Cytotoxicity and gene expression of selected apoptotic markers in the human laryngeal carcinoma cell line (HEp-2) by *Bulbine* spp. fractions

by

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LIST OF ABBREVIATIONS

AIDS: acquired immune deficiency syndrome

agLDL: aggregated low density lipids

ANOVA: analysis of variance

Apaf: apoptosis protease activating factor

ATP: adenosine triphosphate

CALP / CALP I: calpain I inhibitor

CAMP: camptothecin

CCK-8: cell counting kit-8

CCM: complete culture medium

C × CR4: C × C chemokine receptor 4

cDNA: complementary deoxyribonucleic acid

CGNs: cellular granular neurons

CV: coefficient of variation

DMEM: Dulbecco's modified eagle medium

DMSO: dimethyl sulfoxide

DNase: deoxyribonuclease enzyme

DTT: dithiothietol

EDTA: ethylenediamine tetra-acetic acid

EGF: a growth factor that causes continuous proliferation of red blood cancer cells

EGFR: epidermal growth factor receptor

ELISA: enzyme-linked immunosorbent assay

ER: endoplasmic reticulum

FCS: foetal calf serum

GDP: guanosine diphosphate

GTP: guanosine triphosphate

HCl: hydrochloric acid

HIV: human immunodeficiency virus

HPV: human papillomavirus

LDH: lactate dehydrogenase

LDL: low density lipid

MPF: maturation / mitotic phase promoting factor

MPT: mitochondrial permeability transition pore

mRNA: messenger ribonucleic acid

MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

NAD (H): reduced nicotinamide adenine dinucleotide

NADP (H): reduced nicotinamide adenine dinucleotide phosphate

NaOH: sodium hydroxide

PBS: phosphate buffered buffer

PGDF: proliferating growth development factor

PMS: phenazine methylsulfate

PMN: polymorphonuclear neutrophils

qRT-PCR: quantitative reverse transcription-polymerase chain reaction

RNA: ribonucleic acid

RNase: ribonuclease enzyme

RT-PCR: reverse transcription-polymerase chain reaction

SDF-1: ligand stromal cell derived factor-1

SDH: succinate dehydrogenase

SDS: sodium dodecyl sulphate

SMCs: smooth muscle cells

TNF: tumour necrosis factor

TUNEL: terminal deoxynucleotidyl transferase deoxyuridine triphosphate nick end labelling

UPR: unfolded protein response

WST: water soluble tetrazolium salt

XXT: 2,2-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5- carboxanilide)

ABSTRACT

Apoptosis, or programmed cell death, is a process which is pivotal in eliminating damaged, infected, or unwanted cells from the body. It has been studied in numerous types of cell lines ranging from normal to infected cell lines, and there have been a wide range of studies on apoptosis in laryngeal cancer because this type of cancer has become one of the most common types of head or neck cancer due to the high incidence of alcohol consumption, tobacco smoking and chewing of betel quid amongst populations. Laryngeal cancer is usually treated with radiotherapy or is surgically removed, but due to the loss of the function of the larynx after surgery, it has been suggested that alternative strategies or ways of treating laryngeal cancer are required. This has prompted the use of, and research in the field of, plant medicine to combat laryngeal cancer.

Plant medicine has been used for centuries by the Chinese, Indian and Arabian population in Uhani, Ayurveda and Siddha as a form of replacing conventional medicine with complementary and alternative medicine, these include many plants from the family Asphodelaceae, which have become marketable commodities owing to their medicinal values and traditional uses. Amongst these plants, the genus *Bulbine* has been used as a form of natural medicine in rural Africa and they are also exploited for their aloe vera properties as well as their possession of phytochemical compounds such as isoflavanoids, nor-lignans, naphthalene derivatives, anthracene and poly prenylated flavonoids. There has been a compelling amount of literature on the traditional uses of the *Bulbine* spp. because these are linked to the *Bulbine* spp. having secondary metabolites such as pyroles, chromones, coumarins, bianthraceane, benzene as well as alkaloids. However, for *Bulbine natalensis* and *B. frutescens*, the plants of interest in this study, the location of anticancer compounds in them are the only amounts of information available. It has been reported, traditionally, that *B. natalensis* possesses the anticancer potential in the roots, while the anticancer potential for *B. frutescens* is in the leaves. However, this requires scientific clarification. Therefore, this study was conducted to assess programmed cell death or apoptosis by analysing the responses of the human laryngeal carcinoma cell line (HEp-2) to crude aqueous and organic (50% and 100% ethanol) fractions of *B. natalensis* and *B. frutescens*. In order to have achieved this, the HEp-2 cell line was exposed to the above mentioned fractions at three different final concentrations (20, 2 and 1µg/ml) and assessed for cytotoxicity using the 3-(4,5-

dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) cytotoxicity assay as an indicator of cell death after fraction utilisation (3 days) for 5 and 8 days. The differences in the potency of the *Bubline* spp. fractions were confirmed using the non-parametric ANOVA test. Thereafter, selected fractions were screened for apoptotic potential using reverse transcriptase-polymerase chain reaction (RT-PCR) to determine the expression of *bax* and *caspase-3* biomarkers, which are the biomarkers that participate in mitochondrial, endoplasmic reticulum and receptor-ligand mechanism of apoptosis. The fractions of *B. frutescens* were selected relative to those of *B. natalensis* for the RT-PCR procedure (read section 3.4.1. for the selection procedure) and links between the cytotoxicity and gene expression results were analysed.

It was found that the *B. natalensis* fractions had a much greater cytotoxic effect on the HEp-2 cell line compared to fractions of *B. frutescens* by the fifth day of the MTT assay. On the eight day of incubation, there was an increase in HEp-2 cell line proliferation by the fractions of both plant species administered. The fractions selected for *bax* and *caspase-3* gene expression analysis for *B. natalensis* were the: 20 µg/ml root and corm aqueous fractions, 20 µg/ml leaf and corm 100% ethanol fractions, 20 µg/ml corm 50% ethanol fraction, 2 µg/ml root aqueous fraction, 2 µg/ml leaf 100% ethanol fraction and the corm 1 µg/ml aqueous and 50% ethanol fractions. The fractions that were compared to *B. natalensis* were the 20 µg/ml root and leaf aqueous and 100% ethanol fractions respectively, the 2 µg/ml root aqueous fraction and the 2 µg/ml leaf 100% ethanol fraction. It was found from RT-PCR analysis that all of the *B. natalensis* fractions tested induced expression of *caspase-3*, which indicated that those fractions were capable of inducing apoptosis in laryngeal carcinoma *in vitro*, since *caspase-3* is the molecular indicator of apoptosis. The aqueous *B. frutescens* root fraction, did not induce expression of *caspase-3* gene, although it caused expression of *bax*. This implied that the root aqueous *B. frutescens* fraction, may be involved in some other form of cell death, other than apoptosis. It was also found that there was variability in the response of the HEp-2 cell line to the *Bulbine* spp. fractions because of the variation in *bax* expression among fractions of different concentration. It was difficult, from this study, to classify fractions into categories for their mechanism of action, because not all of the fractions that caused the expression of *capase-3*, induced *bax* gene expression. Hence, proper conclusions were unable to be made, more so, because all the mechanisms of apoptosis mentioned, involve *bax* gene activation in order to proceed to completion. Therefore for those *Bulbine* spp. fractions to

which the HEp-2 cell line exhibited a variable response to, it was postulated that cell death or apoptosis occurred through some other unknown mechanism. Overall, the cytotoxicity result didn't correlate to the gene expression results because fractions that promoted HEp-2 cell line growth by day five, expressed apoptotic markers, which highlighted the sensitivity and accuracy of the cells-to-cDNA^{TM} II kit for detecting a few possibly apoptosed cells. This was confirmed by the fact that the HEp-2 cell line used in the MTT cytotoxicity assay and gene expression study had the same passage number and were viable, the latter being achieved because the MTT assay only measures the cytotoxicity of compounds once they have been taken up by viable cells – measuring mitochondrial activities expressed as absorbances. Therefore, the deduction that HEp-2 cell death may be due to *bax*/*caspase-3* expression was valid because the mRNA was isolated from viable HEp-2 cells that had been killed by *Bulbine* spp. fractions of different polarity. Furthermore, the lack of correlation between the cytotoxicity and gene expression results indicated the amount of HEp-2 cell line proliferation by the fraction out-competes those that died, thereby producing a negative cytotoxicity result. There was a relationship between the traditional information about the anticancer potential for *B. natalensis* and *B. frutescens*. For example, the aqueous root fractions of *B. natalensis* were found to be non-toxic to the HEp-2 cell line, but did express *caspase-3*, which indicated the possibility of apoptosis. Similarly, the 100% ethanol leaf *B. frutescens* fractions were non-toxic to the HEp-2 cell line, but were able to induce apoptosis as well. This emphasised that the MTT cytotoxicity assay should be compared with other methods of measuring cytotoxicity when performing studies like this, because although literature has emphasised many advantages of using the MTT cytotoxicity assay in apoptotic studies, this study proved otherwise.

When identical HEp-2 cells were treated with the same extract, only some cells were killed (apoptosis) whereas others proliferated. This was because although the cells were identical phenotypically, they were all probably at different phases of the cell cycle resulting in the HEp-2 cells responding variably to the same fraction at different concentrations. It was also found that the responses were concentration independent. For example, the 1 µg/ml *B. natalensis* corm fraction exhibited the highest toxicity of the three concentrations administered. The lowest cytotoxicity was achieved for the 20 µg/ml fraction – showing a proliferative effect on the HEp-2 cell line. Similarly, the 2 µg/ml aqueous *B. natalensis* leaf fraction induced the highest cytotoxicity level in the HEp-2 cell line followed by the 1 µg/ml

and then the 20 µg/ml fractions. Apart from the genetic variation in identical HEp-2 cells; this indicated that the HEp-2 cell line was selective to particular fractions of the *Bulbine* spp. for utilisation. Concentration independence and HEp-2 cell preferential selection has been reported in many other studies involving plant fractions/extracts and natural products.

This study demonstrated that although all the tested *B. natalensis* fractions were capable of inducing HEp-2 cell death possibly via. apoptosis (*caspase-3* induction), a lack of any link between apoptosis and the cytotoxicity results (hence the 20 µg/ml corm fraction had a negative cytotoxicity but expressed both apoptotic markers), indicated the need for phytochemical screening of both *Bulbine* spp. in future, to determine the compounds that are responsible for the cytotoxicity and gene expression result outcomes of both *Bulbine* spp. fractions. Furthermore, procaspase genes also have to be analysed since genes are expressed to form procaspases, which then form active caspases.

Although normal cells also express *caspase-3* genes during apoptosis, this study focused exclusively on the effect of *Bulbine natalensis* and *B. frutescens* fractions (selected relative to the cytotoxicity results of *B. natalensis*) on the HEp-2 cell line (read cell culture and cytotoxicity discussion for selection of HEp-2 cell line). The validity of this study is confirmed by similar experimental designs that assayed the cytotoxicity of plant-derived or natural compounds on cancer cell lines only, and the detection of apoptosis through *caspase-3* induction and other unrelated methods. This is the first study to report the induction of apoptosis in cancer cell lines by *Bulbine* spp. fractions using cytotoxicity and the expression of *bax* and *caspase-3* apoptotic markers. It provides insight into the interaction between the HEp-2 cell line and the aqueous and organic fractions of *B. natalensis* and *B. frutescens* by analyzing links between cytotoxicity and *bax* and *caspase-3* gene expression; which could probably contribute to drug design with selected *Bulbine* spp. fractions. Further investigations are required in future, to confirm the possible drug targets of the studied *Bulbine* spp. fractions in an attempt of assaying their therapeutic importance.

CHAPTER ONE

Introduction

The Asphodelaceae family of plants contain over 41 species varieties that have been traditionally used and screened for medicinal values, and as a result many have become marketable commodities in Botswana, Ethiopia, Kenya, Tanzania, Uganda, and many Asian and European countries (Dagne *et al*., 1994; Abegaz *et al*., 1999). These species include *Bulbine capitata*, *Bulbine natalensis* as well as *Bulbine frutescens* (Abegaz *et al*., 1999; Coopoosamy, 2011), which are more noted for having phytochemical compounds such as isoflavanoids, nor-lignans, anthracene, napththalene derivatives and poly prenylated flavonoids (Abegaz *et al.,* 1999). The genus *Bulbine* also has aloe vera properties and is used as a form of natural medicine in rural areas in much of Africa by the African community, where modern medicine is inaccessible and too expensive (Coopoosamy, 2011). On the other hand, *Bulbine* spp. is used as an important food source similar to the plant species *Microseris lanceolata* and *M. scapigera* used in Australia by the Aborigines. The roots of *Bulbine* spp. is a rich store for starches and polymers of fructose (an energy source used by inhabitants) (Incoll *et al*., 1989; Englyst and Hudson, 1997; Gott, 2008). *Bulbine abyssinica*, *B. annua*, *B. aspodeloides*, *B. frutescens*, *B. capitata* and *B. latifolia* are known, for many years, to be rich sources of secondary metabolites such as alkaloids, benzene, naphthalene, anthraceane, bianthracene derivatives, chromones, coumarins and pyroles (Abegaz *et al*., 1999). These metabolites have a variety of antibacterial, antifungal, antiviral, anti-inflammatory and antiparasitic properties (HealingAloe, 2012).

There is minimal scientific evidence on *B. natalensis* and *B. frutescens*, which were the plants examined in this study, that support their traditional and herbal uses. The present study therefore focused on screening *Bulbine natalensis* and *Bulbine frutescens* plant organs (roots, corm and leaves) for anticancer or apoptotic potential using organic and aqueous solvent fractions in an *in vitro* study.

Apoptosis, or programmed cell death, is a form of cell death that is pivotal in eliminating damaged, infected, or unwanted cells from the body (Flemming, 1885; Ellis *et al*., 1991). It is 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 *et al*., 1991). The process is characterised by various biochemical and morphological changes (Culotta and Koshland, 1993), which include: cell shrinkage, mitochondrion breakdown, nuclear DNA fragmentation and the release of apoptotic bodies (Culotta and Koshland, 1993; Green and Kroemer, 1998; Häcker, 2000). According to the WST-8 patent No. 2.251,850 (Canada), the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cytotoxicity assay is the most reliable method of measuring cell death, and it was thus employed to measure the cytotoxicity of the *Bulbine* spp. fractions administered to the HEp-2 laryngeal cancer cell line, in order to determine their bioactivity.

The *p53*, bax and caspase-3 genes play an important role in regulating apoptosis (Ellis *et al*., 1991), with *p53* being central to the regulation of the eukaryotic cell cycle (see supplementary information). Studying the expression of apoptotic biomarkers are an important endeavour to determine therapeutic measures of drug design in the pharmaceutical industry (Singh and Reddy, 2012). The expression (and regulation) of these biomarkers (*p53, bax* and *caspase-3*) allow scientists to get a view of the spectrum of drug targets and to determine the action of those drugs in patients with infected cells like cancer *in vitro* (Wang and Yuen, 1994; Singh and Reddy, 2012). Apart from the 3 mentioned apoptotic markers, there are hundreds of biomarkers that participate in regulated forms of cell death (including apoptosis and necrosis) and these have been reviewed in Singh and Reddy (2012) to a great depth. Presently there is a lack of understanding with regard to the proliferation of cancer cells in the human body as well as within the *in vitro* environment. It is believed that reverse transcriptase-polymerase chain reaction (RT-PCR), a molecular biology technique, may help foster a greater understanding and clear our predicaments about apoptotic gene expression with regard to the effects of *Bulbine* spp. (crude) extracts/ fractions in *in vitro* cancer studies.

The aim of this study was therefore to study the induction of apoptosis in the human laryngeal carcinoma cell line, HEp-2, by *Bulbine* spp. fractions by focusing on genes - their expression in relation to the pathways of apoptosis.

The study included four objectives. The first was to prepare *Bulbine* spp. fractions using 3 different types of solvents, two organic and one aqueous (distilled water, 50% ethanol and 100% ethanol). The second was to optimise the dose and time response of *Bulbine* spp. crude

fractions on the HEp-2 cancer cell line by determining the cytotoxicity of the plant fractions using the MTT (3,4,5-dimethylthiazol-2yl)-2-5-diphenyltetrazolium bromide) cytotoxicity assay. Following the optimisation of dose and time response, amplifying cDNA for the analysis of apoptotic gene expression of *caspase-3* and *bax* markers by PCR, was the third objective. And the fourth objective was to analyse the gene expression results and make conclusions about apoptotic gene expression in the light of the mechanisms of apoptosis induced using agarose gel electrophoresis.

CHAPTER TWO
Literature Review

2.1. Brief history and importance of apoptosis

Apoptosis, or programmed cell death, was first conclusively demonstrated in the nematode, *Caenorhabditis elegans* (Ellis *et al*., 1991), where the key genes involved in apoptosis were first characterised (Ellis *et al*., 1991). In the 1800s, scientists believed that throughout development, cell proliferation occurred on a continuous basis, and for the first time, in 1842, Vogt described that cell death was present in toad development (Clarke and Clarke, 1996). Fifty years later, Flemming recognised cell death as part of a physiological function; when he discovered cell death in rabbit Graafian follicles (Flemming, 1885). Later on Flemming further developed the theory and proposed that cell death within the cells involved chemical changes. He observed a similar mechanism of cellular degradation in testicle germ cell populations in 1887, which by the end of the nineteenth century, he termed apoptosis. He documented the morphological features of apoptosis such as the collapse of the cell, protein degradation and DNA fragmentation (Häcker, 2000).

A small number of developmental biologists kept the concept of physiological cell death alive; and by 1914, the idea of continuous cell proliferation was supported by a theorem that compensated for mitotic division occurring during apoptosis so as to maintain homeostasis of healthy verses harmful cells (Majno and Joris, 1995). The Australian pathologist, John Kerr, rediscovered apoptosis in 1974 while studying towards his Ph.D degree. He observed a type of cell death in hepatocytes that could be distinguished from necrosis and he named it shrinkage necrosis. Kerr, Wyllie and Currie wrote a paper in 1972 which described the morphological features that are observed in a wide variety of cell types during physiological episodes of cell death (Kerr *et al*., 1972). In addition, they described that classical, accidental cell death and necrosis was able to be separated through the body, by means of its own ability to control developmental and homeostatic cell death. Another research group found that the DNA in lymphocytes broke down into multiples of approximately 180 - 200 base pairs (bp) (Yamada *et al*., 1981), forming a DNA ladder during apoptosis, which Wyllie considered a hallmark in apoptosis research (Wyllie *et al*., 1984). This, to Wyllie and other scientists, emphasised that there was a molecular mechanism associated with apoptosis, leading to the

rapid expansion of research in apoptosis in respect to the oncogenes and tumour suppressor genes that are found to have a role in apoptosis. This emphasised the role of apoptosis in the control of homeostasis (Cheng, 1997; 1998).

Apoptosis plays a major role from embryonic development to adulthood. During development, apoptosis facilitates sculpturing of the fingers and toes, nervous system maturation, male and female reproductive organ formation (Baker, 1971), and resorption of the tail of tadpoles during metamorphosis (Becker *et al*., 2003). In postnatal life, apoptosis ensures proper functioning of tissues such as the reproductive organs and the immune system by keeping it at a state of homeostasis by counter-balancing cell division and cell migration (Hengartner, 2000). It does this by purging the body of cells that have been invaded by pathogens (Baker, 1971).

It is believed that apoptosis evolved through prokaryotic mechanisms of cell death that involved quorum sensing through endosymbiosis of bacteria and the formation of mitochondria (Ameisen, 1996; 2002; 2004). This opened up therapeutic opportunities in diseases where cell death plays a major role, as in neurodegenerative disease (Ameisen, 1996; 2002; 2004).

2.2. Important considerations about some plant anticancer agents

Apoptosis, as mentioned previously, is a form of programmed cell death that occurs naturally (Zimmerman *et al*., 2001), but can also be studied artificially in the laboratory (Singh and Reddy, 2012). Chinkwo (2005) found that the extracts of *Sutherlandia frutescens*, a plant that is distributed mostly along the west coast of the Western Cape (Van Wyk, 1997), induced a greater apoptotic activity in three different cell lines when they were treated with *S. frutescens* extracts made of plant material that were taken from the native province (Western Cape), compared to the extracts from the same plant species of the Northern Province and the Orange Free State. This suggested that environmental pressures affect plants in different areas, resulting in plants of the same genus or species synthesising different compounds. Soil compositions and the synthesis and accumulation of secondary metabolites, as a protective mechanism against environmental factors, is a cause of plants of similar types producing different compounds (Wink, 1999). However, it is also important for scientists to test extracts from different parts of the plants (such as the seeds, roots, stems, leaves and flowers), in general, to obtain an idea where antimicrobial and other medicinal activities are produced and

accumulate in the plant. Chinkwo (2004) suggests that a plants anticancer properties may be validated when it demonstrates a marked apoptotic activity, due to environmental pressures plants experience in their native areas/regions.

Africa is a rich source of medicinal plants. One known species is *Catharanthus roseus*, which yields antitumour phytochemicals such as vinblastine and vincristine. In other developing countries like India and Sri Lanka, herbs possessing anticancer properties have been exploited for decades. One best known herb is *Nothadocytes foetida* (tata lakda), an import in Germany, France, Switzerland, Japan, the United Kingdom, and the United States of America. In these countries *N. foetida* is used as an anticancer drug source (Hoarean and DaSilva, 1999).

There are four structural classes of anticancer phytochemicals available on the market in the USA. These are: *Catharanthus vinca* alkaloids (vinblastine, vincristine, vinorelbine), epipodophyllotoxins (etoposide, etoposide phosphate, teniposide), taxanes (paclitaxel and dicataxel), and camptothecin derivations (irinotecan and topotecan) (Gragg and Newman, 2012).

Other chemotherapeutic plant products identified are: (1) antimutagenic alkaloids, coumarins, and flavonoids from the seeds of *Casimiroa edulis* (Rutaceae) (Ito *et al*., 1998) (2) with inolides from *Physalis philadaphica* (Solanaceae) (Kennelly *et al*., 1997) (3) antioxidant flavonoids from *Chorizantha diffusa* (Polygonaceae) (Chung *et al*., 1999) and (4) steroidal alkaloids from *Pachysandra procubens* (Baxaceae) (Chang *et al*., 1998). In addition to these cancer chemoprotective agents, the following plant families constitute plant species that have active compounds which act against cultured human cancer cells: Maliaceae, Asteraceae (Cui *et al*., 1997), Euphorbiaceae (Long *et al*., 1997) and Dipterocarpaceae (Kinghorn *et al*., 1999).

In laboratory animal models, extracts and purified constituents of some culinary herbs such as fruit, spices, teas, and vegetables inhibit cancer development (Ghani, 1998; Chung, 1999; Gonzalez, 2006; Chan, 2011). There are five potential mechanisms induced by plant compounds, when treating cancer to the natural benefit of human health. These mechanisms of action are: cytotoxicity, antiproliferation, suppression of apoptosis, antimutagenicity and the enhancement of DNA repair and mutagenic responses (Gonzales and Valerio, 2006). No

results have been reported on the induction of these mechanisms by plant compounds derived from the genus, *Bulbine*.

Kniphofia and *Bulbine* genera are plant taxa that belong to the family Asphodelaceae. These plants are native to Africa and are widely distributed in South Africa. According to researchers at the University of Wuerzburg in Germany, *Kniphofia* and *Bulbine* species have medicinally important substances known as phenyl anthraqinones, which are analogues to the phenyl anthraquinones found in some antitumour agents. This led to the assumption that the substances could be used to treat cancer. In the laboratory, Kniphofia displays excellent inhibitory activity against leukaemia cells (Gerhard Bringmann, University of Wuerzburg).

It is believed that 4 phenyl anthraquinone molecules can join together to form a macromolecule and that even though it becomes a 3-dimensional structure, it may be possible that it interacts with enzymes and the genetic code (DNA). Gerhard Bringmann from the University of Wuerzburg emphasizes this as an important characteristic for future medicinal applications and that the substances of many *Kniphofia* and *Bulbine* spp. require further examination and research.

2.3. Cytotoxicity assay

Cytotoxicity of plant extracts and natural products are important to assay in order to determine just how toxic these compounds are to cells and microorganisms such as viruses and bacteria. There are many well researched and established assays that can be used to screen for the cytotoxicity of plant and drug compounds and even chemicals. Amongst the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) cytotoxicity assay, are the colony formation method, CCK-8 (cell counting kit-8), crystal violet method, XTT (2,2-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay, ^{51}Cr (chromium) method, trypan blue assay, tritium-labelled thymidine uptake method and the WST (water-soluble tetrazolium salt) method, all of which are used to measure the toxicity of compounds by counting live cell numbers. Conversely, the trypan blue assay measures the toxicity of compounds by staining dead cells blue in colour, while availing the unstained cells for counting under the microscope (WST-8 patent No. 2.251,850, Canada). The unstained cells are used in the calculation of cell death as a percentage of the cytotoxicity of the drug, chemical or plant extracts/fractions.

Human cells have a highly specialised structure. They are irregular in shape, but like plant cells, they possess enzymes, cell receptors, mitochondria, co-enzymes and nucleotides that form the bases in their DNA, which is situated within their nuclei. The function of these cellular components predicts and influence the activity of various compounds on cell viability and it is the interaction amongst these components that make the detection of cell viability complicated (WST-8 patent).

Although we may say that all of the cytotoxicity assays are of equal importance, the MTT cytotoxicity assay is the most widely used assay for measuring cell viability and/or cytotoxicity of compounds (based on article citations), and would therefore form a crucial part of the discussion that follows.

The MTT cytotoxicity assay has been reported as being safe, reliable, re-producible and easy to use. It relies on the reduction of a colouring agent or enzyme called the 'dehydrogenases'. These dehydrogenases are found in the mitochondria of living (or viable) cells, and their role in programmed cell death (or apoptosis) is made more apparent when yellow MTT becomes reduced to MTT formazan; which is needle-shaped in morphology, purple in colour and is only soluble in living cells. However, the solubility of MTT formazan occurs by means of using organic solvents like DMSO (dimethyl sulpfoxide), isopropanol or SDS (sodium dodecyl sulphate). The detection of cell viability and the calculation of the cytotoxicity of compounds are achieved by obtaining absorbance readings, using wavelengths that are prescribed and predetermined from published papers, using an ELISA (enzyme-linked immunosorbent assay) plate reader (WST-8 patent).

However in cytotoxicity studies, the recording of absorbance readings from the MTT assay is not always the best option because some MTT crystals float with the cells making it difficult to remove from the cell culture. This provokes the reading of insignificant well-to-well absorbance readings and the wastage of cells (WST-8 patent). As a result, in the present study the media was not removed prior to adding the MTT reagent, so that the absorbance results were able to be reproduced with confidence using the ELISA plate reader.

The MTT cytotoxicity assay depends only on the activity of mitochondrial dehydrogenases. This gives the MTT assay a competitive edge over all of the other mentioned methods. A brief discussion about the other methods (WST-8 patent) in relation to the MTT assay follows below.

The CCK-8 method, unlike the MTT cytotoxicity assay, involves most of the dehydrogenases in the cell; the WST-8 method involves water-soluble tetrazolium salts and as a result it does not form formazan crystals like the MTT assay. The formation of formazan crystals is the hallmark indicator of cell viability after 1 - 4 hrs of incubation in MTT cytotoxicity assays (Berridge and Tan, 1993).

The tritium-based thymidine uptake method is considered the most sensitive, since it relies on the use of radioisotopes to determine the activity of DNA polymerisation once tritium-labelled thymidine is incorporated and taken up by the cell. It has been found that the incorporation of tritium-labelled thymidine causes cells to growth. This growth is measured using a scintillation counter (WST-8 patent).

The ^{51}Cr method only measures low levels of cytotoxicity and causes problems associated with the storage, handling and disposal of cellular material. Furthermore, it utilises adenylate kinase, lactate dehydrogenase and glucose-6-phosphate dehydrogenase cell death enzymes, with lactate dehydrogenase being the only enzymes that is stable during cell death. The measuring of low levels of cytotoxicity and the fact that lactate dehydrogenase is the only stable enzyme during cell death, makes the MTT dehydrogenase enzyme the most suitable for carrying out cell viability and/or cytotoxicity assays, more so in this study, because an objective of this study was to measure high levels of cytotoxicity while ascertaining minimal doses (WST-8 patent) to the larynx cancer cell line.

In the MTT cytotoxicity assay, the MTT reagent enters into the mitochondrion of viable cells because the reagent is a neutral to positively charged organic molecule. The neutral to positively charged state of the MTT reagent prevents any force of repulsion at the surface of the mitochondrion, allowing it to enter the cell. When the reagent is in the mitochondrion of viable cells, it becomes reduced to an insoluble purple-coloured product, called formazan. This product can only be solubilised by means of organic solvents (already mentioned). These solvents release a solubilised formazan reagent that is measured spectrophotometrically and the absorbances are used to calculate the percentage cell viability and the cytotoxicity of the plant fractions.

Once DMSO, SDS or isopropanol have solubilised the formazan crystals, the cell is considered dead. This is the case with cytotoxicity/cell viability assays, except the CCK-8 method in which 90% of cells remain viable after the solvent-treated 96-well plates are

incubated for 24 hours. This is a huge advantage when experimenters want to measure cell viability and use viable cells for further experiments; however those cells must be washed to remove any solvent from their surfaces (WST-8 patent).

The MTT cytotoxicity assay is a strictly dehydrogenase-based assay and it involves many well-known elements that form part of the mitochondrial respiratory chain. These oxidative elements include NAD (H) (reduced nicotinamide adenine dinucleotide) and NADP (H) (reduced nicotinamide adenine dinucleotide phosphate) as well as the activity of the mitochondria. When cells are treated with compounds at different concentrations (pulse drug exposures), they undergo a decline in cell viability and this possibly leads to cell death. However in MTT-treated cells 96-well plates, no colour changes may occur even after a 3 - 5 days of incubation and this means that when those plates are retrieved from a 5% CO_2 incubator, they appear yellow (the colour of the MTT reagent). However, once the MTT-treated plates are treated with an organic solvent, a blue colour forms after a further 1 h of incubation, but no colour change may exists. This occurs because the time taken for the formazan crystals to dissolve differs amongst different cell types and therefore it is expected that absorbances will differ between and amongst different cell types. This also implies that an appropriate incubation time has to still be developed in order to give a proportional relationship between cell numbers and absorbance readings (WST-8 patent).

The organic solvents, DMSO, SDS and isopropanol are used to inhibit colour development in MTT cytotoxicity assay i.e. to stop the MTT reaction. An amount of 10 µl 1% SDS and 100 µl DMSO has been recommended for use in routine cytotoxicity studies. However, a fourth solvent, HCl (hydrochloric acid) has also been shown and proved to stop colour development, however it has been emphasised in the literature that readings must be taken 24 h after the reaction has been stopped (WST-8 patent). To the scientific community, the latter is considered to be time consuming. For the sake of clarity, the use of an alkaline solution like NaOH (sodium hydroxide) to stop the MTT reaction, has proven to give invalid-absorbance readings because alkaline solutions, in general, cause WST-8 and other tetrazolium salts to become unstable.

The importance of the MTT cytotoxicity assay is reiterated by being a ‘parent’ assay from which the XTT assay was born. Ultimately, the principle of both assays are the same, but the XTT assay was developed as a ‘sister’ assay to eliminate the aspiration/solubilisation step

required in the MTT assay (Paull *et al*., 1988). In the XTT assay, the XTT reagent is utilised directly by viable cells to dissolve water-soluble formazan which can be detected optically using ELISA, without any further processing of the sample (Boyd, 2012).

The XTT and MTT assays are similar in that they both have unstable end-points that make use of a large panel of cells. Both assays use tetrazolium assays that have the potential to screen for tumours. XTT and MTT depend on the reduction of tetrazolium to generate NADP and NADPH, which are respiratory chain elements (Vistica *et al*., 1991; Berridge and Tan, 1993).

An MTT assay involving test glucose samples revealed that glucose concentration declined significantly after 7 days of incubation. This is considered an advantage when using the MTT cytotoxicity assay, because in XTT assays, tetrazolium had been found as being incapable of reducing glucose concentration without using an electron transfer reagent, such as phenazine methylsulfate (PMS). It had been found that in MTT assays, the glucose concentration declined with the formation of formazan crystals (Vistica *et al*., 1991; Berridge and Tan, 1993). However, it is also known that the formation of proper formazan crystal depends on optimal pH of the growth medium. For example, in RPMI-1640 medium, the optimum pH to dissolve formazan is between 7 to 9 when XTT/PMS plates are removed from a high 5% CO_2 incubator environment. In contrast, it had been found in MTT/PMS assays, that the erratic optical density measurements are actually attributed to the interaction between phenazine methylsulfate and glutathionine that are found in the growth medium (Vistica *et al*., 1991).

Since the reduction of MTT is affected by the glucose concentration in the media, it has been assumed that the reduction of cellular MTT is a measure of the rate of glycolytic (the process by which glucose is broken down in cells) NADH production (Boyd, 2012). This statement is supported by the fact that cytochlasin B, which is a potent inhibitor of glucose transport into cells has been found to be receptive to cellular MTT responses. This implies that unlike XTT and WST-1 which depend on superoxide dismutase (located on the plasma membrane or extracellularly) for their reduction to formazan crystals, the MTT cytotoxicity assay is insensitive to the function of this enzyme, be it in the presence or absence of PMS (Berridge and Tan, 1993).

PMS enters the cell and/or mitochondrial membrane because of its electronic structure and its association with MTT. When the MTT reagent enters the mitochondrion, 1-methoxy PMS

also enters, but water-soluble tetrazolium salts (WST-8) do not have the ability to enter. 1-methoxy PMS accepts an electron from NADH or NADPH at the inner mitochondrial membrane and the PMS passes the electron to WST-8 which is situated in the outer mitochondrial membrane (Berridge and Tan, 1993).

The XTT and non-tetrazolium assays, on the other hand, requires a standardised medium (at physiological pH 7.4) comprising biotin, L-asparagine, pyruvate, and oxaloacetate for intracellular CO_2 production. There is currently dose-response data available for anticancer drugs such as camptothecin, doxorubicin and taxol using the MTT and non-tetrazolium salt assays only. These results have been obtained by performing MTT and non-tetrazolium salt assays using PDGE basal growth medium or RPMI-1640 under ambient CO_2 and a 5% CO_2 environment (Vistica *et al*., 1991; Boyd, 2012).

In the present study the MTT cytotoxicity assay was used because there is a standard set of anticancer drugs (camptothecin, doxorubicin and taxol) that have already been tested and established using this method. The XTT is not a well-established assay because there has been no standard set of anticancer drugs that have been tested for cytotoxicity against cancer cell lines. In addition, the MTT assay is not affected by reduced pyridine nuceotides (NADH and NADPH) in the absence of cells or cell extracts as is the case with XTT and WST-1, and more concerningly, Dithiothietol (DTT; $C_4H_{10}O_2S_2$), mercaptoethanol, and to a lesser extent, reduced glutathionine, also reduce WST-1 (Boyd, 2012).

In the presence of intermediate electron acceptors, WST-1 and XTT are rapidly reduced by NADH and NADPH in the absence of cells and enzymes. It has been found that the mitochondrion inhibits NADH-dependent reduction and that mitochondrial enzymes were needed for WST-1 and XTT to be reduced by succinate (Berridge and Tan, 1993). MTT differs from WST-1 and XTT because it is particularly reduced by microsomal enzymes that use reduced pyridine nucleotides (NADH and NADPH). However, it also uses succinate as an electron donor in the MTT reduction process through mitochondrial succinate dehydrogenase enzymes. The latter case involving succinate dehydrogenases is slow and contributes little to the total cellular MTT reduction process (Berridge and Tan, 1993) and it is believed that it is the microsomal enzymes that play a more prominent role in the reduction of MTT, making the MTT cytotoxicity assay more reliable and widely used in anticancer studies.

2.4. Studies using the MTT cytotoxicity assay

The MTT assay is commonly used for drug screening and cytotoxicity tests of chemicals and plant extracts on cancer and normal cells. In 2009, Jia together with a few other scientists used MTT cytotoxicity assay to determine growth inhibition and cell viability of K562 cells treated with curcumin. They found a significant decrease in the viability of K562 cells in a time-and-dose dependent manner. The inhibitory rate of curcumin (~20 μM) on the viability of K562 cells reached 42.17 ± 2.48% after 24h and it increased to 82.49 ± 0.78% after 72 h following treatment. This proposed a concentration to them that was able to be used for subsequent cell death studies (Jia *et al*., 2009). They later confirmed this to be apoptosis by studying the cellular morphological changes (such as chromatin condensation and nuclear fragmentation using fluorescent microscopy), as well as confirming curcumin-induced apoptosis by analysing gene expression profiles of Bax, Bid and Bak.

Jia *et al.* (2009) confirmed their MTT results by analysing the activation and expression of Bcl-2 proteins, an anti-apoptotic protein, which protects the mitochondrion from the effects of proapoptotic proteins like Bid, Bak and Bax on the mitochondrial membranes. The Bid cleavage products, t-Bid, increased after 6 h of curcumin treatment in a time-dependent manner while the level of Bcl-2 were found to be reduced 6 h after curcumin treatment. The lowest level of Bcl-2 was observed 24 h after drug treatment. This provided a set-point at which apoptosis could be initiated in K562 cells, by the activation of *bax* gene expression, 6 h after curcumin treatment.

In Bangladesh, the Amoora species, *Amoora rohituka*, an evergreen tree, is believed to have apoptotic activity (Ghani, 1998; Ramachandran *et al*., 2006). Apoptosis using this plant species in breast, colon, cervical and leukaemia cancer cells, through caspase activity has been studied with great success. The discussion that follows focuses on caspase activity in breast cancer cells treated with *Amoora rohituka*.

The medicinal properties of *Amoora rohituka* has been studied, but its anticancer potential has been poorly described, like *Bulbine* spp. This has caused scientists in Bangladesh to study its anticancer properties, while noting that different plant extracts have different levels of cytotoxicity on the same cell line and that different cell lines have different susceptibilities to different plant extracts (Ford *et al*., 2005; Rivenbark *et al*., 2006). These Indian scientists studied the anticancer properties of the genuses, *Amoora rohituka* and *A. chittagonga* on 5

cell lines and one human foreskin fibroblast cancer cell line. The five cell lines that were used in their study were: MCF-7 and HTB-126 (breast cancer), Panc-1, Mia-Paca2, and Capan-1 (pancreatic cancer), and HS68 (fibroblast cells) (Chan *et al*., 2011).

When the MCF-7 breast cancer cell line was treated with *A. chittagonga* they found that an IC_{50} of 42 µg/ml of Pet-ether (petroleum ether) and CH_2CH_2 (dichloro-methanol) extracts respectively, caused MCF-7 cell death. When they treated the same cell line with extracts of *A. rohituka*, the Pet-ether extracts showed an IC_{50} of ~ 41 µg/ml, while the *A. chittagonga* EtoAc (ethyl acetate) and *A. rohituka* MeOH (methanol) extracts did not show any cytotoxic effect on the MCF-7 cancer cells. When the other breast cancer cell line, HTB126 was treated with the CH_2CH_2 extract of *A. chittagonga* cell death occurred at an IC_{50} value of ~ 43 µg/ml. Similarly, all 3 pancreatic cancer cell lines treated with *A. chittagonga* CH_2CH_2 extracts showed significant cytotoxicity with IC_{50} values of ~ 39 µg/ml, 30 µg/ml, and 65 µg/ml, respectively (Chan *et al*., 2011).

The differences in cytotoxicity results among all the cancer cell lines indicated that from a single Amoora plant, many plant extracts using different solvents could be made, like other plants, but that the partitioning of such plant extracts using different solvents may have a significant effect on the biological activity of the resulting fraction. The results obtained from their cytotoxicity study on breast cancer cells have indicated, and it informs researchers, that the cytotoxicity values of *A. rohituka* and *A. chittagonga* may have been due to the content of amooranin, the active ingredient within the crude fractions (Chan *et al*., 2011). The difference in the cytotoxicity of the crude fractions made from *Amoora* in all 5 cell lines and the fibroblast cell lines suggested that certain cancer cell lines are more susceptible to some crude fractions and not others, while some may be resistant to particular fractions and display low or no toxicity (Kavitha *et al*., 2010; Chan *et al*., 2011).

In a study that investigated the cytotoxicity activity of root fractions of *Eurycoma longifolia* Jack root against new and HS27 cells (human foreskin fibroblasts), the extract (TACME) and 4 fractions (TAF273, TAF355, TAF191 and TAF401) of *E. longifolia* root showed high cytotoxicity against HS27 cells compared to Vero cells. No cytotoxicity activity was exhibited by the TAF355 fraction against Vero cells, while the TAF401 showed moderate cytotoxic activity (15.00 µg/ml) against the same cell lines.

The rate at which a cell divides determines the rate at which cells proliferate. Anticancer compounds have been used to prevent cancer cell proliferation by inhibiting the division of cancer cells for decades. The results obtained when testing the cytotoxicity of a particular drug or compound may be different when they are compared amongst and between different testing methods. For example, it has been established that both HeLa (human epithelial carcinoma) and SiHa (cervical carcinoma) cell lines are susceptible to carvacrol at IC_{50} 50mg/L and that its entry into those cancer cell lines increase when the MTT and LDH (lactate dehydrogenase) assays are employed and compared to other assays used for testing cytotoxicity of carvacrol like the XTT method (Mehdi *et al*., 2011).

2.5. Induction and detection of apoptosis in infected cells

2.5.1. Biological aspects of apoptosis

When the cells of the body do not receive an adequate supply of extracellular growth factors and mitogens, the growth and division of those cells are inhibited. This leads to the activation of apoptosis, whereby damaged, infected, or unwanted cells are eliminated from the body (Flemming, 1885; Ellis *et al*., 1991; Conlon and Raff, 1999). However, these are not the only factors that can induce apoptosis. Wyllie (1997) has suggested that when cells experience conflicting signals, the disruption of the normal status of the cell can also trigger apoptosis.

Apoptosis occurs on a continuous basis in the body in order to maintain homeostasis of 'good' verses 'bad' cells. The apoptotic stimuli in this process of normal cell turnover is represented by cytokines or death factors such as, Fas ligand (Fas L) (Nagata, 1994; 1997) or TNF (tumour necrosis factor) (Tartaglia *et al*., 1993; Tabibzadah *et al*., 1999). However, apoptosis can also be induced by some factors that are in fact pathological insults (Bratton and Cohen, 2001; Wahl and Carr, 2001). Staurosporin, an antimicrobial drug, functions as a general kinase and activates apoptosis. However, the exact apoptotic mechanism is unknown (Ojeda *et al*., 1995). Chemical agents like hydrogen peroxide also have the potential to trigger apoptosis in several cell types (Gorman *et al*., 1997; Madesh and Hajnoszky, 2001).

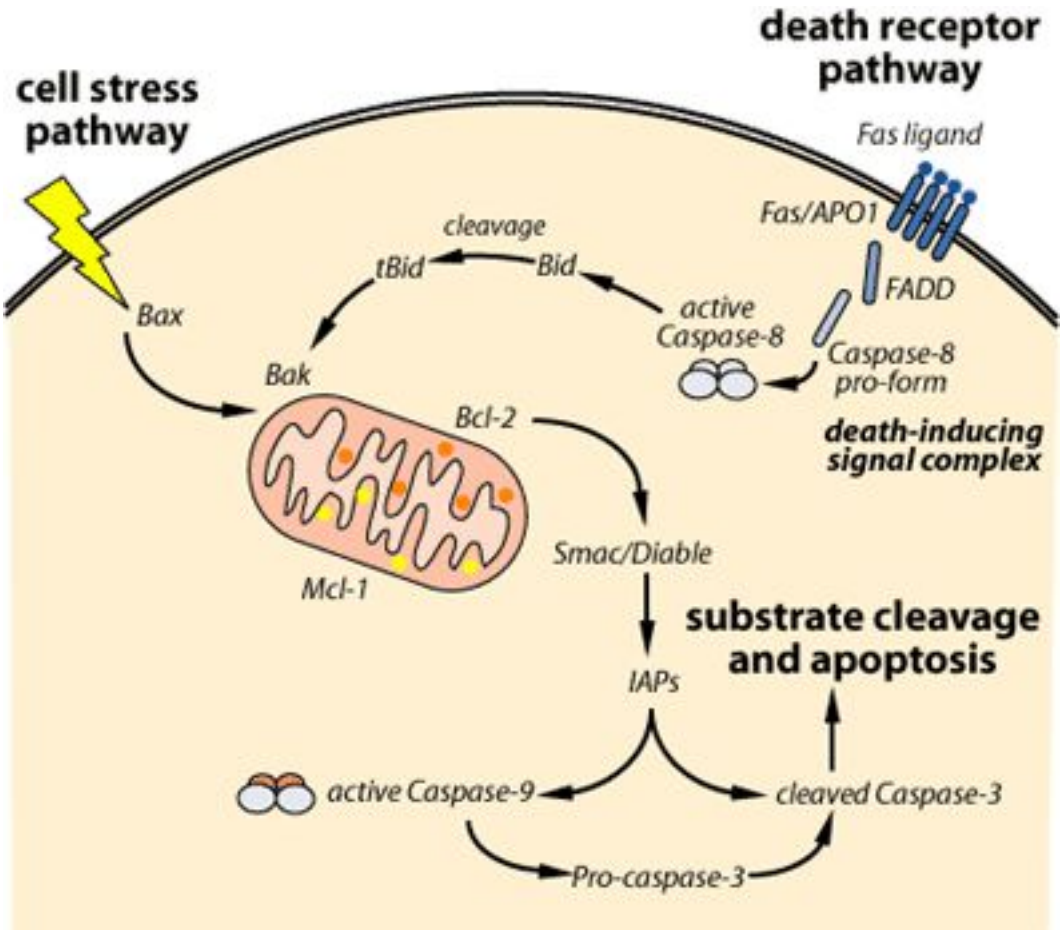


Figure 1: Apoptosis shown by the ligand-receptor and mitochondrion mechanisms (Yau, 2011)

When cells in the human body become infected by viruses, a population of killer lymphocytes are activated and induce the infected cells to initiate apoptosis (Hengartner, 2000). One way by which cells do this is by the binding of a protein CD95, also called Fas ligand, on a specific receptor (CD95 / Fas receptor) that is located on the cell membrane of the infected cell (Saikumar *et al*., 1999). The binding of the CD95/Fas ligand to the CD95/Fas receptor causes the adaptor proteins to cluster (FADD).

The clustering of FADD to the CD95/Fas receptor causes a particular procaspase, called procaspase-8, to assemble at the site of receptor clustering. Procaspase-8 is a protease that can cleave other proteins in apoptosis, including itself. When procaspase-8 cleaves itself, it releases caspase-8, which is activated. In addition, the cleavage of procaspase-3 by procaspase-8 results in the release of the caspase-3, which is also activated (Figure 1). The caspase-8 and caspase-3 play pivitol roles in many steps of the apoptotic pathway (Hengartner, 2000). Caspase-3 is said to be essential for the typical morphology of apoptosis

(Shiokawa *et al*., 1997) while caspase-8 activates proteins that are essential for promoting cell death (Bid) (Figure 1). This is achieved when bid interacts with proteins that are located on the surface of the mitochondrion. These bid proteins belong to the bcl-2 protein family (Hengartner, 2000) (Figure 1).

A more specialised form of receptor-ligand mediated apoptosis has also been understood when the cell membrane of infected cells become perforated with perforins due to Fas-Fas ligand binding caused by cytotoxic T-lymphocytes. This releases the protease granzyme B into the cytoplasm. The granzyme B splices caspases resulting in a caspase cascade causing cellular morphological changes (Martin and Green, 1995).

There are many other forms of receptor-mediated forms of apoptosis, but their mechanisms are still unclear. Apo-3 is a death receptor that induces the activation of caspase-8 by binding to the ligand Apo-3L (Chinnaiyan *et al*., 1995). TRAIL-R2, Apo-4 and Apo-5 have TRAIL as the ligand (Walczak *et al*., 1997). Other death receptors are: Apo-6, CD27, CD134, CD137, CD30 and CD40 (Ashenazi and Dixit, 1999). Ashenazi and Dixit (1999) recently found that the binding of TRAIL or CD95/Fas ligand to certain cell membrane receptors of infected cells do not induce apoptosis. These receptors are called decoy receptors (DcR) and they believe that these decoy receptors are present in normal tissues and may be involved in protecting cells against apoptosis.

