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# APOPTOSIS IN THE HUMAN LARYNGEAL CARCINOMA (HEp-2) CELL LINE BY *BULBINE NATALENSIS* AND *B. FRUTESCENS* FRACTIONS

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**ABSTRACT**

Many plants that belong to the genus *Bulbine* have compounds in their roots and leaves which are considered important for traditional treatments. The stems and roots of *Bulbine* species are believed to contain anticancer compounds such as anthraquinones, including chrysophanol and knipholone. However, in general, people utilise plants of the *Bulbine* genus for the treatment of rashes, itches, wounds, burns, cracked lips and cracked skin. This study assessed the effect of aqueous and organic fractions of *Bulbine natalensis* and *Bulbine frutescens* on the human laryngeal carcinoma cell line (HEp-2) for apoptosis. The MTT assay was used to determine the cytotoxicity of the fractions administered and to select fractions for analysis of *bax* and *caspase-3* gene expression, which are induced during programmed cell death type 1, known as apoptosis. All of the *B. natalensis* fractions induced expression of *caspase-3*, while the tested *B. frutescens* aqueous root fractions failed to induce expression of *caspase-3*. The variation in *bax* gene expression indicated that HEp-2 cell death was due to apoptosis and other unknown forms of cell death that may or may not activate *caspase-3* gene expression..

**Key Words**: Apoptosis, *bax* and *caspase-3* markers, cytotoxicity, autophagy.

## INTRODUCTION

Apoptosis, or programmed cell death, is a process by which old or infected cells are destroyed. Unlike in the past, currently, many forms of cell death are grouped under the term 'apoptosis' and even natural cell death or necrosis, which occurs naturally in the mammalian body, has begun to fall under this group. However, apoptosis is triggered when the nutrient supply to old or infected cells are inadequate or when those cells experience conflicting signals due to an inadequate supply of growth factors or mitogens, for example. The first scientist to have discovered apoptosis was Flemming who, in 1885, worked with rabbit Graafian follicles, and then again observed this type of cell death in testicular germ cell populations in 1887 (Flemming W, 1885). By the end of his lifetime, he discovered the current universally-accepted morphological features of apoptosis, such as the collapse of the cell, protein degradation and DNA fragmentation. However, molecular characterisation of this process was pivotal if one was to understand apoptosis completely, and Ellis and co-workers conclusively demonstrated apoptosis in the nematode, *Caenorhabditis elegans* in 1991, where they had characterised the key genes involved in apoptosis (Ellis RE *et al*., 1991). Today, these genes have become well-known to scientists and in addition to those apoptotic markers, other genes are currently being identified. The *p53*, *bax*, *bcl-2*, and *caspase-3* genes can be considered the most widely studied apoptotic markers because they provide information about the three mechanisms of apoptosis activation (Culotta E and Koshland DE, 1993; Ellis RE *et al*., 1991). *p53* is the initiator gene for apoptosis, *bax* is the

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death-promoting gene, *bcl-2* is the death-inhibiting gene or the *bax* gene antagonist, while *caspase-3* is the hallmark indicator of apoptosis (Green D and Kroemer G, 1998), i.e., it indicates optimal functioning of all genes in the apoptotic pathway should its gene be detected because it is the last gene to be expressed in apoptosis. The latter provides evidence of the morphology of apoptosis described by Flemming (Flemming W, 1885).

All three mechanisms/pathways of apoptosis, namely the receptor-ligand, mitochondrial and endoplasmic reticulum, involve these genes (Hengartner MO, 2000; Holdenrieder S and Stieber S, 2004). When infected cells like cancer, are exposed to apoptotic stimuli, e.g., an anticancer drug or plant extract, the apoptotic mechanism triggered varies, but not much research has been done to distinguish the mechanisms of apoptosis activated when such cells undergo chemical or artificial stress. All the information available on apoptotic mechanisms infer that a mechanism is activated when cells are treated with apoptotic stimuli, without specifying which mechanism in particular.

Apoptosis has been studied in a variety of cell lines and/or cell types, including polymorphonuclear neutrophils (PMN) and smooth muscle cells (SMCs); and K562 (liver carcinoma), HEp-2 (laryngeal carcinoma), A549 (lung carcinoma), and HeLa (cervical carcinoma) cell lines. These cell lines and types have been subjected to apoptosis by a variety of compounds which include, alkaloids of *Catharanthus vinca, Casimiroa edulis* and *Pachysandra procubens* (Ito A *et al*., 1998), flavonoids of *Chorizantha diffusa* (Chung HS *et al*., 1999), the withinolides of *Physalis philadalphica* (Kennelly EJ *et al*., 1997) as well as a range of plant extracts that are used by herbal and traditional healers (Cui B *et al*., 1997; Long L *et al*., 1997; Kinghorn AD *et al*., 1999). The extracts are made using different extraction methods because of the influence of the extraction method on the extract composition, e.g., an extract that is neither polar or non-polar will be prepared effectively in alcohol (e.g. methanol, ethanol, pentanol etc.) because alcohol has polar and non-polar properties. In addition to these cancer chemoprotective agents, several plant families possess active compounds against cultured human cancer cells, *viz.* Maliaceae, Asteraceae (Cui B *et al*., 1997), Euphorbiaceae (Long L *et al*., 1997) and Dipterocarpaceae (Kinghorn AD *et al*., 1999). Within the family Asphodelaceae, research about the anticancer potential of *Bulbine* spp. plant extracts/fractions are ongoing as it is believed that these plants display anticancer potential since they belong to the aloe family, but there has been no properly recorded information about the chemotherapeutic property of *Bulbine* spp. In South Africa, both species are used to treat mosquito bites, blisters, cold sores, pimples, mouth ulcers, cracked skin, bruises, sunburns particularly by traditional healers (Coopoosamy RM, 2011). *B. frutescens* has been commonly known to be used in the treatment of diarrhoea and is less popular compared to *B. natalensis* (Coopoosamy RM, 2011) while *Bulbine natalensis* has been found to raise testosterone levels in men (Roberts A, 2012).

In this study we investigated apoptosis in the human laryngeal carcinoma cell line, HEp-2, by *B. natalensis* aqueous and two organic crude fractions using reverse transcriptase-polymerase chain reaction (RT-PCR). Fractions of *B. frutescens* were selected and compared for anticancer potential relative to those for *B. natalensis.* We found that *B. natalensis* had a greater cytotoxic effect on the HEp-2 cancer cell line compared to *B. frutescens.* Variable responses with regard to the mechanism of activation of apoptosis were observed by analysis of *bax* and *caspase-3* gene expression. These findings may have possible implications for the chemopreventive activities (current drug targets and mode of action) of *Bulbine* spp.

## MATERIALS AND METHODS

### *Cell line*

The adherent human laryngeal carcinoma cell line HEp-2 (Highveld Biological) was used for this study. The cells were grown in Dulbecco's Modified Eagles Medium (DMEM) (Highveld Biological) supplemented with 5% foetal calf serum (GIBCO, Invitrogen) and 1% penicillin/streptomycin solution (GIBCO, Invitrogen) at a temperature of 37$^{o}$C in a humidified incubator with a 5% $CO_2$ atmosphere.

### *Plant material*

The plants used in this study were *Bulbine natalensis* and *Bulbine frutescens*. These plants were collected from the Durban area, and identified by botanist, Professor Himansu Baijnath (University of KwaZulu-Natal, Durban).

The leaves, fibrous roots and corm of *B. natalensis* were separated. For *B. frutescens*, the leaves and roots (with stem portions) were separated. The organs from both plant species were washed thoroughly, rinsed and air-dried.

### *Preparation of Bulbine* spp. *fractions*

Three types of plant fractions from each *Bulbine* spp. were prepared, namely, aqueous, 50% ethanol and 100% ethanol. The plant material was blended into a powder. For the aqueous plant fractions, 25 g of powdered plant material was added to 200 ml of distilled water and shaken at 160 rpm for 24 - 48 h at 37$^{o}$C in a shaking incubator (Infors HT Ecotron). After agitation, the contents of the flask were filtered through a 11 µm filter disk (Whatman No. 1, UK). The filtrate was incubated in a biofreezer at -70$^{o}$C (Snijder Scientific) for 24 h and then freeze-dried (Virtis) for 24 - 48 h. One mg of the freeze-dried aqueous extract was dissolved in 1 ml of 10 mM plant tissue culture grade dimethyl sulfoxide (DMSO)

(Sigma, South Africa), yielding a concentration of 1000 µg/ml.

