Research Article

Cytotoxic and apoptosis-inducing effects of *Sutherlandia frutescens* in neuroblastoma cells

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ABSTRACT

Background: *Sutherlandia frutescens* (*S. frutescens*) is a shrub with attractive flowers used traditionally ~~by~~ in South African to treat a variety of health conditions. Its anti-cancer activity has been reported in a number of cancers, and accordingly, this study was designed to investigate the cytotoxic activity and apoptosis-inducing effects of *S. frutescens* in neuroblastoma ~~cells~~, a type of childhood cancer and a leading cause of death in children. **Method:** In this study, the effect of *S. frutescens* on cell viability and survival of SKNBE(2) and SHSY5Y neuroblastoma cells was determined using the MMT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide) cell viability assay and clonogenic assay respectively. Furthermore, flow cytometry was used to investigate the accumulation of intracellular reactive oxygen species (ROS) as well as loss of mitochondrial membrane potential (MMP). Caspase-9 activation was also used to detect apoptosis in these cells. **Results:** The results from this study show that exposure of *S. frutescens* to SKNBE(2) and SHSY5Y cells led to the inhibition of cell viability and survival and also induced apoptosis, increased intracellular accumulation of ROS and triggered a loss of MMP in these cells. **Conclusion:** Findings from this study validate folkloric claims on *S. frutescens* and demonstrate that this plant might serve as a potential anti-cancer agent in neuroblastoma cells, thus providing the rationale for further investigations into the bioactive components eliciting these activities.

INTRODUCTION

Childhood cancer remains the leading cause of disease-related death in children (van der Pal et al., 2010). Neuroblastoma, an embryonal cancer of the sympathetic nervous system, often occurs in young children (Howman-Giles et al., 2007). Clinically, children with neuroblastoma are designated as high-risk patients and conventional treatment involves a co-ordinated sequence of chemotherapy, surgery, and radiation (Pearson et al., 2008).

Although there have been significant improvement in the outcomes of neuroblastoma patients due to intensified chemotherapy, many children with high-risk neuroblastomas sometimes develop complications, including hearing loss, cardiac dysfunction, infertility, and second malignancies (Zage et al., 2008). Therefore, more effective and less toxic therapeutic strategies need to be developed, including selective and efficient targeting of tumour cells to improve survival.

More than 80% of the world's population make use of complementary and alternative medicines which include herbalism and botanical medicines (Mainardi et al., 2009, Robinson and Zhang, 2011). Plant and plant-derived products have been reported to serve as a template for the discovery of new drugs due to their content of active ingredients which have the same molecular targets as pharmaceutical drugs (Pan et al.,

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2013, Yuan et al., 2016, Ahn, 2017). Many drugs used in cancer chemotherapy including vincristine, irinotecan, etoposide and paclitaxel, are classic examples of medicinal plant-derived compounds (Balunas and Kinghorn, 2005, Nobili et al., 2009). Thus, herbal and botanical medicines represent an enormous medicinal resource at a fraction of the cost of conventional medicine. Due to the numerous gains of plant and plant-derived compounds in cancer research, *S. frutescens* has been selected as the plant of study in this research.

S. frutescens (*unwele* in Zulu; *kankerbos* in Afrikaans; cancer bush in English), a member of the pea family, is a shrub with attractive flowers used traditionally by South Africans to treat a variety of health conditions. The medicinal use probably originated with the Khoi and Nama people of South Africa; who used the plant decoctions externally to wash wounds and internally for fevers and a variety of other ailments. Also, it is used as an old Cape remedy for stomach problems and internal cancers (Van Wyk and Gericke, 2000).

Previous studies have evaluated the effects of *S. frutescens* on several cancer cell lines. The ethanolic extract has been reported to have a concentration-dependent anti-proliferative effect on MCF7 and MDA-MB-468 (breast cancer cells) as well as Jurkat leukemic cells and HL60 (Tai et al., 2004, Stander et al., 2007). The ethanolic extract was also demonstrated to induce an S-phase cell cycle arrest, apoptosis and autophagy in cultured breast adenocarcinoma cells. The aqueous extract of *S. frutescens* was reported to inhibit the growth of MCF-7 cells and induce cytotoxicity in cervical carcinoma (Caski) and Chinese hamster ovary (CHO) cells (Chinkwo, 2005, Steenkamp and Gouws, 2006). Recently, *S. frutescens* was reported to modulate apoptosis induction in colon cancer cells via the phosphatidylinositol 3-kinase pathway as well as cause caspase-independent cell death in melanoma cells (Leisching et al., 2015, van der Walt et al., 2016). *S. frutescens* has been shown in humans to be safe and to have non-toxic or deleterious side effects (Johnson et al., 2007). Therefore, the overall aim of this study was to evaluate the cytotoxic and apoptosis-inducing effects of *S. frutescens* in SK-N-BE (2) and SHSY5Y neuroblastoma cells, with a view to understanding its benefits as a potential anticancer agent.

