INVESTIGATION OF THE PROBABLE ANTI-CANCER EFFECTS OF THE CRUDE METHANOL EXTRACT OF *DICEROCARYUM SENECIOIDES*, (Klotzch) J. Abels, LEAVES ON CERVICAL HeLa CANCER CELLS

BY

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DECLARATION

I declare that the study entitled **INVESTIGATION OF THE PROBABLE ANTI-CANCER EFFECTS OF THE CRUDE METHANOL EXTRACT OF *DICEROCARYUM SENECIOIDES*, (Klotzch) J. Abels, LEAVES ON CERVICAL HeLa CANCER CELLS** is my own work. This report is being submitted for the degree of Master of Science in Biochemistry at the University of Limpopo. This report has not been previously submitted to this or any other University. I further declare that all sources quoted are indicated and acknowledged by means of a comprehensive list of references.

Malemela K.M. (Miss)
Surname and initials (Title).

Date.

Student number.

Signature.

DEDICATION

This work is dedicated to **MYSELF** and my dearest late sister, **TSHEGOFATSO MALEMELA**.

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LIST OF CONFERENCE PRESENTATIONS

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LIST OF ABBREVIATIONS

µg	Microgram
µl	Microliter
µM	Micromolar
ABP	Actin binding protein
AIF	Apoptosis inducing factor
ANOVA	Analysis of variance
Apaf-1	Apoptosis protease activating factor 1
APC	Anaphase promoting complex
ATCC	American type culture collection
Bad	Bcl-2-associated death promoter
Bak	Bcl-2 antagonist killer 1
Bax	Bcl-2 associated X
Bcl-2	B-cell lymphoma-2
Bcl-xL	B-cell lymphoma-extra large
BH-3	Bcl-2 homology 3
CAD	Caspase activated DNase
CARD	Caspase recruitment domain
CDK	Cyclin-dependent kinase
CDKI	Cyclin-dependent kinase inhibitor
cIAP-1/2	Cellular Inhibitor of apoptosis-1/2
CO_2	Carbon dioxide
DED	Death effector domain

DFF	DNA fragment factor
DISC	Death-inducing signalling complex
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
FADD	Fas-associated death domain
FasL	Fas-1 induced apoptosis signal ligand
FasR	Fas-1 induced apoptosis signal receptor
FBS	Foetal bovine serum
FITC	Fluorescein Isothiocyanate
G1	Gap 1
G2	Gap 2
H_2O_2	Hydrogen peroxide
Hdm2	Human double minute 2-homolog
HIF 1-α	Hypoxia-inducible factor 1-alpha
HO-1/2	Heme oxygenase-1/2
HRP	Horseradish peroxidase
Hsp	Heat shock protein
IAP	Inhibitor of apoptosis
ICAD	Inhibitor of caspase activated DNase
M phase	Mitosis phase
ml	Milliliters
MMP	Mitochondrial membrane potential

MPT	Mitochondrial permeability transition
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium
NADH	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
nM	Nanomolar
^{o}C	Degrees Celsius
PARP	Poly (ADP-ribose) polymerase
PBS	Phosphate buffered saline
PI	Propidium Iodide
PON 2	Paraoxonase 2
PS	Phosphatidylserine
RNase	Ribonuclease
ROS	Reactive oxygen species
S phase	DNA synthesis phase
S.D.	Standard deviation
Smac/Diablo	Second mitochondria-derived activator of caspases/direct IAP-binding protein with low pI
TNF	Tumour necrosis factor
TRAIL	Tumour necrosis factor alpha- related apoptosis inducing ligand
UV	Ultraviolet
XIAP	X-linked Inhibitor of apoptosis protein

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ABSTRACT

Dicerocaryum senecioides is a plant widely used as a nutritional source. It is used also for treatment of measles, wounds and to facilitate birth in domestic animal and humans in many parts of southern Africa (Mampuru *et al*., 2012). Findings in our laboratory have shown that a dichloromethane fraction of *D. senecioides* possesses anti-inflammatory properties in human t-lymphocytes (Madiga, 2009), while the methanol crude extract possesses anti-proliferative and proapoptotic properties against Jurkat T cancer cells (Mphahlele, 2008). In this study, the probable anti-cancer effect of *D. senecioides* crude methanol leaf extract was investigated on cervical HeLa cancer cells. Dried powdered leaves of *D. senecioides* were extracted with absolute methanol to obtain a crude extract. To assess the cytotoxicity effect of the extract, KMST-6 and HeLa cell cultures were exposed to various extract concentrations (0 to 600 µg/ml) for 24 and 48 hours and subjected to the MTT assay. The results showed the extract to have no significant increase in the viability inhibition of HeLa cells at all tested concentrations after 24 hours of treatment. However, treatment with 400, 500 and 600 µg/ml of the extract for 48 hours revealed significantly increased HeLa cell viability inhibition. Furthermore, the extract showed to have no effect on the viability of normal human fibroblast KMST-6 cells at concentrations below 600 µg/ml, after 24 and 48 hours of treatment, thus showing selective cytotoxicity of the extract. To determine the mode of cell death associated with the increase in HeLa cell viability inhibition, the Hoechst 33258 nuclear staining assay and inverted light microscopy were employed. The data proposed apoptosis as the mode of cell death associated with the inhibition of HeLa cell viability. This was evidenced by changes in cell morphology such as the loss of HeLa cell radial extensions, cell shrinkage, as well as nuclear morphological features such as chromatin condensation. Apoptosis induction was further confirmed by the annexin-V/PI and multicaspase assays, using flow cytometry. The results showed an increase in the percentage of cells stained with annexin-V/PI, as well as increased caspase activity in extract-treated HeLa cells. To elucidate proapoptotic mechanisms of the extract, Western blotting analysis as well as the human apoptosis antibody array kit were used. This was to measure the expression profile of a number of apoptosis regulatory proteins. The results demonstrated modulation of some anti- and pro-apoptotic proteins, as well as the release of mitochondrial proteins required

for initiation of apoptosis, in the cytoplasm. The *D. senecioides* extract showed to have no effect on the cell division cycle of HeLa cells as determined by the PI staining assay. In conclusion, *D. senecioides* crude methanol leaf extract induced some degree of apoptosis in cervical HeLa cancer cells via the intrinsic apoptosis pathway. This was by modulating some of the members of the Bcl-2 family of proteins, which, facilitated the release of cytochrome *C* and activation of a caspase cascade.

CHAPTER ONE

1. Introduction

It has been reported by the World Health Organisation (WHO), that cancer is one of the leading causes of morbidity and mortality worldwide (Ferlay *et al.*, 2013a). Cancer, according to the National Cancer Institute (NCI) of the United States of America, is described as a collection of associated diseases. It results from uncontrollable cell division which leads to deregulation between cell death and cell proliferation. This leads to formation of tumours, as cells that should have died did not receive the signal to do so (Foo *et al.*, 2015). These tumours can either be malignant or benign (Lodish, 2000). Malignant tumours contain cells that are capable of detaching, migrating and forming secondary tumours in other parts of the body. In contrast, benign tumours have cells that proliferate and remain at the site of origin (Anusha *et al.*, 2016).

A global increase of 1% in cancer-related deaths was reported between the years 2011 and 2013. These cases are expected to increase by 46% over the next 13 years (Moodley *et al.*, 2016). Cervical cancer is reported as the fourth most prevalent cancer across the world (Ferlay *et al.*, 2013a). It is said that 1.4% of women develop cervical cancer between birth and the age of 79, at a global level. It was reported that in the year 2012, 528 000 women had cervical cancer, while 7.6% of cancer deaths, were a result of cervical cancer (Ferlay *et al.*, 2013b). In South Africa, cervical cancer is one of the 5 most prevalent cancers amongst women (Kashafi *et al.*, 2017).

Although substantial progress has been made in understanding the molecular basis, detection and treatment of cancer, the current cancer treatments, such as radiation therapy, chemotherapy and surgery have shown limited survival benefits when used for most advanced stage cancers (Hu and Fu, 2012). Surgical and radiation therapies, depending on the position of the tumour in the body, are rarely capable of the complete removal of the tumour (Praveen *et al.*, 2016). Hence, invasive cervical cancer still threatens the lives of women (Peng *et al.*, 2017).

Chemotherapy is routinely used for cancer treatment. It exploits susceptibility of cancer cells due to the loss of regulatory functions present in normal cells. Currently

used chemotherapeutic treatments however, possess intrinsic problems that may result in various toxicities (Desai *et al.*, 2008). These include, myelotoxicity and cardiotoxicity, caused by 5-fluorouracil (Macdonald, 1999; Rexroth and Scotland, 1994), cardiac, renal and myelotoxicity, caused by doxorubicin D (Afzal *et al.*, 2017; Manil *et al.*, 1995) and pulmonary toxicity, caused by bleomycin (Adamson, 1976). Furthermore, several chemically derived epigenetic drugs have been developed and undergone trials. These drugs exert heritable changes in gene expression, such as DNA methylation and histone modifications, which do not result from alteration of the DNA sequence (Kelly *et al.*, 2010). Examples of these drugs are; 5-azacytidine (azacitidine; Vidaza) and 5-aza-2'-deoxycytidine (decitbine; Dacogen), suberoyanilde hydroxamic acid (SAHA, Vorinostat, Zolinza) and FK228 (Romidespin, Istodax) (Desai *et al.*, 2008). Although epigenetic drugs display a more therapeutic approach in treatment of diseases, there are however, limitations. These limitations include, but are not limited to; diverse assays, large gene lists, few comparative data and the ongoing disagreements in published papers (Lorincz, 2011). Moreover, it is difficult to engineer a chemically derived drug that is specifically toxic to cancerous cells and non-toxic to normal cells (Greenwell and Rahman, 2015).

Due to the high cancer mortality rate, the development of drug resistance, as well as undesirable side effects, there is a pressing need to search for and/or develop new anti-cancer drugs (Praveen *et al.*, 2016). There exist a number of pervasive developments in methods for synthesis of cancer therapeutic drugs in the pharmaceutical industry, however, medicinal plants still represent important sources of new molecular identities. This is because plants can synthesise and produce components that are burdensome to obtain through chemical synthesis and this makes them an important source for the development of new anticancer drugs, that would perhaps selectively kill cancerous cells (Sponchiado *et al.*, 2016).

Studies in our laboratory demonstrated that the crude methanolic extract of *Dicerocaryum senecioides* possesses anti-proliferative and proapoptotic properties against Jurkat T cells (Mphahlele, 2008). The semi-purified extract of *D. senecioides* showed anti-inflammatory properties in human t-lymphocytes. These activities were attributed to the abundance of phenolic compounds and flavonoids in the extract (Madiga *et al.*, 2009). Thus, this study was done to investigate *D. senecioides'* potential as an apoptosis inducer in cervical HeLa cancer cells.

1.1 Plants and cancer treatment

Plant products have been used for treating various disorders for centuries. The intake of plants as medicine and common dietary supplements rich in anti-oxidants has been associated with reduced risks of cancer and other degenerative disorders associated with inflammation and ageing (Madiga *et al.*, 2009). Currently, more than 75% of the world's population depends completely on herbs for their primary health care (Sponchiado *et al.*, 2016). Compounds that are characteristic to the plant kingdom and necessary for plant survival and "housekeeping" of the organism are continually being investigated for the ability to inhibit cancer cell growth and initiate apoptosis of cancerous cells, without affecting normal cells (Greenwell and Rahman, 2015). The selection of plants with potential anticancer properties has been done either randomly or based on the indigenous traditional knowledge of ethnic communities (Katiyar *et al.*, 2012). The biological activity associated with plants is attributed to a number of chemical compounds, such as terpenoids, flavonoids, alkaloids, saponins, tannins and phenolics (Hasler and Blumberg, 1999). Furthermore, plants that have high antioxidant activity have been shown to be potential anti-proliferative and proapoptotic agents (Wang *et al.*, 2012). Hence, there are already a number of therapies put forward for cancer treatment which are plant derived. In the market, there are currently four known classes of plant derived anti-cancer agents, the vinca alkaloids (vinblastine, vincristine and vindesine), the epipodophyllotoxins (etoposide and teniposide), the taxanes (paclitaxel and docetaxel) and the camptothecin derivatives (camptothecin and irinotecan) (Desai *et al.*, 2008). Plants have also been proven useful and effective in prolonging patient survival time, preventing side effects in chemotherapy, and improving the quality of life in cancer patients (Monteiro *et al.*, 2014). Therefore, using a treatment regimen or approach that exploits these medicinal plants as anti-proliferative and proapoptotic agents, may provide a way to stop the growth of cancer cells and also, possibly, render cell death (apoptosis) without damaging normal cells.

The National Cancer Institute-USA has screened roughly 35,000 plant species for potential anticancer activities. Amongst these, approximately 3,000 plant species were found to have anticancer activity (Desai *et al.*, 2008). There is still a large reservoir of bioactive compounds, however, only a few have been examined thus far and continue to be a principal potential source of anticancer agents (Dhanamani, 2011).

1.1.1 *Dicerocaryum senecioides*

Dicerocaryum senecioides subsp. *Transvaalense* (Klotzch) J. Abels [family: Pedaliaceae], commonly known as devil's thorn or lempati, in the Sepedi language, is a reclining perennial with annual creeping stems. It grows widely in sandy soils of the veld in southern Africa. The plant has stems that are virtually hairless or to a moderate extent pubescent and has distinctive fruits armed with a pair of sharp points that easily penetrate in animal hooves, aiding in seed dispersal (Hyde, 2017). It has leaves on opposite sides of the stem, usually narrowly ovate in outline and has flowers that are deep pink or mauve, usually with darker spots on the lower lip and in the throat (figure 1). The leaves of *D. senecioides*, like those of related family members, *D. zanguebarium* (Lour.) Merrill and *D. eriocarpum* (Decne.), are used not only as a food source, but also in folk medicine for treating measles, wounds and to facilitate births in domestic animals and humans (Mampuru *et al*., 2012).

Figure 1: ***Dicerocaryum senecioides*.** (Hyde, 2017)

Unlike necrosis, apoptosis does not trigger an inflammatory or immune response (Elmore, 2007), therefore, this mode of cell death is a preferable way for the execution of cancer cell death during treatment. As such, modulation of apoptotic proteins, pathways and selective induction of apoptosis by natural products are likely to be a promising approach for cancer therapy.

In this study, the crude methanol leaf extract of *D. senecioides* was investigated for its potential as an apoptosis inducer in cervical HeLa cancer cells.

1.2 Apoptosis

Apoptosis is a planned series of events of cell suicide, critical for development, tissue homeostasis and disposal of damaged cells from the body (Sankari *et al.*, 2012). It is characterised by a number of morphological and biochemical features (Elmore, 2007). Eukaryotic cells have internal and external leaflets of lipid bilayer which display an asymmetric lipid distribution. Within the inner leaflet, lies the acidic phospholipid phosphatidylserine (PS). Asymmetry of this phospholipid is conserved by enzymes that are responsible for transporting lipids through membrane leaflets driven by a pre-existing transbilayer lipid gradient.

During early apoptosis, PS becomes translocated to the outer leaflet of the plasma membrane, making the cell a target for scavenger receptors or phagocytes. This serves as an effective apoptosis marker, identified by binding of annexin-V (Rysavy *et al.*, 2014). Furthermore, the cell begins to shrink and chromatin condenses into sharply outlined masses which become marginated against nuclear membranes (Kerr *et al.*, 1972). In the later stages, the nucleus continues to condense and eventually breaks up and the cell detaches from its surrounding tissue and its outline becomes intricately folded, forming extensions. These extensions may also separate causing a plasma membrane to seal, which then creates a separate membrane around the detached solid cellular material, forming what is known as apoptotic bodies (Saraste and Pulkki, 2000).

Moreover, there is a formation of membrane blebs. These blebs are caused by the actin-myosin protein system. This protein system is responsible for the main contraction of all muscular tissue (Coleman *et al.*, 2001). Cardinal actin-binding proteins responsible for membrane blebbing are; the actin binding protein (ABP), which is absent in tumour cells and causes extensive blebs under normal physiological conditions; the talin and α-actinin, which correlate with peroxide-induced blebbing, and the cytoskeleton protein, fodrin, which is cleaved by caspases during apoptosis (Mills *et al.*, 1998). These blebs also lead to alteration of the mitochondrial membrane potential (MMP), the source of signals that initiate apoptotic cell death (Wyllie, 1980).

Late apoptotic cells may also have a loss of membrane integrity, however, these do not become pro-inflammatory like necrotic cells (Patel *et al.*, 2006). In addition, chromosomal DNA may be cleaved into oligonucleosomal sized fragments, a

biochemical hallmark of apoptosis (Wyllie, 1980). The DNA fragment factor (DFF), a heterodimeric protein composed of DNA fragment factors 45 and 40, is responsible for the degradation of chromosomal DNA. It causes chromosomal DNA fragmentation, in the presence of an activated caspase-3. The DFF40 contains the intrinsic DNase activity while the DFF45 serves as an inhibitor of DFF40 activity. Consequent to the activation of apoptosis, DFF45 is cleaved by caspase-3, dissociating from DFF40 and thereby activating DNase. This then leaves DFF40 in a position to cleave DNA into oligonucleosomal sized fragments (Zhang and Xu, 2000).

Many of the features displayed in cells undergoing apoptosis are a result of the activation of a caspase cascade (Elmore, 2007).

1.2.1 Caspases

Caspases are a family of proteolytic enzymes existing as dormant precursors in most nucleated metazoan cells. These enzymes exist in a dormant state because their unregulated activity would be lethal for a cell and thus, they are synthesised as zymogens (Boatright and Salvesen, 2003). Caspases are classified according to the length of their pro-domains (p20 and p10). p20 is a large subunit, while p10 is a small subunit. These subunits correspond to their positions in the apoptotic signalling cascade. In this regard, caspases are divided into initiator caspases and effector caspases. Initiator caspases, which are caspase-1, -2, -4, -5, -8, -9, -10, -11 and -12, have long pro-domains that comprise of one of two characteristic protein-protein interaction motifs; the death effector domain (DED) or the caspase recruitment domain (CARD). These become autoactivated under apoptotic conditions by a process that requires and is facilitated by multicomponent complexes, like the apoptosome. In contrast, effector caspases, which are caspase-3, -6 and -7, have short pro-domains (Li and Yuan, 2008), and are activated by initiator caspases through proteolytic cleavage (Shi, 2004).