For the organic solvent fractions, 25 grams of powered plant material was resuspended in 100 ml of the solvent. The 100% ethanol fractions were left open to allow the ethanol to evaporate completely. For the 50% ethanol extraction, the fraction was rotatory evaporated (after being shaken for 24 - 48 h in a shaking incubator at 37$^{o}$C) (Heidolph) to remove the ethanol, followed by freezing and freeze-drying as described for the aqueous extraction.

***Treatment of cells with plant extracts***

*Bulbine* fractions from each organ was dissolved in 10 mM DMSO (Sigma, South Africa) and was stored in dark-coloured eppendorf tubes at 4$^{o}$C as a stock solution. From the stock concentration (1 mg/ml), a 10-fold and thereafter a 2-fold dilution from the 10-fold dilution was made to prepare 100 µg/ml and 50 µg/ml solutions. This was performed for each of the prepared fractions. The dilutions were performed in DMSO. DMSO was used for the dilutions because it is a supersolvent (Anonymous 1) known to dissolve plant extracts and fractions completely. Although the aqueous plant extracts were soluble in water, DMSO was used to dissolve them to keep all of the experimental parameters in terms of preparing the plant extracts and fractions consistent. Furthermore, administering an extract dissolved in water would have altered the concentration of the HEp-2 cell culture volume i.e. making it more dilute relative to the DMSO-dissolved organic fractions administered. Since the cell culture volume would have been more dilute, it could result in the HEp-2 cell line acquiring more resources from the medium due to their internal hypertonic state. In order to avoid this, and uncontrollable cell death, the aqueous extracts were not dissolved in water (Becker WM *et al*., 2003).

The cells were exposed to each *Bulbine* fraction at 3 different final concentrations (20 µg/ml, 2 µg/ml and 1 µg/ml) and for two different time durations, 5 and 8 days. The amount of fraction to which the cells could be exposed to for each concentration was calculated by taking into account that DMSO was toxic to the cells at a concentration greater than 200 µM. Therefore the fractions were administered at this concentration and the concentration of *Bulbine* spp. used were 20, 2 and 1 µg/ml. HEp-2 cells that were grown in media containing 200 µM (2%) DMSO without fractions served as the negative control (this result was obtained by performing an optimisation experiment involving 200 µM, 500 µM and 1 mM DMSO concentrations).

***Cell death assay***

The cytotoxicity of the plant fractions were assessed by the MTT (3,4,5-dimethylthiazol-2-yl)-2-5-diphenyltetrazolium bromide) cytotoxicity assay, which indicates cell death by turning yellow MTT into a purple formazan product. This reaction is catalysed by the enzyme, mitochondrial succinate dehydrogenase (Mossman T, 1983). Cells ($\pm 1 \times 10^5$) were plated in two 96-well plates (Coastar from Corning). After 24 h they were treated with the 3 different concentrations of each fraction prepared (20, 2 and 1 µg/ml). After 5 and 8 days, each plate was assessed for cytotoxicity. Twenty microlitres MTT (5 mg/ml in PBS) (Invitrogen) was added to each well of the 96-well plate and incubated for 4 h at 37$^{o}$C in a humidified incubator with a 5% $CO_2$ atmosphere (Snijder Scientific). One hundred microlitres of 1% DMSO was added to each well and incubated for a further hour to dissolve the formazan crystals. The amount of formazan was determined by measuring the absorbance at 450 nm (EZ4U, 2000) using an ELISA plate reader (BioHit). The HEp-2 cell line with a passage number of 3 number were used in the cytotoxicity assay and gene expression study, and they were only used once flasks were 80 - 90% confluent.

The equation for calculating % cytotoxicity or % HEp-2 cell death

$$\% \text{ cytotoxicity} = 100 - \left( \frac{\text{Abs of sample}}{\text{Abs of DMSO control}} \times 100 \right)$$

***Gene expression studies***

Two apoptotic genes, *bax* and *caspase-3* were studied using reverse transcriptase-PCR (RT-PCR). The housekeeping gene *β-actin* was used as a control. Reverse transcription was performed using the Cells-to-cDNA Kit (Ambion, Inc, Austin, Texas) in a total reaction volume of 25 µl. Based on an optimisation experiment, 5 µl of RT product was used in PCR reactions. PCR was carried out using gene-specific upstream and downstream primers purchased from Integrated DNA Technologies, USA (see Table 1 for primer sequences). Reverse transcription at 42$^{o}$C for 15 minutes was followed by denaturation at 94$^{o}$C for 2 minutes, annealing at 55$^{o}$C for 30 seconds, and extension at 72$^{o}$C for 30 seconds in the thermocycler (GeneAmp PCR System 9700, Applied Biosystems). The number of cycles for *β-actin*, *bax* and *caspase-3* genes was 35 and this was followed by a final extension of 72$^{o}$C for 5 minutes. PCR products were separated on a 0.8% agarose gel (SeaKem® LE Agarose, Lonza Rockland, USA) and stained with ethidium bromide.

**RESULTS**

***Optimisation of dose and time response of Bulbine* spp. *fractions on the HEp-2 cell line using the MTT cytotoxicity assay***

***Optimisation of DMSO concentration***

Before optimisation of dose and time response of the two *Bulbine* spp. fractions on the HEp-2 cell line, it was important to eliminate the effect of DMSO on the treated HEp-2 cell line. This was considered important so that the effects of the fractions were obtained, and not the

toxic effect of DMSO. The concentration of DMSO that was not lethal to the HEp-2 cell line was obtained by performing an optimisation experiment that involved using DMSO at 3 different concentrations (200 µM, 500 µM and 1000 µM) in the MTT cytotoxicity assay.

Across all of the concentrations or doses of DMSO administered to the HEp-2 cell line, the 200 µM DMSO concentration caused the smallest percentage of cell death after 5 days of incubation. The 16.18% cytotoxicity obtained for the 200 µM DMSO concentration confirmed existing reports that DMSO is toxic to cells and can cause cell death or show higher percentages of cell death when plant fractions are administered to cells by accelerating the cell death process. This has made studies involving apoptosis, that are specifically about medicinal compounds inconclusive.

The 500 µM and 1000 µM DMSO concentrations had a higher lethal effect on the HEp-2 cell line, as evidenced by their cytotoxicity percentages of 51.91 and 46.15%, respectively. Based on these results the 16.18 % cytotoxicity for the 2% DMSO concentration was considered acceptable and was used as the negative control in all of the MTT cytotoxicity studies involving the standards (camptothecin and calpain I inhibitor) and *Bulbine* spp. fractions.

***Optimisation of dose and time response of Bulbine spp. fractions***

Cytotoxicity on HEp-2 cell line treated with crude fractions of root, leaf and corm of *Bulbine natalensis* and *Bulbine frutescens* for five and eight days was measured and is presented in Figures 1-4.

The average percentage cytotoxicity of mean triplicates for the positive control, camptothecin and the apoptotic pathway genetic control, calpain I inhibitor were 11.42 and 2.12%, respectively. The 2 and 1 µg/ml 100% ethanol corm fractions exhibited the highest level of cytotoxicity on the HEp-2 cell line. These fractions had cytotoxicities of 22.56 and 20.69%, respectively. The corm 20 µg/ml aqueous fraction had the lowest killing effect on the HEp-2 cell line, with a cytotoxicity of -4.09% relative to the other corm fractions. The leaf fractions had killing and growth enhancing potential on the HEp-2 cell line after 5 days of incubation. The 2 µg/ml aqueous fraction for the leaf showed the maximum HEp-2 cell line death potential (16.47%) of the leaf fractions only. All of the tested root fractions had a proliferative effect on the HEp-2 cell line, with the 2 µg/ml fraction having the lowest percentage of cytotoxicity (-18.86%).