MATERIALS AND METHODS

Preparation of S. frutescens aqueous extract

Powdered dry leaves of *S. frutescens* R. BR. (variety *Incana* E. Mey.) [family: Fabaceae] were purchased from the suppliers Big Tree Health Products (Fish Hoek, South Africa) and 1 kg of the powdered preparation was soaked in boiling water and left overnight for extraction. The resultant extract was filtered with Whatman filter paper and the filtrate was evaporated in a freeze-dryer to obtain a dried powder product which was stored away at -20°C until needed. Aliquot portions of the product were weighed and dissolved in phosphate buffered saline (PBS, Lonza Group Ltd., Verviers, Belgium) to give a stock solution from which dilutions were made in media for treatment of cells on each day of the experiment.

Cell lines and culture conditions.

The human malignant neuroblastoma cell lines SKNBE(2) and SHSY5Y as well as the non-cancerous KMST6 fibroblast cells were cultured in monolayer using Dulbeco Modified Eagles Medium (DMEM, Lonza Group Ltd., Verviers, Belgium) supplemented with 10% foetal bovine serum (FBS, Gibco, Life Technologies Corporation, Paisley, UK) and 1% 100 U/mL penicillin and 100 μg/mL (Lonza Group Ltd. Verviers, Belgium). Cells were grown at 37°C, in a humidified atmosphere consisting of 5% CO_2 and 95% air, and the culture medium was routinely replaced every two to three days and the cells were sub-cultured using a solution of 0.25 % trypsin EDTA (Lonza Group Ltd., Verviers, Belgium) when 80% confluency was attained.

Measurement of cell viability

Cell viability was determined using the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (MTT) assay kit (Roche, USA) following the manufacturer's protocol. Briefly, SKNBE(2) and SHSY5Y cells were plated in 96-well cell culture plates at a cell density of 2500 and 5000 cells respectively per well and incubated overnight for 24 hours to allow the cells to attach. The medium was thereafter replaced with fresh medium containing increasing concentrations of *S. frutescens* (0 to 5 mg/ml) for 48 hours and untreated cells served as control. After 48 hours incubation, 10 μl of the MTT solution (5 mg/ml) was added to each well and the cells were incubated for an additional 4 hours at 37°C, followed by the addition of 100 μl of the solubilisation buffer (10% SDS in 0.01 M HCl). Plates were then left overnight at 37°C and absorbance of each well was read at 570 nm using a BMG Labtech Omega® POLARStar multimodal plate reader. The percentage cell viability was calculated using the formula below:

$$\% \text{ Cell Viability} = \frac{\text{Absorbance of treated well}}{\text{Absorbance of Untreated well}} \times 100$$

The concentration required to kill 50% of the cells (IC_{50}) was determined via a survival curve using GraphPad

Prism 6 software (GraphPad software, San Diego, CA, USA) and all experiments were conducted in triplicates.

Clonogenic assay

The clonogenic assay was conducted to determine the impact of extract treatment on cell survival. This assay assesses the ability of single cells to divide and form colonies (a colony consists of 50 cells) after being treated with anticancer agents. Briefly, cells were plated in 60 mm dishes and allowed to grow overnight before exposure to two different concentrations of the extract (half the IC_{50} and IC_{50}) for 24 hours. After treatments, cells were harvested and re-plated at a density of 500 cells per dish in 35 mm dishes and were allowed to grow over a period of fourteen days to form colonies. At the termination of the experiment, formed colonies were fixed with a solution of methanol and glacial acetic acid (3:1) and stained with 0.5% crystal violet stain. Images of the dishes were taken and areas covered by colonies were calculated using Image J software and expressed as a percentage of the control which was set at hundred percent.