Caspases may be inhibited from cleaving various cell components by a number of proteins. The major natural cellular anti-caspase factors are inhibitors of apoptosis (IAPs). These proteins contain a Baculovirus IAP repeat (BIR) motif that is important for their anti-caspase function (Salvesen and Duckett, 2002). They inhibit caspases through two distinct mechanisms; through direct interaction with caspases and through ubiquitin and degradation. Direct interaction is carried out by members of the IAP

family, X-linked inhibitor of apoptosis protein (XIAP), cellular inhibitor of apoptosis-1 and -2 (cIAP-1/2) and neuronal apoptosis inhibitory protein (NAIP), which inhibit caspases 3, 7 and 9 (Kasof and Gomes, 2001). Ubiquitination and degradation involves RING-containing IAPs that use their ubiquitination properties to bring about proteasomal degradation of caspases-3 and -7.

The activation of a caspase-cascade occurs in either of two pathways, namely: The intrinsic (mitochondrial) pathway or the extrinsic (death receptor) pathway (Nys and Agostinis, 2012).

1.2.2 The intrinsic pathway of apoptosis

The intrinsic pathway is triggered by a miscellany of stimuli, such as hormone or growth factor deprivation, chemotherapeutic drugs, viral infections and DNA damage (Elmore, 2007). Stimuli causes mitochondrial membrane potential (MMP) decrease, which leads to outer mitochondrial membrane permeabilisation. Once permeable, proapoptotic proteins, such as cytochrome *C*, are released into the cytoplasm, where they trigger cell death. This is by promoting caspase activation or acting as caspase-independent death effectors (Poot and Pierce, 1999). The presence of cytochrome *C* in the cytoplasm promotes caspase activation through the formation of an apoptosome complex. Apoptosome formation begins when cytochrome *C* binds to the C-terminal region of Apaf-1, a nucleotide-binding domain and a C-terminal domain containing 12-13 WD-40 repeats. The binding of cytochrome *C* to Apaf-1 facilitates the association of deoxyadenosine triphosphate (dATP) with Apaf-1, which exposes its N-terminal caspase recruitment domain (CARD). This then creates a platform on which the initiator pro-caspase-9 is recruited through a CARD-CARD interaction and becomes autoactivated, forming an active caspase-9. Caspase-9 then activates executioner caspases (figure 1.2) (Adrain *et al.*, 2001). Mitochondrial proteins that act as caspase-independent death effectors include; second mitochondria-derived activator of caspases/direct inhibitor of apoptosis (IAP)-binding protein with low pI (Smac/Diablo), which is a mitochondrial serine protease that alienates IAPs; the high temperature requirement protein A2 (Omi/HtrA2), which increases the likelihood of apoptosis; the apoptosis inducing factor (AIF) and endonuclease G, a mitochondrion-specific nuclease that translocates to the cytoplasm and cleaves chromatin DNA into nucleosomal fragments independently of caspases (Fulda and Debatin, 2006).

The Bcl-2 family of proteins determines whether or not the apoptosome can assemble (Adams and Cory, 2001). The Bcl-2 protein family is divided into three classes based on structure and function, namely: (i) the multiregion proapoptotic proteins, (ii) the antiapoptotic proteins, and (iii) the BH3-only proteins. The multiregion proapoptotic proteins include Bak and Bax. These consist of three domains, BH1, BH2 and BH3, which undergo conformational activation through oligomerisation. This leads to direct permeabilisation of the mitochondrial outer membrane (MOM) and release of components of the intermembrane space that activate the final effector caspases of apoptosis. The antiapoptotic proteins include Bcl-2, Bcl-xL, MCL-1 and A1/BFL-1. These possess four domains, BH1, BH2, BH3 and BH4, which actively oppose the BH1-3 proteins (Boyd *et al*., 1995), through heterodimerisation between Bcl-2-like cell death agonists and antagonists. This opposition is by insertion of the BH3 α-helix of the proapoptotic proteins, into the hydrophobic clefts of antiapoptotic proteins (Lomonosova and Chinnadurai, 2008). Finally, the BH3-only proteins, are classified as activators or sensitisers due to their affinity for binding to the Bcl-2 multiregion proteins. They sense cellular stress and either directly or indirectly activate the proapoptotic members of the Bcl-2 family of proteins (Shamas-Din, 2013).

The interaction amongst these classes is not well understood and thus, there are several proposed models used to try and understand these interactions. One model, the direct activation model, suggests that the BH3-only proteins are required to directly bind and activate proapoptotic proteins Bax and Bak. The BH3-only activator proteins, tBid, Bim and Puma bind to both the proapoptotic and the antiapoptotic Bcl-2 multiregion proteins, while the BH3-only sensitiser proteins, Bad, Noxa, Bik, Bmf, Hrk and Bnip3 bind only to the antiapoptotic proteins. This liberates the activator BH3-only proteins to promote permeabilisation of the mitochondrial outer membrane. Another model, the displacement model, suggests that the BH3-only proteins do not directly bind to Bak and Bax to cause activation, but rather, Bak and Bax are constitutively active and can therefore be inhibited by the antiapoptotic proteins for the cell to survive. In this case, to initiate apoptosis, the BH3-only proteins displace Bax and Bak from antiapoptotic proteins thereby promoting Bax and Bak-mediated mitochondrial outer membrane permeabilisation (MOMP). (Lomonosova and Chinnadurai, 2008; Shamas-Din *et al*., 2013). Other models are variants and combinations of the already mentioned models above.

The roles of the Bcl-2 protein family in cell fate decision are therefore primarily governed by the relative concentration and affinity of different binding partners available in that specific subcellular membrane (Shamas-Din *et al.*, 2013).

1.2.3 The extrinsic pathway of apoptosis

Binding of a death ligand to its related cell surface receptor triggers apoptosis via the extrinsic signalling pathway. Receptors involved are members of *the tumour necrosis factor (TNF) receptor* gene superfamily. Thus far, the best characterised ligands and corresponding death receptors include; FasL/FasR, TNF-α/TNFR1, Apo3L/DR3, Apo2L/DR4 and Apo2L/DR5 (Elmore, 2007). Members of this receptor family have a cytoplasmic domain of about 80 amino acids known as the death domain (Ashkenazi and Dixit, 1998), which plays a critical role in the transmission of death signals from the surface of the cell to intracellular signalling pathways (Chicheportiche *et al.*, 1997). Upon binding of a ligand to its respective receptor, death domains such as the fas associated death domain (FADD) and/or TNFR1 associated death domain (TRADD) (Muzio *et al.*, 1996), oligomerise, resulting in the formation of a death-inducing signalling complex (DISC) (Kischkel *et al.*, 1995). Pro-caspase-8 is recruited into the DISC through interaction between the death effector domain (DED) of caspase-8 and death domains of the receptors (Li *et al.*, 1998). Once activated, through autocleavage, caspase-8 directly mediates activation of executioner caspases (figure 1.2) (Scaffidi *et al.*, 1998).

1.2.4 Execution of apoptosis

The intrinsic and extrinsic pathways of apoptosis both end at a point of execution. This occurs following activation of executioner caspases, such as caspase-3, -6 or -7, by initiator caspases involved in the extrinsic or intrinsic pathway (figure 1.2). Activation of executioner caspases results in the cleavage of nuclear proteins, such as the cytoskeletal protein alpha fodrin and poly ADP ribose polymerase (PARP), which is an enzyme responsible for the detection and initiation of immediate cellular response to single-strand DNA breaks (Elmore, 2007). Following degradation, cells undergoing apoptosis display distinct morphological and biochemical features (Slee *et al.*, 2001). Activated executioner caspases cleave the inhibitor of caspase activated DNase (ICAD), to release the caspase activated DNase (CAD), which degrades the chromosomal DNA within the nuclei and causes chromatin condensation. Caspase-3

induces cytoskeletal reorganisation and disintegration of the cell into apoptotic bodies (Sakahira *et al*., 1998). These apoptotic bodies are engulfed by phagocytic cells as the last component of apoptosis. The engulfment by phagocytic cells is facilitated by phagocytic phosphatidylserine recognition, which allows early uptake and disposal of apoptotic cells, thus, avoiding an inflammatory response (Martinvalet *et al*., 2005). This early and efficient uptake of apoptotic cells, without release of cellular constituents and lack of inflammation, distinguishes apoptosis from necrosis (Elmore, 2007)

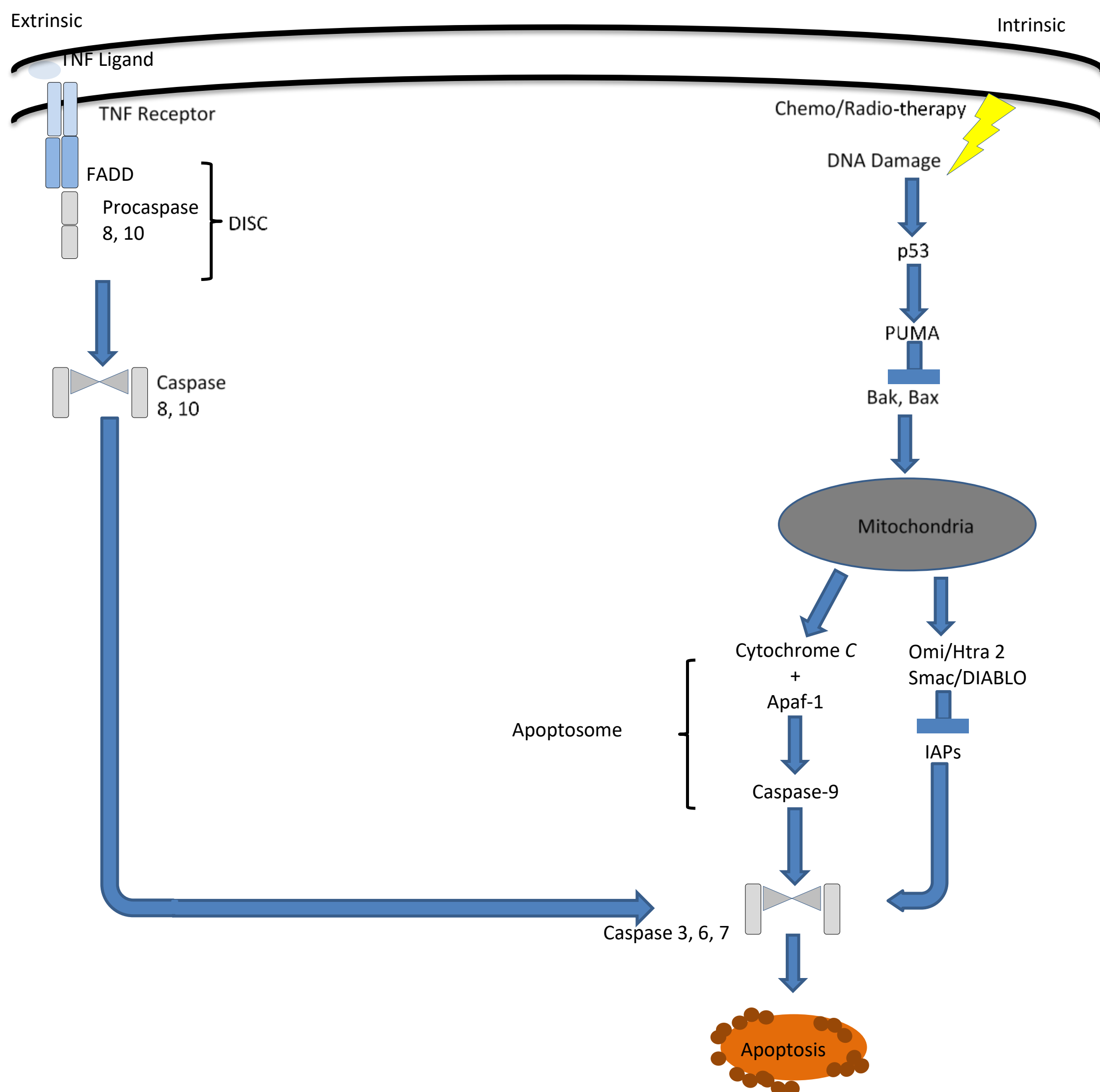


Figure 1.2: Generalised overview of the intrinsic and extrinsic pathways of apoptosis. The intrinsic pathway is triggered by intracellular stress leading to the release of cytochrome *C* from the mitochondria which in turn activates initiator caspase-9. The extrinsic pathway is mediated by death receptor proteins which leads to the activation of initiator caspase-8 and/or -10. Activation of initiator caspases lead to the activation of executioner caspases (caspase-3, 6/7), which ultimately induces apoptosis (Hengartner, 2000).

1.3 The Eukaryotic Cell Division Cycle

The cell division cycle is a systematically arranged and monitored set of events that are liable for proper division of eukaryotic cells into two daughter cells (Li *et al.*, 2015). The cell cycle (figure 1.3) is divided into two major portions; mitosis and interphase. The interphase consists of three coordinated processes, namely; Gap 1 (G1), DNA Synthesis (S) and Gap 2 (G2), which involve cell growth and DNA replication. Mitosis (M) involves the distribution of duplicated chromosomes to daughter cells and cell division. The interphase commences when the cell receives signal to reproduce (Marieb, 2000). During the G1 phase, the cell is metabolically active and continuously growing, but does not replicate its DNA. The S phase, which follows the G1 phase involves DNA synthesis and takes place in preparation for cell division. The completion of DNA synthesis is followed by the G2 phase, during which the cell continues to grow and synthesise proteins in preparation for mitosis (Alam *et al.*, 2013). During the mitosis phase, replicated chromosomes are separated into two new nuclei. The M phase is divided into five stages; the prophase, where chromatin condenses; the prometaphase, where nuclear membrane breaks into a number of “membrane vesicles”; the metaphase, where chromosomes become more condensed and coiled; the anaphase, where replicated chromosomes are split and daughter chromatids pulled to opposite poles of the cell and lastly; the telophase, where there is a formation of two daughter nuclei in each daughter cell (O'Connor, 2008).

The decision for a cell to commit to a new round of the cell division cycle is arbitrated by sequential activation and inactivation of a well-conserved family of serine/threonine protein kinases, known as cyclin-dependent kinases (CDKs). Activity of these kinases depend on a regulatory subunit called a cyclin (Malumbres, 2014). Different cyclin/cdk complexes regulate passage from one phase of the cell cycle to another (Arellano and Moreno, 1997). Of the 21 cdks encoded in the human genome, so far, only seven (cdk1, 2, 3, 4, 6, 10 and 11) have been shown to have a direct role in the progression of the cell cycle (Malumbres, 2014). Cyclin D/cdk complexes are found active in the G1 phase, where they are capable of driving cells into the S phase. This is by phosphorylation of the retinoblastoma protein (pRb), which allows progression of cells into the S phase. Accumulation of cyclins E and A at the G1/S phase boundary further successfully activate cdks 2 and 1, thereby promoting progression of cells into the G2 phase. Once in the G2 phase, B type cyclins (cyclin B1) and cdk 1 drive the cell into

the M phase (Sánchez-Martínez, 2015). The association of cdks with cyclins is controlled by a family of proteins known as cyclin-dependent kinase inhibitors (CDKIs). Members of the cdki family include; p21, p27 and p57, which bind to cdks and inhibit cyclin/cdk complexes. This inhibits cdk activity and prevents phosphorylation of proteins that mediate transition from one phase of the cell cycle to another (Pellegata *et al*., 1996). The rest of the family includes p15, p16, p18 and p19, which act by replacing cyclin D from cdks 4 and 6, also required to promote progression of the cell cycle (Sherr and Roberts, 1999).

Progression of the cell division cycle is dictated by control mechanisms called checkpoints. These serve to ensure that the genome of the cell remains stable and the cell does not acquire genetic alterations. Checkpoints accomplish this through cell cycle arrest and repair or apoptosis (Schwartz and Shah, 2005; Wodarz, 2004). There are currently three principal known checkpoints; The G1/S checkpoint, the G2/M checkpoint and the M checkpoint. At the G1/S checkpoint, the cell assesses the condition and availability of enzymes and nutrients required for DNA synthesis. Given favourable conditions, the cell enters the S phase. However, if the conditions are unfavourable, the checkpoint delays entry of the cells into the S phase and the cells then enter the resting (G0) phase (Boundless, 2016). At the G2/M checkpoint, the cell is examined if it has undergone all the mandatory changes during the S and the G2 phase. If so, the cell is then primed to divide. If faulty DNA is detected, the G2/M checkpoint temporarily prevents entry of the cells into the mitosis phase, to give the cells time to repair their DNA. This prevents replication of damaged DNA that would generate genetic abnormalities (Bode and Dong, 2007). Cyclin B/cdc2 complex primarily governs the transition from the G2 to the M phase. This ultimately activates downstream targets that promote entry of the cell into the M phase. At the M checkpoint, chromosomes are assessed if they have assembled at the mitotic plate and are under bipolar tension before cells can enter the anaphase. This checkpoint ensures that cells, with even a single unaligned chromosome, do not exit the mitosis phase to produce aneuploid cells, by generating an inhibitory signal to block the onset of anaphase (Yen and Lane, 2004). This is accomplished by stimulating the anaphase promoting complex (APC), which becomes free to degrade cyclin B. This essentially influences separase to cause separation to sister chromatids, therefore splitting the cell into its two daughter cells (figure 1.3) (Wodarz, 2004).