The average percentage cytotoxicity of mean triplicates of all the *B. frutescens* crude fractions had not conformed to any of the aqueous root fractions for *B. natalensis* (Figure 1) since all of the fractions for *B. frutescens* had negative cytotoxicity values. Camptothecin displaying a cytotoxicity of 10.7% and this provided evidence that the assay was being conducted was feasible since camptothecin is an anti-cancer/apoptotic control. Calpain I inhibitor, on the other hand, stimulated the proliferation of HEp-2 cell line after 5 days of incubation. Overall, the root 100% ethanol fractions had the lowest cytotoxicity levels. The root 1 µg/ml 100% ethanol fraction had the least cytotoxic effect on the HEp-2 cell line (-23.2%) across all tested fractions. The leaf 2 µg/ml 100% ethanol fraction had the smallest negative cytotoxicity percentage (-0.83%).

The average percentage cytotoxicity of mean triplicates for all *Bulbine natalensis* crude fractions, after 8 days of incubation, were negative i.e. the fractions all promoted HEp-2 cell line proliferation. The controls, camptothecin and calpain I inhibitor were not cytotoxic against the HEp-2 cell line and they has cytotoxicity percentages of -0.89 and -3.39%, respectively. The 1 µg/ml 100% ethanol fraction for corm was the only fraction that was cytotoxic to the HEp-2 cell line after 8 days of incubation (5.51% cytotoxicity). In comparison to the 5 day results (Figure 1), all of the fractions except for the 1 µg/ml 100% ethanol corm fraction, was not cytotoxic against HEp-2. Between 5 and 8 days, there was a further decrease in cytotoxicity of the 20 µg/ml aqueous fraction for corm and the 2 µg/ml 50% ethanol fraction for the leaf, from -4.09 to -11.53% and -0.88 to -14.31%, respectively. The 2 and 1 µg/ml aqueous fractions for the roots gained cytotoxic potential against the HEp-2 cell line between 5 and 8 days. The cytotoxicity values for the 2 µg/ml fraction gained cytotoxic potential on the 8$^{th}$ day (-11.18%) compared to the on the 5$^{th}$ day (-18.86%). Similarly, for the 1 µg/ml fraction the recorded percentage cytotoxicity on the 8$^{th}$ day was -4.01% which was lower than the 5 day cytotoxicity reading (-11.02%).

A similar cytotoxicity trend was observed on the 8th day for *B. frutescens* when compared to the 5 day cytotoxicity results. The samples that initially (5 day results; Figure 2) had the lowest HEp-2 cell proliferative percentages, namely, the leaf 2 µg/ml 100% ethanol fraction, the root 20 µg/ml 50% ethanol fraction, as well as the root 1 µg/ml 50% ethanol fraction, showed a further decline in cytotoxicities on the 8th day. These values were, from -0.83 to -14.41%, from -6.06 to -11.1% and, from -2.38 to -21.76% respectively. Camptothecin and calpain I inhibitor had cytotoxicity values of -4.61 and -5.83%, respectively.

**Selection of *Bulbine* spp. fractions for expression of apoptotic genes**

*Bulbine natalensis* fractions showed the greatest number of positive results for % cytotoxicity against the HEp-2 cell line (as shown in Figure 1) for the 5 day-incubated MTT plates compared to *B. frutescens* fractions. 14 samples in total had positive cytotoxicity results. The three aqueous extract fractions of *B. natalensis* (20, 2 and 1 µg/ml), the leaf 1 µg/ml 50% ethanol fraction and the leaf

2 µg/ml 100% ethanol fraction had a negative cytotoxicity result.

For the 8 day *B. natalensis* plates (Figure 2), only the corm 1 µg/ml 100% ethanol fraction showed some cytotoxic effect against the HEp-2 cell line. Cytotoxicity of all *B. frutescens* fractions (Figures 3 and 4) were negative by day 8.

In order to conserve cost effectiveness, fractions of different concentrations were selected for the gene expression study. Overall, the corm fractions for the 5 day MTT plates had the best cytotoxic potential on the HEp-2 cell line, with an exception of the corm 20 µg/ml aqueous fraction. Based on this result, the 2 and 1 µg/ml fractions were not chosen for the gene expression study. Instead, the 20 and 1 µg/ml fractions were chosen, after looking at the overall 5 day cytotoxicity results for both *Bulbine* spp. The negative corm result was used to validate bax and caspase-3 gene expression of other fractions having no cytotoxicity against the HEp-2 cell line. The corm 1 µg/ml *B. natalensis* aqueous fraction was selected over the 2 µg/ml fraction because they exhibited higher % cytotoxicity on the fifth day of reading the MTT results.

For *B. frutescens*, the leaf 20 and 2 µg/ml 100% ethanol fractions and the root 20 and 1 µg/ml 50% ethanol fractions had a positive proliferative effect on the HEp-2 cell line, with higher levels of proliferative activity exhibited by the HEp-2 cell line by the other *B. frutescens* fractions.

In order to meet the objectives of this study, and to make a comparison between *B. natalensis* and *B. frutescens*, the root 2 µg/ml aqueous root fraction of *B. natalensis*, which had a negative % cytotoxicity result and the leaf 20 µg/ml 100% ethanol fraction which had positive percentage cytotoxicity result were selected for comparison with the roots and leaves of *B. frutescens* aqueous and 100% ethanol fractions of the same concentration.

In addition, the root 2 µg/ml aqueous fraction and leaf 100 % ethanol fraction of *B. frutescens*, was selected for comparison with the root and leaf fractions of *B. natalensis* of the same concentrations. The percentage cytotoxicity results of the 2 µg/ml fraction that was selected for *B. frutescens*, had a less negative % cytotoxicity result compared to *B. natalensis*. The root fractions of *B. natalensis* had the highest negative % cytotoxicity. The *B. natalensis* aqueous leaf fractions were all positive for cytotoxicity. The 2 and 1 µg/ml *B. natalensis* aqueous leaf fractions had a % cytotoxicity that was higher compared to the 50 % ethanol corm fractions of the same species. The leaf 20 µg/ml fraction of *B. natalensis* had a lower percentage cytotoxicity compared to the 50 % ethanol corm fraction of the same concentration (see Table 1).

Overall, selection was performed in an ordered manner such that the fraction of *B. frutescens* that was selected for comparative purposes had the less negative cytotoxicity value of two concentrations (see Table 2).

***Gene expression results***

The HEp-2 cell line passage number used was 3. Total RNA was extracted from the HEp-2 cell line and then reversely transcribed by specific priming to single-stranded cDNA using the Cells-to-cDNA kit purchased from Ambion (Pty) Ltd. The cDNA was amplified by polymerase chain reaction (PCR). PCR products were electrophoresed on a 0.8% agarose gel, followed by soaking in ethidium bromide and visualisation under UV light. All the samples were run relative to a β-actin internal control.

Twelve of the fourteen samples tested displayed up-regulation/expression of the *caspase-3* gene. The samples that did not express the *caspase-3* gene were the 20 and 2 µg/ml *B. frutescens* root aqueous fractions (see K and L in Figure 5). The corm 20 and 1 µg/ml 50% ethanol fractions of *B. natalensis*, together with the leaf 20 and 2 µg/ml 100% ethanol fractions of *B. natalensis* and the leaf 20 µg/ml 100% ethanol fractions of *B. frutescens*, did not result in the expression the *bax* gene.

The *B-actin* control was expressed for 12 tested fractions as well as the 4 controls. The DMSO negative control did not show any bands for *bax* and *caspase-3* expression, while camptothecin and calpain I inhibitor expressed the *bax* and *caspase-3* genes. The reverse transcriptase negative control and negative template controls did not produce any bands.