Detection of apoptosis

The Caspase-9 ApoTarget® colorimetric assay kit (Invitrogen, Life Technologies, USA) was used to determine Caspase-9 activity following manufacturer instructions. Briefly, cells were seeded in 60 mm dishes and allowed to settle overnight before treatment with the IC_{50} of *S. frutescens* for 48 hours. Cells were harvested and lysed for 10 minutes in cold lysis buffer and centrifuged at 10 000 x g for 1 minute. The supernatant was thereafter transferred into microcentrifuge tubes before 50 µl of 2X reaction buffer containing Dithiothreitol (DTT) was added to each sample. Afterwards, 5 µl of 4mM LEHD-pNA substrate was added to the samples and incubated at 37°C for 2 hours before reading at 400nm in a microplate reader.

Measurement of intracellular reactive oxygen species

Intracellular ROS activity was determined using the fluorescent probe 2′,7′-Dichlorofluorescin diacetate (DCFH-DA, Sigma St Louis, USA). Briefly, cells were plated in 60 mm dishes and treated with IC_{50} of *S. frutescens* for 48 hours and thereafter collected by trypsinization. Cells were washed with 1X PBS and centrifuged at 3000 revolutions per minute (rpm). The resultant pellet was stained with DCFH-DA for 1 hour after which cells were resuspended in 500 µl of PBS and fluorescence was acquired with the BD Accuri CSampler Flow Cytometer (BD Biosciences Pharmingen, San Diego, CA, USA).

Measurement of mitochondria membrane potential

Flow cytometry was used to determine loss of mitochondrial membrane potential (MMP) following staining with the fluorescent dye rhodamine 123 (Sigma St Louis, USA). Briefly, cells were seeded at an appropriate density in 60 mm dishes and allowed to attach overnight before exposure to the IC_{50} concentration of *S. frutescens* for 48 hours. Cells were collected after treatment and washed with 1X PBS, centrifuged at 3000 rpm for 3 minutes and the resultant pellets were re-suspended in 10 µm of rhodamine 123 in supplemented DMEM for 30 minutes. The cells were thereafter centrifuged at 3000 rpm for 3 minutes and pellets were resuspended in 500 µl PBS for measurement of fluorescent intensity using the BD Accuri C Sampler Flow Cytometer.

Statistical analysis

Data generated from this study was expressed as means ± standard error of mean (SEM) of three independent experiments and analyzed using the GraphPad Prism 6 software. Groups were compared using the t-test and level of significance was set at $P \leq 0.05$.

RESULTS

S. frutescens inhibits cell viability in SKNBE(2) and SHSY5Y neuroblastoma cells

In this study, the effect of the aqueous extract of *S. frutescens* on cell viability in SKNBE(2) and SHSY5Y neuroblastoma cells was determined using the MTT assay. Cells were plated in 96 well plates and exposed to increasing concentrations (1 to 5 mg/ml) of the extract for 48 hours after which MTT assays were performed. Results show that on both SKNBE(2) and SHSY5Y cells, *S. frutescens* induced a dose-dependent reduction in cell viability when compared to the control. Indeed the IC_{50} values obtained from sigmoidal plots were 2.3 mg/ml and 2.2 mg/ml for SKNBE(2) and SHSY5Y cells respectively (Figure 1 A and B). Furthermore, the selectivity of *S. frutescens* to cancer cells was next determined. The non-tumourigenic fibroblast KMST6 cells were exposed to concentrations of *S. frutescens* as same as those used for the neuroblastomas. Figure 1C shows that the KMST6 cells were less sensitive to the extract and at 5 mg/ml concentration cell survival was 50.9%. Taken together these results show that whereas *S. frutescens* inhibited cell viability in the neuroblastoma cells, it exhibited less cytotoxic activity in the non-cancerous KMST6 cells.

S. frutescens inhibits cell survival in SKNBE(2) neuroblastoma cells

The clonogenic assay was performed to determine the impact of *S. frutescens* treatment on the long-term survival of SKNBE(2) as it assesses the reproductive capacity of cells after treatment. Figure 2 shows that both the half IC_{50} (1.15 mg/ml) and IC_{50} (2.3 mg/ml) of *S. frutescens* significantly reduced colony formation in a

dose-dependent manner in SKNBE(2) cells (Figure 2 A). When colony areas were quantified with the control set at 100%, results obtained show that the colony area reduced to 32.1% and 7.9% for half IC_{50} and IC_{50} respectively (Figure 2B). Together, these results suggest that *S. frutescens* mitigates survival of malignant neuroblastoma cells.

