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Mthembu, Nonkululeko Nomfundo

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SCREENING OF TRADITIONAL MEDICINE FOR RBBP6 ANTI-CANCER THERAPY IN CERVICAL CANCER

NONKULULEKO NOMFUNDO

MTHEMBU

A dissertation submitted in fulfilment of the requirements for the degree of Masters in Science in the School of Molecular and Cell Biology, University of the Witwatersrand

Johannesburg, 2013

DECLARATION

I declare **" SCREENING OF TRADITIONAL MEDICINE FOR RBBP6 ANTI-CANCER THERAPY IN CERVICAL CANCER"** to be my work which has not been submitted for any degree or examination at any other University and that all the sources I have used or quoted have been indicated and acknowledged by complete references.

Nonkululeko Nomfundo Mthembu

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Signature…  ……

On this……10th ……day of……..……May…….……..…….2013

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JOHANNESBURG

"Greatness is achieved through discipline, inspiration and passion"
~author unknown

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DEDICATION

This dissertation is dedicated to my mother, Nompumelelo who on the 22 September 2011 passed on from cancer and to my father Thandwa who has continuously supported and believed in me throughout the course of my studies.

I salute you!

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ABSTRACT

Cervical cancer is a gynaecological malignant disorder and is a common cause of death in women of the sub-Saharan Africa, striking nearly half a million of females each year worldwide. Cervical cancer is due to the persistence infection of human papillomavirus (HPV), a formidable virus that targets the cervix and is present in most cancers of the cervix. In South Africa, plants used to treat cancer are rare and there is a need for screening further plant extracts in order to identify potentially new anti-cancer drug discovery leads.

The purpose of this study was to screen *Tulbaghia violacea* (TV) and *Agave palmeri* (AG) for anti-cancer therapy in the cervical cancer cell lines HeLa and ME-180 and in the fibroblast cell line KMST-6. Staurosporine (ST) was used as a positive control. AG and TV crude plant extracts were screened for apoptosis induction, followed by elucidation of the role of Bax, Bcl-2, p53, Rb, RBBP and Mdm2 genes in cervical cancer. Plant extracts of TV and AG were time (24 hours) and dose (50, 100, 150 µg/ml) dependently screened against cervical cancer cell lines HeLa, ME-180 and in KMST-6 for anti-cancer activity using the thiazolyl blue test (MTT) assay. With an IC_{50} ~ 150 µg/ml, *T. violacea* extract exhibited significant cytotoxicity on both HeLa and ME-180 cancer cell lines, whilst *A. palmeri* was cytotoxic to ME-180 cells and 25nM ST as a positive control had a cytoxicity effect on all cell lines including the KMST-6, yet TV and AG had no cytotoxic effect on KMST-6.

The annexin-V/FITC detection assay was performed to evaluate the occurrence of apoptosis. Crude extracts of TV and AG together with ST induced significant apoptosis of HeLa, ME-180 and KMST-6 cells. The crude extracts were further analysed for DNA fragmentation, protein expression and gene expression by Western Blot and RT-PCR respectively, to investigate

whether these extracts have an effect on the on the expression of Bax, Bcl-2, p53, Rb, RBBP6, Mdm2 and the relationship between p53 and RBBP6. Morphological and biochemical changes were seen in this study. A further mixed response by several genes was observed following treatment with the two plant extracts, where RBBP6 was seen to be spliced in cancer cells while Bax was induced and Bcl-2 was inhibited, but the levels of p53 remained the same. Preliminary, the extracts of TV and AG induce cell death by down-regulating Bcl-2 and Mdm2. Quantitative RT-PCR showed that when p53 was silenced RBBP6 was up-regulated and vice versa. From these results it was deduced that RBBP6 gene interacts with p53 during cervical cancer development.

The anti-proliferative activity together with the characterization of p53, RBBP6 and Mdm2 and concentrations of these plant extract could be manipulated as diagnostic markers and potential therapeutic targets for cancer treatment; however, further studies on these plant extracts need to be performed to validate results obtained in this study.

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KEYWORDS:

Apoptosis	Cervical cancer
Tulbaghia violacea	*Agave palmeri*
Staurosporine	Bax
Bcl-2	p53
Rb	RBBP6
Mdm2	

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NOMECLATURE

AIDS	Acquired Immunodeficiency Syndrome
ADP	Adenosine Diphosphate
Amp	Ampicillin
Apaf-1	Apoptosis protease activating factor 1
BCA	Bicinchoninic Assay
BSA	Bovine Serum Albumin
BCNU	1-3-bis 92- chloroethyl) - 1 nitrosoure
Bp	Base pair
BID	BH 3 interacting domain
cDNA	Complementary DNA
CLL	Chronic Lymphocytic leukemia
Cdk	cyclin-D Dependent Kinase
CIN	Cervical Intraepithelial neoplasia
CTL	Cytotoxic T cell
dATP	2’ Deoxyadenosine Triphosphate
DEPC	diethylpyrocarbonate
DMEM	Dulbecco’s Modified Medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic Acid
dsH_2O	deionized water

DWNN	Domain With No Name
ECL	Enhanced Chemiluminescennce Luminol
EDTA	Ethylene Diamine Tetra Acetic acid
FAS	Fas Associated death domain
FACS	Fluorescence- activated cell sorting
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
FITC	Flourescein Isothiocynate
G1	Gap 1
HCl	Hydrochloric Acid
HeLa	Henrietta Lacks
HepG2	Hepatocellular carcinoma
HIV	Human immunodeficiency virus
HPV	Human Papillomavirus
HRP	Horseradish Peroxidase
Kb	Kilo base
kDa	Kilo Dalton
KMST-6	Normal Human fibroblast cell line (skin)
L	Litre
LFS	Li-Fraumeni Syndrome
MCF-7	Michigan Cancer Foundation 7

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ME-180	Invasive squamous cell carcinoma
MEM	Minimum Essential Media
Mdm2	Mouse Double Minute 2
MDG	Methyl-%-D-glucopyranoside
mL	Milliliter
mRNA	Messenger RNA
MHC	Major Histocompatibility complex
MTT	(3-(4, 5- dimethylthiazol-2-yl-) - 2, 5- diphenyl tetrazolium bromide
MW	Molecular weight
NCBI	National Centre for Biotechnology Information
nm	nanometer
PARP	Poly (ADP-ribose) Polymerase
P2P-R	Potential Related Protein
PACT	P53- Associated Cellular Protein
p53	protein 53 (tumour protein 53)
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
pRb	Retinoblastoma protein
PS	Phosphatidylserine
PVDF	Polyvinylidene flouride
RBBp6	Retinoblastoma binding protein 6

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RNA	Ribonucleic acid
RT-PCR	Real Time Polymerase Chain reaction
S	Synthesis
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
ST	Staurosporine
SRB	Sulforhodamine B
TAP-1	Antigen peptide transporter 1
TNF	Tumour Necrosis Factor
TSG	Tumour Suppressor Protein
WR	Working Reagent

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CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1. Introduction

This chapter provides a very brief introduction and literature review on the screening of traditional medicine for RBBP6 anti-cancer therapy in cervical cancer. There is no significant amount of literature available regarding the treatment of cervical cancer using traditional medicine, although there is literature available regarding cervical cancer and its screening and treatment with some traditional medicine in other countries but not in the South Africa perspective. This chapter will describe the epidemiology and natural history of cancer of the cervix and finally describe the use of traditional medicine in treating cervical cancer and other types of cancer. In a surge of combating and dealing with such a disease in the developing country like South Africa by using the natural resources such as plants as most people still consult traditional healers when sick.

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1.2. Cervical cancer

1.2.1. Statistics

Cervical cancer is a gynaecological malignant disorder and is the most common cause of cancer death in women of the Sub-Saharan Africa, Melanesia, South Central and South East Asia, the Caribbean and Latin America (Parkin *et al*, 1999; Hougardy *et al*, 2005; Danny, 2006). In 2002, it was globally estimated that 493 000 new cases were diagnosed with 274 000 women dead within the year (Danny, 2006). Most cases (83%) were reported in developing countries where cervical cancer accounted for 15% of all cancers, with a lifetime risk of 3% when compared to 3.6%- 4.4% in developed countries, with a lifetime risk of 1.1% and low incidence rate (age-standardized rate) which is less than 14 per 100 000 (Parkin *et al*, 1999).

According to the Cancer Registry of South Africa, in 1986 the total number of cancer cases reported was 16 559, of which 2897 (17.4%) were new cases of histologically confirmed cervical cancer. Whereas in 1992 about 25 143 cases of cancer were reported and cervical

cancer cases remained at the same proportion of 17.8% when compared to 1989. An average of 3387 new cases of cancer of the cervix were then reported each year in 1993-1995 and the age-specific incidence rate (ASIR) of cervical cancer was 22 per 100 000, 27 per 100 000 for black women in disadvantaged communities. The lifetime risk of developing cervical cancer is 1 in 34 in black women and 1 in 93 in white women of Southern Africa (van Schalkwyk *et al*, 2002; Walker *et al*, 2002). Possibly the lifetime risk is high in black women due to limited access to healthcare and these women normally present the case when it's too late. To date, according to the World Health Organization (WHO, 2010) a South African population with 16.84 million women ages 15 and above are at high risk of developing cervical cancer with 5743 diagnosed and 3027 dying from cervical cancer. It is the most common cancer to affect black women, accounting for about 25% of cancer deaths and with 13.3% of South African females living with human immunodeficiency virus infection (HIV)/ acquired immunodeficiency syndrome (AIDS) because they are more susceptible to opportunistic diseases such as cervical cancer and have increased risk of contracting HPV (Kirsten, 2009). Cervical cancer is a third leading cause of death among women in Southern Africa with an overall age standardized incidence rate of 30 per 100 000 per year (Francis *et al*, 2010). Invasive cervical cancers are attributed to Human Papillomavirus (HPV) 16 and 18 according to WHO and the recent study indicated that worldwide HPV prevalence in cervical carcinoma is 99.8% (Schneider, 2004; Botha *et al*, 2010).

1.2.2. Epidemiology

The cause of cervical cancer is mainly due to the persistent infection of human papillomavirus which has more than 100 genotypes with varying virulence (i.e. HPV 16, 18, 31, and 45) with HPV 16 accounting for more than 50% of cervical cancers, HPV 18 with 10% followed by subtype 31 and 45 in most countries (Parkin *et al*, 1999; Hougardy *et al*, 2005). Other co-factors of cervical cancer include multiple sex partners related to early onset of sexual activity which is thought to be associated with high risk because, during puberty, cervical tissue undergoes a variety of changes that may make the area more vulnerable to damage. Recurring sexually transmitted disease, smoking, oral contraceptive, family history, and molecular genetic factors such as loss of function in the tumour suppressor gene p53 to name but few factor that contribute to development of cervical cancer (Parkin *et al*, 1999; Lu, 2004; Hougardy *et al*, 2005).

It is from the precursor lesions known as cervical dysplasia or cervical intraepithelial neoplasia (CIN) that, cervical cancer develops and can be classified as a mild (CIN I), moderate (CIN II) or severe (CIN III) or carcinoma-in-situ in terms of regression (Fletcher, 1993, Soliman *et al*, 2004). The human papilloma virus consists of a double stranded circular DNA genome of 8 kilobases (kb), a non-enveloped virion and an icosahedral capsid which can infect mucosae and cutaneous surfaces together with epithelial cells thereby replicating episomally utilizing two of its virus- encoded proteins, E1 and E2 and causing warts and epithelial tumours (Chakrabarti and Krishna, 2003; Soliman *et al*, 2004; Hougardy *et al*, 2005). It is rare for HPV to integrate into its host DNA but when it does integrate, it does so in a monoclonal fashion, which results in the loss of expression of the E2 protein. E2 suppresses the expression of E6 and E7. Its loss results in the E6 and E7 overexpression (Finzer *et al*, 2002; Hougardy *et al*, 2005). The repression of both E6 and E7 in the study by Morrison *et al*, (2011) was previously shown to result in activation of their respective tumor suppressor targets, tumour protein (p53) and retinoblastoma protein (pRb), and subsequent senescence induction in cervical cancer cells. The E6 and E7 protein of oncogenenic HPV subtypes are co-expressed and facilitate degradation of cellular proteins that regulate the cell cycle and apoptosis and their oncogenes are essential in initiation and maintaining cervical cancer in high risk HPV (Morrison *et al*, 2011). The ability of viral DNA to integrate into the host is selective advantage towards progression of the HPV, because E6/E7 will continuously be expressed without losing the viral templates due to extrachromosomal segregation or encapsidation (Finzer *et al*, 2002). This will lead to the generation of virus-cell-fusion transcripts, whereby the own poly-A signal is lost and these E6/E7 mRNA are highly stable. This will, in turn, raise the amount of viral oncogenes resulting in the effect of cellular signalling or/and particular cellular transcription factors both at transcriptional and translational levels, whose function favour host cell transformation (Slebos *et al*, 1994; Finzer *et al*, 2002). Thus, E6 and E7 proteins of 'high risk type' of HPV have been shown to be essential for cellular transformation via *in vitro* and *in vivo* studies (Chakrabarti and Krishna, 2003).

The E6 protein inactivates the p53 pathway while the E7 protein inactivates the retinoblastoma (Rb) pathway, both of which are essential in initiating and maintaining the proliferative state of cervical-cancer cells and thus an increased likelihood of malignant transformation (Soliman *et al*, 2004; Hougardy *et al*, 2005; Koushik *et al*, 2005). Together *E6* and *E7* regions of the viral genome are also vital for maintaining the malignant phenotype

(Hamada *et al*, 1996). The HPV E6 has an ability to form a complex with and inactivate p53 thereby stimulating p53 degradation through a selective [(E6-AP) ubiquitin ligase] ubiquitin-dependent proteolytic pathway where the proteasome detects the proteins that have been targeted for degradation because ubiquitin peptides are attached to them (Figure 1.1) (Soliman *et al*, 2004; Hougardy *et al*, 2005). High oncogenic risk types of HPV (i.e. 16 and 18) E6 proteins have a high affinity for p53 when compared to low-oncogenic risk types such as HPV 6 and 11. HPV 16 E7 interacts with various proteins, most of which are vital regulators of cell growth, these include retinoblastoma tumour suppresser family protein (Rb, p10, p130, histone deacetylases (HDAC), AP-1 transcription factors, TATA box binding protein (TBP), cyclins, cyclin-dependent kinases (cdks) and cdk inhibitors, M2 pryruvate kinase (M2-PK) which allows this protein to deregulate the cell cycle and increased cell proliferation, immortalization and finally transformation (Chakrabarti and Krishna, 2003). Alterations in the p53 gene, including deletion, insertion and point mutation are the most frequent genetic events in many different carcinomas, such as in colon, breast and lung (Bartek *et al*, 1990; Rodrigues *et al*, 1990; Takahashi, 1992).

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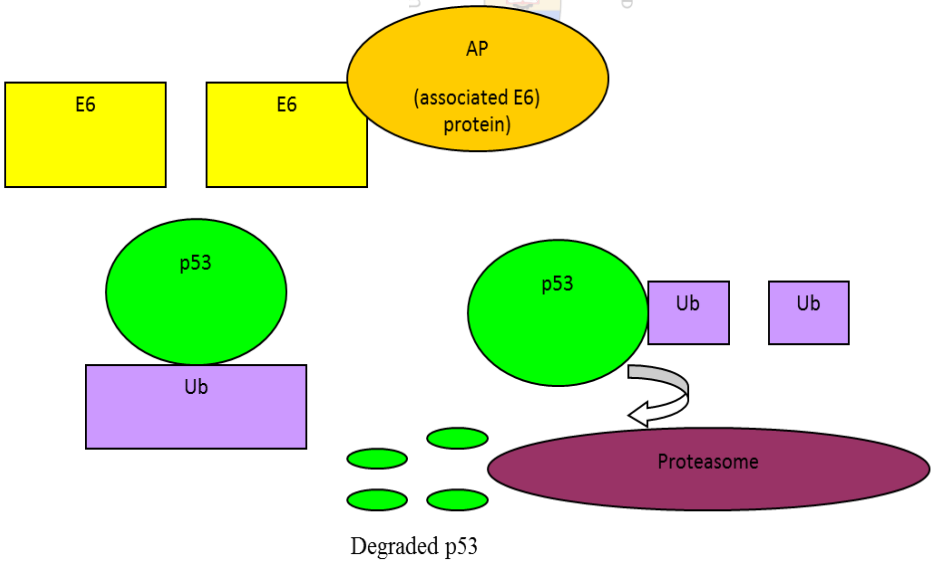


Figure 1.1: The schematic diagram showing degradation of p53 by HPV E6 protein through the ubiquitin (Ub)-dependent proteolytic pathway. Normally, p53 expression is very low in cervical cancer cells but in response to DNA damage stress it is stabilized and induced to exert its various cellular responses including apoptosis (Lu, 2004; Soliman *et al*, 2004; Singh *et al*, 2007).