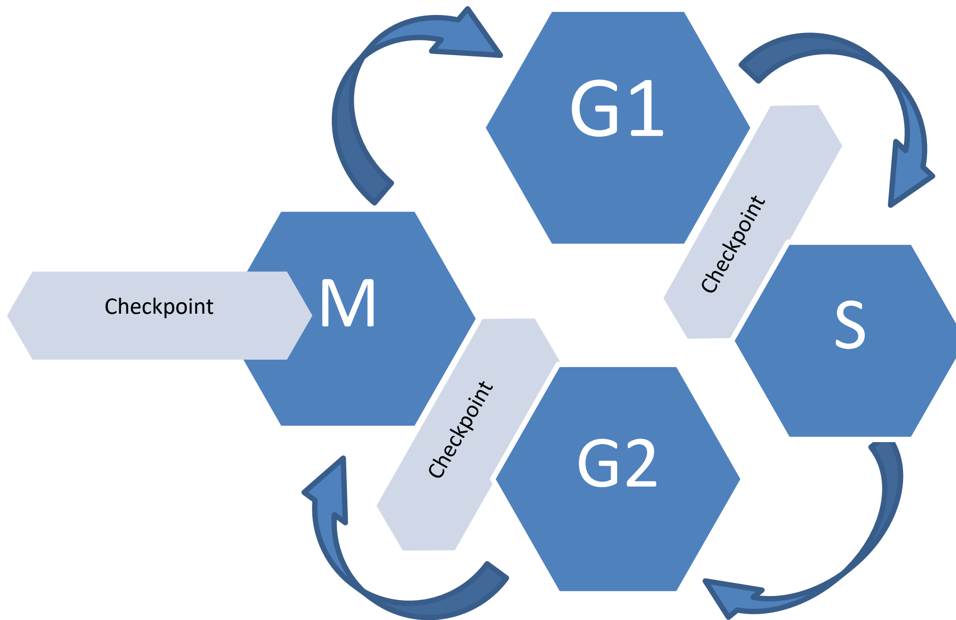


Figure 1.3: The cell division cycle and checkpoints. The eukaryotic cell cycle consists of four phases, (G1, S, G2 and M). Cyclin/cdk complexes govern the transition of the cell from one phase to another. These complexes are in turn negatively regulated by a group of proteins called cyclin-dependent kinase inhibitors (CDKIs), which act at the checkpoint to ensure proper division of cells (Schwartz and Shah, 2005).

1.4 p53 and cancer

Unlike normal cells, tumour cells undergo uncontrolled proliferation (Malumbres and Barbacid, 2001). This is due to mutations in genes that cause an acceleration in cell division rates and/or inhibit normal control systems, such as cell cycle arrest or apoptosis (Baba and Câtoi 2007). Many cancer cells carry two mutant genes that code for the tumour suppressor gene, *p53*, which normally functions by sensing DNA damage and acts as the transcription factor for genes involved in the cell cycle and checkpoint controls (Nowsheen and Yang, 2013). Under physiological conditions, p53 level is kept low and its function repressed. However, under stressful conditions, such as DNA damage by radiation or anticancer drugs, the p53 protein becomes stabilised and activated by post-translational modifications (Huang *et al.*, 2015). These include phosphorylation at the N-terminus resulting in reduced p53 affinity for its primary

negative regulator, human double minute 2 homologue (Hdm2). Phosphorylation of p53 at the N-terminus has been reported to promote recruitment of transcriptional co-activators. While phosphorylation at the C-terminal, in response to UV light, activates DNA binding through stabilisation of the p53 tetramer (Foo *et al.*, 2015). Studies have shown that, in response to UV radiation, p53 becomes phosphorylated at serine residues 15 (S15) and 392 (S392). Phosphorylation at S15 prevents binding of the negative regulator of p53, Hdm2, while phosphorylation at S392 induces p53 DNA-binding function. These modifications may appear at the same molecule of p53 (Kapoor *et al.*, 2000). Once activated, p53 regulates cellular differentiation and apoptosis by activating transcription of a number of genes (Yoneda *et al.*, 1999). This includes transcriptional upregulation of *p21*, a gene whose product causes cell cycle arrest at various checkpoints, to give time for assessment of the DNA damage extent (Foo *et al.*, 2015). Once DNA damage is repaired, the cell progresses through the cell cycle, however, if the damage is irreparable, p53 may then induce apoptosis (Kroemer *et al.*, 2007), via the intrinsic pathway, by modulating a number of proapoptotic genes encoding members of the Bcl-2 protein family (Amaral *et al.*, 2010).

Mutated *p53* gene found in most cancers, produces a stable mutant protein whose accumulation is regarded as a hallmark of cancer (Gualberto *et al.*, 1998). Mutant p53 proteins often lose their activity as tumour suppressors and, in turn, gain oncogenic functions that help cells have growth and survival advantages (Rivlin *et al.*, 2011). The *p53* gene mutation in tumourigenesis is three-fold; (i) changes that eliminate function of the wild-type p53, (ii) changes that generate dominant negative activity, through tetramer formation with wild-type p53, and (iii) changes that cause *p53* to exude oncogenic function, through selective growth advantages of cells with mutations, transactivation of new target genes or through inappropriate interaction with other cellular proteins (Foo *et al.*, 2015). Its inactivation as a tumour suppressor protein is a recurrent event in tumourigenesis (Rivlin *et al.*, 2011). Therefore, over time, these cancer cells become progressively resistant to controls that maintain normal tissue. As a result, cancer cells divide more rapidly than their progenitors and become less dependent on signals that regulate the cell division cycle (Cooper, 2000).

1.5 Aim

The aim of the study was to investigate the probable anti-cancer effects of a crude methanol leaf extract of *Dicerocaryum senecioides*.

1.6 Objectives

The objectives of the study were to:

I. Investigate the effect of *D. senecioides* crude methanol leaf extract on HeLa cell viability using the MTT assay.

II. Assess morphological and biochemical features associated with apoptosis in HeLa cells exposed to *D. senecioides* crude methanol leaf extract using qualitative and quantitative apoptosis assays.

III. Determine the effect of *D. senecioides* crude methanol leaf extract on the cell division cycle of cervical HeLa cancer cells using flow cytometry.

IV. Assess the effect of *D. senecioides* crude methanol leaf extract on the expression profiles of apoptosis regulatory proteins using the human apoptosis antibody array kit and Western blotting assay.

CHAPTER TWO

2. Methodology and analytical procedures

2.1 Materials and Equipment

- Waring Commercial Blender (Model 32BL79, Dynamics Corporation, New Hartfort, Connecticut, USA)
- CO_2 incubator (HERA CELL 150i) and Bench top centrifuge (Micro CL 17R), ThermoScientific, Rockford, USA)
- Horeseradish peroxidase (HRP) and Bicinchoninic acid (BCA) assay kit (Pierce®) (ThermoScientific, Rockford, USA)
- Microtiter-Plate Reader, GloMax®-Multi+Detection system (Promega, Madison, USA)
- Inverted light and fluorescent microscope (Nikon ECLIPSE T*i*, Tokyo, Japan)
- Muse® Cell Analyser, Muse™ Cell Cycle Kit and Muse™ MultiCaspase Kit (Merck, Darmstadt, Germany)
- Chemidoc-XRS, PowerPac™ and Trans-Blot®Turbo™ System (Bio-Rad Laboratories, Hercules, USA)
- Sodium dodecyl sulfate (SDS) (Bio-Rad Laboratories, Hercules, USA)
- iBind™ Western Device, iBind™ Cards, iBind™ Solution Kit and Bolt 4-12% Bis-Tris Plus (novex®, lifetechnologies™), (ThermoFisherScientific, Waltham, USA)
- Taxol, Hoechst 33258 (Invitrogen™) (ThermoFisherScientific, Waltham, USA)
- Methanol and Ethanol (Rochelle Chemicals, SA)
- Dimethyl sulfoxide (DMSO), tween-20 and glycine (Merck Chemicals, (PTY) LTD, Darmstadt, Germany)
- HeLa (ATCC® CCL-2™) (biosafety level 2) and KMST-6 (ATCC® PCS-201-012™) (biosafety level 1) cell lines (ATCC, Rockville, USA)
- Dulbecco's Modified Eagle Medium (DMEM) and foetal bovine serum (FBS) (Hyclone, Cramlington, UK)
- Phosphate buffered saline (PBS), Trisaminomethane-hydrochloric acid (Tris-HCl) and 0.25% Trypsin-Ethylenediaminetetraacetic acid (EDTA) (Lonza, Basel, Switzerland)

- 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (life technologies™) (ThermoFisherScientific, Waltham, USA)
- Annexin-V-FITC Apoptosis Detection Kit (eBioscience®, Vienna, Austria)
- Propidium iodide (PI), Ribonuclease A (RNase A), Sodium deoxycholate (Na-deoxycholate), nonyl phenoxypolyethoxylethanol (NP-40) and TRIZMA® (tris base) (Sigma-Aldrich, Saint Louis, USA)
- Human Apoptosis Antibody Array Kit (R&D Systems, Minneapolis USA)
- Caspase-9 antibody (p10, F-7) (mouse monoclonal IgG_{2a}) and Secondary antibody (goat, anti-mouse polyclonal IgG) (Santa Cruz Biotechnology) (Anatech Instruments, Gauteng, South Africa)

2.2 Plant collection and extraction

Dicerocaryum senecioides plant leaves were collected from the University of Limpopo, in the Limpopo province. Plant samples were deposited at the Larry Leach Herbarium (UNIN), at the University of Limpopo, for authentication and voucher specimen. The plant leaves were air-dried and ground to powder using a commercial blender. The powdered leaves were then extracted with absolute methanol over a three day period in a shaker incubator, at ambient temperature. Following extraction, the extract was filtered with filter paper (Whatman no.1) and air-dried using an industrial fan, to obtain the most stable, non-volatile compounds. The dried extract was then dissolved in DMSO to prepare a stock solution of 250 mg/ml, which was aliquoted in 0.5 ml microcentrifuge tubes and kept at -20°C until use.

2.3 Cell culture maintenance and treatment

Cervical HeLa (ATCC® CCL-2™) (biosafety level 2) cancer and human fibroblasts KMST-6 (ATCC® PCS-201-012™) (biosafety level 1) cells were purchased from the American Type Culture Collection (ATCC). The cells were grown in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% (v/v) heat inactivated foetal bovine serum (FBS) and maintained in a tissue culture incubator at 37°C in humidified air containing 5% CO_2. The cells were treated with various concentrations (0-600 µg/ml) of *D. senecioides* crude methanol leaf extract, prepared by diluting the stock solution (250 mg/ml) with culture media. Taxol at 25 nM from a stock solution of 100

nM was diluted in 1x phosphate buffered saline (PBS) and served as a positive control for qualitative and quantitative apoptosis assays.

2.4 Cytotoxicity assay

The effect of *D. senecioides* crude methanol leaf extract on HeLa cancer cells and normal KMST-6 cells was evaluated by measuring the metabolic activity of cells via the (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (MTT) assay. The assay uses a yellow tetrazolium salt (MTT), that is reduced by the activity of NAD(P)H-dependent cellular oxidoreductase enzymes in the mitochondria of viable cells. This reduction leads to a change in colour from yellow to purple (Berridge, 2005). The assay was based on a method described by Mosmann, (1983), with modifications. Cells were seeded in 96-well plates at a density of $1x10^4$/well in 100 µl and allowed to attach in an incubator overnight. The cells were treated with 200 µl of varying concentrations (0-600 µg/ml) of the *D. senecioides* extract for 24 and 48 hours. Following treatment, the supernatant was removed and 100 µL of 1 mg/ml MTT in 1x PBS was added to the cells and incubated for 30 minutes in a tissue culture incubator at 37°C. The MTT solution was then removed and 200 µL of dimethyl sulfoxide (DMSO) added to dissolve the purple formazan salts formed within the cells. For quantification, the absorbance was measured at 560 nm using a GloMax®-Multi+Detection System microtiter plate reader. Data was expressed as percentage viability for KMST-6 cells and percentage viability inhibition for HeLa cells, calculated using the following formulae;

$$\%\,Viability = \frac{Absorbance\ of\ treated\ cells}{Absorbance\ of\ untreated\ cells} \times 100$$

$$\%\,Inhibition = ((1 - \frac{Absorbance\ of\ treated\ cells}{Absorbance\ of\ untreated\ cells}) \times 100)$$

2.5 Qualitative and quantitative apoptosis assays

2.5.1 Cell morphology assay

Changes in the morphology of HeLa cells exposed to the extract were examined by viewing under an inverted light microscope. Cells undergoing apoptosis exhibit various morphological changes such as the shrinking in cell size and formation of irregular cell extensions (Kerr *et al.*, 1972). Cells seeded in 6-well plates at a density of $3.0x10^5$/well, in 3 ml, were allowed to attach in an incubator overnight and treated with 3 ml of 0, 400 and 500 µg/ml of the *D. senecioides* extract and 25 nM taxol, after removal of spent media, for 24 and 48 hours. Following treatment, the cells were washed with 300 µl of 1x PBS by gentle tilting. The cells were then observed and photographed under an inverted light microscope. The data was presented as pictures, with arrows highlighting differences in healthy (untreated) cells and unhealthy (treated) cells.

2.5.2 Cell nuclear morphology assay

The assessment of nuclear morphology by fluorescence microscopy was performed using the cell-permeable nucleic acid stain Hoechst 33258 as described by Cummings *et al.*, (2004), with slight modifications. Cells undergoing apoptosis display condensed chromatin which when bound to by nucleic acid stains emits fluorescence based on the degree of condensation under a fluorescent microscope (Cummings *et al.*, 2004). Cells ($3.0x10^5$/well), in 3 ml, were seeded in 6-well plates, allowed to attach in an incubator overnight and treated with 3 ml of 0, 400 and 500 µg/ml of the *D. senecioides* extract and 25 nM taxol, after careful removal of the spent media, for 24 and 48 hours. The cells were washed twice by gentle tilting in 1 ml of 1x PBS and stained with 25 µg/ml of Hoechst 33258 solution in 1x PBS for 10 minutes at room temperature, in the dark. The cells were then washed twice, as above, viewed and photographed under a fluorescent microscope, excitation/emission (352/461 nm). The data was presented as pictures with arrows highlighting degree of fluorescence in untreated and treated cells.

2.5.3 Percentage determination of apoptotic cells

To determine the onset of apoptosis and quantitatively assess the extent of the *D. senecioides* extract-induced apoptosis, the annexin-V-FITC apoptosis detection kit was used, following the manufacturers protocol. During the early stages of apoptosis, phosphatidylserine (PS) is translocated from the inner to the outer surface of the cell membrane. On the outer surface, PS is recognised and bound to by annexin-V and therefore emits fluorescence (Rysavy *et al.*, 2014), detected by flow cytometry in this assay. Cells at a density of 3.0×10^5/well, in 3 ml, were seeded in 6-well plates and allowed to attach in an incubator overnight. After removal of the spent media, the cells were treated with 3 ml of 0, 400 and 500 µg/ml of the *D. senecioides* extract and 25 nM taxol for 6 and 24 hours. Following treatment, detached cells in the supernatant were collected and attached cells harvested using 300 µl of 1x trypsin. The combined cells were washed by centrifugation at 1000 rpm in 1x PBS and resuspended in binding buffer by gentle pipetting. The cells were then stained with annexin-V-FITC for 10 minutes in the dark, washed as above and resuspended in binding buffer. The cells were counter stained with 20 µg/ml of propidium iodide (PI) and analysed using the Muse® Cell Analyser, recording 2000 events. The data was expressed as percentage of early apoptotic cells (Annexin-V+ and PI-), late apoptotic cells (Annexin-V+ and PI+) and necrotic cells (Annexin-V- and PI+).

2.5.4 Western blotting analysis of caspase-9 protein expression

Effect of the *D. senecioides* extract on protein expression level of caspase-9 was assayed by Western blotting, as described by Hong *et al.*, (2014), with modifications. Caspases play an important role in the process of apoptosis (Abraham, 2004). These caspases are able to initiate an intracellular event cascade and further direct cellular breakdown through cleavage of structural proteins (Portera, 1999). The Western blotting assay is used to detect expression of specific proteins. Cells (5.0×10^6), in 15 ml, were seeded in 75 cm^3 flasks and allowed to attach in an incubator overnight. Following treatment with 15 ml of 0, 400 and 500 µg/ml of the *D. senecioides* extract and 25 nM taxol, cells were harvested by scraping into the media, washed after centrifugation as described in section 2.5.3 and total cellular protein obtained by lysing the cells using the RIPA buffer [50 mM Tris-HCl, 150 mM NaCl, 1.0% (v/v) NP-40, 0.5% (w/v) Na-deoxycholate and 0.1% (w/v) SDS]. Cell lysates were centrifuged at

13,000 x g for 5 minutes at 4°C and protein concentration determined by the bicinchoninic acid (BCA) protein assay kit, following the manufacturer's protocol. Briefly, diluted bovine serum albumin (BSA) standards, in triplicate, as well as extracted protein samples were loaded into a 96-well microtiter plate and the BCA working reagent added at a ratio of 1:8 (v/v, sample to working reagent) onto the standards and samples. The microtiter plate was then covered with a lid and incubated at 37 ºC for 30 minutes, following which, the absorbance was measured at 570 nm using a microtiter plate reader. Forty micrograms of the protein from treated and untreated cells was then resolved on a Bolt 4-12% sodium dodecylsulfate (SDS) polyacrylamide gel at 150 Volts for 30 min in running buffer consisting of 0.1% SDS, 190 mM glycine and 25 mM Tris base at pH 8.3. Following electrophoresis, the proteins were transferred using a blotting buffer [25 mM Tris-HCl (pH 8.3) and 20% methanol] onto a nitrocellulose membrane by electro-blotting for 30 minutes at 25 Volts, using a Trans-Blot®Turbo™ System. The membrane was blocked by incubating with blocking buffer [3% BSA in 0.05% PBS-Tween 20 (PBST) buffer] for 30 minutes, to prevent nonspecific interactions. The membrane was further incubated with a primary antibody (diluted 1:1 000 in PBST) specific for caspase-9, wash buffer and a horseradish peroxidase (HRP)-conjugated secondary antibody (diluted 1:20 000 in PBST) overnight at room temperature using the iBind™ system. The iBind™ system allows for loading of all solutions at the start of the procedure from which point all steps proceed automatically by sequential lateral flow technology (SLF). This was followed by washing of the membrane 3 times with PBST, for 15 minutes on a rocking platform shaker, at room temperature. The membrane was then visualised using the Chemidoc XRS after addition of a substrate for HRP, following the manufacturer's instructions. The data was expressed as expression fold of the untreated control, from densitometric analysis done using Quantity One Software.

