respectively.
OFT showed no change in activity from 0–70 min but a slight increase in activity of extract from NP at 75 min ($A_{155} > 0.04$).

3.4. Sutherlandia frutescens extract induced DNA fragmentation is similar to staurosporine and ceramide

Initial results showed that Sutherlandia frutescens extract induces apoptosis, and it was intended to compare the DNA fragmentation pattern with other known inducers of apoptosis (Fig. 4). DNA fragmentation was observed when genomic DNA extracted from staurosporine or ceramide treated CHO cells (Fig. 4, lanes 2, 4 and 5). Genomic DNA extracted from cells treated with Sutherlandia frutescens extract from the WCP also displayed fragmentation (Fig. 4, lane 3) and as expected no fragmentation was observed with DNA extracted from untreated cells (Fig. 4, lane 6). This result further suggests that Sutherlandia frutescens extract from the WCP had apoptotic activity due to the fragmentation of genomic DNA.

3.5. Analysis of Sutherlandia frutescens induced apoptosis on CHO cells using flowcytometry

Flowcytometry was used to evaluate the behaviour of cells treated with an apoptotic agent or Sutherlandia frutescens extract from the WCP to determine apoptosis. A control experiment was designed where Jurkat T cells were grown and untreated or treated with 3.5 mg/mL of Sutherlandia frutescens extract or 1.3 $\mu$m staurosporine for 6 h prior to flowcytometry using the annexin-V dye (Fig. 5B). Normal cells remain in the first decade and the percentage indicates the number of normal cells in the population. The second decade indicates cell death and the percentage indicates the number of cells that died naturally within the population. A shift along the X-axis (relative fluorescence) therefore indicates apoptosis and shift to the third decade indicates necrosis. The percentage of cells in the untreated sample that are normal is >90% (Fig. 5A). Cells treated with staurosporine showed more than 80% of apoptotic cell death (Fig. 5B). Apoptosis is further indicated by the histogram result where about 14% of the cells population were viable (M1) while more than 84% of the cells were apoptotic (M2) induced by Sutherlandia frutescens extract (Fig. 5C). The flowcytometric results indicated that WCP extract had apoptotic activity. The dot plot (Fig. 6) indicated that cells in the A region were normal (FSC pattern) while cells in the B region (SSC pattern) were apoptotic.

4. Discussion and conclusion

Apoptosis is a form of programmed cell death that occurs naturally in cells and can be beneficial to cancer therapy as previously studied (Zimmermann et al., 2001). Several apoptotic tests were utilized to validate the claim of cancer therapeutic benefit of Sutherlandia frutescens as used by herbalists. The results show that extracts of Sutherlandia frutescens from South Africa had apoptotic activity in three different cell lines; CHO, Caski and Jurkat T lymphoma cells. These cells displayed morphological disintegration (Fig. 1) and other cellular membrane change indicative of apoptosis. Furthermore, it was demonstrated that apoptosis occurred as a result of flip-flop translocation of phosphotidyl serine (Fig. 2), a cellular phospholipid membrane (Martin et al., 1994; Savill and Fadok, 2000). There are some similarities between apoptosis and normal mitosis such as chromatin condensation, dissolution of nuclear membrane and activation of cyclin-dependent kinases (Anton, 1999). Therefore apoptotic fragments usually contain condensed chromatin shown in Fig. 2. There is a further proposition that during apoptosis there is DNA cleavage at the matrix attachment region where loops of chromosomal DNA are attached to the loops of the nuclear matrix resulting in chromatin condensation (Anton, 1999).