Together E6 and E7 vastly contribute to carcinogenesis by suppressing apoptosis through targeting the host tumour suppressor gene products p53 and pRB together with senescence and by stimulating cellular proliferation and acceralating proteasomal degradation and inactivation (Figure 1.1) contributing to cellular immortalisation and transformation (Hougardy *et al*, 2005).

1.2.3. Apoptosis and other cell death pathways

Cell death occurs by various morphologically and biochemically distinct pathways which include apoptosis, necrosis, and autophagy. Apoptosis plays a vital role in embryogenesis and maintains homeostasis in multicellular organisms and is described as programmed cell death, a conserved mechanism directed by genes to eliminate damaged cells. (Taraphdar *et al*, 2001; Ghobrial *et al*, 2005; Sundquist *et al*, 2006; de Bruin and Medema, 2008). Apoptosis is induced by many stimuli, including growth factor withdrawal, ultraviolet or gamma radiation, chemotherapeutic agents, immunotherapy, or activation of death receptors (intracellular stress and extracellular ligand signals) (Hougardy *et al*, 2005; de Bruin and Medema, 2008). It is characterized by a series of distinct morphological and biochemical changes which include condensation, fragmentation of the nuclear chromatin, compaction of the cytoplasmic organelles with cell shrinkage and loss of positional organization of organelles in the cytoplasm, dilation of the endoplasmic reticulum, a decrease in cell volume and alteration to the plasma membrane resulting in the recognition and phagocytosis of the apoptotic cells (Lee *et al*, 2009; Taraphdar *et al*, 2001). Although apoptosis refers to a purely morphological change, biochemical events accompany these morphological changes and these events occur during the intrinsic and extrinsic pathway (Sundquist *et al*, 2006).

The intrinsic and extrinsic signaling pathways initiate apoptosis. The intrinsic pathway which is due to intracellular stress signals, such as growth factor withdrawal, DNA damage, oxidative stress or oncogene activation. Which upon signal response lead to permeabilization of the mitochondrial outer membrane and is regulated by pro- and anti-apoptotic members of the Bcl-2 family in which members are either death antagonists or death agonists either repressing apoptosis. In turn, blocking the release of cytochrome-*c* or acting as a promoter of apoptosis by releasing cytochrome-*c* from the mitochondria (Ghobrial *et al*, 2005; De Bruin and Medema, 2008). In normal uterine cervix tissue cell proliferation and apoptosis is balanced, the imbalance of proliferation together with apoptosis may lead to squamous cell

carcinoma because these processes remain crucial for normal development and maintenance of tissue homoeostasis by eliminating damaged, virally infected, or otherwise harmful cells (Hougardy *et al*, 2005). Finzer *et al*, 2002 defines apoptosis due to viral infection (HPV) as a genetically determined programme where replicating or persisting viruses that are not yet packaged into mature viral capsid are cleaved. This process is mediated by activation of initiator and effector proteases called caspases which function to cleave specific death substrates, resulting in cellular disassembly (Lowe and Lin, 2000; Dlamini *et al*, 2004; Hougardy *et al*, 2005). This is very much dependent on the balance between the anti-apoptotic protein Bcl-2 and the pro-apoptotic Bax than on Bcl-2 quantity alone according to the rheostat model that determines whether a cell will live or die in response to an apoptotic stimulus (Nickells, 2010). Bax therefore be activated to initiate mitochondrial dysfunction that is critical during the intrinsic apoptotic pathway. These proteins also undergo post-transcriptional modification, desphosphorylation and cleavage, which result to their mitochondrial activation and translocation leading to apoptosis (Scorrano and Korsmeyer, 2003).

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Once the apoptotic stimuli is sent, the outer mitochondrial membrane becomes permeable and this leads to the release of cytochrome-*c* from the membrane space and the second mitochondrial-derived activator of caspase in the cytosol where cytochrome-c interacts with Apaf-1, leading to the activation of caspase-9 pro-enzyme (Ghobrial *et al*, 2005; Kang and Reynolds, 2009). This occurs in a cascade manner where caspase-9 activates caspase-3, which activates the rest of the caspases and this result into apoptosis (Dlamini *et al*, 2004). In a nutshell, cytochrome *c* will then bind to the protein apoptosis-protease-activating-factor 1 (Apaf-1) forming a complex called the apoptosome since it is bound to dATP as a cofactor and it contains cytochrome *c*, Apaf-1 and initiator caspase 9 which is auto-induced within this complex (Purring-Koch and McLendon 2000; De Bruin and Medema, 2008). Active caspase-9 cleaves and activates other executioner caspases thus turning on a protease cascade which will eventually lead to cleavage of caspase-3 causing apoptosis (Li *et al.* 1997).

The extrinsic pathway on the other hand, involves cell surface death receptors together with inducers and suppressors, which will aid in determining whether the cell will undergo mitosis or apoptosis (Ghobrial *et al*, 2005; Hougardy *et al*, 2005). In most cancers, the ability of the

cells to either undergo mitosis or apoptosis is impaired causing uncontrolled cell proliferation, which leads to tumour formation. The extrinsic apoptotic pathway commences with the activation of death receptors on the cell membrane, which belong to the tumour-necrosis-factor-receptor super-family (Hougardy *et al*, 2005). The binding of specific tumour-necrosis-factor-receptor ligand such as FASL or tumour-necrosis-factor-related apoptosis-inducing ligand TRAIL, to the cognate receptors FAS and DR4 or DR5 respectively triggers apoptosis in this pathway (de Bruin and Medema, 2008). As a result of this activation an intracellular death-inducing signaling complex composed of trimerised receptor molecules, recruited FAS-associated death domain molecules and procaspase-8 molecules, causes activation of the caspase-8 intracellular apoptotic cascade leading to the cleavage of several substrates in the cytoplasm and nucleus and completion of the apoptotic process (Hougardy *et al*, 2005). Caspase-3 is also involved in this pathway as it is activated by caspase-8 directly or indirectly by cleaving BID (a death agonist protein from the Bcl-2 family). Cleaved or truncated BID translocates into the mitochondria whereby it triggers the apoptotic pathway (Dlamini *et al*, 2004; Hougardy *et al*, 2005; de Bruin and Madema, 2008). In both pathways, apoptotic biochemical events occur after activation of the effector caspases and this includes the phosphatidylserine exposure on the external surface of the plasma membrane, cleavage of poly (ADPribose) polymerase (PARP) and internucleosomal DNA fragmentation (Sundquist *et al*, 2006).

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The capability to interfere with these apoptotic pathways allows human papillomavirus oncoproteins to interfere with normal balance between proliferation and apoptosis in HPV-infected cervical epithelial cells. The virus escapes detection by the immune system during progression to cervical cancer and strategically modulates the apoptotic response upon infection (Finzer *et al*, 2002; Hougardy *et al*, 2005). For example, escape from the host immune system can be favored, by the fact that HPV is able to down-regulate the major histocompatibility complex (MHC) class I expression and the loss of TAP-1 transporter protein expression together with the ability to present foreign antigens (i.e. E2 and E6) to cytotoxic T cells (CTL) (Cromme *et al*, 1994; Bartholomew *et al*, 1997; Bontkes *et al*, 1998; O'Brien and Campo, 2003). If the viral infection persists, its oncoproteins degrade *p53* and inactivate *Rb* tumour suppressor genes thus HPV proteins are vital for cervical carcinogenesis (Hamada *et al*, 1996). Thus, DNA viruses are strategically able to modulate apoptosis e.g. adenovirus-infected cells are able to overcome the antiviral host defense by

degrading the CD95-receptor and interfere with normal function of tumour suppressor genes (Finzer *et al*, 2002).

1.2.4. Tumour suppressor genes (TSG)

The tumour suppressor genes not only regulate the growth of cells but they function to inhibit tumour growth. When they are inactivated or altered by i.e. genetic and epigenetic mutations and their inhibitory function is lost thus causing cancer. When proteins encoded by these genes are no longer expressed, cell proliferation cannot be inhibited. There are various types of these genes; some are responsible for directing information to the cell so that it stops dividing (cell cycle arrest), others control DNA damage by repairing it whereas others are responsible for apoptosis a form of cell death (Ghobrial *et al*, 2005). Genes such as p53 and retinoblastoma (*Rb*) gene, to name but few, play a major role in cancer.

1.2.4.1. p53

Approximately about 70% of all human cancers carry p53 mutations, and recent studies suggest that many tumours that retain wild-type p53 are defective in either the ability to induce or the ability to respond to p53 (Oren, 1999; Bertram, 2001). The mechanism of inactivation or loss of wild type p53 in the development of cervical carcinoma is attributed to either mutation within the genome or the presence of a virally encoded p53 binding protein such as E6 thus causing carcinogenesis (Hougardy *et al*, 2005). The tumour suppressor gene, *p53* encodes a 53kDa recessive loss-of-function protein (an oligomeric transcription factor), and, by binding to specific DNA target sequences, either activates or represses expression of a number of genes that regulate the cell cycle, differentiation, DNA repair and apoptosis (Classon and Harlow, 2002; Dlamini *et al*, 2004; Motadi *et al*, 2007). The function of the *p53* gene was confirmed through the study of germ-line mutations of *p53* gene in families with Li-Fraumeni syndrome (LFS), in which affected individuals were predisposed to early-onset malignancies including breast carcinoma, sarcomas, and brain tumors (Frebourg *et al*, 1995).

The mechanism of p53 activation in response to DNA damage is dependent on DNA protein kinase and other oncogenic proteins such as Myc, Ras, adenovirus E1A, and β-catenin (Oren, 1999; Abd El All *et al*, 1999; Ryan *et al*, 2001). The *p53* gene negatively regulates the cell cycle and requires ‘‘loss of function’ mutations for genomic instability, impaired cell cycle regulation and inhibition of apoptosis results into tumour formation whereas the activation

induces cell cycle arrest, initiates apoptosis and regulates cell senescence to defend the host cell against cancer (Soliman *et al*, 2004; Ghobrial *et al*, 2005). Thus, the pivotal role of p53 is to maintain the genome's integrity either by inducing cell cycle arrest or apoptosis hence the nickname "guardian of the genome" (Oren, 1999; Bertram, 2001). Post mitosis, during the G_1 phase of the cell cycle, p53 levels are normally low and protein levels are phosphorylated by the M phase cyclin-dependent kinase (CDK) pathway which includes a major gene target of p53, the CDK inhibitory protein, p21. This is a negative regulator growth factor that protects cells against apoptosis by invoking the cyclin-dependent kinase and caspase pathways (Motadi *et al*, 2007; Ryan *et al*, 2001).

If p53 is activated this will result in an increased expression of p21 and the cell cycle will arrest at G_1 and G_2 phase. Apoptosis is regulated by the activation of down-stream targets that are directly involved in the control of apoptosis, where p53 binds to mouse double minute (Mdm2). In turn, p53 levels are regulated via a negative feedback loop in which p53 stimulates the expression of mdm2 protein which in turn inhibits p53 by binding to its transcriptional domain, acting as an ubuquitin ligase, which promotes p53 degradation, by binding p53 and exporting it to the nucleus (Motadi *et al*, 2007). On the other hand, the binding of MDM2 to the amino-terminal transactivation domain of p53 directly inhibits the transcriptional activation and repression by p53 and this results into retinoblastoma protein (pRb) forming a trimeric complex with Mdm2 and p53, preventing p53 degradation. Thus, Rb may enhance p53-dependent cell death (Ryan *et al*, 2001).

1.2.4.2. Retinoblastoma (*Rb*)

Retinoblastoma (*Rb*) gene, encoding protein pRb was the first tumour suppressor gene to be cloned and was discovered to be mutated in human cancers, such as small-cell lung carcinoma and asteosarcoma (Classon and Harlow, 2002). Rb is a member of 'pocket proteins', which include p107 and p130 with main sequence similarity in the domain that mediates interaction with many proteins including viral oncoproteins. The gene product, Rb is a target of oncoproteins that are expressed by DNA tumour viruses, i.e. adenovirus E1A, SV40 T antigen and the E7 protein of HPV although in cervical carcinomas that do not express the E7 protein, the activity of Rb is inactivated by somatic mutation in order to cause cancer (Classon and Harlow, 2002; Sherr and McCormick, 2002).

The cell is upheld at a checkpoint after DNA damage. This lasts until the damage is repaired and if this damage is irreversible, apoptosis is triggered (Ghobrial *et al*, 2005). Loss of function of Rb protein which regulates the G_1/ S phase checkpoint by binding to E2F family transcription factor and inhibiting promoters of expression genes required for transition results in uncontrolled cell proliferation or cancer (Ahlander and Bosco, 2009; Poznic 2009). These findings suggest that Rb is a regulator of cell proliferation as this was shown through overexpression studies where Rb was overexpressed and the cells underwent arrest in the G_1 phase of the cell cycle, whereas cells deficient of Rb underwent an acceralated G_1 transition implying the role of Rb as a cell proliferation inhibitor (Classon and Harlow, 2002). The ability of Rb to repress E2F- mediated transcription is controlled by the phosphorylation of Rb by the cyclin-dependent kinase (CDKs) such as CDK1 where the interaction between Rb and E2F is disrupted (Buchkovich *et al*, 1989). Rb is also implicated in playing a pivotal role in DNA replication, differentiation, and in apoptosis. Therefore, the decrease in differentiation potential with an increase in proliferation rate in Rb deficient cells, contribute to tumourigenesis. Rb deficiency is lethal at E1A. In knockout mice it was shown that after 14 to 15 days of embryonic development they die (Sherr and McCormick, 2002).

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The roles of Rb and p53 are interconnected because both these tumour suppressor genes and their protein products play similar roles. Proteins p53 and Rb require specific protein-to-protein interactions to mediate their respective functions and they interact with RBBP6, a retinoblastoma binding protein 6 (RBBP6) *in vivo* and *in vitro* (Li *et al*, 2007). This interaction concurrently promotes p53 degradation via a proteasome pathway enhancing the activity of Mdm2 gene (figure 1.2) (a p53 inhibitor with ubiquitin ligase activity) a key p53 negative regulator thereby suppressing p53 binding to DNA (Soliman *et al*, 2004; Pugh *et al*, 2006; Ntwasa, 2008). On the other hand RBBP6 may also block the binding of adenovirus E1A protein to Rb (Pugh *et al*, 2006).

1.2.4.3. RBBP6

RBBP6 is a 250kDA multi-domain splicing-associated protein which adopts a ubiquitin-like fold (N-terminal domain DWNN) as shown in figure 1.2, and has a conserved zinc finger like motif with an ability to localize itself into the chromosome during mitosis and to nuclear speckles, which are believed to be the main site of activity for pre-mRNA splicing and processing, during interphase. Over-expression leads to cell cycle arrest and apoptosis (Pugh

et al, 2006; Ntwasa, 2008). This tumour binding protein in its truncated form may also be referred to as a potential related protein (P2P-R) or p53-associated cellular protein (PACT) due to its ability to bind to p53 (Simon *et al*, 1997; Li *et al.* 2007). Such interactions interfere with ability of p53 and Rb to bind to damaged DNA and EF2 resulting into apoptosis inhibition and induced cell proliferation respectively (Chibi *et a*l, 2010). RBBP6 negatively regulates pre-mRNA processing proteins by covalently modifying them with an ubiquitin-like moiety and localizing to chromosomes during the mitotic cell cycle and to nuclear speckles, active sites for pre-mRNA splicing and processing, during interphase (Pugh *et al*, 2006). This later led to the discovery that P2P-R is actually an alternatively spliced form of PACT which lacks the 34 amino acid exon (Faro, 2004). P2P-R protein levels are highly expressed during mitosis, and are phosphorylated by the mitotic cdc2 kinase. Over-expression results into mitotic arrest at prometaphase and mitotic apoptotic (Scott *et al*, 2005). Over expression of the pro-apoptotic region discovered within the P2P-R in breast cancer cell lines (MCF-7) promoted camptothecin induced apoptosis because this region overlapped the region responsible for p53 and single stranded DNA binding (Faro, 2004).