2.5.5. Determination of caspase activity

To determine the presence of active caspases in cells treated with the *D. senecioides* extract, the Muse™ MultiCaspase Kit was used, following the manufacturer's protocol. The multicaspase assay detects the presence of multiple caspases (caspase-1,-3,-4,-5,-6,-7,-8 and -9) and simultaneously determines the percentage of cells with caspase activity (Cohen, 1997). Cells (3.0×10^5/well), in 3 ml, were seeded in 6-well plates and

allowed to attach in an incubator overnight. After removal of the spent media, cells were treated with 3 ml of 0, 400 and 500 µg/ml of the *D. senecioides* extract and 25 nM taxol for 24 hours. Following treatment, cells were harvested and washed as described in section 2.5.3 and resuspended in 1x caspase buffer by gentle pipetting. To each tube, 50 µl of the cell suspension and 5 µl of the Muse™ MultiCaspase Reagent working solution were added. The samples were then mixed by gentle pipetting and incubated for 30 minutes at 37ºC in a tissue culture incubator. Following incubation, 150 µl of Muse™ Caspase-7-AAD working solution was added, mixed as above and incubated for 5 minutes at room temperature in the dark. The samples were then analysed using the Muse® Cell Analyser and the data expressed as percentage fold of total caspase activity.

2.5.6 Analyses of apoptotic protein expression using the proteome profiler array

The expression profile of apoptosis regulatory proteins in treated and untreated HeLa cells were analysed using the human apoptosis antibody array kit. The array is used to detect expression of a number of proteins. Cells ($5.0x10^6$), in 15 ml, were seeded in 75 cm^3 flasks and allowed to attach in an incubator overnight. Following removal of the spent media, cells were treated with 15 ml of 0, 400 and 500 µg/ml of the *D. senecioides* extract and 25 nM taxol for 24 hours. To extract total protein from treated and untreated cells, the cells were harvested by scraping into the media, washed after centrifugation as described in section 2.5.3 and lysed following the manufacturer's instructions. Extracted proteins were quantified using the BCA assay kit as described in section 2.5.5. Array membranes were blocked using the provided blocking buffer for 30 minutes. Three hundred microgram of protein from treated and untreated cells was added to respective array membranes. The membranes were incubated at 4°C overnight on a rocking platform shaker, washed 3 times with wash buffer for 10 minutes on a rocking platform shaker and a detection antibody cocktail diluted in array buffer was added. The membranes were then incubated for an hour on a rocking platform shaker. The membranes were washed again as above, after which, diluted streptavidin-HRP was added and the membranes incubated for 30 minutes at room temperature, on a rocking platform shaker. Lastly, the membranes were washed as above and a chemiluminescent-reagent mix added for developing spots which were

then visualised using the Chemidoc-XRS. The data was expressed as a percentage of the untreated control.

2.6 Cell division cycle analysis

The effect of the *D. senecioides* extract on the cell division cycle of HeLa cells was assessed by the Muse™ Cell Cycle kit, following the manufacturer's protocol, with a few modifications. Cells undergoing apoptosis tend to undergo cell cycle arrest. These cells are stained using nucleic acid stains such as PI and classified based on their size and DNA content into respective cell cycle phases (Pucci *et al.*, 2000). Cells ($3.0x10^5$/well), in 3 ml, were seeded in 6-well plates and allowed to attach in an incubator overnight. Following removal of the spent media, cells were treated with 0, 400 and 500 µg/ml of the *D. senecioides* extract and 25 nM taxol for 6 and 24 hours. Following treatment, detached cells in the supernatant were collected and adhering cells harvested and washed as described in section 2.5.3 and fixed in 70% ice-cold ethanol for overnight at 4°C. The combined cells were again washed as above and stained with the Muse™ Cell Cycle reagent for 30 minutes in the dark, at room temperature. The cells where then analysed using a population profile of cell size index vs DNA content index using the Muse® Cell Analyser, with a recording of 5000 events. The data was expressed as percentage of cells in each cell cycle phase.

2.7 Statistical analysis

The data were expressed as mean ± standard deviation (S.D). Statistically significant differences between the untreated control and treatments were determined using the GraphPad InStat 3 Software by one-way ANOVA, followed by Dunnett's comparison test. Differences between means of untreated, taxol-treated and *D. senecioides* extract-treated cells were considered significant at $p \leq 0.05$ (*) and highly significant at $p \leq 0.01$ (**).

CHAPTER THREE

3. Results

3.1 Effect of *Dicerocaryum senecioides* crude methanol leaf extract on human fibroblast KMST-6 viability and viability inhibition of cervical HeLa cancer cells

Cytotoxicity is considered as the first step in the path to understanding a drug's potential use against cancer and hence there is an ongoing search for potent and selective anti-cancer agents (Arora and Tandon, 2015; Badisa *et al*., 2009). Effect of the *D. senecioides* extract on the viability of normal human fibroblast cells, KMST-6, and cervical HeLa cancer cells was evaluated using the MTT assay. The results showed no significant increase in percentage viability inhibition of HeLa cells at the tested concentrations after 24 hours (Figure 3.1A). However, a significant ($p \leq 0.01$) increase in percentage viability inhibition of HeLa cells was observed after 48 hours in cells treated with concentrations from 400 µg/ml and above (figure 3.1C). On the other hand, the extract was shown not to be cytotoxic to KMST-6 cells at concentrations ranging from 0 – 500 µg/ml as the cells were shown to have their viability unaffected, after 24 and 48 hours of treatment (figure 3.1B and 3.1D). The data suggests that at the tested concentrations, the extract is selectively toxic to HeLa cancer cells and not normal KMST-6 cells.

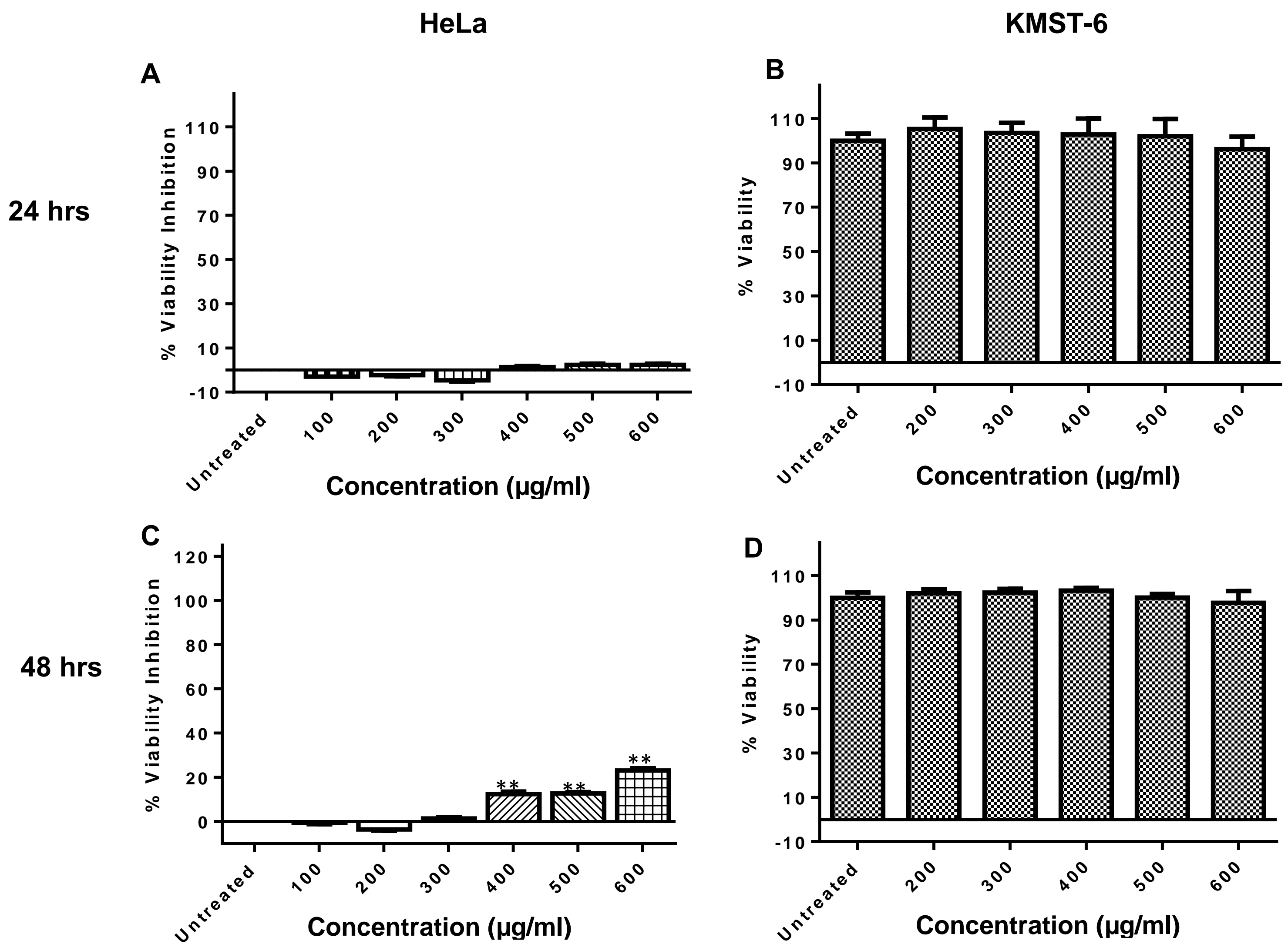


Figure 3.1: Effect of *Dicerocaryum senecioides* crude methanol leaf extract on viability of human fibroblast (KMST-6) cells and viability inhibition of cervical HeLa cancer cells. Cells were incubated with the extract at the indicated concentrations for 24 hours **(A and B)** and 48 hours **(C and D)**. The effect of the extract was then determined using the MTT assay. Each data point represents the mean± S.D of three independent experiments, performed in sextuplicate. *$p \leq 0.05$, ** $p \leq 0.01$, indicate significant differences to the untreated control.

3.2 Effect of *Dicerocaryum senecioides* crude methanol leaf extract on nuclear and cell morphology of HeLa cells

Dying cells exhibit various nuclear and morphological changes that can be used in characterising the type of cell death (Fink and Cookson, 2005). *D. senecioides* extract-induced nuclear and cell morphology changes in cervical HeLa cancer cells were assessed using inverted light microscopy and the Hoechst 33258 staining assay. Extract-treated cells appeared to have shrunk in size and had irregular extensions, typical of apoptotic cell death, when compared to the untreated cells (figure 3.2A). Treatment also induced nuclear morphological changes associated with apoptosis after staining with Hoechst 33258. Chromatin condensation was made apparent by a higher degree of bright blue fluorescence in extract-treated cells when compared to the untreated cells. Furthermore, fluorescence intensity was shown to increase with increasing time and concentration (figure 3.2B). The above mentioned was also observed in taxol-treated cells, however, extract-treated cells showed more intense features associated with apoptosis than those treated with the taxol concentration used in this study. The data therefore suggests that the observed percentage viability inhibition of HeLa cells in section 3.1 is due to the induction of apoptosis.

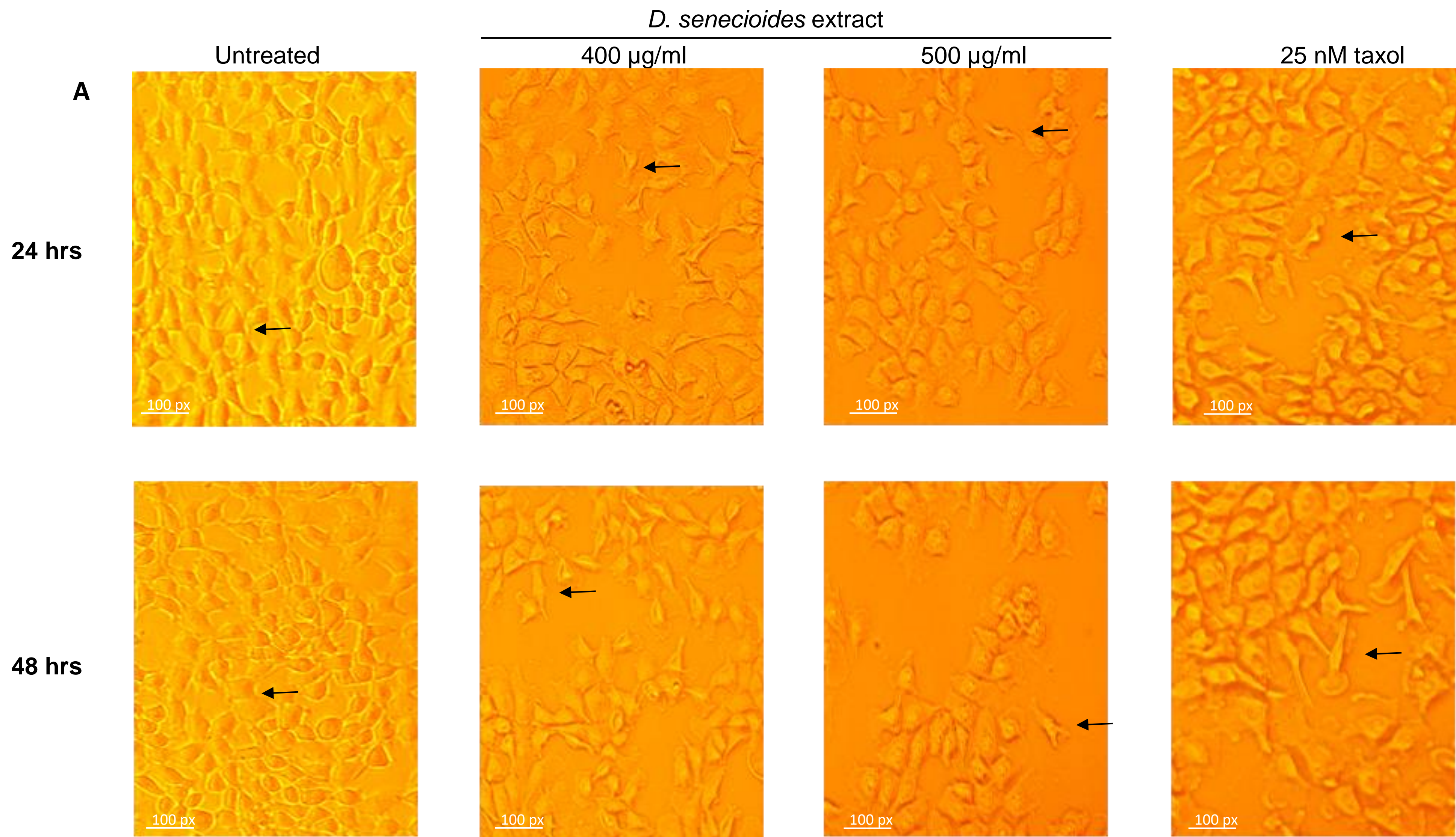


Figure 3.2 A: HeLa cancer cell morphological changes mediated by *Dicerocaryum senecioides* crude methanol leaf extract. Cells were treated with 0, 400 and 500 µg/ml of the extract and 25 nM taxol for 24 and 48 hours. Cells were observed and photographed under an inverted light microscope (40X). Arrows illustrate the difference in morphology of untreated and treated cells. Photographs represent one of three independent experiments.