Figure 2. *S. frutescens* inhibits colony formation. (A) *S. frutescens* shows a dose-dependent reduction in colony formation in SKNBE(2) cells following staining of cells with crystal violet. (B) Quantification of colony area using Image J. Bars represent means ± SEM. *$p \leq 0.05$.
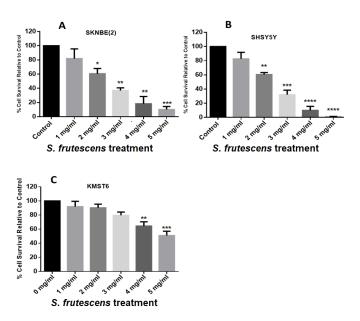

Figure1. *S. frutescens* inhibits cell viability cell viability in neuroblastoma cells. *S. frutescens* induced a dose-dependent response in (A) SKNBE(2) (B) SHSY5Y and (C) KMST6. Bars represent means ± SEM of three independent experiments performed in quadruplicate wells. *$p \leq 0.05$.

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S. frutescens activates Caspase-9 activity in neuroblastoma cells

S. frutescens has previously been reported to induce cytotoxicity and inhibit cell viability in cancer cells via apoptosis (Chinkwo, 2005, van der Walt et al., 2016). Caspases are known as the molecular machinery that

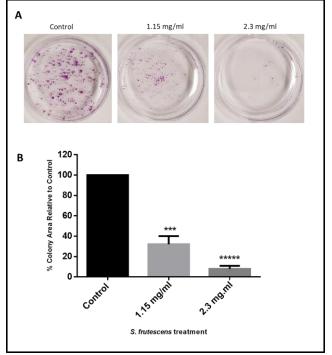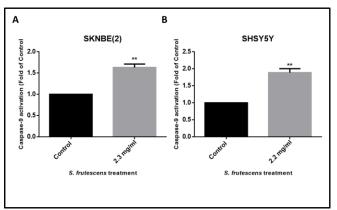drives apoptosis and caspase-9 activation is widely reported to be involved in the cascade of events in the mitochondrial apoptotic pathway (Wang and Xu, 2005, Shao et al., 2014). In this study, Caspase-9 activity was investigated in neuroblastoma cells treated with *S. frutescens*. Results show that *S. frutescens* significantly induced apoptosis in SKNBE(2) and SHSY5Y neuroblastoma cells by the increase in caspase-9 activity when compared to treated cells (Figure 3). Altogether, findings from this study suggest that apoptosis is involved in the cytotoxicity induced by *S. frutescens* in both neuroblastoma cell lines.

S. frutescens increases intracellular ROS generation in neuroblastoma cells

Excessive ROS production is widely known to induce cytotoxicity in cells and is largely due to an imbalance between ROS production and intracellular antioxidant defence mechanisms (Schumacker, 2006). To understand the apoptotic mechanism induced by *S. frutescens* in SKNBE(2) and SHSY5Y neuroblastoma cells, the level of intracellular ROS was investigated as previously described. The SKNBE(2) and SHSY5Y cells were treated with 2.3 and 2.2 mg/ml concentrations of *S. frutescens* respectively and stained with DCFH-DA. Results show that *S. frutescens* treatment on both

cell lines caused a shift in fluorescence to the right when compared to the control, indicating increased accumulation of intracellular ROS (Figure 4A and B).

Fig. 3: *S. frutescens* activates Caspase-9 activity**.** SKNBE(2) (A) and SHSY5Y (B) treated with 2.3 and 2.2 mg/ml of *S. frutescens* respectively for 48 hours showed a significant activation of Caspase-9 when expressed as fold of control. Each bar represents mean ± SEM.*$p \leq 0.05$*.

When the mean fluorescence intensity of treated cells was expressed as fold of control, SKNBE(2) and SHSY5Y treated cells showed an increase in fluorescence intensity (Figure 4 C and D). Taken together, these results suggest that *S. frutescens* treatment led to the accumulation of intracellular ROS in neuroblastoma cells and may in part contribute to the apoptosis observed in the cells.