A more recent study by Li, *et al*, 2007 revealed a biological function of *Pact*, where knockout mice were generated and it was discovered that the disruption of PACT led to early embryonic lethality before embryonic day 7.5 accompanied by widespread apoptosis. To study the biological functions of *Pact*, PACT knockout mice were generated and found that disruption of PACT leads to early embryonic lethality before embryonic day 7.5 (E7.5) accompanied by widespread apoptosis together with p53 accumulation *in vivo*. Therefore, PACT was deduced to be involved in the negative regulation of p53 function through ubiquitin-dependent proteolysis by Mdm2 and implies a critical role for PACT in development and a considerable implication in tumourogenesis (Li *et al*, 2010). Therefore Mdm2 almost plays a similar role as RBBP6, in the sense that it functions as E3 ubiquitin ligase that is responsible for ubiquitination and degradation of p53 inactivating apoptosis and causing cell cycle arrest (Iwakuma and Lazano, 2004). In certain studies RBBP6 was shown to be upregulated in oesophageal cancer cells, and this high expression level went hand in hand with high rate of proliferation in these cells, thus cytotoxic T cells and antibodies specific against RBBP6 could successfully be utilized in immunotherapy against this cancer to allow the regression in mice xenograft models leading to decrease in tumour size (Pugh *et al*, 2006 and Muleya, 2010).

RBBP6, therefore, is involved cancer development due to the presence of RING finger domain in all homologues and the DWNN domain, which regulate pre-mRNA processing of proteins by covalently modifying them with an ubiquitin-like moiety. Similarly playing a role as an ubiquitin-like modifier thereby promoting cell proliferation because ubiquitination leads to targeted break-down (figure 1.2) (Pugh *et al*, 2006). The p53-binding domain is known to interact with p53, this resulting into a suppression of p53 binding to DNA. On the other hand, the Rb domain binds to Rb, blocking the binding of E2F transcription factors (Figure 1.2) (Pugh *et al*, 2006). Most cancers begin with the loss of function of some tumour suppressor genes followed by the accumulation of mutations in oncogenes and in tumour suppressor genes. The loss of function of p53, Rb including high expression of RBBP6 in some human tumours may alter the response to chemotherapeutic agents (Bertram, 2001). Restoring of p53 function together with alteration the function of many other tumour suppressor genes make an attractive target for gene therapy. For example, in pre-clinical studies it has been shown that introducing a wild- type p53 into prostate and lung tumour cells with mutated p53 results in apoptosis and injection of p53 vectors into tumours of immune-suppressed nude mice results into inhibition of cell proliferation (Bertram, 2001).

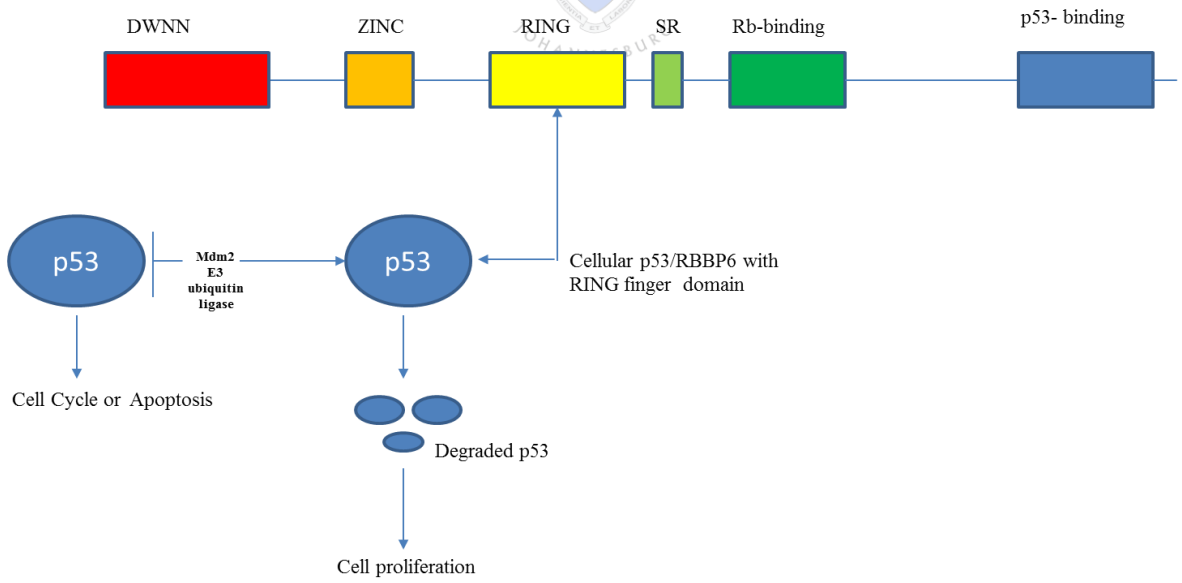


Figure 1.2: The structure showing RBBP6 protein family domain arrangement in Homo sapiens The homologues of RBBP contain a DWNN domain, a zinc knuckle and a RING finger with a p53 and Rb-binding domain. RBBP6 with RING finger domain binds to cellular p53 to facilitate the degradation of p53 or ubiquitination through Mdm2 (E3) to promote cell proliferation or Mdm2 may inhibit p53 resulting into cell cycle or apoptosis activation (Moll and Petrenko, 2003 and Pugh *et al*, 2006)

Chemotherapeutic agents provide a cure to cancer and comprise a diverse group of compounds with different mechanisms of action. But their ultimate ability to induce apoptosis represents a unifying concept for the mechanism of chemoprevention and treatment (Taraphdar *et al*, 2001). For instance, prevention or treatment of cervical cancer for example, is categorized into three areas of primary, secondary, and tertiary. The primary strategy aims to reduce the incidence of disease by targeting the entire population without symptoms and diseases. In cervical cancer, this includes preventive vaccines and risk reduction. The secondary strategy involves early detection or screening in an aim to reduce prevalence of the cervical cancer by Papanicolau smear and HPV detection. The tertiary strategy involves reduction of recurrence or early detection of recurrence, which includes therapeutic vaccines (Sasieni, 2006; Sitas *et al*, 2008, Frazer, 2008). Cervical cancer treatment regimens depend on several factors including the type and stage of cancer, possible side effects and individual preference. Surgically the tumour or surrounding tissue can be removed during an operation if the cancer has not spread beyond the cervix. Methods like conization which remove microinvasive cervical cancer have been used; hysterectomy, radition and chemotherapy have been vastly used if the cancer has spread beyond the cervix (Chien *et al*, 2011). It is therefore vital to investigate other chemoprevention strategies such as traditional medicine as a potential target for anti-cancer therapy.

1.3. Traditional medicine in apoptotic induction

More than 50% of all modern drugs in clinical use are derived from natural products, many of which have an ability to control cancer cells and even HIV/AIDS (Richter, 2003; Madhuri and Pandey, 2009). In the United States, 25% of the pharmaceutical prescriptions contain at least one plant-derived ingredient; therefore plants are a vital source of medicine (Samy and Gopalakrishnakone, 2007). There are about 69% of anti-cancer drugs, which were approved between the 1980's and 2002 and were evidently derived from natural products or developed on the knowledge gained from natural products (Newman and Cragg, 2007). In industrialized countries, medicinal herbs gradually lost importance during the course of chemistry's progression in the 20th century but they are currently experiencing an impressive revival (Efferth *et al*, 2008). Various plant products are known to induce apoptosis in neoplastic cells but not in normal cells. Since apoptosis is pivotal mode of action for anti-tumour agents, such as ionizing radiation, alkylating agents [cisplatin and 1-3-bis (2-chloroethyl)-1 nitrosourea (BCNU)], topoisomerase inhibitor etiposide, cytokine tumour necrosis factor (TNF), taxol, and N-substituted such as metoclopramide and 3-chloroprocainamide are able to induce

apoptosis without affecting normal cells (Taraphdar *et al*, 2001). It is thus considered vital to screen apoptotic inducers from plants, either in the form of crude extracts or as components isolated from them.

1.3.1. Plant extracts treating cancer and other diseases

A number of scientific investigations have highlighted the importance of many plant families (i.e. Apocynaceae, Vitaceae, Nyssaceae, to name but few) used as medicinal plants that play a vital role in the development of new drugs where bioactive compounds such as viniblastine, vincristine which treat lymphocytic leukemia, small cell lung, cervical and breast cancer can be exported or imported in the current market (Fabricant and Farnsworth, 2001; Samy and Gopalakrishnakone, 2007; Efferth *et al*, 2008). Natural products such as *Vinca* alkaloids obtained from *Catharanthus roseus* G. Don, family Apocynaceae; the DNA topoisomerase I inhibitor camptothecin from *Camptotheca acuminate* Decne. (Nyssaceae) and Resveratrol, which is derived from Vitis vinifera belonging Vitaceae family, possess anti-inflammatory and anti-mutagenous activities that are potential cancer chemopreventive agents (Hutching *et al*, 1996; Neuwinger, 1996; Jang *et al*, 1997; Lin *et al*, 1999; Cragg and Newman, 2005). Plants are therefore utilized in producing novel or known structures as lead compounds for semisynthesis to produce patentable entities of higher activity and/or lower toxicity such as taxotere, galegine. Using these agents as pharmacological tools or the whole plant/part of it as herbal remedies (e.g. cranberry, Echinacea, feverfew, garlic, ginkgo bibola, St. John's wort, saw palmetto) is advantageous. Since majority of chemotherapeutants have been reported to severely affect normal host cells and plant "natural" are considered ineffective to normal cells (Fabricant and Farnsworth, 2001, Taraphdar *et al*, 2001).

Experimentally and clinically, medicinal plants have been found to be effective against various types of cancers or tumours such as sarcoma, lymphoma, carcinoma and leukemia i.e. *Saphora flavescens* and *S. subprostrata* are effective in treating leukemia (and cervical cancer cells) and Indian *Nothapodytes nimmoniana* (*Mappia foetida*) is used to treat cervical cancer in Japan (Samy and Gopalakrishnakone, 2007; Madhuri and Pandey, 2009). Some of these plants possess immunomodulatory stimulating non-specific immunity together with specific immunity and antioxidant properties that may prevent and cure cancer and other diseases by protecting cells from highly reactive oxygen compound damage (Madhuri and Pandey, 2009).

Thus, much plant- derived products have been reported to exhibit strong anti-tumour activity against several rodent and human cancer cell lines.

In the studies done on *S. frutescens* by Zhao *et al*. (2003) and Nile *et al*. (2003), plant extracts were able to induce apoptosis in cancer cells. Thus, this plant can be utilized to generate therapeutic drugs because it induces apoptosis as this is one approach in cancer therapy. In Japan, sho-saiko-to consisting of crude ingredients extracted from *Bupleuri radix*, *Pinelliae tuber*, *Scutellariae radix*, *Zizyphi fructus*, *Ginseg radix*, *Glycyrrhizae*, and *Zingiberis rhizome* has been reported to suppress proliferation of human HCC cell line and cholangiocarcinoma cells because ingredients such as glycyrrhizin and baicalein can interfere with the cell cycle at the G_0G_1 phase *in vitro* and with DNA synthesis. This herbal medicine was reported to have not affected normal PBL human cells when water-soluble ingredients of sho-saiko-to were used (Yano *et al*, 1994). On the other hand, Opuku *et al*. (2000) demonstrated that aqueous extracts form medicinal plants of Vitaceae in South Africa showed anti-proliferative activities of up to 97% inhibition *in vitro* against HepG2 cells. Ethanolic extracts of *S. frutescens* in the study by Stander *et al*, 2007 inhibited proliferation of about 50% of MCF-7 mammary adenocarcinoma cells over the period of 72 hours at a concentration of 1.5mg/ml.

Southern African plants such as *Tulbaghia* (known as a wild garlic herb) species are known to treat various ailments such as fever, cold, asthma, tuberculosis, stomach-ache and even cancer of the esophagus (Van Wyk and Gericke, 2000). The sulfur-containing compounds found in this species were discovered to attribute to their characteristic smell and taste and other compounds are essential for antibacterial activity (Lyantagaye, 2011). Compounds 2,4,5,7-tetrathiaoctane-2,2- dioxide and 2,4,5,7-tetrathiaoctane found on leaves of *T. violacea* were found to have antibacterial activity and their crude aqueous extracts have been shown to exhibit apoptosis inducing ability, and so potentially these extract can be used as anticancer agents (Burton, 1990; Lyantagaye and Rees, 2003; Davison *et al*, 2012). Derived compounds of *T. violacea* containing a methyl-%-D-glucopyranoside (MDG) moiety in their structure are postulated to interfere with the bioactivities of *hexokinase*, as well to induce reactive oxygen species, which cause cellular damage and result in apoptotic cell death in the study by Lyantagaye *et al*, 2005. The Agavaceae families of plants, such as *Agave americana*, have been reported to traditionally treat high blood pressure in Africa and their concentrated sap

and extract have anti-bacterial activity and anti-inflammatory effect respectively (Anajwala *et al*, 2010). In the study by Anajwala *et al*, 2010 on the MCF-7 breast cancer cell line, it was reported that methanolic extract of *A. americana* show potent toxicity by MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide and SRB (sulforhodamine B) assay. Crude extracts on the other hand were discovered to possess two compounds that are similar to the neurotransmitter; acetylcholine and these compounds may be involved in cytotoxicity (Hackman *et al*, 2006).

There has been some success in the use of traditional medicinal products approach to anti-cancer drug discovery. Natural products like staurosporine (ST) isolated from *Streptomyces stausporeus* are known successfully induce cell cycle arrest or apoptosis at varying concentration in many types of cancers (Bernard *et al*, 2001). But little is known about Southern African plants used to treat cancer, thus screening various plant extracts to identify potentially new anti-cancer drugs is ideal. The molecular mechanisms underlying cancer progression and cell death has been intensively studied. Yet, facilities for diagnosis and treatment or even palliation of cancer in developing countries like South Africa are extremely limited and many people are dependent on traditional medicine because of their accessibility and affordability (Denny, 2006; Steenkamp, 2003; Fouche et al, 2008). It is therefore vital to screen traditional medicinal products together with RBBP6 for anti-cancer action in cervical cancer.

1.4. Objectives and Rationale

The purpose of this study is to screen several South African traditional medicinal plants for anti-cancer therapy in cervical cancer. It is necessary to test whether there are any natural products that can be used in combination with several apoptosis inducing genes such as RBBP6 to act as anti-tumour agents. The understanding of the role of RBBP6 and apoptosis in the development of cervical cancer (especially HPV-induced cervical cancer) might lead to the development of new anti-cancer therapeutic drugs/vaccines that may guide tumour cells to apoptosis without being toxic to normal cells.

- To treat human cervical carcinoma cells lines (HeLa and ME-180) and normal human fibroblasts (KMST-6) with different concentrations of traditional crude plant extract of *Tulbaghia violacea* and *Agave palmeri* and then evaluate the cytotoxic activity of these plant extracts by measuring cell viability.

- To determine the mode of cell death induced by plant extracts of *T. violacea* and *A. palmeri* (whether apoptotic or necrotic) on HeLa, ME-180 and KMST-6 using flow cytometry.

- To extract protein of untreated and treated HeLa, ME-180, and KMST-6 cell lines after treatment with plant extracts that induce cell death and assess protein expression of Bax, Bcl-2, p53, Rb, Mdm2 and RBBP6 by immunoblotting.

- To elucidate the role of RBBP6 and p53 genes in cervical cancer.

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CHAPTER 2

MATERIALS AND METHODS

2.1. Introduction

In this current chapter procedures together with principals of each technique used in this study will be further discussed before elaborating on the protocol of each technique. Techniques used in this study include the following: Cell Cytototixity Assay (MTT Assay), Flow Cytometry (FITC Annexin V Apopotosis Detection kit I) using FACSDiva version 6.2, Polymerase Chain Reaction (PCR), RT-PCR and immunoblot analysis (western blot). Chemicals, supplies, recipes for solutions together with equipment used will be given appendix A and B.

2.2. Materials

2.2.1. Cell lines

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In this study, both non-tumourigenic and tumourigenic cell lines were used to characterize the role of various South Africa medicinal plants. These cell lines were purchased from the Health Science Research Resources Bank of the Japanese Collection of Research Bio-resources (JCRB). They include the following: HeLa (carcinoma of cervix), ME180 (cervical carcinoma), and the non-tumourigenic immortalized human cell lines, KMST-6.

Cell lines were cultured in 25cm² cell culture flasks and maintained in Eagle's minimum essential media (MEM with L-glutamine)/ Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10 % Fetal Bovine Serum (FBS) and 1% Penicillin streptomycin (pen/strep) (Sigma Aldrich). Cell cultures were maintained at 37ºC in an incubator with 5% carbon dioxide (CO_2) atmosphere. Cells were grown to 70-80% confluence as a monolayer, the medium was discarded and cells were washed with sterile Phosphate Buffered Saline (PBS). The cells were trypsinised using 2ml of trypsin/ethylenediaminetetra-acetic acid (EDTA) (Sigma Aldrich) and incubated at 37ºC to allow detachment. Medium was added to stop the trypsin reaction.