**Table 1. Primer sequences used in RT-PCR**

| Primer | Forward | Reverse |
|---|---|---|
| ***β-actin*** (accession number ×00351) | 5'-CTGTCTGGCGGCACCACCAT-3' | 5'-GCAACTAAGTCATAGTCCGC-3' |
| ***bax*** (accession number L22473) | 5'-AAGCTGAGCGAGTGTCTCAAGCGC-3' | 5'-TCCCGCCACAAAGATGGTCACG-3' |
| ***caspase-3*** (accession number U26943) | 5'-TTTGTTTGTGTGCTTCTGAGCC-3' | 5'-ATTCTGTTGCCACCTTTCGG-3' |

**Table 2. Overview of fractions selected for RT-PCR: samples tested and comparisons made**

| Final concentration in cell volume (µg/ml) | Fraction | *Bulbine natalensis* organ | *Bulbine frutescens* organ |
|---|---|---|---|
| 20 | aqueous | roots | roots |
| 20 | aqueous | corm | no comparison |
| 20 | 50% ethanol | corm | no comparison |
| 20 | 100% ethanol | leaves | leaves |
| 20 | 100% ethanol | corm | no comparison |
| 2 | aqueous | roots | roots |
| 2 | 100% ethanol | leaves | leaves |
| 1 | aqueous | corm | no comparison |
| 1 | 50% ethanol | corm | no comparison |

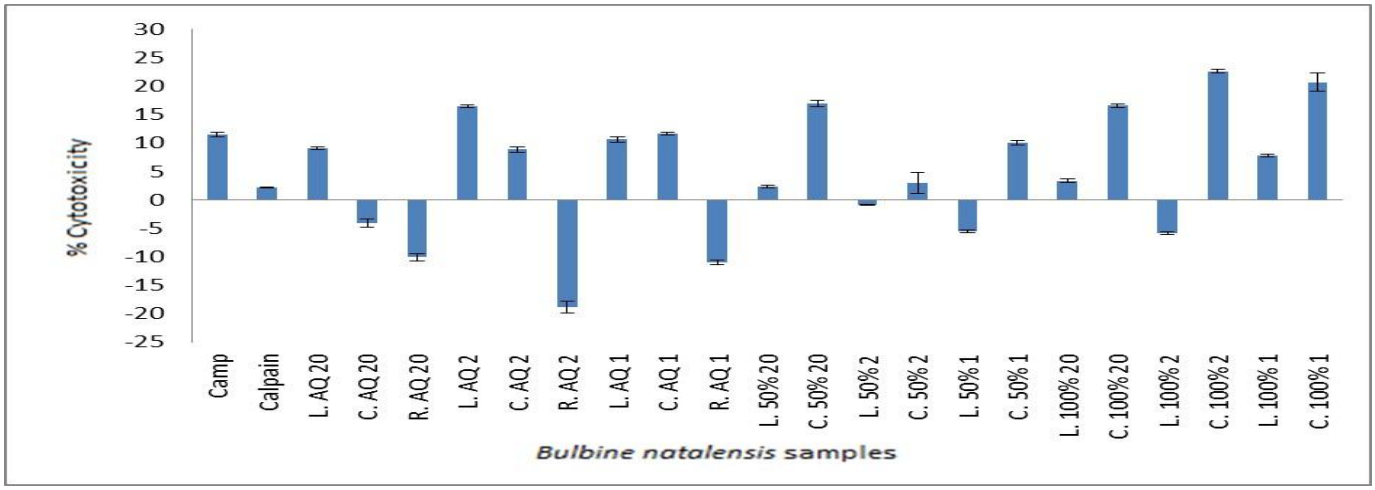



**Figure 1.** Cytotoxicity spectrum of aqueous and organic *Bulbine natalensis* crude fractions (leaf, roots and corm) on the HEp-2 cell line (5 day result). Cytotoxicity is derived from three experiments done in triplicate. The values are represented as mean ± S.D. The controls are: Camp: camptothecin and Calpain: Calpain I inhibitor. The samples are: C: corm, L: leaf, and R: roots. The solvents of extraction used are: AQ: Aqueous fraction, 50%: 50% ethanol fraction, and 100%: 100% ethanol fraction. The concentrations of each sample are: 20: 20 µg/ml, 2: 2 µg/ml, and 1: 1 µg/ml. Therefore, L.AQ20 is interpreted as being the 20 µg/ml leaf aqueous fraction for *Bulbine natalensis.*

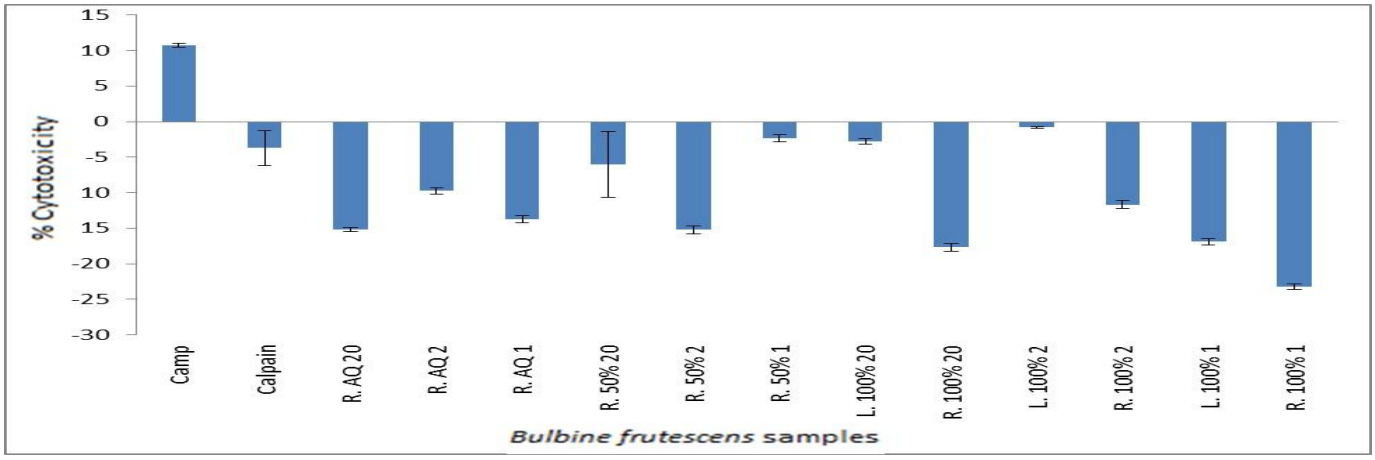



**Figure 2.** Cytotoxicity spectrum of aqueous and organic *Bulbine frutescens* crude fractions (leaf and roots) on the HEp-2 cell line (5 day result). Cytotoxicity is derived from three experiments done in triplicate. The values are represented as mean ± S.D. The controls are: Camp: camptothecin and Calpain: Calpain I inhibitor. The samples are: R: root and L: leaf. The solvents of extraction used are: AQ: Aqueous fraction, 50%: 50% ethanol fraction, and 100%: 100% ethanol fraction. The concentrations of each sample used are: 20: 20 µg/ml, 2: 2 µg/ml, and 1: 1 µg/ml. Therefore, R.AQ20 is interpreted as being the 20 µg/ml root aqueous fraction for *Bulbine frutescens.*