The mitochondria plays a role in a second pathway of apoptosis (Hengartner, 2000). In this pathway, cytosolic agents such as nitrogen monoxide and radiation (Boscá and Hortelano, 1999; Mathieu *et al*., 1999) induce apoptosis via the activity of a protein known as p53 (Hengartner, 2000). Apoptosis in this pathway is characterised by changes in mitochondrial membrane permeability, leading to the release of cytochrome c and other mitochondrial proteins into the cytosol (Culotta and Koshland, 1993).

p53 is an important regulator of the cell cycle and its pathway can activate other death-promoting proteins such as Bax (Hengartner, 2000). This gene is mainly expressed when DNA damage occurs by γ-radiation or chemotherapeutic agents (Kamesaki, 1998; Mathieu *et al*., 1999). Cytochrome c is localised on the outerside of the inner mitochondrial membrane and in the intermembrane space of the mitochondrion (Hirsch *et al*., 1997). Cytochrome c plays a crucial role in the intercellular electron transport chain for the production of adenosine triphosphate (ATP) (Hirsch *et al*., 1997). At the outer mitochondrial membrane

surface, the balance between death-promoting and death-inhibiting proteins determine whether the mitochondrion releases cytochrome c (Marzo *et al*., 1998; Green and Kroemer, 1999; Hengartner, 2000). The bax (death-promoting protein) and p53 protein are structurally related to each other, and to the anti-apoptotic protein called Bcl-2 (Hengartner, 2000). When the balance shifts towards death-promoting members of the Bcl-2 family (bid, bax, p53 and Bcl-2), a cell is more likely to undergo apoptosis (Hengartner, 2000). Rossé *et al.* (1998) found that bcl-2 inhibits the working of cytochrome c rather than its release.

During apoptosis, cytochrome c is released into the cytosol where it binds to Apoptosis protease activating factor (Apaf-1) (Li *et al*., 1997). The release of cytochrome c recruits proteins such as Apaf-1 and other procaspases, like procaspase-9. The association of Apaf-1 and procaspase-9 forms a complex called the apoptosome (Hengartner, 2000). Procaspase-9 becomes cleaved into activated caspase-9 (Hengartner, 2000), which in turn results in the activation of procaspase-3 into caspase-3 (Li *et al*., 1997). Caspase-3 is responsible for cellular morphological changes (Martin and Green, 1995).

There have been some controversy as to how cytochrome c enters into the cytosol via the mitochondrial permeability transition pore (MPT). Some authors have suggested that the MPT is regulated by *bax* and *bcl-2* (Green and Kroemer, 1998; Marzo *et al*., 1998). Proteins of the inner and outer mitochondrial membrane, as well as intermembrane proteins, make up the MPT (Shimizu *et al*., 1999). When the channels open, ions enter. This causes the mitochondria to swell, which in turn causes breaks in the outer membrane, leaving the inner membrane intact because of its larger surface area. The cytochrome c escapes through the outermembrane breaks and enters the cytosol (Green and Kroemer, 1998). In contrast, Eske *et al* (1998) assume that the *bax*-induced release of cytochrome *c* may be dependent on magnesium ion (Mg^{2+}) concentration rather than on the mitochrondrial permeability transition pore. Also, it is believed that the formation of a megachannel may only occur in the later stages of apoptosis (Saikumar *et al*., 1999).

Recent research has found a third mechanism by which the apoptotic process occurs via the Endoplasmic Reticulum (ER). Agents that stress the ER such as kunicamycin and thapsigargin have shown recognisable apoptosis in embryonic fibroblasts of normal (heterozygous) mice (Nakagawa *et al*., 2000). The ER-mediated stressing agents such as Fas-

specific antibodies or dexamethasone, activates the receptor-ligand mechanism and the mitochondrial mechanism independently (Nakagawa *et al*., 2000).

In all three mechanisms of apoptosis (receptor-ligand mechanism, mitochondrial pathway and the mechanism by which the endoplasm plays a role), the morphological changes seen during apoptosis in infected cells are the same. These morphological characteristics are: cell shrinkage, collapse of the cytoskeleton, nuclear envelope breakdown, chromatin condensation and DNA degradation (Culotta and Koshland, 1993) (Figure 2).

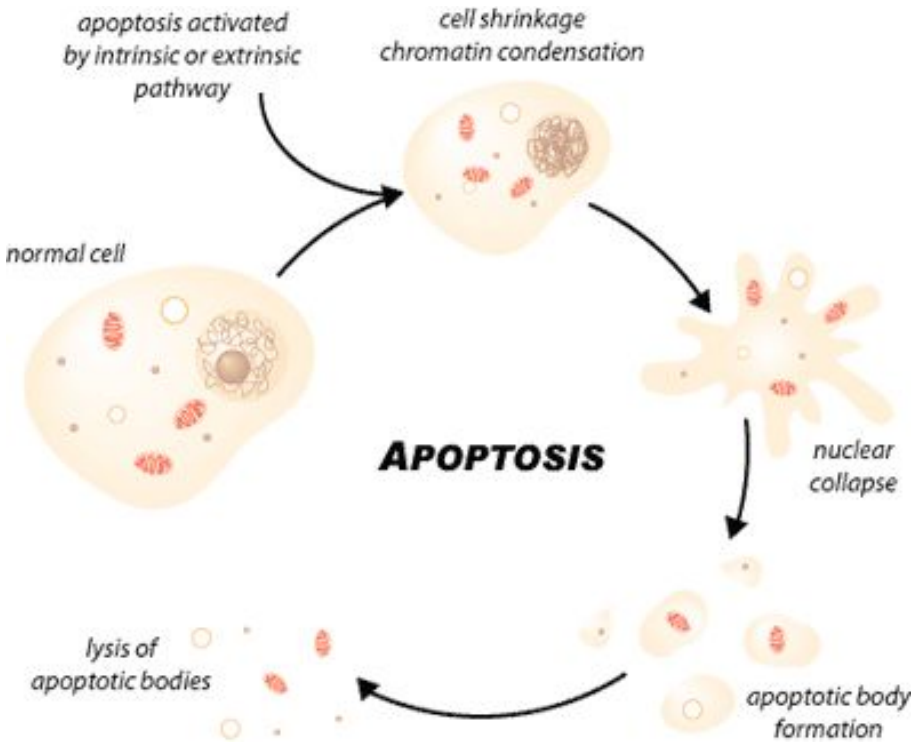


Figure 2: Apoptosis induced by intrinsic and extrinsic stimuli:
Morphological changes occurring during apoptosis (Yau, 2011)

The advantage of the function of p53 is that it serves as a molecular stop light during apoptosis by preventing infected cells from proliferating and being passed on to daughter cells. This means that without p53, the development of cancer would be much greater by allowing it to reproduce with the damaged DNA (Culotta and Koshland, 1993).

The inherited defects in the p53 gene generally increase a person's chance of developing cancer. The mutations in the p53 are not inherited; instead this is caused by environmental pressure such as DNA-damaging chemicals. Some cancer viruses such as human papillomavirus (HPV) induces the development of cancer by producing proteins that block

the action of the proteins encoded by Rb and p53 tumour suppressor genes (Culotta and Koshland, 1993).

In conclusion, the main function of apoptosis is to destroy cells that contain damaged DNA thereby preventing the proliferation and genetic spread from one generation to another. An important concern though is the high concentration of Bcl-2 proteins when the Bcl-2 oncogenes are transcribed and translated into those proteins because this contributes to the survival of genetically damaged cells like cancer (Culotta and Koshland, 1993).

2.5.2. Expression, regulation and detection of apoptosis

Several detection techniques are used to detect apoptosis. The techniques that can be used to study the morphology of apoptotic cells are: electron, light and fluorescent microscopy (McCarthy and Evans, 1998). In addition, and most relevant to the present study, reverse transcriptase-polymerase chain reaction (RT-PCR) and gel electrophoretic analysis of DNA amplicons are also used, but for the molecular detection of apoptosis (Chen *et al*., 2010). RT-PCR is a particularly important technique since modern biological investigations have indicated that the proliferation of some neoplasmic cells are related to the induction of apoptosis which is also regulated by the expression of oncogenes of the same neoplasmic cells (Chen *et al*., 1998). Recently, Chen *et al.* (1998) has grouped apoptotic genes into living and dead genes. The living genes include the cells proliferation and *bcl-2* genes (Chen *et al*., 1998), while the death genes include suppressing cell proliferation (*p53*) and *bax* genes (Cheng *et al*., 1997). *bax* and *bcl-2* genes have recently been studied extensively in gastric, lung and prostate cancer and have shown to be important in the regulation of apoptosis (Xue *et al*., 1999; Pillai *et al*., 2004).

Gel electrophoresis results have shown that apoptosis is linked to an interchromosomal DNA ladder which is specific for the detection of apoptosis (Dong *et al*., 1997; Saikumar *et al*., 1999). The characteristic of 'laddering' has also been observed when cancer cell lines undergo apoptosis that are triggered by anticancer agents which are derived from plants such as *Sutherlandia* spp. (Chinkwo, 2005).

Vinblastine, a plant anticancer agent derived from the plant *Catharanthus roseus* G. Don (Apocynaceae), has been found to be effective against leukaemia, lymphomas, advanced testicular, breast and lung cancer, as well as Kaposi's sarcoma; while vincristine, derived

from the same plant species, is used mostly for the treatment of leukaemia's (Cragg and Newman, 2012). This has indicated that even though different plant extracts can be derived from one plant species, their action to therapeutic treatment in cancer could possibly vary. However, the mechanism of apoptosis may be the same (i.e. have the same morphological and genetic result outcomes). Differences in genetic outcomes may be apparent because of the cytotoxicity and/or potency of the administered compounds/fractions (Gragg and Newman, 2012).

Bulbine and *Kniphofia* are closely related genera that belong to the sub-family of perennial herbs, Asphodeloideae. These extracts may target and/or 'switch on' death signaling genes such as *bax* resulting in cell death. This may be represented by DNA fragmentation. The reason for this has to do with the relation of the 2 plant species by possessing the same anticancer compounds, anthraquinones. Chrysophanol, aloe-emodin, knipholone, knipholone anthrone, and islandicin, are the anthraquninones, found in the roots of *Bulbine frutescence* (L.) Willd and *Bulbine abyssinica* (Dagne and Yenesew, 1994).

The inhibitory effects of anticancer compounds and the promotion of apoptosis in cancer cell lines can be quantified by RT-PCR (Chen *et al.*, 2010). This is a sensitive method of analysing gene expression. It consists of cDNA synthesis, DNA amplification, and signal detection. However, the accuracy of quantification is dependent on the efficiency across these steps (RT and cDNA amplification) (Shiao, 2003). Since RNA is a very unstable molecule because of its single-strandedness, it is not appropriate to conduct RNA studies in cancer cell lines treated with plant compounds of unknown cytotoxicity or that are new to anticancer research. One should use, instead, a protocol that is independent of RNA isolation in order to obtain high thoroughput results. RT-PCR is used to study the expression of genes, such as those involved in apoptotic signaling (Shiao, 2003). *B-actin* or *GADPH* are used as internal RT-PCR controls in apoptotic / cell death studies and its expression in cell lines indicate that the cell line is viable at the time of performing the RT-PCR experiment (Shiao, 2003).

In South Africa, *B. natalensis* and *B. frutescens* are best known to treat wounds, burns, rashes and cracked skin (Jager, 1996). Current research interests are on *B. natalensis* crude fractions and its action against cancer cell lines in relation to apoptotic mechanisms because this is not well understood, nor has it been established. This has been better understood for cancer cell

lines treated with natural products (Pillai *et al*., 2004) and the fractions of other plant spp. (Chikwo, 2005).

2.6. Medicinal plants, aloe and the genus *Bulbine*

Throughout the world various strategies are currently being developed for the selection of plant species for scientific examination. One of those strategies involve the isolation and identification of substances from plants that have positive activity in assays they are used in e.g. a plant that has good apoptotic potential will be selected if the other plants tested have poorer apoptotic potential in an apoptotic assay (Abegaz *et al*., 1999). Many plant species such as *Hagenia abyssinica*, *Salsola somalensis*, *Taverniera abyssinica*, *Bulbine capitata, Rhamnus prinoides* and *Borstenia* spp. have become marketable commodities because they have already been screened by traditional methods (Abegaz *et al*., 1999). These plant species are well-known for their isoflavanoids, nor-lignans, anthracene, napththalene derivatives and poly prenylated flavonoid biological activities (Abegaz *et al.,* 1999). Like *Bulbine capitata* there are several other Bulbine species that have traditional uses and are currently being screened for their medicinal values (Dagne and Yenesew, 1994; Abegaz *et al*., 1999). These include *Bulbine natalensis* and *Bulbine frutescens*, which belong to the family Asphodelaceae (Abegaz *et al*., 1999; Coopoosamy, 2011).

Asphodelaceae is the family to which the aloe belongs and therefore, traditionally, the uses of *Bulbine* spp. mimic those of the aloe. It has been believed that aloe vera is a natural medicine for cancer, ulceritus, Crohn's and Celiac disease, cholesterol, diabetes, cardiovasicular diseases, inflammation and other health conditions (Adams, 2007), and this property of aloe vera has become particularly important in developing countries where traditional medicine plays a pivitol role in health care (Abegaz *et al*., 1999; Adams, 2007). In addition, aloe vera is also used as a natural food preservative in the food industry (Adams, 2007). The uses of *Bulbine* spp. vary in different parts of the world, for example, in south-eastern Australia, Bulbine is an indigenous plant that is used by Aborigines as a food source or life necessity, while in most of Africa, selected species are used for their medicinal properties (Incoll *et al*., 1989; Englyst and Hudson, 1997; Gott, 2008). The genus *Bulbine* is known for their storage property of carbohydrates in their roots in the form of starches, with amylose and amylopectin in varying proportions, however, their effect on pre-European Aborigean are quite drastic in that consuming *Bulbine* spp. was found to cause type 2 diabetes because of

the fructans (polymers of fructose) that are highly concentrated in the roots (Incoll *et al*., 1989; Englyst and Hudson, 1997; Gott, 2008). This dietary effect has also been found in other indigenous European plants such as *Microseris lanceolata* and *M. scapigera* (Incoll *et al*., 1989; Englyst and Hudson, 1997; Gott, 2008).

Bulbine spp. have been used as a medicinal source since the early eighteenth century, ever since the Dutch and British settled in South Africa (Coopoosamy, 2011). *Bulbine* spp. contain novel natural products and more insight into their gained marketed commodity status have been acquired through scientific visits to markets in Botswana, Ethiopia, Kenya, Tanzania and Uganda (Abegaz *et al*., 1999). This implies that the genus *Bulbine* has become more wide spread since the eighteenth century, where it was most abundant in South Africa. The species that belong to the genus *Bulbine* (Asphodelaceae) are mostly distributed in South Africa, where approximately 41 species have been recorded (Abegaz *et al*., 1999). *Bulbine abyssinica*, *B. annua*, *B. aspodeloides*, *B. frutescens*, *B. capitata* and *B. latifolia* are a rich source of secondary metabolites and these metabolites include: alkaloids, benzene, naphthalene, anthraceane, bianthracene derivatives, chromones, coumarins and pyroles (Abegaz *et al*., 1999), which have a variety of activities ranging from antibacterial to antifungal to antiviral and antiparasitic (HealingAloe, 2012).

Some *Bulbine* spp. like *B. natalensis* (Figure 3) contain plant sterols such as campesterol, β-sitosterol, lupeol and cholesterol which are used as natural analgesics to reduce inflammation and thus pain (HealingAloe, 2012). *B. natalensis* and the other mentioned species have been reported to contain medicinal compounds that are similar to those found in the *Knipholone* spp. (Abegaz *et al*., 1999). These compounds include anthraquinones, islandicin, knipholone and knipholone anthrone (Abegaz *et al*., 1999). Anthraquinones are antimicrobial agents that include barbaloin-10, isobarbaloin and anthrone c (glycosides and chromones) (HealingAloe, 2012). In addition, since *Bulbine* spp. resembles the aloe, it is believed that they probably contain sallyic acid, an aspirin-like compound, which has anti-inflammatory and antimicrobial properties similar to the sterols (HealingAloe, 2012). Similarly they contain the 12 essential amino acids that are present in aloe: alanine, arginine, asparagine, cystenine, glutamic acid, glycine, histidine, proline, serine, tyrosine, glutamine and aspartic acid which have different functions in the human and animal body (HealingAloe, 2012). The sap of aloe contains important enzymes such as aliiase, amylase, certalase, bradykinase, lipases and proteases and therefore they aid in cleansing the circulatory system (HealingAloe, 2012).

This cleansing action is further enhanced by the present of saponins, detergent-like substances, which also act as an anticarcinogen (a substance that counteracts a cancer-producing substance) (HealingAloe, 2012).

Aloe sap also contains the glycoproteins, aloctin A and B (Imanishi *et al*., 1981; Abegaz *et al*., 1999), however, no recent studies have been performed to clarify if these compounds are present in *Bulbine* spp. The presence of these compounds was first discovered from *Aloe ferox* and *A. vera* (Abegaz *et al*., 1999). The true natural product is believed to be aloctin B since it has a glucose moiety in the α-orientation from which aloctin A is derived (Abegaz *et al*., 1999). Studies have reported that aloctin B is derived from the attachment of glucose to aloe-emodinanthrone (Abegaz *et al*., 1999).

Figure 3: a) *Bulbine natalensis* and b) Star-shaped flowers of *B. natalensis* (African aromatics, 2012)

In Botswana, Ethiopia, Kenya, Tanzania and Uganda, the roots and corms of *Bulbine* spp. are sold as a commodity because of their medicinal value (Abegaz *et al*., 1999). *B. natalensis* is known as Ibhucu (Zulu) and Rootwortel (Afrikaans) in South Africa (Coopoosamy, 2011). This is an evergreen perennial that has broad sharp pointed fleshy yellow-green leaves. It has star-shaped flowers and is drought resistant (Figure 3). In contrast, *B. frutescens* has narrower green pointed leaves which are not as fleshy as *B. natalensis* (Coopoosamy, 2011) (Figure 4). *B. frutescens* only has two plant organs, roots and leaves, whereas *B. natalensis* has three organs, namely, roots, corm (bulbotuber) and leaves. Both these plant species are used by herbalists and traditional healers in South Africa to treat mosquito bites, blisters, cold sores, mouth ulcers, pimples, cracked skin, and to heal blisters among many others (Jager, 1996; HealingAloe, 2012). It has also been widely researched that *B. natalensis* is used by

bodybuilders to boost testosterone levels, to lower oestrogen levels, burn fat, build muscle and gain strength (HealingAloe, 2012). Although more general information exists on *B. frutescens*, *B. natalensis* is the more frequently researched/studied species.

Figure 4: *Bulbine frutescens* showing flowers (Colours of Creation, 2012)

To date, apart from the general analgesic, anti-inflammatory, wound healing, immune modulating, antitumour, antiviral, antibacterial and antifungal activities (HealingAloe, 2012), there are no recorded studies on the induction of apoptosis in cancer cell lines by *Bulbine* species. All the information that does exist on *B. natalensis* and *B. frutescens* are on their uses in traditional and herbal medicine, nothing with any scientific clarity. There is a reservoir of knowledge on the general uses of *B. frutescens*. However, very little information exists for *B. natalensis.* Recent studies at the Department of Biotechnology and Food Technology at the Durban University of Technology found that *B. natalensis* has cell killing and apoptotic potential against cancer cell lines (Padayachee and Reddy, 2009; Kasumbwe and Reddy, 2010; Singh and Reddy, 2012).

CHAPTER THREE

Cell Culture and Cytotoxicity

3.1. Introduction

When scientists are in search for new medicinal discoveries, they are unable to use human models for testing their research products because of ethical reasons and because, while normal cell lines have intact programmed cell death mechanisms, cancer cells (like HEp-2), lack such mechanisms. However, treating infected cells in the body also cause normal cells to encounter the treatment type, resulting in side effects to organs including the gastrointestinal tract, bone marrow and circulatory system etc. (Balasubashini *et al*., 2006). This makes the use of animal models like rat and mice more convenient. When there is a new pharmaceutically discovered product, tests are first performed on infected (and/or normal) cells to evaluate their effectiveness before commercialising them as a therapeutic source. This is the principle employed when testing the cytotoxicity of newly discovered or plant-derived compounds, and depending on the compound that is being tested, either normal or infected cell lines are used when performing laboratory tests (Burdall *et al*., 2003; Balasubashini *et al.,* 2006).

There are advantages and disadvantages of using cell lines and these have been described in considerable detail by Burdall *et al.* (2003). The two most common advantages are that cell lines are easy to handle and are available in infinite quantities because of their unlimited ability to selfreplicate. They are also replacable if lost due to contamination from frozen stock cultures (Burdall *et al*., 2003). The disadvantage using cell lines is that culturing/subculturing causes gentotypic (karyotype and clonogenicity) and phenotypic (morphological) drift, and this has been found to be common in frequently used cell lines such as the HeLa (cervical cancer) and MCF-7 (breast cancer) cell lines (Osborne *et al*., 1987), as well as some cell lines that have been deposited in cell banks for many years. Subculturing has been shown to result in selecting subpopulations of rapidly growing clones within a population which may cause phenotypic changes over time (Burdall *et al*., 2003). Because of the genotypic and phenotypic drift amongst established cell lines, it has become evident that there is a lot of heterogeneity within established cell lines of the breast, cervix, larynx and the oesophagus (Burdall *et al*., 2003).

Cytotoxicity is the measure of the how toxic a compound is to a particular organism or cell culture system (Mossman, 1983; WST-8 patent). It is usually expressed as a percentage of 100, minus the ratio of cell viability i.e. absorbance of treated cells (nm): absorbance of untreated cells (Balasubashini *et al*., 2006; Padma *et al*., 2007) or DMSO negative control (nm) (as in the present study). The MTT cytotoxicity assay is the most widely used method for measuring the cytotoxicity of natural and unnatural compounds (WST-8 patent), and is the most published method in articles relating to cytotoxicity. However there are other methods that have become less widely used as well, and these are: colony formation, CCK-8, crystal violet, Trypan blue, XTT assay, ^{51}Cr (chromium), tritium-labelled thymidine uptake and the WST methods (WST-8 patent) (refer to literature review).

In the Chinese, Indian and Arab systems of medicine, plants have been for used for centuries in Uhani, Ayurveda and Siddha – as a form of replacing conventional medicine with complementary and alternative medicine (Shafi *et al*., 2009). Laryngeal carcinoma has become one of the most common types of head or neck cancers, and its high incidence among people is attributed to alcohol consumption, smoking of tobacco and the chewing of betel quid. This type of cancer is treated with radiotherapy or is surgically removed – treatments which often cause the larynx to lose its function (Day *et al*., 2003). This highlighted the need of the present study to screen fractions of the indigenous species *Bulbine* for anticancer properties using the MTT cytotoxicity assay, given the fact the Kasumbwe and Reddy (2010) found that organic and aqueous fractions (similar to those in the present study) of the species, *Bulbine natalensis* were cytotoxic. The effect of plant compounds on laryngeal cancer has been extensively studied, with a profound amount of success to improve the prognosis of laryngeal cancer treatment (Ghashm *et al*., 2010), particularly because plant products serve as vital sources of supressing agents that interfere with the carcinogenic process (Patel *et al*., 2011; Shilpa *et al*., 2012). *Braccharis dracunculifolia, Euphorbia hirta, Nigella sativa, Rubia cordifolia*, ceramide and green tea are but a few examples that have shown promising anticancer activity, using the MTT cytotoxicity assay, against the HEp-2 cell line (Ali *et al*., 2003; Hsu *et al*., 2004; Kummaline *et al*., 2007; Lin *et al*., 2009; Búfalo *et al.,* 2010; Maleek *et al*., 2010; Sidambaram *et al*., 2011; Shilpa *et al*., 2012). However, although fractions from those plant species exhibited only cytotoxic effects on the HEp-2 cell line in a dose and time dependent manner (followed by an increased release of lactate dehydrogenase, in a dose and time dependent manner, into the complete culture medium), there have been conflicting

reports about delayed apoptosis or cell death upon the treatment of HEp-2 with *Nigella sativa* or black cumin seeds, ceramide and the organic extracts of *Playcodon grandiflorum* (Lee *et al*., 2004; Maleek *et al*., 2010). These are three cases where polarity-guided fractionation, of those plant compounds, resulted in proliferative and antiproliferative effects on laryngeal carcinoma, highlighting the selective growth inhibitory and apoptotic effect of those fractions on cancer cells (Worthen *et al*., 1998; Shioeb *et al*., 2003). However, in the HEp-2 and HeLa cell lines, the potent properties of compounds from venoms for example, are always hindered by their high toxicities (Balasubashini *et al*., 2006). This emphasised the need to perform new scientific research in field of laryngeal cancer, to envisage the link between cytotoxicity and apoptosis-inducing potential in the HEp-2 cell line (Lee *et al*., 2004).

In this chapter the MTT assay was used to measure the cytotoxicity of aqueous and ethanol crude fractions of *B. natalensis* and *B. frutescens.* When using the MTT cytotoxicity assay, the cytotoxicity of compounds are based on the ability of an organism or cell to takeup a compound. The less the production of purple formazan crystals is an indication of enhanced cell death by that compound, with the inverse relationship being valid (Mossman, 1983; Vistica *et al*., 1991; Berridge and Tan, 1993). However, a compound administered with same concentration to different cells or organism types may exhibit different levels of cytotoxicity due to many reasons, one being the genotypic drift caused during the subculturing process (Osborne *et al*., 1987). Pillai *et al.* (2004) found that the growth inhibitory effect of curcumin on A549 (small cell lung carcinoma) and H1299 (large cell lung carcinoma) cells was 50 and 40 µM, respectively. This highlighted the differences in the sensitivity or susceptibility of the two cell lines to curcumin administered at the same concentration. Say an organism or cell is genetically programmed not to consume a particular compound or substance (i.e. it is resistant), then reading the absorbance of such compounds will not produce valid cytotoxicity readings. This means that there will be no reading on the ELISA plate reader causing it to go off balance i.e. the ELISA plate reader will have to be re-calibrated (obtained from performing such experiments). In this chapter, a negative cytotoxicity reading means that the fraction had a proliferative effect, while a positive reading would indicate that the fraction possesses an antiproliferative / cell killing effect on the HEp-2 cell line.

3.2. Materials and Methods

3.2.1. Plant material

Plant material, *Bulbine natalensis* and *Bulbine frutescens* were collected from the Durban area, and identified by botanist, Professor Himansu Baijnath (University of KwaZulu-Natal, Durban) (Figures 3 and 4).

Figure 5: a) *Bulbine natalensis* b) *Bulbine frutescens*

The leaves, fibrous roots and corm (bulbotuber) were removed individually from *B. natalensis* (Figure 5). For *B. frutescens*, the leaves and roots (with stem portions) were removed individually (Figure 5). All plant organs were washed and rinsed thoroughly so as to ensure that all the sand from them were removed. The plant organs were then left in a rack over the sink to drip-dry. Thereafter, the plant organs were placed on a table, covered with newspaper, where they were exposed to sunlight, before air-drying. Once dried, the plant organs were stored in separate transparent plastic bags (Figure 6).

Figure 6: *B. natalensis* leaves (a) and corm (b) and *B. frutescens* leaves (c) and roots (d) in plastic bags after air-drying

3.2.2. 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 (Figures 7 and 8). 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 stored in a biofreezer at -70^{o}C (Snijder Scientific) for 24 h and then freeze-dried (Virtis) for 24 - 48 h (Figure 9). One mg of the freeze-dried aqueous extract was dissolved in 1 ml of 10 mM plant tissue culture grade dimethyl sulfoxide (DMSO) (Sigma Inc), 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 (Figure 9). 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 (Figure 9).

Figure 7: *Bulbine natalensis* dried organs ground into powder during the preparation of the crude fractions. a and b) leaves,c and d) corm/bulbotuber, e and f) roots.

Figure 8: *Bulbine frutescens* dried organs ground into a powder during the preparation of the crude fractions. a and b) leaves, c and d) roots with stem portions.

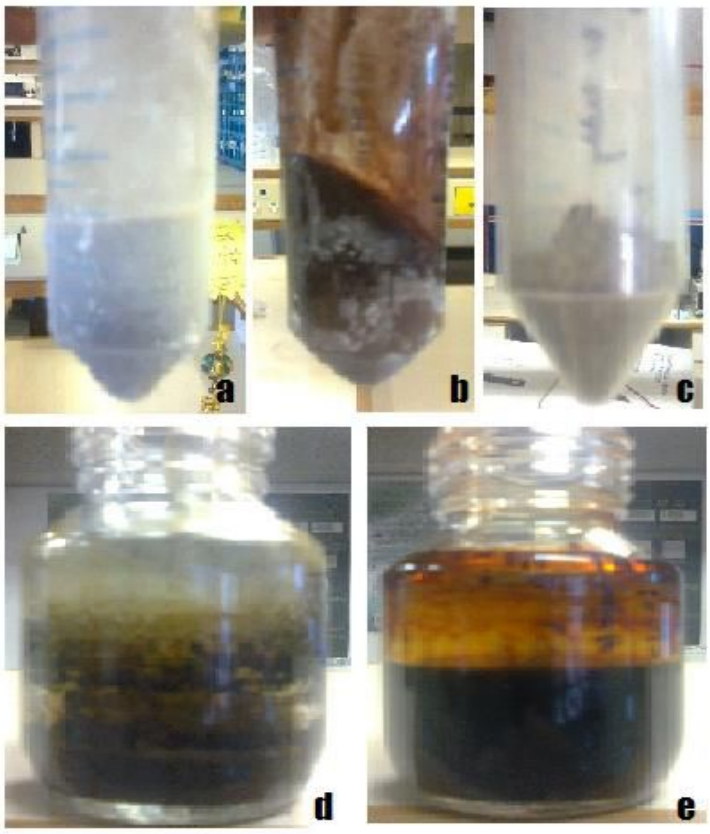


Figure 9: Biofreezed samples (a and b), free-dried sample (c), 100% ethanol samples (d and e) left to air-dry.

3.2.3. Cell Culture

3.2.3.1. Cell culture and maintenance

The adherent spindle-shaped human laryngeal carcinoma cell line HEp-2 (ATCC no. CCL-23) was used in this study (Figure 10). This cell line was first established in 1952 by A.E. Moore, H.W. Toolan and C. Sabachewsky when weanling rats underwent tumourigensis after they had been treated with epidermoid carcinoma tissue from the larynx of a 56-year old man (Grem and Fisher, 1989). Their study further showed that HEp-2 cells were resistant to fluctuations in temperature, nutrition and environmental stress and it possessed a high proliferation rate of a 23 hour cell cycle (Grem and Fisher, 1989). Since 1952, the HEp-2 cell line has supported the growth of 10 of 14 arboviruses including the measle virus, and in addition, it has been used for experimental studies of tumour production in rats, hamsters, mice, embryonated eggs and terminal cancer patient volunteers (Sidambaram *et al.*, 2011). HEp-2 cells contain HeLa marker chromosomes, and they are derived via HeLa

contamination. They are also positive for keratin by immunoperoxidase staining (ATCC, 2012).

The HEp-2 cell line was supplied by Highveld Biological in two flasks placed in a polystyrene box, which had parafilmed caps. When the cells had arrived, each flask was split into two new flasks under the laminar flow hood (Scientific Engineering Inc). The Ultraviolet light of the laminar flow was switched on 30 minutes before performing cell culture work and 30 minutes after cell culturing and maintenance. The laminar flow as well as hands were swabbed with 70% ethanol (Merck) before, after and during cell culturing and maintenance. This ensured that the environment of the laminar flow was sterile and free of contaminating organism.

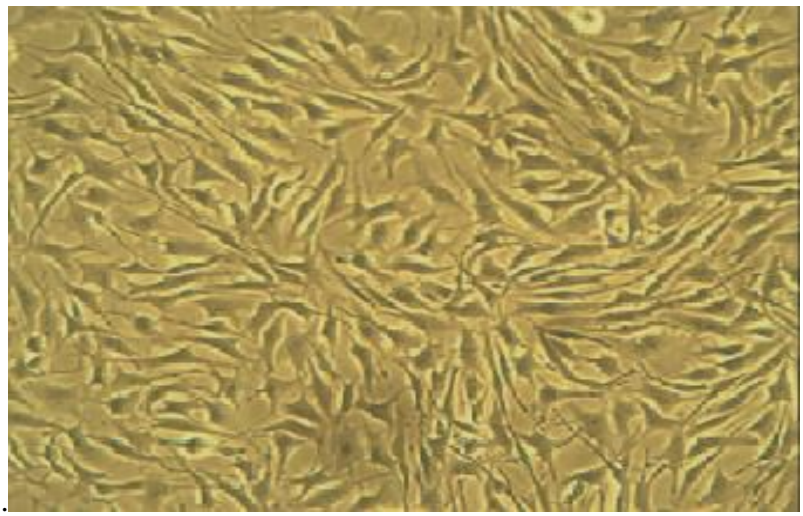


Figure 10: Spindle-shaped HEp-2, laryngeal carcinoma cells, supplied by Highveld Biological (magnification: 100 X).

HEp-2 cells were grown asceptically in 250 ml canted-necked 75 cm^3 tissue culture flasks (Corning Inc) containing DMEM (Dulbecco's Modified Eagle Medium) (Highveld Biological) that had already been supplemented by the supplier with glucose (4,5 g/l), L-glutamine (1 mM) and sodium pyruvate (0.110 g/l). The medium was further augmented with 10% heat-activated foetal calf serum (FCS) (GIBCO, Invitrogen) and 1% streptomycin/penicillin solution (GIBCO, Invitrogen). This medium was termed the Complete Culture Medium (CCM). The quantities of supplements used for every 100 ml of CCM made, was 5 ml of FCS and 1 ml of antibiotic solution to every 96 ml of DMEM that was used. The CCM was filter-sterilised at the time of usage. However, for the stock solution

no filter-sterilisation was required. The cells were grown in CCM that were at room temperature.

The tissue culture flasks, containing cells with CCM, were placed in an incubator (Snijder Scientific) containing a 5% CO_2 atmosphere and a temperature of 37^{o}C. The flasks were monitored daily using an inverted light microscope (Nikon). When the cells had reached the exponential growth phase and were between 80 - 90% confluent, they were subcultured. The estimated time noted for subculturing was between 2 - 3 days.

3.2.3.2. Subculturing and changing of media

When the tissue culture flasks were 80 - 90% confluent, CCM was removed from the flasks and the monolayer of cells were washed twice with 5 ml phosphate saline buffer (PBS). The PBS solution was made by dissolving one PBS tablet (Sigma-Aldrich Inc) in 200 ml autoclaved-distilled water. The pH of the PBS solution was checked using the pH meter and adjusted using NaOH and HCl solutions, until pH 7.4 was obtained. This pH was recommended by Sigma-Aldrich Inc.

An aliquot of 1 ml filter-sterilised trypsinising solution (0.25% trypsin with 0.1% EDTA (ethylenediamine tetra-acetic acid) (1:1 v/v) and 0.1% glucose) (Highveld Biological) was thereafter added to the flasks and placed into the above mentioned incubator for about 3 minutes. These flasks were not capped tightly. From working with these cells, it was noticed, that loosely tightened-capped flasks aided the HEp-2 cells from detaching from the flask.

After 3 minutes, the caps of the flasks were immediately tightened and hit gently on their side to aid the detachment process (Figure 11). This took place in the tissue culture laminar hood because of the sterile and aseptic environment. Once the trypsin solution turned turbid, it was taken as an indication that the HEp-2 cells had detached from the flasks. The flasks were then viewed under the inverted microscope (Nikon) to ensure that the cells were floating in the trypsinising solution and that they were healthy for subculturing.

Thereafter, the flasks were opened and the HEp-2 cells containing trypsinising solution were treated with 10 ml of (prewarmed at room temperature) CCM each. This assisted to stop the effect of the trypsin on the HEp-2 cells. It was personally observed from prior investigations dealing with this project that HEp-2 cells lose their spindle-shaped morphology when left in trypsin for too long.

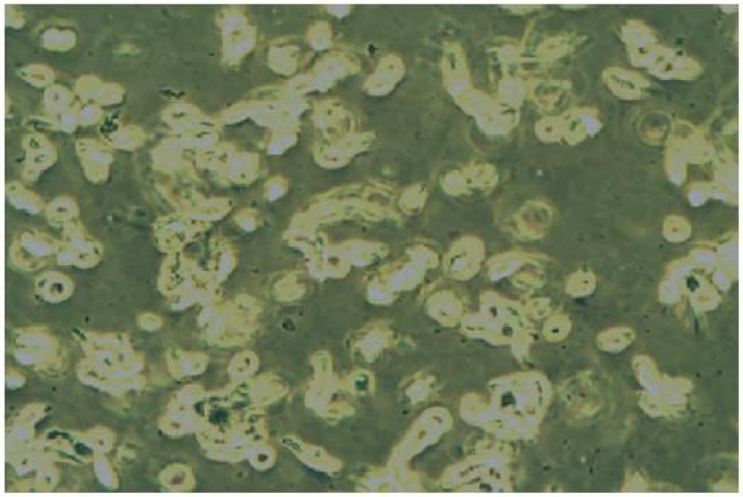


Figure 11: Detached HEp-2 cells in the presence of trypsin (magnification: 100 X).

From each canted-necked flask, 5.5 ml of CCM containing HEp-2 cells were serologically pipetted into a new 250 ml 75 cm^3 tissue culture flask. The original flask as well as the new flask was topped up with 15 ml of filter-sterilised CCM to bring the volume to 20 ml in each flask. The flasks were thereafter placed in the mentioned incubator (Snijder Scientific) (Figure 12).

In instances where cells in flasks were growing too slowly, no subculturing was performed. Instead the old CCM was removed and replaced by new filter-sterilised, prewarmed, CCM. They were thereafter microscopically monitored daily until they reached the exponential growth phase (80 - 90% confluent) and were ready for subculturing (Figure 13).

Figure 12: Changes in colour of CCM. a) pink CCM immediately after subculturing or when media has been changed, b) dark orange CCM after two days of subculturing or medium has been changed, c) completely orange CCM when HEp-2 cells were left in medium for too long (acidifying media).

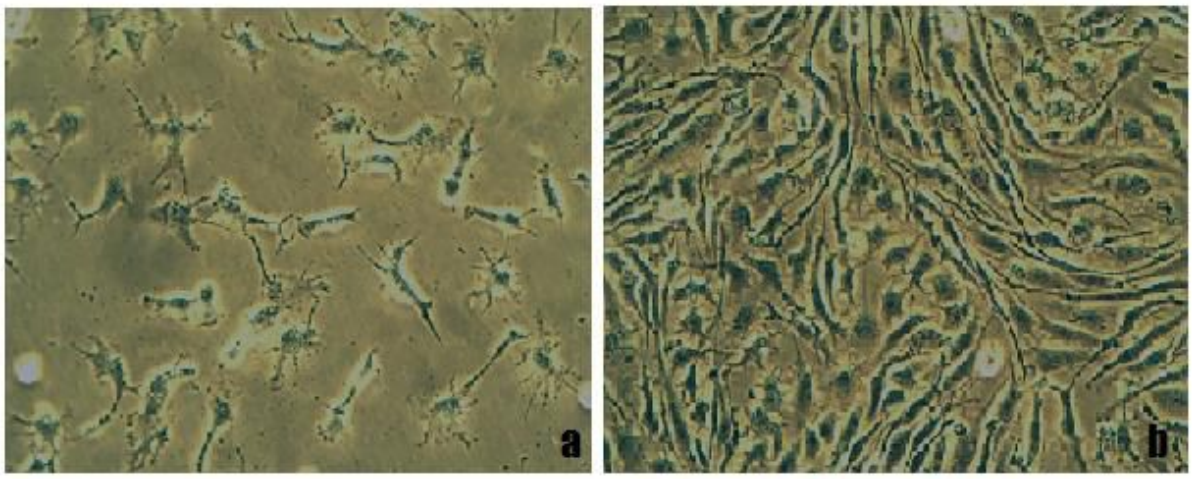


Figure 13: HEp-2 cell growth after subculturing. a) HEp-2 cell confluency following one day of incubation after subculturing (acclimatisation phase), b) 80 - 90% cell confluency after 2 days of incubation following subculturing (exponential phase) (magnification for a and b: 100 X).

The turbidity ('milky nature') of CCM in the flasks were monitored to ensure that contaminated flasks were thrown away. This also ensured that contamination of the incubator and associated HEp-2 culture flasks were prevented. Cell growth was monitored using an inverted light microscope (Nikon) at all times.

3.2.3.3. Storage of HEp-2 cell line

In cases where there were many flasks of cells available, some flasks were stored away. The initial procedure during the storage of cells was the same as subculturing. The difference was that instead of the 5.5 ml of CCM being serologically pipetted into a new flask, the entire 10 ml (and 1 ml trypsin) of CCM from the original was serologically pipetted into 2 (2 × 5.5 ml) 50 ml centrifuge tubes (Corning Inc) and capped tightly. The tubes were thereafter centrifuged at 1.500 rpm (Merck) for approximately 7 minutes. The centrifuged tubes were handled with hands that were swabbed with 70% ethanol.

Once the pellet was visible, the supernatant was removed aseptically and the pellet was reconstituted with 1 - 1.5 ml of filter-sterilised, prewarmed at room temperature, cryopreservative-freezing solution. This solution was made up of 90% DMEM (Highveld Biological), 10% FCS (GIBCO, Invitrogen) and 10% DMSO (Sigma-Aldrich Inc). The cells together with the cryopreservative were mixed gently using a micropipette and placed into

2.0 ml cryogenic vials (Corning Inc). The cryovials were thereafter placed in a box that was placed into the biofreezer at -70^{o}C (Snijder Scientific) immediately.

The cryovials were labeled with the type of cell line, the date they were stored, as well the type of subculture that was stored (for example, if the flask of HEp-2 cells that was stored was already subcultured three times, the cryovial was labeled 3 × to indicate this; Figure 14). Only cell culture flasks that went through 3 subcultures were used

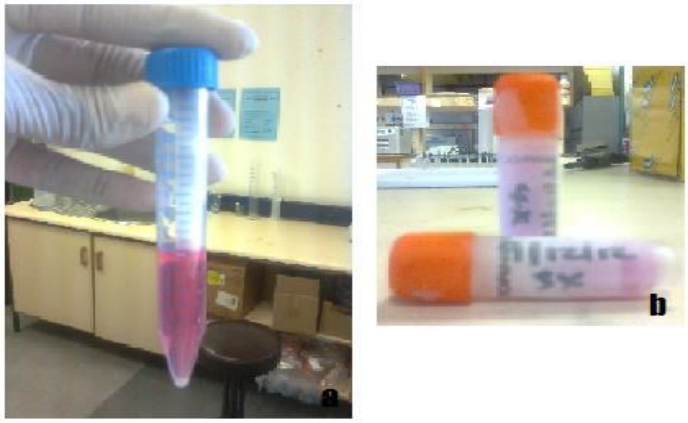


Figure 14: Storage of HEp-2 cells. a) the pellet obtained after centrifuging with CCM, b) cryovials used to store HEp-2 cells noting the cell line, level of subculturing (3 ×, 4 ×, etc.), and date of storage.

3.2.3.4. Regeneration of stored HEp-2 cells

Stored cells were regenerated by massaging the cryovials between hands, and once melted, transferring the HEp-2 cells to 20 ml of pre-warmed CCM in flasks. These flasks were incubated at 37^{o}C in a humidified incubator with a 5% CO_2 atmosphere.

3.2.3.5. Cell count

Cell enumeration for subsequent experiments (the MTT cytotoxicity assay and apoptotic gene expression study) were carried out using the dye-exclusion method of Freshney (1987) on a Neubauer counting chamber (haemocytometer).

The chamber was first cleaned with 70% ethanol. The Neubauer chamber contains a grid of 4 sets of 16 squares. The cell suspension (i.e. cells that had been trypsinised and to which 10 ml CCM had been added to stop the reaction between trypsin and membrane components of the HEp-2 cells), was mixed and approximately 1 ml was placed into an eppendorf tube. 100 µl of cell suspension from the eppendorf tube was placed into another eppendorf tube to which

equal volume (i.e. 100 µl) of trypan blue dye was added and mixed properly using a pipette. A cover slip was placed over the chamber and 40 - 60 µl of cell suspension with trypan blue dye, was run on the edge of the grooves using a Gilson tip. The chamber was thereafter observed under the phase contrast microscope (Nikon).

Only healthy HEp-2 cells were counted. These cells had a 'glowing' appearance. The cells that were blue were ignored because they had taken up the trypan blue dye and were therefore considered dead according to Freshney (1987).

The following equation was used to obtain the cell count:

Total cell count = 16 squares × 4

= cell count in 4 sets of 16 squares

16 squares = 2×10^4 /ml

therefore cells per ml = $\frac{\text{total cell count}}{4} \times 2 \times 10^4$ per ml

= $\frac{\text{total cell count}}{2} \times 10^4$

= cells per ml

3.2.4. Treatment of HEp-2 cell line with *Bulbine* spp. fractions

Bulbine fractions from each organ was dissolved in 10 mM DMSO (Sigma) (final concentration of 1000 µg/ml) and was stored in dark-coloured eppendorf tubes at 4°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. Although the aqueous plant fractions were soluble in water, DMSO was used to dissolve them to keep all of the experimental parameters in terms of preparing the plant fractions consistent. Furthermore, administering a fraction 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 possibly

uncontrollable cell death, the aqueous fractions were not dissolved in water (Becker *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 of greater than 200 µM. Therefore the fractions were administered at this concentration and the final 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) (optimisation experiment results follow). Initially a 3 day MTT cytotoxicity study was performed for the *Bulbine natalensis* fractions so that some idea about the utilisation of the fractions by the HEp-2 cell line could be ascertained. The protocol used is the same as explained above.

3.2.5. MTT cytotoxicity assay

The MTT (3,4,5-dimethylthiazol-2yl)-2-5-diphenyltetrazolium bromide) cytotoxicity assay is a method that is used to evaluate the cytotoxicity of compounds or to measure cell viability. The mitochondrial activity of cells are measured 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. The greater the purple colour of the reaction, the less the extent at which a particular compound (crude fraction) induces cell death in that specific reaction (WST-8 patent).

Crude fractions are mostly dissolved in DMSO because DMSO is a supersolvent. Papers have not reported the cytotoxicity of DMSO when treating cells with crude fractions. Therefore, the first step in this study was to eliminate the toxic effect of DMSO on the HEp-2 cell line to obtain results that are about the crude fraction of *Bulbine* spp. in particular. The procedure used for the optimisation of the DMSO control (200, 500, 1000 µM) was performed in triplicate and followed the same procedure as indicated below.

Cells (±1 × 10^5) were plated in two 96-well plates (Coastar from Corning) (Figure 15). 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 (Figure 16). The amount of formazan was determined by measuring the absorbance at 450 nm (EZ4U, May 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.

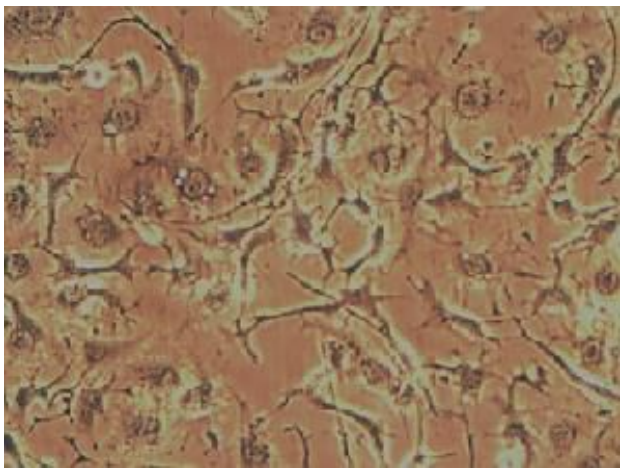


Figure 15: HEp-2 cells in the wells of the 96-well tissue culture plate at ± 1 × 10^5 cell titer before treatment with the MTT reagent (magnification: 100 X).