2.2.2. Plant material and extract preparation

Agave palmeri and *Tulbaghia violacea* stem and leaves were collected from the arid highland region of South Africa (SA), in Limpopo. Some of the plants material was obtained from Prof L.J. Mampuru from the University of Limpopo, Turfloop (School of Molecular and Life Science). Stems and leaves were dried in an oven. Using liquid nitrogen, the plant material was blended and the powder mixture was then weighed and mixed with distilled water (dsH_2O). The suspension was filtered through Whatman® qualitative filter paper (Sigma Aldrich) overnight. The filtrate was then weighed and homogenized with Dimethyl sulfoxide (DMSO) to a required concentration of 40mg/ml. This was further be passed through the 0.45µm and 0.22µm filters to obtain a purified stock solution that was further diluted with MEM and DMEM supplemented with 10% FBS and 1% pen/strep to make 50, 100, and 150µg/ml (w/v) for the treatment of cell lines.

2.3. Methods

2.3.1. Cell Cytotoxicity Assay (MTT Assay)

The MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide] is a colorimetric assay method used to measure cell cytotoxicity of potential medicinal agents such as *A. palmeri* and *T. violacea* plant extract because of their ability to stimulate and inhibit cell viability and growth. Cell proliferation (cell culture assay) together with viability (cell counting) can be measured by the capability of enzyme activity that reduces MTT in metabolically active cells (a pale yellow coloured substrate) to an insoluble formazan dye which is insoluble and gives a purple colour once solubilised for spectrophotometer measurement (Mossman, 1983).

Ninety six well tissue culture plates were used to culture cells for the MTT assay. The 5×10^4 cells of HeLa, ME180, and KMST-1 in 90µl media were seeded into each well and incubated overnight at 37^oC prior to treatment with varying concentrations of plant extract of *A. palmeri* and *T. violacea* (50, 100, 150µg/ml (w/v)). Other wells were left for a non-treatment control and a blank with only cells and media and the other well seeded with cells to be treated with 25nM staurosporine (ST) a known apoptosis inducer (positive control). Cells were treated with plant extract and ST for 24 hours. Later, 10 µl of MTT (prepared to 5mg/ml PBS)

(Appendix A1) was added to each well except for the blank and the plates were further incubated for 4 hours. To dissolve the formazan crystals formed, 90 µl of DMSO was added and the optical density was measured using a spectrophotometer (Bio-Rad Microplate reader) with a reference of 570nm wavelength to obtain absorbance (Abs) values, which is a direct representation of viable cells.

Cell viability was calculated as follows:

$$\%\boldsymbol{Cell\ Viability} = \frac{[\boldsymbol{Abs\ of\ treated\ cells} - \boldsymbol{Abs\ of\ blank}]}{[\boldsymbol{Abs\ of\ untreated\ cells} - \boldsymbol{Abs\ \ of\ blank}]} * \mathbf{100}$$

2.3.2. Flow Cytometry (Annexin V- FITC Staining)

This assay aids in measuring membrane alteration of an individual cell in a homogenous population in order to distinguishing live, apoptotic (early/ late) and necrotic cells (Rahman, 2005). A number of cell surface changes occur during apoptosis i.e. phospholipid distribution, which is asymmetrical with the inner membrane containing anionic phospholipids such as phosphatidyl-serine plus an outer membrane with neutral phospholipid in normal cells. The amount of phosphatidylserine (PS) on the outer surface of the membrane increases in the apoptotic cell, exposing PS to the surrounding liquid (Lassota, 1992). After the MTT assay, flow cytometry was used to analyse the cytotoxic effect of *T. violacea*, *A. palmeri* and staurosporine on HeLa, ME-180 and KMST-6 cell lines.

To quantitatively determine the percentage of cells within the population that are undergoing apoptosis, the annexin V-FITC apoptosis detection kit from BD Biosciences was used. Annexin V (a calcium-dependent phospholipid-binding protein) conjugated to a flourochrome probe, flourescein isothiocyanate (FITC) is used in conjunction with propidium iodide staining solution for flow cytometric analyses of cells undergoing apoptosis or necrosis respectively (Hawley and Harley, 2005). This is because annexin V has a high affinity for PS exposed on surface of apoptotic cell and will not bind to living cells. Because 2 flourochromes were used in this experiment and emission profiles are known to coincide in such cases, fluorescence compensations and gating procedures were set by having flask of cells of untreated cells and treated cells which were FITC annexin V only stained, PI stained

only, unstained, annexin V/PI stained to obtain control readings which eliminate results of unwanted particles e.g. debris (Rahman, 2005).

About $1x10^5$ HeLa, ME180, and KMST-6 cells were incubated overnight at 37^oC to 70% confluency in $25cm^2$ cell culture flask (section 2.2.1) and treated with 150µg/ ml concentrations of natural plant extract of *A. palmeri*, *T. violacea* and 25nM staurosporine (positive control) for 24 hours because in these concentrations approximately 50% cell death was induced according to MTT assay studies. A negative control of untreated and singled stained annexin V/PI cells was also included to eliminate cell debris and quantify each dye with which a particular cell is labeled. After which, the cell population of interest was selected by creating a gate around the population of about 5000 cells of interest per run. Cells were then detached by trypsinization (Trypsin-EDTA) (section 2.2.1), 1ml cold PBS was added and cells were transferred into 1ml eppendorf tube, then centrifuged at 400 g for 5minutes. The supernatant was discarded and the pellet was wash in cold PBS twice. After these washes the pellet was resuspended in 100µl 1X binding annexin V binding buffer at a concentration of $1x10^5$ cells/ml. Into a eppendorf with 100µl of the solution 5µl of FITC annexin V and 5µl PI was added then incubated in the dark at room temperature for 10-15 minutes to allow binding. After this period 400µl 1X binding buffer was added to each tube and analysis using BD FACScalibur Flow Cytometer was done within 1 hour.

2.3.3. Total Ribonucleic Acid (RNA) extraction

Total RNA was extracted from both treated and untreated cell lines (HeLa, ME180 and KMST-6) using High Pure RNA Isolation Kit (Roche, German-RSA). Cultured cell were trypsinized, pelleted and resuspended in 200µl PBS. Cells were then lysed in 400µl of lysis-binding buffer and then vortexed for 15seconds. The sample was then transferred into the High Pure Filter tube, incubated for 1 minute and then centrifuged for 15 seconds at 8000X g at room temperature. The flow-through liquid was discarded to leave behind the sample attached into the High Pure Tube. Samples remaining in the High Pure Tube membrane and in the collection tube were incubated with a mixture of incubation buffer and DNAase I for 15 minutes at room temperature. The High Pure Tube together with collection tube was further washed with 500µl of wash buffer I and centrifuged for 15 seconds at 8000X g. The flow-through was discarded and the tubes reassembled and the washing was repeated with 500µl wash buffer II. A third wash was done with 200µl Wash Buffer II at 12 000X g for 2 minutes at room temperature. The High Pure Tube was transferred into a sterile 1.5 ml micro

centrifuge/ eppendorf tube. Then 50µl of RNA Elution Buffer was added to the assembled micro centrifuge tube and High Pure Tube to elude the total RNA and incubated for 1 minute. The elution was carried out by centrifuging at 12 000X g for 2 minutes.

The total RNA was quantified using a Nanodrop (NanoDrop technologies, USA) at 260 and 280nm as per manufactures guide. The quality of 260/280 ratio more than 2.0 was regarded as pure and this was further accessed with RNA electrophoresis (Appendix B17). Remaining RNA was aliquoted into 10µl of aliquots and stored at – 80 ºC for future use.

2.3.4. RNA electrophoresis

Once, RNA had been extracted, 1% (w/v) agarose gel (dissolved in 10ml 10X 3-(N-morpholino) propanesulfonic acid buffer, MOPS and 72 ml sterile water) was made and allowed to cool to about 50ºC, 18ml of 37% (v/v) formaldehyde was added and mixture was stained with 3 µl of 100 µg/ml ethidium bromide (EtBr). Ten microlitres of total extracted RNA together with 10 µl of freshly prepared formaldehyde gel loading buffer will be mixed and heated at 65ºC for 5 min allowed to cool on ice for 2 minutes and the loaded on the gel which will be ran at 100Volts for ± 2 hours in 1X MOPS (Appendix B3).

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2.3.5. Total Deoxyribonucleic Acid (DNA) extraction

Fragments of DNA released from the untreated and treated cells during necrosis or cell-mediated cytotoxicity, or within the cytoplasm of apoptotic cells can be analyzed by extracting DNA. A Quick-gDNA™ MiniPrep kit, from Zymo Research was used to extract DNA from untreated and treated cells (HeLa, ME-180 and KMST-6). Cells were harvested into sterile 15 ml tube and washed with 1X PBS centrifuged for 10 minutes at 10° C (1200 rpm). The supernatant was discarded and the cell pellet was resuspended in 1X PBS. Cells pellet was washed twice with 10 ml 1X PBS and centrifuged between the washes. The pellet was resuspend in 200µl Genomic Lysis buffer, vortexed and allowed to stand for 5-10minutes at room temperature. The mixture was transferred to Zymo- Spin™ Column in a collection tube. This was centrifuged at 10 000X g for 1 minute and the collection tube with the flow through was discarded. The Zymo-Spin™ Column was transferred into a new Collection Tube then 200 µl of DNA Pre-Wash Buffer was added to the spin column,

centrifuging the column at 10,000 x g for 1 minute. 500 µl of g-DNA Wash Buffer to the spin column was added and further centrifugation at 10,000 x g for one minute. The spin column was transferred to a clean microcentrifuge tube and ≥50 µl DNA Elution Buffer was added to the spin column. This was incubated for 2-5 minutes at room temperature and then centrifuge at top speed for 30 seconds to elute the DNA. The eluted DNA was used immediately to investigate whether DNA had been fragmented after treating cells with 150µg/mL *T. violacea* and *A. palmeri* for 24 hours or stored ≤-20ºC for future use. DNA concentration was measured using a nanodrop (NanoDrop technologies, USA) as per manufactures guide. 5 µl of each DNA sample was run on 1% agarose gel electrophoresis.

Gel electrophoresis is used as a visualisation technique, to investigate on whether treated and untreated cells have DNA fragments, smears or one band when undergoing apoptosis, necrosis or cell proliferation, respectively.

2.3.6. Agarose gel electrophoresis of DNA

The principle of agarose gel electrophoresis involves separation of nucleic acids according to their size and conformation. During this technique the DNA fragments can be visualized by staining the gel with ethidium bromide, that intercalates between bases of DNA and illuminating the gel under UV light on a transluminator. Nuclear changes such fragmentation of DNA occur during apoptosis. Agarose gel electrophoresis is a technique used for separation of nucleic acids according to size and charge. The DNA fragments were then visualized by staining the gel with ethidium bromide at a concentration of 1µg/ml, which intercalates between bases of DNA and illuminating the gel under UV light using a transluminator. Agarose gel (1%) (Appendix B2) was prepared in 1 X TBE and contained 2µl ethidium bromide in order to electrophorese the DNA samples. The DNA molecular weight marker used was from Fermentas Mass Ruler DNA ladder mix, Ready-to-use, 80-10 000bp (Appendix B, Figure 1.19). Gels were then subjected to 70 volts for 90 minutes in 1X TBE buffer.

2.3.7. Reverse transcription (RT)

Promega's cDNA synthesis kit for RT-PCR (Promega, USA) was used to synthesize cDNA for gene expression studies. The enzyme, reverse transcriptase, in the kit synthesizes a new

cDNA strand at the site determined by the type of primer used and the resulting cDNA is then used as a DNA template for normal PCR.

Reserve transcription was therefore performed as per manufacturer instructions as follows: A total RNA sample of 2µg was mixed with 0.5µg per reaction oligo (dT)15 primer and incubated at 70 ºC for five minutes to denature any secondary structures and to allow the primers to anneal. The template preparation was then placed in ice for 5 minutes. The preparation (Appendix B) was then mixed with the rest of the components of the cDNA synthesis reaction tabulated in table 1.2 (Appendix B). The samples were mixed and briefly centrifuged. Following this samples were incubated at 25 ºC for 10 minutes to allow the primers to anneal at their sites. This was followed by incubation of samples at 42 ºC for 60 minutes to allow reverse transcription. Reverse transcriptase was inactivated by incubation at 70 ºC for 10 minutes and the sample were cooled on ice. The sample were either used immediately for qRT-PCR or PCR or stored at -20 ºC for later use.

2.3.8. Polymerase Chain Reaction

2.3.8.1. Primers

To amplify a fragment of the translated regions and to measure the expression of TP53, Retinoblastoma Binding Protein 6 (RBBP6) and RB1 genes, the following primers were designed using the National Centre for Biotechnology Information (NCBI) website and once the primer sequences were identified, the sequence was sent to Inqaba Biotechnological Industry, (SA) for synthesis.

p53 primers:

Forward primer: 5´ GGC CCA CTT CAC CGT ACTA A 3´

Reverse primer: 5´ GTG GTT TCA AGG CCA GAT GT 3´

RBBP6 primers:

Forward primer: 5´ CAG CGA CGA CTA AAA GAA GAG TCT 3´

Reverse primer: 5´ GGT AAT TGC GGC TCT TGC CT 3´

Rb1 Primers:

Forward primer: 5´ TGC ATG GCT CTC AGA TTC AC 3´

Reverse primer: 5´ AAG GCT GAG GTT GCT TGT GT 3´

Polymerase chain reaction (PCR) is a technique that allows logarithmic amplification of short DNA sequences of between 100 to 600 bases pair within a longer double stranded DNA molecule. Primers for genes of interest were designed using Nucleotide sequences from NCBI. The cDNA obtained through reverse transcription was used as a DNA template. The PCR was performed using a master mix from Promega (USA). The master mix contains deoxyribonucleotides, 2X PCR buffer, $MgCl_2$ and Taq Polymerase. A total of 25µl reactions required exactly 12.5µl master mix; 1µl (10pmoles) of each of the sequence specific primers; 2µl 25mM MgCl2; 1µl template DNA (1pg-1µg) and 7.5µl nuclease-free water (Appendix B). The reactions were then subjected to approximately 30-35 cycles of the three PCR steps (denaturation, annealing and extension) after the initial denaturation step and followed by a final extension step. The steps were as follow with some variation in annealing temperature and extension time depending on the nucleotide sequence of the gene of interest:

95 ºC for 2 Min

94 ºC for 30 sec

Tm-5 for 30 sec → 30-35 cycles

72 ºC for 30 sec

72 ºC for 10 Min

The products were stored at 4 ºC until further use for RT-PCR.

2.3.9. Quantification of mRNA using RT-PCR

Real time polymerase chain reaction is the most sensitive technique for simultaneous mRNA detection and quantification. It is used to quantitate changes in gene expression patterns. It can also be used to quantify mRNA levels from much smaller samples, such as mRNA from a single cell.

To monitor cDNA amplification in RT-PCR, SYBR Green JumpStart Taq Ready Mix (Sigma) was used in our study. The mixture contains a JumpStart Taq DNA polymerase and only requires addition of template, primers and water. It is a fluorescent intercalating dye which presents the simplest and cheapest way of detecting PCR product in real time. The dye will only fluoresce when bound to double stranded DNA, thus allowing for fluorescence to increase as the number of double stranded DNA increases, this allows for DNA concentration to be quantified at each cycle. The real-time PCR cocktail together with the parameters are explained in Appendix B. Quantitative analysis of the mRNA transcripts was carried out. The real-time PCR cocktail together with the parameters taken into account were analyzed on the Roche LightCycler machine used for quantitative analysis of mRNA transcript explained in Appendix B in duplicate. The melting point was analysed using a RT-PCR to confirm the success of our amplification and it was indicated by a single peak at a melting temperature point of the product.

2.3.10. Western blot/ Immunoblotting

Western blotting, also referred to as immunoblotting, is well established and widely used technique for the detection and analysis of proteins. It is based on building an antibody protein complex via specific binding of an antibody to proteins immobilized on a membrane (such as nitrocellulose or polyvinylidene fluoride (PVDF) and detecting the bound antibody by various methods based on chemiluminescence, chemiflourescence, fluorescence, chromogenic or radioisotopic detection (Towbin *et al*, 1979). A workflow is giving below:

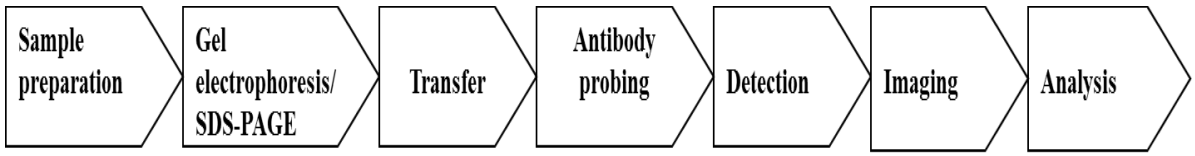


Figure 1.3: A workflow underlining basic steps followed in Western blotting

2.3.10.1. Protein extraction

Protein was extracted from cultured cells in monolayer using the Thermo Scientific RIPA buffer. This buffer allows protein extraction of cytoplasmic, membrane and nuclear protein.