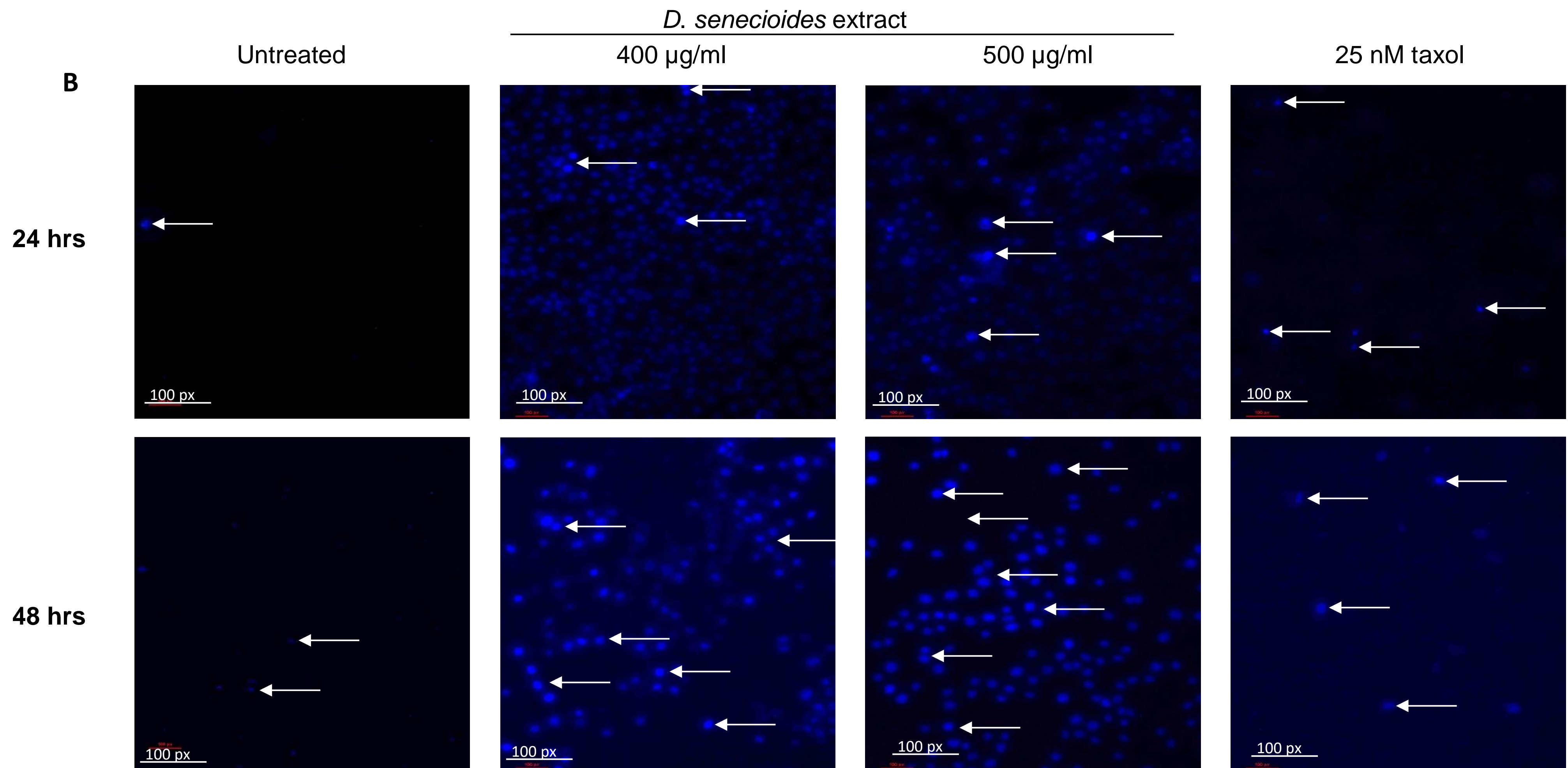


Figure 3.2 B: HeLa cancer cell nuclear morphological changes mediated by *Dicerocaryum senecioides* crude methanol leaf extract. Cells were treated with 0, 400 and 500 µg/ml of the extract and 25 nM taxol for 24 and 48 hours. The cells were stained with Hoechst 33258, viewed and photographed under a fluorescence microscope (20X). Arrows illustrate the difference in bright blue fluorescence between treated and untreated cells. Photographs represent one of three independent experiments.

3.3 Determination of the onset of apoptosis and percentage of HeLa cells undergoing apoptosis following treatment with *Dicerocaryum seneciodes* crude methanol leaf extract

During the early stages of apoptosis, phosphatidylserine (PS) is translocated from the inner surface to the outer surface of the cell's membrane for recognition by phagocytic cells (Wlodkowic *et al*., 2012). In this assay, annexin-V is used to detect the onset of apoptosis by binding onto the exposed PS of the cell. Annexin-V and PI combination is used to detect cells in late stages of apoptosis as PI stains the nucleus of cells whose membrane is no longer intact and thus PI alone detects necrotic cells. The data showed a significant ($p \leq 0.01$) increase in percentage of cells stained with annexin-V and annexin-V/PI following 6 hours of treatment with 400 µg/ml of the extract (figure 3.3B). Treatment with the extract after 24 hours revealed no significant increase in percentage of cells stained with either annexin-V, annexin-V/PI or PI, when compared to the untreated cells (figure 3.4B). Taxol-treated cells also showed no significant increase in percentage of cells stained with annexin-V and annexin-V/PI and PI, following 6 and 24 hours of treatment, when compared to the untreated cells (figure 3.3B and 3.4B). Figures 3.3A and 3.4A serve as a representation of the apoptosis profile taken from one experiment. The data therefore suggest induction of apoptosis in extract-treated cells as early as 6 hours.

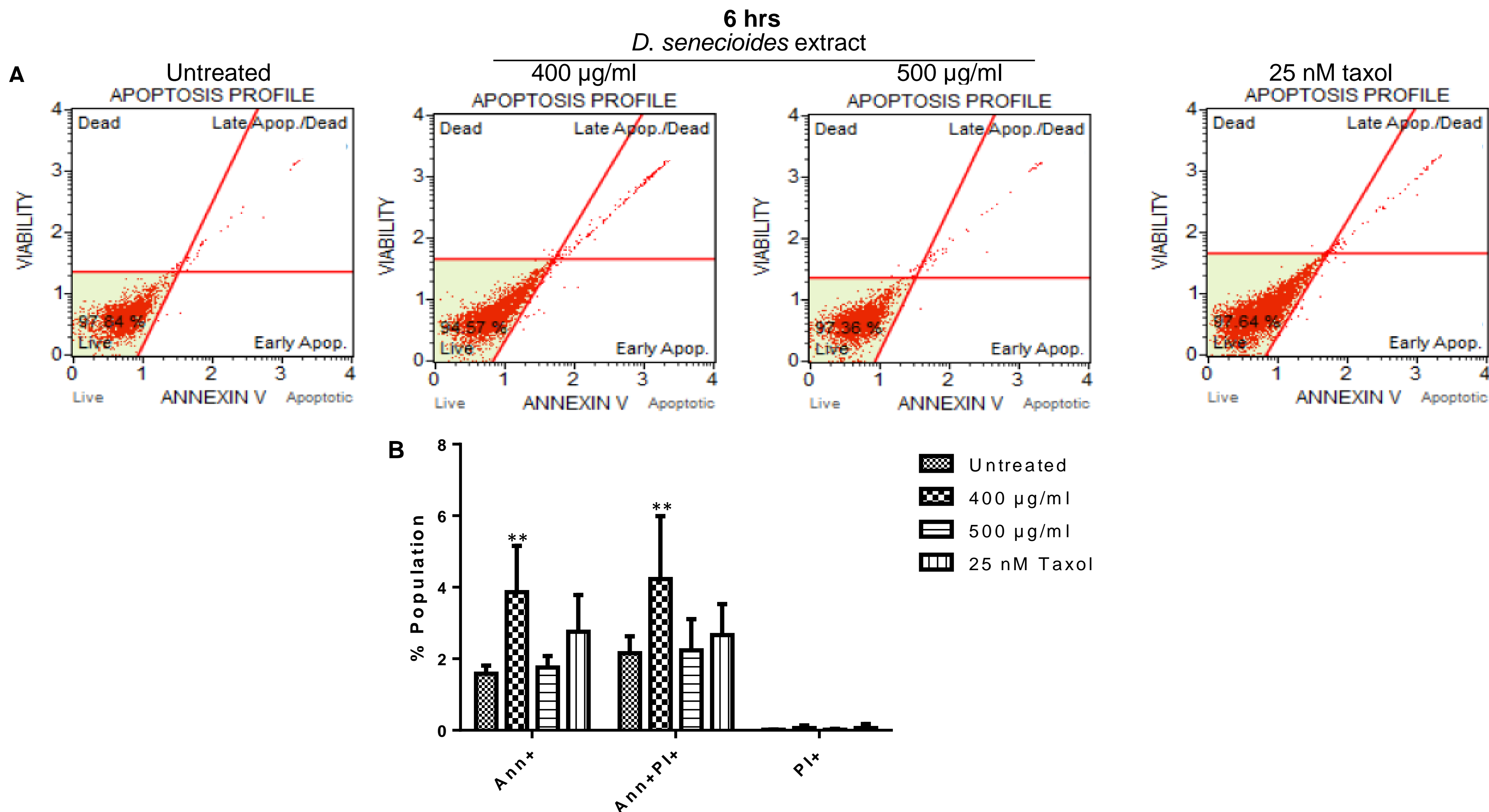


Figure 3.3: Effect of *Dicerocaryum* senecioides crude methanol leaf extract on the onset and percentage of apoptosis in cervical HeLa cancer cells. Cells were seeded and treated with 0, 400 and 500 µg/ml of the extract and 25 nM taxol and incubated for 6 hours. Following treatment, cells were harvested, stained with annexin-V and PI and subjected to flow cytometric analysis. Graph shows percentages of cells stained by annexin-V and/or PI. The data shown represents the mean± S.D. of three independent experiments, performed in duplicate. *p ≤ 0.05, ** *p* ≤ 0.01, indicate significant differences to the untreated control.

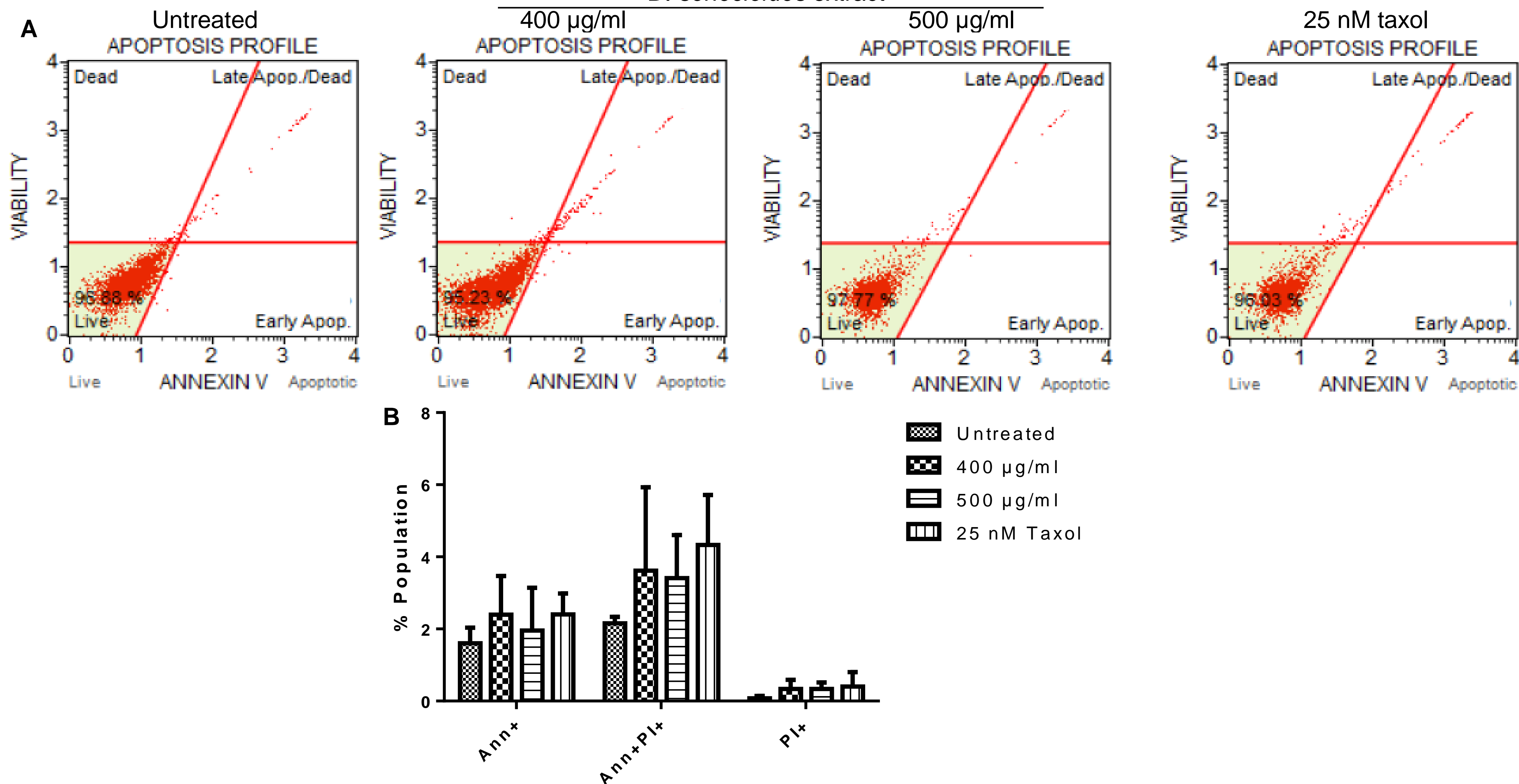


Figure 3.4: Effect of *Dicerocaryum* senecioides crude methanol leaf extract on the onset and percentage of apoptosis in cervical HeLa cancer cells. Cells were seeded and treated with 0, 400 and 500 µg/ml of the extract and 25 nM taxol and incubated for 24 hours. Following treatment, cells were harvested, stained with annexin-V and PI and subjected to flow cytometric analysis. Graph shows percentages of cells stained by annexin-V and/or PI. The data shown represents the mean± S.D. of three independent experiments performed in duplicate. $*p \leq 0.05$, $** p \leq 0.01$, indicate significant differences to the untreated control.

3.4 Effect of *Dicerocaryum senecioides* crude methanol leaf extract on expression level of the Bcl-2 protein family

The Bcl-2 family of proteins are known to play a vital role in either inhibition or promotion of apoptosis (Hassan *et al.*, 2014). To investigate the effect of the *D. senecioides* extract on expression patterns of some of the members of the Bcl-2 family of proteins in cervical HeLa cancer cells, the human apoptosis antibody array kit was used. The results revealed that the proapoptotic, Bax, protein expression level was significantly ($p \leq 0.01$) upregulated after 24 hours of treatment, with 400 µg/ml of the extract (figure 3.5). The Bcl-2 protein expression level showed no significant difference following extract and taxol treatment, when compared to untreated cells. Furthermore, pro- and antiapoptotic proteins Bad and Bcl-x, respectively, showed no significant change following extract-treatment, when compared to untreated cells. Taxol-treated cells on the other hand showed a significant ($p \leq 0.01$) downregulation of Bad protein expression level and no change in expression level of Bcl-x when compared to untreated cells. The data, therefore, suggest modulation in Bax to Bcl-2 ratio (table 1) and membrane pore formation, typical of apoptosis, in extract-treated cells.

Table 1: Bax:Bcl-2 ratio.

Bax:Bcl-2			
Untreated	400 µg/ml	500 µg/ml	25 nM Taxol
1	1.8	0.7	0.8

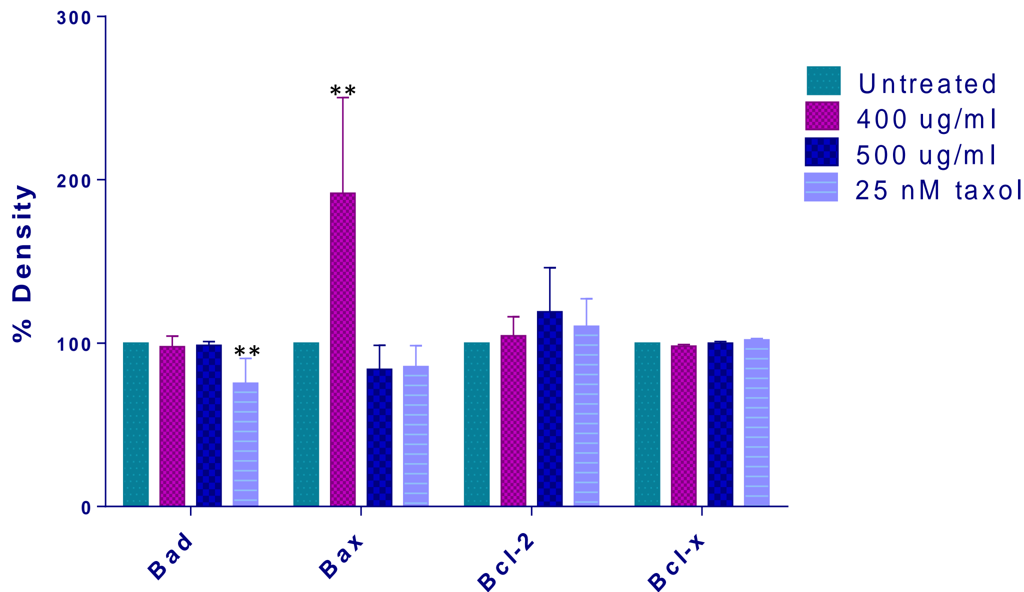


Figure 3.5: Effect of *Dicerocaryum senecioides* crude methanol leaf extract on the expression profile of pro- and anti-apoptotic proteins, **in HeLa cancer cells**. Cells were treated with 0, 400 and 500 µg/ml of the extract and 25 nM taxol for 24 hours. The expression level of the proteins was determined using the human apoptosis antibody array kit. The density of each spot relative to untreated control is shown by bar graphs. Each data point represents the mean± S.D of two independent experiments performed in duplicate. $*p \leq 0.05$, $** \ p \leq 0.01$, indicate significant differences to the untreated control.

3.5 Effect of *Dicerocaryum senecioides* crude methanol leaf extract on the release of mitochondrial proteins

Following modulation of the Bcl-2 family of proteins, the mitochondria becomes porous and releases proapoptotic proteins (cytochrome *C*, Omi/HtrA2 and Smac/Diablo) which participate in activation of a caspase cascade, that ultimately leads to apoptosis (Elmore, 2007). Exposure of cells to the extract for 24 hours resulted in a significant ($p \leq 0.01$) release of the proapoptotic protein cytochrome *C* into the cytosol, for both extract concentrations (figure 3.6). Omi/HtrA2 and Smac/Diablo, however, were seen to be present in no significant amounts in the cytoplasm, following treatment with 400 and 500 µg/ml of the extract, when compared to the untreated cells. Treatment with taxol showed a marked, but not significant decrease in cytochrome *C* and Omi/HtrA2 levels. The data confirms mitochondrial membrane permeabilisation and therefore, suggest involvement of the intrinsic pathway of apoptosis.