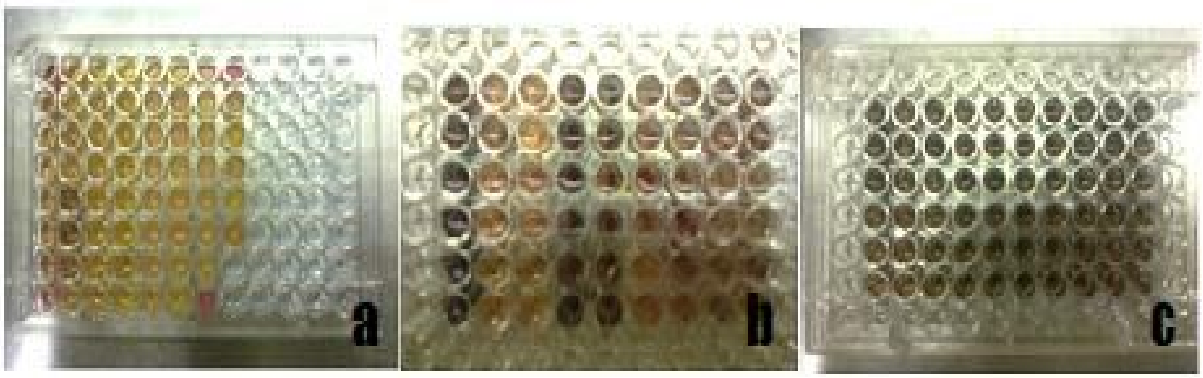


Figure 16: Colour changes in MTT 96-well plates. a) yellow colour when MTT reagent is added, b) violet-blue colour after 3 hours of incubation following MTT addition, c) dissolution of violet-blue colour after 1 h of incubation following 100 µl DMSO addition.

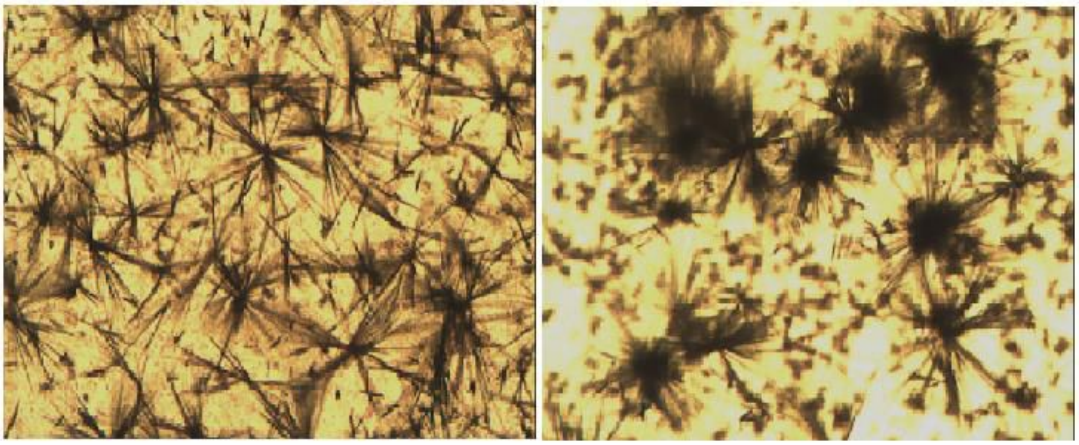


Figure 17: Undissolved violet-blue formazan crystals in the wells of the 96-well tissue culture plate (magnification: 100 X).

3.2.6. Data Analysis

Each fraction for the MTT cytotoxicity assay was run in triplicate in 96-well plates. The standard mean of the triplicate absorbances at 450 nm were used in the calculation of percentage HEp-2 cell death by the different *Bulbine* species fractions. For the triplicate absorbance values, which essentially represent HEp-2 mitochondrial activity, after 5 and 8 days, the standard deviation as well as the standard error was calculated as indicated in Townend (2002) and confirmed using Microsoft Excel version 2010. The standard error indicated whether the sample mean fell within a certain range of the true population from which the HEp-2 cell line was taken (Townend, 2002).

The absorbance results of fractions and controls were analysed using the nonparametric ANOVA (Analysis of Variance) calculating the H statistic, to determine whether the 20, 2 and 1 µg/ml fractions differed in terms of their concentrations/potency amongst each other. A probability level of $P \leq 0.05$ was considered significant. The nonparametric ANOVA was chosen because the data was not representive of a typical normal distribution (Townend, 2010). Fractions were categorised in Table 1 below.

Table 1: Percentage cytotoxicity range, description and codes.

% Cytotoxicity	**Description**	**Code**
20 - 30	dead / good cytotoxicity	D
10 - 20	cytotoxicity	C
1 - 10	poor cytotoxicity	PC
-10 - 1	no effect	NE
-25 - -10	growth enhancer	GR

The equation:

$$H = \frac{12}{N(N+1)} \times \frac{\sum Ri^2}{n_1} - 3(N+1)$$

was used to calculate the H statistic, where n_1 was the number of data values for each concentration; R_1 was the sum of rank values; and N was the total number of data values. Significance was obtained by comparing the calculated value to the value on the x^2 table at p = 0.05.

% Cytotoxicity bar graphs indicated the mean ± S.D. #*P* <0 .001. There were 3 sets of results for each control and *Bulbine* spp. fraction. The percentage cytotoxicity of each fraction was calculated using the equation below (Freshney, 2005) and the mean absorbances of the triplicates at 450 nm were used in the calculation.

$$\%\ \text{cytotoxicity / HEp-2 cell death} = 100 - \left(\frac{\text{absorbance of sample at 450 nm}}{\text{absorbance of DMSO control at 450 nm}} \times 100 \right)$$

For the reliability testing, tests on mean absorbances as well as mean cytotoxicity results for each fraction of both *Bulbine* spp was performed. The IBM SPSS (Statistical Package for the Social Sciences) version 19 was used for data analysis. The reliability of replicates were tested by calculating the coefficient of variation (CV) of the triplicate measures for each fraction at 5 and 8 days separately. The control fractions were not included in the reliability testing. The CVs were expressed as percentages, and the mean CV was calculated for each species, organ and concentration. The higher the mean CV was, the higher the reliability of the result.

The mean absorbance and cytotoxicity results for each fraction were tested for normality of distribution using the one-sample Kolmogorov-Smirnov tests as described in Townend (2002). When the fraction was normally distributed, the one-way ANOVA was employed and compared between independent groups. A $p < 0.05$ was considered as being statistically significant.

Differences in the cytotoxicity between the organ fractions at 5 and 8 days for *B. natalensis* were also measured using the one-way ANOVA, and analysed for each organ using the Bonferroni statistical test (Townend, 2002). This test examined whether there were any significant differences in the cytotoxicity values of all organs between each other at 5 and 8 days.

3.3. Results

3.3.1. Preparation of *Bulbine* spp. crude fractions (aqueous, 50% and 100% ethanol)

All the plant fractions of both plant species were prepared successfully. For HEp-2 cell treatment, all of the fractions were used with an exception of the *Bulbine frutescens* leaf aqueous fraction and the 50% and 100% ethanolic root fractions of *Bulbine natalensis*. Fractions were completely dissolved in DMSO.

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

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 effect 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.

3.3.2.1. Optimisation of DMSO concentration

Table 2.1: Mean absorbance at 450nm ± standard deviation and % cytotoxicity for DMSO in the HEp-2 cell line (105 000 ± 6 244.1 SD cells/well).

Final concentration in cell volume (µg/ml)	**Untreated cells**		**200 µM DMSO**		**500 µM DMSO**		**1000 µM DMSO**	
	Absorbance	**% Cytotoxicity**	**Absorbance**	**% Cytotoxicity**	**Absorbance**	**% Cytotoxicity**	**Absorbance**	**% Cytotoxicity**
20	1.05548 ± 0.106561	0.00	0.81585 ± 0.168504	22.70	0.7246 ± 0.079055	31.35	0.52145 ± 0.083368	50.60
2	1.05548 ± 0.102318	0.00	0.8847 ± 0.026163	**16.18**	0.50755 ± 0.013506	57.91	0.56835 ± 0.00601	46.15
1	1.05548 ± 0.038113	0.00	0.5625 ± 0.660438	46.71	0.6345 ± 0.095601	39.89	0.44735 ± 0.030052	57.62

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 2% (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.

The cytotoxicity in the HEp-2 cell line treated with crude fractions of root, leaf and corm of *Bulbine natalensis* and *Bulbine frutescens* for five and eight days were measured and is presented in Figures 19-22. A 3 day MTT cytotoxicity assay was initially performed for the experiemental plant, *B. natalensis*, to determine the duration required by the HEp-2 cell line to utilise a the fraction maximally across the 8 day period (Figure 18).

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

A. Three day cytotoxicity results: Fraction utilisation (*Bulbine natalensis*)

The average percentage cytotoxicity of mean triplicates for the controls, camptothecin and calpain I inhibitor were -0.059% and -0.029%, respectively. The *Bulbine natalensis* samples (the experimental plant) induced no cytotoxicity in the HEp-2 cell line after 3 days of incubation with the MTT reagent, except the 20 µg/ml leaf fraction, which had a negligible cytotoxicity percentage of 0.029%. All the *B. natalensis* fractions had a cytotoxicity percentage of below -0.2% (Figure 18). Figure 18 shows that the HEp-2 cell line responded very little to treatment after day 3, as evidenced by Figures 19, 20, 21 and 22 which show positive and negative cytotoxicity values. Since *Bulbine natalensis* was the experimental plant, and the results in Figure 18 was achieved, performing the MTT cytotoxicity assay for

B. frutescens was not required (read selection of *Bulbine* spp. crude fractions for expression of apoptotic genes).

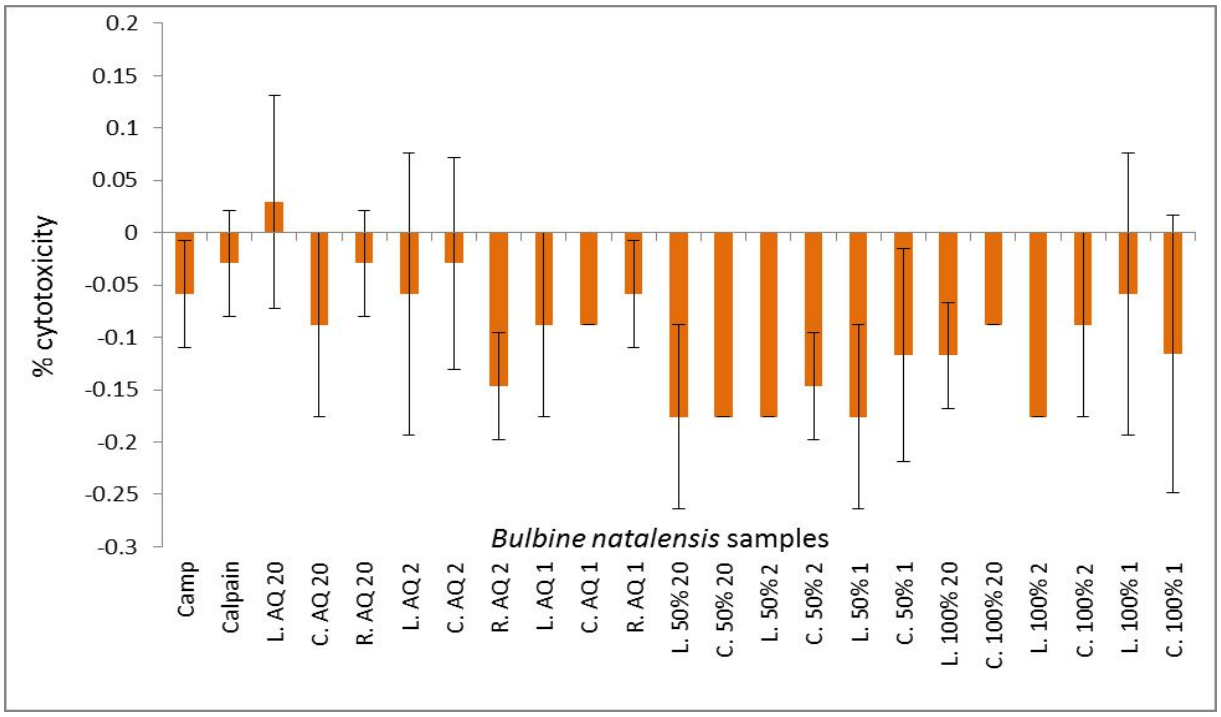


Figure 18: Cytotoxicity spectrum of aqueous and organic *Bulbine natalensis* crude fractions (leaf, roots and corm) on the HEp-2 cell line (114 000 ± 10583 SD cells/well) (3 day result). Cytotoxicity is derived from three experiments done in triplicate. The values are represented as mean ± S.D. The controls 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 concentration 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.*

B. Five day cytotoxicity results

The average percentage cytotoxicity of mean triplicates for the controls, camptothecin and calpain I inhibitor were 11.42% and 2.12%, respectively. The *Bulbine natalensis* 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%) (Figure 19).

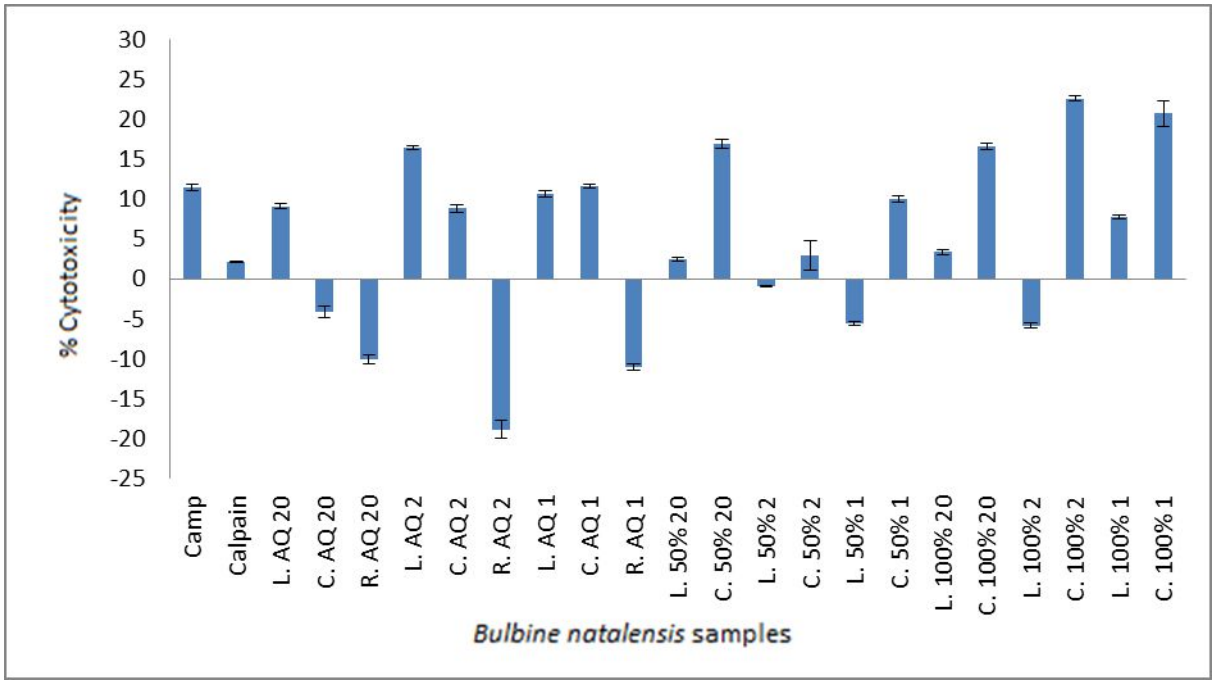


Figure 19: Cytotoxicity spectrum of aqueous and organic *Bulbine natalensis* crude fractions (leaf, roots and corm) on the HEp-2 cell line (114 000 ± 10583 SD cells/well) (5 day result). Cytotoxicity is derived from three experiments done in triplicate. The values are represented as mean ± S.D. The controls 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 concentration 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.*

The average percentage cytotoxicity of mean triplicates of all the *B. frutescens* crude fractions had not conformed with any of the aqueous root fractions for *B. natalensis* (Figure 19) since all of the fractions for *B. frutescens* had negative cytotoxicity values (Figure 20). Camptothecin displayed a cytotoxicity of 10.7% and this provided evidence that the assay was being conducted was feasible since camptothecin is an anticancer/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 cytotoxic 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%) (Figure 20).

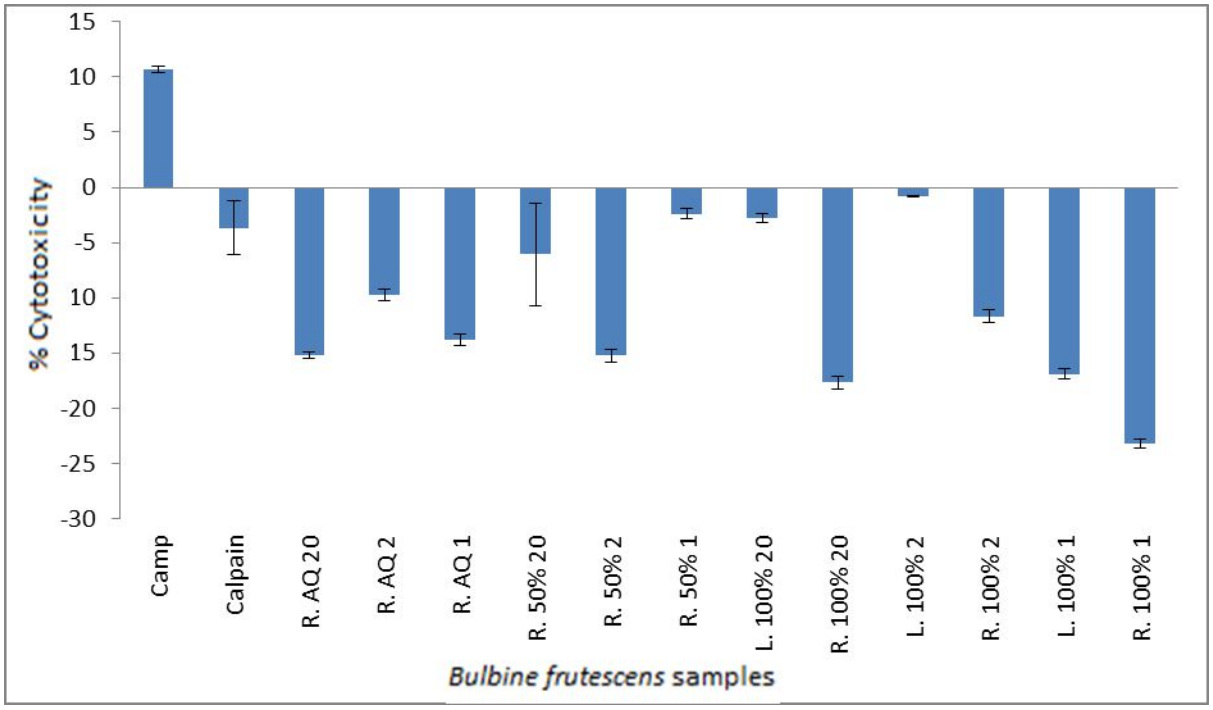


Figure 20: Cytotoxicity spectrum of aqueous and organic *Bulbine frutescens* crude fractions (leaf and roots) on the HEp-2 cell line (114 000 ± 10583 SD cells/well) (5 day result). Cytotoxicity is derived from three experiments done in triplicate. The values are represented as mean ± S.D. The controls 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 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.*

C. Eight day cytotoxicity results

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 (Figure 21). The controls, camptothecin and calpain I inhibitor were not cytotoxic against the HEp-2 cell line and they had 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 19), all of the fractions except for the 1 µg/ml 100% ethanol corm fraction, was not cytotoxic against the HEp-2 cell line. 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 8th day (-11.18%) compared to the 5th day (-18.86%) result. Similarly for the 1 µg/ml fraction, the recorded percentage cytotoxicity on the 8th day was -4.01% and this was lower than the 5 day cytotoxicity reading (-11.02%) (Figure 21).

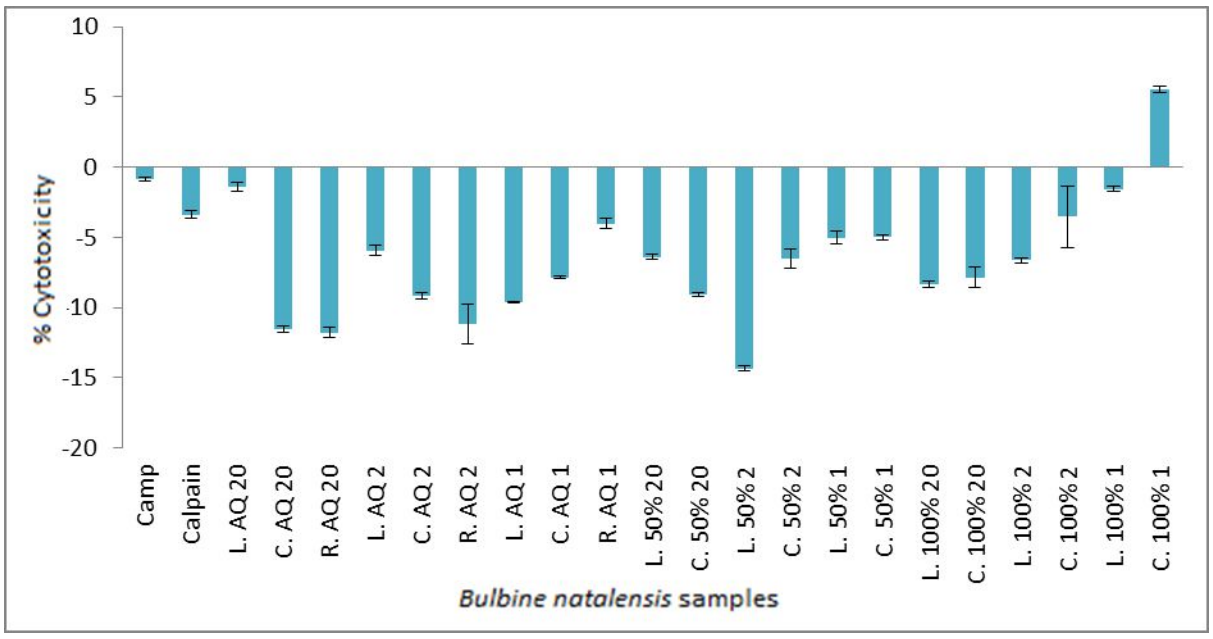


Figure 21: Cytotoxicity spectrum of aqueous and organic *Bulbine natalensis* crude fractions (leaf, roots and corm) on the HEp-2 cell line (114 000 ± 10583 SD cells/well) after 8 days. Cytotoxicity is derived from three experiments done in triplicate. The values are represented as mean ± S.D. The controls 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 concentration 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.*

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 (Figure 20) 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 (Figure 22). 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.

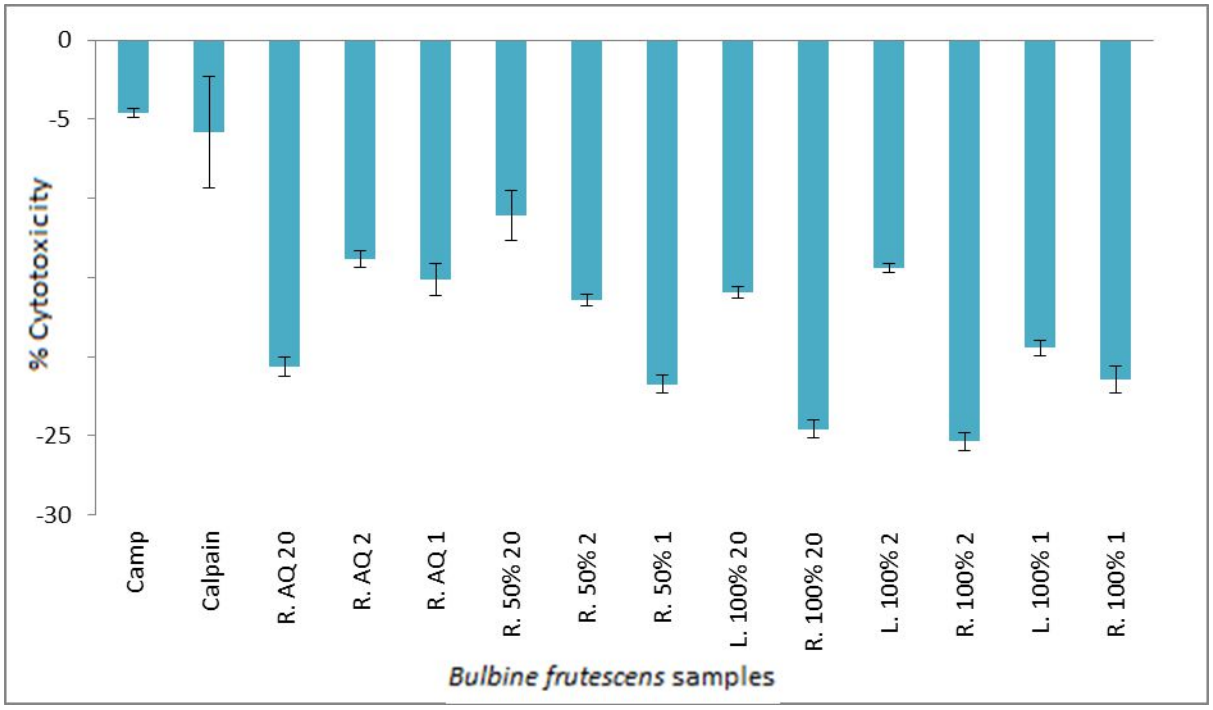


Figure 22: Cytotoxicity spectrum of aqueous and organic *Bulbine frutescens* crude fractions (leaf, roots and corm) on the HEp-2 cell line (114 000 ± 10583 SD cells/well) (8 day result). Cytotoxicity is derived from three experiments done in triplicate. The values are represented as mean ± S.D. The controls 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.*

(the statistics for chapter 3 can be found in Appendix 1)

3.4. Discussion

Cytotoxicity refers to how toxic a compound (i.e. a drug or other source of apoptotic stimuli like *Bulbine* spp. fractions in this case) is to a particular cell line, micro-organism, animal model or other living organism (Mossman, 1983). The MTT cytotoxicity assay, out of six other methods (WST-8 patent), has probably become the most widely used method of measuring cytotoxicity because it has been found to be the most frequently reported method in scientific articles. This was a reason as to why the MTT cytotoxicity assay was selected for this study (WST-8 patent). However, in order to obtain good cytotoxicity results using the MTT assay, the cell system used has to respond to the compound it is exposed to. Therefore the adherent HEp-2 (laryngeal carcinoma) cell line was selected, and it was found to be susceptible to the prepared aqueous and organic fractions of *Bulbine natalensis* and *B. frutescens*, which were completely dissolved in dimethyl sulfoxide, a supersolvent. The ratio of organic and aqueous solvents used for the extraction of polar and non-polar compounds from the ground *Bulbine* material was different. It was found that 200 ml of distilled water was required to get 25 g of ground leaf material dissolved and filtered adequately, while 100 ml of organic solvents were sufficient to dissolve and filter the ground material of both plant species and of all the tested organ fractions of leaf, root (*B. natalensis* and *B. frutescens*) and corm (*B. natalensis*)). The difference in rations arose because of the ability of the ground leaf material, of both plant species to absorb water, thereby causing the leaf particles to swell and thus become difficult to filter.

The organic fractions were successfully made using 50% and 100% ethanol, while the aqueous fractions were made with distilled water. The idea was that since distilled water is the most polar of the three solvents used, more polar compounds will be extracted from the crude material during the preparation of this fraction, followed by the 50% and then 100% ethanol fractions. However, from this study, it was difficult to decipher which contained the most polar compounds because all the fractions dissolved completely in DMSO (which is polar as well as non-polar). There is generally a discrepancy in the views of scientists, which have been reported, saying that aqueous fractions of plant compounds don't dissolve completely in DMSO and remain as a residue at the bottom of the eppendorf tube (Gaylord Chemical Company L.L.C., 2007), but this was not the case with the *Bulbine* spp. aqueous fractions tested. The concentrations of fractions used in this study were 20, 2 and 1 µg/ml.

Initially, at the beginning of this study, cell culture handling and cytotoxicity experiments were being conducted on three different cell lines at different times. These cell lines were the HeLa (adherent, cervical carcinoma), HEp-2 and K562 (suspension, liver carcinoma). The HeLa cell line multiplied rapidly after only one day of subculturing and reached a confluency of 100% by the second day. Although there have been many cytotoxicity studies conducted using the HeLa cell line, it was not used for this study because the *Bulbine* spp. fractions tested had not been reported before, and therefore using this cell line would have introduced another variable into this study. Therefore, a cell line that was more stable and multiplied at a lower rate was required, more so, because no significant differences in percentage cytotoxicity results of each tested *Bulbine* spp. organ after 5 days of incubation and the difference after 8 days of incubation were only for *B. frutescens*. Many of the percentages were almost the same on the 5 and 8 day plates, but differed between each other (Figures 1 and 3; Figures 2 and 4 – Appendix 1: HeLa Cytotoxicity Graphs and Tables). This implied that another cell line was required. The suspension cell line K562, was selected but not used. The K562 cell line was easy to grow, subculture, maintain and store, however, unlike contaminated HeLa and HEp-2 cell lines, the contaminated K562 cell line was difficult to decontaminate using extra penicillin/streptomycin antibiotics in CCM. This resulted in the study being less cost effective.

When a flask of K562 became contaminated moderately, attempts were made to decontaminate and re-suspend them in CCM that contained 2 ml of antibiotics instead of the standard 1 ml for every 100 ml of CCM prepared. It was found that centrifuging those cells, re-suspending the pellet in PBS, centrifuging again, and then re-suspending the pellet in 20 ml CCM, resulted in no change from the initial contaminated flasks after 2 days of incubation. This was attributed to the first centrifugation step, in which debris and other contaminants from the contaminated CCM get incorporated into the pellet of the K562 cells. This also caused many other flasks of K562 cells to get contaminated and thrown away. Eventually, this cell line was not considered as being the best even though it was considered to be easy to work with. It was found that the K562 cell line was p53 deficient (Li *et al.*, 2012), making its mechanism of multiplication/proliferation inconclusive. Since apoptotic markers *bax* and *caspase-3* are regulated by *p53*, this made the K562 cell line not appropriate for usage in this study because those were the genes of interest.

The risk of contamination was much lower with the HeLa and HEp-2 cell lines because they were adherent and this allowed for adequate removal of debri and contaminants from flasks, by washing with PBS and re-suspending them in new prewarmed CCM. The HEp-2 cell line had a much lower rate of multiplication after subculturing. It was found that CCM flasks became 80 - 100% confluent after subculturing or resuscitation of a frozen cryovial of HEp-2 cells, after 2 days of incubation at 37^{o}C in a 5% CO_2 incubator. The HEp-2 cell line was, therefore the best option relative to the HeLa and K562 cell lines and was used during the MTT cytotoxicity assay producing valid results.

Detachment of these HEp-2 cells from tissue culture flasks was initially a challenge and the amount of trypsinising solution had to be optimised. It was found that 30 ml trypsin detached all HEp-2 (and HeLa cells), but caused the morphology of the HEp-2 cell line to change to the extent that they adopted the shape of the K562 cell line (cocci). Since *caspase-3* which is an important molecular indicator for the morphology of apoptosis, and the fact that 30 ml of trypsin possibly caused damage to the cell line, lower amounts were tested (10, 5 and 1 ml). It was found that 1 - 2.5 ml trypsin was sufficient to detach the HEp-2 cells from tissue culture flasks without causing them to lose their spindle shape. The HEp-2 cell line was selected because, in addition to the above, in the absence of particular stimuli, HEp-2 rarely undergoes autocrine apoptosis or cell death (Chatterjee *et al*., 2001). Autocrine apoptosis, by definition, is a form of cell death whereby the cell line secretes their own products which causes or accelerates their own cell death, whereas paracrine apoptosis is defined as cell death that can be induced either by a transported product that was synthesised elsewhere in the body and transported to the cell line, or by some added agent or chemical such as a drug compound (Chatterjee *et al*., 2001).

In animal cells, ceramide forms an important component of the cell membrane, which is made up of a combination of sphingosine and fatty acid. In addition to the ceramide, cell membranes are also composed of major phosphoshingolipids called sphingomyelin. Sphingomelin is abundant in the outer leaflets of the plasma membranes and it constitutes 30% of all lipids (Rothman and Lenard, 1977). Sphingomyelin as phospholipids are formed when ceramide reacts with phosphatidylcholine to form sphingomyelin and diacylglycerol in the golgi apparatus of the cell, and to a lesser extent in the plasma membrane. Sphingomyelin is restricted to the lumen of the cell, in organelles that are involved in secretory and endocytic

processes (Galcheva *et al*., 2005). It has been confirmed in numerous cell types that ceramide functions as a second messenger in several cellular processes, including apoptosis, growth suppression, differentiation, transformation, proliferation, regulation of cell-to-cell and cell-to-substrate interactions and cell senescence (CM, 2007). Therefore when a cell comes in contact with a cytotoxic or non-cytotoxic substance, the substance first interacts with ceramide, which then generate a signal into the cell allowing the compounds to be utilized by the cells either through membrane proteins, ion exchange channels or the receptor-ligand mechanism (Becker *et al*., 2003). This is the first step that determines how the compound would be taken into the cell. This results either in cell growth or cell death (or apoptosis) (Singh and Reddy, 2012).

Once the HEp-2 cell line was selected for this study, the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cytotoxicity assay was performed. The MTT cytotoxicity assay is the most reliable, re-producible and easy to use method for measuring the cytotoxicity of compounds and it is a strictly, but not limited to, dehydrogenase-based assay (WST-8 patent). There are two types of dehydrogenases involved in the MTT cytotoxicity assay *viz*. lactate dehydrogenase (LDH) (Balasubashini *et al.,* 2006) and succinate dehydrogenase (SDH) (Berridge and Tan, 1993) and the one that is reported depends on the type of results one wants to present and the type of data that is available. In this study, the cell titre (count) used in all MTT experiments was based on the optimal cell count that was required for the gene expression study (114 000 ± 10 583 SD cells/well). The cell count was performed using the method suggested by Freshney (2005), which utilised the Trypan blue assay. Only HEp-2 that had a bright appearance were counted. Those HEp-2 cells that were the colour of trypan blue were not counted, since they were considered non-viable (Mossman, 1983; Freshney, 2005). Each crude fraction was prepared at three different concentrations and each fraction was administered to the HEp-2 cell line in triplicate per 96-well plate i.e. three absorbance readings were obtained for each crude fraction at 450 nm using the ELISA plate reader, followed by the dissolving of the purple formazan crystals, after 1h incubation with 100 µl DMSO (Pillai *et al*., 2004). It was noted that purple formazan crystals were formed after HEp-2 was exposed to the *Bulbine* spp. crude fractions and incubated at 37^{o}C in a 5% CO_2 atmosphere in the presence of the MTT reagent for 3 hrs (Pillai *et al.,* 2004). The purple formazan was attributed to the fact that the HEp-2 cell line was viable and that the MTT reagent had entered the mitochondria of those cells (Mossman, 1983; WST-8 patent). The

ELISA plate reading of each fraction in triplicate was used to calculate the percentage cytotoxicity of that fraction by using the formula given in the materials and methods section. In order to obtain triplicate cytotoxicity results, the experiment was conducted twice more and each time percentage cytotoxicity results were calculated and noted. The three cytotoxicity percentages were used to calculate the standard deviation between triplicate data sets (extraspecific), whereas, the standard deviation for each set of triplicate absorbance readings was also calculated and noted (intraspecific) (see Appendix 1).

Positive and negative percentage cytotoxicity results for both *Bulbine* spp. against the HEp-2 cell line was obtained. This was significant because it indicated that the prepared crude fractions were hydrophobic and that they passed through the plasma membrane and entered into the HEp-2 cell line easily (Pillai *et al*., 2004). However, the initial problem was that the cytotoxicity results were not reproducible. After numerous failed attempts of reproducing the cytotoxicity results, it was decided that the HEp-2 cell line used for the cytotoxicity as well as the gene expression study be at a certain level of subculture. The cells were noted as being at level 0 subculture from the time they arrived from Highveld Biological. Only HEp-2 that underwent subculturing 3 times (passage number of 3) followed by storage at -70^{o}C, resuscitation and incubation in new, prewarmed, CCM for 2 days, were used in the cytotoxicity and gene expression experiments. The level of subculture at which the HEp-2 cell line was at on delivery, was not noted.

The MTT cytotoxicity assay was performed using the HEp-2 cell line at three incubation periods. The first time cytotoxicity results were taken after 3 days of incubation and noted in triplicate. It was noted from those results that the *B. natalensis* crude fractions had no effect on the HEp-2 cell line after 3 days (Figure 18). This did not imply that the cell line was resistant to the fractions or that the potency of the fractions were too weak, but simply that the HEp-2 cell line had not yet started utilising the fractions administered. This was evidenced by the cytotoxicity results obtained after 5 days of HEp-2 cell line incubation with *B. natalensis* fractions at all 3 dose concentrations, with a few exceptions. A different cytotoxicity spectrum developed after 8 days of incubation in that all of the *B. natalensis* fractions, except the corm 1 µg/ml 100% ethanol fraction, promoted HEp-2 cell line growth. This was due to the rate at which the HEp-2 cell line utilised the fractions of *Bulbine natalensis* and this was evidenced by the calculated cytotoxicity results, that were negative.

Negative cytotoxicity results are seldomly reported, but Lee *et al.* (2004), provided confidence that negative cytotoxicity results are reportable and publishable. The negative cytotoxicity results for some fractions suggested that those fractions were not potent enough. In order to have proceeded with this study it was important to know if there were differences amongst the 3 concentrations of each fraction administered to the HEp-2 cell line. This was achieved using the non-parametric ANOVA that utilised the H statistic (Appendix 1). It was found that there was a significant difference between the percentage cytotoxicity results of the 3 concentrations at which *Bulbine* spp. crude fractions were administered to the HEp-2 cell line after 5 days of incubation ($p=0.05$). After 8 days, there was a variable response whereby the *B. frutescens* fractions did not show any difference between the percentage cytotoxicity results at which the 3 concentrations are advantageous at (i.e. cause cell death), while for *B. natalensis* there was a difference. This meant that the 5 day triplicate MTT cytotoxicity results for *B. natalensis* and *B. frutescens* were more reliable than the 8 day cytotoxicity results.

Lactate dehydrogenase (Balasubashini *et al*., 2006) and succinate dehydrogenase (Mossman, 1983; WST-8 patent) are enzymes that participate in the reduction of yellow coloured MTT to purple formazan after incubation of treated cell lines with the MTT reagent for a particular period of time, usually taken as three to four hours in anticancer studies (Mossman, 1983). The fact that both positive and negative cytotoxicity results were obtained indicated a variable response of the dehydrogenase enzymes that cleave MTT. In all of the cases where *Bulbine* spp. fractions induced HEp-2 cell death (positive cytotoxicity value), the MTT was cleaved by the tetrazolium ring of succinate dehydrogenase in active mitochondria (viable HEp-2 cells) i.e. even though the cells were undergoing antiproliferative ability induced by the fraction, only those that were viable and utilised the fraction (generated the formazan product) were measured at 450 nm using the ELISA plate reader. Since those fractions induced HEp-2 cell death, those cells was less metabolically active in the wells of the 96-well plate that contained those fractions. Those fractions produced a higher calculated percentage cytotoxicity value (*B. natalensis* corm and leaf 1 µg/ml fraction, corm 20 µg/ml 50% ethanol fraction, corm 2 µg/ml 100% ethanol fraction, and leaf 2 µg/ml aqueous fraction, to name a few) compared to those fractions that promoted HEp-2 cell proliferation (*B. frutescens* root 20 and 1 µg/ml 100% ethanol fraction, root 2 µg/ml 50% ethanol fraction, leaf 1 µg/ml 100% ethanol fraction, and the *B. natalensis* corm 20 µg/ml aqueous fraction, to name a few). It has

been recently reported that viable cells also use microsomal enzymes to reduce MTT by using reduced energy carriers such as NADH and NADPH (Berridge and Tan, 1993) as an additional factor, and this has been considered an advantage when using the MTT cytotoxicity assay (WST-8 patent).

Although the MTT cytotoxicity assay does not differentiate between the different types of cell death such as necrosis, apoptosis, autophagy and several known types, pertinent information have been reported regarding these processes using this assay, mainly in terms of the involvement of LDH and SDH. It has been discovered that SDH is an enzyme that indicates whether apoptosis has occurred while LDH indicates whether cell death occurred via the necrotic pathway. Balasubashini *et al.* (2006) found that when the HEp-2 cell line is exposed to stimuli (antiproliferative peptide (7.6 kDa) from lionfish (*Pterios volitans*), the production of less LDH suggests that the event of cell death is by apoptosis. However, in the present study, reverse transcriptase polymerase chain reaction was used to determine whether cell death of selected fractions were induced by the apoptotic pathway or not, and if so, the mechanism induced was postulated. The *Bulbine* spp. fractions that induced HEp-2 cell death by day 5, could have done so by the loss of intracellular LDH and its release into the complete culture medium (CCM) because the cell membrane must have increased with time and dose during that cytotoxic period (Kotakis and Timbrell, 2006). During this phase of cell death, the cell shrinkage is abrupt and involves adjacent cells losing contact with each other followed by the convolution of the cell membrane due to a net outward movement of fluid from the cell. This outward fluid movement occurs because of the inhibitor of the Na^+, K^+, Cl^- co-tansporter system (Wilcock and Hickman, 1988). Geerl and Vaux (2005) have suggested that a cytotoxic compound will not die because of the direct effects of the compound, but rather because the cell will activate its suicide mechanism to die earlier by apoptotis. Cytotoxicity has been measured at a variety of wavelengths. These wavelengths are: 450 (EZ4U, 2000), 490 (Balasubashini *et al*., 2006), 540 (Pillai *et al*., 2004) and 570 nm (George *et al*., 2010). In this study all readings were performed at 450 nm, and this was because this filter was readily available.

The controls, camptothecin as well as the calpain I inhibitor, exhibited variable cytotoxic effects against the HEp-2 cell line. There was a more variable response of the HEp-2 cell line to calpain I inhibitor. This variable activity of calpain I inhibitor was evidenced by having the

least reliable coefficient of variation result of triplicate absorbances out of the four controls tested (untreated, 200 µM DMSO, camptothecin and calpain I inhibitor). Calpain I inhibitor had a mean standard deviation of 2.22947 ± 1.650191 SD after 5 days of incubation (Appendix 1 Table 2 - reliability testing I). Camptothecin, on the other hand, had only a cytotoxic effect on the HEp-2 cell line with a percentage cytotoxicity value of 11.42% ± 0.420 SD (Appendix 1 Table 1.4) and 10.70% ± 0.300 SD (Appendix 1 Table 2.4) after 5 days. After 8 days of incubation, camptothecin began to enhance HEp-2 proliferation with cytotoxicity values of -0.89% ± 0.142 SD (Appendix 1 Table 3.4) and -4.61% ± 0.291 SD (Appendix 1 Table 4.4). This could have been due to the cell death machinery of the HEp-2 cell line (Lee *et al*., 2004, Balasubashini *et al*., 2006, Padma *et al*., 2007).

In contrast, although calpain I inhibitor initially showed to have a cytotoxic effect on the HEp-2 cell line after 5 days of incubation (2.12 ± 0.081 SD; Appendix 1 Table 1.4), later experiments using the same passage number and same stored calpain I inhibitor, revealed that calpain I inhibitor enhanced HEp-2 cell growth after 5 days of incubation (-3.69% ± 2.448 SD; Appendix 1 Table 2.4). On the 8th day of incubation, calpain I inhibitor, exhibited negative cytotoxicity percentages for both set of results. These were -3.39% ± 0.264 SD and -5.83% ± 3.521 SD, respectively (Appendix 1 Tables 3.4 and 4.4, respectively). The inhibition of apoptosis in cancer cell lines by calpain I inhibitor has recently been shown to exhibit variable responses in hepatocellular carcinoma cell lines (such as SK-HEP-1 and HLF) and colorectal cell lines (RKO and DLD-1) (Machiels *et al*., 1997). In the present study, this was evidenced by camptothecin having a more reliable coefficient of variation compared to calpain I inhibitor across the eight day incubation period. For camptothecin this was 3.2421% ± 0.61800 SD (5 day) and 11.1544% ± 6.85669 SD (8 day), while for calpain I inhibitor it was 35.0653% ± 44.14825 SD (5 day) and 34.0761% ± 37.17168 SD (8 day) (Appendix 1 Table 2 - reliability testing II). The camptothecin control was specific to indicate that some level of apoptosis could have been induced in the HEp-2 cell line, and this was achieved. The calpain I inhibitor control was used for genetic purposes in that its role is usually to inhibit the function of the proteasome in cells and thus, usually prevents apoptosis from occurring (Machiels *et al*., 1997). The 200 µM DMSO (negative control) had an acceptable cytotoxicity result akin to the cytotoxicity result of the untreated HEp-2 cell line. 200 µM DMSO was considered optimal after having performed a successful optimisation experiment involving 500 and 1000 µM DMSO concentrations. From this experiment, the 500 and 1000 µM

DMSO concentrations were cytotoxic to the HEp-2 cell line, and were therefore not selected for usage in this study. Camptothecin and calpain I inhibitor can be used as controls in apoptotic studies, including the MTT cytotoxicity assay (Hsiang *et al*., 1985; Altznauer *et al*., 2004; Legaraz and Yang, 2006; Li *et al*., 2006; Sareen *et al*., 2007).

The HEp-2 cell line showed a variable response to calpain I inhibitor and this could have been due to various unknown reasons. It has been found in dexamethasone-induced thymocyte apoptosis, that calpain I inhibitors slowed down the upstream regulation of apoptosis by acting on calpain proteins which formed part of the calpain/calpastatin system. The calpain/calpastatin system is responsible for various membrane fusion events in the body including, neural vesicle exocytosis, platelet and red cell aggregation (Alverna *et al.,* 2003). These two proteins have been found to affect the expression levels of genes encoding structural and regulatory proteins, including those involved in apoptosis such as *p53, bax and caspase-3* (Raynaud *et al*., 2005). The genes involved in this pathway exist in many spliced transcript variants, but not all have been determined (Raynaud *et al*., 2005). The fact that calpain I inhibitor began to promote HEp-2 cell growth on the eighth day of incubation indicates that it possibly induced a downregulation of the critical effector *caspase-3*. However, because the cytotoxicity for one of the two set of five day incubated plates showed a negative cytotoxic effect on the HEp-2 cell line (Figure 20), indicated that calpain I inhibitor had switched from having a cytotoxic effect to having the opposite effect. This could have been due to calpain I inhibitor instability or it could even imply that the sensitivity of the HEp-2 cell line for calpain I inhibitor decreased. The exact action of calpain I inhibitor on the HEp-2 cell line is not yet fully understood, but it simply means that, if calpain I inhibitor exclusively promotes cell growth, then both five day plate cytotoxicity results should have been negative because cell killing activity for one of them causes uncertainty regarding the therapeutic action of calpain I inhibitor in the treatment of laryngeal carcinoma.

The active compounds in crude fractions, lose their effect when they are stored for too long or thawed and used too frequently (Crowley and Martini, 2001). Cytotoxicity experiments were conducted at different times using the same passage HEp-2 cell number, and probably this could have caused calpain I inhibitor to have lost its stability. However, this has to still be confirmed by other studies. Since each set of cytotoxicity results included three similar absorbance readings, it indicated that the results were reproducible. However, calpain I

inhibitor, stability and action on calpain I in the calpain I/calpastatin system are not the only factors that determine whether a compound can induce apoptosis or not.

It has been found that some cancer cell lines like the prostate cancer cell line, contain proteasomes that degrade p53 (Zhu *et al*., 1995, Kubbutat and Vousden., 1997). It is unknown at present as to whether the mechanism of p53 protection apply to HEp-2 cell lines. If it does, then the calpain I inhibitor probably stabilises endogenous p53, causing proper regulation of the eukaryotic cell cycle (G1, S, G2, M (mitosis) phases).

In this study, it was assumed that all HEp-2 cells (p53 proficient) plated were of the same *p53* status. However, the exact phase at which the HEp-2 cell line was at in the cytotoxicity and gene expression studies were not noted. It has been found in human fibroblast, hepatocellular carcinoma and colorectal cells, that calpain I inhibitor targets cells that are in the G2/M phase of the cell cycle (Machiels *et al*., 1997; Dietrich *et al*., 1999) i.e. cells that are about to undergo anaphase (Becker *et al*., 2003). Furthermore, those cells were reported to have been arrested when exposed to 50 - 100 µg/ml of calpain I inhibitor. This arrest occurs because of the ability of calpain I inhibitors to inactive proteasomes (Atencio *et al*., 2000). Conversely, at concentrations of between 1 - 20 µg/ml, calpain I inhibitor was found to inhibit G2/M cell arrest (Atencio *et al*., 2000). Therefore, if this was true for the HEp-2 cell line (since it was administered at 20 µl/ml), then no cell death should have been detected in the MTT cytotoxicity assay at all. However, if the HEp-2 cell line shifted from the G1 to S phase without any interruptions, and then to the G2 phase at the time of performing the first five day MTT assay, then there is a possibility that the absence of nuclear fusion events, resulted in there being a cytotoxic result for the one set of calpain I inhibitor results.