Cells treated and untreated with plant extracts and staurosporine were washed with ice-cold phosphate buffer saline (PBS) twice, suspended in 500µl cold RIPA extraction buffer (Thermo Scientific, Rockford, USA) and kept in ice for 5 minutes, swirling the plate occasionally for uniform spreading. Using a cell scraper, a lysate was gathered and transferred into a 1ml eppendorf tube and centrifuged at ~14 000x g for 15 minutes to pellet the cell debris. The supernatant was transferred into a new eppendorf and stored at 70^{o}C for further analysis.

2.3.10.2. Protein estimation assay

Protein estimation for calorimetric detection and quantification of total protein was done using the Pierce® Bicinchoninic (BCA) protein Assay Kit (Thermo Scientific, Rockford, USA). Protein concentration was determined and reported with reference to standards of bovine serum albumin (BSA). A series of diluted standards of BSA were prepared, where one ampule of BSA was serially diluted with distilled water into 1.5ml eppendorf tube in a working range of 20- 2000µg/ml in a volume of 3 replicates. Concurrently a serial dilution of the unknown sample of protein extracted from HeLa, ME-180 and KMST-6 cell line both treated and untreated with plant extract and staurosporine was prepared for measurement. Into a microplate well, 25µl of the solution from each test tube (standard and unknown sample replicate) was pipetted. 200µl working reagent (WR) containing a 50:1, BCA reagent A: B was added to each well and mixed thoroughly on a plate shaker for 30 seconds, then covered and incubated at 37^{o}C for 30 minutes. The plate was cooled to room temperature the absorbance at or near 560nm was read using a plate reader. The absorbance of the standards versus their concentration was plotted and the extinction coefficient was computed and the unknown protein sample concentration was calculated.

2.3.10.3 Antibodies

RBBP6, p53, Mdm2, Rb, Bax, Bcl 2 and β-actin mouse monoclonal primary antibodies (IgG) and goat anti mouse coupled with horseradish peroxidase (HRP) secondary antibody from SantaCruz Biotechechnology were used in the immunoblot experiments.

2.3.10.4 Immunoblot analysis

After the proteins were quantified, approximately 30-40µg of total cellular protein were boiled in 2X sodium dodecyl sulphate (SDS) sample loading buffer [(125mM Tris HCL, 4% (w/v) sds, 20% glycerol, 10% (V/V) 2- mercaptoethanol, pH 6.8)].The proteins were

resolved in 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Appendix B) according to Laemmli (1970) at a constant voltage of 90 V with SDS-PAGE 1X running buffer (Appendix B). One gel was stained with Coomassie Brilliant Blue R250 after electrophoresis to check the transfer efficiency (Appendix B). Another gel with protein samples was then electro-blotted onto polyvinylidene fluoride transfer (PVDF) membrane 0.45µm (Pall Corporation, Pensacola, FL, USA) using the Biorad mini trans-blot system at 400milliamperes (mA) for 60 minutes at 4^{o}C in a western blot transfer buffer (Appendix B). Upon completion of the protein transfer, the membrane was blocked with 0.05% fat free milk (Appendix B) for 1 hour shaking slowly at room temperature in order to block non-specific binding sites After blocking, the membrane was incubated in blocking buffer containing a specific primary mouse monoclonal antibodies: p53 (1:750), P2P-R (1:500), Rb (1:2500, Mdm2 (1:250), Bax (1:750), Bcl-2 (1:500) and β- Actin (1:1000) at 4^{o}C overnight. 1: 2000 primary antibody of interest and incubated overnight at 4^{O}C in the shaker. β actin was utilized as an internal control. To remove residual antibody, the membrane was washed with 0.1%PBS+ Tween 20 3 times at 10 minute intervals. Then the membrane was ready for incubation with goat anti-mouse (IgG) HRP conjugated secondary antibody at 1: 10 000 in blocking buffer for 2 hours at room temperature. The membrane was then washed with 1X PBS + 0.1 % Tween as described above. The membrane was then exposed to Thermo Scientific SuperSignal West Dura Substrate, a luminol-based enhanced chemiluminescence (ECL) HRP substrate (Thermo Scientific, Rockford, USA) for 10minutes in order to detect immunoreactive proteins. These were then visualized using a ChemiDoc XRS Image Analyzer (Biorad Laboratories, Inc., USA). Each immunoblot was repeated 3 times.

2.3.11. ***Statistical analysis***

The results of each series of experiments (performed in triplicates) were expressed as the mean values ± standard deviation of the mean (SD). Levels of the statistical significance were calculated using the paired t-test when comparing two groups (Graph Pad Instat program found on http://www.graphpad.com/scientific-software/instat/) and the P-values, $P<0.05$ were considered significant.

CHAPTER 3

RESULTS

3.1. Introduction

The purpose of this study was to screen *Tulbaghia violacea* and *Agave palmeri* plant extract for RBBP-6 anti- cancer therapy in cervical cancer. However, with little knowledge on these two plant extracts' mode of action and their chemical profiles together with active compounds, we had to first identify one suitable solvent to extract active compounds that might be of importance in activating cell death in cancer cells.. The results obtained in this chapter address the following hypothesis:

I) Plants extracts of *Tulbaghia violacea* and *Agave palmeri* have anti-proliferative properties and induce cell death in cervical cancer cell line (HeLa and ME180).

To prove this hypothesis a variety of experimental procedures were carried out; these involved MTT assay (cell cytotoxicity/ viability assay), Flow Cytometry (Annexin V)/ apoptosis detection, and immunoblotting for the detection of apoptotic protein expression.

3.2. *Tulbaghai violacea* and *Agave palmeri* crude plant extracts have a cytotoxic effect on cervical cancer cells

To examine whether *Tulbaghia violacea* and *Agave palmeri* extracts affects the viability of HeLa, ME-180, and KMST-6 (non-tumourogenic human embryo fibroblast) cell lines, the cells were treated with plant extract at 50-150 µg/mL for a period 8- 24 hours. As shown in figure 1.4 (A-D) and in figure 1.5 (A-B), cell viability was slightly decreased following treatment by *T. violecea* and *A. palmeri* in a dose-dependent manner over 24 hours. Sensitivity at the highest concentration tested (150 µg/ml) was high in the following order: HeLa (figure 1.4 A and B), ME-180 (figure C and D) and KMST-6 (Figure 1.5 A and B) to crude plant extract of *T. violecea* and *A. palmeri*. The ever green leaves of *T. violacea* extracts in this study, have shown to have more anti-proliferative effect in HeLa cells at a concentration dependent cytotoxic activity with half maximal inhibitory concentration (IC_{50}) value greater than 150µg/ml exhibiting 58% cell viability and 42% cell death following 24 hour treatment (Figure 1.4A). However, in ME-180 and KMST-6 at the same concentration it exhibit a weak cytotoxicity activity with both 21% and 18% cell death respectively after

treatment (figure 1.4C and 1.5A).

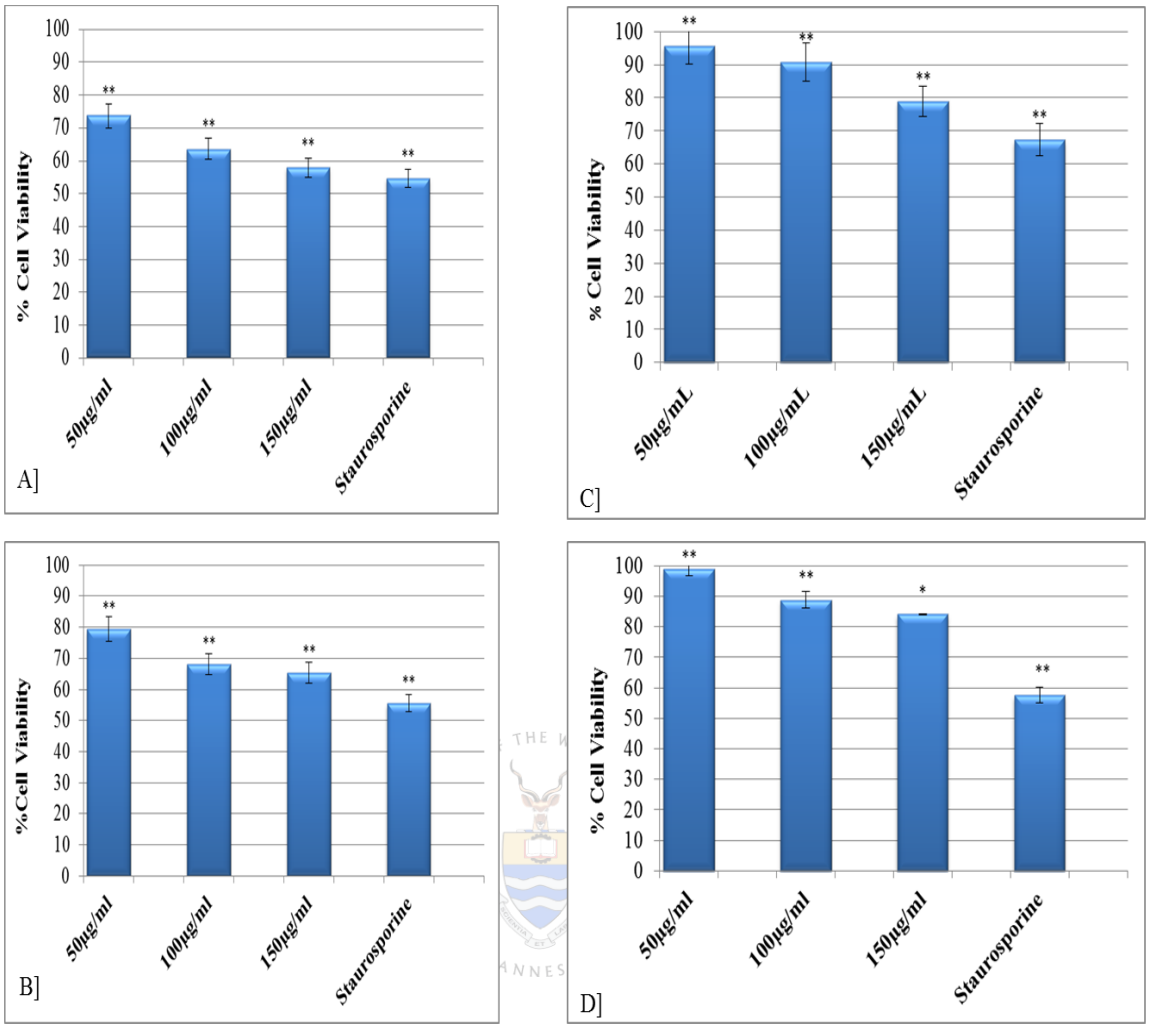


Figure 1.4: Cell viability assay of the herbal extract A] *T. violecea,* B] *A. palmeri* at increasing concentration of (50, 100 and 150µg/ml) on HeLa (carcinoma of cervix) and C] *T. violecea* D] *A. palmeri* on ME-180 (cervical carcinoma) cell line together with 25nM staurosporine for 24 hours. Cell viability was determined in triplicate from three independent experiments by MTT assay. Data represented as mean ± SD, **P<0.10, *P< 0.05

On the other hand, after treatment with *A. palmeri*, results obtained suggested that crude extracts did not exhibit our expected anti-proliferative activity in both ME-180 (figure 1.4 D) and KMST-6 (figure 1.5B) cell lines, but showed very little cytotoxic activity on HeLa cell line (figure 1.4B) at >150µg/ml which was our maximum concentration in which only 34% cell death was induced. However, following treatment at 100µg/mL for 24 hours only 32% cell death was induced in HeLa cell (figure 1.4 B). In summary, the crude plant extract of *T. violecea* and *A. palmeri* highly reduced cell viability in HeLa but not in ME-180 and KMST-6 cell line at the concentration of 150µg/ml as compared to control experiments **p<0.10.

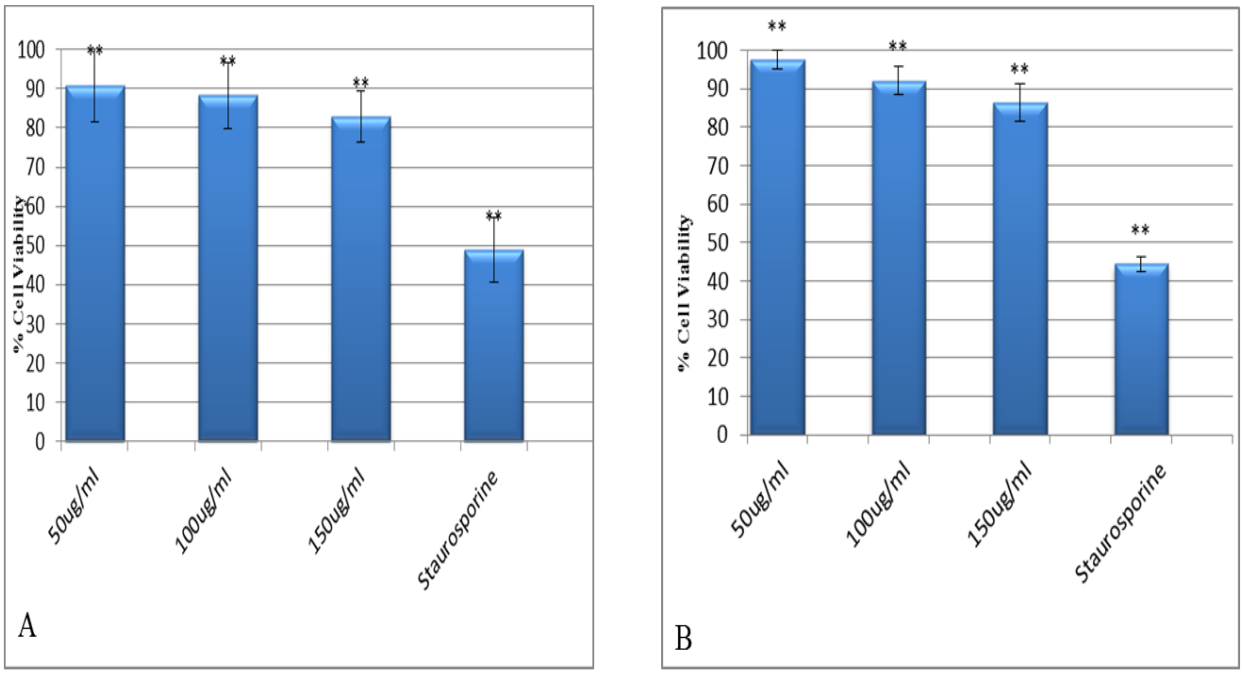


Figure 1.5: Cell viability assay of the herbal extract A] *T. violecea*, B] *A. palmeri* at increasing concentration of (50, 100 and 150µg/ml) on KMST-6 (human embryo non-tumourigenic) cell line together with 25nM staurosporine for 24 hours. Cell viability was determined in triplicate from three independent experiments by MTT assay. Data represented as mean ± SD, **P<0.10

ST was shown to inhibit cell viability at a concentration of 25nM in HeLa, ME-180 and KMST-6 (figure 1.4 and 1.5) cell lines after 24-hour treatment. Consequently, to determine whether the inhibition of cell viability by these plants extract (TV and AG) and ST resulted from apoptosis, a measurement of the cell population after 24-hour treatment was done by flow cytometry.

3.3. Tulbaghia violacea and *Agave palmeri* crude plant extracts trigger DNA fragmentation in HeLa and ME-180 cervical cancer cell line

To gain an insight on the effect of crude plant extract of *T. violacea* and *A. palmeri* on the nuclear alteration, the hallmark of apoptosis, DNA fragmentation. The effect of these were analysed on HeLa, ME-180 and KMST-6 cell lines. To detect these two changes, cells were treated with 150µg/ml of *T. violacea* and *A. palmeri* together with 25nM of staurosporine for 24 hours.

Figure 1.6 shows that the occurrence of DNA fragmentation is indicated by the distinct smear of nuclear material which is indicative of DNA fragmentation on the wells of the gel. Normally, this is represented by distinct multiple bands on the agarose gel. From our results with untreated cells as represented in lanes 2, 6 and 10 there seem to be no apoptosis that was initiated as the is still one distinct single band as compare to other lanes that have shown a smear or DNA fragment especial from those cells treated with ST (figure 1.6).