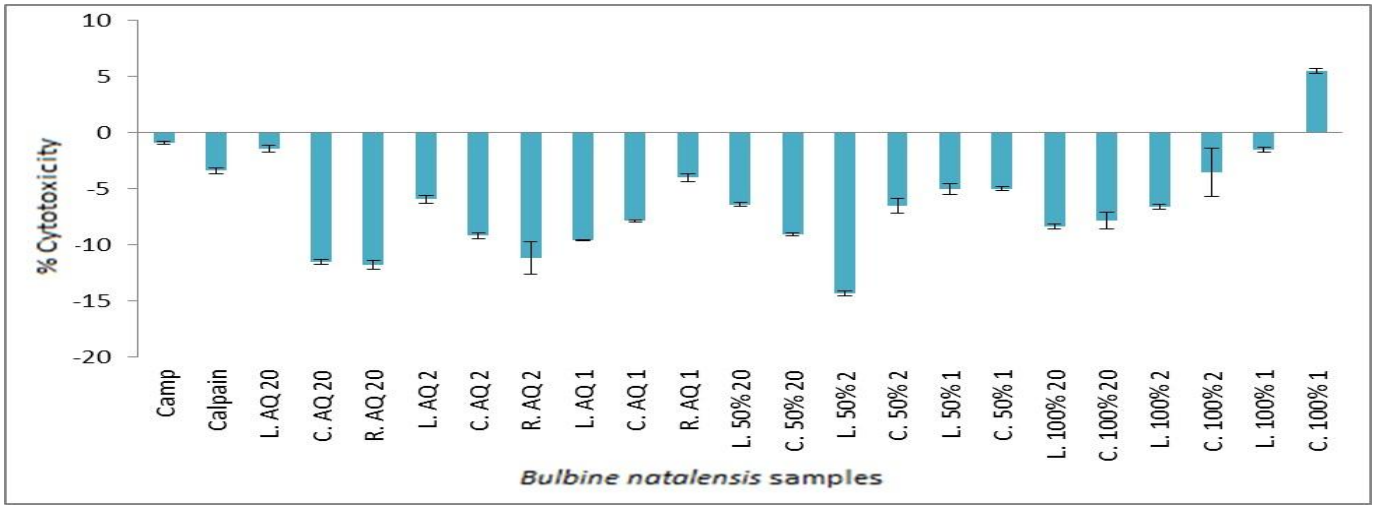



**Figure 3.** Cytotoxicity spectrum of aqueous and organic *Bulbine natalensis* crude fractions (leaf, roots and corm) on the HEp-2 cell line after 8 days. Cytotoxicity is derived from three experiments done in triplicate. The values are represented as mean ± S.D. The controls are Camp: camptothecin and Calpain: Calpain I inhibitor. The samples are: C: corm, L: leaf, and R: roots. The solvents of extraction are: AQ: Aqueous fraction, 50%: 50% ethanol fraction, and 100%: 100% ethanol fraction. The concentrations of each sample are: 20: 20 µg/ml, 2: 2 µg/ml, and 1: 1 µg/ml. Therefore, L.AQ20 is interpreted as being the 20 µg/ml leaf aqueous fraction for *Bulbine natalensis.*

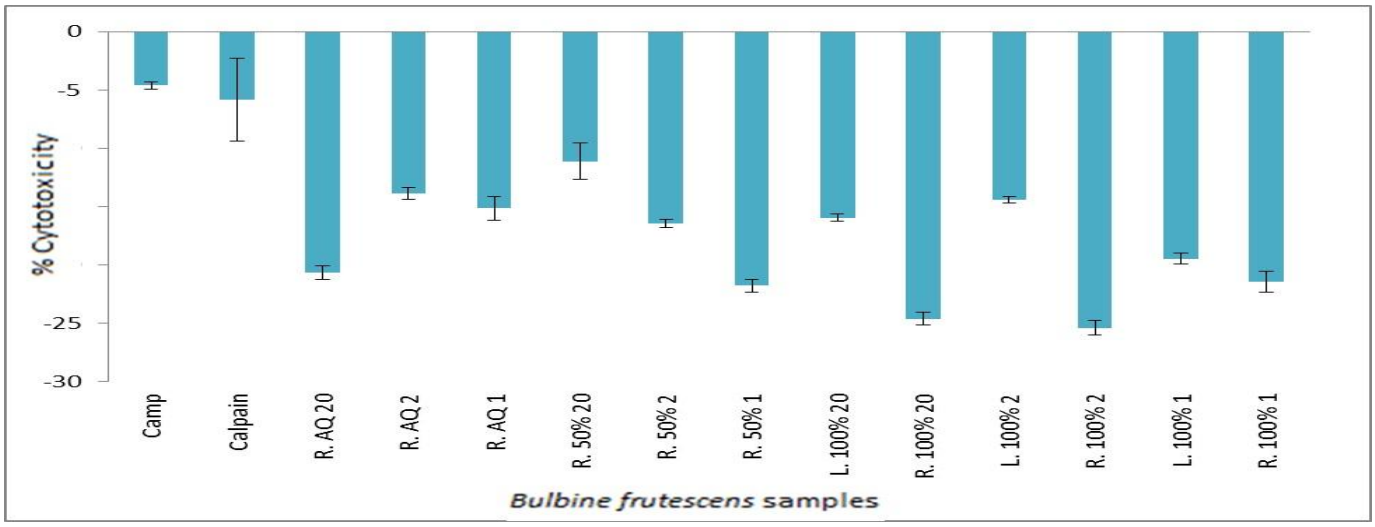
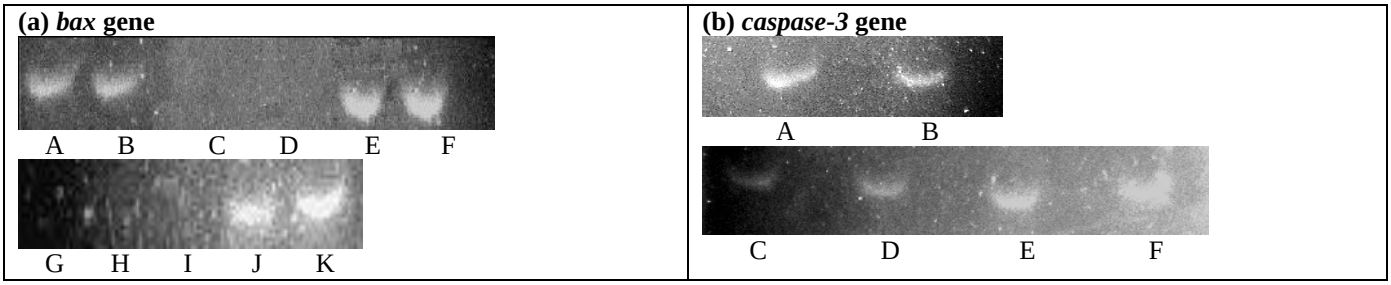



**Figure 4.** Cytotoxicity spectrum of aqueous and organic *Bulbine frutescens* crude fractions (leaf, roots and corm) on the HEp-2 cell line (8 day result). Cytotoxicity is derived from three experiments done in triplicate. The values are represented as mean ± S.D. The controls are: Camp: camptothecin and Calpain: Calpain I inhibitor. The samples are: R: root and L: leaf. The solvents of extraction used are: AQ: Aqueous fraction, 50%: 50% ethanol fraction, and 100%: 100% ethanol fraction. The concentration of each sample used are: 20: 20 µg/ml, 2: 2 µg/ml, and 1: 1 µg/ml. Therefore, R.AQ20 is interpreted as being the 20 µg/ml root aqueous fraction for *Bulbine frutescens.*