In addition to the above, calpain I inhibitor have also been used in a variety of pathological indications to protect cells from death as well as to promote cell death in some cancer cell lines (Wang and Yuen, 1994, Bartus *et al*., 1995, Robinson, 1996). Therefore it could be concluded that calpain I inhibitor can stabilise factors needed for cell survival, apoptosis and cell cycle control.

The role of camptothecin in apoptotic studies is more definitive when compared to calpain I inhibitor. Camptothecin is a cytotoxic alkaloid isolated from the plant *Camptotheca*

acuminata (family: Nyssaceae). Camptothecin just like the other anticancer drugs, doxorubicin and methotrexate, induce apoptotsis via the FasL/Fas complex (Wesselborg *et al*., 1999). FasL is a membrane protein that forms part of the tumour necrosis factor (TNF) (Siegel *et al*., 2000). Therefore, the positive cytotoxicity results for camptothecin on the HEp-2 cell line after five and eight days of incubation indicates the activation of the FasL/Fas pathway for cell death. This means that when HEp-2 cells are exposed to 20 µl/ml of camptothecin for 5 or 8 days, CD95 receptors on the cell membranes became upregulated and result in the induction of the expression of FasL i.e. the activation of FasL/Fas-dependent apoptosis. However, the role of camptothecin has also been highly reported in molecular studies as being a topoisomerase inhibitor i.e. it inhibits DNA synthesis (i.e. causes DNA double strand breaks; Becker *et al*., 2003) preventing cells that have moved from the G1 phase of the cell cycle from being replicated in the S phase. This causes cells to undergo arrest. However some cells escape the cytotoxic effect of camptothecin and enter into the G2 and M phases unharmed or probably replicate mutant form of cells. However, this has yet to be confirmed. Rapid RNA synthesis cessation before transcription has also been reported as being induced by camptothecin treatment i.e. an S-phase independent cell death pathway exists. Hayward *et al.* (2003) also demonstrated in SN38 that cell death by camptothecin is induced by an increase in pro-apoptotic factors such as p53, Bax, Bcl-XL, and p-21/WAF-1 in colon cancer cells (Shao *et al*., 1995). Furthermore, in leukaemia cancer cell lines exposed to camptothecin, pro-apoptotic proteins: Bax, bF11, Bak, pRb2, c-jun and jun-b become upregulated, while cell cycle regulatory proteins such as cdk4, cyclinB1, CRAF-1 and DPI become downregulated.

Cell proliferative activity of camptothecin has not been reported in cell lines where the receptor-ligand mechanism of apoptosis has been apparent. The negative cytotoxicity result for one set of camptothecin results (Figure 20) could be due to the reason of stability that was discussed for calpain I inhibitor, which in this case, is able to cause Fas and FasL to remain in different cytoplasmic compartments within HEp-2 cells i.e. preventing the trimerisation of FasL (Meza-Lamas *et al*., 2006). Trimerisation of FasL is an important apoptotic event, and it involves the formation of three clefts at the interface of the monomers. It is at these three clefts that the Fas receptor molecules binds to and triggers a cascade of events for apoptotic cell death (Meza-Lamas *et al*., 2006). Previous studies have reported that some cell lines, like the human leukaemia cancer cell line, survives after exposure to camptothecin by means of

them possessing cell survival genes such as *NF-KB* transactivation and epidermal growth factor receptor (EGFR) genes, which are expressed on camptothecin exposure (Carson *et al*., 2004).

Camptothecin, like doxorubicin, is a natural antibiotic that has been found to be cytotoxic against human tumour cell lines (oral squamous cell carcinoma HSC-2, HSC-3, submandibular gland carcinoma HSG, promyelocytic leukaemia HL-60) that against normal cells (gingival fibroblast HGF, pulp cell HPC, periodontal ligament fibroblast HPLF) (Kawase *et al*., 2003). This cytotoxic alkaloid (has pharmacokinetic, chemotherapeutic and macrophage-mediated immunomodulatory properties) induces its cytotoxic effect on cell lines through its ability to intercalate with DNA, interact with the plasma membrane as well partake in oxidation-reduction reactions (Amcamone, 1969; Arena, 1971; Razek, 1971; Tritton and Yee 1982; Mihich and Ehrke, 1984, Simpkins *et al*., 1984; Youngman and Elstner, 1984). Kawase *et al.* (2003) found there to be no close relationship between tumour-specific cytotoxicity with doxorubicin, and the apoptosis-inducing activity in chemically-defined compounds (such as steroidal saponins, coumarins, flavonoids, vitamin C, gallic acid, catechin, dopamine, α,β-unsaturated ketones, styrylchromones) in the above mentioned tumour and normal cells. This indicated that it is possible for a compound or plant extract to have a high tumour specificity, but only have apoptosis-inducing potential on some cell types. Therefore, the low percentage cytotoxicity of camptothecin on day 5, 11.42% ± 0.420 SD, could have been due to the robustness of the HEp-2 cell line and not that camptothecin had any apoptosis-inducing potential on the HEp-2 cell line. The above explanation lead to the speculation that perhaps utilising another cell line under the same laboratory conditions and experimental design set up, would induce a higher cytototic level for camptothecin and calpain I inhibitor controls and different cytotoxicity results for the *Bulbine* spp. fractions administered.

The cytotoxicity percentages for *B. natalensis* and *B. frutescens* fractions were variable, with all *B. frutescens* fractions failing to exhibit any antiproliferative effect on the HEp-2 cell line. The statistical parameters for reliability between 5 and 8 day absorbance readings for *B. natalensis* and *B. frutescens* revealed that they were reliable at 5 days but at 8 days, *B. natalensis* was the least reliable because it produced the highest mean standard deviation of the two species on that day i.e. 8.6225 ± 13.33016 SD (Appendix 1 Table 1 - reliability

resting I). In order to determine if there were any significant differences in the absorbance readings at 5 or 8 days between the two *Bulbine* spp. crude fractions, the non-parametric one-sample Kolmogorov-Smirnov test was employed. It was found that there was a highly significant difference in the absorbance readings after 8 days of incubation at $p < 0.001$. The mean standard deviation after 8 days were -6.11810 ± 4.516475 SD (*B. natalensis*) and 1.11186 ± 0.042602 SD (*B. frutescens*), while at 5 days, the mean standard deviation was at a p value of 0.215 (> 0.05) and was insignificant (Appendix 1 - Kolmogorov-Smirnov test: Absorbences A).

B. frutescens cytotoxicity results had the most reliability on the eighth day of incubation (3.9663% ± 3.54736 SD), whereas *B. natalensis* was most reliable on the fifth day (8.7270% ± 13.27633 SD) (Appendix 1 Table 1 - reliability testing II). The fractions of *B. natalensis* that had no cytotoxic effect against HEp-2 after 5 days of incubation, were all root aqueous fractions (20 µg/ml: -10.09% ± 0.589 SD, 2 µg/ml: -18.86% ± 1.115 SD, and 1 µg/ml: -11.02% ± 0.416 SD), the corm 20 µg/ml aqueous fraction (-4.09% ± 0.700 SD), the leaf 2 and 1 µg/ml 50% ethanol fractions (-0.88% ± 0.107 SD and -5.55% ± 0.191 SD respectively), and the leaf 2 µg/ml 100% ethanol fraction (-5.87% ± 0.303 SD) (Appendix 1 Table l.4). After a further 3 days of incubation, only the corm 1 µg/ml 100% ethanol fraction killed HEp-2, but at a lower cell killing potential (5.51% ± 0.242 SD) compared to day 5 (20.69% ± 1.616 SD) (Appendix 1 Table 3.4). Therefore it was concluded that 14 out of the overall 21 crude fractions for *B. natalensis* was cytotoxic against the HEp-2 cell line by the fifth day. Contradictingly, all of the *B. frutescens* fractions enhanced HEp-2 cell line proliferation after 5 days of incubation (Appendix Table 2.4), with a much greater increase in its HEp-2 cell proliferative potential seen 3 days later. Those crude fractions of *B. frutescens* were the, root 1 µg/ml 50% fraction [a 19.38% (21.76% - 2.38%) decline in cytotoxic potential], root 2 µg/ml 100% ethanol fraction [a 13.69% decline (25.37% - 11.68%)], and the leaf 20 and 2 µg/ml 100% ethanol fractions [a 13.12% (15.93% - 2.81%) and 13.58% (14.41% - 0.83%) decline respectively] (Appendix 1 Table 4.4).

There were also fractions that pointed more toward HEp-2 cell death and those fractions did not exhibit a sharp decline in percentage cytotoxicity between the fifth and eight day MTT plate incubations. For *B. natalensis* those fractions were the, leaf 2 µg/ml 100% ethanol fraction [a 0.78% decline (6.65% - 5.87%)] and the root 20 µg/ml aqueous fraction [a 1.69%

decline (11.78% - 10.09%)] (Appendix 1 Tables 1.4 and 3.4), while for *B. frutescens* the fractions were the, leaf 1 µg/ml ethanol fraction [a 2.55% (19.45% - 16.9%) decline], root 2 µg/ml 50% ethanol fraction [a 1.01% (16.43% - 15.24%) decline], and the root 1 µg/ml aqueous fraction [a 1.36% (15.15% - 13.76%) decline] (Appendix 1 Tables 2.4 and 4.4). The only evident relationships in the cytotoxic effect between the fractions of both *Bulbine* spp. were the root aqueous crude fractions, because they had a proliferative effect on the HEp-2 cell line. However as evidenced from above, although the root fraction of *B. natalensis* had a similar cytotoxic effect on the HEp-2 cell line as the root fraction of *B. frutescens*, they were of different concentrations.

The cytototic effect of the *Bulbine* spp. fractions were time and dose independent in the HEp-2 cell line exposed to them. Maleek (2010) found the same relationship when he tested the effect of purified ceramide on HEp-2, AMGM5 and normal REFAM3 cells. He found that at 7 µM of ceramide, the % cytotoxicity in the HEp-2 cell line was the lowest, being only 1%, whereas when he tested 15 µM, 30 µM and 60 µM, the cytotoxic effect against HEp-2 cells were 13.2%, 13.1% and 12.5% respectively. Similarly he found that RD cell viability was influenced by the concentration of ceramide they were exposed to. At 7 and 15 µM, the HEp-2 cell percentage inhibition was 6.7% and 7.5% respectively. While at 30 µM, ceramide produced a higher toxic effect of 26.3%, followed by a reduction to 18.8% at 60 µM. This means that at higher concentrations, it is possible for cell lines to become resistant to compounds and begin multiplying as a survival strategy (Singh and Reddy, 2012).

The AMGM5 ceramide-treated cells, underwent different levels of toxic changes when they were exposed to concentrations of 7, 15 and 30 µM respectively. At these concentrations, the % inhibition was 21%, 47% and 79% accordingly. At 60 µM of ceramide, the % inhibition decreased to 50%. It was apparent that AMGM5 cells treated with 30 µM of ceramide should have suffered a significant reduction in cell viability. In the present study, similar situations were present. For example the 20 µg/ml aqueous corm fraction of *B. natalensis* had a % inhibition of -4.09% on the fifth day, whereas the 2 and 1 µg/ml fractions had a HEp-2 % inhibition of 8.83% and 11.58% respectively. Maleek (2010) demonstrated a similar dose independent relationship in normal REFAM3 cells. He found that cells treated with 30 and 60 µM of ceramide were inhibited at a cytotoxicity percentage of 16.7% and 15.5% respectively, while exposure of REFAM3 cells to 7 and 15 µM of ceramide was inhibited at a low level of

toxicity (11.3% and 3.3% respectively). Ultimately the cytotoxic effect of compounds and plant fractions such as those present in *Bulbine*, depend on the composition of compound or plant fraction and whether the treated cell has the ability to generate ceramide-1-phosphate by the action of ceramide kinase (Sugiura et al., 2002) since ceramide, as mentioned, is an important cell membrane component. Subsequently, ceramide-1-phosphate in the plasma membrane can be hydrolysed by phosphatase (Boudker and Futerman, 1993). Rile *et al.* (2003) reported that ceramide-1-phosphate can also be formed intracellularly. Plant fractions that contain ceramide may play a greater role in cell proliferation because they may bind to the CD95 receptors on the cell and inhibit apoptosis from occurring by causing cell proliferation and senescence (Rothman and Lenard, 1977).

Comparison of fractions of the same concentration between *Bulbine* spp. revealed cytotoxicity percentages that were different. For example, the root 2 µg/ml fraction of *B. natalensis* had a larger proliferative effect (-18.86%) compared to the same fraction for *B. frutescens* (-9.77%) on the HEp-2 cell line. This was found to be precarious since it has been reported that phenylanthraquinones, an anticancer substance, is found predominantly in the roots of *Bulbine* and *Kniphophia* spp. (Dagne and Yenesew, 1994; Habtemariam, 2010). Due to the presence of these substances it was expected that the HEp-2 cell death by the root 20, 2 and 1 µg/ml aqueous fractions of one *Bulbine* spp. would correspond to the other species. Failure of this expectation led to the belief that there was possibly a difference in the partitioning of cell killing substances in the same organs of plants that belong to the same genus and thus the same family. Furthermore, there is no evidence that suggest that phenylanthraquinones are extracted from plants of the genus *Bulbine* and *Kniphophia* using aqueous fractions. If phenylanthraquinones were able to be extracted using aqueous solvents (like water in this case), then this result indicates that they are probably present in minimal quantities in *Bulbine* spp. to induce cell death that could otherwise be detected using the MTT cytotoxicity assay.

The wide difference in percentage cytotoxicity readings between the two *Bulbine* spp. indicate that perhaps phenylanthraquinones are found at a much lower concentration in the roots of *B. natalensis* as compared to *B. frutescens.* Interestingly though, it has been reported in traditional and herbal information, that the leaves of *B. frutescens*, have the greater anticancer potential while the roots of *B. natalensis* have a much higher anticancer potential

(HealingAloe, 2012). Scientifically, it has been shown that all the corm fractions tested were cytotoxic against the HEp-2 cell line (except the corm 20 µg/ml aqueous fraction). When the aqueous fractions of *B. natalensis* were compared to the corm fractions, it was the corm fractions that probably had the highest anticancer potential (due HEp-2 cell death induced by those fractions) and not the roots. If anthraquinones are predominately anticancer in the roots of *B. natalensis*, then the low cytotoxicity results obtained for the tested root fractions poses the assumption that the phenylanthraquinones are probably concentrated in the corm of *B. natalensis* and that during root formation, those substances are translocated into the roots which then interact with the root-soil-water continuum (Dagne and Yenesew, 1994, Abegaz *et al*., 1999).

The idea that translocation of cell killing substances (anticancer/apoptotic stimuli) within and between plant organs differ, create controversy when analysing cytotoxicity results. In this study, the plants were all native to South Africa and therefore the results were analysed keeping this in mind. However, a researcher in some other part of the world may want to perform this experiment and may find that the cytotoxicity results are different resulting in subsequent results of confirmatory experiments being different. This has been found to be linked to the conditions at which *Bulbine* spp. plants in those countries have become acclimatised to over time (Chinkwo, 2005). There is currently no results/data on the cytotoxicity of *Bulbine* spp. plants from another country and therefore a comparison was unable to be made at this stage. It was however, discovered that although *B. frutescens* had more growth promoting compounds in the fractions prepared, there were no significant differences in the organ absorbance readings at 5 or 8 days between that species (p: 0.220 - 0.461) and *B. natalensis* (p: 0.314 - 0.839) from the one-sample Kolmogorov-Smirnov test i.e. between the leaf and root fractions that were compared (Appendix 1 - Kolmogorov-Smirnov test: Absorbences B).

The larger proliferative effect of the root *B. frutescens* fraction was seen in the 20 µg/ml sample. This could have been due to either different compounds being present in the roots of both *Bulbine* spp. fractions or the different partitioning of cytotoxic phytochemicals in the root, leaf and corm of *B. natalensis* and the leaf and root (with stem portions) of *B. frutescens* (as mentioned previously). Similarly, although the leaf 2 µg/ml 100% ethanol fraction of both species promoted HEp-2 cell proliferation by the eight day, the *B. frutescens* fraction showed

a very negligible cell proliferative potential after 5 days of MTT plate incubation (-0.83%). The corm 100% ethanol *Bulbine natalensis* aqueous fraction as well as the leaf aqueous fractions induced HEp-2 cell death, while the 2 and 1 µg/ml leaf 50% ethanol fractions caused the cell line to proliferate. Since the corm 100% ethanol *Bulbine natalensis* fractions produced the highest cytotoxic effect relative to all the *Bulbine* spp. fractions after 5 days, the greatest amount of phytochemicals that induce HEp-2 cell death is expected in that fraction (Singh and Reddy, 2012), however, in addition to the properties which Bulbine fractions possess (anti-inflammatory, immune modulating, antitumour, antiviral, antibacterial and antifungal activities) (Búfalo *et al*., 2010; HealingAloe, 2012), anti-oxidants (or the production of reactive oxygen species, vitamins E, C or reactive oxygen species scavengers) can also induce cell signalling events which result in apoptosis and necrotic cell death in cell lines (Balasubashini *et al*., 2006). The HEp-2 cells that were plated out during the MTT cytotoxic assay in our study were respiring and viable and this was confirmed by the formation of formazan crystals during the MTT cytotoxicity assay (Mossman, 1983). During the incubation phase, after treatment with the *Bulbine* spp. fractions, the cells continue to respire until the fractions induce an effect on the HEp-2 cell (cytotoxic and proliferative effect). This respiration process can result in hypoxia or hypotension which may contribute to the cytotoxicity percentage of *Bulbine* spp. fractions indirectly (Loguercio and Federico, 2003). This hypoxic event can be enhanced by reactive oxygen species present in plant fractions (Loguercio and Federcio, 2003). Reactive oxygen species cause cell death or cell injury by causing lipid peroxidation of cell membranes, protein and DNA oxidation (Jashiek *et al*., 1988; Gavin *et al*., 1999; Chien *et al*., 2001; Yu *et al*., 2005) resulting in a cascade of events which lead to apoptosis. The reactive oxygen species (or hypotension) also has the potential to trigger the translocation of nuclear factor-kappa β (NF-kβ) and activator protein I to the nucleus (Li *et al*., 1997). In addition these fractions may activate inflammatory cytokines, chemokines, and adhesion molecules that can contribute to a further production of reactive oxygen species (Bremer *et al*., 1994; Ferret *et al*., 2001; Loguercio and Federcio, 2003) and uninterruptedly activate the cascade of Bax and cytochrome c translocation and caspases (apoptosis) (Chien *et al*., 2005). It is possible for plant fractions to also contain various kinds of anti-oxidants that decrease NF-kβ activation, which reduces cell death (Jaeschke *et al*., 1988; Bremer *et al*., 1994; Gavin *et al*., 1999; Ferret *et al*., 2001; Loguercio and Federcio, 2003; Chien *et al*., 2005). This could have been possible for fractions of *B. frutescens* and *B. natalensis* that promoted HEp-2 cell growth by inhibiting cell death by

treatment with those fractions. This is because some anti-oxidants contain reactive oxygen species scavengers which have the ability to prevent cellular damage. These scavengers include the enzymes catalase and superoxide dismutase (Wu *et al*., 1997) which offer cell lines protection against the reduction of plasma, cytokine levels, preserved anti-oxidant and anti-apoptotic protein content, and a decreased reactive oxygen species level in cell lines (Wu *et al*., 1997). This results in pro-apoptotic signalling (Wu *et al*., 1997), in which the ration of Bcl-2/Bax proteins remain in high proportions in the cell, as well as the expression of CCP32, the cleavage of poly-(ADP-ribose)-polymerase (PARP) and a reduction in the fragmentation of DNA (Hengartner, 2000; Liu *et al*., 2009). Reactive oxygen species generation plays a key role in the induction of apoptosis in HEp-2 cells (Shilpa *et al*., 2012).

The difference in cytotoxicity percentages between the aqueous and organic fractions of both Bulbine spp. could be attributed to the different compounds that are found in those fractions. Furthermore, whereas one would expect similar cytotoxic effects on the HEp-2 cell line for the 50% and 100% ethanol fractions, this was not the case. Balasubashini *et al.* (2006) found that the antiproliferative effect of venom was reduced due to heating over an extended period of time (36h) in HEp-2 and HeLa cell lines and that further investigations were required. Similarly, the compounds present in the fractions that induced cell death in HEp-2 cell line, could have had a diluted cytotoxic effect compared to the compounds present in the crude plant material because of the differences in fraction preparation and furthermore, heating at 37oC over an extended period of time, required to rotatory evaporate the ethanol from the ethanol fractions, could have altered the cytotoxic compounds present (Lipps, 1999).

There was a marked difference in the cytotoxic effect of the root 2 and 1 µg/ml aqueous *B. natalensis* fractions from 5 to 8 days of incubation. For the 2 µg/ml fraction, there was a decrease in HEp-2 proliferative potential from -18.82% to -11.18%, while for the 1 µg/ml fraction, the HEp-2 killing potential increased from -11.02% to -6.4% by the 8th day. This increase could have been due to the potency of the actual prepared aqueous fractions as well as the responsiveness of the HEp-2 cell line to those fractions, but because the DMSO dilutions of these fractions were made from their respective stock solutions (i.e. the 20 µg/ml fraction), this was not considered important. Furthermore, although the percentage cytotoxicity increase only occurred for the 1 µg/ml fraction, the 5 day cytotoxicity result alleviated the possibility of there being a dilution error during fraction preparation (that could

have been related to the potency of the crude fraction because the crude fraction samples to which the HEp-2 cell line were exposed to for 5 days, were the same as those to which the HEp-2 cell line was exposed to for the 8 day MTT cytotoxicity experiments). Moreover, the HEp-2 cell line used during the 5 and 8 day cytotoxicity experiments were taken from the same 75 cm^3 tissue culture flasks and those plates were incubated on the same day. When the root 20 and 1 µg/ml aqueous fraction results were compared, there seemed to have been a strong obvious indication that HEp-2 cell death depends on the potency of the crude fraction as well as the preference of the cell line for the crude fraction at that potency.

Similarly, the root 1 µg/ml ethanol fraction of *B. frutescens* showed a decline in cytotoxicity potential by the 8^{th} day. This decline was negligible because there was only a 1.78% (23.2% - 21.42%) increase in HEp-2 cell line proliferation (Appendix 1 Tables 2.4 and 4.4). The difference between the 5 and 8 day cytotoxicity results of *B. frutescens*, indicated the selective preference of this cell line for certain crude fractions. It also reiterated that although preferential selection is dose and time dependent, the potency of the actual prepared/administered fraction determines the extent of cell death as well as the rate at which cell death is induced. The rate and extent of cell death differs between and amongst various cell lines that are exposed to the same plant fraction or natural product (Talin and Mahasneth, 2010).

The spectrum of cytotoxicity results for 5 and 8 day incubated MTT plates, indicated that samples had to be objectively selected for *bax* and *caspase-3* evaluation. Based on the fact that all of the 8 day fractions had the potential to enhance the growth potential of the HEp-2 cell line, those samples were not selected for PCR analysis. This means that only selected *B. natalensis* fractions were chosen and that relative to those some *B. frutescens* fractions were selected. This was the sensible thing since the this project looked at the apoptotic potential of *Bulbine* spp. Fractions on the HEp-2 cell line, but the methods of analysis was representative of analysing the data of other cell lines.

Since *B. natalensis* was the reference plant in this study, reliability testing as well as the one-sample Kolmogorov-Smirnov tests were limited to this plant species. It was found that, between organ fraction comparisons (leaf, root and corm), that the 8 day corm fractions showed the lowest reliability with coefficient of variation of 10.7645 ± 19.36225 when

compared to the 5 day samples (Appendix 1 Table 1 - reliability testing III). This was reinterated when the percentage cytotoxicity coefficient of variation for corm showed the least reliability on the 8th day (10.7645 ± 19.36225 SD). The leaf fraction was the most reliable on the 5th day (6.6242 ± 4.29828 SD), with the root fraction being the most reliable on the eighth day (5.4525 ± 4.23934 SD) (Appendix 1 Table 1 - reliability testing IV).

In terms of the solvent system, it was found that the 8 day 100% ethanol fractions were the least reliable (8.4803 ± 17.21614 SD). Different compounds were present in the 50% ethanol fractions (3.7594 ± 3.53525 SD). This was deduced when the coefficient of variation of the aqueous (4.9687 ± 6.65819 SD) and 100% ethanol fractions were compared to the 50% ethanol fraction (Appendix 1 Table 1 - reliability testing III). This was validated when the percentage cytotoxicity coefficient of variation of the 8 day 100% ethanol fraction was 9.2092% ± 16.89564 SD and that of the aqueous and 50% ethanol fractions were, 6.0178% ± 6.19595 SD and 5.3487% ± 4.73881 SD respectively. At 5 days, the 50% ethanol fraction was the least reliable (21.9488% ± 28.01136 SD) while the 100% ethanol fraction was the most reliable (5.5352% ± 3.99749 SD) (Appendix 1 Table 2 - reliability testing IV).

When the one-sample Kolmogorov-Smirnov test was employed between the cytotoxicity readings of *Bulbine natalensis* organ fractions at 5 or 8 days, it was found that there was an overall difference between them since the test produced a p value of < 0.001 (5 day) and 0.001 (8 day). The Bonferroni (one-way ANOVA) showed that the percentage cytotoxicity values of all organs differed significantly from one another after 5 days of incubation, however, after a further 3 days of incubation the percentage cytotoxicity results of the corm and leaves, as well as the roots and leaves, were not significantly comparable. Hence, since there weren't any discrepancy in the cytotoxicity readings for the two sets of organs mentioned, only samples from the 5 day *B. natalensis* crude fractions were used for the apoptotic gene expression study (Appendix 1 Table 2 - One-sample Kolmogorov-Smirnov test: Cytotoxicities).

The cytotoxic properties of *Bulbine* spp. fractions may also be explained due to the presence of flavonoids or other phenolic substances in the fractions (Moreno *et al*., 2005), which contribute to other properties that *Bulbine* spp possess. These properties include: anti-inflammatory, immune modulating, antitumour, antiviral, antibacterial and antifungal

activities (Búfalo *et al*., 2010; HealingAloe, 2012). Phenol compounds are present in all plant material, including *Bulbine* spp., and these phenols are secondary metabolites which belong to a large heterogeneous group of biologically active non-nutrients (Búfalo *et al*., 2010). Secondary metabolites are synthesized in response to plant stress and are catalysed by enzymes which are involved in morphological and biochemical regulatory patterns that are caused by pathogens, wound affects and adverse environmental conditions. When plant material is prone to infection in the environment, the production of phenolic compounds in response to plant defence mechanisms become hindered (Búfalo *et al*., 2010). The *Bulbine* spp. plant material used in this study were collected at different time intervals and from different environmental sites, all of which are in the vicinity of different environmental stress/es. Therefore the chemical composition of the fractions prepared in this study were complex and could have possibly varied in the proportion of phytochemicals which brought about the cytotoxicity and proliferative effect on the HEp-2 cell line (Búfalo *et al*., 2010; Singh and Reddy, 2012) for the same fractions at different administered concentrations. An example where this is evident is the 5 day corm 2 µg/ml 100% ethanol fraction of *Bulbine natalensis* where the highest percentage cytotoxicity of 22.56% was recorded in relation to the highest and lowest administered concentrations to the HEp-2 cell line (16.54% and 20.69% respectively). A similar trend was observed for the leaf aqueous fractions of *Bulbine natalensis.* It can therefore be deduced that the cytotoxic effect of the *Bulbine* spp. fractions on HEp-2 cells may be the result of the synergistic affect of its constituents, which may induce cellular growth or enhance cell growth inhibition at the same time (Kujumgiev *et al*., 1999; Búfalo *et al*., 2010; Singh and Reddy, 2012). Kujumgiev *et al*. (1999) and Búfalo *et al*. (2010) found that the cytotoxic activity of isolated compounds from the plant *Baccharis drucunculifolia*, an important vegetal source of propolis in southeast Brazil, were exhibited in the HEp-2 cancer cell line at higher concentrations, whilst their effect on cell viability *in vitro* was nullified at lower concentrations. The isolated compounds cimmamic acid (5, 10 and 25 µg/10 µl of cells) and caffeic acid (5 µg/10 µl of cells) brought about these effects on the HEp-2 cell line at the above mentioned concentrations. In contrast, Búfalo *et al*. (2010) also found that the propolis of *B. druncunculifolia* had a greater cytotoxic effect on the HEp-2 cell line compared to the extracts that were prepared from the vegetal source itself. The HEp-2 cells were treated with propolis and vegetal source extracts at an elevated concentration of 50 and 100 µg/ml per 100 µl of HEp-2 cells to induce the cytotoxic effect in a concentration-dependent manner, because at lower concentrations of 5, 10 and 25 µg/10 µl, the *B.*

drucunculifolia extracts and essential oils, the propolis as well as the caffeic and cinnamic acid showed no cytotoxic activity against the HEp-2 cells (Búfalo *et al*., 2010). Fukuda, Ohzoshi, Makina and Fugimoto (2006) also found that the sesquiterpene and terpene phenols from *B. druncunculifolia* exhibited a potent cytotoxic activity against leukaemia cells. These studies indicated that there is contradicting evidence that plant fractions could induce different levels of cytotoxicity or no cytotoxicity at all in infected cells because of the different type of phenolic substances within the plant and the prepared fractions, and that situations of irregular cytotoxicity levels can also come to fruition in many cell types (Búfalo *et al*., 2010; Singh and Reddy, 2012).

Selective cytotoxicity has been widely researched in human epithelial cell lines such as CaCo (colon cancer), HEp-2 and HeLa cell lines (Calvert *et al.*, 2005), and there is a profound amount of information about phytochemicals having potential chemopreventative or chemotherapeutic efficacy against various types of cancer (Surh, 2003). The HEp-2 cell line, as mentioned, is derived from HeLa cell contamination and therefore it is a hardy cell line (Sidambaram *et al*., 2011; Singh and Reddy, 2012) which resists phenotypic and genotypic changes to chemicals present in the growth medium (Chatterjee *et al*., 2001). Calvert *et al*. (2005) found that when the cytotoxicity of 3 *Fusarium* toxins, deoxynivalenol (DON), T-2 toxins and zearalenone (ZON), were tested on those epithelial cell lines using the MTT cytotoxicity assay, HeLa cell lines were the most sensitive to T-2 toxin and ZON treatment at concentrations of 0.1 µg/ml and 1.0 µg/ml respectively, after 2 days of exposure to the toxin. Their study also showed that after a further 2 days of exposure, the HeLa cell line evoked rapid cell death responses to DON at 0.2 µg/ml. In the preliminary observations to the present study, the HeLa cell line was found to be very sensitive to *Bulbine natalensis* fraction treatment after 3 days of incubation, and that its rate of multiplication was very rapid – reaching 100% confluency after only one day of incubation. This finding was in agreement with Calvert *et al*. (2005). Calvert *et al*. (2005) has shown and Singh and Reddy (2012) have re-interated that the treatment of cells with plant fractions may not necessarily be dose and time dependent. Calvert *et al.* (2005) found that the HEp-2 cell line was sensitive to DON treatment after 2 days exposure at 0.1 µg/ml with complete cell death occurring after 2 days at 1.0 µg/ml and complete cell death occurring at day 4 with a concentration of 0.1 µg/ml. Hence from this study that at day four a lower concentration of DON induced HEp-2 cell death while 2 days prior to this cytotoxicity reading, a concentration 10 times more was

required to induce cell death. This indicated that the cytotoxic effect of mycotoxins and possibly plant fractions on cell lines can be time dependent, but concentration independent in the HEp-2 cell line because the HEp-2 cell line does not undergo autocrine apoptosis in the absence of apoptotic stimuli (Chatterjee *et al*., 2001). The response of cell types to apoptotic stimuli when performing cytotoxicity studies depend on the replication machinery of the cell line (Calvert *et al*., 2005; Singh and Reddy, 2012). Calvert *et al*. (2005) found that DON, ZON and T-2 mycotoxins failed to induce cell death in CaCo and this indicated to them that there was some type of cell type mycotoxin specificity. Similarly our study found that this type of specificity existed when the HEp-2 cell line was exposed to the leaf aqueous and corm 100% ethanol fractions of *Bulbine natalensis.* Those fractions were cytotoxic to the HEp-2 cell line, with the root aqueous fractions causing cell proliferation after 5 days of incubation. The HEp-2 cell line was the most sensitive to the fractions mentioned above in the light of cytotoxicity. However, this cell line was sensitive to all the fractions for 5 and 8 days. Reubal *et al*. (1989) suggested that compounds can be less cytotoxic to mammalian cell lines and that on occasion the same compounds can enhance the cleavage activity of the cell types – the latter referring to cell proliferation. Reubal *et al*. (1989) made this suggestion from experiments conducted using the MTT cytotoxic assay, which many authors considered to be the best screening method since it is reproducible, accurate, and a rapid tool for assaying the viability of various cell lines on exposure to toxins (Holt *et al*., 1989; Visconti *et al*., 1991; Kitabatake *et al*., 1993; Hanelt *et al*., 1994; Widestrand *et al.,* 2003).

In conclusion, percentage cytotoxicity results were variable amongst the two *Bulbine* species and they were concentration independent per solvent fraction and final concentration in HEp-2 cell volume. This was ascribed to HEp-2 cell line preference for crude fractions of certain *Bulbine* species organs and potencies.

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

Bulbine natalensis fractions showed the greatest number of positive results (cell killing) for % cytotoxicity against the HEp-2 cell line (Figure 19) for the 5 day-incubated MTT plates compared to *B. frutescens* fractions (Figure 20). 14 samples in total had positive cytotoxicity results. The three aqueous 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 21), 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 20 and 22) were negative by day 8.

In order to conserve cost effectiveness, fractions of different concentrations were selected for the apoptotic 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 confirm the *bax* and *caspase-3* gene expression results of other fractions not having any cytotoxic effect 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 (Figure 19).

Selection was performed in an ordered manner such that the fraction of *B. frutescens* that was selected for comparative purposes, with the fractions of *B. natalensis*, had the less negative cytotoxicity value of two concentrations (Table 3).

Table 3: 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

CHAPTER FOUR

Apoptotic gene expression

4.1. Introduction

The basic principle of gene expression lies in the interpretation of the genetic code (Mathews *et al*., 2000; Becker *et al*., 2003). The genetic code is based on a set of rules which highlights the relationship between the nucleotide base sequence (purines and pyrimidines) of the DNA molecule and the linear sequence of amino acids in the protein molecule (Mathews *et al*., 2000; Becker *et al*., 2003). During information transfer within living organisms, the information encoded in the nucleotide base sequence of DNA is transcribed into messenger RNA (mRNA) through the process of complementary base pairing (Mathews *et al*., 2000; Becker *et al.,* 2003). The sequence of purine and pyrimidine bases in the mRNA transcript is thereafter translated into a string of amino acids which becomes recognisable as a polypeptide (protein) (Mathews *et al*., 2000; Becker *et al*., 2003). Since information is transferred from DNA to mRNA and then to proteins, any deformity or mutation in the nucleotide sequence of the DNA, induced by environmental or endogenous factors, will result in polypeptides chains that have either altered or different functions (Becker *et al*., 2003). However, some may even be non-functional (Mathews *et al*., 2000; Becker *et al*., 2003).

The fact that there is a strong relationship between mutations in DNA and function of a protein, was discovered in 1940 by George Beadle and Edward Tatum while working on a self-sufficient bread mold, *Neurospora crassa* (Becker *et al*., 2003). *N. crassa* grows in minimal medium containing sugar, inorganic salts and the vitamin biotin and since the mold is self-sufficient, all other requirements needed by it are provided by the organism's metabolic pathways. Beadle and Tatum subjected the mold to X-rays, while in minimal medium, to induce mutations in the nucleotide sequence of the DNA that encode the organism's metabolic pathways (Becker *et al*., 2003). Beadle and Tatum found that mutant strains lost their ability to grow in minimal medium and that those strains had to be transferred into complete culture medium to grow at the rate they normally do in minimal medium. This suggested that the mutant strains, lost their ability to synthesis certain amino acids, nucleotides and vitamins, and therefore those amino acids, nucleotides and vitamins had to be supplied in the culture medium (Becker *et al*., 2002). In order to characterise which amino acid was needed by each mutant strain, Beadle and Tatum allowed them to grow on

selective medium that was supplemented with a specific amino acid (Becker *et al*., 2003). This led to the discovery that one mutant strain required tyrosine, for example, while another required arginine and so on. Since this was not completely conclusive, Beadle and Tatum took their investigation further by looking at particular steps in the metabolic pathway of *N. crassa* (Becker *et al*., 2003). This was a complicated task and it involved supplementing minimal medium with metabolic precursors of a given amino acid or vitamin as opposed to the actual amino acid or vitamin itself. By finding out which precursors stimulated the growth of a particular mutant *N. crassa* strain, they were able to conclude that a single enzyme catalysed step leading to the synthesis of a specific compound was disabled. This means they were able to deduce that there was a relationship between a mutated gene and the lack of a specific protein in the metabolic pathway (Becker *et al*., 2003). It was from this discovery that Beadle and Tatum formulated the hypothesis that for every gene there is an associated protein or enzyme (central dogma of molecular biology) (Mathews *et al*., 2000; Becker *et al*., 2003). Since the 1940s, the genetic code is used as the basis for understanding and analysing gene expression results of DNA that contain regulatory sequences, chromosomal structural areas, intergenic segments and other non-coding DNA that contribute greatly to the phenotype of an organism (Mathews *et al*., 2000). In 1911 Peyton Rous discovered the first known tumour virus which caused tumours in chickens. However, it was only in the late 1980s when emphasis was placed on human cancers by Robert Weinberg. Prior to the 1980s, research on human cancers were being carried out, but they were mainly noted as being unusual infections. Wienberg recognised that human tumours consisted of transforming genes, as was the case with tumour viruses, and this was a significant finding since it allowed other scientists to unify the theories of carcinogenesis (Mathews *et al*., 2000). In the absence of exogenous viruses, Bruce Ames found that chemicals were able to induce carcinogensis in cells by mutating proto-oncogenes (Temin, 1988; Mathews *et al*., 2000). However, there have been other reported genetic alterations that have been found in tumour tissue and these have involved the detection of tumour suppressor genes (Knudson, 1993). These genes suppress tumour/cancer progression within tissues and have the ability to promote cancer development if a gene encoding the tumour suppressor becomes mutated by a chemical or drug compound (Mathews *et al*., 2000). The tumour suppressor gene, *p53*, is a 53 kilodalton gene which participates in the process of programmed cell death, but in general, it also regulates the cell cycle by preventing inappropriate movement of cells from the G1 to S phase. This prevents cells from multiplying uncontrollably (cancer development) (Knudson, 1993). Research is

currently being undertaken in a variety of research institutes throughout the world to elucidate the true biochemical role of *p53* in a variety of cancer cell lines (Mathews *et al*., 2000), including the HEp-2 cell line. However, what is known, through x-ray crystallographic experiments since 1994, is that in order for *p53* to function optimally, proper complementary base pairing between *p53* and the *p53* binding site of an oligonucleotide must occur (Knudson, 1993; Burley, 1994). The functioning of *p53* in tumour suppression and cell cycle regulation, is attributed to the bending and twisting of the DNA, which accounts for the variability in its expression levels (Mathews *et al*., 2000). It has been reported that the amino acid residues in close contact with DNA, are those that are mutated in mutant *p53* proteins (Mathews *et al*., 2000).

There are many kinds of chemotherapeutic agents that are currently being used to treat oral cancers (Shilpa *et al*., 2012). Amongst these are cisplatin and 5-fluorouracil which are associated with side-effects, thereby making the screening of apoptotic inducers from plants (either the crude extract or as an active compound isolated from those) an imperative (Shilpa *et al*., 2012). Hsu *et al*. (2004) found that most cancers are resistant cisplatin and 5-fluorouracil either because of their developed resistance to these drug therapies or because they are not dependent on a single receptor or signal transduction pathway for the growth and progression of the cancer. Therefore the use of crude plant material may activate multiple pathways, and may provide a greater chance for effective treatment of patients with oral cancers (Shilpa *et al*., 2012).

Apoptosis, or programmed cell death, occurs during normal development as a highly regulated process of cell suicide, which disturbs the physiology and metabolism of the cell (Vaux and Strasser, 1996; White, 1996). In mitochondrial and endoplasmic reticulum apoptosis, the apoptotic signals that are received converges to a central pathway that involves a family of aspartate-specific cysteinyl proteases (cysteine aspartate, or caspases), which are activated by proteolytic cleavage during the cell death process (Alnemri *et al*., 1996; Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997; Cryns and Yuan, 1998; Green, 1998; Thornberry and Lazebnik, 1998). Caspase activation leads to the processing of various cytoplasmic and nuclear targets, by a subclass of caspases. These caspases include caspase-3, caspase-6, and caspase-7 (Villa *et al.,* 1997; Salvesen and Dixit, 1997). The DNA repair enzyme, poly (ADP-ribose) polymerase (PARP), the DNA fragmentation factor (DFF) and

scaffolding proteins (lamins and actin) are the cleavage targets (Nicholson and Thornberry, 1997; Villa *et al.,* 1997; Salvesen and Dixit, 1997; Hengartner, 2000; Van Cruchten and Van den Broeck, 2002). In general, the process of apoptosis involves the processing of caspase-3, DFF and PARP.

Apoptosis has been detected by many methods. These have been reviewed in Van Cruchten and Van den Broeck (2002) and include: light, fluorescent (using acridin orange and Hoechst stains) and electron microscopy using nucleic acid-staining dyes like hematoxylin to detect the morphological changes of apoptosis expressed by *caspase-3*; gel electrophoresis to detect internucleosomal DNA fragmentation with low reliability because of the fragmentation of oncotic cells; *in situ* histochemistry that uses TUNEL to detect *caspase-3* (however, TUNEL can be coupled with fluorescent microscopy or flow cytometry but with low reliability since those techniques detect oncotic cell death, therefore detection of *caspase-3* is reliable by TUNEL since it has not been detected in oncosis, thereby making the technique very specific) and flow cytometry using the double staining technique with annexin V and propidium iodide (in this technique apoptotic cells are only coloured by annexin V while the oncotic cells stain both annexin V and propidium iodide). In addition, fluorescent lipophilic molecules can also be used to measure decreased mitochondrial transmembrane potential in apoptotic cells by remaining in their monomeric form in the cytoplasm with a green fluorescence, whereas in normal cells those lipophilic molecules aggregate in the mitochondrial and emit a red fluorescence. Although this technique can be coupled with microscopy and flow cytometry, the detection of oncotic cells causes the latter two methods to be unreliable as stated in Van Cruchten and Van den Broeck (2002).

Gel electrophoresis has become the most reliable means of detecting apoptosis in old and infected cell lines using RT-PCR (Holdenrieder and Stieber, 2004). RT-PCR uses a viral enzyme called reverse transcriptase that synthesises DNA from a single-stranded RNA template (Mathews *et al*., 2000). In the present study, RNA was isolated from the HEp-2 cell line and transcribed into cDNA using the Cells-to-cDNA^{TM} II kit (Ambion, Inc). The cDNA was thereafter used to measure the expression of *caspase-3* and *bax* mRNA from a large number of HEp-2 cells. The Cells-to-cDNA^{TM} II kit is convenient for measuring cellular mRNAs from a variety of cell types since it performs the RNA isolation and thereby prevents the loss of RNA material, allowing for many samples to be analysed by RT-PCR, much faster

and simpler. An advantage of using the Cells-to-cDNA™ II Kit is that it inactivates all RNases that may be present in cultured mammalian cells by heating with Cell Lysis II buffer. This buffer ensures that mRNA is not cleaved and is intact for gene expression analysis (Cells-to-cDNA™ II Kit manual). The use of RNase was discovered by Klebe *et al.* (1996) while experimenting with placental RNase inhibitor and crude cell lysates that were only active against members of the RNase A superfamily (Cells-to-cDNA™ II Kit manual).

The Cells-to-cDNA™ II kit has a wide variety of applications ranging from analysing a large number of differentially-treated cells to measuring mRNA expression levels in cell lines treated with varying concentrations of compounds to screening for candidates that have a desired effect on particular mRNAs over a period of time (Cells-to-cDNA™ II Kit manual). It has been found in extracts of *Braccharis dracunculifolia, Euphorbia hirta, Nigella sativa* and *Rubia cordifolia* that the expression of *caspase-3* and *bax* occurs in a dose and time dependent manner in HEp-2 cell lines (Ali *et al*., 2003; Hsu *et al*., 2004; Kummaline *et al*., 2007; Lin *et al*., 2009; Búfalo *et al.,* 2010; Shilpa *et al*., 2012). To the best of our knowledge, there is no scientific report on the apoptosis inducing effect of *Bulbine natalensis* and *Bulbine frutescens* on human laryngeal carcinoma. Hence, the present study was performed using the Cells-to-cDNA™ II Kit to elicit the apoptosis inducing effect of selected aqueous and organic fractions of *B. natalensis* and *B. frutescens in vitro*, using the HEp-2 cell line as the experimental model. The results were correlated with the mechanisms of apoptosis for each fraction. Those results were further compared to the 2% (200 µM) DMSO, camptothecin and calpain I inhibitor controls.

4.2. Materials and Methods

4.2.1. Reverse-transcription polymerase chain reaction

RT-PCR is a method used to analyse gene expression in plant and animal models such as bacteria, fungi and archaea. It uses primers that bind to mRNA templates to amplify full length cDNA. This process of obtaining cDNA is catalysed using reverse transcriptase. Depending on the type of primers used (oligo-dT, site-specific primers or random hexanucleotides), the length of the cDNA generated varies. Reverse transcriptase enzyme and thermostable *Taq* polymerase, are used to catalyze two separate reactions (Shiao, 2003).

RT-PCR was used to study the *bax* and *caspase-3* related genes. The *β-actin* gene was used as the internal/housekeeping control. cDNA was generated using the cells-to-cDNA^{TM} II kit, which was purchased from Ambion, Inc. This kit involves cDNA synthesis from mRNA without the initial RNA isolation. The steps that follow indicate the preparatory steps that were undertaken to perform RT-PCR.

4.2.2. Washing and lysis of HEp-2 cell line

The CCM (200 µl) containing the *Bulbine* spp. fractions in the wells of the 5 day incubated 96-well plates were first removed using sterile serological pipettes. Only 96-well plates that were incubated at 37^{o}C in a 5% CO_2 atmosphere for 5 days were used to study apoptotic gene expression. The cells were (114 000 ± 10 583 SD cells/well) counted using the trypan blue dye exclusion method in Freshney (1987) (see cytotoxicity chapter) in each well were thereafter washed with 0.2 ml cold PBS (1 tablet dissolved in 200 ml autoclaved distilled water, cooled and adjusted to pH 7.4) (i.e. added, pipetted up and down and removed) before the 96-well plate was placed onto ice. 100 µl of ice cold Cell Lysis II buffer was added to each well and mixed by pipetting. After all the samples had Cell Lysis II buffer, the 96-well plate was transferred to an iron block and incubated at 75^{o}C (Laboratory Supplies, Johannesburg) for 15 minutes.

Once the 15 minutes had elapsed, the plates were placed on ice and 2 µl of DNase I (2U/ul) from the Cells-to-cDNA^{TM} II kit was added to all the samples and mixed by gently shaking the plate. The plates were then incubated at 37^{o}C (Series 2000 Scientific) for 15 minutes. The plates were then incubated at a temperature of 75^{o}C (Laboratory Supplies) for 5 minutes to inactivate the DNase enzyme.

4.2.3. One-step RT-PCR

There were two negative controls used in the RT-PCR. One negative control contained all the reaction components except for the reverse transcriptase enzyme, while the negative template control contained water instead of the HEp-2 cell lysate.

Thin walled centrifuged tubes were placed on ice. The following was added to each tube: 5 µl cell lysate (for the negative template control, nuclease-free water was added), 2.5 µl 10 X RT-Buffer, 4 µl dNTP mix (2.5 mM each), 1 µl RNase inhibitor, 1 µl MMLV reverse transcriptase (for the negative RT control, nuclease-free water was added), 1 µl PCR primers supplied by IDT: Integrated DNA Technologies (a mixture of 10 µM of each primer), 2 U thermostable *Taq* DNA polymerase supplied by Fermentas Life Sciences (0.4 µl of the 5 U/ul), and 10.1 µl nuclease-free water was added to each tube to bring reaction volume to 25 µl.