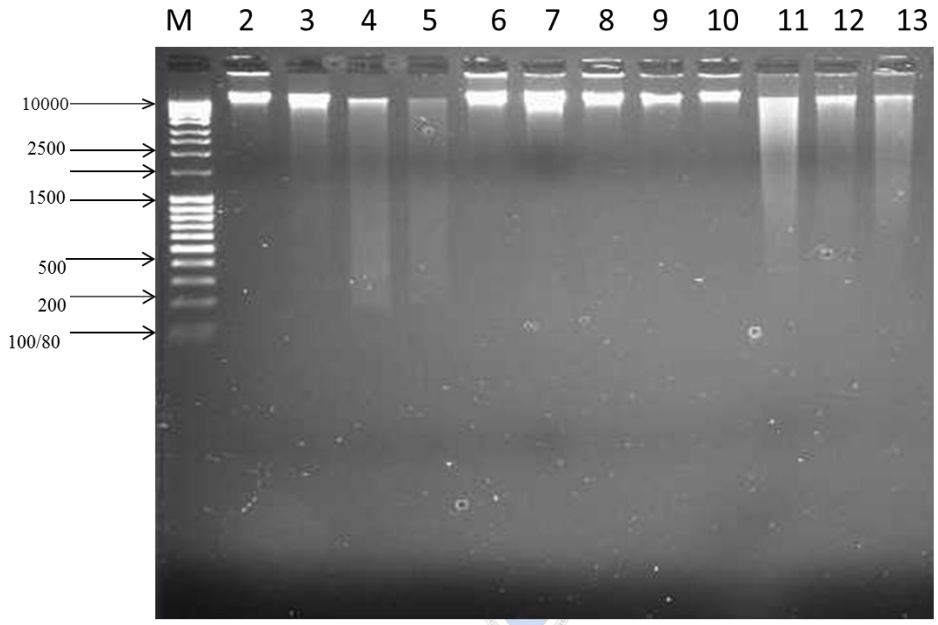


Figure 1.6: DNA fragmentation in HeLa, KMST-6 and ME-180 cell line following treatment with *T. violecea* (TV), staurosporine (ST), *A. palmeri* (AG). A 1.5% Agarose gel electrophoresis demonstrating apoptotic DNA fragmentation together with necrosis characteristics. Lane-M represents the marker for DNA size while lane 2, 3, 4 and 5 represents HeLa cell line of untreated control sample and treated HeLa cell line with, staurosporine, *T. violecea* and *A. palmeri* respectively. Lane 6, 7, 8, and 9 represents KMST-6 cell line untreated control sample and treated with staurosporine, TV, AG respectively and lane 10, 11, 12, and 13 ME-180 cell line untreated sample and treated ST, TV and AG for 24 hours.

3.4. Tulbaghia violacea and *Agave palmeri* crude plant extracts trigger apoptosis in HeLa and ME-180 cervical cancer cell line

Advances in DNA fragmentation are preceded by the alteration in PS where during the early stages of apoptosis, the PS asymmetry is lost because of its externalization (Wong and Kadir, 2011). This particular feature change was investigated using the Annexin V/PI staining kit, where cells treated and untreated with plant extract were double stained with Annexin V-FITC/PI or unstained and analyzed by flow cytometry (figure 1.7, 1.8 and 1.9), described in chapter 2, section 2.3.2. It was vital to include negative and positive controls in this

experiment, in order to make sure that the selected population to be analyzed was a true reflection of the events that were taking place after treatment with plant extracts and that cell debris was eliminated from the calculation of cells undergoing apoptosis as well as eliminating those that are alive or necrotic. The graphs below are representative of the flow cytometry apoptosis analysis.

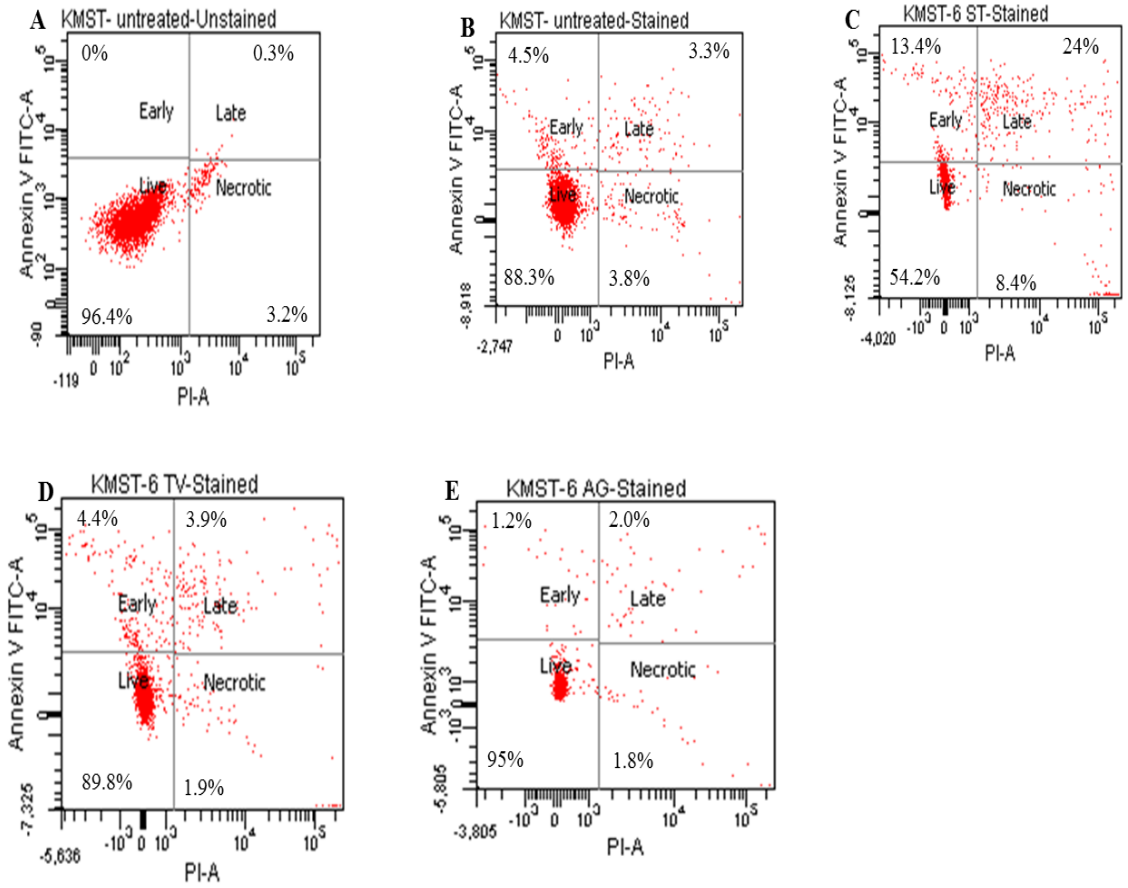


Figure 1.7: Scatter analysis showing the non-tumorogenic immortalized human cell lime KMST-6 after treatment with *Tulbaghia violecea* and *Agave palmeri* at a concentration of 150µg/ml for 24 hours. A] KMST-6 untreated and unstained; B] KMST-6 untreated stained with Annexin V-FITC and Propidium Iodide (PI); C] KMST-6 treated with 25nM staurosporine (ST); D] KMST-6 treated with *Tulbaghia violecea* (TV) and E] KMST-6 treated with *Agave palmeri* (AG).

According to figure 1.7 (A) and 1.7 (B) untreated KMST-6 cells, which were not stained with Annexin V/PI showed low or negative staining for both AnnexinV/FITC and PI (AnnexinV-FITC/PI), indicating live cells (96.4% live). The untreated stained (AnnexinV-FITC/PI) had low staining for both AnnexinV/FITC and PI indicating more viable cells (88.3% live). After treatment with 25nM ST, 150µg/ml TV or AG for 24 hours, the cells started to undergo apoptosis, shown in figure 1.7 (C), 1.7 (D), and 1.7 (E) respectively. Early (Annexin V +/PI-) and late (Annexin V +/PI +) apoptotic cells represented was approximately 13.4% of KMST-

6 cells at early apoptosis, 24.0% at late apoptosis and 8.4% undergoing necrosis after treatment with staurosporine (ST). After treatment with TV 4.4% of the cells underwent early apoptosis, 3.9% were at late apoptosis and 1.9% at a necrotic stage whereas treated with AG induced early apoptosis (1.2%), late apoptosis (2.0%) and necrosis (1.8%) in KMST-6 cell line.

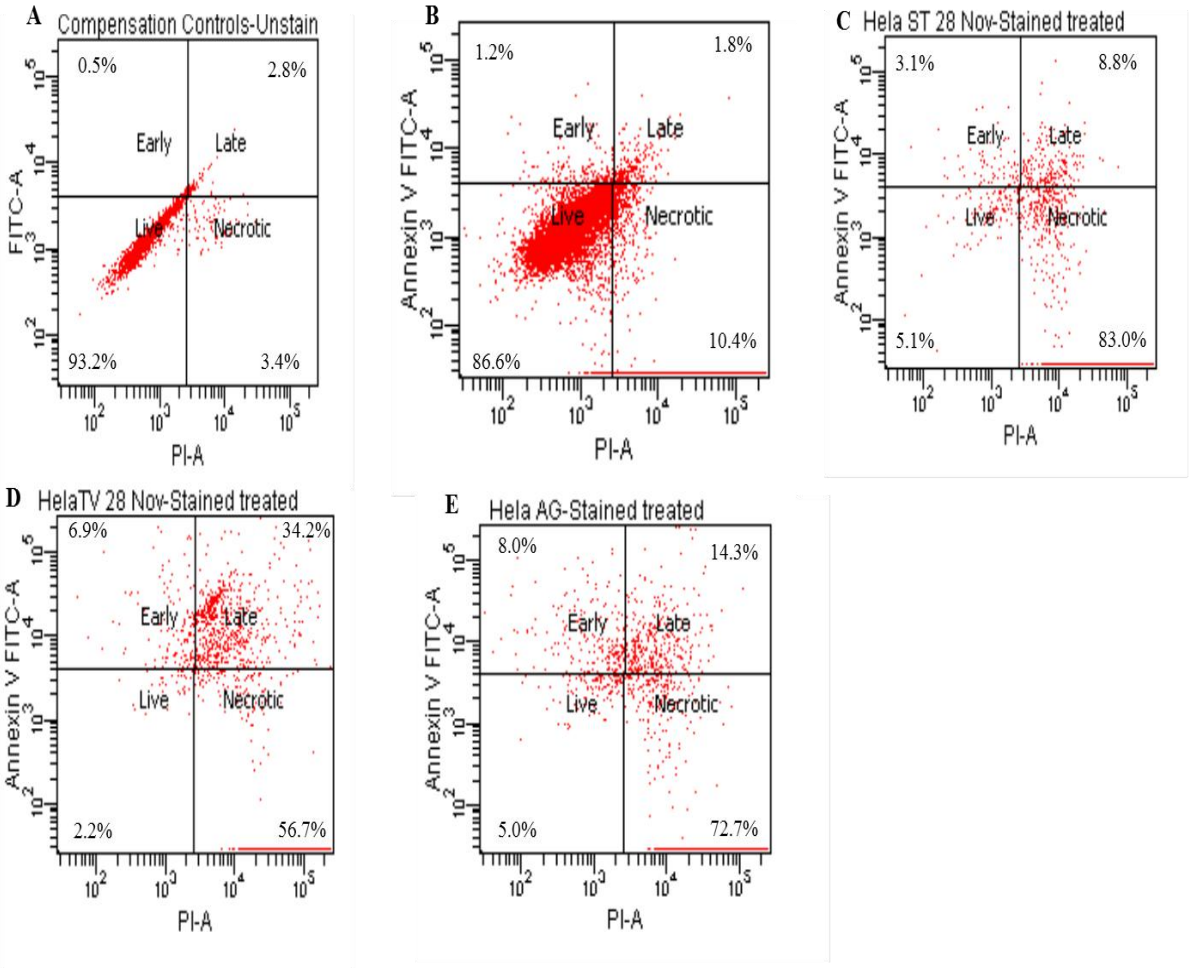


Figure 1.8: Scatter analysis showing the HeLa cell line (carcinoma of cervix) after treatment with *Tulbaghia violecea* and *Agave palmeri* at a concentration of 150µg/ml for 24 hours. A] HeLa untreated and unstained; B] HeLa untreated stained with Annexin V-FITC and Propidium Iodide (PI); C] HeLa treated with 25nM staurosporine (ST); D] HeLa treated with *Tulbaghia violecea* (TV) and E] HeLa treated with *Agave palmeri* (AG).

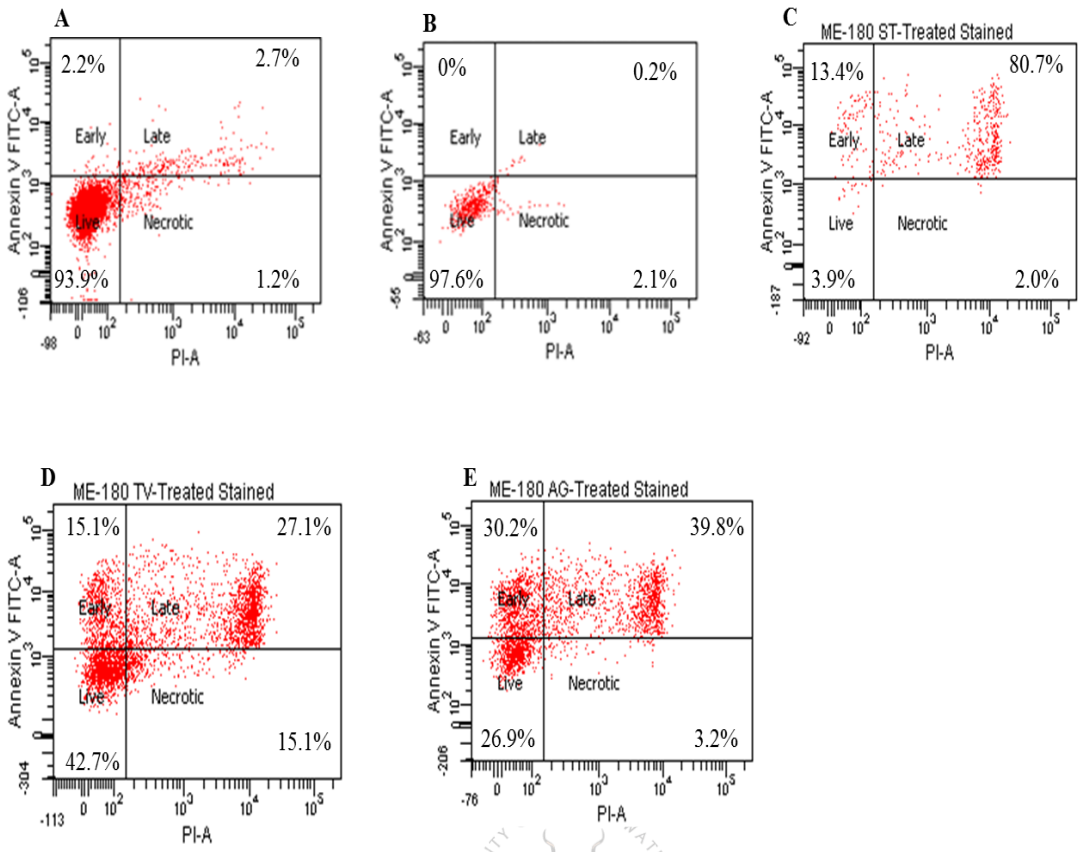


Figure 1.9: Scatter analysis showing the ME-180 cell line (cervical carcinoma) after treatment with *Tulbaghia violecea* and *Agave palmeri* at a concentration of 150µg/ml for 24 hours. A] ME-180 untreated and stained with Annexin V-FITC and Propidium Iodide (PI); B] ME-180 untreated unstained; C]ME-180 treated with 25nM staurosporine (ST); D] ME-180 treated with *Tulbaghia violecea* (TV) and E] ME-180 treated with *Agave palmeri* (AG).