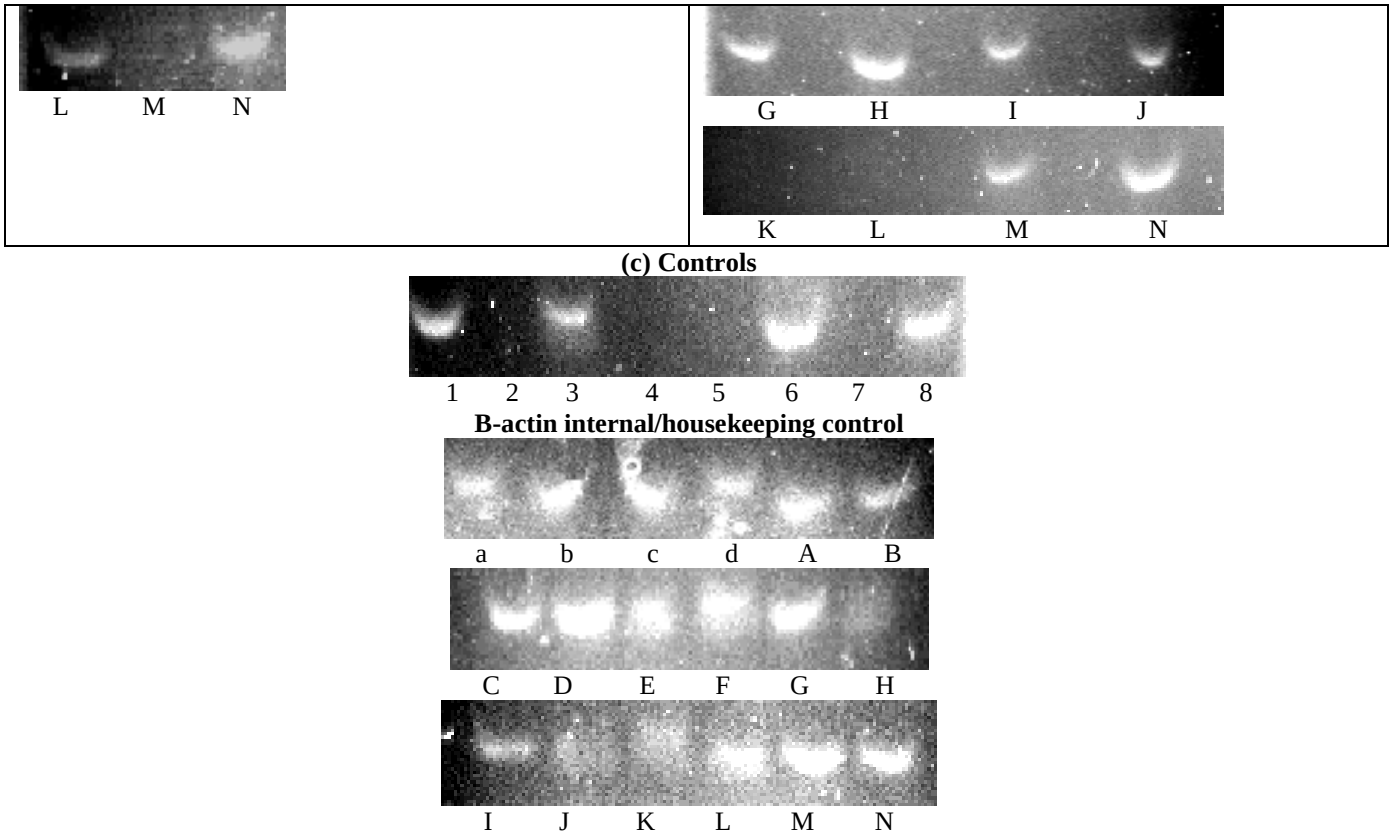



**Figure 5**. Reverse Transcription-Polymerase Chain Reaction analysis of apoptosis-related gene (a) *bax* (b) *caspase-3* and (c) controls treated with 14 *Bulbine* spp. fractions. These results are representative of RT-PCR results in triplicate. The fractions tested for *B. natalensis* were: A: corm 20 µg/ml aqueous, B: corm 1 µg/ml aqueous, C: corm 2 µg/ml 50% ethanol, D: corm 1 µg/ml 50% ethanol, E: corm 20 µg/ml 100% ethanol, F: corm 1 µg/ml 100% ethanol, G: root 20 µg/ml aqueous, H: root 2 µg/ml aqueous, I: leaf 20 µg/ml 100% ethanol, J: leaf 2 µg/ml 100% ethanol. For *B. frutescens*, the fractions tested were: K: root 20 µg/ml aqueous, L: root 2 µg/ml aqueous, M: leaf 20 µg/ml 100% ethanol, N: leaf 2 µg/ml 100% ethanol. The controls tested against the HEp-2 cell line were: 1: 20 µg/ml camptothecin (bax), 2: reverse transcriptase negative control, 3: 20 µg/ml camptothecin (caspase-3), 4: 2% DMSO (bax), 5: 200 µM DMSO (caspase-3), 6: 20 µg/ml calpain I inhibitor (bax), 7: negative template control, 8: 20 µg/ml calpain I inhibitor (caspase-3). For the β-actin control: a: untreated HEp-2 cells, b: 200 µM DMSO, c: 200 µM camptothecin, d: 200 µM calpain I inhibitor, A - N represent the sample fractions.

## DISCUSSION AND CONCLUSION

Apoptosis, or programmed cell death, is a form of cell death that is pivotal in eliminating damaged, infected, or unwanted cells from the body (Ellis RE *et al*., 1991; Flemming W, 1985). Apoptosis is well understood in a variety of cell lines such as neuron, muscle and blood cell lines, and it is known to be genetically regulated since 1991 (Ellis RE *et al*., 1991). During apoptosis cells undergo various biochemical and morphological changes (Culotta E, Koshland DE, 1993). These changes include: cell shrinkage, mitochondrion breakdown, nuclear DNA fragmentation and the release of apoptotic bodies (Häcker G, 2000). Apoptosis is controlled by a *p53* suppressor gene which activates other genes by acting as a transcriptional factor (Culotta E, Koshland DE, 1993; Hengartner MO, 2000). The other genes activated are involved in cell-cycle regulation, the induction of apoptosis and senescence. Apoptosis requires the cleavages of various proteases, one of the most important ones being the caspases (Reimertz C *et al*., 2003). Caspase-3 is the molecular indicator of apoptosis (Ellis RE *et al*., 1991; Hengartner MO, 2000). This study assessed apoptosis in the HEp-2 cancer cell line using aqueous and organic (50 and 100% ethanol) fractions of *Bulbine natalensis* and *Bulbine frutescens* by reverse-transcription polymerase chain reaction after performing MTT cytotoxicity assay on the tested fractions.

To date, some fractions/extracts of this genus have been proven to be cytotoxic against cancer cell lines and antibacterial against various bacterial strains (Coopoosamy RM, 2011; Padayachee B and Reddy L, 2009; Kasumbwe K and Reddy L, 2010). The final concentration of fractions used for the cytotoxicity and gene expression study, for each organ of each *Bulbine* spp. were 20, 2 and 1µg/ml. For *B. natalensis*, the roots, corms and leaves were evaluated for apoptotic potential whereas for *B. frutescens*, the leaves and roots with stem portions

(herein referred to as the roots) were evaluated. The fractions selected for RT-PCR were based on their cytotoxicity results for each *Bulbine* spp.

The prepared *Bulbine* spp. fractions were hydrophobic against the HEp-2 cell line and the hydrophobic nature was confirmed by the positive and negative cytotoxicity results because the MTT cytotoxicity assay only measures the toxicity of compounds once a cell has utilised the compound and the compound had interfered with the permeability of the mitochondrial membrane resulting in a decline in the mitochondrial transmembrane potential (Holdenrieder S and Stieber, 2004; Häcker G, 2000; Sareen SR *et al*., 2007; Jia Y-Ji *et al*., 2009). In this study, the *Bulbine* spp. fractions promoted HEp-2 cell line growth as well as cell death through apoptosis and it also other cell death pathways. For the corm samples, the amount of formazan produced was observed as being far less (less purple colour intensity) compared to the root and leaf fractions of both *Bulbine* spp. and this was ascribed to the higher toxicity of the *B. natalensis* corm fractions. The gene expression results also confirmed that the fractions were hydrophobic because the HEp-2 cell line either expressed *bax*, *caspase-3* or both of these genes for particular fractions. This highlighted the principle of the MTT cytotoxicity assay (Mossman T, 1983; Reddy L *et al*., 2006) in its role of indicating cell death by expressing those apoptotic markers once *Bulbine* spp. fractions interfered with the activity of the mitochondria. The MTT cytotoxicity assay measures the activity of the mitochondria spectrophotometrically through the reduction of a yellow-coloured MTT salt to purple formazan crystals by the enzyme succinate tetrazolium reductase or through mitochondrial succinate dehydrogenase, both of which are found in viable cells (Mossman T, 1983; Reddy L *et al*., 2006). Once the transmembrane potential of the cell is affected, those apoptotic markers, which participate in cell death, are either expressed or not.

Gene expression results of the *Bulbine* spp. fractions were confirmed by the DMSO negative control which did not produce any *bax* and *caspase-3* expression. Camptothecin as well as calpain I inhibitor induced apoptosis in the HEp-2 cell line after 5 days of incubation and it was concluded that further studies are required to confirm the action of calpain I inhibitor on the HEp-2 cancer cell line because calpain I inhibitor has been discovered to exhibit different effects on different cell lines and this has been ascribed to the *p53* status of the cell as well as the phase in the cell cycle at which such cells are exposed to calpain I inhibitor. For example, in fibroblasts, calpain I inhibitor initiated apoptosis during the G1/S (first growth/replication) phase of the cell cycle, while in the hepatocellular carcinoma lines (SK-HEP-1 and HLF) and colorectal cell lines (RKO and DLD-1) this was not the case, but both cell lines differed in the levels of *p53* expression (Atencio IA *et al*., 2000). The HEp-2 cell line used in this study of *bax* and *caspase-3* expression was *p53* proficient to eliminate any uncertainty in the results obtained with regard to the regulation of the HEp-2 cell cycle. Viable HEp-2 cells were used during the gene expression and cytotoxicity study. β-actin was used as the RT-PCR control.