Figure 4. *S. frutescens* induces Intracellular ROS accumulation. Representative flow cytometric profile of SKNBE(2) (A) and SHSY5Y (B) treated with 2.3 and 2.2 mg/ml of *S. frutescens* respectively for 48 hours. The black histogram shows the profile of control cells and coloured histogram shows the profile of treated cells. The mean fluorescence intensity of treated cells was expressed as fold of control (C) SKNBE(2) and (D) SHSY5Y. Each bar represents mean ± SEM. *$p \leq 0.05$*.

S. frutescens induces loss of MMP in neuroblastoma cells
Mitochondrial function is highly critical for cell survival, and an interruption of MMP is known to induce apoptosis in cells via the mitochondrial apoptotic pathway (Shao et al., 2014). Following exposure to *S. frutescens*, SKNBE(2) and SHSY5Y neuroblastoma cells were stained with the rhodamine 123 fluorescent probe, and MMP was measured using the flow cytometer. Figures 5 A and B show a reduction in fluorescence intensity in the *S. frutescens*-treated cells as evident by a shift in fluorescence to the left when compared to the control. Furthermore, the mean

fluorescence intensity (expressed as fold of control) was much lower in both SKNBE(2) and SHSY5Y cells (Figures 5 C and D). Taken together, these results indicate that treatment with *S. frutescens* led to a disruption of MMP in neuroblastoma cells and might play a role in the cytotoxicity observed in these cells.

Figure 5. *S. frutescens* disrupts MMP. Flow cytometric assay of MMP changes detected with Rhodamine 123 dye in SKNBE(2) (A) and SHSY5Y (B) treated with 2.3 and 2.2 mg/ml of *S. frutescens* respectively for 48 hours. The change in the fluorescence intensity of Rhodamine 123 indicated the loss of MMP and the mean fluorescence intensity of treated cells were expressed as fold of control (C) SKNBE(2) and (D) SHSY5Y. Each bar represents mean± SEM. *$p \leq 0.05$*.

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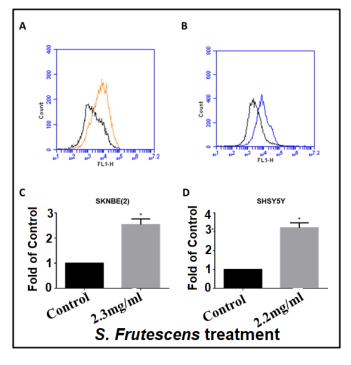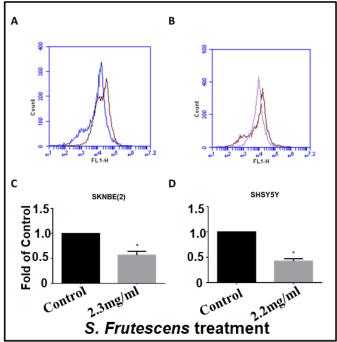
Fig.4: *S. frutescens* induces Intracellular ROS accumulation. Representative flow cytometric profile of SKNBE(2) (A) and SHSY5Y (B) treated with 2.3 and 2.2 mg/ml of *S. frutescens* respectively for 48 hours. The black histogram shows the profile of control cells and coloured histogram shows the profile of treated cells. The mean fluorescence intensity of treated cells was expressed as fold of control (C) SKNBE(2) and (D) SHSY5Y. Each bar represents mean ± SEM. *$p \leq 0.05$. **Fig.4:** *S. frutescens* induces Intracellular ROS accumulation. Representative flow cytometric profile of SKNBE(2) (A) and SHSY5Y (B) treated with 2.3 and 2.2 mg/ml of *S. frutescens* respectively for 48 hours. The black histogram shows the profile of control cells and coloured histogram shows the profile of treated cells. The mean fluorescence intensity of treated cells was expressed as fold of control (C) SKNBE(2) and (D) SHSY5Y. Each bar represents mean ± SEM. *$p \leq 0.05$.

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evident by a shift in fluorescence to the left when compared to the control. Furthermore, the mean fluorescence intensity (expressed as fold of control) was much lower in both SKNBE(2) and SHSY5Y cells (Figures 5 C and D). Taken together, these results indicate that treatment with *S. frutescens* led to a disruption of MMP in neuroblastoma cells and might play a role in the cytotoxicity observed in these cells.