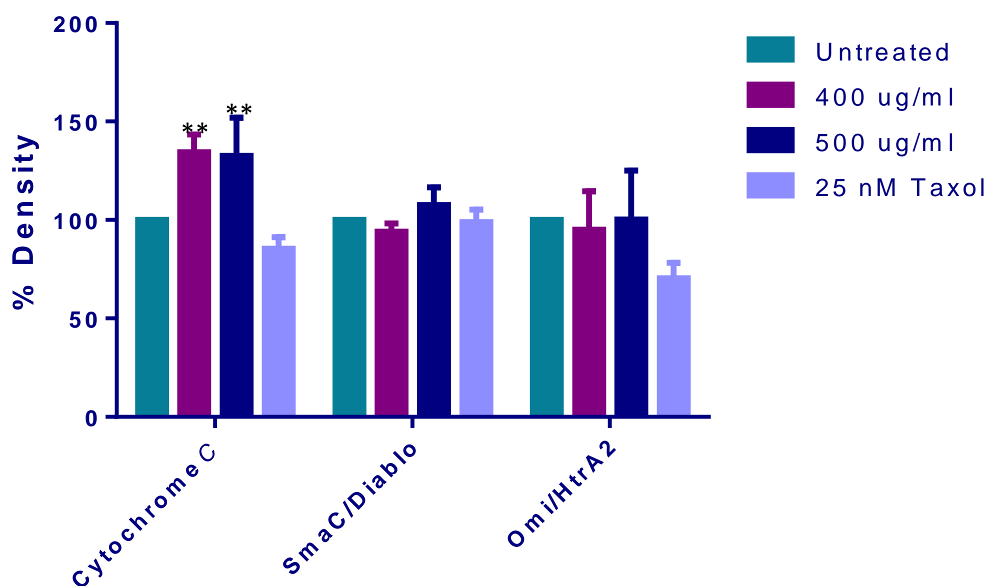


Figure 3.6: Effect of *Dicerocaryum senecioides* crude methanol leaf extract on the release of mitochondrial proapoptotic proteins in HeLa cancer cells. Cells were treated with 0, 400 and 500 µg/ml of the extract and 25 nM taxol for 24 hours. The expression level of the proteins was determined using the human apoptosis antibody array kit. The density of each spot relative to untreated control is shown by bar graphs. Each data point represents the mean± S.D of two independent experiments performed in duplicate. *$p \leq 0.05$, ** $p \leq 0.01$, indicate significant differences to the untreated control.

3.6 Effect of *Dicerocaryum senecioides* crude methanol leaf extract on caspase expression level and activity

The release of mitochondrial proteins leads to the activation of initiator caspase-9 which in turn participates in the cleavage of pro-caspase-3 to caspase-3, in order to execute apoptosis (Chandra and Tang, 2003). Western blot results showed an upregulation in caspase-9 protein expression level with 500 µg/ml of the extract as well as with 25 nM taxol after 24 hours of treatment (figure 3.17A). Treatment with 400 µg/ml of the extract, however, led to the downregulation of caspase-9 protein expression level. Pro-caspase-3 showed a significant ($p \leq 0.01$) upregulation following 24 hours of treatment with 500 µg/ml, while cleaved/active caspase-3 showed no significant change following extract treatment (figure 3.7B). On the other hand, treatment with taxol revealed a significant downregulation of pro-caspase-3 ($p \leq 0.01$) and cleaved caspase-3 ($p \leq 0.05$) expression levels. Furthermore, multicaspase assay revealed that cells treated with 500 µg/ml of the extract showed an increase in caspase activity of ~2 fold (figure 3.8B). Figure 3.7C serves as a loading control for the Western blotting results and figure 3.8A represents total caspase activity from one independent experiment. The data therefore suggests participation or activity of initiator and executioner caspases involved in the intrinsic pathway of apoptosis in extract-treated cells.

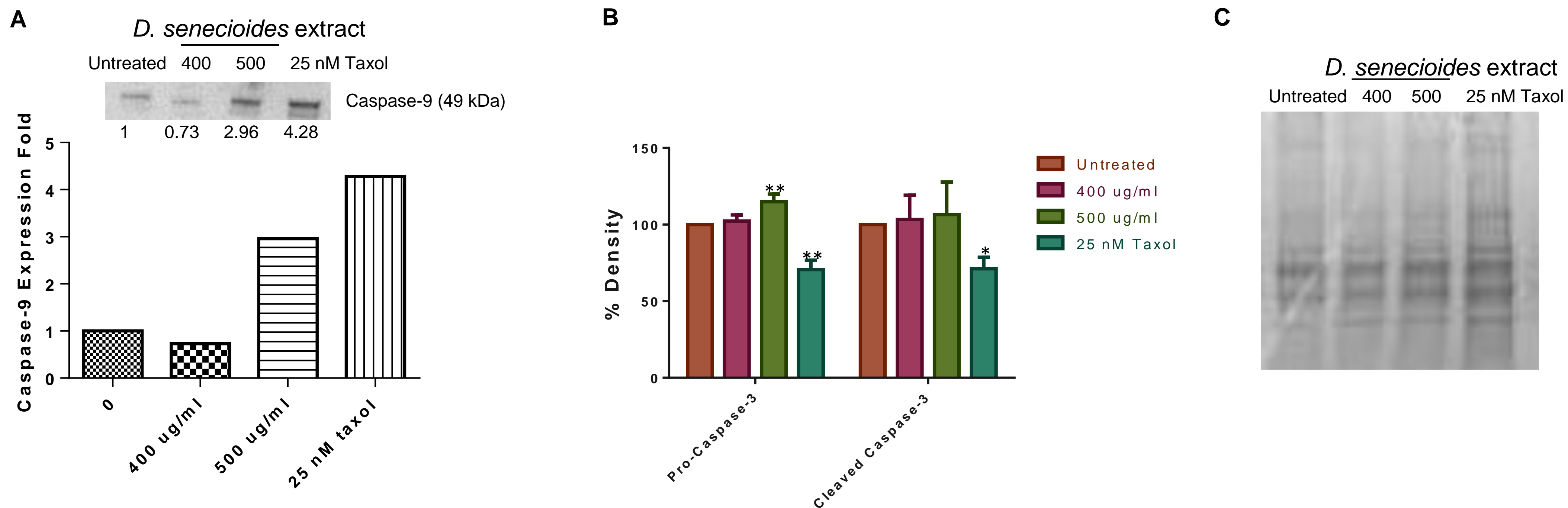
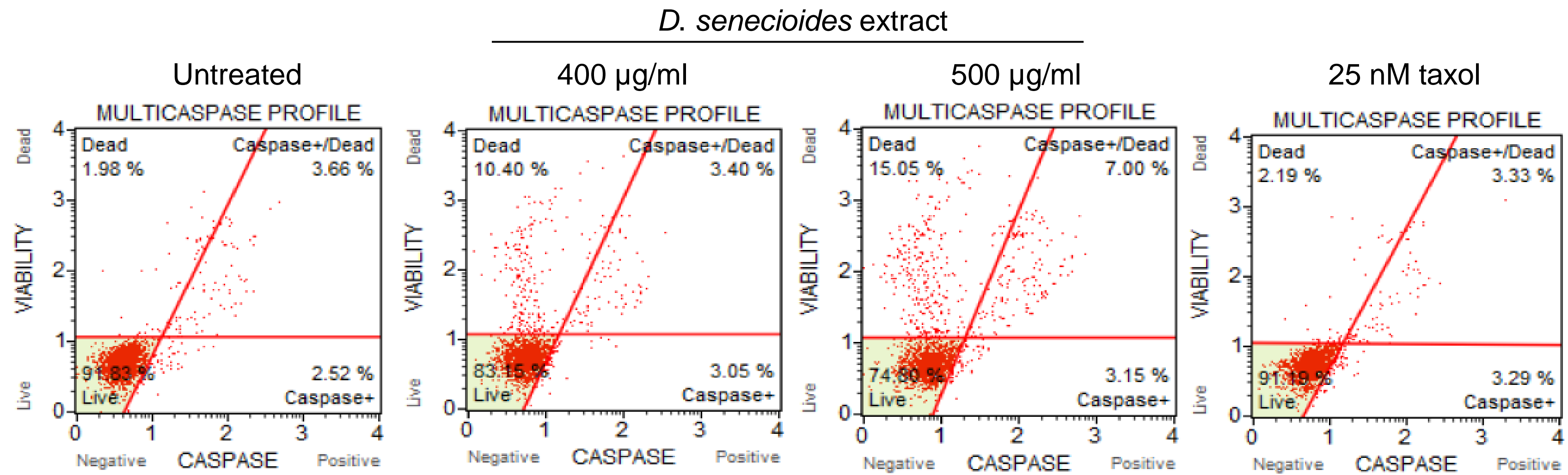


Figure 3.7: Effect of *Dicerocaryum senecioides* crude methanol leaf extract on caspase expression in HeLa cancer cells. Cells were treated with 0, 400 and 500 µg/ml of the extract and 25 nM taxol for 24 hours. Caspase expression was determined using Western blotting **(A)** and the human apoptosis antibody array kit **(B)**. The density of each spot relative to untreated control is shown by bar graphs. Each data point represents the mean± S.D of two independent experiments, performed in duplicate **(B)**. The SDS protein gel **(C)** was used as a loading control for Western blotting **(A)**. *$p \leq 0.05$, ** $p \leq 0.01$, indicate significant differences to the untreated control.



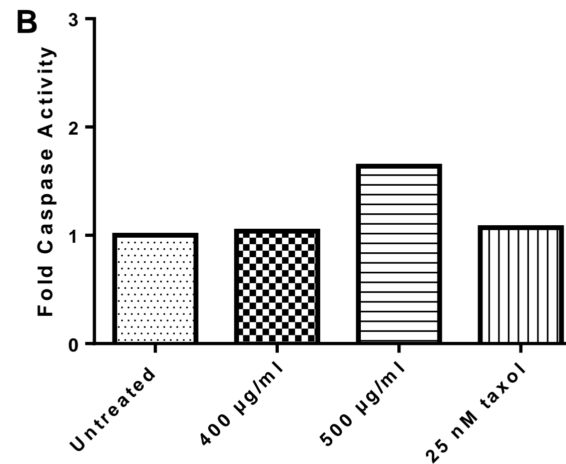


Figure 3.8: Effect of *Dicerocaryum senecioides* crude methanol leaf extract on caspase activity of HeLa cancer cells. Cells were treated with 0, 400 and 500 µg/ml of the extract and 25 nM taxol for 24 hours. Caspase activity was determined using the Muse™ MultiCaspase Kit and analysed using the Muse® Cell Analyser. Percentage fold of total caspase activity is shown by bar graphs. Each data point represents the mean of two independent experiments.

3.7 Effect of *Dicerocaryum senecioides* crude methanol leaf extract on apoptosis inducing receptors and death domains

The extrinsic pathway of apoptosis is mediated by a number of receptors that transmit death signals from the surface of the cell through associated death domains (Guicciardi and Gores, 2009). Thus far, best characterised ligands and corresponding death receptors include FasL/FasR and TNF-α/TNFR1 (Elmore, 2007). Treatment with 400 µg/ml of the extract for 24 hours had no effect on the expression level of TRAIL-R1, while treatment with 500 µg/ml showed no significant difference when compared to the untreated cells (figure 3.9). The Fas-associated death domain protein (FADD) showed no significant change in expression level, following treatment with 400 µg/ml of the extract, when compared to the untreated cells. Treatment with 500 µg/ml of extract, however, showed FADD expression to be markedly, but not significantly upregulated. No significant effect was observed for the tumour necrosis factor receptor-1 (TNF-R1) following treatment with 400 and 500 µg/ml of the extract, when compared to the untreated cells. Moreover, FasR, a member of the tumour necrosis factor (TNF), also showed no significant difference, following treatment with 400 and 500 µg/ml of the extract, when compared to the untreated cells. Taxol-treated cells, on the other hand, showed marked downregulated expression levels of all the proteins investigated except for FasR, which showed a significant ($p \leq 0.01$) downregulation. The data therefore suggest no involvement of the extrinsic pathway in the extract-induced apoptosis.

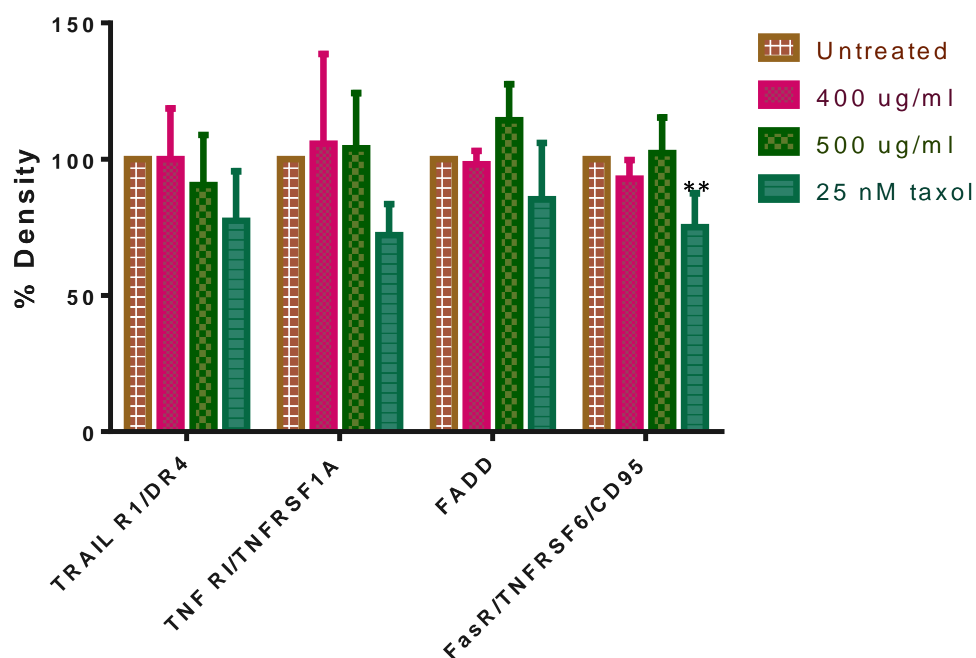


Figure 3.9: Effect of *Dicerocaryum senecioides* crude methanol leaf extract on the expression profile of apoptosis ligands and receptors, **in HeLa cancer cells**. Cells were treated with 0, 400 and 500 µg/ml of the extract and 25 nM taxol for 24 hours. The expression level of the proteins was determined using the human apoptosis antibody array kit. The density of each spot relative to untreated control is shown by bar graphs. Each data point represents the mean± S.D of two independent experiments performed in duplicate. *$p \leq 0.05$, ** $p \leq 0.01$, indicate significant differences to the untreated control.

3.8 Effect of *Dicerocaryum senecioides* crude methanol leaf extract on the expression profile of inhibitors of apoptosis

Signalling and progression of apoptosis is highly suppressed by inhibitors of apoptosis (IAPs). These act by binding and inhibiting caspase activity and thus apoptosis (Kocab and Duckett, 2016). The cellular inhibitor of apoptosis protein-2 (cIAP-2) was markedly, but not significantly upregulated, following 24 hours of treatment with the extract (figure 3.10). Inhibitors of caspase activity, Survivin and XIAP, showed no notable change in expression level, when compared to the untreated cells. The antiapoptotic and caspase inhibitor protein, Livin showed a concentration-dependent marked, but not significant upregulation, while the promoter of tumour survival protein, Clusterin, showed no significant change following treatment with the extract, when compared to the untreated cells. On the other hand, taxol-treated cells showed a significant ($p \leq 0.01$) downregulation of all the proteins investigated, except for Livin, where the decrease was not significant. The data therefore suggest a limited role of IAPs under current experimental conditions.

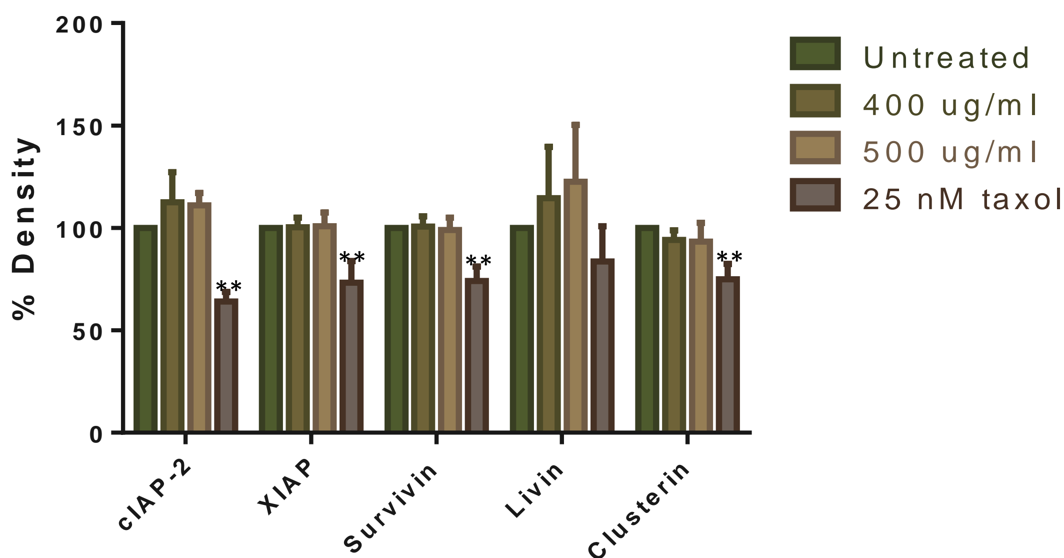


Figure 3.10: Effect of *Dicerocaryum senecioides* crude methanol leaf extract on the expression profile of inhibitors of apoptosis and caspase activity in HeLa cancer cells. Cells were treated with 0, 400 and 500 µg/ml of the extract and 25 nM taxol for 24 hours. The expression level of the proteins was determined using the human apoptosis antibody array kit. The density of each spot relative to untreated control is shown by bar graphs. Each data point represents the mean± S.D of two independent experiments performed in duplicate. *$p \leq 0.05$, ** $p \leq 0.01$, indicate significant differences to the untreated control.