There were 14 fractions altogether that were assessed for apoptotic potential using RT-PCR. Of the 14 fractions, only 2 fractions did not induce apoptosis in the HEp-2 cell line. This was evidenced by a lack in *caspase-3* expression in the HEp-2 cell line. *caspase-3* is the main gene which indicates whether apoptosis had occurred and this has been demonstrated in numerous cell lines (Van Cruchten S and Van den Broeck W, 2002; Cregan SP *et al*., 1999; Nakamura M *et al*., 2004; Puerto HLD *et al*., 2010). The two fractions that had not expressed c*aspase-3* were the root 20 and 2 µg/ml *B. frutescens* aqueous fractions. The remaining 12 fractions expressed *caspase-3* in the HEp-2 cell line, indicating the apoptotic potential of those fractions. 6 out of these 12 fractions did not express the *bax* gene, the first activated gene by the *p53* tumour suppressor gene in the mitochondrial and endoplasmic reticulum-mediated apoptotic signalling pathways (Culotta E, Koshland DE, 1993). These fractions were, the root 20 and 2 µg/ml aqueous fractions, the corm 20 and 1 µg/ml 50% ethanol fractions and the leaf 20 µg/ml 100% ethanol fraction of *B. natalensis*, as well as the *B. frutescens* leaf 2 µg/ml 100% ethanol fraction.

Although similarities between the gene expression results of both *Bulbine* spp. in terms of their organ and solvents used to extract compounds were expected, the only similarity was in terms of whether apoptosis had occurred or not. This was evident between the 100% ethanol leaf fractions of both *Bulbine* spp. However, the mechanism by which apoptosis was induced, and confirmed by the literature, by those fractions were different. Since the leaf 20 µg/ml 100% ethanol fraction of *B. natalensis* did not express the *bax* gene, it indicated that apoptosis occurred through some other unknown mechanism and that some other process like autophagy could have stimulated apoptotic cell death. Jia *et al* (2009) reported a similar apoptotic event in K562 (liver cancer cell line), whereby autophagy stimulated apoptosis on exposure to curcumin. Our conclusion for the leaf 20 µg/ml 100% ethanol fraction was reported like above since all three apoptotic mechanisms (mitochondrial, ER, and receptor-ligand) involve *bax* gene expression either directly or indirectly. Indirect *bax* expression is well understood in the receptor-ligand mechanism since death promoting proteins (Bid), which includes Bax, are activated by *caspase-8* gene expression, which causes *caspase 3* to be expressed (Shiokawa D *et al*., 1997; Marzo I *et al*., 1998). This not so well established mechanism could also explain the lack of *bax* gene expression for the leaf 2 µg/ml 100% ethanol *B. frutescens* fraction. Therefore, in cases where the same fraction induced *bax*

and *caspase-3* gene expression in the HEp-2 cell line, autophagy cannot be eliminated as a possibility that could have occurred (Jia Y-Ji *et al*., 2009). Autophagy as defined in Jia *et al* (2009) is an intracellular degradation system that sequestrates the cytoplasm and organelles into double-membrane vesicles which are then degraded within the lysosome of the cell. It is also referred to as type 2 programmed cell death, whereas type 1 cell death refers to apoptosis (Häcker G, 2000).

The expression of the *bax* and c*aspase-3* genes for the tested root fractions of *B. frutescens* were in contrast to the root fractions of *B. natalensis*. The root 20 and 2 µg/ml *B. frutescens* aqueous fractions had no apoptotic potential on the HEp-2 cell line, but it caused this cell line to express the *bax* gene. The *bax* gene is therefore only related to apoptotic potential when *caspase-3* is expressed; otherwise it participates in other forms of cell death. However its expression with *caspase-3* does not eliminate forms of cell death other than apoptosis. Since no previous cytotoxicity or gene expression studies have been conducted using *Bulbine spp*. fractions, conclusions about the effect of these fractions on other cell lines were unable to be made. This suggested that although *bax* is an apoptotic cell death gene, it has the potential to participate in other cell death mechanisms. As mentioned, recent evidence has indicated that *bax* gene activation could trigger autophagy, instead of apoptosis, but this is a newly researched area of interest. However *bax* gene activation is not a necessary event for autophagy (Jia Y-Ji *et al*., 2009).

It was important to note as to whether the percentage cytotoxicity and RT-PCR were correlated in the sense that a fraction that was cytotoxic should have expressed *bax* and *caspase-3*, if it were to have apoptotic potential or induce cell death. It was found that *bax* gene expression occurred between the positive and negative percentage cytotoxic fractions in this study. For example, the corm 20 (16.56%) and 1 (9.98%) µg/ml 50% ethanol fractions showed apoptotic potential in the HEp-2 cell line, while at the same administered concentration (and with positive cytotoxicity result), the corm 100% ethanol fraction also expressed the *bax* gene. This could have been due to the different compounds (some of which are: anthraquinones, islandicin, knipholone and knipholone anthrone) present in different proportions in those fractions (Dagne E and Yenesew A, 2004).

It was also found that the corm 20 µg/ml aqueous fraction (-4.09% cytotoxicity) induced apoptosis in the HEp-2 cell line because of *caspase-3* expression. However, this occurred even though this fraction had a proliferative effect on the HEp-2 cell line after 5 days of incubation. This meant that the proliferative effect of the 20 µg/ml aqueous fraction on the HEp-2 cell line was much greater than its cytotoxic effect on this cell line, resulting in *caspase-3* expression. This result also highlights the sensitivity of RT-PCR in detecting particular genes. The same RT-PCR result was obtained for the leaf 2 µg/ml 100% ethanol fraction of *B. natalensis* and the leaf 20 µg/ml 100% ethanol fractions of *B. frutescens.* All three of those fractions showed an increased growth effect on the HEp-2 cell line by the 8$^{th}$ day of incubation for cytotoxicity. This was an important observation since it increased the reliability of the RT-PCR result of the corm fraction in relation to the two tested leaf fractions since the corm fraction was not cytotoxic after the fifth day of incubation.

The differences in the expression of the *bax* and *caspase-3* genes are believed to be due to the different compounds present in the *Bulbine* spp. fractions (Dagne E and Yenesew A, 2004) and this was confirmed by other scientists who have performed RT-PCR experiments. The following comparisons between percentage cytotoxicity and gene expression results were made:

The root 20 µg/ml aqueous *B. natalensis* fraction had an average cytotoxicity of -10.09% (proliferative potential) and did not express *bax* in the RT-PCR study. However when compared to the corm aqueous fraction at the same concentration, the *bax* gene was expressed in spite of it having a low cytotoxic effect (-4.09%) on the HEp-2 cell line. Similarly, fractions with positive cytotoxicity results also showed inconsistent *bax* gene expression. For example the corm 20 µg/ml 100% ethanol fraction (16.54%) induced *bax* gene expression in the HEp-2 cell line while the leaf fraction did not (3.34%). In this situation, it could be that the leaf fraction induced some other form of cell death first that ultimately resulted in apoptosis and other forms of cell death because its cytotoxic effect is much smaller on the HEp-2 cell line compared to the corm fraction which is five times more cytotoxic. This was also evident for the fractions of *B. frutescens* selected for comparative purposes with *B. natalensis* in the RT-PCR.