As shown in figure 1.8 (A), 1.8 (B) and 1.9 (A), 1.9 (B), before HeLa and ME-180 cells were treated, 93.3% and 96.0% respectively of the cells were viable, with mostly negative staining for both AnnexinV/FITC and PI respectively. When HeLa cells were treated with ST, most of the cell population underwent cell death (83%), with a small percentage of cells (3.1%) in early apoptosis and late (8.8%) apoptosis. When these cells (figure 1.8) were exposed to TV and AG they showed a similar pattern to those that were treated with ST. Most of the population were undergoing cell death that resembled that of a necrotic cell, with TV treated cells showing 56.2% necrosis, 6.9% early apoptosis, 34.2% late apoptosis and AG treated cells at 72.7% necrotic, 8.0% early apoptosis, 14.3% late apoptosis stage (figure 1.8 (D), 1.8(E). It is important to note that, at every treatment dose and exposure time of HeLa cell lines, the necrotic cell population was higher. This indicates that necrosis was the preferential form of cell death induced by plant extract TV, AG in HeLa cell lines. This is in contrast to

effects on the ME-180 cell line, as shown in figure 1.9 (C), 1.9 (E), and 1.9 (F), where the cell population after treatment at dose and exposure time had greater amounts of cells undergoing apoptosis. When cells were treated with the positive control (ST), 13.4% of the cell population underwent early apoptosis, 80.7% late apoptosis, 2.0% necrosis and 3.9% of them were living (figure 1.9 C). After TV and AG plant extract treatment, 15.1% of the cells were in early apoptosis, 27.1% in late apoptosis, 15.1% necrosis, 42.7% live and 30.2% early apoptosis, 39.8% late apoptosis, 3.2% necrosis, 26.9% live respectively (figure 1.9 (D), 1.9 (E). This suggests that externalized phosphatidylserine residues were present and that apoptosis was a preferred form of cell death with the necrotic population as small as, between 2.0%- 15.1% after treatment.

Apoptosis is controlled by a numerous number of cascades of events closely monitored by a number of genes. These involve a network of genes and their products, which play a vital role in this process. In most cancers p53 function is impaired and its role as a tumour suppressor gene is lost, whereas RBBP and other similar genes such as Mdm2 which are highly expressed with an ability to interact with p53 together with other genes such as Rb perpetuating cancer development. The balance in the Bcl-2 family of proteins such as the Bax and Bcl-2 ratio is also important. In this study, flow cytometry analysis was followed by an analysis of the expression levels of proteins Bax, Bcl-2, p53, Rb, RBBP6 and Mdm2 which partake a role in apoptosis at a molecular level, using immunoblotting or Western blot analysis.

3.5. The effects of *Tulbaghia violacea* and *Agave palmeri* crude plant extracts on Bax, Bcl-2, p53, Rb, RBBP6 and Mdm2 protein

In this study, the effects of *T. violacea* and *A. palmeri* extract effect on Bax, Bcl-2, p53, Rb, RBBP6 and Mdm2 protein expression profiles of HeLa and ME-180 tumourigenic cell lines after exposure to 150µg/ml for 24 hours were assessed by Western blotting. Members of the tumour suppressor proteins (p53 and Rb) together with the Bcl-2 family of proteins such as Bax, Bcl-2 and other nuclear proteins such RBBP6 and Mdm2 are well known in playing a crucial role promotion or inhibition of apoptosis and cell proliferation. Exposure of HeLa and ME-180 cell lines to plant extracts (TV and AG) may either result into up-regulation or down-regulation of various proteins which either plays a crucial role in induction or suppression of apoptosis or in the inhibition cell proliferation. β- Actin was utilized to normalize protein loading during western blotting.

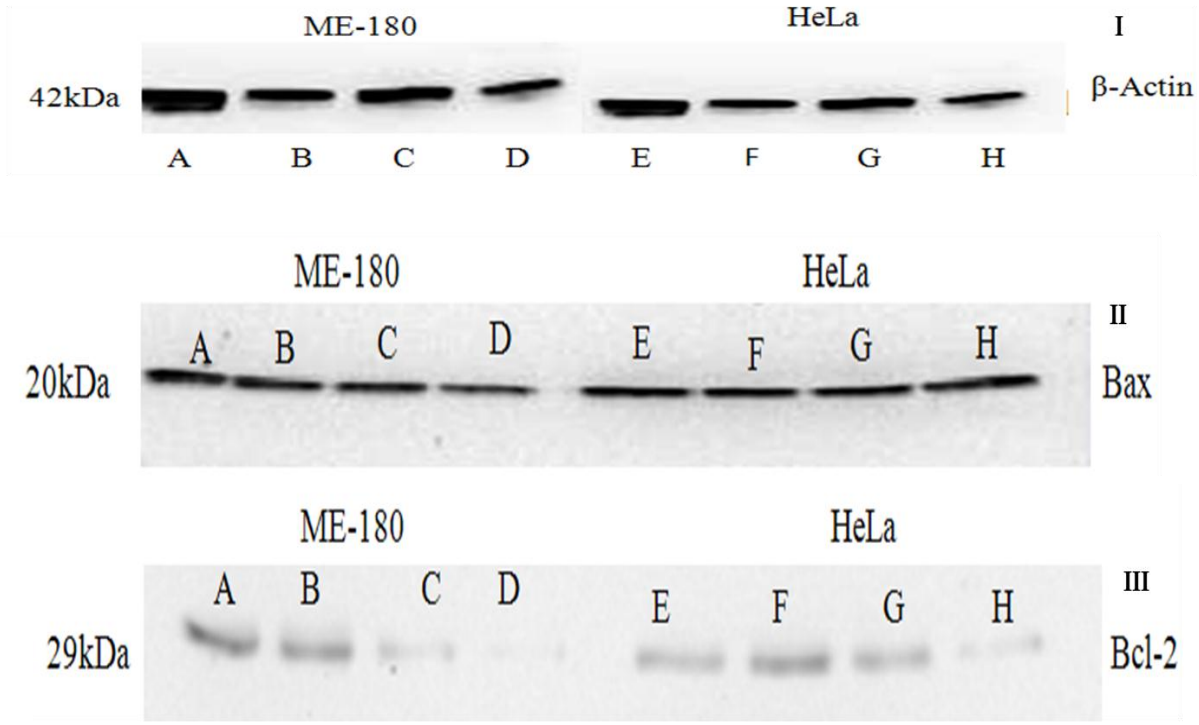


Figure 1.10: (I) Effects of *T. violacea* (TV) and *A. palmeri* (AG) on β-Actin protein in ME-180 and HeLa cell lines after 150µg/ml exposure and 25 nM staurosporine for 24 hours. Protein expression was analyzed by western blotting using β-Actin (1:1000) antibody to probe. A and E represent untreated controls, B and F (ST), C and G (TV), D and G (AG) (II) Shows the results of Western blot, which detected a ~20 kDa Bax protein. (III) Shows the results of Western blot, which detected a ~29 kDa Bcl-2 protein. The band size detected by the antibody does correspond with that of expected known size of Bcl-2 when proteins were probed with antibody to Bcl-2 (1:1000).

The results (Figure 1.10) demonstrates that the expression level of Bax was well expressed in ME-180 and HeLa cell lines treated and untreated with *T. violacea, A. palmeri* and in positive control staurosporine. The Western blot also demonstrates that the expression level of Bcl-2 is differentially expressed in ME-180 and HeLa cell lines treated and untreated with *T. violacea, A. palmeri* and a positive control staurosporine.

Figure 1.10 I, II, II represent HeLa and ME-180 cells treated with plant extracts and probed with β- Actin, Bax and Bcl-2 antibodies following treatment with TV (Figure 1.10 I, II, III C & G), AG (Figure 1.10 I, II, III D & H) and ST (Figure 1.10 I, II, III B & F) respectively. The results indicate there was no notable effect on Bax protein expression in all the treatments whereas with Bcl-2 protein as indicated in figures 1.10 III showing a reduction in protein expression following treatment with TV and AG in both ME- 180 (C&D) and HeLa (G&H).

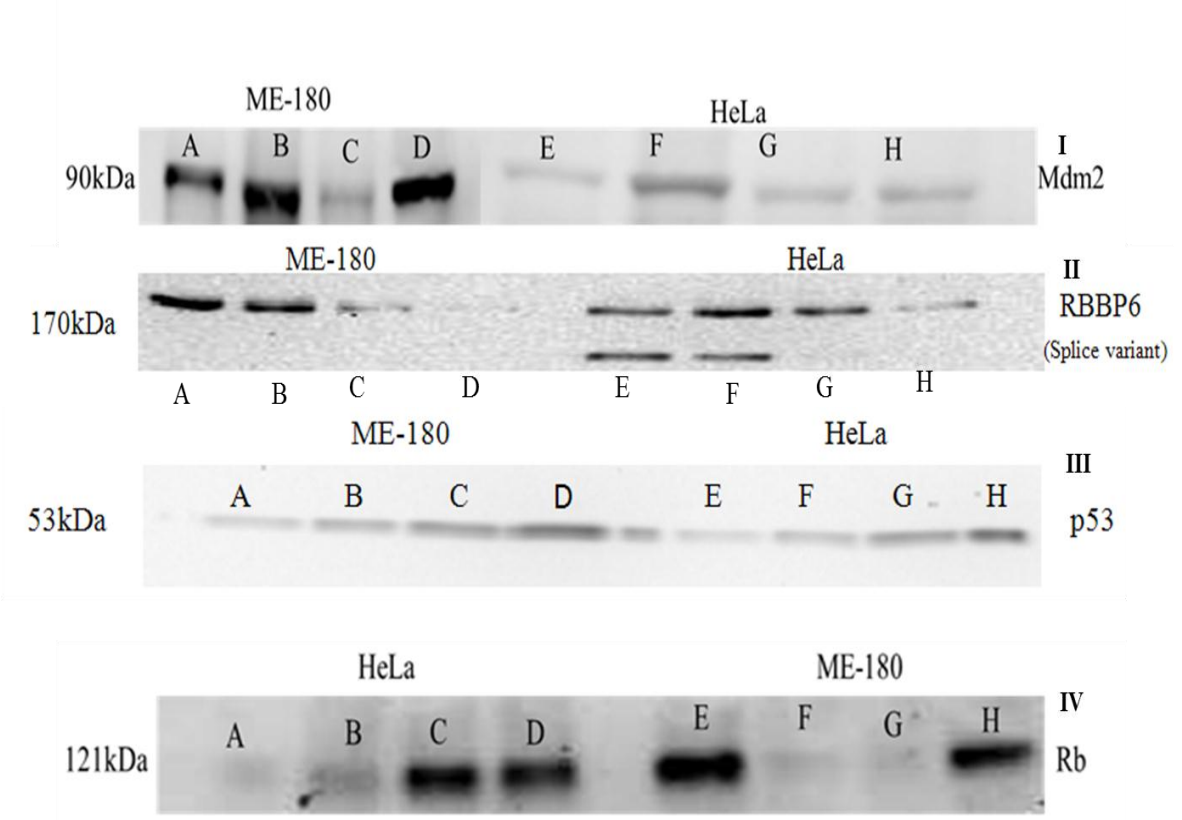


Figure 1.11: (I) Effects of *T. violacea* (TV) and *A. palmeri* (AG) on Mdm2 protein in ME-180 and HeLa cell line after 150 µg/ml exposure and 25 nM staurosporine for 24 hours. Protein expression was analyzed by western blotting using Mdm2 (1:500) antibody to probe. Lane A and E represent untreated cells, B and F (ST), C and G (TV), D and H (AG). (II) Effects of *T. violacea* (TV) and *A. palmeri* (AG) on RBBP6 protein (1:250) in ME-180 and HeLa cell lines after 150µg/ml exposure and 25nM staurosporine for 24 hours. The results suggest that two spliced variants were detected in untreated and ST treated HeLa cell line. (III) Effects of TV and AG on p53 protein in ME-180 and HeLa cell lines after 150µg/ml exposure and 25 nM staurosporine for 24 hours. Protein expression was analyzed by western blotting using p53 (1:750) antibody to probe. (IV) Effects of TV and AG on Rb protein in ME-180 and HeLa cell lines after 150µg/ml exposure and 25 nM staurosporine for 24 hours. Protein expression was analyzed by western blotting using Rb (1:500) antibody to probe.

To further predict the mechanism of how *T. violacea* (TV) and *A. palmeri* (AG) induce apoptosis, western blots were performed to determine expression of p53, Rb, RBBP6 and Mdm2. When compared to controls (Figure 1.11 IIIA & E), the expression of p53 was higher in ME-180 and HeLa cells after treatment with ST (Figure 1.11 III B & F), TV (Figure 1.11 III C & G) and AG (Figure1.11 III D & H). Thus, TV, AG and ST treatment increased the expression of p53, which highly favors apoptosis as a tumour suppressor protein. When ME-180 and HeLa cell lines were treated with plant extract and staurosporine, the retinoblastoma (pRb) protein was upregulated in HeLa cells by the two plant extract as compared to ME-180 (Figure 1.11 IV B, C, E). When the expression of the cell proliferating protein RBBP6 was analyzed by western blotting, we observed high levels of this protein in untreated ME-180

and HeLa cell lines (Figure 1.11 II A, B, E & F) as compared to treated cell lines. A phosphorylation event resulting into two splice variants (indicated by 2 bands) of RBBP6 was seen in figure 1.11 II (E) and (F) in HeLa cell lines that were untreated and treated with ST respectively. RBBP6 was down regulated following treatment with TV and AG in both ME-180 and HeLa cell line (Figure 1.11 II C, D, and 1.11 II G, F). When we looked at Mdm2 in ME-180 and HeLa cells following treatment with TV there was a decrease in the expression of Mdm2 protein (Figure 1.11 I C& G). When ME-180 cells were treated with AG the expression of Mdm2 was similar to the expression seen in untreated cells and ST ME-180 cell lines. In untreated and ST treated HeLa cell lines Mdm2 was slightly up regulated but down regulated in TV and AG treated cells (Figure 1.11 I E, F, G and H).

3.6. The effects of *Tulbaghia violacea* and *Agave palmeri* crude plant extracts on p53 and RBBP6 mRNA expression

In order to confirm our earlier results on the expression levels of RBBp6 and p53 proteins, we conducted qRT-PCR to confirm the results obtained in our western blot analysis. Our results indicate that when RBBP6 mRNA increases p53 decreases as indicated in figure 1.17 following treatment with both AG (A) and TV (B) respectively.

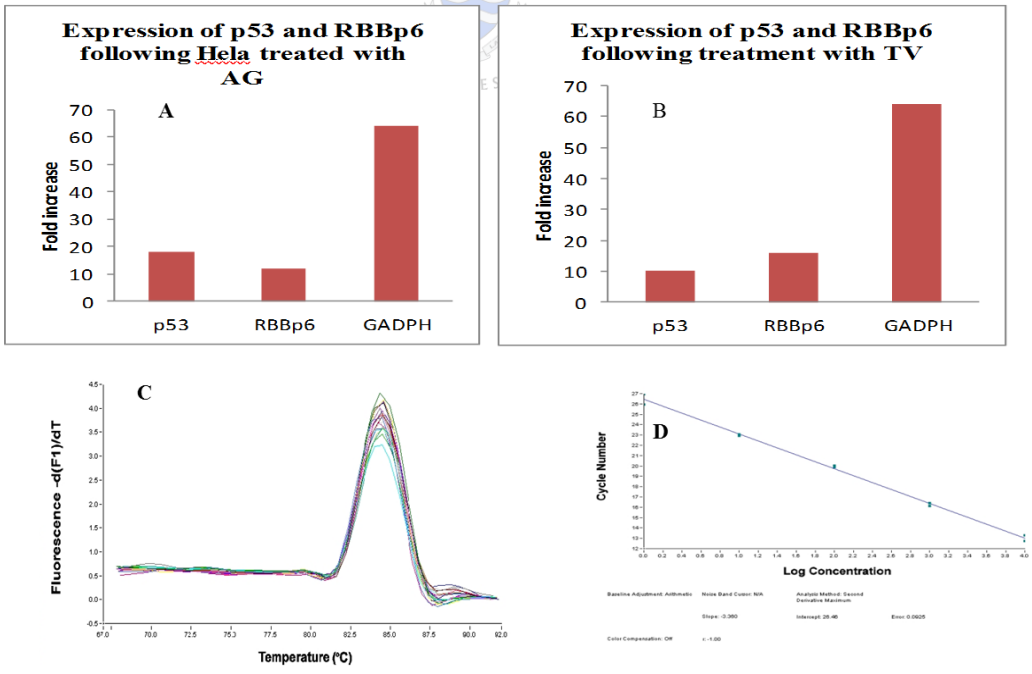


Figure 1.12: **A)** and **B)** Quantitative RT-PCR is showing a proposed relationship between p53 and RBBP6. The results suggest that when p53 and RBBP6 are inversely proportional to one another. **C)** The above figure illustrates the Melting point with RBBP6 product at ~ 84 °C. **D)** To access the efficiency of the primers, a standard curve was prepared based on serially dilution of HeLa cDNAs. All samples were performed in duplicates.

CHAPTER 4

DISCUSSION

Agents, which are mostly derived from plants and have potential ability to suppress cell proliferation at the same time enhancing apoptosis, have attracted a vast interest as anti-cancer agents. Because many current available anticancer agents have side effects and even affect normal cells, traditional plants and their compounds have been screened for anticancer activities because in many studies they have been discovered to be safer (less toxic), easily available and cheaper when compared to modern (allopathic) drugs hence many people especially in the developing countries such as Southern Africa still rely on them to treat various ailments (Agarwal *et al*, 2012). Anticancer drugs like those derived from *C. roseus* (Vinblastine) have fuelled the search for more safe affective plant derived compounds that can be potentially utilized as anticancer agents (Fabricant and Fransworth, 2001).