To confirm that Sutherlandia frutescens extracts induced apoptosis, a dose response and time course experiment with the different selected plant populations was made. The results show that extracts from WCP have more apoptotic activity than the other extracts (NP and OFT). This result may indicate that the action of Sutherlandia frutescens extract from WCP is both time and dose dependent. The observation that the extracts from the other selected population did not have apoptotic activity suggests that certain environmental challenges on the different plants may cause them to synthesize different or new compounds and thus making them distinct in different areas. These different compounds present in the same species could be attributed to plants which differ from their area of collection based on soil composition, protection against environmental factors (e.g. high UV) leading to the synthesis and accumulation of secondary metabolites (Wink, 1999).
The DNA fragmentation technique was applied, as it is known to be hallmark of apoptosis (Wyllie, 1980) to compare the Sutherlandia frutescens extract with other known inducers of apoptosis such as staurosporine and ceramide as positive controls, DNA fragmentation, coupled with morphological appearance of chromatin condensation, is a direct result of DNA cleavage. It occurs as a result of activation of endogenous Ca^{2+} and Mg^{2+} endonucleases, which are enzymes that selectively cleave DNA at sites located within the nucleosomal subunits generating strand breaks of multiple integers of 50–200 bp fragments (Wyllie, 1980). Since the Sutherlandia frutescens extract produced comparable results to the known apoptotic inducers staurosporine and ceramide (Haefena et al., 2002; McKeaguel et al., 2003), it provides further support for its apoptotic activity.

It was seen in the initial results that Sutherlandia frutescens extract from WCP has the ability to cause apoptotic cell death in cultured cells as shown by morphological observation (microscopy), phosphotidyl serine externalisation (apoPercentage™ assay), chromatin condensation (common trichrome) and nuclear fragmentation (agarose gel electrophoresis). Flow cytometry was used subsequently to evaluate the behaviour of cells treated with an apoptotic agent or Sutherlandia frutescens extract from the WCP to determine apoptosis. Flow cytometry is a more accurate, qualitative and quantitative method to analyse apoptosis (Vermeulen et al., 2002). In flow cytometry, fluorescent dye is added to cells treated with apoptotic agents (plant extract) and the cells passed through an argon laser. This causes the cells to emit fluorescence as well as expressing physical properties. The analyses of the fluorescence and physical properties indicate whether cells are apoptotic or not, shown by the addition of fluorescent dye (Fig. 5). The result showed that more than 80% of the cells died by apoptosis induced by Sutherlandia frutescens extract, which is in close comparison of more than 80% apoptotic cell death induced by staurosporine indicating that Sutherlandia frutescens extracts induce apoptosis.

Hence together, the results reported here indicate that extracts from Sutherlandia frutescens grown in specific regions can induce apoptosis, which could be beneficial to cancer therapy. With the concept that certain plants can treat different forms of diseases as claimed by traditional healers (Bieseubach, 1998), in certain instances plants remain the only source of medicine for rural health care. In addition, such plants can provide pharmaceutical companies with the opportunity to generate new drugs. Sutherlandia frutescens locally known as “cancer bush” or “kankerbos” is one plant that has caught the interest of medicinal plant dealers due to the claim that it has a broad therapeutic potential and more specifically, it is perceived by some to have the ability to treat cancer (Van Wyk, 1997). The data from this study demonstrates that Sutherlandia frutescens extracts bioactivity varies between different selected plant populations studied. The extract from the WCP demonstrated the most apoptosis on cultured cells. In order to extract more of the apoptotic compounds and to identify which part of the plant contains a higher concentration of these compounds, it will be vital to evaluate extracts from different parts of Sutherlandia frutescens (seeds, roots, stems, leaves and flowers).

In summary, the claim by traditional healers that Sutherlandia frutescens has anti-cancer properties has been partially validated by identifying an extract in the plant that has marked apoptotic activity. The mechanism of apoptotic action is not fully understood but one could speculate that this extract activates the central death caspase 3, which is regulated by apoptosome complex (Hengartner, 2000). However, this apoptotic activity seems not to be universally present in all Sutherlandia frutescens extracts tested in the selected populations studied (or present at very low levels). It can be anticipated that some of these compounds in Sutherlandia frutescens extracts, if structurally identified and characterized (Mass Spectroscopy, 1H nuclear magnetic resonance) may be candidates for anti-cancer drug development.

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