The leaf 2 µg/ml 100% ethanol fraction of *B. frutescens* did not express the *bax* gene even though it had an extremely low proliferative effect, whereas the 20 µg/ml fraction which had a much lower percentage cytotoxicity, upregulated *bax*. Since the percentage cytotoxicity values of both fractions reached almost the same value by the 8$^{th}$ day and the HEp-2 cell culture was the same (i.e. taken from the same tissue culture flask), indicated that the expression of *bax* by the 20 µg/ml fraction and not by the 2 µg/ml fraction, was probably due to the HEp-2 cell line not being susceptible to the 2 µg/ml fraction by the 5$^{th}$ and 8$^{th}$ day of incubation. This highlighted that the HEp-2 cell line was probably selective in consuming fractions that were concentration independent i.e. the cells may consume a fraction made of one *Bulbine* spp. organ at a high concentration, while another organ at a lower concentration. Furthermore, it was postulated at the HEp-2 cell line probably required more time to consume the 2 µg/ml fraction. Similar situations have been reported in other cell lines such as leukemia (Nakamura M *et al*., 2004), breast (Sareen D *et al*., 2007) and hepatocellular

carcinoma cells (Atencio IA *et al*., 2000). Calculation of the non-parametric ANOVA and H statistic revealed that the five day percentage cytotoxicity results of each final concentration of fractions administered to the HEp-2 cell line, were different from one other and that only by the 8$^{th}$ day did the 3 dilutions of *B. frutescens* fractions not differ from each other. The 8 day finding was not significant since those results were not taken into account for the gene expression study. Therefore the downregulation of *bax* for the leaf 20 µg/ml 100% ethanol fractions of *B. frutescens* suggested that the compounds in those fractions that induce apoptosis were probably in a lesser quantity compared to the 2 µg/ml fraction because of the lower cytotoxicity result. This could have been a possibility even though the 20 µg/ml fraction induced apoptosis by upregulating both *caspase-3* and *bax*. However, it was found that the 20 µg/ml fraction did not necessarily have to activate *bax* for *caspase-3* to be expressed i.e. apoptosis could have been stimulated through autophagy (Jia Y-Ji *et al*., 2009) or the receptor-ligand mechanism that activates death promoting proteins other than Bax. This meant that the *caspase-3* upregulation by this fraction obscures whether *bax* is entirely involved in apoptosis because for the 2 µg/ml fraction, the *bax* gene was downregulated. The alternative explanation could be that cell damage (caused during tapping of the flasks during trypsinisation and damage to the cell membrane by subculturing subcultured cells using trypsin) could have occurred to a great extent that the induction of the *bax* gene by both of these fractions, could have contributed to apoptosis as well as autophagy i.e. the mechanism of apoptosis is not possible to be classified. The latter was further emphasised by the 1 µg/ml fraction having showed the lowest cytotoxicity of the three tested concentrations i.e. it could have had the least apoptotic compounds compared to the 20 µg/ml fraction.

The downregulation of *bax* and upregulation of *caspase-3* were observed for the root 20 and 2 µg/ml aqueous fractions of *B. natalensis* at proliferative percentages (-10.09 and -19.19% respectively). Similarly, the corm 20 and 1 µg/ml fractions had percentage cytotoxicity results of 16.56 and 9.98% respectively, and had the same RT-PCR results as the root fractions. The low percentage cytotoxicity result of the corm 2 µg/ml fraction suggested the possibility that that fraction had the potential of inducing cell death in the HEp-2 cell line through *bax* gene activation. Since the cytotoxicity result of the corm 2 µg/ml fraction was the average of three independent cytotoxicity experiments (as was the case with all the tested fractions), with the HEp-2 cell line passage number being 3, the percentage cytotoxicity could have been due to a mistake made during the preparation of the fraction (dilution performed). This means that an incorrect amount of the stock concentration could have been pipetted and went unnoticed.

The corm 100% ethanol fractions (20 and 1 µg/ml) had percentage cytotoxicity values of approximately 20%, and they may have induced apoptosis by either the ER or mitochondrial pathways, in addition to other cell death pathways. The corm 20 µg/ml aqueous fractions (-4.09%) upregulated *bax* while the 1 µg/ml fraction also upregulated *bax*. This result suggested that although at a higher concentration this fraction promoted HEp-2 cell growth, there were still HEp-2 cells that had undergone apoptosis as evidenced by *caspase-3* expression. The Cells-to-cDNA Kit is accurate in detecting apoptosis using site-specific primers because even if a single cell had undergone apoptosis or *bax* gene expression, it would have been detected [taken from the kit]. However, autophagy could have been induced by this fraction as well, for the reasons previously given. The 11.58% cytotoxicity of the 1 µg/ml fraction suggested cell death, vaguely, and it could be assumed, that apoptosis would also be possible for the 2 µg/ml fractions as it was for the 20 and 1 µg/ml fractions. The RT-PCR result of the 1 µg/ml fraction was not ascribed to a dilution error because all three fractions including the 2 µg/ml fractions were made from the same stock concentration (1 mg/ml).

The leaf 2 µg/ml 100% ethanol fraction of *B. natalensis* could have induced apoptosis through the receptor-ligand mechanism due to *bax* downregulation since this mechanism is not dependent on *bax* gene expression. However, the 2 µg/ml fraction had upregulated *bax* at a negative cytotoxicity. This could have been given the same explanation as the corm 20 µg/ml aqueous fraction, but does not completely true since the 1 µg/ml fraction had the highest cytotoxicity (7.78%), and was made from the same stock. One possibility is that the HEp-2 cell line probably responds to fractions at different levels depending on its sensitivity to a particular concentration of that fraction i.e. it may utilise the fraction to a greater degree when it is at a lower concentration (1 µg/ml) causing the cytotoxicity of the fraction to be higher or it may take a very long time to utilise the stock concentration (20 µg/ml) giving a lower cytotoxicity when compared to the cytotoxicity of the 1 µg/ml fraction after 5 days of incubation. It was important to know that although the cells used for cytotoxicity were at the same confluency and level of subculture, they each have their own identity and needs. This could have been the reason for the cytotoxicity and gene expression results of the corm 20 and 1 µg/ml fractions.

One of the challenges faced during this study was that there were no existing information on the cytotoxicity or expression on *bax* and *caspase-3* molecular markers for aqueous, and ethanol *Bulbine* spp. fractions that could be used to validate these results further. These results showed that apoptosis could have occurred through any one of three pathways (mitochondrial, endoplasmic reticulum and receptor-ligand (through the activation of death promoting members) because the aim was not to determine the expression of the *p53* tumour suppressor gene. Thus, in future the *p53* gene needs to be analysed for its expression

in order to understand why some fractions are capable of expressing *bax* and not *caspase-3* (for example, the *B. frutescens* root aqueous fractions).

*B. frutescens* is a well-known *Bulbine* spp. compared to *B. natalensis* in terms of its medicinal properties. In this study *Bulbine* spp. fractions caused the HEp-2 cell line to exhibit a mixed response (i.e. cell death and growth) and therefore the use of an unaffected (normal) cell was not required, mainly because the fractions of *B. frutescens* selected for the gene expression study were those relative cytotoxic *B. natalensis* fractions. Therefore the study was limited to treating a cancer cell only with *Bulbine* spp. fractions. Therefore, limitations of this study, is that this is an *in vitro* study and the results may only be extrapolated to *in vivo* effects in animal models because phytochemical screening of the fractions are required to identify the apoptotic compounds in the fractions before tests on animal models can be performed. In general, different cell lines respond differently to the same compound, and so it is undetermined as to whether similar results would present itself if another cell line was used concurrently with the HEp-2 cell line. For example the anticancer potential of peptide (7.6 kDa) from lionfish (*Pterios volitans*) was found to induce different degrees of apoptosis, characterised by different morphological and *Bcl-2* profiles, in HEp-2 and HeLa cell lines (Balasubashini MS *et al*., 2006). This is evidence that apoptotic stimuli administered to different cell lines will evoke different responses amongst each other, possibly because of the genetic machinery amongst the different cell lines, for example although the HEp-2 as well as K562 cell lines undergo apoptosis characterised by the same morphological features, the HEp-2 cell line is *p53* proficient (Amaral JD *et al*., 2009) while the K562 cell line is *p53* deficient (Pillai GR *et al*., 2004). Furthermore, the rate of cell proliferation, as well as the phase at which the cells are in their cell cycle (interphase (G1, S and G2), prophase, prometaphase, metaphase, anaphase and telophase) (Mitchison TJ and Salmon ED, 2001) can also result in different responses of cell lines to different apoptotic stimuli.

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