3.9 Effect of *Dicerocaryum senecioides* crude methanol leaf extract on the expression profile of ROS-dependent, heat shock and cell survival proteins

As a result of stressful conditions, cells produce heat shock proteins (hsp), to make them resistant to stress-induced cell damage, thereby regulating apoptosis (Fulda *et al.*, 2010). Treatment with the extract for 24 hours showed to have no noteworthy effect on Hsp60 and Hsp70 expression level, when compared to the untreated cells (figure 3.11). Expression level of the human enzyme, paraoxonase-2 (PON-2), responsible for cell survival was increased significantly ($p \leq 0.01$) with treatment at 400 µg/ml and markedly with 500 µg/ml of the extract. Heme oxygenases 1 and 2 (HO-1 and HO-2) were shown to be markedly, but not significantly downregulated, following treatment with 400 µg/ml of the extract, while treatment with 500 µg/ml of the extract had no effect on the expression level of HO-1 and HO-2. On the other hand, treatment with taxol downregulated the expression level of HO-1 and HO-2 significantly ($p \leq 0.01$), when compared to the untreated cells. The data therefore suggest possible promotion of cell survival by PON 2 in extract-treated cells, especially with the concentration of 400 µg/ml.

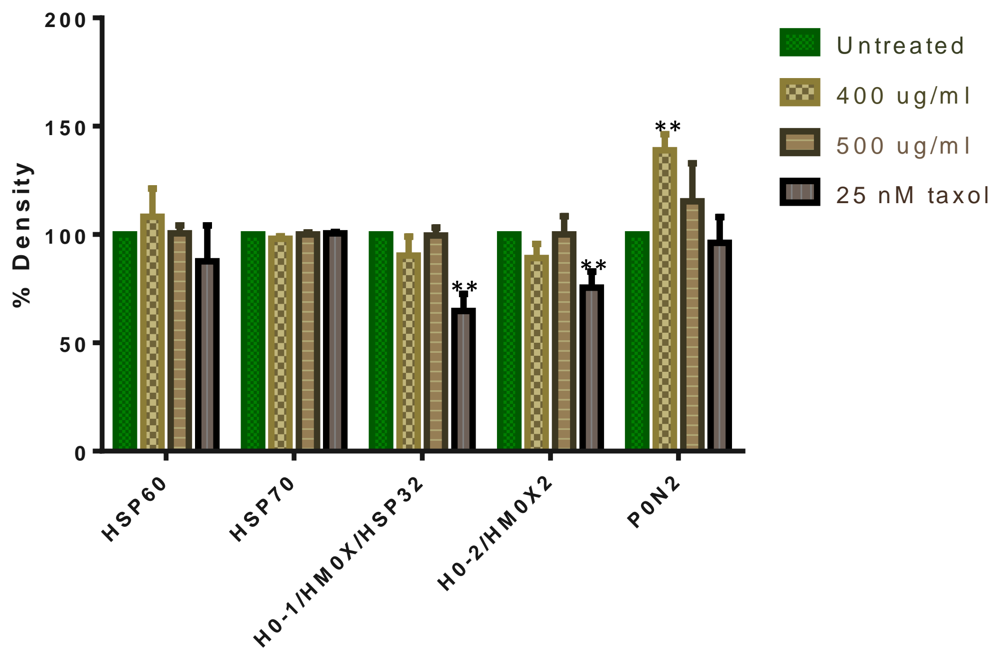


Figure 3.11: Effect of *Dicerocaryum senecioides* crude methanol leaf extract on the expression profile of ROS dependent, **heat shock and cell survival proteins in HeLa cancer cells.** Cells were treated with 0, 400 and 500 µg/ml of the extract and 25 nM taxol for 24 hours. The expression level of the proteins was determined using the human apoptosis antibody array kit. The density of each spot relative to untreated control is shown by bar graphs. Each data point represents the mean± S.D of two independent experiments performed in duplicate. *$p \leq 0.05$, ** $p \leq 0.01$, indicate significant differences to the untreated control.

3.10 Effect of *Dicerocaryum senecioides* crude methanol leaf extract on p53 phosphorylation

Phosphorylation of the p53 protein is essential for its stability and function as a tumour suppressor protein (Ashcroft *et al*., 1999). Following 24 hours of treatment with the extract, data revealed significantly ($p \leq 0.01$) increased levels of p53 phosphorylation at serine residue 46 (S46). A significant ($p \leq 0.01$) increase in p53 phosphorylation at serine residue 392 (S392) was observed only in cells treated with 500 µg/ml of the extract (figure 3.12). On the other hand, taxol-treated cells showed markedly, but not significantly decreased levels of phosphorylation of p53 at S46 and at S392 showing a significant ($p \leq 0.05$) decrease in phosphorylation. The data therefore suggest extract-induced activity of p53 and further confirms the induction of apoptosis via the intrinsic pathway, in extract treated cells.

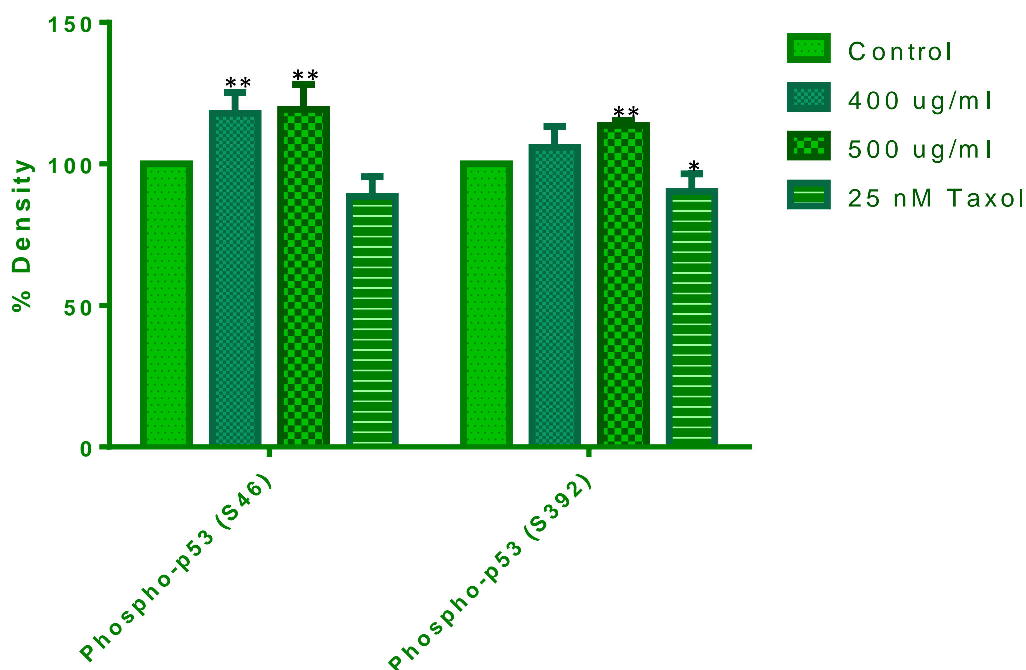


Figure 3.12: Effect of *Dicerocaryum senecioides* crude methanol leaf extract on p53 phosphorylation in HeLa cancer cells. Cells were treated with 0, 400 and 500 µg/ml of the extract and 25 nM taxol for 24 hours. The expression level of the proteins was determined using the human apoptosis antibody array kit. The density of each spot relative to untreated control is shown by bar graphs. Each data point represents the mean± S.D of two independent experiments performed in duplicate. *$p \leq 0.05$, ** $p \leq 0.01$, indicate significant differences to the untreated control.

3.11 Effect of *Dicerocaryum senecioides* crude methanol leaf extract on the cell division cycle of cervical HeLa cancer cells

The emergence of apoptosis corresponds with arrest of the cell division cycle (Pucci *et al*., 2000). In order to assess if the induction of apoptosis in extract-treated cells is associated with arrest of cells, the effect of the extract on the cell division cycle was investigated. Cells treated with the extract showed a significant ($p \leq 0.01$) decrease in percentage of cells distributed at the G0/G1 phase, following 6 hours of treatment with 500 µg/ml of the extract and taxol. However, there was no subsequent increase in percentage of cells distributed within the S nor the G2/M phase, when compared to the untreated cells to suggest cell cycle arrest (figure 3.13B). Furthermore, cells treated with the extract and taxol for 24 hours showed no significant change in cell distribution at any of the cell cycle phases (figure 3.14B). Figures 3.13A and 3.14A serve as a representation of the DNA profile in the cell distribution cycle taken from one experiment. The observed data suggests no apparent cell cycle arrest in extract- or taxol-treated cells, after 6 and 24 hours.

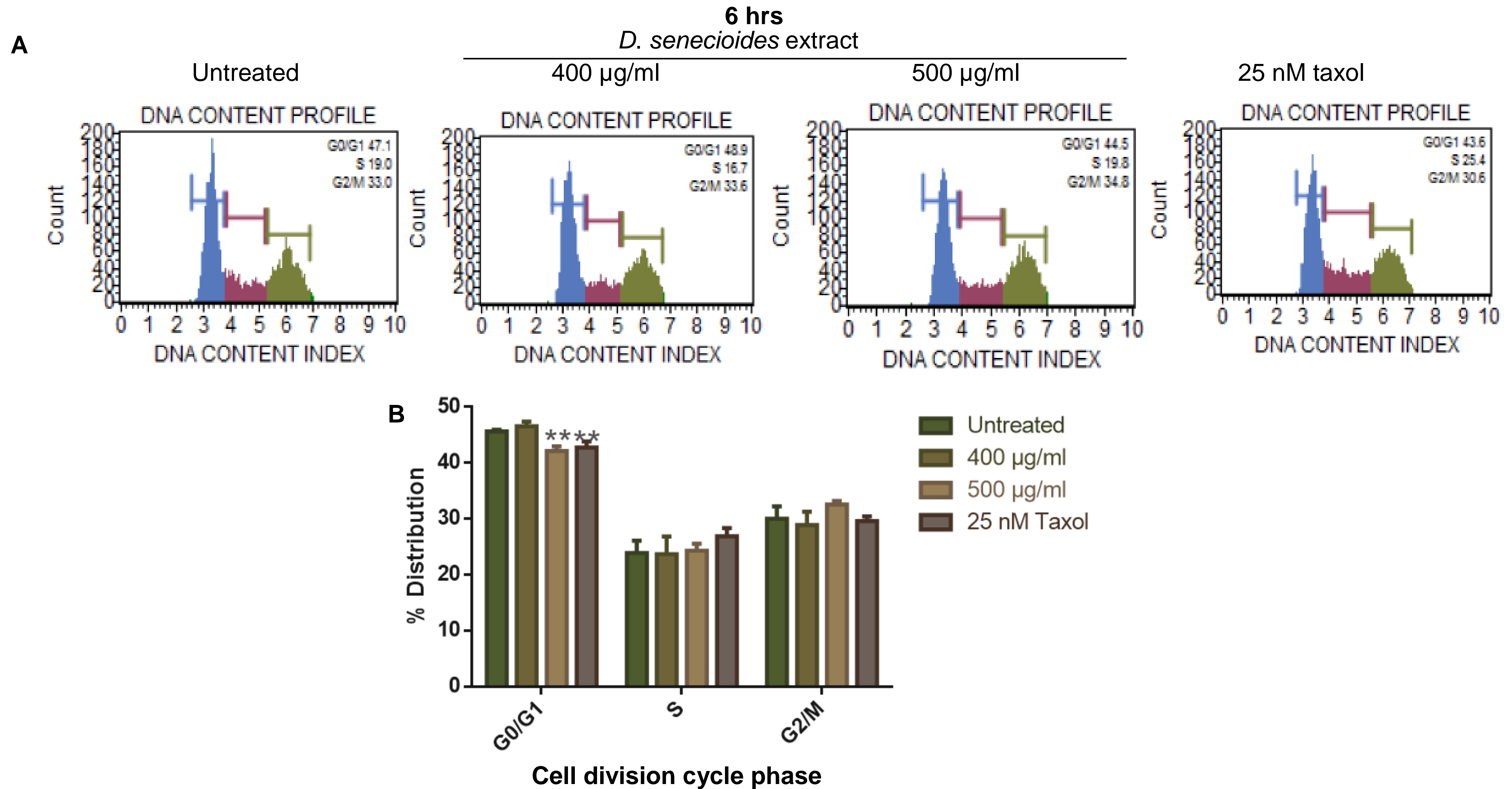


Figure 3.13: Effect of *Dicerocaryum senecioides* crude methanol leaf extract on the cell division cycle of HeLa cancer cells. Cells were seeded and treated with 0, 400 and 500 µg/ml of the extract and 25 nM taxol and incubated for 6 hours. Following treatment, cells were harvested, fixed in 70% ethanol and stained with PI. Cells were then subjected to flow cytometric analysis. Graph shows percentage of cells in various cell cycle phases. The data shown represents the mean± S.D. of three independent experiments, performed in duplicate. $*p \leq 0.05$, $**\ p \leq 0.01$, indicate significant differences to the untreated control.

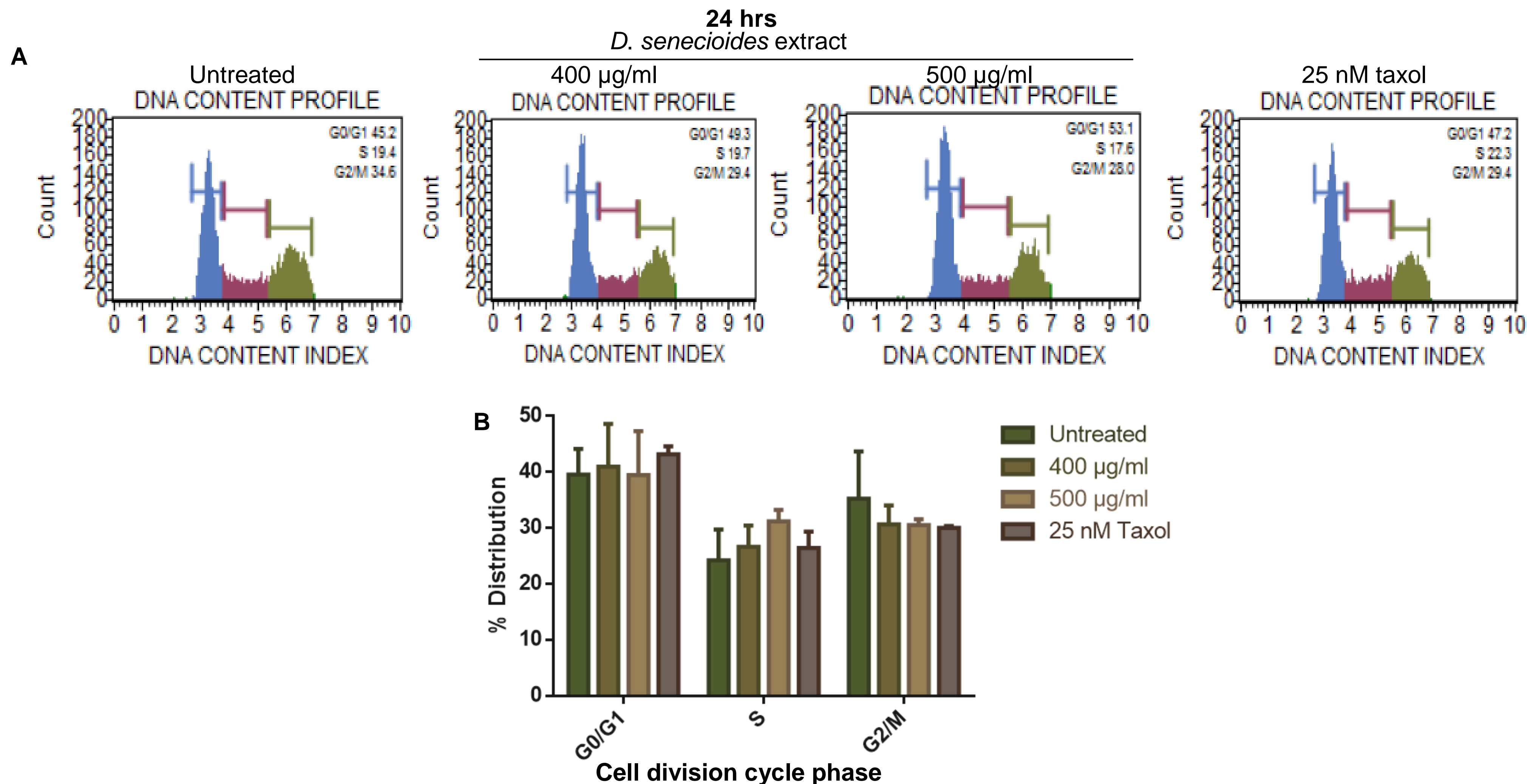


Figure 3.14: Effect of *Dicerocaryum senecioides* crude methanol leaf extract on the cell division cycle of HeLa cancer cells. Cells were seeded and treated with 0, 400 and 500 µg/ml of the extract and 25 nM taxol and incubated for 24 hours. Following treatment, cells were harvested, fixed in 70% ethanol and stained with PI. Cells were then subjected to flow cytometric analysis. Graph shows percentage of cells in various cell cycle phases. The data shown represents the mean± S.D. of three independent experiments, performed in duplicate. *$p \leq 0.05$, ** $p \leq 0.01$, indicate significant differences to the untreated control.

3.12 Effect of *Dicerocaryum senecioides* on expression levels of checkpoint activation and cell cycle arrest proteins

Cell cycle phase transitioning is governed by a number of checkpoints activated by proteins such as Phosphorad17 (RAD17) (Delacroix *et al.*, 2007) and Claspin (Sierant, 2010). The activator of the G1/S checkpoint, RAD17, following 24 hours of treatment with 500 µg/ml of the extract was found significantly (p≤0.01) upregulated (figure 3.15). However, the activator of the G2/M checkpoint, Claspin, showed no significant difference, when compared to the untreated cells. p27, a cyclin-dependent kinase inhibitor that regulates transition from the G0 to the S phase (Chu *et al.*, 2008), also showed no significant difference following treatment with the extract, when compared to untreated cells. In contrast, p21 protein, which also acts as a cyclin-dependent kinase inhibitor (CDKI), was significantly (P≤0.05) upregulated in cells treated with 500 µg/ml of the extract. On the other hand, taxol-treated cells revealed no difference in expression level when compared to the untreated cells. The data therefore suggest inhibition of CDKs by p21.