DISCUSSION

S. frutescens is an indigenous plant of South Africa and has been of benefit to many cultures with its origin linked to the Khoi people of South Africa. It has been used in ancient times for various disease conditions including internal cancers (Van Wyk and Albrecht, 2008). Previous studies have confirmed that this plant has potent anti-cancer properties in breast, ovarian, oesophageal, cervical and leukaemia cancerous cells (Tai et al., 2004, Chinkwo, 2005, Stander et al., 2009, Skerman et al., 2011). In the present study, the cytotoxic effect of *S. frutescens* was investigated in SKNBE(2) and SHSY5Y neuroblastoma cells and results obtained show that the aqueous extract of this plant induced dose-dependent cytotoxicity in both these cell lines but was selectively less sensitive to the non-cancerous KMST6 cells. Findings from the clonogenic assay showed that *S. frutescens* inhibited cell proliferation and survival as evident by the reduction in colony area in the treated cells when compared to the control, thus suggesting a loss of reproductive capacity after treatment. These findings are supportive of the cytotoxicity results.

Apoptosis, or programmed cell death, plays an essential role in regulating a broad range of biological processes, such as cell differentiation and proliferation, development, immunity, and tissue homeostasis and

dysregulation of apoptosis is associated with a variety of human diseases, including cancer, autoimmune diseases, infections, and neurodegenerative diseases (Fuchs and Steller, 2011, Han and Ravichandran, 2011, Portt et al., 2011). Apoptosis is the most important therapeutic target and the most common mechanism by which chemotherapeutic agents induce death in cancer cells to eradicate tumour tissue. The induction of apoptosis is regarded as the most effective strategy for eliminating cancer cells (Pore et al., 2013, Signore et al., 2013). The results of the present study show that *S. frutescens* induced apoptosis in both neuroblastoma cell lines possibly via the mechanisms of loss of MMP and accumulation of ROS both of which have been reported to play a role in the induction of apoptosis in cancer cells. The findings from this study align with previous studies which showed that the observed death of cancer cells following treatment with *S. frutescens* was mediated via apoptotic pathways (Stander et al., 2009, Skerman et al., 2011, van der Walt et al., 2016). The loss of MMP observed in both the SKNBE(2) and SHSY5Y cells in this study following treatment with *S. frutescens,* has been linked to cytotoxicity and apoptosis in cancer cells (Shao et al., 2014).

ROS are strongly involved in the regulation of many cellular functions and a moderate rise in ROS could result in enhanced cell growth and proliferation. However, overproduction of ROS beyond levels which endogenous antioxidants can cope with, will result in oxidative stress and cell death (Trachootham et al., 2009). Most tumour cells have been reported to contain high levels of ROS and as such rely on endogenous antioxidants to regulate redox balance in order to continue promoting cell survival (Wang et al., 2017). Thus, a fragile imbalance in ROS-antioxidant levels could be a possible target for anti-cancer agents (Raj et al., 2011). Medicinal plants and plant-derived natural products have been previously reported to induce cytotoxicity by promoting oxidative stress in cancer cells and depleting endogenous antioxidants (Srinivas et al., 2004, Badmus et al., 2015). In the current study, treatment with *S. frutescens* resulted in the accumulation of intracellular ROS in SKNBE(2) and SHSY5Y neuroblastoma cells, which could possibly have contributed to the observed cytotoxicity. Furthermore, an increase in ROS accumulation could trigger a disruption in mitochondrial function (Ishikawa et al., 2008).

The biological activity of *S. frutescens* has previously been attributed to its chemical composition including its large amounts of amino acids (notably l-arginine and l-canavanine), pinitol, flavonol glycosides and triterpenoid saponins (Moshe et al., 1998, Van Wyk and Albrecht, 2008, Shaik et al., 2011). The presence of the compound l-canavanine and its major metabolite canaline in *S. frutescens* could be among the contributing factors to its *in vitro* anti-proliferative and apoptotic activity (Chinkwo 2005). Both l-canavanine and canaline have previously been reported to exert anti-cancer properties (Tai et al., 2004).

CONCLUSION

This study investigated the cytotoxic and apoptosis-inducing effects of *S. frutescens* in SKNBE(2) and SHSY5Y neuroblastoma cells. Findings show *S. frutescens* differentially induced cytotoxicity on cancerous and non-cancerous cells and the underlying mechanism was associated with apoptosis mediated via the ability of this plant extract to increase the accumulation of ROS and a loss of MMP in these cells. Studies to isolate the bioactive components present in the aqueous extract of *S. frutescens* responsible for eliciting the reported effects need to be done. In addition, more molecular studies to elucidate other possible mechanisms involved in *S. frutescens*-induced cell death need to be done.

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