Table 4: Primer sequences used in RT-PCR (Pillai *et al*., 2004)

Primer	**Forward**	**Reverse**
β-actin	5'-CTGTCTGGCGGCACCACCAT-3'	5'-GCAACTAAGTCATAGTCCGC-3'
bax	5'-AAGCTGAGCGAGTGTCTCAAGCGC-3'	5'-TCCCGCCACAAAGATGGTCACG-3'
caspase-3	5'-TTTGTTTGTGTGCTTCTGAGCC-3'	5'-ATTCTGTTGCCACCTTTCGG-3'

The reaction microfuge tubes were thereafter placed into the thermocycler (GeneAmp PCR System 9700, Applied Biosystems) under the conditions in Table 5. The PCR products were stored in cryotubes at -20^{o}C.

Table 5: Thermocycling conditions for RT-PCR (Cells-to-cDNA Kit ™)

Cycle	Temperature (°C)	Duration
Reverse transcription	42	15 min
Denaturation	94	2 min
35 cycles	94	30 sec
	55	30 sec
	72	30 sec
Final extension	72	5 min

4.2.4. Analysis of PCR products

4.2.4.1. Preparation of 50 X TAE stock solution

A 50 X TAE stock solution was prepared. It involved adding 242 g 2 M Tris base (Sigma-Aldrich, Inc), 57.1 ml acetic acid (Merck) and 100 ml 0.5 M EDTA (Merck) to 700 ml of distilled water. The pH of the solution was adjusted to pH 8 using a 1N NaOH solution. The TAE was stored at room temperature.

To make a 1 X TAE, to perform gel electrophoresis, 20 ml of the 50 X TAE stock solution was added to 980 ml of distilled water.

4.2.4.2. Agarose gel electrophoresis

A 0.8% agarose gel was prepared for agarose gel electrophoresis because this was the concentration at which *bax* and *caspase-3* genes showed proper separation. 0.8 grams of agarose powder (SeaKem® LE Agarose, Lonza Rockland, USA) was added to 100 ml of 1 × TAE buffer in a 250 ml bottle and warmed in the microwave. The bottle was swirled every 30 seconds.

The casting tray with the gel was placed in the electrophoretic apparatus. 1 X TAE buffer was put over the gel. The comb was removed to expose the wells. 10 µl of each sample from the microfuge tubes were added to the wells and the samples were electrophoresed at 100 V (BIO-RAD) for one and a half hours. Thereafter, the gel was removed from the casting tray and placed in a 0.5 µg/ml ethidium bromide solution. The gel was stained for 20 minutes. The

gel was thereafter visualised using UV light that was supplied by the UV transluminator that was located within the BIO-RAD gel documentation system (BIO-RAD Laboratories, Inc).

4.2.4.3. Analysing gene expression results

PCR products were analysed using a cross-sector approach. The presence and absence of bands were noted and compared between and amongst the different *Bulbine* spp. fractions that were tested. The % cytotoxicity results were used to validate the gene expression results and to established, if any links exist between the cytotoxicity and gene expression results. In addition to formulating a link between these two aspects, the cytotoxicity and gene expression results were used to understand the mechanisms of action of *Bulbine* crude fractions on HEp-2 cancer cell lines.

4.2.5. Calculations and Statistics

The binomial distribution statistical method was employed as suggested by Townend (2002) for determining the percentage upregulation and downregulation, the standard error and confidence limit of the *bax* and *caspase-3* genes for the *Bulbine* spp. fractions, organs, solvent system as well as of the different fractions for the *B. natalensis* corm and the root aqueous and 100% ethanol fractions of this species.

4.3. Results

The HEp-2 cell line passage number used was 3. Total RNA was extracted from the treated HEp-2 cell line and then transcribed using RT-PCR by using site-specific primers. 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. For fractions that induced *bax* and *caspase-3* gene expression, only one band appeared on the agarose gel. For fractions which did not induce the expression of one or both of those genes, no bands appeared on the agarose gel. No banding patterns were observed.

4.3.1. *bax* gene expression

There were 14 *Bulbine* spp. fractions which were tested on the HEp-2 cell line for *bax* gene expression. Not all of those fractions induced *bax* gene expression in the HEp-2 cell line, as indicated in Figure 23. The *Bulbine natalensis* 20 and 1 µg/ml corm aqueous fractions (A and B, respectively), and 20 and 1µg/ml corm 100% ethanol fractions (E and F, respectively) expressed *bax*. The 2 µg/ml 100% ethanol leaf fraction for *B. natalensis* expressed *bax* (J) while the 20 µg/ml 100% ethanol fraction did not express *bax* (I). Similarly, the *B. frutescens* 20 ug/ml 100% ethanol leaf fraction did not express *bax* (M), while the 2 µg/ml fraction expressed *bax* (N). The *B. frutescens* 20 µg/ml (K) and 2 µg/ml (L) root aqueous fractions expressed *bax*.

There were four other fractions, apart from the two mentioned above (I and M) which did not induce *bax* gene expression in the HEp-2 cell line. These fractions were the *Bulbine natalensis* corm 2 and 1 µg/ml 50% ethanol fractions (C and D) and the root 20 and 2 ug/ml aqueous fractions (G and H).

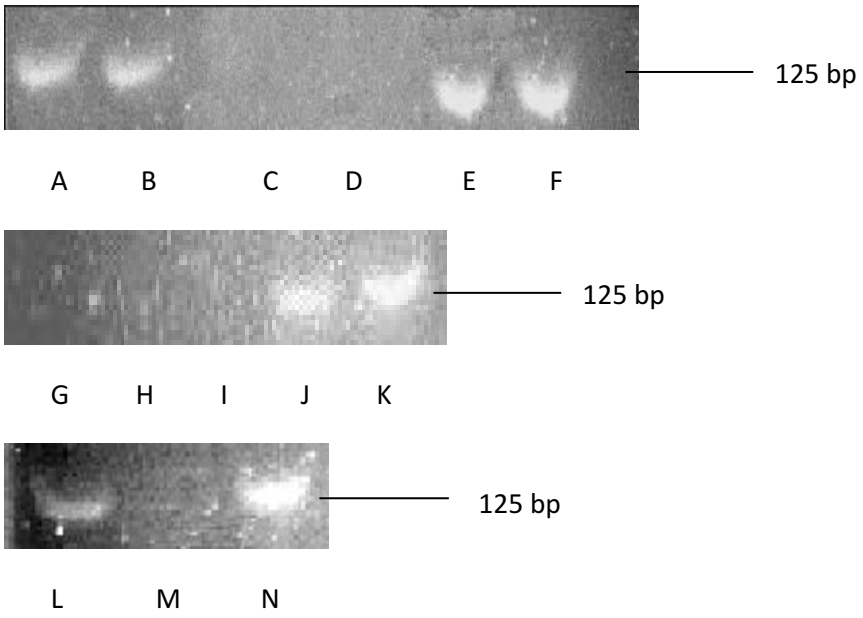
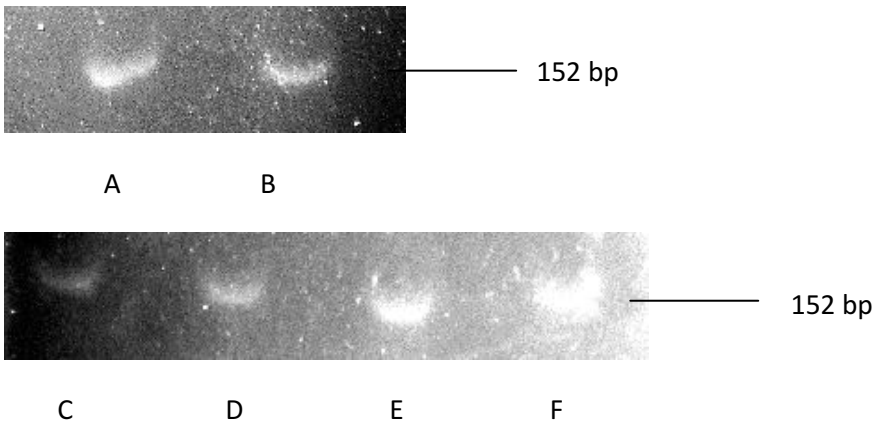


Figure 23: Reverse Transcription-Polymerase Chain Reaction analysis of *bax* gene in HEp-2 (114 000 ± 10583 SD cells/well) treated with 14 *Bulbine* spp. fractions. These results are representative of RT-PCR results in triplicate. The fractions tested for *B. natalensis* were: A: corm 20 µg/ml aqueous, 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.

4.3.2. *caspase-3* gene expression

The 14 *Bulbine* spp. fractions that were tested for *bax* gene expression in the HEp-2 cell line (Figure 23) were tested for *caspase-3* gene expression (Figure 24).



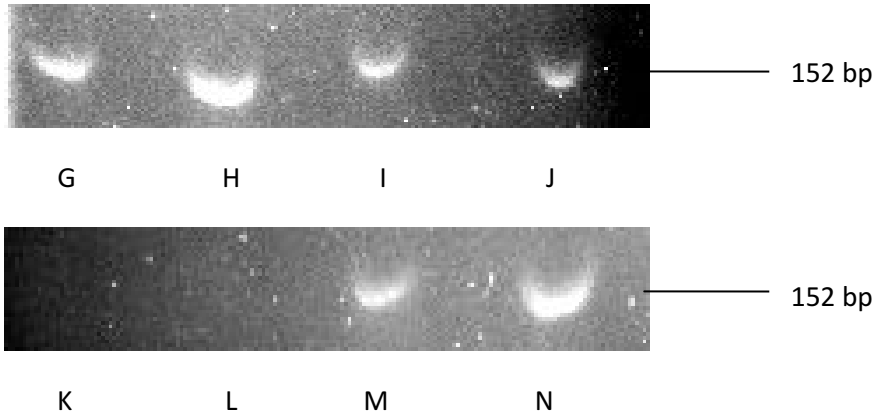


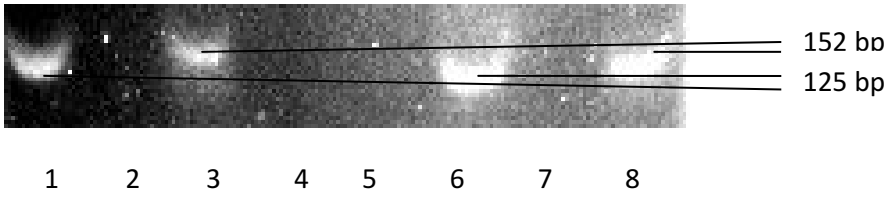
Figure 24: Reverse Transcription-Polymerase Chain Reaction analysis of *caspase-3* gene in HEp-2 (114 000 ± 10583 SD cells/well) treated with 14 *Bulbine* spp. fractions. The fractions tested for *B. natalensis* were: A: corm 20 µg/ml aqueous, B: corm 1 µg/ml aqueous, C: corm 2 µg/ml 50% ethanol, D: 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. These results are representative of RT-PCR carried out in triplicate.

Twelve fractions in total induced the expression of *caspase-3*. The two fractions which did not express *caspase-3* were the *B. frutescens* 20 and 2 µg/ml aqueous root fractions (K and L, respectively), while all the *B. natalensis* fractions including the leaf 20 µg/ml 100% ethanol fraction (I) which did not express *bax*, expressed *caspase-3*. Some other fractions which did not express *bax*, but which induced *caspase-3* expression in HEp-2 were: C and D (corm 2 and 1 µg/ml 50% ethanol), G and H (root 2 and 20 µg/ml aqueous) and M (leaf 20 µg/ml 100% ethanol) fractions.

4.3.3. *bax* and *caspase-3* gene expression for the apoptotic controls

There were 8 controls that were tested in total apart from the *B-actin* internal control sample fractions (Figure 25). These controls included the negative reverse transcriptase and the negative template RT-PCR controls which did not show any bands (2 and 7, respectively) and indicated that the RT-PCR procedure was working.

Apoptotic and RT-PCR controls



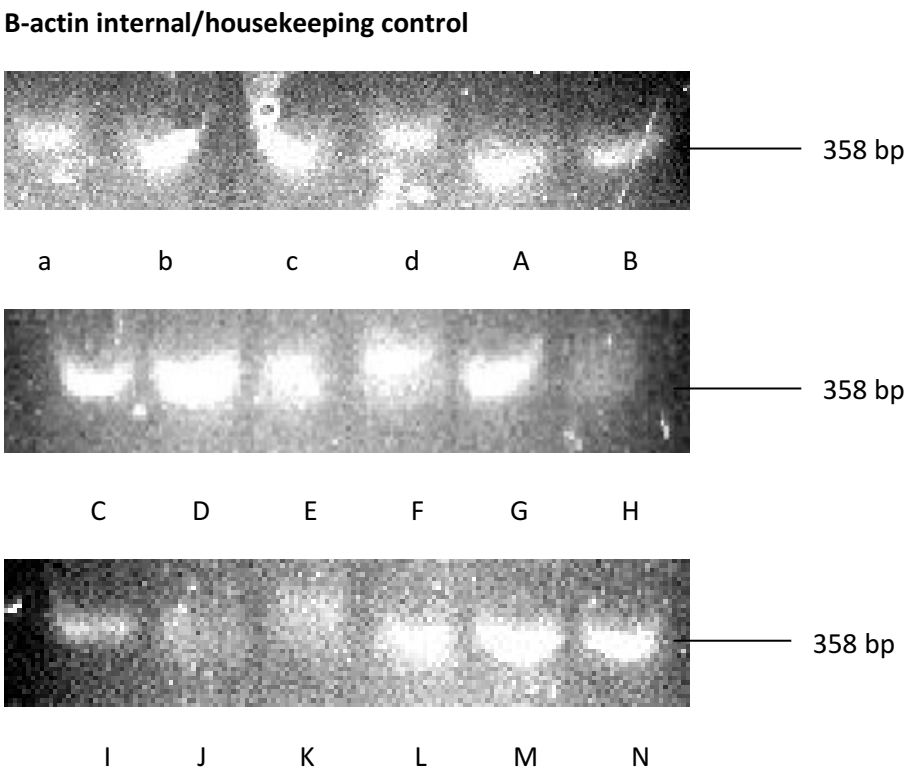


Figure 25: Reverse Transcription-Polymerase Chain Reaction analysis of *β-actin* (control), *bax* and *caspase-3* genes in the HEp-2 cell line (114 000 ± 10583 SD cells/well). These results are representative of RT-PCR results in triplicate. The apoptotic and RT-PCR controls tested against the HEp-2 cell line were: 1: 20 µg/ml camptothecin (bax), 2: reverse transcriptase negative control, 3: 20 µg/ml camptothecin (caspase-3), 4: 200 µM 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 inibitor, A - N represent the sample fractions. These sample fractions 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.

Camptothecin induced the expression of *bax* and *caspase-3* as shown as 1 and 3 respectively in Figure 25. The 200 µM DMSO control did not express *bax* and *caspase-3* (4 and 5, respectively), while calpain I inhibitor expressed *caspase-3* and *bax* in the HEp-2 cell line (6 and 8, respectively). Figure 25 also shows the expression of the *β-actin* gene for the untreated HEp-2 cell line (a) as well as HEp-2 treated with the controls (b: 200 µM DMSO, c: 200 µM camptothecin and d: 200 µM calpain I inhibitor). β-actin served as an RT-PCR control. Although for all the HEp-2 treated cells (A - N), the *β-actin* gene was expressed, it was expressed more clearly for 11 samples. These samples were the *Bulbine natalensis* corm 20 and 1 µg/ml aqueous fractions (A and B), the corm 2 and 1 µg/ml 50% ethanol fractions (C

and D), the corm 20 and 1 µg/ml 100% ethanol fractions (E and F), the root 20 µg/ml aqueous fraction (G) and the leaf 20 µg/ml 100% ethanol fraction (I). For *Bulbine frutescens*, *β-actin* gene expression was clear for the root 2 µg/ml aqueous fraction (L), the leaf 20 µg/ml 100% ethanol fraction (M) and the leaf 2 µg/ml 100% ethanol fraction (N). H, J and K bands were not completely clear (Figure 25).

4.3.4. Overview of results

Overview of bax and caspase-3 expression is presented in Tables 6 and 7 below.

Table 6: Overview of *bax* and *caspase-3* expression in HEp-2 cell line by the controls

Control	***bax* gene**	***caspase-3* gene**
200 µM DMSO	-	-
Camptothecin	+	+
Calpain I inhibitor	+	+
Negative RTase	-	-
Negative template	-	-

Table 7: Overview of *bax* and *caspase-3* expression in HEp-2 cell line by *Bulbine* spp. crude fractions (the treatment, PCR as well as gels were performed in triplicate)

***Bulbine* spp. organ**	**Fraction**	**Final concentration in cell volume (µg/ml)**	***bax* gene**		***caspase-3* gene**	
			Bulbine natalensis	*Bulbine frutescens*	*Bulbine natalensis*	*Bulbine frutescens*
corm	aqueous	20	+	n/a	+	n/a
corm	aqueous	1	+	n/a	+	n/a
corm	50% ethanol	20	-	n/a	+	n/a
corm	50% ethanol	1	-	n/a	+	n/a
corm	100% ethanol	20	+	n/a	+	n/a
corm	100% ethanol	1	+	n/a	+	n/a
roots	aqueous	20	-	+	+	-
roots	aqueous	2	-	+	+	-
leaves	100% ethanol	20	-	-	+	+
leaves	100% ethanol	2	+	+	+	+

4.3.5. Statistics Results

For *Bulbine natalenss*, there was a 95% chance that the mean percentage upregulation of *bax* success lied between 12.1% and 76.45%. There was also a 95% chance that the mean percentage upregulation of *caspase-3* success lied between 68.63% and 109.13% (Table 8).

For *B. frutescens*, there was a 95% chance that the mean percentage upregulation of *bax* success lied between 32.86% and 117.14%. There was also a 95% chance that the mean percentage upregulation of *caspase-3* success lied between 1% and 99% (Table 8).

In conclusion, there was an overall 95% chance that the mean percentage upregulation of *bax* success lied between 23.84% and 76.16%, and that there was a 95% chance that the mean percentage of *caspase-3* upregulation success lied between 10.61% and 60.81% (Table 8).

Table 8: Binomial distribution statistics for *bax* and *caspase-3* genes of *Bulbine* spp. crude fractions

Genes	Description	*Bulbine natalensis*	*Bulbine frutescens*	Overall
bax	Percentage downregulated	5/9 (55.55%)	1/4 (25%)	7/14 (50%)
	Percentage upregulated	4/9 (44.44%)	3/4 (75%)	7/14 (50%)
	Estimated Standard deviation	1.47	0.86	1.87
	% upregulation ± Standard Error	± 16.33 SE	± 21.5 SE	± 13.36 SE
	95% confidence limits	± 32.01 CL	± 42.14 CL	± 26.16 CL
caspase-3	Percentage downregulation	1/9 (11.11%)	2/4 (50%)	5/14 (35.71%)
	Percentage upregulation	8/9 (88.88%)	2/4 (50%)	9/14 (64.29%)
	Estimated Standard deviation	0.93	1	1.79
	% upregulation ± Standard Error	± 10.33 SE	± 25 SE	± 12.80 SE
	95% confidence limits	± 20.25 CL	± 49 CL	± 25.1 CL

For the *B. natalensis* corm fractions, there was a 95% chance that the mean percentage upregulation of *bax* success lied between 29.42% and 103.9%. There was also a 95% chance that the mean percentage upregulation of *caspase-3* success lied between 0.00% and 100.0% (Table 9).

For the *B. natalensis* root fractions, there was a 95% chance that the mean percentage upregulation of *bax* success lied at 0.00%. There was also a 95% chance that the mean percentage upregulation of *caspase-3* success lied between 0.00% and 100.0%. In contrast to the *B. natalensis* root fractions, the *B. frutescens* root fractions showed that there was a 95% chance that the mean percentage upregulation of *bax* success lied between 0.00% and 100.0% and that there was a 95% chance that the mean percentage upregulation of *caspase-3* success lied at 0.00% (Table 9).

For the *B. natalensis* leaf fractions, there was a 95% chance that the mean percentage upregulation of *bax* success lied between -19.58% and 119.6%. There was also a 95% chance that the mean percentage upregulation of *caspase-3* success lied between 0.00% and 100.0%. For the *B. frutescens* leaf fractions, there was a 95% chance that the mean percentage upregulation of *bax* success lied between -19.58% and 119.6%. There was a 95% chance that the mean percentage upregulation of *caspase-3* success lies between 0.00% and 100.0% (Table 9).

Overall. there was a 95% chance that the mean percentage upregulation of *bax* success lied between 29.42% and 103.9% and that there was a 95% chance that the mean percentage upregulation of *caspase-3* success lied between 0.00% and 100.0% for the corm samples. For the root fractions, there was a 95% chance that the mean percentage upregulation of *bax* success lied between -48.00% and 148.0%. There was a 95% chance that the mean percentage of *caspase-3* upregulation success lied between -48.00% and 148.0%. The leaf fractions showed a 95% chance that the mean percentage upregulation of *bax* success lied between -48.00% and 148.0% and that there was a 95% chance that the mean percentage of *caspase-3* upregulation success lies between 0.00% and 100.0% (Table 9).

Table 9: Binomial distribution statistics for *bax* and *caspase-3* genes of *Bulbine* spp. organs

Genes	**Description**	***Bulbine natalensis***			***Bulbine frutescens***		**Overall**		
		Corm	Roots	Leaves	Roots	Leaves	Corm	Roots	Leaves
bax	Percentage downregulated	2/6 (33.33%)	2/2 (100.0%)	1/2 (50.00%)	0/2 (0.00%)	1/2 (50.00%	2/6 (33.33%)	2/4 (50.00%	2/4 (50.00%)
	Percentage upregulated	4/6 (66.66%)	0/2 (0.00%)	1/2 (50.00%)	2/2 (100.0%)	1/2 (50.00%)	4/6 (66.66%)	2/4 (50.00%)	2/4 (50.00%)
	Estimated Standard Deviation	1.14	0.00	0.71	0.00	0.71	1.14	1.00	1.00
	% upregulation ± Standard Error	± 19.00 SE	± 0.00 SE	± 35.50 SE	± 0.00 SE	± 35.50 SE	± 19.00 SE	± 50.00 SE	± 50.00 SE
	95% confidence limits	± 37.24 CL	± 0.00 CL	± 69.58 CL	± 0.00 CL	± 69.58 CL	± 37.24 CL	± 98.00 CL	± 98.00 CL
caspase-3	Percentage downregulated	0/6 (0.00%)	0/2 (0.00%)	0/2 (0.00%)	2/2 (100.0%)	0/2 (0.00%)	0/6 (0.00%)	2/4 (50.00%)	0/4 (0.00%)
	Percentage upregulation	6/6 (100.0%)	2/2 (100.0%)	2/2 (100.0%)	0/2 (0.00%)	2/2 (100.0%)	6/6 (100.0%)	2/4 (50.00%)	4/4 (100.0%)
	Estimated Standard Deviation	0.00	0.00	0.00	0.00	0.00	0.00	1.00	0.00
	% upregulation ± Standard Error	± 0.00 SE	± 0.00 SE	± 0.00 SE	± 0.00 SE	± 0.00 SE	± 0.00 SE	± 50.00 SE	± 0.00 SE
	95% confidence Limits	± 0.00 CL	± 0.00 CL	± 0.00 CL	± 0.00 CL	± 0.00 CL	± 0.00 CL	± 98.00 CL	± 0.00 SE

Based on Table 10.1 (next page), there was a 95% chance that the mean percentage upregulation of *bax* success for *B. natalensis* corm lied between 19.42% and 103.9%. There was a 95% chance that the mean percentage upregulation of *caspase-3* for *B. natalensis* corm was 100%

Table 10.1: Binomial distribution statistics, showing the different fractions, for *bax* and *caspase-3* genes of *Bulbine natalensis* corm

Genes	**Description**	***Bulbine natalensis***			
		Corm			
		Overall	Aqueous	50% ethanol	100% ethanol
bax	Percentage downregulated	2/6 (33.33%)	0	2	0
	Percentage upregulated	4/6 (66.66%)	2	0	2
	Estimated Standard Deviation	1.14			
	% upregulation ± Standard Error	± 19.00 SE			
	95% confidence limits	± 37.24 CL			
caspase-3	Percentage downregulated	0/6 (0.00%)	0	0	0
	Percentage upregulation	6/6 (100.0%)	2	2	2
	Estimated Standard Deviation	0.00			
	% upregulation ± Standard Error	± 0.00 SE			
	95% confidence Limits	± 0.00 CL			

Based on Table 10.2 (next page), there was a 95% chance that the mean percentage upregulation of *bax* success for *B. natalensis* and *B. frutescens* leaves lied between -19.58% and 119.58%. There was a 95% chance that the mean percentage upregulation of *caspase-3* for *B. natalensis* and *B. frutescens* leaves was 100%. In contrast, there was a 95% chance that the mean percentage upregulation of *bax* for *B. natalensis* and *B. frutescens* roots was 0.00%. There was also a 95% chance that the mean percentage upregulation of *caspase-3* for *B. natalensis* and *B. frutescens* leaves was 100%.

Table 10.2. Binomial distribution statistics for *bax* and *caspase-3* genes of the 100% ethanol leaf and aqueous root fraction of *Bulbine* spp

Genes	**Description**	Leaves				Roots			
		Bulbine natalensis		*Bulbine frutescens*		*Bulbine natalensis*		*Bulbine frutescens*	
		Overall	100% ethanol	Overall	100% ethanol	Overall	Aqueous	Overall	Aqueous
bax	Percentage downregulated	1/2 (50.00%)	1	1/2 (50.00%)	1	2/2 (100.0%)	2	0/2 (0.00%)	0
	Percentage upregulated	1/2 (50.00%)	1	1/2 (50.00%)	1	0/2 (0.00%)	0	2/2 (100.0%)	2
	Estimated Standard Deviation	0.71		0.71		0.00		0.00	
	% upregulation ± Standard Error	± 35.50 SE		± 35.50 SE		± 0.00 SE		± 0.00 SE	
	95% confidence limits	± 69.58 CL		± 69.58 CL		± 0.00 CL		± 0.00 CL	
caspase-3	Percentage downregulated	0/2 (0.00%)	0	0/2 (0.00%)	0	0/2 (0.00%)	0	2/2 (100.0%)	2
	Percentage upregulation	2/2 (100.0%)	2	2/2 (100.0%)	2	2/2 (100.0%)	2	0/2 (0.00%)	0
	Estimated Standard Deviation	0.00		0.00		0.00		0.00	
	% upregulation ± Standard Error	± 0.00 SE		± 0.00 SE		± 0.00 SE		± 0.00 SE	
	95% confidence Limits	± 0.00 CL		± 0.00 CL		± 0.00 CL		± 0.00 CL	

For the aqueous fractions, there was a 95% chance that the mean percentage upregulation of *bax* success lied between -45.06% and 178.4%. There was also a 95% chance that the mean percentage upregulation of *caspase-3* success lied between -5.06% and 178.4%. In contrast, there was a 95% chance that the mean percentage upregulation of *bax* success lies at 0.00% for the 50% ethanol fractions and that there was a 95% chance that the mean percentage upregulation of *caspase-3* success lied between 0.00% and 100.0% for those fractions. In addition, there was a 95% chance that the mean percentage upregulation of *bax* success lied between -45.06% and 178.4% for the 100% ethanol fractions and that those fractions had a 95% chance that the mean percentage of *caspase-3* upregulation success lied between 0.00% and 100.0% (Table 11, next page).

Table 11: Binomial distribution statistics for *bax* and *caspase-3* genes of the solvent system

Genes	Description	Aqueous	50.00% Ethanol	100.0% Ethanol
bax	Percentage downregulated	2/6 (33.33%)	2/2 (100.0%)	2/6 (33.33%)
	Percentage upregulated	4/6 (66.66%)	0/2 (0.00%)	4/6 (66.66%)
	Estimated Standard deviation	1.14	0.00	1.14
	% upregulation ± Standard Error	± 57.00 SE	± 0.00 SE	± 57.00 SE
	95% confidence limits	± 111.7 CL	± 0.00 CL	± 111.7 CL
caspase-3	Percentage downregulation	2/6 (33.33%)	0/2 (0.00%)	0/6 (0.00%)
	Percentage upregulation	4/6 (66.66%)	2/2 (100.0%)	6/6 (100.0%)
	Estimated Standard deviation	1.14	0.00	0.00
	% upregulation ± Standard Error	± 57.00 SE	± 0.00 SE	± 0.00 SE
	95% confidence limits	± 111.7 CL	± 0.00 CL	± 0.00 CL

4.4. Discussion

bax and *caspase-3* gene expression was studied using specific primers and the reverse transcription polymerase chain reaction procedure. The HEp-2 passage number was three and this was the same passage number that was used in the cytotoxicity. The HEp-2 cell line was treated with the same *Bulbine natalensis* and *Bulbine frutescens* fractions that were administered during the cytotoxicity study. Based on the cytotoxicity results of the 5 and 8 day MTT cytotoxicity assay for both *Bulbine* spp. fractions, and the fact that all 8 day fractions enhanced HEp-2 cell line proliferation, *bax* and *caspase-3* gene analysis was restricted to fractions that induced cell death by the fifth day of incubation. Since this study was focussed on apoptosis and the effect of *Bulbine* spp. extracts/fractions on cancer cell lines, the fractions of *B. frutescens* that were selected for gene expression analysis were those that could be compared to *B. natalensis*, which possessed cell killing potential. *B. frutescens* had no HEp-2 cell killing potential (refer to previous chapter).

The fractions that were selected for *bax* and *caspase-3* expression were the, *B. natalensis* corm 20 and 1 µg/ml aqueous fractions, corm 2 and 1 µg/ml 50% ethanol fractions, corm 20 and 1 µg/ml 100% ethanol fractions, *B. natalensis* root 20 and 2 µg/ml aqueous fractions, *B. natalensis* leaves 20 and 2 µg/ml 100% ethanol fractions, *B. frutescens* root 20 and 2 µg/ml aqueous fractions, and *B. frutescens* leaf 20 and 2 µg/ml 100% ethanol fractions. Overall, there was a greater variability in HEp-2 cell line *bax* gene expression since six out of the fourteen selected fractions did not express *bax*. However, twelve out of those 14 fractions expressed *caspase-3*. At the same time, all samples show positive for β-actin gel products. The HEp-2-treated cell lines produced the same gene expression results in triplicate and it indicated that the method employed to assess *bax* and *caspase-3* gene expression was standardised. Standardisation was confirmed by the total cell count number used in the gene expression study (114000 ± 10583 SD cells/well) which was akin to that of the cytotoxicity study (as indicated in the methodology); and the fact that Pillai *et al*. (2004) used *B-actin* as a correlate to the number of total cell number in their RT-PCR study. The intensity of the *β-actin* control in the present study confirms that the methodology utilised cells which were viable only, and that the method employed was standardised in relation to the fractions used. Furthermore, the fact that certain fractions expressed *bax* and *caspase-3* and that the 2% (200µM) DMSO (negative control) did not express those genes confirmed that the DMSO

concentration that was used to dissolve the extracts were not capable of inducing the expression of the *bax* and *caspase-3* genes. This indicated that the fractions were prepared in the correct concentration of DMSO, which was suitable to study gene expression in the HEp-2 cell line using RT-PCR. Moreover, HEp-2 expressed *bax* and *caspase-3* genes when this cell line was exposed to the controls, camptothecin and calpain I inhibitor, which indicated that apoptosis was capable of being induced in this cell line. Dong *et al.* (1997), Eske *et al.* (1998), Atencio *et al*. (2000) and Pillai *et al*. (2004) found either of or both of these compounds capable of inducing apoptosis in tumour cancer cell lines. Therefore when a fraction resulted in *bax* and *caspase-3* expression, it was postulated that apoptosis was induced in the HEp-2 cell line by that fraction. *B-actin* served as an internal RT-PCR control and its expression confirmed that the HEp-2 cell line was viable at the time of performing the RT-PCR experiment since none of the *B. natalensis* fractions induced 100% cell death. This confirmed that the fractions were hydrophobic and had entered the HEp-2 cell line, either promoting or inhibiting its growth. This was also confirmed in the cytotoxicity experiments in which the *Bulbine* spp. fractions produced positive and negative cytotoxicity results in the MTT cytotoxicity assay, for example most of the corm samples induced HEp-2 cell death, except for the aqueous 20 µg/ml fraction which promoted HEp-2 cell proliferation. All the root fractions of both *Bulbine* spp. induced HEp-2 cell proliferation. The fractions were hydrophobic because the MTT cytotoxicity assay would only measure the toxicity of compounds once it has been taken up by the HEp-2 cell line, resulting in a decline in its mitochondrial transmembrane potential (Häcker, 2000; Holdenrieder and Stieber, 2004). Pillai *et al.* (2004) reported in human lung cancer cell lines that since curcumin induced the expression of *caspase-3* and *bax/bcl-2* in a dose-dependent manner, that the extracts made were hydrophobic. Alternatively, hydrophobicity of the fractions could be that since the fractions contain growth inhibitory and/or stimulatory substances, which were unknown at the time of performing this study, they could have entered the HEp-2 cell line through cell membrane proteins (Becker *et al.*, 2003). This is being in the mind that the cytotoxic / proliferative effect of the fractions were obtained after excluding the effect of DMSO on the HEp-2 cell line. Given the latter statement, the hydrophobicity of the fraction could also be attributed to the fact that DMSO probably imparts some metallic or ionic properties to the fraction and serves as a vehicle or ligand for them to enter into the cytoplasmic regions of the HEp-2 cell line through ion exchange channels on the HEp-2 cell membrane (Mathew *et al*., 2000; Becker *et al*., 2003).

It has been shown in some cell lines that apoptosis by ionising radiation, ultraviolet light, heat shock, kinase inhibition and anticancer drug such as camptothecin and calpain I inhibitor and, is induced through the *bcl-2*-regulated mitochondrial pathway (Strasser *et al*., 1995). *bax*, the death-promoting gene, in addition to *bid* and *p53*, belong to the *bcl-2* gene family and are indicators of apoptosis (Hengartner, 2000). Therefore the *bcl-2* gene possesses anti-apoptotic functions and its decreased expression i.e. upregulation/expression of *bax,* ultimately affects the lifespan of cells (Hengartner, 2000). Calpain I inhibitor, as the name suggests, has some association with calpain gene-encoded proteins, that participate in apoptosis. Calpain proteins within cell lines are associated with the calpain/calpastatin system, which encode structural and regulatory proteins such as *bid*, *bax* and *caspase-3*, however, although the role calpain I inhibitor was to inhibit the expression of the *calpain* gene and thus *bax* and *caspase-3* genes subsequently in the HEp-2 cell line (Altznauer *et al*., 2004; Raynaud *et al*., 2005), calpain I inhibitor induced the expression of *bax* and *caspase-3* genes (and thus *calpain*) even though its cytotoxic effect to this cell line was uncertain. This implied that calpain I inhibitor induces some other apoptotic signalling pathway in the HEp-2 cell line. Alternatively, the expression of *bax* and *caspase-3* genes is not directly associated with cytotoxicity as the information in the following pages. This, as far as the literature is concerned, has not been reported because the physiological role of calpain proteins itself, is poorly understood (Atencio *et al*., 2000; Altznauer *et al.*, 2004; Sareen *et al*., 2007). However, since calpain proteins are present and conserved in almost all mammalian cell lines (such as fibroblasts, neutrophils, tumour cell lines, hepatocellular carcinoma cell lines, colorectal cell lines, Jurkat cells) (Atencio *et al*., 2000; Altznauer *et al*., 2004), studying this enzyme through calpain inhibition is worthwhile because the conserved nature of calpain proteins suggests that this cysteine protease is an important enzyme (Alverna *et al*., 2003). Calpain I inhibitor was selected as an apoptotic/cell cycle regulatory control in this study because it had been believed that taxol was less effective than calpain I inhibitor. Furthermore, the controversies surrounding calpain I inhibitor became clear once the RT-PCR results were obtained using the HEp-2 cell line. One of the major arguments that arose after obtaining this result in this study was to determine the mechanism by which calpain I inhibitor induced apoptosis in the HEp-2 cell line because if the HEp-2 cell line possesses a calpain/calpastatin system, then *bax* and *caspase-3* genes should have not been expressed. In this system, the activation of calpain proteins are dependent on the levels of cytosolic free calcium (Ca^{2+}) levels, which are directly proportional to the sodium ion (Na^{+}) levels in the cytosol of the cell. Ca^{2+} passes through the

Na^+ / Ca^{2+} exchangers that are in the mitochondrial membrane (Häcker, 2000; Van Cruchten and Van den Broeck, 2002; Sareen *et al*., 2007; Jia *et al*., 2009). When the cytosolic Ca^{2+} levels are sufficiently high, caspases become activated which result in the cleavage of calpastatin. This cleavage produces Bax which is unable to interact with anti-apoptotic Bcl-$X_{L,}$ and releases cytochrome c from the mitochondria. Cytochrome c provides the energy for the cleavage of procaspase-3 into caspase-3, resulting in apoptosis via. the mitochondrial or endoplasmic reticulum pathway (Van Cruchten and Van den Broeck, 2002; Puerto *et al*., 2010). It also has recently been found that $S_{mac,}$ a newly discovered pro-apoptotic factor, is released from the mitochondria into the cytosol during the induction of apoptosis (Atencio *et al*., 2000; Nakamura *et al*., 2004). This implies that the levels of calpastatin is regulated by calpain I activity and thus caspases, and that the levels of calpastatin could possibly change within a cell line when exposed to a death stimulus. The calpain I inhibitor, served as an activator of apoptosis instead of an inhibitor, if the explanation above were to hold true for the HEp-2 cell line. However, the Ca^{2+} levels were not determined for the HEp-2 cell line. If the levels of Ca^{2+} were normal or high within the HEp-2 cell line that was assessed for *bax* and *caspase-3* gene expression, then it could be assumed that the calpain I inhibitor reduced the levels of calpastatin as an early event upon apoptosis induction that involved an increase in the overall proteolytic activity that is generally seen during late apoptosis. Conversely, if the levels of Ca^{2+} were low (due to depletion of ER stores, for example), then calpain I inhibitor may have had nothing to do with *bax* and *caspase-3* expression after 5 days of incubation. However, this presumption was not considered by us since viable (trypan blue dye exclusion assay) trypsin-detached, spindle-shaped, HEp-2 cells in fresh CCM were plated out prior to treatment with the controls and *Bulbine* spp. fractions. The fact that the HEp-2 cell line had probably gone through mutational events during subculturing, or were at a different point in the cell cycle when treated with the *Bulbine* spp. fractions, for calpain protein activity was substantiated by the fact that the gene expression results and cytotoxicity results were obtained in triplicate using the same passage number and at different times. Calpain I inhibitor was found to be cytotoxic as well as antiproliferative for the day 5 MTT cytotoxicity assay, thereby making the mechanisms of apoptosis induced by calpain I inhibitor inconclusive. This, as mentioned in the cell culturing and cytotoxicity chapter, was probably because the HEp-2 cell line was not very susceptible to calpain I inhibitor by day 5. However, it was only after 5 days of incubation, that calpain I inhibitor had a cytotoxic effect on the HEp-2 cell line. A 3 day MTT preliminary study involving calpain I inhibitor was

performed in this study as well, and it was found that after three days of incubation, the *Bulbine* spp. fractions as well as the calpain I inhibitor and camptothecin controls were not cytotoxic to the HEp-2 cell line (Figure 18). Furthermore, all the controls were administered at the same concentration to the HEp-2 cell line so as to keep the experiments standardised. It is not possible to measure the cell number after performing the MTT cytotoxicity assay, to get a clear and exact estimate of the cell number at day 5 and day 8 to confirm the response of the different treatment. Using another method, to measure the treatment response, from the review by Singh and Reddy (2012), would not give a true indication of the responses that are obtained using the MTT cytotoxicity assay because of the differences in the sensitivity of the methods. It has been shown in several gene expression and cytotoxicity studies that the results obtained in flow cytometer studies, for example, may not necessarily correlate to the results in an MTT or RT-PCR study because of the sensitivity differences the exist between the methodologies (Singh and Reddy, 2012).

As can be understood from Altznauer *et al.* (2004) and Sareen *et al.* (2007), calpain and caspases work in a cooperative manner to bring about apoptosis. However, there has been reported, contradicting data that suggests uncertainty as to whether they independently act in different apoptotic pathways. For example, in Jurkat and HL-60 cell lines, it has not been understood as to whether apoptosis is induced directly or indirectly by the Fas receptor-mediated death pathway, and since the mitochondria involves *bax* activation for *caspase-3* expression, with *bax* being a target of calpain, it was unclear as to whether Fas receptor-mediated apoptosis in those cells required Bax cleavage (Altznauer *et al*., 2004). Therefore, with the HEp-2 cell line, Jurkat and HL-60 cell lines, the apoptotic mechanism induced or inhibited by calpain I inhibitor still remains unclear. It has also been found in hepatocellular carcinoma cells and colorectal cell lines that calpain I inhibitor did not lead to G2/M phase arrest since the anticancer compound did not have any effect in the proteasome at 1 - 20 µM. However, in the present study 20 µM of calpain I inhibitor was administered to the HEp-2 cell line and it was found to be cytotoxic i.e. cell death was induced by calpain I inhibitor at some point in the eukaryotic cell cycle, and RT-PCR confirmed this to be apoptosis, to some extent (Kubbutat and Vousden, 1997). This suggested that the role of calpain I inhibitor differs between different cell lines and that much needs to known about the involvement of calpain inhibitors in tumour cell death. Therefore, the exact pathway or mechanism of

induction of apoptosis in the HEp-2 cell line by calpain I inhibitor needs to be confirmed by future, further studies.

Camptothecin, the apoptotic control, showed apoptotic potential in the HEp-2 cell line by inducing the expression of *bax* and *caspase-3*. This was expected since camptothecin is a topoisomerase I inhibitor which inhibits DNA and/or RNA synthesis resulting in cell cycle arrest (Hsiang *et al*., 1985; Legarza and Yang, 2006; Li *et al*., 2006). Camptothecin thus inhibited DNA or RNA synthesis by intercalating between the topoisomerase/DNA complex. Hsiang *et al.* (1989) found that when this complex collided with the replication fork during S-phase, double-stranded breaks persisted, which eventually led to cell death. In the present study, the cells were in the exponential phase (and 80 - 90% confluent flasks) prior to plating out for the gene expression study, but as to whether they were all in the S-phase of interphase is unknown and probably impossible to achieve. Nevertheless, apoptosis occurred after 5 days i.e. most of the cells were dead, with DNA repair probably occurring 3 days later, possibly because by 8 days the topoisomerase/DNA complex no longer bonded strongly to the complementary strand of DNA, allowing for non-lethal collisions to occur (Li *et al*., 2006). This result was evidenced by the HEp-2 cell killing potential after five days of incubation in the MTT cytotoxicity assay, and the cell proliferating effect of camptothecin after a further 3 days of incubation. Other reasons have also been reported which are independent of the S-phase mechanisms. These speak about the interruption of transcription and chromosomal aberations in the G2 phase of the cell cycle caused by camptothecin (Hsiang *et al*., 1985 & 1989; Shao *et al*., 1995; Häcker, 2000; Becker *et al*., 2003; Hayward *et al*., 2003; Meza-Lamas *et al*., 2006).

In the HEp-2 cell line, camptothecin initiated Fas L/Fas-dependent apoptosis, by inducing the expression of Fas/CD95 receptors (Becker *et al.,* 2003) on their surfaces upon treatment and incubation at 37^{o}C for 5 days. Once the FasL domain of camptothecin bonded to the trimeric binding site of Fas on the surface of the HEp-2 cell line, spontaneous cell death occurred i.e. apoptosis. This was concluded from studies conducted involving HEp-2 and camptothecin, which showed that pro-apoptotic genes *FasL*, *Fas/CD95*, *caspase-3* and *DFF40* are overdriven by camptothecin treatment (Meza-Lamas *et al*., 2006). In lymphocytes, on the other hand, the shortened RNA chains produced by the inhibition of RNA synthesis, are able to be reversed by camptothecin removal, while shortened DNA chains are irreversible. In the

HEp-2 cell line, this has been found to be the opposite, however, camptothecin is unable to cleave purified DNA (Hsiang *et al*., 1985; Meza-Lamas *et al*., 2006). DNA fragmentation as well as nucleic acid synthesis inhibition, induced by camptothecin is currently a popular field of research since the cellular targets of camptothecin are still unknown (Legarza and Yang, 2006; Li *et al*., 2006). Camptothecin induced apoptosis in HEp-2 by recruiting adaptor FADD proteins to the CD95/Fas receptor (Becker *et al.,* 2003). This caused procaspase-8 (precursor to caspase) to assemble at the site of recruitment where it first cleaved itself into caspase-8 and then caspase-3. *caspase-3* expression indicated apoptosis (Shiokawa *et al*., 1997) while *caspase-8* (not studied), activated death promoting proteins like Bid, which belongs to the Bcl-2 family (Hengartner, 2000) and brought about apoptosis. However, it is important to note from the results obtained that the CD95/Fas receptor pathway induced by campothecin, is not the only mechanism induced because this mechanism also reverts back to the mitochondria, and therefore the mitochondrial pathway is also involved (Marzo *et al*., 1998; Hengartner, 2000; Singh, Rishan, personal deduction, 2012). This was evidenced by the expression of *bax* and also implied proper regulation of the cell cycle by *p53*, which is an activated gene in the apoptotic pathway that is situated before *bax* and *bcl-2*. Therefore, in the HEp-2 cell line, cytochrome c release from the mitochondria was also expected to be released so as to drive apoptosis to completion, and furthermore, *caspase-3* expression is also the result of the formation of an apoptosis protease activating factor (Apaf-1) (Li *et al*., 1997) and its association with procaspase-9 (because of cytochrome c release). This is because procaspase-9 in the apoptosome becomes cleaved into caspase-9 (Hengartner, 2000), which results in the cleavage of procaspase-3 into caspase-3.

Although HEp-2 DNA was cleaved and resulted in apoptosis after 5 days of performing cytotoxicity tests, it is uncertain if *bax* and *caspase-3* genes would have been expressed after a further 3 days of performing camptothecin cytotoxicity tests because those percentage cytotoxicity results appeared to have a proliferative effect on the HEp-2 cell line. The above deduction could be considered correct because any single-stranded breaks that may have occurred upon camptothecin treatment, had to have been irreversible since the incubation temperature had not been lowered from $37^{o}C$ to $0^{o}C$, at intervals, after each day of incubation, as suggested by several authors (Meza-Lama *et al*., 2006; Legarza and Yang, 2006; Li *et al*., 2006). This implied that when the HEp-2 cell line was exposed to camptothecin, after the fifth day, DNA polymerase I no longer was covalently linked to the 3′

end of the drug-induced DNA strand break, thereby preventing its replication. Since camptothecin behaves in the manner of an uncompetitive inhibitor, its role in destabilising the topoisomerase/DNA complex is well understood (Li *et al*., 2006). Camptothecin has also been shown to inhibit the replication of two DNA viruses, adenoviruses and vaccinia virus, within HeLa cells and this is a reason why camptothecin was used as an anticancer agent; another being that treatment with camptothecin leads to more tolerable side effects and a persistence of DNA breaks in cancer cells, which might increase the chances of killing them via. FasL/Fas / Fas/CD95, mitochondrial, endoplasmic reticulum etc. mechanisms of apoptosis (Balasubashini *et al*., 2006; Meza-Lamas *et al*., 2006).

Apoptosis might have been induced in the HEp-2 cell line by the *B. natalensis* corm aqueous and 100% ethanol fractions, as well as the *B. natalensis* leaf 20 µg/ml and *B. frutescens* leaf 2 µg/ml 100% ethanol fractions. This was deduced since these fractions expressed *bax* and *caspase-3* genes. Although the two *B. frutescens* leaf fractions did not induce *caspase-3* gene expression, *bax* was expressed and this suggested that the compounds present in those 100% ethanol fractions are not involved in triggering apoptosis in HEp-2, but are involved in some other form of cell death. In contrast, there were also fractions that induced *caspase-3* gene expression in HEp-2, but not *bax.* Those fractions were five *B. natalensis* and one *B. frutescens* fractions. These were: corm *B. natalensis* 2 and 1 µg/ml 50% ethanol fractions, root *B. natalensis* 20 and 2 µg/ml aqueous fractions, and the leaf *B. natalensis* and *B. frutescens* 20 µg/ml 100% ethanol fractions. Surprisingly the leaf *B. frutescens* and *B. natalensis* 20 µg/ml 100% ethanol fraction did not express *bax* while the 2 µg/ml fraction expressed *bax.* This created uncertainty as to whether the compounds in this fraction would induce the same mechanism of apoptosis when administered to HEp-2 cell line at different concentrations because *bax* was expressed at a lower concentration of the fraction administered. Thus if expression of the studied apoptotic markers are concentration independent of the fraction administered in RT-PCR, then there should either be expression of *bax* for both fractions or no expression at all since the compounds in both fractions were the same, but only differed in the final concentration. However, comparing the two fractions with each other suggested that the compounds in those fractions induce apoptosis by the same mechanism. This could have been due to the presence of the same or similar compounds in those fractions, especially since both species belong to the same genus and family, but this needs to be phytochemically confirmed. Binomial distribution statistics for the *bax* and

caspase-3 apoptotic markers of *Bulbine* spp. crude fractions proved that the percentage upregulation of the *bax* gene for *B. natalensis* was 44.44% (4/9 fractions) ± 16.33SE, while for *B. frutescens* percentage upregulation was 75% (3/4 fractions) ± 21.5SE. Similarly the *caspase-3* gene upregulation of *B. natalensis* was 88.88% (8/9 fractions) ± 10.33SE, while for *B. frutescens* it was 50% (4/4 fractions) ± 25SE (Table 8).