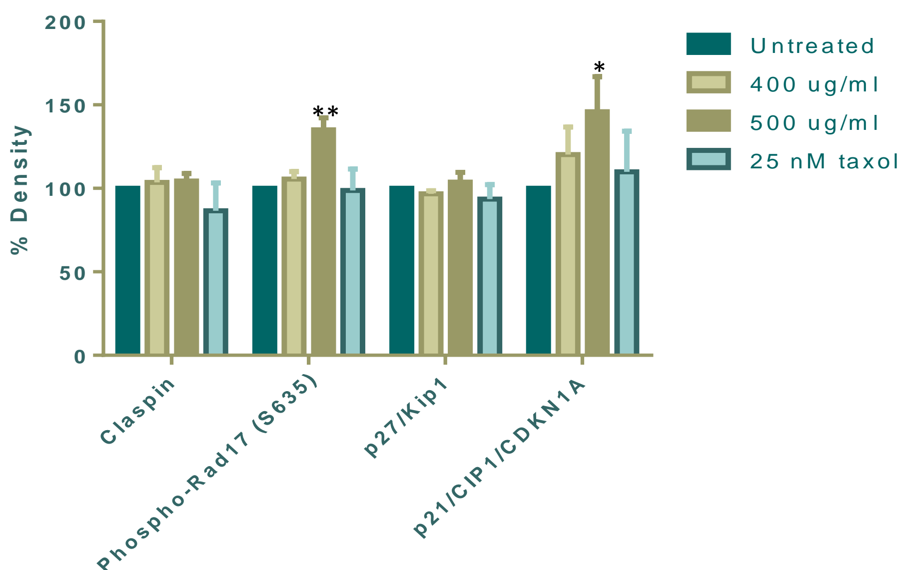


Figure 3.15: Effect of *Dicerocaryum senecioides* crude methanol leaf extract on the expression profiles of checkpoint activators and cell cycle arrest proteins in HeLa cancer cells. Cells were treated with 0, 400 and 500 µg/ml of the extract and 25 nM taxol for 24 hours. The expression level of the proteins was determined using the human apoptosis antibody array kit. The density of each spot relative to untreated control is shown by bar graphs. Each data point represents the mean± S.D of two independent experiments performed in duplicate. *p ≤ 0.05, ** p ≤ 0.01, indicate significant differences to the untreated control.

CHAPTER FOUR

4. Discussion and Conclusion

Millions of women across the globe continue to suffer and die from cervical cancer (Ferlay *et al.*, 2013b). This is because the current cancer treatments have shown limited survival benefits, especially when used for most advanced stage cancers (Hu and Fu, 2012).

The crude methanol leaf extract of *Dicerocaryum senecioides* has demonstrated anti-proliferative and proapoptotic activity against Jurkat T cancer cells (Mphahlele, 2008). In this study, the extract was investigated for its potential use as an anti-cancer agent against cervical HeLa cancer cells. The first step in understanding the potential use of a drug against cancer involves evaluating its cytotoxicity (Arora and Tandon, 2014).

A well sought after cancer drug is one that exerts maximal toxicity to cancer cells while sparing normal cells (Kuroda *et al.*, 2015). In the evaluation of potential selective cytotoxicity of the *Dicerocaryum senecioides* crude methanol leaf extract, viability of the human fibroblasts (KMST-6) and cervical HeLa cancer cells treated with the extract was assessed, using the MTT assay. The increase in viability inhibition of HeLa cells (figure 3.1A and 3.1C) suggested selective cytotoxicity, as the viability of KMST-6 cells was shown not to be affected (figure 3.1B and 3.1D).

Necrosis and apoptosis are distinct modes of cell death characterised by varying morphological and biochemical features. Necrosis occurs in response to a number of events such as, severe cell injury and depletion of the cell's energy and is characterised by swelling of intracellular organelles and cell lysis (Kaminski *et al.*, 2002; Kanduc *et al.*, 2002). In contrast, apoptosis is induced by a variety of physiological stimuli and pharmacological agents (Kerr *et al.*, 1972), and displays distinct morphological changes and biochemical features. These include chromatin condensation, nuclear fragmentation, membrane blebbing and cell shrinkage. Furthermore, loss of membrane integrity and ultrastructural modification of the cytoplasm organelles are observed in the later stages of apoptosis (Wong, 2011).

In this study, the increase in HeLa cell viability inhibition was associated with the induction of apoptosis, as seen by the presence of cell morphological changes, such

as cell shrinkage and the presence of irregular shaped extensions (figure 3.2A). Hoechst 33258 is a nucleic acid stain that emits blue fluorescence when bound to double-stranded DNA and is used to assess the integrity of cell nuclei. Since apoptotic cells have condensed chromatin, nuclei of these cells would appear bright blue as opposed to nuclei of cells that are not undergoing apoptosis. The increase in the number of cell nuclei emitting bright blue fluorescence observed in extract-treated cells (figure 3.3B), further suggested apoptosis as the mode of cell death.

Another feature of cells undergoing apoptosis is externalisation of phosphatidylserine and loss of membrane function (Elmore, 2007). Under physiological conditions, a cell's membrane has phosphatidylserine (PS) on its inner leaflet. In the early stages of apoptosis, the PS is translocated to the outer surface (Wlodkowic *et al.*, 2012). Annexin-V binds to PS of apoptotic cells whose membrane has not raptured and when used with PI, which is excluded by apoptotic cells with intact membrane, is used to distinguish apoptotic cells from necrotic cells (Crowley *et al.*, 2016). Increase in percent of cells stained with annexin-V in this study thus confirmed the induction of apoptosis in extract-treated cells and further proposed the induction of apoptosis as early as 6 hours (figure 3.3B). Furthermore, PI staining the nuclear of treated cells indicated loss of membrane function and therefore, late stages of apoptosis (figure 3.4B).

The process of apoptosis occurs in either of two pathways; the mitochondrial (intrinsic) or the trans-membrane receptor mediated (extrinsic) pathway. The intrinsic pathway is largely regulated by the Bcl-2 family of proteins consisting of pro- and anti-apoptosis members. An increase of the proapoptotic proteins, such as Bax, over antiapoptotic proteins, such as Bcl-2, induce outer mitochondrial membrane permeabilisation. This results in the release of mitochondrial proteins, such as cytochrome *C*, Smac/Diablo and Omi/HtrA2 (Elmore, 2007; Vaux, 2011). The detection of proapoptotic proteins, cytochrome *C*, Smac/Diablo and Omi/HtrA2 in the cytoplasm (figure 3.6), suggested mitochondrial membrane permeability. As a result of the increase in Bax expression level (figure 3.5), despite a significant shift in ratio of Bax to Bcl-2 (table 1). In the cytoplasm, cytochrome *C* triggers caspase activation by forming an apoptosome complex with dATP, Apaf-1 and pro-caspase-9. This complex autoactivates caspase-9. Caspase-9 activation then leads to activation of executioner caspases that cleave

various cellular components resulting in features associated with apoptosis (Elmore, 2007). Western-blot results revealed an increase in initiator caspase-9 expression levels by ~3 fold in HeLa cells treated with 500 µg/ml of the extract (figure 3.7A). However, there was no significant upregulation of the executioner caspase, caspase-3 for the 500 µg/ml treatment (figure 3.7B). This suggested that, although a significant increase in the level of pro-caspase-3 expression was detected for the 500 µg/ml (figure 3.7B), there was either minimal or no activation of the active form, caspase-3. The lack of significant caspase-3 expression therefore suggests the initiator caspase, caspase-9 was not significantly activated, as may be portrayed in the multicaspase assay (figure 3.8B). Thus resulting in the minimal degree of apoptosis execution observed in this study (figure 3.3B and 3.4B).

Caspase activity may be inhibited by inhibitors of apoptosis (IAPs). These proteins act by inhibiting both initiator and executioner caspases such as caspase-9 and caspase-3/7, respectively (Martins *et al.*, 2002). Cellular inhibitors of apoptosis, (cIAP), cIAP-1 and cIAP-2, as well as the X-chromosome-linked IAP (XIAP), prevent caspase activation by targeting caspases for ubiquitin mediated proteasomal degradation (Vaux and Silke, 2005). Livin interacts with executioner caspases, such as caspase-3 and targets them for degradation (Xue *et al.*, 2013). Survivin is another protein reported to block caspase activity and subsequently inhibits apoptosis (Jaiswal *et al.*, 2015; Xu *et al.*, 2013). Survivin binds to Smac/Diablo to inhibit IAP-Smac/Diablo interactions. This then leaves IAPs such as XIAP free to interact with caspase-9 and inhibit apoptosis (Song *et al.*, 2003). In this study, no apparent effect in XIAP and Survivin protein expression level was observed in extract-treated cells (figure 3.10). This then ruled out Survivin-Smac/Diablo interaction and inhibition of caspase-9 activity by XIAP. However, caspase activity could have been inhibited by the observed upregulation of cIAP-2 (figure 3.10), which is able to directly bind to the zymogen form of caspase-9 and prevent its proteolytic processing of executioner caspases (Deveraux, 1998). Moreover, Kasof and Gomes, (2001), reported that HeLa cells may be protected from apoptosis induced by the expression of FADD, Bax, RIP, RIP3 and DR6 in cells transfected with the *livin* gene. Thus the minimal degree of apoptosis induced by the extract observed in this study, may be, in part, attributed to the marked upregulation of livin protein expression level.

The extrinsic pathway of apoptosis involves transmembrane receptors of the *tumour necrosis factor (TNF) receptor* gene superfamily. These receptors interact with their respective ligands (Locksley *et al.*, 2001). This superfamily has a cytoplasmic domain that plays a critical role in the transmission of death signals, from the surface of the cell to the intracellular signalling pathways (Chicheportiche *et al.*, 1997). Upon binding of a ligand to its receptor, the oligomerisation of a death domain, fas-associated death domain (FADD), or the tumour necrosis factor receptor type 1-associated death domain (TRADD), results in the formation of a death-inducing signalling complex (DISC). The DISC recruits and activates the initiator caspase, caspase-8, which in turn mediates downstream caspase-cascade, by activating executioner caspases (Scaffidi *et al.*, 1998). Activation of caspase-8 by FADD is a requirement for TRAIL receptors (TRAIL-R1 and TRAIL-R2). TNF-R1 and fasR are adaptors of the apoptotic signal transduction (Hongxia, 2005). In this study, no significant change in protein expression level of TRAIL-R1, FADD, TNF-R1 and fasR were observed in extract-treated cells (figure 3.9). This then suggested that, the induction of apoptosis in extract-treated cells did not involve the extrinsic pathway.

When cells are exposed to stressful conditions, a number of heat shock proteins (hsp) are produced to make cells resistant to stress-induced cell damage (Daugaard *et al.*, 2007). Hsp60 acts by forming a complex with the proapoptotic protein, Bax, which inhibits apoptosis. However, under hypoxic conditions, Hsp60 dissociates from Bax, allowing Bax translocation to the mitochondria to participate in the induction of apoptosis (Gupta and Knowlton, 2005). Hsp70 is reported to interact with the apoptosis inducing factor (AIF) to negatively interfere with caspase independent apoptosis (Garrido *et al.*, 2001). As the findings of the study revealed no significant change in Hsp60 and Hsp70 protein expression level (figure 3.11), this suggested no involvement of apoptosis interference by Hsps investigated in this study. Furthermore, reactive oxygen species (ROS) may increase greatly in response to stress, thereby causing damage to cells (Cederbaum, 2000). These are products of normal metabolism and depending on the concentration, can be beneficial or harmful to cells and tissues (Circu and Aw, 2010). These products can also function as signalling molecules that regulate many physiological processes, including the induction of apoptosis (Kamogashira *et al.*, 2015). Following treatment with the extract, heme oxygenases 1 and 2 (HO-1 and HO-2), proteins whose expression is entirely

dependent on ROS production, showed no significant change when compared to untreated cells. This therefore suggested no production of harmful levels of ROS to cause a response from these ROS-dependent proteins in extract-treated cells. In addition, plants possess antioxidant activities, making them a good source for protection of cells from damage by ROS. The human enzyme paraoxonase 2 (PON 2) acts the same way. As an antioxidant, PON 2 reduces cellular oxidative damage and influences redox signalling, which influences cell survival (Altenhofer *et al.*, 2010). In this study, only a small degree of apoptosis was observed. The observed upregulation of PON 2 in extract-treated cells could have further prevented significant apoptosis execution.

The occurrence of apoptosis is in close association with the cell division cycle arrest (Pucci *et al.*, 2000). Within the eukaryotic cell division cycle, cells are examined for irregularities, including DNA damage. If damage of the cell is detected, the cell undergoes cell cycle arrest to give time for the damage to be repaired by DNA repair mechanisms. Once the cell is clear of damage, the cell splits into two cells, during the mitotic phase. However, if the detected damage is irreparable, the cell undergoes programmed cell death, apoptosis (Alam *et al.*, 2013). Accumulation of cells at the G0/G1 phase would suggest cell cycle arrest in response to unfavourable conditions such as lack of nutrients which leads to the inhibition of the synthesis of enzymes required for DNA synthesis, thereby preventing entry into the S-phase. While arrest at the G2/M phase would suggest failure of cells to undergo mandatory changes that prime cells to divide (Jaiswal *et al.*, 2015). The G1/S checkpoint is governed by the phosphorylation or expression of RAD17, which participates in cell cycle regulation and G1/S checkpoint activation. The G2/M checkpoint is influenced by the expression level of Claspin, which, as a requirement for the G2/M checkpoint activates and promotes cell proliferation (Lin *et al.*, 1996). Activation of the G1/S checkpoint was proposed by the observed significant increase in RAD17 expression level in cells treated with 500 µg/ml of the extract (figure 3.15). However, no significant effect was observed regarding levels of Claspin protein expression in extract-treated cells (figure 3.15).

Moreover, upon stress by DNA damaging agents and/or mitogenic stimulations, p53, a tumour suppressor protein that exists in low amounts in cells is briefly activated

through phosphorylation (Ashcroft *et al*., 1999). Phosphorylation of p53 on a number of serine residues modifies the tumour suppressor protein. Phosphorylation at S15, S46 and S392, in response to stress signals is observed in cells directed towards apoptosis (Lane, 2016). Furthermore, when activated, p53 is involved in the regulation of cellular differentiation and apoptosis by activating transcription of genes such as *p21* (Foo *et al*., 2015). The protein p21, a cyclin-dependent kinase inhibitor (CDKI), negatively regulates the cell cycle and thus acts as a tumour suppressor (Seo *et al*., 2003). p21 is involved in the G1 phase cell cycle arrest, through dependent and independent mechanisms (Katori *et al*., 2006). Although there was apparent activation of p53 in extract-treated cells (figure 3.12), as well as p21 upregulation (figure 3.15), there was no cell cycle arrest in extract-treated cells. Since treatment with the extract revealed no noteworthy change in percentage of cells accumulated in the G0/G1 phase, the S-phase, the G2 phase nor the M phase (figure 3.13B and 3.14B), this then suggested that the extract did not induce cell cycle arrest in experimental cells. The inability of the extract to induce cell cycle arrest was also supported by the decline in expression level of the cyclin-dependent kinase inhibitor (CDKI), p27, in cells treated with 400 µg/ml of the extract. The CDKI p27 is said to be highly expressed in quiescent cells and it's expression declines before cellular proliferation, in response to mitogenic signals (Hiromura *et al*., 1999) (figure 3.15). Clusterin is a chaperone-like protein that promotes tumour cell survival induced by chemotherapy, by binding to apoptotic and DNA repair proteins (Zhang *et al*., 2005). Therefore, its downregulation suggests uncompromised activity of DNA repair proteins and as such, may be a reason for the lack of cell cycle arrest (figure 3.13B and 3.14B), as DNA damage that may have resulted from treatment with the extract could have been repaired.

Treatment with taxol at 25 nM did not show significant apoptosis insult either, as only some apoptotic features were observed at this taxol concentration. These apoptotic features are changes in cell and nuclear morphological features (figure 3.2A and 3.2B), downregulation of the inhibitors of apoptosis (figure 3.10) and downregulation of HO-1 and HO-2 (figure 3.11). This could be attributed to the fact that taxol-induced apoptosis in HeLa cells is serum dependent (Maldonado *et al*., 1996), and for each morphological and biochemical feature, a different taxol concentration is required, for each cell line (Wang, 2000).

In summary, treatment with *D. senecioides* crude methanol leaf extract selectively inhibited viability of cervical HeLa cancer cells. The extract induced cell shrinkage and altered the cell morphology of HeLa cells, causing irregular extensions, indicative of some degree of apoptosis induction. This is attributed to modulation of the Bcl-2 protein family, release of cytochrome *C*, upregulation of caspase-9 protein expression, activation of some caspases, as well as increased phosphorylated level of p53, at serine residues 46 and 392.

Despite the presence of some of the features associated with apoptosis, it can be concluded that *Dicerocaryum senecioides* crude methanol leaf extract did not induce significant apoptotic insult for HeLa cancer cells to undergo apoptosis, at the tested concentrations. It is also known that crude extracts, unlike semi-purified, possess a number of compounds that would either synergistically enhance a common function or interfere with functions possessed by other compounds present in the crude. Therefore, it is possible that this plant extract contains compounds with opposite anti- and pro-apoptotic properties that would ultimately cancel each other out and may be the cause for the observed minimal apoptosis in extract-treated cells.

However, due to its selectivity, specific compounds in *D. senecioides*, that may be responsible for HeLa cell sensitising, should be purified and further evaluated *in vitro*.

CHAPTER FIVE

5. References

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