It was observed that the root *B. natalensis* and *B. frutescens* 20 and 2 µg/ml aqueous fractions caused the HEp-2 cell line to behave differently. For the *B. natalensis* tested root fractions, apoptosis occurred and this was evidenced by *caspase-3* gene expression since it is a molecular indicator of apoptosis (Becker *et al*., 2003). There have been many studies conducted on apoptosis, but non of these conclude apoptosis strictly on *caspase-3* expression or any other method, even when other methods like flow cytometry and immunohistochemistry are being performed in conjunction with RT-PCR studies (Reimertz *et al*., 2003; Balasubashini *et al*., 2006; Amaral *et al.,* 2009). However, for the *B. frutescens* tested fractions *caspase-3* was not expressed and this indicated that apoptosis is not induced in HEp-2 by this fraction. The expression of *bax* by this fraction questioned as to whether it could actually induce HEp-2 cell death because *bax* gene expression is considered central to three pathways of apoptosis i.e. the receptor-ligand pathway (through activation of Bid/death promoting proteins), mitochondrial pathways and endoplasmic reticulum pathways. Further misunderstandings occurred because all of the *B. frutescens* fractions had no cytotoxic potential against the HEp-2 cell line.

Overall the results showed that there was a possibility that *Bulbine* spp. fractions could induce apoptosis in HEp-2 cell lines, and that the type of cell death mechanism activated depends on the compounds present within the fractions for the fractions that were cytotoxic. The type of compounds present differed between the different solvents used during the extraction procedure. From this study, it was inconclusive as to whether apoptosis was induced in the HEp-2 cell line by the fraction in a dose-and-time dependent manner because of the variation in *bax* gene expression, in particular. Apoptosis is characterised by the activation of caspases, and it requires the activation of *caspase-3*, the cleavage of Bid, as well as the downregulation of *bcl-2* gene expression (Cregan *et al*., 1999; Van Cruchten and Van den Broeck, 2002; Becker *et al*., 2003; Holdenrieder and Stieber, 2004; Nakamura *et al*., 2004; Pillai *et al*., 2004; Puerto *et al*., 2010). The *B. natalensis* 2 µg/ml 100% ethanol leaf

fraction, the aqueous as well as the 100% ethanol corm fractions were prpbably the best representation of apoptosis in this study, because activation of the *bax* gene orchestrated apoptosis through *p53* regulation of the HEp-2 cell cycle, and the upregulation of the *caspase-3* gene of the ER or mitochondrial pathway (this deduction being made exclusively on the basis of *bax* and *caspase-3* expression induced by the mentioned fractions). The activation of *bax* and *caspase-3* genes in apoptosis are mediated through the regulation of *p53* in cancer cell lines (Hengartner, 2000; Amaral *et al*., 2009). *p53* induction was evidenced by the β-actin expression for those fractions because β-actin is only expressed in viable cells and the genetic machinery in those cells are mediated by *p53*. When binomial distribution statistics was performed (Table 9) for *bax* and c*aspase-3* apoptotic markers for each *Bulbine* spp. organ, it was found that the standard error of the corm fractions for *bax* gene upregulation was the lowest i.e. 66.66% (4/6 fractions of which two were fractions made using water and the other two 100% ethanol) ± 19.00%, with that of the leaves having a higher standard error of 50.00% (1/2 fractions) ± 35.50SE. This SE was equivalent to the tested *B. frutescens* leaf fractions. There was also a 0.00% SE for all organ fractions of *B. natalensis* assessed for *caspase-3* induction since the percentage upregulation was 100%. It is possible to provide the percentage upregulation and downregulation binomial statistic information given the studied method, but like in all scientific studies, a larger data set would obviously provide a set of results with a greater reliability and which would be representative of the sample size studied. The reported results are thus valid, but further studies could be used to improve the data set. In terms of the six corm fractions of *B. natalensis* that showed an upregulation for *caspase-3,* it was found that 2 were prepared using water, another two using 50% ethanol and the last two was made using 100% ethanol. However, the six corm fractions had the same percentage upregulation as was for all organ fractions of this species assessed for c*aspase-3* expression. However for *B. frutescens* only the leaf fractions had a SE of 0.00% with the percentage upregulation being 100%. The root fractions for this species did not express *caspase-3*.

Although the corm fractions were a good representation of mitochondrial and ER mechanisms of apoptosis, it did not necessarily eliminate the receptor-ligand mechanism because some compounds in those fractions may have simultaneously induced that mechanism of apoptosis, which also upregulates the *caspase-3* gene. More conclusively, the receptor-ligand mechanism was observed for the *B. natalensis* corm 50% ethanol fractions,

aqueous root fractions, 20 µg/ml 100% ethanol leaf fraction and the *B. frutescens* 2 µg/ml 100% ethanol leaf fraction due to the absence of *bax* activation in those samples. The difference in the mechanism of apoptosis between the corm 50 and 100% ethanol fractions, suggested not only that they contain compounds that activate different apoptotic pathways, but also that if they contain the same compounds, then those compounds are in different proportions in those fractions that brought about the *caspase-3* gene expression result obtained.

It has been suggested that compounds causing ER stress could also possibly activate the mitochondrial pathway of apoptosis (Häcki *et al*., 2000; Annis *et al*., 2001; Wei *et al*., 2001). However, the effect of the *Bulbine* spp. fractions that induced HEp-2 cell death in this study, via. *bax* and *procaspase-3* activation (the latter of which is confirmed by *caspase-3* expression), had not been screened for their effect on the endoplasmic reticulum, and furthermore, it is uncertain as to whether the expressed *bax* and *caspase-3* apoptotic markers is an accurate indication of apoptosis by the mitochondrial pathway. This is because the mitochondria has also been discovered to act as an orchestrator of apoptotic and autophagy activation, which are integrated events and depend on the collapse of mitochondrial transmembrane potential (Van Cruchten and Van den Broeck, 2002; Sareen *et al*., 2007; Jia *et al*., 2009). Autophagy, by definition, refers to the intracellular digestion of old or unwanted organelles or other cell structures as it occurs within autophagic lysosomes and it is also another form of cell death (Becker *et al*., 2003). There is a possibility that when the HEp-2 cell line was exposed to *Bulbine* spp. fractions that expressed *bax* and *caspase-3*, that both autophagic and apoptotic signals converged at the surface of the mitochondrial membrane that provoked a mitochondrial permeability transition resulting in cytochrome c release into the cytosol, thereby triggering caspase-dependent apoptosis or autophagy (Jia *et al*., 2009). The activation of both types of cell death could have occurred at the mitochondrial surface because the fractions that were administered to the HEp-2 cell line were hydrophobic and interefered with the permeability of the mitochondrial/cell membrane (the reason for the hydrophobicity of the studied *Bulbine* spp. fractions and controls have previously been discussed). The integration of autophagy and apoptosis to bring about cell death is still a new event and further investigations are required using known compounds to study this further. However, autophagy as well as apoptosis are significant to report in *Bulbine* spp.–induced death of the HEp-2 cell line, as well as to note that autophagy probably even lends itself to

promote apoptosis in the HEp-2 cell line. However, in order for the mitochondrial apoptotic pathway to be activated by ER stress, there are two other genes that must be targeted by *p53*. These genes are *bbc3/PUMA* which has been found to be expressed in SH-SY5Y neuroblastoma cells (Reimertz *et al*., 2003). Han *et al.* (2001) found that *bbc3/PUMA* has a role in *p53*-independent regulation. Luetjens *et al.* (2001) found that *bbc3/PUMA* expression in SH-Neo and SH-Bcl-xL cells were upregulated when exposed to tunicamycin. Similarly, those genes were upregulated after an exposure to thapsigarin, which induces ER stress by depleting ER Ca^{2+} stores. In general, p53 also mediates cell death through the transcriptional induction of the *bax* gene in neuron cells. Miyashita *et al.* (1994) and Miyashita and Reed (1995) suggested that this was due to the *bax* gene possessing *p53*-responsive sequences, and that *bax* induction has been observed during *p53*-mediated cell death in certain cell systems like the HEp-2 cancer cell line (Selvakumaran et *al*., 1994; Zhan *et al*., 1994; Brady et *al*., 1996; Atencio *et al*., 2000).

Assuming that the *Bulbine* spp. fractions induced ER stress or *caspase-3* gene expression within the HEp-2 cell line, then unfolded and malfolded proteins possibly existed within the lumen of the ER (Kaufman, 1999), which resulted in the activation of a controlled transcriptional response or unfolded protein response (UPR) (Perez-Sala and Mollinedo, 1995). This has yet to be suggested. Molecular chaperones contain the target genes of the UPR and they help to alleviate ER stress by inhibiting the aggregation of abberant proteins. For these fractions, ER stress alleviation was probably not possible that it induced a caspase-dependent death programme. Nakagawa *et al.* (2000) found that ER stress in murine cells, activate *caspase-12*, that subsequently activates executioner caspases such as *caspase-3*. An exception, in the present study were the *B. frutescens* aqueous root fractions, which failed to express the *caspase-3* gene. This could have been due to the HEp-2 cell line having the ability to reverse cell death by repairing itself after being subjected to the apoptotic stimuli, hence only *bax* was expressed. This means that the apoptotic effect of the fraction administered was not strong enough to complete the apoptotic process such that *p53* favoured the repair process. Another reason could be that this fraction does not promote sufficient release of cytochrome c from the mitochondria into the cytosol to drive the process of apoptosis to completion via. any one of the three pathways causing the HEp-2 cell line to proliferate through the process of reverse apoptosis. Another possibility is that because the decision made by a cell to grow or die is controlled by *bcl-2* which prolongs cell death

through apoptosis because of it being a proto-oncogene, there could be a possibility that although *bax* was expressed in RT-PCR for that fraction, the *bcl-2* anti-apoptotic gene could have been expressed to a much greater degree like in the study performed by Pillai *et al.* (2004). However, this protective action of *bcl-2* is still unclear and it has been discovered that in colorectal cancer cells the *bcl-2* gene up-regulation suppresses *p53*-dependent apoptosis (Atencio *et al*., 2000). The effect of the *B. frutescens* aqueous root fractions on the HEp-2 cell line also highlighted the importance of the 15 bcl-2 family members in mammalian cells by highlighting their role as heterodimers. These 15 bcl-2 family members function as either pro-apoptotic members (Bax, Bak, Bad) or anti-apoptotic (Bcl-2, Bcl-X_L) regulators that titrate each other's function (functions defined previously by Hengartner, 2000). Therefore although all of the *Bulbine* spp. fractions possibly induce homodimerisation or heterodimerisation within the HEp-2 cell line, the *B. frutescens* aqueous root fractions confirms this possibility.

Reimertz *et al.* (2003) reported that ER stress may result in a highly ordered gene regulation process that began with an up-regulation of BiP, GRP24 (molecular chaperones) to keep nonglycosylated proteins in a folding-competent state, and ended with an improvement of the secretory pathway. These authors further stated that when stress within the ER persisted, the endoplasmic reticulum apoptotic death pathway was activated so as to target unfolded proteins to degradation via. the proteasome. Reimertz *et al.* (2003) provided reasons as to what type of response cell lines can exhibit when exposed to stimuli that cause ER stress. They suggested that prolonged ER stress can result in either an adaptive response that promotes cell survival or the induction of apoptotic cell death. Reverse apoptosis in the HEp-2 cell line exposed to *B. frutescens* aqueous root fractions, could have been to an adaptive response by the cell line. This highlighted the selective preference of cell lines to certain plant fractions and not others.

In future more apoptotic markers need to be assessed in addition to the *FADD, FasL/Fas, p53, bbc3/PUMA, bcl-2,* and procaspase genes (Han *et al*., 2001; Nakano and Vousden, 2001; Yu *et al*., 2001). Furthermore, although binomial statistical tests were performed using the gene expression results, it was unclear whether it was really relevant to report *bax* and *caspase-3* expression results per solvent system and organ fraction for each *Bulbine* spp. This was because even if similarities did persist in the gene expression results amongst different

organ fractions for the same solvent system, or same organ fractions between the two *Bulbine* spp. for the same solvent system (e.g. the tested *B. natalensis* and *B. frutescens* root aqueous fractions and *B. natalensis* and *B. frutescens* 100% ethanol leaf fractions), the fact that the same compounds are probably bringing about the cell death event will remain unknown phytochemically. Furthermore, depending on the point at which the HEp-2 cell cycle (interphase: G1, S1, G2, M, prophase, metaphase, anaphase, telophase and cytokinesis) was at the time of *Bulbine* spp. fraction exposure, the possibility of obtaining an accurate perspective with regard to the *bax* and c*aspase-3* gene expression results in HEp-2 cell line was difficult. This is logical because the HEp-2 cell line exposed to *Bulbine* spp. apoptotic stimuli are also possibly prone to mutational events (point mutation, DNA rearrangement, gene amplification and chromosomal translocation), replication and transcriptional error events (Becker *et al*., 2003) that are probably similar or different between the tested *Bulbine* spp. organ fractions. What was reported from previous herbal and traditional medicinal studies, was that the leaf of *B. frutscens* possessed anticancer potential which was consistent with literature that stated that the anticancer potential of *B. natalensis* was found in the roots while in *B. frutescens*, this potential was in the leaves. Although it was found that a somewhat similar effect was observed with the *B. natalensis* and *B. frutescens* root aqueous fractions and the *B. natalensis* and *B. frutescens* leaf 100% ethanol fractions on the HEp-2 cell line, it was unable to be concluded as to whether this result was correct because traditional reports lack scientific clarification.

Ultimately, the position of the HEp-2 cell cycle, whether cells are exposed to camptothecin, calpain I inhibitor or *Bulbine* spp. fractions, determines cellular responses to the administered compound. For example, in a study the accumulation of aphidicolin-dependent expression of *p53* in the nucleus and induction of *bax* was seen in S-phase cells, while aphidicolin independent *p53* expression and induction of *p21* was seen in G1-phase cells (Becker *et al*., 2003). Probably this may lead to the understanding about why some cells are responsive to some compounds while others appear to be resistant and/or repair DNA damage. More studies need to be performed to determine whether homodimerisation or heterodimerisation of pro-apoptotic and anti-apoptotic genes in favour of apoptosis (Pillai *et al*., 2004) is one of the suggested mechanisms of apoptosis in the HEp-2 cell line by *Bulbine* spp. fractions or its compounds.

CHAPTER FIVE

General Discussion

Apoptosis, or programmed cell death, occurs during normal development as a highly regulated process of cell suicide, which disturbs the physiology and metabolism of the cell (Ellis *et al*., 1991; Vaux and Strasser, 1996; White, 1996), but can be studied *in vitro* (Singh and Reddy, 2012). This process is triggered by a family of aspartate-specific cysteinyl proteases (cysteine aspartate, or caspases), which become activated by proteolytic cleavage during cell death (Alnemri *et al*., 1996; Nicholson and Thornberry, 1997; Salvesen and Dixit, 1997; Cryns and Yuan, 1998; Green, 1998; Thornberry and Lazebnik, 1998). In general, the process involves caspase-3 processing (Nicholson and Thornberry, 1997; Villa *et al.,* 1997; Salvesen and Dixit, 1997) and is controlled by a *p53* suppressor gene which activates other genes by acting as a transcriptional factor (Culotta and Koshland, 1993; Hengartner, 2000). The other genes activated are involved in cell-cycle regulation, the induction of apoptosis and senescence (Reimertz *et al*., 2003). Caspase-3 is the molecular indicator of apoptosis (Ellis *et al*., 1991; Hengartner, 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, Kasumbwe and Reddy (2010) found that the ethanolic extracts of *Bulbine natalensis* showed promise for anticancer potential. In the present study, the final concentration of fractions used for the cytotoxicity and gene expression study, for each organ of each *Bulbine* spp. were 20, 2 and 1 µg/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.

mRNA, unlike DNA, is a single-stranded nucleic acid molecule and it is short-lived. In this study, there was no initial isolation of mRNA (which was an advantage) becaue the Cells-to-cDNA^{TM} II kit isolated mRNA, and cDNA amplicons were generated immediately, followed by storage at -20^{o}C in cryotubes. The methodology was similar to Pillai *et al.* (2004). The HEp-2 cells that were used in the gene expression and cytotoxicity studies were viable and

this was confirmed using the cell counting method that was developed by Freshney (1987), in which only healthy 'glowing' cells are counted. Therefore, the RNA that was isolated to determine apoptosis through *caspase-3* induction, were from HEp-2 cells that were killed (or stimulated) by exposure to the *Bulbine* spp. fractions. Furthermore, HEp-2 cells do not undergo autocrine apoptosis (Chatterjee *et al*., 2001).

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 (Häcker, 2000; Holdenrieder and Stieber, 2004; Sareen *et al*., 2007; Jia *et al*., 2009; Singh and Reddy, 2012). In this study, the *Bulbine* spp. fractions promoted HEp-2 cell line growth as well as cell death through apoptosis and 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, 1983; Reddy *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, 1983; Reddy *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 (Kerr and Harmon, 1991; Singh and Reddy, 2012).

The HEp-2 cells were identical, but when they were treated with the same fraction, some cells died whereas others proliferated. This indicated that the death of the HEp-2 cell line was concentration independent. For example, the corm 20 µg/ml aqueous fraction had a proliferative effect on the HEp-2 cell line after 5 days of the MTT assay, whereas the 2 and 1

µg/ml fraction were cytotoxic. This result also indicated that the HEp-2 cell line may preferentially select fractions for utilisation at different rates. Depending on the constituents of the fraction, it could have been that the 20 µg/ml fraction contained a higher concentration of growth stimulatory substances, resulting in the HEp-2 proliferative response. The fact that the 1 µg/ml fraction had a greater cytotoxic effect on the HEp-2 cell line compared to the 2 µ/ml fraction, indicated that the concentration of growth stimulatory substances in the 20 µg/ml fraction was higher and that once subsequent dilutions were made (2 and 1 µg/ml fractions), the proliferative effect became diluted, resulting in HEp-2 cell death. It can also be speculated that if the cytotoxicity result was not due to the concentration of growth stimulatory compounds in the 20 µg/ml fraction, then it is possible that when this fraction is too concentrated (with cell killing phytochemicals), they have a proliferative effect on the cell line. Therefore, the response of the HEp-2 cell line to *Bulbine* spp. fractions depend on the proportion of growth inhibitory/stimulatory substances in the fraction. This is a reason why the HEp-2 cell line is able to die at a higher cytotoxicity level for a lower *Bulbine* spp. fraction exposure concentration and vice versa (for some fractions).

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, 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 (Cregan *et al*., 1999; Van Cruchten and Van den Broeck, 2002; Häcker, 2000; Nakamura *et al*., 2004; Puerto *et al*., 2010; Singh and Reddy, 2012). 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 and Koshland, 1993; Singh and Reddy, 2012). 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 mechanisms 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 *et al*., 1997; Green and Kroemer, 1998; Marzo *et al*., 1998; Hengartner, 2000). 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 *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, 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 *et al*., 2009).

It was important to note 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 they 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 results), 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 and Yenesew, 1994).

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 8th 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 and Yenesew, 1994) and this was confirmed by other scientists who have performed RT-PCR experiments (Culotta and Koshland, 1993). 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 was much smaller on the HEp-2 cell line compared to the corm fraction which was 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 8th 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 5th and 8th 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 that 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 *et al*., 2004), breast (Sareen *et al*., 2007) and hepatocellular carcinoma cells (Atencio *et al*., 2000). Calculation of the non-parametric ANOVA and H statistic (Appendix 1) 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 8th 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 fraction 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 *et al*., 2009) or the receptor-ligand mechanism that activates death promoting proteins other than Bax (Culotta and Koshland, 1993). 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 those 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 which had 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^{TM} II 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 fraction 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 is 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 their 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 of *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 to understand more clearly why some fractions are capable of expressing *bax* and not *caspase-3* (for example, the *B. frutescens* root aqueous fractions). Furthermore, procaspase genes also have to be analysed since genes are expressed to form procaspases, which them form active caspases.

Limitations of this study, is that this was 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 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 *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 *et al*., 2009) while the K562 cell line is *p53* deficient (Pillai *et al*., 2004). The locality and geographical region from which the plants are taken as well as the climate and time of day that they are uprooted, also has an affect on the type of compounds that plants of the same species or genus possess (Van Wyk, 1997; Wink, 1999). 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 and Salmon, 2001) can also result in different responses of cell lines to different apoptotic stimuli.

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 to the cytotoxic fractions of *B. natalensis.* Normal cells also express caspase genes (see articles by Weinmann *et al*., 1999; Singh and Reddy, 2012) and although this study does not represent a case of no expression vs. expression of caspases in HEp-2, its validity is confirmed by similar experimental designs that assayed the cytotoxicity of plant-derived or natural compounds on cancer cell lines only, and the detection of apoptosis through *caspase-3* induction and other unrelated methods (Cregan *et al*., 1999; Wu *et al*., 2002; Nakamura *et al*., 2004; Padma *et al*., 2007; Sareen *et al*., 2007; Bornschein *et al*., 2008; Liao *et al*., 2008; Mehdi *et al*., 2011; Ive *et al*., 2012). Such scientific studies have possible implications for the chemopreventative activities (current drug targets and modes of actions) of plant compounds, and provides an idea about the behaviour of infected cell lines on exposure to such compounds, as initial studies, without having any intention of commercialising or manufacturing them in the form of modalities. Sensibly, following these initial studies, the introduction of normal cell lines into experimental designs, requires that the administered compound be maintained at effective doses against infected (cancer) cells, while their effect against normal cells should be minimised. This is a challenge to achieve by scientists.

In this study, it was found that the relationship between the cytotoxicity results and apoptotic biomarkers were inconclusive because not all of the fractions that expressed *caspase-3* and/or *bax* apoptotic markers were cytotoxic. Susuki *et al.* (2005) found that the anthracycline antibiotic, doxorubicin, induced apoptotic markers *caspase-3, 8* and *9* in HSC-2 cells, but did not induce DNA fragmentation – a hallmark in apoptosis research. The authors suggested that the induction of internucleosomal DNA fragmentation required the activation of caspases over a certain threshold level and that the induction of apoptosis by doxorubicin may be incomplete because they had not observed any changes in the intracellular concentration of Bcl-2, Bax and Bad proteins. Similarly, the induction of apoptosis in the HEp-2 cell line by *Bulbine* spp. fractions could have been over a certain threshold for some fractions which were not cytotoxic. For example the leaf 20 and 2 µg/ml 100% ethanol fractions of *Bulbine frutescens* induced *caspase-3* expression in HEp-2, but these fractions were not cytotoxic and induced HEp-2 cell proliferation as evidenced by the cytotoxicity results.

This is the first study conducted to interpret and make conclusions on the link between cytotoxicity and expression of *bax* and *caspase-3* genes in apoptotic studies. It allows for the analyses of similar links between the cytotoxicity and expression of other genes in the apoptotic pathways of other cell lines in an attempt of determining possible drug targets as well as in assaying the therapeutic value of plants.

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APPENDIX 1

Cell Culture and Cytotoxicity

Bulbine natalensis 3 day MTT results in triplicate

Table 1A: Mitochondrial activities (absorbance) of aqueous and organic ***Bulbine natalensis*** crude fractions on HEp-2 cell line read at 450 nm after **3 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
Bulbine natalensis roots	aqueous	20	1.138	1.138	1.135	1.137 ± 0.002	-0.088
		2	1.139	1.139	1.136	1.138 ± 0.002	-0.176
		1	1.138	1.138	1.136	1.137 ± 0.001	-0.088
Corm	aqueous	20	1.137	1.139	1.136	1.137 ± 0.002	-0.088
		2	1.138	1.138	1.136	1.137 ± 0.001	-0.088
		1	1.137	1.138	1.137	1.137 ± 0.001	-0.088
	50% EtOH	20	1.138	1.140	1.138	1.319 ± 0.001	-0.176
		2	1.141	1.138	1.137	1.139 ± 0.002	-0.176
		1	1.137	1.139	1.137	1.138 ± 0.001	-0.176
	100% EtOH	20	1.139	1.138	1.135	1.137 ± 0.002	-0.088
		2	1.141	1.139	1.135	1.138 ± 0.003	-0.176
		1	1.139	1.139	1.135	1.136 ± 0.002	-0.260
Leaves	aqueous	20	1.137	1.138	1.137	1.137 ± 0.001	-0.088
		2	1.137	1.138	1.137	1.137 ± 0.001	-0.088
		1	1.138	1.139	1.136	1.138 ± 0.002	-0.176
	50% EtOH	20	1.137	1.139	1.136	1.137 ± 0.002	-0.264
		2	1.138	1.139	1.138	1.138 ± 0.001	-0.176
		1	1.137	1.138	1.137	1.137 ± 0.001	-0.264
	100% EtOH	20	1.138	1.140	1.138	1.139 ± 0.001	-0.088
		2	1.138	1.138	1.136	1.137 ± 0.001	-0.176
		1	1.141	1.138	1.137	1.139 ± 0.002	-0.176

untreated HEp-2 cell line	n/a	n/a	1.115	1.101	1.112	1.109 ± 0.007	n/a
200 µM DMSO	n/a	20 (µl/ml)	1.136	1.137	1.134	1.135 ± 0.002	n/a
camptothecin	n/a	20	1.139	1.138	1.135	1.137 ± 0.002	-0.088
calpain I inhibitor	n/a	20	1.138	1.138	1.136	1.137 ± 0.001	-0.088

Table 1B: Mitochondrial activities (absorbance) of aqueous and organic ***Bulbine natalensis*** crude fractions on HEp-2 cell line read at 450 nm after **3 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
Bulbine natalensis roots	aqueous	20	1.135	1.135	1.134	1.135 ± 0.001	0
		2	1.136	1.136	1.135	1.136 ± 0.001	-0.088
		1	1.136	1.136	1.137	1.242 ± 0.005	-0.088
Corm	aqueous	20	1.136	1.135	1.134	1.135 ± 0.001	0
		2	1.136	1.136	1.135	1.136 ± 0.001	-0.088
		1	1.137	1.135	1.136	0.963 ± 0.004	-0.088
	50% EtOH	20	1.138	1.138	1.136	1.137 ± 0.001	-0.176
		2	1.137	1.137	1.136	1.137 ± 0.001	-0.176
		1	1.137	1.137	1.137	1.137 ± 0.000	-0.176
	100% EtOH	20	1.135	1.136	1.136	1.136 ± 0.001	-0.088
		2	1.135	1.135	1.134	1.135 ± 0.001	0
		1	1.135	1.135	1.136	1.135 ± 0.001	0
Leaves	aqueous	20	1.135	1.134	1.134	1.134 ± 0.001	0.088
		2	1.137	1.137	1.136	1.137 ± 0.001	-0.176
		1	1.137	1.137	1.135	1.136 ± 0.001	-0.088
	50% EtOH	20	1.136	1.137	1.138	1.137 ± 0.001	-0.176
		2	1.138	1.137	1.137	1.137 ± 0.001	-0.176
		1	1.137	1.136	1.137	1.137 ± 0.001	-0.176
	100% EtOH	20	1.138	1.137	1.137	1.137 ± 0.001	-0.176

		2	1.136	1.137	1.137	1.137 ± 0.001	-0.176
		1	1.137	1.136	1.136	1.136 ± 0.001	-0.088
untreated HEp-2 cell line	n/a	n/a	1.111	1.109	1.109	1.110 ± 0.001	n/a
200 µM DMSO	n/a	20 (µl/ml)	1.135	1.134	1.136	1.135 ± 0.001	n/a
Camptothecin	n/a	20	1.135	1.137	1.134	1.135 ± 0.002	0
calpain I inhibitor	n/a	20	1.135	1.132	1.137	1.135 ± 0.003	0

Table 1C: Mitochondrial activities (absorbance) of ***Bulbine natalensis*** crude fractions on HEp-2 cell line read at 450 nm after **3 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
Bulbine natalensis roots	aqueous	20	1.138	1.138	1.136	1.137 ± 0.001	0
		2	1.184	1.187	1.183	1.185 ± 0.002	-0.176
		1	1.138	1.138	1.136	1.137 ± 0.001	0
Corm	aqueous	20	1.139	1.139	1.138	1.137 ± 0.001	-0.176
		2	0.952	0.954	0.951	0.952 ± 0.002	0.088
		1	0.963	0.967	0.965	0.965 ± 0.002	-0.088
	50% EtOH	20	1.140	1.138	1.139	1.139 ± 0.001	-0.176
		2	1.138	1.138	1.137	1.138 ± 0.001	-0.088
		1	1.137	1.137	1.136	1.137 ± 0.001	0
	100% EtOH	20	1.138	1.137	1.140	1.139 ± 0.001	-0.088
		2	1.139	1.139	1.136	1.138 ± 0.002	-0.088
		1	1.139	1.137	1.138	1.138 ± 0.001	-0.088
Leaves	aqueous	20	1.138	1.136	1.134	1.136 ± 0.002	0.088
		2	1.015	1.017	1.012	1.015 ± 0.003	0.088
		1	1.095	1.098	1.091	1.095 ± 0.004	0
	50% EtOH	20	1.139	1.139	1.137	1.138 ± 0.001	-0.088
		2	1.139	1.139	1.139	1.139 ± 0.000	-0.176

		1	1.138	1.138	1.137	1.138 ± 0.001	-0.088
	100% EtOH	20	1.140	1.138	1.136	1.138 ± 0.002	-0.088
		2	1.138	1.139	1.141	1.139 ± 0.002	-0.176
		1	1.138	1.132	1.137	1.136 ± 0.003	0.088
untreated HEp-2 cell line	n/a	n/a	1.105	1.007	1.101	1.071 ± 0.056	n/a
200 µM DMSO	n/a	20 (µl/ml)	1.137	1.136	1.138	1.137 ± 0.001	n/a
Camptothecin	n/a	20	1.138	1.136	1.139	1.138 ± 0.002	-0.088
calpain I inhibitor	n/a	20	1.138	1.138	1.136	1.137 ± 0.001	0

Table 1D: Mean % cytotoxicity of *Bulbine natalensis* crude fractions after 3 days of incubation

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**% cytotoxicity in triplicate**			**% cytotoxicity (mean ± Standard Deviation)**
			1	**2**	**3**	
Bulbine natalensis roots	aqueous	20	-0.088	0	0	-0.029 ± 0.051
		2	-0.176	-0.088	-0.176	-0.147 ± 0.051
		1	-0.088	-0.088	0	-0.059 ± 0.051
Corm	aqueous	20	-0.088	0	-0.176	-0.088 ± 0.088
		2	-0.088	-0.088	0.088	-0.029 ± 0.102
		1	-0.088	-0.088	-0.088	-0.088 ± 1.7^{-17}
	50% EtOH	20	-0.176	-0.176	-0.176	-0.176 ± 3.4^{-17}
		2	-0.176	-0.176	-0.088	-0.147 ± 0.051
		1	-0.176	-0.176	0	-0.117 ± 0.102
	100% EtOH	20	-0.088	-0.088	-0.088	-0.088 ± 1.7^{-17}
		2	-0.176	0	-0.088	-0.088 ± 0.088
		1	-0.260	0	-0.088	-0.116 ± 0.132
Leaves	aqueous	20	-0.088	0.088	0.088	0.029 ± 0.102
		2	-0.088	-0.176	0.088	-0.059 ± 0.134

		1	-0.176	-0.088	0	-0.088 ± 0.088
	50% EtOH	20	-0.264	-0.176	-0.088	-0.176 ± 0.088
		2	-0.176	-0.176	-0.176	-0.176 ± 3.4^{-17}
		1	-0.264	-0.176	-0.088	-0.176 ± 0.088
	100% EtOH	20	-0.088	-0.176	-0.088	-0.117 ± 0.051
		2	-0.176	-0.176	-0.176	-0.176 ± 3.4^{-17}
		1	-0.176	-0.088	0.088	-0.059 ± 0.134
untreated HEp-2 Cell line	n/a	n/a	n/a	n/a	n/a	n/a
200 µM DMSO	n/a	20 (µl/ml)	n/a	n/a	n/a	n/a
Camptothecin	n/a	20 µg/ml	-0.088	0	-0.088	-0.057 ± 0.051
calpain I inhibitor	n/a	20 µg/ml	-0.088	0	0	-0.029 ± 0.051

***Bulbine natalensis* 5 day MTT results in triplicate**

Table 1.1: Mitochondrial activities (absorbance) of aqueous and organic ***Bulbine natalensis*** crude fractions on HEp-2 cell line read at 450 nm after **5 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
Bulbine natalensis roots	aqueous	20	0.937	0.980	1.105	1.007 ± 0.087	-10.42
		2	1.110	0.940	1.180	1.079 ± 0.123	-18.07
		1	0.909	1.124	1.019	1.017 ± 0.108	-11.5
corm	aqueous	20	1.507	1.023	1.069	1.199 ±0.267	-4.08
		2	1.112	0.966	1.071	1.049 ± 0.075	8.94
		1	1.106	0.947	1.000	1.018 ± 0.081	11.63
	50% EtOH	20	0.958	0.987	0.940	0.962 ± .0.024	16.49
		2	1.168	1.090	1.067	1.108 ± 0.053	3.82
		1	0.935	1.211	0.970	1.039 ± 0.150	9.81
	100% EtOH	20	0.897	1.061	0.922	0.960 ± 0.088	16.7
		2	0.920	0.831	0.923	0.891 ± 0.052	22.65
		1	0.898	0.921	0.882	0.900 ± 0.020	21.88
leaves	aqueous	20	1.083	0.983	1.075	1.047 ± 0.056	9.11
		2	0.825	1.012	1.045	0.960 ± 0.118	16.67
		1	0.992	0.943	1.155	1.030 ± 0.111	10.59
	50% EtOH	20	1.116	1.159	1.096	1.124 ± 0.032	2.43
		2	1.061	1.134	1.294	1.163 ± 0.119	-0.95
		1	1.196	1.071	1.388	1.218 ± 0.160	-5.73
	100% EtOH	20	1.027	1.22	1.098	1.115 ± 0.098	3.21
		2	1.185	1.081	1.399	1.222 ± 0.162	-6.08
		1	1.062	1.079	1.043	1.061 ± 0.018	7.89
untreated HEp-2 cell line	n/a	n/a	1.014	1.038	1.429	1.160 ± 0.233	0
200 µM DMSO	n/a	20 (µl/ml)	1.000	1.229	1.227	1.152 ± 0.131	0

camptothecin	n/a	20	0.843	1.177	1.038	1.019 ± 0.168	11.5
calpain I inhibitor	n/a	20	1.022	1.063	1.300	1.128 ± 0.150	2.08

Table 1.2: Mitochondrial activities (absorbance) of aqueous and organic ***Bulbine natalensis*** crude fractions on HEp-2 cell line read at 450 nm after **5 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
Bulbine natalensis roots	aqueous	20	0.954	0.994	0.930	1.023 ± 0.032	-9.41
		2	1.193	0.954	1.201	1.116 ± 0.140	-19.31
		1	0.930	1.142	1.036	1.036 ± 0.107	-10.80
corm	aqueous	20	1.522	1.084	1.048	1.218 ± 0.264	-3.40
		2	0.983	1.136	1.089	1.069 ± 0.078	9.25
		1	1.130	0.963	1.023	1.039 ± 0.085	11.80
	50% EtOH	20	0.979	1.004	0.956	0.980 ± .0.024	16.81
		2	1.112	1.185	1.089	1.129 ± 0.050	4.16
		1	0.949	1.229	0.987	1.055 ± 0.152	10.44
	100% EtOH	20	0.918	1.078	0.943	0.980 ± 0.086	16.81
		2	0.848	0.937	0.941	0.909 ± 0.053	22.84
		1	0.949	0.909	1.010	0.956 ± 0.051	18.85
leaves	aqueous	20	1.098	1.097	1.010	1.068 ± 0.051	9.34
		2	0.853	1.033	1.062	0.983 ± 0.113	16.55
		1	1.010	0.959	1.176	1.048 ± 0.113	11.04
	50% EtOH	20	1.136	1.186	1.119	1.147 ± 0.035	2.63
		2	1.097	1.152	1.311	1.187 ± 0.111	-0.76
		1	1.217	1.094	1.412	1.241 ± 0.160	-5.35
	100% EtOH	20	1.043	1.235	1.125	1.134 ± 0.096	3.71
		2	1.206	1.099	1.423	1.243 ± 0.165	-5.52
		1	1.069	1.085	1.099	1.084 ± 0.015	7.98
untreated Hep	n/a	n/a	1.030	1.057	1.450	1.179 ± 0.235	0

2 cell lin							
200 µM DMSO	n/a	20 (µl/ml)	1.025	1.254	1.255	1.178 ± 0.133	0
camptothecin	n/a	20	0.864	1.194	1.059	1.039 ± 0.166	11.8
calpain I inhibitor	n/a	20	1.047	1.087	1.321	1.152 ± 0.148	2.21

Table 1.3: Mitochondrial activities (absorbance) of ***Bulbine natalensis*** crude fractions on HEp--2 cell line read at 450 nm after **5 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
Bulbine natalensis roots	aqueous	20	0.955	0.998	1.126	1.026 ± 0.089	-10.44
		2	1.195	0.960	1.194	1.116 ± 0.135	-20.13
		1	0.930	1.204	1.134	1.029 ± 0.102	-10.76
corm	aqueous	20	1.528	1.046	1.094	1.223 ± 0.266	-4.80
		2	1.133	0.985	1.092	1.070 ± 0.076	8.31
		1	1.122	1.016	0.966	1.035 ± 0.080	11.31
	50% EtOH	20	0.973	1.003	0.911	0.962 ± .0.047	17.57
		2	1.104	1.183	1.086	1.158 ± 0.052	0.77
		1	0.951	1.227	0.984	1.054 ± 0.151	9.68
	100% EtOH	20	0.916	1.079	0.942	0.979 ± 0.088	16.11
		2	0.936	0.847	0.941	0.908 ± 0.053	22.19
		1	0.918	0.942	0.896	0.918 ± 0.023	21.34
leaves	aqueous	20	1.101	1.004	1.091	1.065 ± 0.053	8.74
		2	1.035	0.836	1.064	0.978 ± 0.124	16.20
		1	1.011	0.963	1.191	1.048 ± 0.120	10.19
	50% EtOH	20	1.135	1.175	1.116	1.142 ± 0.030	2.14
		2	1.072	1.150	1.311	1.178 ± 0.122	-0.94
		1	1.210	1.399	1.086	1.232 ± 0.158	-5.57
	100% EtOH	20	1.235	1.042	1.115	1.131 ± 0.097	3.08

		2	1.200	1.096	1.415	1.237 ± 0.163	-6.00
		1	1.081	1.095	1.064	1.080 ± 0.016	7.46
untreated HEp-2 cell line	n/a	n/a	1.229	1.052	1.318	1.200 ± 0.135	0
200 µM DMSO	n/a	20 (µl/ml)	1.243	1.247	1.012	1.167 ± 0.135	0
camptothecin	n/a	20	1.196	0.864	1.058	1.039 ± 0.167	10.97
calpain I inhibitor	n/a	20	1.034	1.080	1.316	1.143 ± 0.151	2.06

Table 1.4: Mean % cytotoxicity of *Bulbine natalensis* crude fractions after 5 days of incubation

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**% cytotoxicity in triplicate**			**% cytotoxicity (mean ± Standard Deviation)**
			1	**2**	**3**	
Bulbine natalensis roots	aqueous	20	-10.42	-10.44	-9.41	-10.09 ± 0.589
		2	-18.07	-20.13	-18.36	-18.86 ± 1.115
		1	-11.5	-10.76	-10.80	-11.02 ± 0.416
corm	aqueous	20	-4.08	-4.80	-3.40	-4.09 ± 0.700
		2	8.94	8.31	9.25	8.83 ± 0.479
		1	11.63	11.31	11.80	11.58 ± 0.249
	50% EtOH	20	16.49	17.57	16.81	16.96 ± 0.555
		2	3.82	0.77	4.16	2.92 ± 1.867
		1	9.81	9.68	10.44	9.98 ± 0.407
	100% EtOH	20	16.7	16.11	16.81	16.54 ±0.376
		2	22.65	22.19	22.84	22.56 ± 0.334
		1	21.88	21.34	18.85	20.69 ± 1.616
leaves	aqueous	20	9.11	8.74	9.34	9.06 ± 0.303
		2	16.67	16.20	16.55	16.47 ± 0.244
		1	10.59	10.19	11.04	10.61 ± 0.425
	50% EtOH	20	2.43	2.14	2.63	2.40 ± 0.246

		2	-0.95	-0.94	-0.76	-0.88 ± 0.107
		1	-5.73	-5.57	-5.35	-5.55 ± 0.191
	100% EtOH	20	3.21	3.08	3.74	3.34 ± 0.350
		2	-6.08	-6.00	-5.52	-5.87 ± 0.303
		1	7.89	7.46	7.98	7.78 ± 0.278
untreated HEp-2 Cell line	n/a	n/a	n/a	n/a	n/a	n/a
200 µM DMSO	n/a	20 (µl/ml)	n/a	n/a	n/a	n/a
camptothecin	n/a	20 µg/ml	11.5	10.97	11.80	11.42 ± 0.420
calpain I inhibitor	n/a	20 µg/ml	2.08	2.06	2.21	2.12 ± 0.081

***Bulbine frutescens* 5 day MTT results in triplicate**

Table 2.1: Mitochondrial activities (absorbance) of ***Bulbine frutescens*** crude fractions on HEp-2 cell line read at 450 nm after **5 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
Bulbine natalensis roots	aqueous	20	1.120	1.007	1.026	1.051 ± 0.061	-15.24
		2	1.031	0.991	0.989	1.004 ± 0.024	-10.09
		1	1.067	1.046	1.010	1.041 ± 0.029	-14.14
	50% EtOH	20	1.027	0.900	0.999	0.925 ± 0.067	-1.43
		2	1.069	1.067	1.034	1.057 ± 0.020	-15.89
		1	0.902	0.934	1.120	0.936 ± 0.118	-2.63
	100% EtOH	20	1.153	1.063	1.008	1.075 ± 0.073	-18.31
		2	0.925	0.972	1.166	1.021 ± 0.128	-11.95
		1	1.092	1.253	1.037	1.127 ± 0.112	-23.57
leaves	100% EtOH	20	1.033	0.850	0.933	0.939 ± 0.092	-2.96
		2	0.906	0.875	0.979	0.920 ± 0.053	-0.88
		1	1.104	1.066	1.038	1.069 ± 0.033	-17.21
untreated HEp-2 cells	n/a	n/a	0.978	0.975	1.071	1.008 ± 0.055	0
200 µM DMSO	n/a	20 (µl/ml)	0.883	0.930	0.922	0.921 ± 0.025	0
camptothecin	n/a	20	0.781	0.871	0.784	0.812 ± 0.051	10.96
calpain I inhibitor	n/a	20	0.960	0.938	0.902	0.933 ± 0.029	-2.30

Table 2.2: Mitochondrial activities (absorbance) of ***Bulbine frutescens*** crude fractions on HEp-2 cell line read at 450 nm after **5 days of incubation period**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
Bulbine natalensis roots	aqueous	20	1.049	1.008	1.005	1.074 ± 0.025	-14.87
		2	1.085	1.061	1.027	1.021 ± 0.029	-9.20
		1	0.954	0.994	1.122	1.058 ± 0.088	-13.16
	50% EtOH	20	1.044	0.916	1.016	0.992 ± 0.067	-6.10
		2	1.093	1.084	1.051	1.076 ± 0.022	-15.08
		1	0.920	0.950	0.987	0.952 ± 0.034	-1.82
	100% EtOH	20	1.171	1.080	1.035	1.095 ± 0.069	-17.11
		2	0.942	0.988	1.183	1.038 ± 0.128	-11.05
		1	1.113	1.270	1.062	1.148 ± 0.108	-22.78
leaves	100% EtOH	20	1.054	0.875	0.941	0.957 ± 0.091	-2.35
		2	0.927	0.902	0.998	0.942 ± 0.050	-0.75
		1	1.125	1.087	1.053	1.088 ± 0.036	-16.36
untreated HEp-2 cells	n/a	n/a	0.999	0.994	1.085	1.026 ± 0.051	0
200 µM DMSO	n/a	20 (µl/ml)	0.901	0.955	0.950	0.935 ± 0.030	0
camptothecin	n/a	20	0.799	0.900	0.814	0.838 ± 0.055	10.37
calpain I inhibitor	n/a	20	0.960	1.008	1.021	0.996 ± 0.032	-6.52

Table 2.3: Mitochondrial activities (absorbance) of ***Bulbine frutescens*** crude fractions on HEp-2 cell line read at 450 nm after **5 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
Bulbine natalensis roots	aqueous	20	1.141	1.033	1.044	1.073 ± 0.059	-15.50
		2	1.050	1.010	1.007	1.022 ± 0.024	-10.01
		1	1.086	1.064	1.028	1.059 ± 0.029	-13.99
	50% EtOH	20	1.045	1.017	1.023	1.028 ± 0.015	-10.66
		2	1.084	1.091	1.024	1.066 ± 0.037	-14.75
		1	0.919	0.992	0.951	0.954 ± 0.037	-2.69
	100% EtOH	20	1.171	1.077	1.031	1.093 ± 0.071	-17.65
		2	0.939	0.997	1.187	1.041 ± 0.130	-12.06
		1	1.110	1.269	1.055	1.145 ± 0.111	-23.25
leaves	100% EtOH	20	1.049	0.871	0.954	0.958 ± 0.089	-3.12
		2	0.924	0.893	0.993	0.937 ± 0.051	-0.86
		1	1.125	1.081	1.057	1.088 ± 0.034	-17.12
untreated HEp-2 cells	n/a	n/a	0.999	0.989	1.082	1.023 ± 0.052	0
200 µM DMSO	n/a	20 (µl/ml)	0.898	0.946	0.943	0.929 ± 0.027	0
camptothecin	n/a	20	0.797	0.890	0.800	0.829 ± 0.053	10.76
calpain I inhibitor	n/a	20	0.976	0.956	0.918	0.950 ± 0.029	-2.26

Table 2.4: Mean % cytotoxicity of *Bulbine frutescens* crude fractions after 5 days of incubation

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**% cytotoxicity in triplicate**			**% cytotoxicity (mean ± Standard Deviation)**
			1	**2**	**3**	
Bulbine frutescens roots	aqueous	20	-15.24	-14.87	-15.50	-15.20 ± 0.317
		2	-10.09	-9.20	-10.01	-9.77 ± 0.492
		1	-14.14	-13.16	-13.99	-13.76 ± 0.528
	50% EtOH	20	-1.43	-6.10	-10.66	-6.06 ± 4.615
		2	-15.89	-15.08	-14.75	-15.24 ± 0.587
		1	-2.63	-1.82	-2.69	-2.38 ± 0.486
	100% EtOH	20	-18.31	-17.11	-17.65	-17.69 ± 0.601
		2	-11.95	-11.02	-12.06	-11.68 ± 0.571
		1	-23.57	-22.78	-23.25	-23.20 ± 0.397
leaves	100% EtOH	20	-2.96	-2.35	-3.12	-2.81 ± 0.406
		2	-0.88	-0.75	-0.86	-0.83 ± 0.07
		1	-17.21	-16.36	-17.12	-16.90 ± 0.467
untreated HEp-2 cell line	n/a	n/a	n/a	n/a	n/a	n/a
200 µM DMSO	n/a	20 (µl/ml)	n/a	n/a	n/a	n/a
camptothecin	n/a	20	10.96	10.37	10.76	10.70 ± 0.300
calpain I inhibitor	n/a	20	-2.30	-6.52	-2.26	-3.69 ± 2.448

***Bulbine natalensis* 8 day MTT results in triplicate**

Table 3.1: Mitochondrial activities (absorbance) of ***Bulbine natalensis*** crude fractions on HEp-2 cell line read at 450 nm after **8 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
B. natalensis *r*oots	aqueous	20	1.013	1.041	1.065	1.039 ± 0.026	-12.20
		2	1.077	1.057	0.983	1.039 ± 0.050	-12.20
		1	0.964	0.988	0.936	0.963 ± 0.026	-3.99
corm	aqueous	20	1.272	1.148	1.065	1.162 ± 0.104	-11.62
		2	1.122	1.134	1.147	1.134 ± 0.013	-8.93
		1	0.945	1.238	1.186	1.123 ± 0.156	-7.88
	50% EtOH	20	1.128	1.153	1.131	1.137 ± 0.014	-9.22
		2	1.125	1.071	1.110	1.102 ± 0.028	-5.86
		1	1.059	1.034	1.186	1.093 ± 0.082	-4.99
	100% EtOH	20	1.169	1.114	1.066	1.116 ± 0.052	-7.20
		2	0.969	0.936	1.285	1.063 ± 0.193	-2.11
		1	0.985	1.005	0.963	0.984 ± 0.021	5.48
leaves	aqueous	20	0.982	1.033	1.159	1.058 ± 0.091	-1.63
		2	1.065	1.110	1.133	1.103 ± 0.035	-5.69
		1	1.177	1.136	1.110	1.141 ± 0.034	-9.61
	50% EtOH	20	1.151	1.135	1.034	1.107 ± 0.063	-6.34
		2	1.205	1.178	1.189	1.191 ± 0.014	-14.31
		1	1.156	1.037	1.081	1.092 ± 0.060	-4.80
	100% EtOH	20	1.067	1.212	1.104	1.127 ± 0.075	-8.26
		2	1.142	1.045	1.141	1.109 ± 0.056	-6.53
		1	1.090	1.023	1.054	1.056 ± 0.034	-1.44
untreated HEp-2 cell line	n/a	n/a	1.210	1.177	1.221	1.203 ± 0.023	0
200 µM DMSO	n/a	20 (µl/ml)	1.092	1.035	0.996	1.041 ± 0.048	0

camptothecin	n/a	20	1.049	1.136	0.966	1.050 ± 0.085	-0.86
calpain I inhibitor	n/a	20	1.143	1.040	1.042	1.075 ± 0.059	-3.26

Table 3.2: Mitochondrial activities (absorbance) of ***Bulbine natalensis*** crude fractions on HEp-2 cell lines read at 450 nm after **8 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
B. natalensis roots	aqueous	20	1.034	1.062	1.082	1.061 ± 0.024	-11.45
		2	1.102	1.083	1.008	1.064 ± 0.050	-11.76
		1	0.991	1.012	0.959	0.987 ± 0.027	-3.68
corm	aqueous	20	1.288	1.169	1.089	1.182 ± 0.100	-11.72
		2	1.146	1.161	1.168	1.158 ± 0.011	-9.45
		1	0.965	1.254	1.207	1.142 ± 0.155	-7.94
	50% EtOH	20	1.144	1.174	1.145	1.154 ± 0.017	-9.07
		2	1.146	1.139	1.097	1.127 ± 0.026	-6.52
		1	1.052	1.080	1.207	1.113 ± 0.083	-5.20
	100% EtOH	20	1.190	1.087	1.139	1.139 ± 0.052	-7.66
		2	0.990	1.306	0.957	1.084 ± 0.193	-2.46
		1	1.001	1.026	0.978	1.002 ± 0.024	5.29
leaves	aqueous	20	1.006	1.028	1.174	1.069 ± 0.091	-1.04
		2	1.086	1.131	1.157	1.125 ± 0.036	-6.33
		1	1.205	1.150	1.125	1.160 ± 0.041	-9.64
	50% EtOH	20	1.178	1.156	1.049	1.128 ± 0.069	-6.62
		2	1.228	1.199	1.210	1.212 ± 0.015	-14.56
		1	1.185	1.061	1.105	1.117 ± 0.063	-5.58
	100% EtOH	20	1.082	1.229	1.125	1.145 ± 0.076	-8.22
		2	1.157	1.066	1.169	1.131 ± 0.056	-6.90
		1	1.111	1.048	1.071	1.077 ± 0.032	-1.80
untreated	n/a	n/a	1.231	1.205	1.242	1.226 ± 0.019	0

HEp-2 cells							
200 µM DMSO	n/a	20 (µl/ml)	1.108	1.052	1.014	1.053 ± 0.047	0
camptothecin	n/a	20	1.066	1.157	0.984	1.069 ± 0.086	-1.04
calpain I inhibitor	n/a	20	1.164	1.063	1.063	1.097 ± 0.058	-3.69

Table 3.3: Mitochondrial activities (absorbance) of ***Bulbine natalensis*** crude fractions on HEp-2 cell lines read at 450 nm after **8 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
Bulbine natalensis roots	aqueous	20	1.027	1.052	1.072	1.050 ± 0.023	-11.70
		2	1.019	1.064	1.008	1.030 ± 0.030	-9.57
		1	0.981	1.007	0.954	0.981 ± 0.027	-4.36
corm	aqueous	20	1.289	1.164	1.079	1.177 ± 0.106	-11.25
		2	1.143	1.162	1.160	1.155 ± 0.010	-9.17
		1	0.963	1.253	1.207	1.141 ± 0.156	-7.84
	50% EtOH	20	1.143	1.170	1.145	1.153 ± 0.015	-8.98
		2	1.142	1.139	1.122	1.134 ± 0.011	-7.18
		1	1.076	1.050	1.200	1.109 ± 0.080	-4.82
	100% EtOH	20	1.083	1.185	1.183	1.150 ± 0.058	-8.70
		2	0.990	1.425	0.951	1.122 ± 0.263	-6.05
		1	1.017	0.996	0.979	0.997 ± 0.019	5.77
leaves	aqueous	20	0.999	1.050	1.173	1.074 ± 0.089	-1.51
		2	1.081	1.125	1.150	1.119 ± 0.035	-5.77
		1	1.198	1.150	1.128	1.159 ± 0.036	-9.55
	50% EtOH	20	1.168	1.152	1.052	1.124 ± 0.063	-6.24
		2	1.222	1.210	1.192	1.208 ± 0.015	-14.18
		1	1.172	1.055	1.098	1.108 ± 0.059	-4.78
	100% EtOH	20	1.086	1.229	1.131	1.149 ± 0.073	-8.60

		2	1.163	1.057	1.162	1.127 ± 0.061	-6.52
		1	1.111	1.038	1.069	1.073 ± 0.037	-1.42
untreated HEp-2 cell line	n/a	n/a	1.225	1.192	1.240	1.219 ± 0.025	0
200 µM DMSO	n/a	20 (µl/ml)	1.108	1.053	1.014	1.058 ± 0.047	0
camptothecin	n/a	20	1.065	1.151	0.981	1.066 ± 0.085	-0.76
calpain I inhibitor	n/a	20	1.159	1.058	1.059	1.092 ± 0.058	-3.21

Table 4.4: Mean % cytotoxicity of *Bulbine natalensis* crude fractions after 8 days of incubation

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**% cytotoxicity in triplicate**			**% cytotoxicity (mean ± Standard Deviation)**
			1	**2**	**3**	
B. natalensis roots	aqueous	20	-12.20	-11.45	-11.70	-11.78 ± 0.382
		2	-12.20	-11.76	-9.57	-11.18 ± 1.409
		1	-3.99	-3.68	-4.36	-4.01 ± 0.340
corm	aqueous	20	-11.62	-11.72	-11.25	-11.53 ± 0.248
		2	-8.93	-9.45	-9.17	-9.18 ± 0.260
		1	-7.81	-7.94	-7.84	-7.86 ± 0.068
	50% EtOH	20	-9.22	-9.07	-8.98	-9.09 ± 0.121
		2	-5.86	-6.52	-7.18	-6.52 ± 0.660
		1	-4.99	-5.20	-4.82	-5.00 ± 0.190
	100% EtOH	20	-7.20	-7.66	-8.70	-7.85 ± 0.768
		2	-2.11	-2.46	-6.05	-3.54 ± 2.181
		1	5.48	5.29	5.77	5.51 ± 0.242
leaves	aqueous	20	-1.63	-1.04	-1.51	-1.39 ± 0.3112
		2	-5.69	-6.33	-5.77	-5.93 ± 0.349
		1	-9.61	-9.64	-9.55	-9.60 ± 0.046
	50% EtOH	20	-6.34	-6.62	-6.24	-6.40 ± 0.170
		2	-14.31	-14.56	-14.18	-14.35 ± 0.193

		1	-4.80	-5.58	-4.73	-5.04 ± 0.472
	100% EtOH	20	-8.26	-8.22	-8.60	-8.36 ± 0.209
		2	-6.53	-6.90	-6.52	-6.65 ± 0.217
		1	-1.44	-1.80	-1.42	-1.55 ± 0.214
untreated HEp-2 cell line	n/a	n/a	n/a	n/a	n/a	n/a
200 µM DMSO	n/a	20 (µl/ml)	n/a	n/a	n/a	n/a
camptothecin	n/a	20	-0.86	-1.04	-0.76	-0.89 ± 0.142
calpain I inhibitor	n/a	20	-3.26	-3.69	-3.21	-3.39 ± 0.264

***Bulbine frutescens* 8 day MTT results in triplicate**

Table 5.1: Mitochondrial activities (absorbance) of ***Bulbine frutescens*** crude fractions on HEp-2 cell lines read at 450 nm after **8 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
B. frutescens roots	aqueous	20	1.170	1.150	1.040	1.12 ± 0.07	-20.95
		2	1.019	1.082	1.071	1.057 ± 0.034	-14.15
		1	1.083	1.087	1.043	1.071 ± 0.024	-15.66
	50% EtOH	20	1.015	1.063	0.984	1.021 ± 0.040	-10.26
		2	1.113	1.204	1.105	1.081 ± 0.055	-16.74
		1	1.033	1.222	1.138	1.131 ± 0.095	-22.14
	100% EtOH	20	1.229	1.142	1.099	1.157 ± 0.066	-24.95
		2	1.088	1.17	1.237	1.165 ± 0.075	-25.80
		1	1.202	1.154	1.035	1.130 ± 0.086	-22.03
leaves	100% EtOH	20	1.086	1.112	1.03	1.076 ± 0.042	-16.19
		2	1.105	1.048	1.030	1.061 ± 0.039	-14.57
		1	1.078	0.991	1.258	1.109 ± 0.136	-19.76
untreated HEp-2 cells	n/a	n/a	0.996	0.957	1.034	0.996 ± 0.039	0
200 µM DMSO	n/a	20 (µl/ml)	0.953	0.911	0.914	0.926 ± 0.023)	0
camptothecin	n/a	20	0.953	1.039	0.914	0.969 ± 0.064	-4.64
calpain I inhibitor	n/a	20	0.934	1.015	0.936	0.962 ± 0.046	-9.89

Table 5.2: Mitochondrial activities (absorbance) of ***Bulbine frutescens*** crude fractions on HEp-2 cell lines read at 450 nm after **8 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
B. frutescens roots	aqueous	20	1.193	1.173	1.061	1.142 ± 0.071	-19.96
		2	1.040	1.103	1.092	1.078 ± 0.034	-13.24
		1	1.104	1.108	1.043	1.085 ± 0.036	-13.97
	50% EtOH	20	1.036	1.188	1.000	1.075 ± 0.100	-12.92
		2	1.141	1.049	1.126	1.105 ± 0.049	-16.07
		1	1.058	1.238	1.162	1.153 ± 0.090	-21.11
	100% EtOH	20	1.256	1.163	1.122	1.180 ± 0.069	-23.95
		2	1.109	1.191	1.261	1.187 ± 0.076	-24.68
		1	1.231	1.181	1.056	1.156 ± 0.090	-21.43
leaves	100% EtOH	20	1.110	1.138	1.051	1.100 ± 0.044	-15.55
		2	1.126	1.075	1.056	1.086 ± 0.036	-14.08
		1	1.099	1.015	1.281	1.132 ± 0.136	-18.91
untreated HEp-2 Cell line	n/a	n/a	1.024	0.978	1.055	1.019 ± 0.039	0
200 µM DMSO	n/a	20 (µl/ml)	0.974	0.940	0.941	0.952 ± 0.019	0
camptothecin	n/a	20	0.980	1.066	0.932	0.993 ± 0.068	-4.31
calpain I inhibitor	n/a	20	0.961	1.026	0.960	0.986 ± 0.038	-3.57

Table 5.3: Mitochondrial activities (absorbance) of ***Bulbine frutescens*** crude fractions on HEp-2 cell lines read at 450 nm after **8 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
B. frutescens roots	aqueous	20	1.188	1.056	1.171	1.138 ± 0.072	-21.06
		2	1.033	1.089	1.098	1.073 ± 0.035	-14.15
		1	1.097	1.108	1.058	1.088 ± 0.026	-15.74
	50% EtOH	20	1.028	1.001	1.076	1.035 ± 0.038	-10.11
		2	1.040	1.126	1.120	1.095 ± 0.048	-16.49
		1	1.048	1.238	1.156	1.147 ± 0.095	-22.02
	100% EtOH	20	1.246	1.113	1.163	1.174 ± 0.067	-24.89
		2	1.102	1.185	1.255	1.181 ± 0.077	-25.64
		1	1.216	1.168	1.051	1.145 ± 0.085	-21.81
leaves	100% EtOH	20	1.044	1.103	1.126	1.091 ± 0.042	-16.06
		2	1.064	1.185	1.043	1.077 ± 0.077	-14.57
		1	1.093	1.005	1.277	1.125 ± 0.139	-19.68
untreated HEp-2 cells	n/a	n/a	1.011	0.973	1.052	1.012 ± 0.040	0
200 µ DMSO	n/a	20 (µl/ml)	0.965	0.925	0.931	0.940 ± 0.022	0
camptothecin	n/a	20	0.970	1.058	0.931	0.986 ± 0.065	-4.89
calpain I inhibitor	n/a	20	0.951	1.027	0.957	0.978 ± 0.042	-4.04

Table 5.4: Mean % cytotoxicity of *Bulbine frutescens* crude fractions after 8 days of incubation

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**% cytotoxicity in triplicate**			**% cytotoxicity (mean ± Standard Deviation)**
			1	**2**	**3**	
B. frutescens roots	aqueous	20	-20.95	-19.96	-21.06	-20.66 ± 0.606
		2	-14.15	-13.25	-14.15	-13.85 ± 0.520
		1	-15.66	-13.97	-15.74	-15.12 ± 1.000
	50 % EtOH	20	-10.26	-12.92	-10.11	-11.10 ± 1.581
		2	-16.74	-16.07	-16.49	-16.43 ± 0.339
		1	-22.14	-21.11	-22.02	-21.76 ± 0.563
	100 % EtOH	20	-24.95	-23.95	-24.89	-24.60 ± 0.561
		2	-25.80	-24.68	-25.64	-25.37 ±0.606
		1	-22.03	20.43	-21.81	-21.42 ± 0.867
leaves	100 % EtOH	20	-16.19	-15.55	-16.06	-15.93 ± 0.338
		2	-14.57	-14.08	-14.57	-14.41 ± 0.283
		1	-19.76	-18.91	-19.68	-19.45 ± 0.469
untreated HEp-2 cells	n/a	n/a	n/a	n/a	n/a	n/a
200 µM DMSO	n/a	20 (µl/ml)	n/a	n/a	n/a	n/a
camptothecin	n/a	20	-4.64	-4.31	-4.89	-4.61 ± 0.291
calpain I inhibitor	n/a	20	-9.89	-3.57	-4.04	-5.83 ± 3.521

Statistical Parameters

Reliability testing I

5 and 8 day absorbance readings of *Bulbine natalensis, Bulbine frutescens* and controls

Table 1: 5 and 8 day coefficient of variation of triplicate absorbencies of *B. natalensis* and *B. frutescens*

Coefficient of variation of triplicate absorbencies	*Bulbine natalensis*		*Bulbine frutescens*	
	N	mean ± SD	N	mean ± SD
Day 5	21	1.24099 ± 0.565287	12	1.38740 ± 1.246053
Day 8	21	8.6225 ± 13.33016	12	1.1791 ± 0.48256

Since the SD of *B. frutescens* was ± 0.48256 at day 8 and the SD of *B. natalensis* and *B. frutescens* was 0.565287 and 1.246053 at day 5 respectively, it was concluded that all the species were relatively reliable at 5 days. However, at 8 days, *B. natalensis* was the least reliable (± 13.33016 SD).

Table 2: 5 and 8 day coefficient of variation of triplicate absorbencies of positive and negative controls

Coefficient of variation of triplicate absorbencies	**Untreated cells**		**200 (2%) µM DMSO**		**CAMP**		**CALP I Inhibitor**	
	N	mean ± SD	N	mean ± SD	N	mean ± SD	N	mean ± SD
Day 5	2	1.32124 ± 0.530130	2	1.20447 ± 0.119975	2	1.35819 ± 0.338924	2	2.22947 ± 1.650191
Day 8	2	1.0690 ± 0.14065	2	1.1084 ± 0.39157	2	1.1091 ± 0.20782	2	1.1565 ± 0.13643

Overall, all the controls were reliable. The 5 day calpain I (CALP) sample was the least reliable with a SD of ± 1.650191.

Reliability testing II

5 and 8 day cytotoxicity readings of *Bulbine natalensis, Bulbine frutescens* and controls

Table 1: 5 and 8 day coefficient of variation of triplicate cytotoxicities of *B. natalensis* and *B. frutescens*

Coefficient of variation of triplicate absorbencies	*Bulbine natalensis*		*Bulbine frutescens*	
	N	mean ± SD	N	mean ± SD
Day 5	21	8.4265 ± 13.32867	12	12.2500 ± 20.87871
Day 8	21	8.7270 ± 13.27633	12	3.9663 ± 3.54736

At 5 days, the *B. natalensis* was the most reliable (± 13.32867 SD), while at 8 days, the *B. frutescens* was the most reliable (± 3.54736 SD). *B. frutescens* was the least reliable at 5 days with a SD of ± 20.87871.

Table 2: 5 and 8 day coefficient of variation of triplicate cytotoxicities of positive and negative controls

Coefficient of variation of triplicate absorbencies	**CAMP**		**CALP I Inhibitor**	
	N	mean ± SD	N	mean ± SD
Day 5	2	3.2421 ± 0.61800	2	35.0653 ± 44.14825
Day 8	2	11.1544 ± 6.85669	2	34.0761 ± 37.17168

The camptothecin (CAMP) control was the most reliable at 5 and 8 days (± 0.61800 and 6.85669 SD respectively). The calpain I control was the least reliable with very high standard deviation values of ± 44.14825 (5 day) and ± 37.17168 (8 day).

One-sample Kolmogorov-Smirnov test: Absorbances

MTT Absorbances

A.

H_o: There is no difference in absorbance readings at 5 or 8 days between *Bulbine natalensis* and *Bulbine frutescens* crude fractions.

H_A: There is a difference in absorbance readings at 5 or 8 days between *Bulbine natalensis* and *Bulbine frutescens* crude fractions.

Table 1: Comparison of 5 and 8 day absorbencies between *B. natalensis* and *B. frutescens* crude fractions

Average absorbencies	*Bulbine natalensis*		*Bulbine frutescens*		**Total**		***p value**
	N	mean ± SD	N	mean ± SD	N	mean ± SD	
Day 5	21	1.06967 ± 0.095569	12	1.02983 ± 0.064922	33	1.05518 ± 0.086810	0.215
Day 8	21	-6.11810 ± 4.516475	12	1.11186 ± 0.042602	33	-3.48902 ± 5.022328	< 0.001
* a $p \leq 0.05$ was considered as being statistically significant.							

A p value of < 0.001 indicated that there was a highly significant difference in the absorbance readings between the two plant crude fractions after 8 days of incubation. The average absorbance of *B. frutescens* (1.11186) on the 8th day was much higher than that of *B. natalensis* (-6.11810). Due to this significant difference, the alternative hypothesis was accepted.

B.

H_0: There is no difference in organ absorbance readings at 5 or 8 days between *Bulbine natalensis* and *Bulbine frutescens*.

H_A: There is a difference in organ absorbance readings at 5 or 8 days between *Bulbine natalensis* and *Bulbine frutescens*.

Table 1: Comparison of 5 and 8 day absorbencies between *B. natalensis* and *B. frutescens* organs (intraspecific)

Average absorbencies		*Bulbine natalensis*		*Bulbine frutescens*	
		N	mean ± SD	N	mean ± SD
Day 5	leaves	9	1.11711 ± 0.87722	3	0.98867 ± 0.81060
	roots	3	1.05100 ± 0.438691	9	1.04356 ± 0.057493
	corm	9	1.02844 ± 0.099855	n/a	n/a
Total		21	1.06967 ± 0.095569	12	1.02983 ± 0.064922
*p value		0.134		0.220	
Day 8	leaves	9	-6.58593 ± 3.994871	3	1.09522 ± 0.024272
	roots	3	-4.71222 ± 6.143507	9	1.11741 ± 0.047009
	corm	9	-6.11889 ± 4.516475	n/a	n/a
Total		21	-6.11810 ± 4.516475	12	1.11186 ± 0.042602
*p value		0.839		0.461	
* a $p \leq 0.05$ was considered as being statistically significant.					

For *B. natalensis*, the p value for the average absorbance after 5 or 8 days of incubation was 0.314 and 0.839 respectively. A p value of 0.220 and 0.461 was noted for the *B. frutescens* 5 and 8 day read samples respectively. Since the p value for each Bulbine species was greater than 0.05, the null hypothesis was accepted i.e. there was no significant difference in organ absorbance readings at 5 or 8 days between *B. natalensis* or *B. frutescens.*

No statistical tests were performed to see if there were any differences in absorbance readings between *Bulbine natalensis* and *Bulbine frutescens* or amongst their organs at 5 or 8 days because from the cytotoxicity results these were quite obvious. Since *Bulbine natalensis* extract fractions were selected as the reference to which the corresponding fractions of *B. frutesecens* were compared to during the gene expression study, the reliability tests as well as the one-sample Kolmogorov-Smirnov test were limited to *Bulbine natalensis.*

Reliability testing III

5 and 8 day absorbance readings of *Bulbine natalensis* and controls

Table 1: 5 and 8 day coefficient of variation of triplicate absorbencies of *B. natalensis* crude organ fractions

Coefficient of variation of triplicate absorbencies	**Corm**		**Leaves**		**Roots**	
	N	mean ± SD	N	mean ± SD	N	mean ± SD
Day 5	9	1.41747 ± 0.739940	12	1.04192 ± 0.127518	12	1.45411 ± 1.274490
Day 8	9	10.7645 ± 19.36225	12	5.4494 ± 6.66375	12	2.7457 ± 3.77889

Due to the high standard deviation of ± 19.36225 at 8 days for the corm crude fraction, it was concluded that the corm samples showed the lowest reliability while, overall, the 5 day samples showed reliability to some extent. The 8 day leaf and root fractions had the second and third lowest reliability, respectively.

Table 2: 5 and 8 day coefficient of variation of triplicate absorbencies of *B. natalensis* aqueous and organic fractions

Coefficient of variation of triplicate absorbencies	**Aqueous**		**50% Ethanol**		**100% Ethanol**	
	N	mean ± SD	N	mean ± SD	N	mean ± SD
Day 5	12	1.15855 ± 0.352526	9	1.58836 ± 1.463462	12	1.2031 ± 0.606557
Day 8	12	4.9687 ± 6.65819	9	3.7594 ± 3.53525	12	8.4803 ± 17.21614

Due to the SD of ± 17.21614 for the 8 day 100% ethanol fraction, it was concluded that the 100% ethanol samples were the least reliable followed by the 8 day aqueous and 50% ethanol fractions (± 6.65819 and 3.53525 SD, respectively).

Table 3: 5 and 8 day coefficient of variation of triplicate absorbencies of positive and negative controls

Coefficient of variation of triplicate absorbencies	**Untreated cells**		**2% DMSO**		**CAMP**		**CALP I Inhibitor**	
	N	mean ± SD	N	mean ± SD	N	mean ± SD	N	mean ± SD
Day 5	2	1.32124 ± 0.530130	2	1.20447 ± 0.119975	2	1.35819 ± 0.338924	2	2.22947 ± 1.650191
Day 8	2	1.0690 ± 0.14065	2	1.1084 ± 0.39157	2	1.1091 ± 0.20782	2	1.1565 ± 0.13643

Overall, all the controls were reliable. The 5 day calpain I inhibitor (CALP) sample was the least reliable with a SD of ± 1.650191.

Reliability testing IV

5 and 8 day cytotoxicity readings of *Bulbine natalensis* and controls

Table 1: 5 and 8 day coefficient of variation of triplicate cytotoxicities of *B. natalensis* crude organ fractions

Coefficient of variation of triplicate absorbencies	**Corm**		**Leaves**		**Roots**	
	N	mean ± SD	N	mean ± SD	N	mean ± SD
Day 5	9	11.9549 ± 20.10176	12	6.6242 ± 4.29828	12	11.4059 ± 20.95491
Day 8	9	10.7645 ± 19.36225	12	5.7126 ± 6.49245	12	5.4525 ± 4.2393

Due to the low SD of ± 4.29828 relative to the ± 20 SD for corm and roots, it was concluded that the leaves were the most reliable at 5 days. For 8 days, the *B. natalensis* root fraction was the most reliable (± 4.23934), while the corm was the least reliable (± 19.36225 SD).

Table 2: 5 and 8 day coefficient of variation of triplicate cytotoxicities of *B. natalensis* aqueous and organic fractions

Coefficient of variation of triplicate absorbencies	**Aqueous**		**50% Ethanol**		**100% Ethanol**	
	N	mean ± SD	N	mean ± SD	N	mean ± SD
Day 5	12	4.9994 ± 4.08785	9	21.9488 ± 28.01136	12	5.5352 ± 3.99749
Day 8	12	6.0178 ± 6.19595	9	5.3487 ± 4.73881	12	9.2092 ± 16.89564

At 5 days, the 100% ethanol fraction was the most reliable (± 3.99749 SD) while the 50% ethanol fraction was the least reliable (± 28.01136). However, at 8 days, the 50% ethanol fraction was the most reliable (± 4.73881 SD) with the 100% ethanol fraction being the least reliable (± 16.8964 SD).

One-sample Kolmogorov-Smirnov test: Cytotoxicities

% cytotoxicity of Bulbine natalensis

A.

Ho: There is no overall difference between the cytotoxicity readings of each *Bulbine natalensis* organ at 5 or 8 days.

HA: There is an overall difference between the cytotoxicity readings of each *Bulbine natalensis* organ at 5 or 8 days.

Table 1: 5 and 8 day triplicate average cytotoxicities of *B. natalensis* crude organ fractions (extraspecific)

Average absorbencies		*Bulbine natalensis*	
		N	mean ± SD
Day 5	leaves	12	1.4022 ± 8.94386
	roots	12	-12.9122 ± 5.69015
	corm	9	11.7733 ± 856992
Total		33	-0.9745 ± 12.57985
*p value		0.001	
Day 8	leaves	12	1.4022 ± 8.94386
	roots	12	-12.9122 ± 5.69015
	corm	9	-6.1188 ± 4.9612
Total		33	-10.952 ± 7.1477
*p value		< 0.001	
* a $p \leq 0.05$ was considered as being statistically significant.			

The p value for the triplicate average cytotoxicity of *B. natalensis* was < 0.001, whereas after 8 days it was 0.001. Since the p value was less than 0.05, it was concluded that there was an overall difference between the cytotoxicity readings of each *B. natalensis* organ at 5 or 8 days i.e. the alternative hypothesis was accepted. However, in order to find out if there were any significant differences amongst individual *B. natalensis* organ crude fractions, Bonferroni adjusted post hoc comparisons. These results (one-way ANOVA) are shown in table 2.

Table 2: Comparison of 5 and 8 day average cytotoxicity amongst *B. natalensis* organs using the Bonferroni method (one-way ANOVA)

Dependent variable	**Organ (I)**	**Organ (J)**	**Mean difference (I - J)**	**Sig.**	**95% confidence interval**	
					Lower bound	**Upper bound**
Average cytotoxicity Day 5	corm	leaves	10.37111	.016	1.6533	19.0890
		roots	24.68556	.000	15.9677	33.4034
	leaves	corm	-10.37111	.016	-19.0890	-1.6533
		roots	**-14.31444**	.000	6.2433	22.3856
	roots	corm	-24.68556	.000	-33.4034	-15.9677
		leaves	**-14.31444**	.000	-22.3856	-6.2433
Average cytotoxicity Day 8	corm	leaves	2.9697	.773	-3.5546	9.494136
		roots	10.3222	.001	3.79780	16.84663
	leaves	corm	-2.96972	.773	-9.49413	3.554691
		roots	***7.35250***	.013	1.31206	13.39293
	roots	corm	-10.322	.001	-16.84663	-3.797808
		leaves	***-7.3525***	.013	-13.3929	-1.31206

At 5 days, all organs showed significantly different values from each other. However, at 8 days, the corm and leaves, as well as the roots and leaves were not significantly different, but all other comparisons were significant. Since at 8 days, there weren't any discrepancy in the cytotoxicity readings for the 2 sets of organs mentioned, only samples from the 5 day *B. natalensis* crude organ fractions were used for gene expression studies.

To study the effect of *Bulbine* spp. crude fractions on the HEp-2 cell line, the cell death of the HEp-2 cell line treated with different concentrations of *Bulbine* spp. crude fractions, was assessed using the MTT cytotoxicity assay after 5 and 8 days of incubation. The results were categorised for the final concentration of crude fractions used (20, 2, and 1 µg/ml) as indicated in the table below. The non-parametric ANOVA and calculation of the H statistic was employed.

Key: percentage range, description and codes for % cytotoxicity results for *Bulbine natalensis* and *frutescens* crude fractions

% cytotoxicity	Description	Code
20 - 30	dead / good cytotoxicity	D
10 - 20	cytotoxicity	C
1 – 10	poor cytotoxicity	PC
-10 - 1	no effect	NE
-25 - -10	growth enhancer	GR

H_o: There is a significant difference between the percentage cytotoxicity results of the 3 concentrations of *Bulbine* spp. crude fractions administered to HEp-2 cell lines after 5 and 8 days of incubation ($p \leq 0.05$).

H_A: There is no significant difference between the percentage cytotoxicity results of the 3 concentrations of *Bulbine* spp. crude fractions administered to HEp-2 cell lines after 5 and 8 days of incubation ($p \geq 0.05$).

Include 2 tables on non-parametric ANOVA.

Table 1: Non-parametric ANOVA and calculation of the H statistic for *Bulbine natalensis* and *Bulbine frutescens* 5 day results

	Bulbine natalensis 5 day												*Bulbine frutescens* 5 day											
Triplicate concentration	D	C	PC	NE	GR	n_i	R_i	N	d.f.	H. Value	Critical Value	*p value	D	C	PC	NE	GR	n_i	R_i	N	d.f.	H. value	Critical value	*p value
Stock: 1	0	3	3	1	0	3	7	13	2	-39.63	5.99	< 0.05	0	0	0	2	2	2	4	6	2	-35.14	5.99	< 0.05
2	0	1	3	2	0	3	6	12					0	0	0	2	2	2	4	6				
3	0	2	3	1	1	4	7	13					0	0	0	1	3	2	4	6				
100×: 1	1	1	2	2	1	5	7	13	2	-36.38	5.99	< 0.05	0	0	0	1	3	2	4	6	2	-14.14	5.99	< 0.05
2	1	1	2	2	1	5	7	12					0	0	0	2	2	2	4	6				
3	1	1	1	3	1	5	7	13					0	0	0	1	3	2	4	6				
50×: 1	1	2	2	1	1	5	7	13	2	-39.74	5.99	< 0.05	0	0	0	1	3	2	4	6	2	-14.14	5.99	< 0.05
2	0	4	1	1	1	4	7	12					0	0	0	1	3	2	4	6				
3	0	3	2	1	1	4	7	13					0	0	0	1	3	2	4	6				

A calculated H value < critical value was considered as being significant at 2 degrees of freedom, d.f. (3 dilution categories - 1).

Table 2: Non-parametric ANOVA and calculation of the H statistic for *Bulbine natalensis* and *Bulbine frutescens* 8 day results

Triplicate concentration	*Bulbine natalensis* 8 day												*Bulbine frutescens* 8 day											
	D	C	PC	NE	GR	n_i	R_i	N	d.f.	H. Value	Critical Value	*p value	D	C	PC	NE	GR	n_i	R_i	N	d.f.	H. value	Critical value	*p value
Stock: 1	0	0	0	5	2	2	7	6	2	0.021	5.99	< 0.05	0	0	0	2	4	1	4	3	2	36	5.99	> 0.05
2	0	0	0	5	2	2	7	6					0	0	0	2	4	1	4	3				
3	0	0	0	5	2	2	7	6					0	0	0	1	4	1	4	3				
100×: 1	0	0	0	5	2	2	7	6	2	0.021	5.99	< 0.05	0	0	0	1	4	1	4	3	2	36	5.99	> 0.05
2	0	0	0	5	2	2	7	6					0	0	0	2	4	1	4	3				
3	0	0	0	6	1	2	7	6					0	0	0	1	4	1	4	3				
50×: 1	0	0	1	6	0	2	7	6	2	0.021	5.99	< 0.05	0	0	0	1	4	1	4	3	2	36	5.99	> 0.05
2	0	0	1	6	0	2	7	6					0	0	0	1	4	1	4	3				
3	0	0	1	6	0	2	7	6					0	0	0	1	4	1	4	3				

A calculated H value < critical value was considered as being significant at 2 d.f. (3 dilution categories - 1).

HeLa CYTOTOXICITY GRAPHS AND TABLES

Dose and time response of *Bulbine* spp. fractions on the HeLa cell Line after 5 and 8 days

Five day cytotoxicity results

The average percentage cytotoxicity of mean triplicates for the controls, camptothecin and calpain I inhibitor, and the *Bulbine natalensis* fractions are shown in Figure 1. The figure shows that after 5 days, the HeLa cell line showed almost the same level of cytotoxicity for fractions made from the same organ (for example the leaf 50% ethanol and 100% ethanol corm fractions) at the three administered final concentrations.

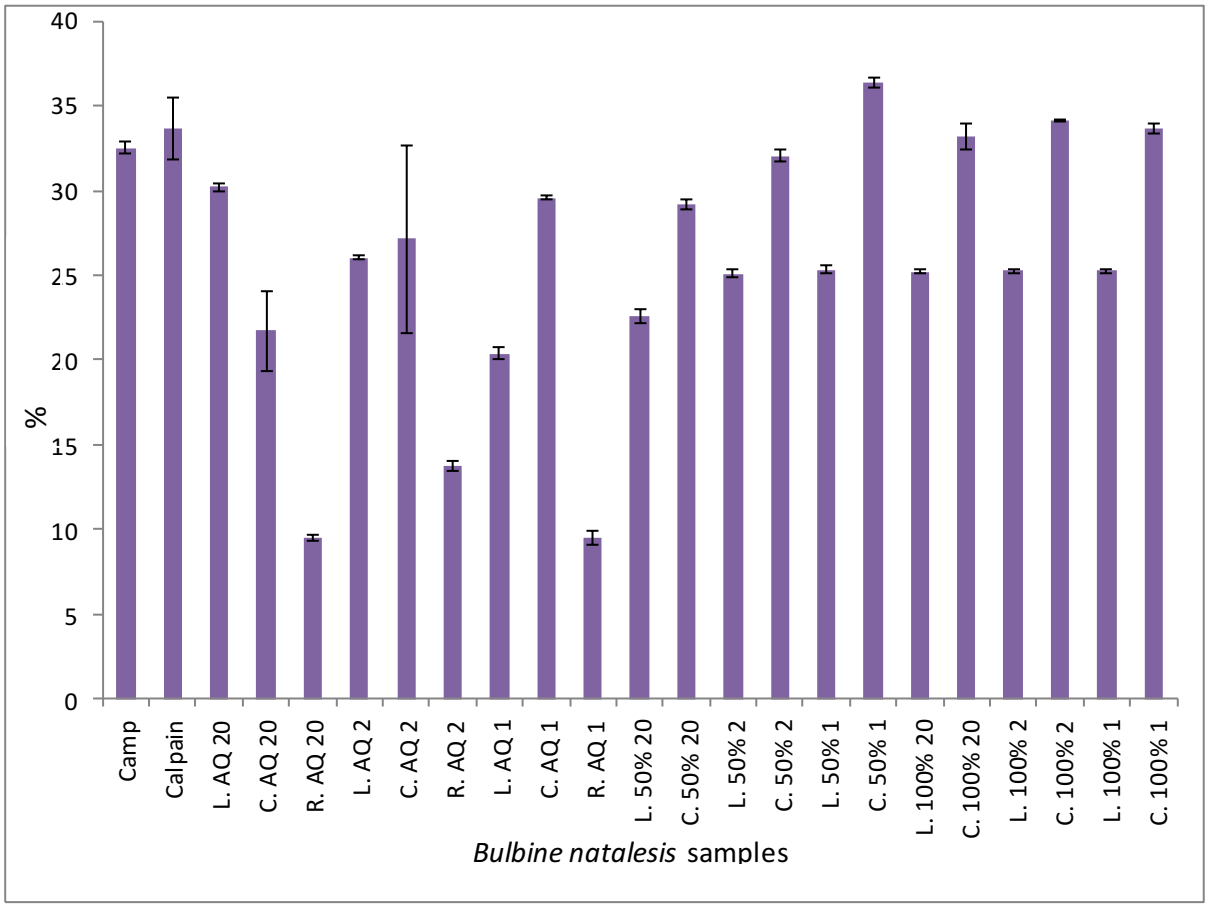

Figure 1: Cytotoxicity spectrum of aqueous and organic *Bulbine natalensis* crude fractions (leaf, roots and corm) on the HeLa cell line (114 000 ± 10583 SD cells/well) (5 day result). Cytotoxicity is derived from three experiments done in triplicate. The values are represented as mean ± S.D. The controls 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 concentration 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.*

The average percentage cytotoxicity of mean triplicates of all *B. frutescens* crude fractions did not show variability in their cytotoxicity reading across the 5 day incubation period as shown in Figure 2. The root aqueous fractions showed almost the same cytotoxicity level for all three final concentrations. However there was more variation in the response of the HeLa cell to the *B. frutescens* fractions compared to the *B. natalensis* fractions (Figure 1). Keeping in mind that *B. natalensis w*as the experimental plant in this study, although the *B. frutescens* readings are more useable, they were not used because of the HeLa cell response to the *B. natalensis* fractions on day 5.

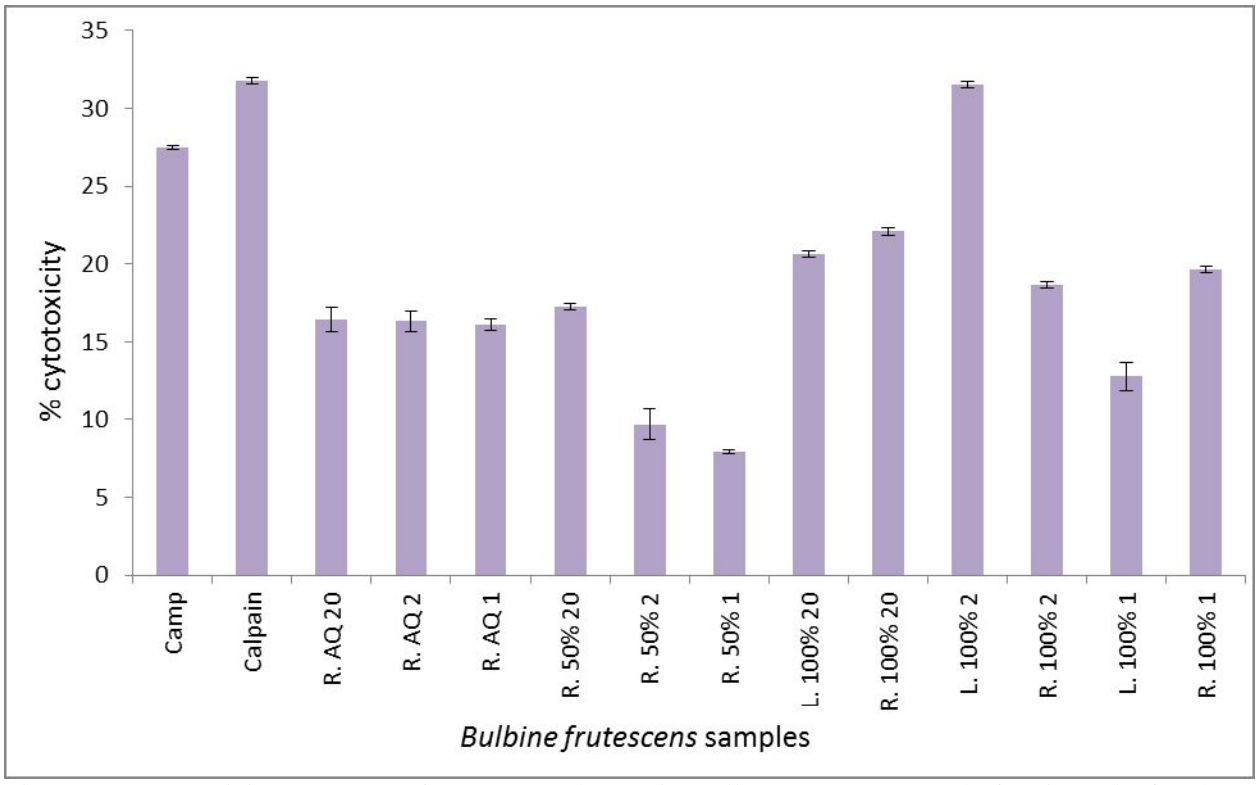

Figure 2: Cytotoxicity spectrum of aqueous and organic *Bulbine frutescens* crude fractions (leaf and roots) on the HeLa cell line (114 000 ± 10583 SD cells/well) (5 day result). Cytotoxicity is derived from three experiments done in triplicate. The values are represented as mean ± S.D. The controls 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 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.*

Eight day cytotoxicity results

The average percentage cytotoxicity of mean triplicates for all *Bulbine natalensis* crude fractions after 8 days of incubation, were all positive for the HeLa cell line. As shown below, the similarity in the cytotoxicity percentages for the leaf 50% ethanol and 100% ethanol corm fractions (Figure 3 were highly variable. There was a huge fluctuation in the cytotoxicity percentages of those results as seen in Figure 3.

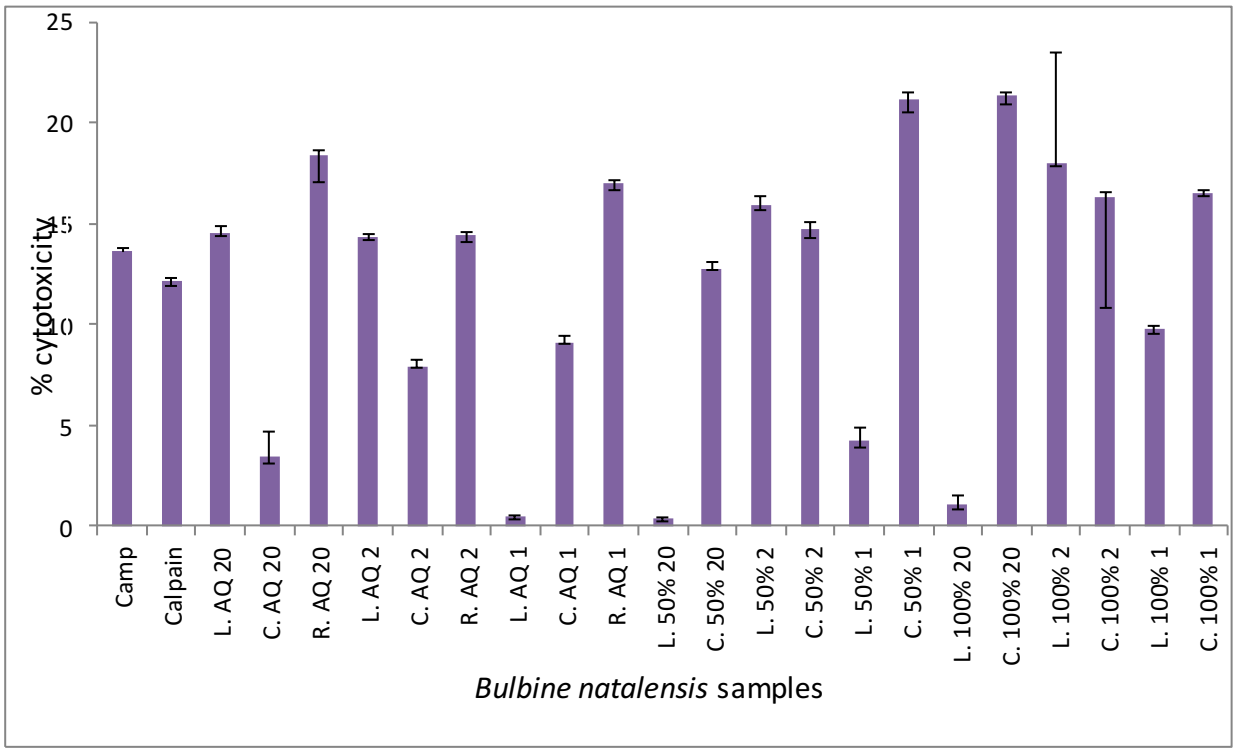

Figure 3: Cytotoxicity spectrum of aqueous and organic *Bulbine natalensis* crude fractions (leaf, roots and corm) on the HeLa cell line (114 000 ± 10583 SD cells/well) (8 day result). Cytotoxicity is derived from three experiments done in triplicate. The values are represented as mean ± S.D. The controls are: Camp: camptothecin and Calpain: Calpain I inhibitor. The samples are: 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 concentration 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.*

There was a great variability in the cytotoxicity percentages for *B. frutescens* compared to the five day readings (Figure 2). The root aqueous cytotoxic fractions began to differ amongst each final concentration in the HeLa cell line, with the 50% ethanol root fractions having similar cytotoxicity percentages amongst each other. Only the 2 and 1 µg/ml 100% ethanol leaf fractions began to invoke a proliferative effect on the HeLa cell line after 8 days of incubation (Figure 4).

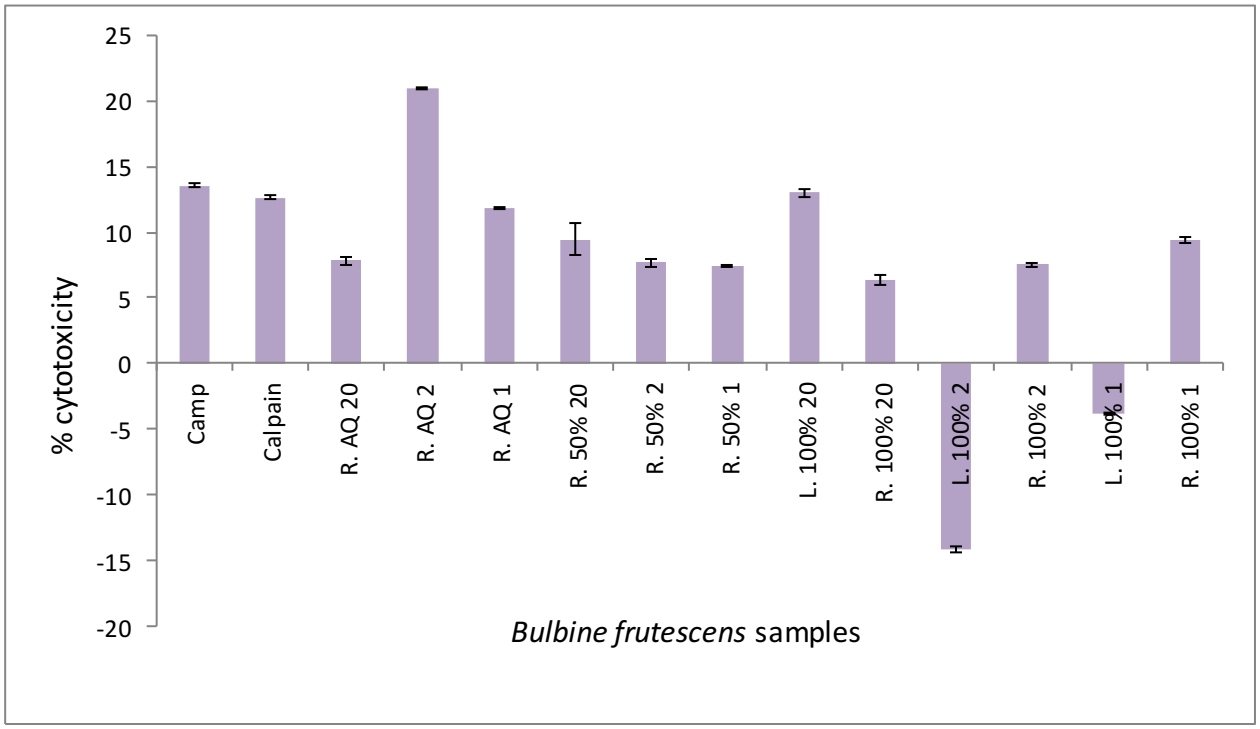

Figure 4: Cytotoxicity spectrum of aqueous and organic *Bulbine frutescens* crude fractions (leaf and roots) on the HeLa cell line (114 000 ± 10583 SD cells/well) (5 day result). Cytotoxicity is derived from three experiments done in triplicate. The values are represented as mean ± S.D. The controls 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 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.*

***Bulbine natalensis* 5 day MTT results in triplicate for the HeLa cell line**

Table B1: Mitochondrial activities (absorbance) of aqueous and organic ***Bulbine natalensis*** crude fractions on HeLa cell line read at 450 nm after **5 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
Bulbine natalensis roots	aqueous	20	1.239	1.244	1.234	1.239 ± 0.005	9.56
		2	1.174	1.187	1.170	1.177 ± 0.009	14.09
		1	1.237	1.242	1.234	1.234 ± 0.004	9.93
Corm	aqueous	20	1.080	1.076	1.079	1.078 ± 0.002	23.31
		2	0.967	0.96	0.962	0.963 ± 0.004	20.71
		1	0.967	0.962	0.968	0.966 ± 0.003	29.49
	50% EtOH	20	0.964	0.961	0.970	0.965 ± 0.005	29.56
		2	0.927	0.930	0.922	0.926 ± 0.004	32.41
		1	0.867	0.870	0.862	0.867 ± 0.004	36.72
	100% EtOH	20	0.908	0.904	0.901	0.904 ± 0.004	34.01
		2	0.901	0.904	0.903	0.903 ± 0.002	34.09
		1	0.901	0.904	0.908	0.904 ± 0.004	34.01
Leaves	aqueous	20	0.957	0.966	0.950	0.958 ± 0.008	30.07
		2	1.012	1.003	1.016	1.013 ± 0.007	26.06
		1	1.088	1.078	1.089	1.085 ± 0.006	20.80
	50% EtOH	20	1.059	1.046	1.061	1.055 ± 0.008	22.99
		2	1.023	1.030	1.033	1.029 ± 0.005	24.89
		1	1.021	1.016	1.026	1.021 ± 0.005	25.47
	100% EtOH	20	1.024	1.028	1.018	1.023 ± 0.005	25.33
		2	1.024	1.023	1.018	1.022 ± 0.003	25.40
		1	1.037	1.032	1.034	1.034 ± 0.003	24.53
untreated HEp-2 cell line	n/a	n/a	0.910	0.913	0.909	0.911 ± 0.002	0
200 µM DMSO	n/a	20 (µl/ml)	1.370	1.379	1.372	1.374 ± 0.005	0

camptothecin	n/a	20	0.926	0.934	0.901	0.920 ± 0.017	32.85
calpain I inhibitor	n/a	20	0.901	0.861	0.877	0.880 ± 0.002	35.77

Table B2: Mitochondrial activities (absorbance) of aqueous and organic ***Bulbine natalensis*** crude fractions on HeLa cell line read at 450 nm after **5 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
Bulbine natalensis roots	aqueous	20	1.242	1.247	1.236	1.242 ± 0.006	9.34
		2	1.182	1.185	1.176	1.181 ± 0.005	13.80
		1	1.241	1.247	1.237	1.242 ± 0.005	9.34
Corm	aqueous	20	1.110	1.070	1.150	1.110 ± 0.040	18.98
		2	0.952	0.961	0.957	0.957 ± 0.005	30.15
		1	0.959	0.963	0.967	0.963 ± 0.004	29.71
	50% EtOH	20	0.970	0.971	0.976	0.972 ± 0.003	29.05
		2	0.930	0.937	0.938	0.935 ± 0.004	31.75
		1	0.870	0.873	0.877	0.873 ± 0.004	36.28
	100% EtOH	20	0.918	0.920	0.923	0.920 ± 0.003	32.85
		2	0.903	0.904	0.900	0.902 ± 0.002	34.16
		1	0.907	0.909	0.910	0.909 ± 0.002	33.65
Leaves	aqueous	20	0.951	0.961	0.947	0.953 ± 0.007	30.44
		2	1.013	1.014	1.007	1.011 ± 0.004	26.20
		1	1.093	1.087	1.097	1.092 ± 0.005	20.29
	50% EtOH	20	1.063	1.066	1.069	1.066 ± 0.003	22.19
		2	1.025	1.020	1.027	1.024 ± 0.004	25.26
		1	1.023	1.027	1.031	1.027 ± 0.004	25.04
	100% EtOH	20	1.027	1.018	1.024	1.023 ± 0.005	25.33
		2	1.028	1.021	1.024	1.024 ± 0.004	25.26
		1	1.037	1.041	1.032	1.037 ± 0.005	24.31
untreated Hep	n/a	n/a	0.915	0.914	0.909	0.913 ± 0.003	0

2 cell lin							
200 µM DMSO	n/a	20 (µl/ml)	1.372	1.364	1.378	1.371 ± 0.001	0
camptothecin	n/a	20	0.930	0.932	0.927	0.930 ± 0.003	32.12
calpain I inhibitor	n/a	20	0.921	0.925	0.918	0.921 ± 0.004	32.77

Table B3: Mitochondrial activities (absorbance) of ***Bulbine natalensis*** crude fractions on HeLa cell line read at 450 nm after **5 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
Bulbine natalensis roots	aqueous	20	1.236	1.240	1.238	1.238 ± 0.002	9.64
		2	1.184	1.187	1.183	1.185 ± 0.002	13.50
		1	1.242	1.247	1.245	1.245 ± 0.003	9.12
Corm	aqueous	20	1.070	1.090	1.010	1.057 ± 0.042	22.85
		2	0.952	0.954	0.951	0.952 ± 0.002	30.51
		1	0.963	0.967	0.965	0.965 ± 0.002	29.56
	50% EtOH	20	0.971	0.977	0.971	0.973 ± 0.003	28.98
		2	0.932	0.929	0.934	0.932 ± 0.003	31.97
		1	0.872	0.875	0.879	0.875 ± 0.004	36.13
	100% EtOH	20	0.920	0.922	0.925	0.922 ± 0.003	32.70
		2	0.901	0.903	0.904	0.903 ± 0.002	34.09
		1	0.911	0.917	0.907	0.912 ± 0.005	33.43
Leaves	aqueous	20	0.958	0.960	0.957	0.958 ± 0.002	30.07
		2	1.015	1.017	1.012	1.015 ± 0.003	25.91
		1	1.095	1.098	1.091	1.095 ± 0.004	20.07
	50% EtOH	20	1.066	1.061	1.062	1.063 ± 0.003	22.41
		2	1.027	1.023	1.025	1.025 ± 0.002	25.18
		1	1.024	1.021	1.022	1.022 ± 0.002	25.40
	100% EtOH	20	1.029	1.019	1.032	1.027 ± 0.007	25.04

		2	1.024	1.025	1.027	1.025 ± 0.002	25.18
		1	1.034	1.037	1.035	1.035 ± 0.002	25.45
untreated HEp-2 cell line	n/a	n/a	0.913	0.909	0.902	0.908 ± 0.006	0
200 µM DMSO	n/a	20 (µl/ml)	1.374	1.362	1.380	1.372 ± 0.009	0
camptothecin	n/a	20	0.924	0.921	0.927	0.924 ± 0.003	32.55
calpain I inhibitor	n/a	20	0.924	0.926	0.925	0.925 ± 0.001	32.48

Table B4: Mean % cytotoxicity of *Bulbine natalensis* crude fractions after 5 days of incubation

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**% cytotoxicity in triplicate**			**% cytotoxicity (mean ± Standard Deviation)**
			1	**2**	**3**	
Bulbine natalensis roots	aqueous	20	9.56	9.34	9.64	9.51 ± 0.155
		2	14.09	13.80	13.50	13.80 ± 0.295
		1	9.93	9.34	9.12	9.46 ± 0.419
Corm	aqueous	20	23.31	18.98	22.85	21.71 ± 2.38
		2	20.71	30.15	30.51	27.12 ± 5.56
		1	29.49	29.71	29.56	29.59 ± 0.112
	50% EtOH	20	29.56	29.05	28.98	29.20 ± 0.317
		2	32.41	31.75	31.97	32.04 ± 0.336
		1	36.72	36.28	36.13	36.38 ± 0.307
	100% EtOH	20	34.01	32.85	32.70	33.19 ± 0.719
		2	34.09	34.16	34.09	34.11 ± 0.040
		1	34.01	33.65	33.43	33.70 ± 0.293
Leaves	aqueous	20	30.07	30.44	30.07	30.19 ± 0.214
		2	26.06	26.20	25.91	26.07 ± 0.145
		1	20.80	20.29	20.07	20.39 ± 0.374
	50% EtOH	20	22.99	22.19	22.41	22.53 ± 0.413

		2	24.89	25.26	25.18	25.11 ± 0.195
		1	25.47	25.04	25.40	25.30 ± 0.231
	100% EtOH	20	25.33	25.33	25.04	25.23 ± 0.167
		2	25.40	25.26	25.18	25.28 ± 0.111
		1	24.53	24.31	25.45	24.76 ± 0.604
untreated HEp-2 Cell line	n/a	n/a	n/a	n/a	n/a	n/a
200 µM DMSO	n/a	20 (µl/ml)	n/a	n/a	n/a	n/a
camptothecin	n/a	20 µg/ml	32.85	32.12	32.55	32.51 ± 0.367
calpain I inhibitor	n/a	20 µg/ml	35.77	32.77	32.48	33.67 ± 1.822

***Bulbine frutescens* 5 day MTT results in triplicate**

Table C1: Mitochondrial activities (absorbance) of ***Bulbine frutescens*** crude fractions on HeLa cell line read at 450 nm after **5 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
Bulbine natalensis roots	aqueous	20	1.254	1.257	1.251	1.254 ± 0.003	15.57
		2	1.253	1.257	1.254	1.255 ± 0.002	15.60
		1	1.263	1.270	1.267	1.267 ± 0.004	15.70
	50% EtOH	20	1.245	1.246	1.248	1.246 ± 0.002	17.10
		2	1.367	1.370	1.365	1.367 ± 0.003	9.05
		1	1.388	1.389	1.381	1.386 ± 0.004	7.78
	100% EtOH	20	1.173	1.178	1.171	1.174 ± 0.004	21.88
		2	1.225	1.230	1.221	1.225 ± 0.005	18.50
		1	1.209	1.213	1.207	1.210 ± 0.003	19.49
Leaves	100% EtOH	20	1.194	1.191	1.197	1.194 ± 0.003	20.56
		2	1.028	1.030	1.033	1.030 ± 0.003	31.47
		1	1.308	1.311	1.304	1.308 ± 0.004	12.97
untreated HEp-2 cells	n/a	n/a	0.967	0.954	0.978	0.966 ± 0.012	0
200 µM DMSO	n/a	20 (µl/ml)	1.504	1.490	1.516	1.503 ± 0.013	0
camptothecin	n/a	20	1.093	1.089	1.091	1.091 ± 0.002	27.41
calpain I inhibitor	n/a	20	1.030	1.032	1.021	1.028 ± 0.006	31.60

Table C.2: Mitochondrial activities (absorbance) of ***Bulbine frutescens*** crude fractions on HeLa cell line read at 450 nm after **5 days of incubation period**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
Bulbine natalensis roots	aqueous	20	1.251	1.250	1.247	1.249 ± 0.002	16.99
		2	1.253	1.254	1.251	1.253 ± 0.002	16.85
		1	1.261	1.257	1.263	1.260 ± 0.003	16.39
	50% EtOH	20	1.244	1.241	1.247	1.244 ± 0.003	17.45
		2	1.363	1.364	1.361	1.343 ± 0.002	10.88
		1	1.386	1.387	1.381	1.385 ± 0.003	8.10
	100% EtOH	20	1.171	1.172	1.169	1.171 ± 0.002	22.30
		2	1.224	1.221	1.220	1.222 ± 0.002	18.91
		1	1.21	1.207	1.204	1.207 ± 0.003	19.91
Leaves	100% EtOH	20	1.195	1.193	1.191	1.193 ± 0.002	20.84
		2	1.030	1.027	1.031	1.029 ± 0.002	31.72
		1	1.306	1.301	1.302	1.303 ± 0.003	13.54
untreated HEp-2 cells	n/a	n/a	0.964	0.952	0.970	0.962 ± 0.009	0
200 µM DMSO	n/a	20 (µl/ml)	1.507	1.501	1.513	1.507 ± 0.006	0
camptothecin	n/a	20	1.090	1.092	1.091	1.091 ± 0.001	27.60
calpain I inhibitor	n/a	20	1.027	1.022	1.026	1.025 ± 0.003	31.98

Table C3: Mitochondrial activities (absorbance) of ***Bulbine frutescens*** crude fractions on HeLa cell line read at 450 nm after **5 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
Bulbine natalensis roots	aqueous	20	1.251	1.246	1.253	1.250 ± 0.004	16.72
		2	1.254	1.251	1.253	1.253 ± 0.002	16.52
		1	1.258	1.256	1.260	1.258 ± 0.002	16.19
	50% EtOH	20	1.245	1.241	1.246	1.244 ± 0.007	17.12
		2	1.363	1.367	1.360	1.363 ± 0.004	9.19
		1	1.386	1.382	1.379	1.382 ± 0.004	7.93
	100% EtOH	20	1.173	1.170	1.168	1.170 ± 0.003	22.05
		2	1.221	1.219	1.223	1.221 ± 0.002	18.65
		1	1.211	1.207	1.204	1.207 ± 0.004	19.59
Leaves	100% EtOH	20	1.195	1.194	1.191	1.193 ± 0.002	20.52
		2	1.027	1.030	1.033	1.030 ± 0.003	31.38
		1	1.367	1.301	1.305	1.324 ± 0.037	11.79
untreated HEp-2 cells	n/a	n/a	0.968	0.953	0.970	0.964 ± 0.009	0
200 µM DMSO	n/a	20 (µl/ml)	1.501	1.498	1.504	1.501 ± 0.003	0
camptothecin	n/a	20	1.087	1.089	1.093	1.090 ± 0.003	27.38
calpain I inhibitor	n/a	20	1.024	1.021	1.026	1.024 ± 0.003	31.78

Table C4: Mean % cytotoxicity of *Bulbine frutescens* crude fractions after 5 days of incubation

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**% cytotoxicity in triplicate**			**% cytotoxicity (mean ± Standard Deviation)**
			1	**2**	**3**	
Bulbine frutescens roots	aqueous	20	15.57	16.99	16.72	16.43 ± 0.754
		2	15.60	16.85	16.52	16.32 ± 0.648
		1	15.70	16.39	16.19	16.09 ± 0.355
	50% EtOH	20	17.10	17.45	17.12	17.22 ± 0.197
		2	9.05	10.88	9.19	9.70 ± 1.019
		1	7.78	8.10	7.93	7.94 ± 0.160
	100% EtOH	20	21.88	22.30	22.05	22.08 ± 0.211
		2	18.50	18.91	18.65	18.69 ± 0.207
		1	19.49	19.91	19.59	19.66 ± 0.219
leaves	100% EtOH	20	20.56	20.84	20.52	20.64 ± 0.174
		2	31.47	31.72	31.38	31.52 ± 0.176
		1	12.97	13.54	11.79	12.77 ± 0.893
untreated HEp-2 cell line	n/a	n/a	n/a	n/a	n/a	n/a
200 µM DMSO	n/a	20 (µl/ml)	n/a	n/a	n/a	n/a
camptothecin	n/a	20	27.41	27.60	27.38	27.46 ± 0.119
calpain I inhibitor	n/a	20	31.60	31.98	31.78	31.79 ± 0.191

***Bulbine natalensis* 8 day MTT results in triplicate**

Table D1: Mitochondrial activities (absorbance) of ***Bulbine natalensis*** crude fractions on HeLa cell line read at 450 nm after **8 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
B. natalensis roots	aqueous	20	0.852	0.860	0.857	0.856 ± 0.004	18.32
		2	0.890	0.912	0.892	0.898 ± 0.012	14.31
		1	0.867	0.870	0.873	0.870 ± 0.003	16.98
Corm	aqueous	20	1.002	1.005	1.009	1.005 ± 0.004	4.10
		2	0.959	0.963	0.961	0.961 ± 0.002	8.30
		1	0.947	0.952	0.949	0.949 ± 0.003	9.45
	50% EtOH	20	0.909	0.912	0.914	0.912 ± 0.003	12.98
		2	0.887	0.891	0.893	0.890 ± 0.003	15.08
		1	0.823	0.827	0.821	0.824 ± 0.003	21.37
	100% EtOH	20	0.823	0.819	0.827	0.823 ± 0.004	21.47
		2	0.877	0.871	0.879	0.876 ± 0.004	16.41
		1	0.872	0.876	0.879	0.876 ± 0.004	16.41
Leaves	aqueous	20	0.893	0.887	0.895	0.892 ± 0.004	14.89
		2	0.897	0.899	0.894	0.897 ± 0.003	14.41
		1	1.042	1.045	1.041	1.043 ± 0.002	0.48
	50% EtOH	20	1.043	1.040	1.047	1.043 ± 0.004	0.48
		2	0.876	0.880	0.873	0.876 ± 0.004	16.41
		1	0.999	1.012	0.979	0.997 ± 0.017	4.87
	100% EtOH	20	1.033	1.030	1.037	1.033 ± 0.004	1.43
		2	0.925	0.923	0.927	0.925 ± 0.002	11.74
		1	0.945	0.950	0.947	0.947 ± 0.003	9.64
untreated HEp-2 cell line	n/a	n/a	0.855	0.833	0.845	0.844 ± 0.011	0
200 µM DMSO	n/a	20 (µl/ml)	1.048	1.048	1.052	1.048 ± 0.005	0

camptothecin	n/a	20	0.904	0.907	0.905	0.905 ± 0.002	13.65
calpain I inhibitor	n/a	20	0.919	0.921	0.927	0.922 ± 0.004	12.02

Table D2: Mitochondrial activities (absorbance) of ***Bulbine natalensis*** crude fractions on HeLa cell lines read at 450 nm after **8 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
B. natalensis roots	aqueous	20	0.852	0.851	0.853	0.852 ± 0.001	18.23
		2	0.891	0.894	0.892	0.892 ± 0.002	14.40
		1	0.866	0.866	0.869	0.867 ± 0.002	16.79
Corm	aqueous	20	1.001	1.003	1.001	1.002 ± 0.002	1.92
		2	0.960	0.961	0.963	0.961 ± 0.002	7.77
		1	0.950	0.951	0.949	0.950 ± 0.001	8.83
	50% EtOH	20	0.910	0.912	0.913	0.912 ± 0.002	12.48
		2	0.890	0.897	0.892	0.893 ± 0.004	14.30
		1	0.824	0.827	0.825	0.825 ± 0.002	20.83
	100% EtOH	20	0.821	0.822	0.824	0.822 ± 0.002	21.11
		2	0.877	0.876	0.873	0.875 ± 0.002	16.03
		1	0.870	0.871	0.873	0.871 ± 0.002	16.41
Leaves	aqueous	20	0.891	0.890	0.892	0.891 ± 0.001	14.49
		2	0.896	0.895	0.891	0.894 ± 0.003	14.20
		1	1.041	1.042	1.043	1.042 ± 0.001	0.00
	50% EtOH	20	1.041	1.043	1.044	1.043 ± 0.002	-0.10
		2	0.880	0.883	0.881	0.881 ± 0.002	15.45
		1	0.999	1.007	1.006	1.004 ± 0.004	3.65
	100% EtOH	20	1.034	1.034	1.037	1.035 ± 0.002	0.67
		2	0.824	0.827	0.873	0.826 ± 0.002	20.73
		1	0.941	0.942	0.940	0.941 ± 0.001	9.69
untreated	n/a	n/a	0.852	0.831	0.842	0.842 ± 0.011	0

HEp-2 cells							
200 µM DMSO	n/a	20 (µl/ml)	1.041	1.043	1.042	1.042 ± 0.001	0
camptothecin	n/a	20	0.901	0.901	0.903	0.902 ± 0.002	13.44
calpain I inhibitor	n/a	20	0.916	0.917	0.917	0.917 ± 0.001	12.00

Table D3: Mitochondrial activities (absorbance) of ***Bulbine natalensis*** crude fractions on HeLa cell lines read at 450 nm after **8 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
Bulbine natalensis roots	aqueous	20	0.854	0.853	0.850	0.852 ± 0.002	18.62
		2	0.892	0.894	0.897	0.894 ± 0.003	14.61
		1	0.868	0.865	0.867	0.867 ± 0.002	17.19
Corm	aqueous	20	1.003	1.003	1.002	1.003 ± 0.001	4.20
		2	0.966	0.967	0.966	0.966 ± 0.001	7.74
		1	0.952	0.950	0.957	0.953 ± 0.003	8.98
	50% EtOH	20	0.912	0.913	0.911	0.912 ± 0.001	12.89
		2	0.891	0.894	0.890	0.892 ± 0.002	14.80
		1	0.825	0.823	0.824	0.824 ± 0.001	21.30
	100% EtOH	20	0.823	0.823	0.824	0.823 ± 0.001	21.39
		2	0.875	0.875	0.871	0.874 ± 0.002	16.52
		1	0.871	0.870	0.874	0.872 ± 0.002	16.71
Leaves	aqueous	20	0.893	0.911	0.891	0.898 ± 0.011	14.23
		2	0.897	0.896	0.896	0.896 ± 0.001	14.42
		1	1.043	1.042	1.040	1.042 ± 0.002	0.48
	50% EtOH	20	1.042	1.041	1.047	1.043 ± 0.003	0.38
		2	0.881	0.880	0.883	0.881 ± 0.002	15.85
		1	1.002	1.003	1.002	1.002 ± 0.001	4.30
	100% EtOH	20	1.034	1.035	1.032	1.034 ± 0.002	1.24

		2	0.821	0.822	0.820	0.821 ± 0.001	21.59
		1	0.942	0.941	0.945	0.943 ± 0.002	9.93
untreated HEp-2 cell line	n/a	n/a	0.856	0.838	0.846	0.847 ± 0.009	0
200 µM DMSO	n/a	20 (µl/ml)	1.049	1.046	1.045	1.047 ± 0.002	0
camptothecin	n/a	20	0.902	0.901	0.907	0.903 ± 0.003	13.75
calpain I inhibitor	n/a	20	0.918	0.920	0.916	0.918 ± 0.002	12.32

Table D4: Mean % cytotoxicity of *Bulbine natalensis* crude fractions after 8 days of incubation

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**% cytotoxicity in triplicate**			**% cytotoxicity (mean ± Standard Deviation)**
			1	**2**	**3**	
B. natalensis roots	aqueous	20	18.32	18.23	18.62	18.39 ± 0.204
		2	14.31	14.40	14.61	14.44 ± 0.154
		1	16.98	16.79	17.19	16.99 ± 0.200
Corm	aqueous	20	4.10	1.92	4.20	3.407 ± 1.288
		2	8.30	7.77	7.74	7.94 ± 0.315
		1	9.45	8.83	8.98	9.09 ± 0.323
	50% EtOH	20	12.98	12.48	12.89	12.78 ± 0.267
		2	15.08	14.30	14.80	14.73 ± 0.395
		1	21.37	20.83	21.30	21.17 ± 0.294
	100% EtOH	20	21.47	21.11	21.39	21.32 ± 0.189
		2	16.41	16.03	16.52	16.32 ± 0.257
		1	16.41	16.41	16.71	16.51 ± 0.173
Leaves	aqueous	20	14.89	14.49	14.23	14.54 ± 0.332
		2	14.41	14.20	14.42	14.34 ± 0.124
		1	0.48	0.42	0.48	0.46 ± 0.035
	50% EtOH	20	0.48	0.43	0.38	0.43 ± 0.05
		2	16.41	15.45	15.85	15.9 ± 0.48

		1	4.87	3.65	4.30	4.27 ± 0.610
	100% EtOH	20	1.43	0.67	1.24	1.113 ± 0.40
		2	11.74	20.73	21.59	18.02 ± 5.456
		1	9.64	9.69	9.93	9.75 ± 0.156
untreated HEp-2 cell line	n/a	n/a	n/a	n/a	n/a	n/a
200 µM DMSO	n/a	20 (µl/ml)	n/a	n/a	n/a	n/a
camptothecin	n/a	20	13.65	13.44	13.75	13.61 ± 0.158
calpain I inhibitor	n/a	20	12.02	12.00	12.32	12.11 ± 0.179

***Bulbine frutescens* 8 day MTT results in triplicate**

Table E1: Mitochondrial activities (absorbance) of ***Bulbine frutescens*** crude fractions on HeLa cell lines read at 450 nm after **8 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
B. frutescens roots	aqueous	20	0.969	0.971	0.973	0.971 ± 0.002	8.05
		2	0.833	0.837	0.840	0.834 ± 0.004	21.02
		1	0.928	0.931	0.934	0.931 ± 0.003	11.84
	50% EtOH	20	0.961	0.965	0.967	0.964 ± 0.003	8.71
		2	0.973	0.977	0.971	0.974 ± 0.003	7.77
		1	0.978	0.981	0.974	0.978 ± 0.004	7.39
	100% EtOH	20	0.987	0.997	0.983	0.989 ± 0.007	6.34
		2	0.977	0.978	0.973	0.976 ± 0.003	7.58
		1	0.957	0.956	0.954	0.956 ± 0.002	9.47
Leaves	100% EtOH	20	0.917	0.918	0.913	0.916 ± 0.003	13.26
		2	1.205	1.207	1.201	1.204 ± 0.003	-14.02
		1	1.093	1.095	1.094	1.094 ± 0.001	-4.00

untreated HEp-2 cells	n/a	n/a	0.984	0.982	0.982	0.983 ± 0.001	0
200 µM DMSO	n/a	20 (µl/ml)	1.059	1.054	1.056	1.056 ± 0.003	0
camptothecin	n/a	20	0.911	0.914	0.917	0.914 ± 0.003	13.45
calpain I inhibitor	n/a	20	0.924	0.921	0.920	0.922 ± 0.002	12.69

Table E2: Mitochondrial activities (absorbance) of ***Bulbine frutescens*** crude fractions on HeLa cell lines read at 450 nm after **8 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
B. frutescens roots	aqueous	20	0.970	0.971	0.969	0.970 ± 0.001	7.88
		2	0.831	0.832	0.834	0.832 ± 0.002	20.99
		1	0.929	0.930	0.926	0.928 ± 0.002	11.87
	50% EtOH	20	0.957	0.958	0.900	0.938 ± 0.033	10.92
		2	0.971	0.970	0.969	0.970 ± 0.001	7.88
		1	0.976	0.974	0.972	0.974 ± 0.002	7.50
	100% EtOH	20	0.984	0.981	0.982	0.982 ± 0.002	6.74
		2	0.971	0.973	0.974	0.973 ± 0.002	7.60
		1	0.954	0.951	0.953	0.953 ± 0.002	9.50
Leaves	100% EtOH	20	0.916	0.917	0.919	0.917 ± 0.002	12.92
		2	1.201	1.202	1.203	1.202 ± 0.001	-14.15
		1	1.091	1.093	1.094	1.093 ± 0.002	-3.80
untreated HEp-2 Cell line	n/a	n/a	0.986	0.980	0.984	0.983 ± 0.003	0
200 µM DMSO	n/a	20 (µl/ml)	1.055	1.053	1.051	1.053 ± 0.002	0
Camptothecin	n/a	20	0.909	0.910	0.906	0.908 ± 0.002	13.77
calpain I inhibitor	n/a	20	0.921	0.920	0.917	0.919 ± 0.002	12.73

Table E3: Mitochondrial activities (absorbance) of ***Bulbine frutescens*** crude fractions on HeLa cell lines read at 450 nm after **8 days of incubation**

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**Absorbance in triplicate**			**Mean absorbance at 450 nm ± SD**	**% Cytotoxicity**
			1	**2**	**3**		
B. frutescens roots	aqueous	20	0.971	0.970	0.974	0.972 ± 0.002	7.52
		2	0.833	0.830	0.831	0.831 ± 0.002	20.93
		1	0.930	0.927	0.925	0.927 ± 0.003	11.80
	50% EtOH	20	0.959	0.961	0.957	0.959 ± 0.002	8.75
		2	0.973	0.977	0.971	0.974 ± 0.003	7.33
		1	0.971	0.974	0.972	0.972 ± 0.002	7.52
	100% EtOH	20	0.987	0.990	0.988	0.988 ± 0.002	5.99
		2	0.973	0.975	0.974	0.974 ± 0.001	7.33
		1	0.954	0.956	0.955	0.955 ± 0.001	9.13
Leaves	100% EtOH	20	0.917	0.918	0.915	0.917 ± 0.002	12.75
		2	1.203	1.204	1.201	1.203 ± 0.002	-14.46
		1	1.090	1.093	1.091	1.091 ± 0.002	-3.81
untreated HEp-2 cells	n/a	n/a	0.988	0.984	0.986	0.986 ± 0.002	0
200 µ DMSO	n/a	20 (µl/ml)	1.053	1.048	1.052	1.051 ± 0.003	0
camptothecin	n/a	20	0.910	0.911	0.908	0.910 ± 0.002	13.42
calpain I inhibitor	n/a	20	0.920	0.923	0.918	0.920 ± 0.003	12.46

Table E4: Mean % cytotoxicity of *Bulbine frutescens* crude fractions after 8 days of incubation

Plant organ	**Fraction**	**Final concentration in cell volume (µg/ml)**	**% cytotoxicity in triplicate**			**% cytotoxicity (mean ± Standard Deviation)**
			1	**2**	**3**	
B. frutescens roots	aqueous	20	8.05	7.88	7.52	7.82 ± 0.271
		2	21.02	20.99	20.93	20.98 ± 0.046
		1	11.84	11.87	11.80	11.84 ± 0.035
	50 % EtOH	20	8.71	10.92	8.75	9.46 ± 1.264
		2	7.77	7.88	7.33	7.66 ± 0.291
		1	7.39	7.50	7.52	7.47 ± 0.070
	100 % EtOH	20	6.34	6.74	5.99	6.36 ± 0.375
		2	7.58	7.60	7.33	7.50 ± 0.150
		1	9.47	9.50	9.13	9.37 ± 0.206
Leaves	100 % EtOH	20	13.26	12.92	12.75	12.98 ± 0.260
		2	-14.02	-14.15	-14.46	-14.21 ± 0.226
		1	-4.00	-3.80	-3.81	-3.87 ± 0.113
untreated HEp-2 cells	n/a	n/a	n/a	n/a	n/a	n/a
200 µM DMSO	n/a	20 (µl/ml)	n/a	n/a	n/a	n/a
camptothecin	n/a	20	13.45	13.77	13.42	13.55 ± 0.193
calpain I inhibitor	n/a	20	12.69	12.73	12.46	12.63 ± 0.146

APPENDIX 2

SUPPLEMENTARY INFORMATION

The eukaryotic cell cycle

Life on Earth is maintained and sustained by cellular growth and divisions that are controlled by various mechanisms that are regulated by genes and proteins (Murray and Hunt, 1993; Becker *et al*., 2003). When a cell in the mammalian body loses control of those mechanisms, uncontrolled cell proliferation causing tumourigenesis results (Murray and Hunt, 1993; Becker *et al*., 2003). This indicates the onset of cancer that is detected by the presence of oncogenes (Murray and Hunt, 1993; Becker *et al*., 2003). However, oncogenes are not expressed until normal genes called proto-oncogenes are transformed by mutations and contribute toward cancer development (Murray and Hunt, 1993; Becker *et al*., 2003). Proto-oncogenes are mutated and transformed into oncogenes by DNA-damaging chemicals, radiation, spontaneous DNA mutations and replication errors (Becker *et al*., 2003). Since mutagenesis was not an important part of this project, details into the four types of mutations (point mutations, DNA rearrangement, gene amplification and chromosomal translocation) that transform proto-oncogenes into oncogenes, will not be discussed in detail. It is important to note that once a normal cell becomes exposed to a mutagenic agent or chemical, the functioning of that cell becomes altered in such a way that oncogenes for example, produce proteins that stimulate the growth of the altered cell in excess, or those oncogenes may, in addition, code for structurally abnormal proteins (Becker *et al*., 2003). In addition to oncogenes, tumour suppression genes and DNA repair genes are the two other classes of genes that are produced from cancer-inducing mutations (Culotta and Koshland, 1958; Becker *et al*., 2003).

When a normal cell is exposed or subjected to a carcinogen or mutagen, the abnormal cell (mutated or cancer cell) generated, proliferates by the same sequence of events as the normal eukaryotic cell cycle. The difference being that the cancer cell has proteins factors produced by oncogenes that participate in the cell cycle (Becker *et al*., 2003). These proteins factors include: growth factors, receptors, plasma membrane G proteins, protein kinases, transcription factors and Cdk-cyclins. Normal and infected cells multiply by the process of mitosis whereby two identical daughter cells are produce with a diploid chromosome number (Mitchison and Salmon, 2001; Becker *et al*., 2003). However,

uncontrolled proliferation occurs with cancer cells due to problems in the cell cycle that are induced by a mutagen. In order for a parent cell to accomplish complete mitosis, it has to bypass six steps (*viz.* interphase, prophase, prometaphase, metaphase, anaphase and telophase) efficiently, without any interruptions (Mitchison and Salmon, 2001; Becker *et al*., 2003). Interruptions in even a single step, can cause cells to multiply non-stop, and this is what causes the spread of cancer in mammalian systems (Becker *et al*., 2003). The first step of mitosis is interphase and it is this phase which one refers to when speaking about the cell cycle (Mitchison and Salmon, 2001; Murray and Hunt, 1993). The cell cycle (interphase) is divided into 4 phases starting with the G1 phase, followed by the S or DNA synthesis, G2 and M phases (Mitchison and Salmon, 2001). It is believed that interphase is the step in which cells spend most their time in, while they spend the least time in the M (mitotic) phase, and that the other steps follow towards the end of the G2 phase (Mitchison and Salmon, 2001; Becker *et al*., 2003). G1 cells can either divide or enter the S phase or they may remain as they are, unmultiplying, in the G1 phase (Murray and Hunt, 1993). Those cells that do not divide are said to be in the G0 state and like nerve cells, they may never divide again. Similarly some cells of the G2 phase, which have passed the replication S phase, may also never divide again (Mitchison and Salmon, 2001).

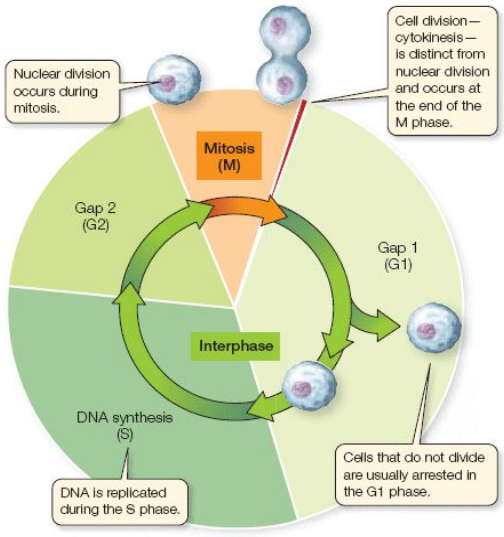

Figure A: Cell cycle in eukaryotic cells consisting of a mitotic (M) phase, during which mitosis and cytokinesis occurs, and a long period of growth known as interphase. Interphase has three subphases (G1, S, and G2) (Purves *et al*., no year supplied).

The passing of cells from the G1 to S phase is considered to be the main step that a cell takes to enter cell division, and because of this there are various factors involved (Becker *et al*., 2003). These factors include the availability of nutrients, external growth factors, cell size and target proteins such as Rb proteins (Becker *et al*., 2003). The Rb protein initiates DNA replication by binding to another protein called the E2F transcription factor which activates genes to produce enzymes and proteins for the cells to enter the S phase (Weinberg, 1995). Immediately after binding this transcription factor, the Rb protein becomes detached so as to activate Cdk-cyclin complexes which catalyses the phosphorylation of the Rb protein (Weinberg, 1995). However, in cancer cells, the *Rb* gene, the first discovered tumour suppressor gene in hereditary retinoblastoma (Weinberg, 1995), results in uncontrolled proliferation when both copies of the gene become mutated (Weinberg, 1995). This has been detected in lung, breast, bladder and several other types of cancers that are produced from environmentally induced mutations.

In addition to the Rb protein, some cancers proliferate when the tumour suppressor gene, *p53*, becomes mutated (Culotta and Koshland, 1993; Weinberg, 1995). Proliferation occurs because cells that have damaged DNA are allowed to pass into the G1 phase and then the S phase, causing more and more abnormal cells to be produced and enter the G2 phase in preparation for mitosis (Murray and Hunt, 1993). It is during the S phase that the cell's main organising center called the centromere is duplicated for prophase of mitosis (Glover *et al*., 1993). Furthermore, since eukaryotic chromosomes are linear, there are multiple DNA replication initiation sites called replicons (Murray and Hunt, 1993). These replicons determine the time spent in the S phase of the cell cycle because certain replicon clusters will replicate early whereas others will replicate later (Becker *et al*., 2003). The concept of aneuploidy has been described recently in cancer cells that have lost the function of *p53* (Becker *et al*., 2003). Such cells have been identified by their abnormal centromere numbers, which are said to interrupt chromosome sorting (Becker *et al*., 2003).

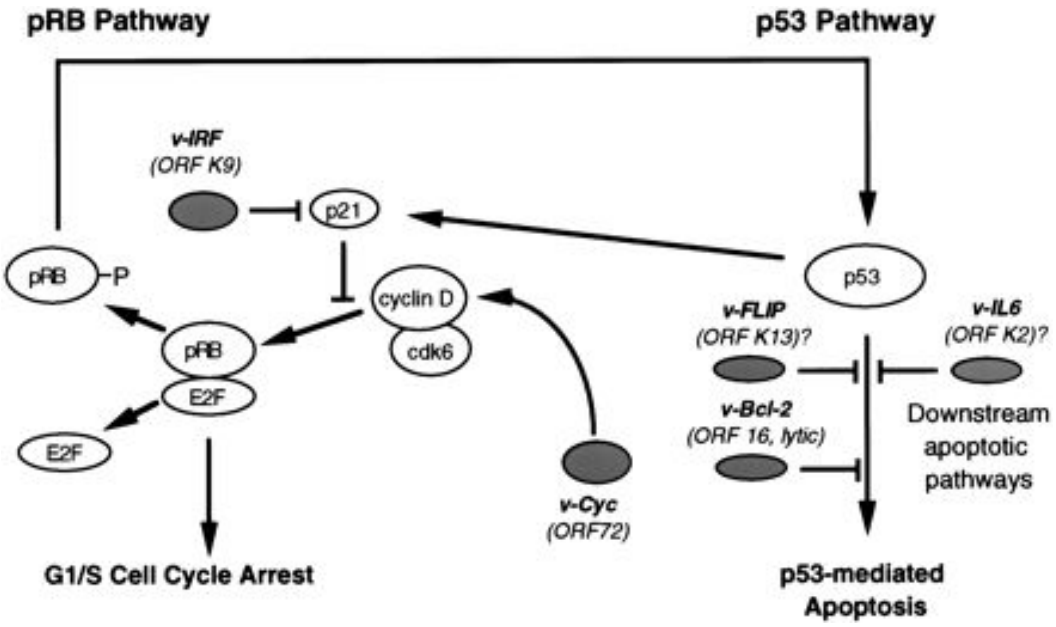

Figure B: Interaction between Rb protein and p53 (Moore and Chang, 1998)

DNA replication G2 phase cells must be completed in order for them to enter into mitosis. G1 and G2 play an important role in regulating the cell cycle and the extent to which they regulate the cell cycle differs between cell type and organism (Murray and Hunt, 1993; Becker *et al*., 2003). Experiments performed on frog eggs have suggested that molecules within the cytoplasm of cells promote cells to move from G1 to G2 checkpoints. These experiments found that the egg cells arrested in the G2 phase, only became matured after hormone treatment, when they were ready for fertilisation. These researchers claimed that there was a cytoplasmic chemical involved, which they called maturation promoting factor (MPF) (Becker *et al*., 2003).

MPF later became known as a universal term, mitosis-promoting factor (or M-phase promoting factor) in order for it to relate to many cell types (Becker *et al*., 2003). The cells that have completed DNA replication from the S phase, enter into the M phase by cyclin-dependent kinase molecules, or Cdk, called mitotic cyclins (Nigg, 2001). Similarly, G1 cyclins aid in the movement of cells from the G1 to S phase. These Cdk's are a product of a yeast gene, *cdc2*, bound to a cyclin protein and functions with ossilating protein levels within a cell (Nigg, 2001; Becker *et al*., 2003). However, the function of mitotic Cdk's is a bit more complicated because they are initially inactive. Once they become activated by an active phosphate group that binds to an amino acid of the mitotic Cdk, it functions as an active MPF, triggering mitosis (Mitchison and Salmon, 2001; Nigg, 2001). It is at this stage when

chromosome condensation, mitotic spindle assembly and nuclear envelope breakdown occurs, so as to bring the cell into prophase of mitosis (Mitchison and Salmon, 2001).

Interestingly enough, when a cell is treated with an inhibitor that targets the G2 phase of interphase, MPF does not get activated and the cell is halted at the G2 checkpoint (Mitchison and Salmon, 2001). The chromosomes during prophase are comprised of two sister chromatids since they are condensed, and they are attached to the centromere (Glover *et al*., 1993; Murray and Hunt, 1993; Mitchison and Salmon, 2001). When prophase begins the two centromeres separate and each chromatid of the pair moves to opposite poles of the cell in preparation for prometaphase, which involves a special type of DNA sequence, *CEN*, that is located at the centromere where they bind to specific proteins to form a kinetochore (Glover *et al*., 1993; Murray and Hunt, 1993; Mitchison and Salmon, 2001; Nigg, 2001). There is one kinetochore attached to each chromatid, which attaches the chromosome to the spindle microtubules (Nigg, 2001). When the spindle microtubule experiences a force, they push the chromosomes toward the center of the cell (Mitchison and Salmon, 1993). The spindle microtubules (a component of the cells structural framework) exist because when prometaphase begins, the breakdown of the nuclear envelope facilitates entry of this cytoskeletal element into the nuclear area which makes contact with the centromere (Glover *et al*., 1993). Once the chromosomes are at equidistant from each pole of the cell, the cell is said to be in metaphase (Mitchison and Salmon, 2001).

Colchicine, a drug which reportedly interferes with the spindle in cells that are in metaphase, allows for scientists to analyse the karyotype (differences in size and shape) of cells (Becker *et al*., 2003). The two chromatids of such cells separate, during anaphase, which is characterised by two types depending on the movement of chromatids (type A and type B) (Becker *et al*., 2003). During anaphase A, as the kinetochore gets shorter and shorter, the chromosomes are pulled, centromere first, to the opposite pole, while in anaphase B, as the microtubules lengthens, the poles move away from each other (Becker *et al*., 2003). In reality both type may occur at the same time or type A occurs first followed by type B (Becker *et al*., 2003). Spindle assembly is considered the third checkpoint of the cell cycle (after G1 and G2 checkpoint) because it ensures that each daughter cell receives a complete set of chromosomes. In addition, during anaphase there are also anaphase-promoting complexes, which control telophase of mitosis and involve a specialised protein, Mad2, which is associated with the kinetochore (Nigg, 2001; Becker *et al*., 2003). It is only when the spindle

fibres are attached to the kinetochore that Mad2 is released from the kinetochore and results in the activation of the anaphase-promoting complex responsible for initiating anaphase. Once the chromosomes have moved to the opposite poles of the cell, they uncoil into chromatin, for the beginning of interphase once again (Becker *et al*., 2003). The spindle dissembles, nucleoi form around nuclei organising centers, and a nuclear envelope also forms around the daughter cells, during telophase. Through the process of cytokinesis, the two daughter cells separate (Becker *et al*., 2003).

Proteins factors produced by oncogenes that participate in the cell cycle

Both normal and infected cells multiply through the same phases involved in mitosis. However, in order for abnormal cells to continuously proliferate, as is the case with cancer, oncogenic proteins are required (Becker *et al*., 2003). Mitogens are growth factors which normal cells require to multiply through the six phases involved in mitosis (Mitchison and Salmon, 2001). However, when a cell acquires an oncogene due to a mutational event in the proto-oncogene, that cell can produce the mitogen without the administration or acquisition of the mitogen externally. In this case, such a protein causes a continueous proliferation of cells (Becker *et al*., 2003). An example of such an oncogene is the *sis* oncogene which encodes for PGDF proteins, that control its own growth, and stimulates cells to produce their own active PGDF (abnormal PGDF), causing continuous growth of those cells (Becker *et al*., 2003). In contrast, in normal cells, only cells in the vicinity of platelets that produce PGDF would grow by acquiring the mitogen (Becker *et al*., 2003).

Mitogens will only bind to specific types of receptors in specific type of cells (Becker *et al*., 2003). In red blood cell cancer cells in chickens, it has been found that growth factor receptors, encoded by the *erb-B* gene*,* alter themselves in such a way, due to the acquisition of an *erb*-B oncogene, that tyrosine kinase (amino acid involved in energy production) become permanently activated even in the absence of the mitogen, EGF (Becker *et al*., 2003). This means that the Erb-B protein is an altered version of the receptor but retains tyrosine kinase activity (Becker *et al*., 2003). This implies that external acquisition of EGF will bind to the receptor binding sites only when EGF is in the vicinity of the receptor, but even without external acquisition, the red blood cancer cells would multiply continuously causing a wider spread of the cancer because of the pseudo tyrosine kinase activity (Becker *et al*., 2003).

In addition to *erb-B* encoded receptors, within eukaryotic plasma membranes there are embedded proteins (Becker *et al*., 2003). One of these is the Ras proteins, which exist in cancers in the mutant form. Ras oncogenes prevent GTP from being hydrolysed into GDP (Becker *et al*., 2003). This causes mutant Ras to remain in its active state, without requiring a mitogen from being bound to a receptor, causing cells to pass through the cell cycle continuously causing cancer proliferation (Becker *et al*., 2003). The main function of Ras proteins is to activate protein kinases such as Raf, that phosphorylates the amino acids, serine, threonine, and tyrosine (Becker *et al*., 2003). Many cancer viruses have *Raf* oncogenes that encode for abnormal Raf proteins that are active. This causes the cell cycle to proceed, keeping the protein kinase levels high in the cell (Becker *et al*., 2003). In all the cases described above, transcription factors are required to regulate the process of transcription from a gene to protein (Becker *et al*., 2003). However, some transcription factors are required to regulate the level of protein kinase e.g. *myc*, *fos*, and *jun* are genes that are also encoded by oncogenes. When these transcription factors are available in cancer cells, the cell cycle occurs at a fast rate causing accelerated cell proliferation (Becker *et al*., 2003). On the other hand, transcription factors also activate genes coding for Cdk and cyclin molecules which were discussed as being important molecules that govern cells through the G1 checkpoint into the S phase (Nigg, 2001; Becker *et al*., 2003). There are two types of oncogenes that cause a continuous proliferation of cells in cancer by producing Cdk-cyclin complexes (Nigg, 2001). These are the Cdk oncogene called *Cdk4* and the cyclin oncogene, *CYCD1*, which have been overexpressed in several types of cancers, including breast cancer (Nigg, 2001).

The process of apoptosis, or programmed cell death, which is regulated by the *p53* gene, is one means by which a cancer cell is subjected to, when it does not receive an adequate supply of factors needed to promote its own growth or survival (Culotta and Koshland, 1993). Currently, research into the development of drug and plant-derived compounds for various types of cancers are being conducted by many scientists in various parts of the world. The actual role of *p53* will not be discussed here because it was not a major part of this project, but it will be covered to some degree in the literature review. It is important to know though, that since the *p53* gene is central to apoptosis and control of the cell cycle, mutation in the *p53* gene and proto-genes do not exclusively contribute to the development of cancer (Culotta and Koshland, 1993; Becker *et al*., 2003).

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