It is important to understand the molecular and signaling pathways that are activated by traditional plants such as *Kedrosis foetidissima*, which was reported to possess anti-proliferative effects and also to induce apoptosis in a p53 independent manner in MCF-7 and YMB-1 breast cancer cells in the study by Choene and Motadi, 2012. Thus, it is due to such discoveries that *Tulbaghia violacea* (TV) and *Agave palmeri* (AG) were evaluated for their anti-cancer activity on HeLa and ME-180 cervical cancer cell lines in this study. The first objective was to evaluate the significant variation in the cytotoxic effects of TV and AG on the HeLa and ME-180 tumourogenic cell lines by measuring cell viability using MTT assay. This assay measures the concentration at which 50% of the cells died after exposure to crude TV and AG plant extract after 24 hours. The half maximal inhibitory concentration (IC_{50}) of 150µg/mL *T. violacea* exhibited 58% cell viability/ 42% cell death in HeLa cells (Figure 1.4A) and weak cytotoxicity activity on both ME-180 and KMST-6 cells at 21% and 18% cell death respectively after treatment (Figure 1.4C and 1.5A) treated for 24 hours.

This investigation correlates with that carried out by (Bungu *et al*, 2006), where 250µg/ml methanolic *T. violacea* leaf and bulb extracts inhibited cell proliferation due to apoptosis induction in HeLa, MCF-7, and WHCO3 and HT29 cell lines. The 150µg/ml of TV leaf extract in this study reduced cell viability in the HeLa cell line, in comparison with 250µg/ml

TV leaf extract inhibiting growth of HeLa (37.5±2.7%) with $p<0.001$ for all cell lines (Bungu *et al*, 2006). Further studies on the same plant also showed induction of cell death in oesophageal cancer (Van Wyk and Gericke, 2000; Lyantagaye, 2011). This action is possibly attributed by the presence of compounds such as C-S lyase, S-substituted cysteine sulfoxide derivatives, 2,4,5,7-tetrathiaoctane-2,2-dioxide and 2,4,5,7- tetrathiaoctane which have been extracted from leaves of this plants and have been reported to inhibit cell proliferation (Jacobson *et al*, 1968; Burton and Kaye, 1992; Srivastava and Singh, 2004).

Not much has been reported on *A. palmeri* species, but ethanol plant extracts from the same subfamily such as *Agave lechuguilla* together with crude/ crystallized saponin extracted from this plant have been reported to respectively have a cytotoxic effect on HeLa cell lines with IC_{50} of 89µg/ml and 78-95µg/ml, but had a higher IC_{50} value of 171.6 and 172.2µg/ml when normal control Vero cells were treated *in vitro* (Casillas *et al*, 2012). In other studies like that of Yokosuka *et al*, (2000) and another by (Barret, 2001), cytotoxic effect of hecogenin and tretragylcosides saponin extracted from *A. americana* against Leukemia cell line HL-60 have been reported. These reports suggest that *A. palmeri* which belongs to the same family as *A. lechuguila* and *A. americana* may be harboring compounds similar to those that were responsible for cytotoxic effect on HL-60 and HeLa cells, confirming the response observed in this study where *A. palmeri* was shown to slightly reduce cell viability in HeLa cells (approximately 34% cell death) but not in the ME-180 and KMST-6 normal fibroblast cell line where 15% cell death was observed after 24 hour treatment. We further suggest that, *A. palmeri* has little cytotoxic effect on HeLa, ME-180 tumourogenic cells but none in KMST-6 cell lines.

Staurosporine, a known inducer of apoptosis, is a potent inhibitor of a number of protein kinases and known to promote intracellular stress-induced apoptosis in cell culture (Benard *et al*, 2001; Feng and Kaplowitz, 2002). As a positive control, cells treated with staurosporine (ST) were included in this study together with untreated cells. From the results, it can be deduced that HeLa and ME-180 cells together with KMST-6 non-tumourogenic normal fibroblast cell lines consistently entered apoptosis after exposure to ST and there was no significance between *T. violecea*, *A. palmeri* and ST ($P<0.10$) treated HeLa, ME-180 and KMST-6 cell line shown in Chapter 3 (Figure 1.4 and 1.5) when looking at the cell cytotoxity which was between 34-58% induced by 25nM ST. These results are in line with those from Bernard *et al*, 2001, where they reported irreversibly induced apoptosis in HeLa, Caski (HPV

positive, wild-type p53) and C33A (HPV negative, mutated p53) after 24 hours treatment with ST at concentrations between 2-40nM. Because calorimetric assays, such as MTT used in this study can be disadvantageous in the sense that it may underestimate cellular damage and detect cell death at a later stage of apoptosis when metabolic activity of the cells is reduced, it was therefore pivotal to extract DNA of treated and untreated HeLa, ME-180 and KMST-6 cell lines which was analysed using gel electrophoresis.

Initially apoptosis is characterized by numerous morphological and biochemical features, these include condensation and fragmentation of the nuclear chromatin, nuclear fragmentation compaction of the cytoplasmic organelles with cell shrinkage and loss of positional organization of organelles in the cytoplasm, dilation of the endoplasmic reticulum, a decrease in cell volume and alteration to the plasma membrane resulting in the recognition and phagocytosis of the apoptotic cells (Lee *et al*, 2009; Taraphdar *et al*, 2001; Wong and Kadir, 2011). The DNA fragmentation, results also confirmed that ST induces apoptosis in HeLa, ME-180 cervical and KMST-6 normal fibroblast cell lines at concentration 25nM after 24 hour treatment when DNA was extracted and analyzed through gel electrophoresis (Figure 1.6 lane 3, 7, 11). During apoptosis especially in the later stage, the endonucleases provoke degradation of nuclear DNA, which results in the fragments of DNA strand breaks (DSBs) which have exposed hydroxyl ends (Compton, 1992). Anti-cancer drug mechanism of action is based on the ability to induce apoptosis and in some cases inhibit apoptosis (Lee *et al*, 2009).

Based on the statement above, it was therefore vital that *T. violacea* and *A. palmeri* induce apoptosis in order to be considered potential anti-cancer plant extracts. From the results in figure 1.6 TV and AG were able to induce cell death as shown with a distinct smear indicative of a fragmented DNA. However in KMST-6 cells which are normal fibroblast cells the two plant extracts did not induce any form of cell death, which suggested that the plant extracts do not interfere normal cells (Figure 1.6). This pattern was similar to that obtained when MCF-7, WHCO3, HT29 and HeLa cell lines were treated with *T. violacea* bulb and leaf methanol extract, where fragments were seen as one of the hallmarks of apoptosis and nuclear condensation was observed after treatment (24 hours) and staining with Hoechst 33342 with a number of cells significantly apoptotic and even more so after 48 hours of treatment (Bunge *et al*, 2006). This fragmentation pattern is a sign of growth inhibitory action associated with apoptosis induction induced by TV and AG plant extract.

Cells death is defined by a decrease in cell viability and DNA fragmentation in this study, is known to occur in 2 distinct modes: apoptosis, which starts with a blebbing of the plasma membrane, which breaks up into membrane-enclosed particles, called apoptotic bodies, containing intact organelles as well as portions of the nucleus and followed by a well-choreographed sequence of morphological events characterized by features such as DNA fragmentation, a laddered size distribution to DNA cleavage between nucleosomes (Kerr *et al*, 1972; Kastan *et al*, 1995). Necrosis, a form of cell death shown by cytoplasmic swelling and vacuolation, rupture of the plasma membrane, dilation of organelles such as the mitochondria, endoplasmic recticulum and Golgi apparatus, as well as moderate chromatic condensation (Festjens *et al*, 2006).

Therefore to characterize the mode of cell death associated with *T. violacea* and *A. palmeri* induced growth inhibitory activity in HeLa and ME-180 tumourogenic cell lines, the morphological changes associated with apoptosis were assessed using Annexin V/PI staining kit. During apoptosis, the distribution of neutral phospholipids and anionic phospholipids such as phosphatidylserine (PS) in the cell membrane changes (Rahman, 2005). Annexin V coupled to FITC is a dye that can be added to the cells and is specific for PS, and will bind to PS on the outer membrane of apoptotic cells whereas PI is an intercalating dye used to identify cells that are at a late stage of apoptosis in a population (Rahman, 2005). So when HeLa and ME-180 cells together with normal fibroblast KMST-6 cell lines were dual stained with AnnexinV/FITC and PI before and after treatment with TV, AG and ST (the positive control), apoptosis was induced. HeLa, ME-180 tumourogenic and KMST-6 cells treated with TV and AG at a concentration of 150μg/mL demonstrated phosphatidylserine residue externalization as indicated by percentage of cells in the population which were either at early or late stage of apoptosis. KMST-6 cell lines treatment with a known inducer of apoptosis ST, resulted in only 37.4% PS residue externalization whereas 8.3% when treated with TV and 3.2% with AG (Figure 1.8). On the other hand HeLa when treated with ST (83.3%), TV (41.1%), and AG (22.3%) and ME-180 treated showed ST (94.1%), TV (42.2%), and AG (70%) PS residue externalization.

The rest of the cells were either alive or undergoing necrosis, as they stained negative for both AnnexinV-FITC/PI (live) and (-) AnnexinV/FITC (+) PI respectively. This demonstrated that treatment of HeLa; ME-180 and KMST-6 cells with staurosporine resulted

in significant induction of apoptosis. When KMST-6 normal fibroblasts were treated with TV and AG relatively no significant toxicity to the cells was observed with only 8.3% and 3.2% PS residue externalization suggesting normal proliferating cells could be protected from TV and AG plant extract. This correlates with the statement made by (Chen *et al*, 2000) about a good chemotherapeutic agent being able to induce apoptosis without being toxic to normal proliferating cells. Notably, ST induced apoptosis in KMST-6 cells lines by 37.3%, suggesting that proliferation was inhibited in normal cells just as in a study by Chan *et al*, 2000 where 0.5nM staurosporine treatment of normal breast epithelial cells inhibited growth by between 40-55% ad arresting cell cycle at a G_0G_1 phase. The mechanism of apoptotic induction by ST is poorly understood, but Zhang *et al*, 2004 suggest that the caspase-dependent and independent apoptotic pathways play a crucial role in induction of apoptosis in melanoma cell lines. We deduce that the significant response of HeLa and ME-180 cells to ST is potentially attributed via caspase-dependent and independent apoptotic pathway.

Potentially, TV and AG crude extracts contain anticancer agents. However, not much has been reported about the extracts of AG, whereas TV crude aqueous extracts have been shown to exhibit apoptosis inducing ability (Lyantagaye, 2011). Methyl-α-D-glucopyranoside (MDG) compound from *T. violacea*, which interferes with *hexokinase* bioactivities and reactive oxygen species have been reported to induce apoptotic death. In other studies, the flavonoids kaempferol, querocetin and myricetin found in TV reduced cell proliferation *in vivo* and reduced risk of pancreatic cancer by 23% (Pastorino *et al*, 2002; Lyantagaye, 2005; Ncube *et al*, 2011). The flavonoid, kaempferol, was reported to inhibit chemoaltract protein (MCP-1) in the study by Kowalski *et al*, 2005. Whereas, total phenolics, gallotanin, flavonoid and saponin content found in micropropagated plants of *T. violacea* had antifungal activity and their secondary metabolites can be used for medicinal purposes to treat cancer (Ncube *et al*, 2011). Thus, a number of compounds that are present in plants such as *T. violacea* and *A. palmeri* possibly interfere with numerous genes together with their protein products to exhibit apoptosis. Looking at the results, isolation of biologically active compounds from plant extract of TV and AG could yet provide an insight on the mode of apoptotic action.

Apoptosis is known to occur through 2 signaling pathways: the extrinsic pathway mediated by TNF and the intrinsic pathway resulting into cytochrome *c* release (Fulda, 2009). Commitment of a cell to apoptosis by either of the pathways is dependent on the balance

between pro-apoptotic and anti-apoptotic protein (Fulda, 2010). In this study, the protein member of the Bcl-2 family, Bax and Bcl-2 were investigated. Bcl-2 is known to regulate permeability of Bax and the ratio of these two proteins is vital for apoptosis to occur (Kang and Reynolds, 2009). Bax is reported to translocate to the mitochondria where it is reported to antagonize the anti-apoptotic activity of Bcl-2 or form a channel that leads to mitochondrial release of cytochrome c (Hervouet *et al*, 2006 *et al*, 2007; Youle and Strasser, 2008). In the cytosol, the presence of cytochrome *c* initiates the formation of apoptosome complex which will serve to recruit and activate initiator caspase -9 which in turn proteotically cleaves and activate caspase 3 thus inactivating various cellular proteins such as PARP (Letai, 2002; Kang and Reynolds, 2009). A potential cytotoxic drug therefore needs to be able to initiate a release of proteins such a cytochrome *c* from a mitochondrial space to allow it to combine to adaptor molecule such as apoptosis protease activating factor 1 (Apaf-1), and also inactivate procaspase 9 within the apoptosome to turn on the cascade activation of caspase (3, 6, 7), resulting in morphological and biochemical changes associated with apoptosis (Kang and Reynolds, 2009). The ratio of anti-apoptotic and pro-apoptotic Bcl-2 family members is their crucial rather than the expression level of one member of the family to regulate apoptosis sensitivity (Fulda, 2009).

In many cancers there is a reported increase in the ratio of anti- to pro-apoptotic Bcl-2 proteins. This imbalance confers cancer cells an apoptotic resistance and allows cells to continue proliferating (Fulda, 2009). Western blot analyses in this study revealed a down regulation of the Bcl-2 protein with an up-regulation and potentially an accumulation of Bax in cells treated with TV and AG (Figure 1.10 II and III). Increase in Bcl-2/Bax ratio promotes cell survival in this study as observed down-regulation of Bcl-2 will result in the ratio being reduced in this case favoring apoptosis. These results suggest that, TV and AG induced apoptosis. Agents targeting the Bcl-2 family of proteins have been reported to have chemo sensitizing effects when treatment was combined with conventional chemotherapeutic drugs in chronic lymphocytic leukemia (CLL) patients (Kang and Reynolds, 2009). An up-regulation of Bax (Figure 1.10 II) implies a subsequent accumulation of Bax protein in the mitochondria therefore potential release of cytochrome *c* to activate caspase cascade. Therefore TV and AG modulate Bax and Bcl-2 expression levels at a protein level. In addition the balance of these proteins potentially activates caspase 9, 3, and possibly induces PARP cleavage in the cells.

Other main mediators of apoptosis include a tumour suppressor protein p53; where an induction of p53 protein levels by various stress types such as (TV and AG crude plant extracts) may potentially prevent inappropriate propagation of cells carrying DNA damage, and mutation (Moll and Petrenko, 2003). Not only does p53 together with Rb gene products control apoptosis, they also play a role in cell cycle progression (Kastan *et al*, 1995). Most notably, p53 upregulation induces a transcription of cyclin dependent kinase (cdk) p21, and Mdm2 inhibiting Cdk2 activity, which controls a transition and arrest of G_2/M phases in the cell cycle resulting in apoptosis (Kastan *et al*, 1995).

Crude extracts of *Tulbaghia violacea* and *Agave palmeri* induce apoptosis in both HeLa and ME-180 tumorougenic cell lines. Western blot analysis indicated the mode of action of these axtracts and we deduce that the action is independent of p53. However, when both HeLa and ME-180 were treated with TV and AG they both had reduced the expression of RBBP6 but increased the level of p53 expression which was in line with several other studies that looked at the relationship between these two genes (Motadi *et al* 2011). Apoptosis is control by a vast number of genes and signaling molecules, thus TV and AG potentially control other genes and molecules within this apoptotic pathway in HeLa and ME-180 cell lines. *T. violacea c*ompounds have been extracted in other research and these compounds can be used to study the mode of action. On the other hand *A. palmeri* compounds have not been isolated but in other species like *A. Americana* compounds have been isolated, an isolation and identification of compounds in this plant can also aid in understanding the molecular mechanism of this plant that follows apoptosis induction observed in this study.

4.1. Conclusion and Future Perspectives

Recently several medicinal plants have been identified as having potential for the treatment of cancer. However the understanding of the molecular mechanism and signaling pathway that they elicit still remain a mystery. In this study the molecular mechanism of cell death induction by TV and AG on HeLa and ME-180 cell lines was determined. From the results we can suggest that the two extracts could be inducing cell death by interfering with the Bcl-2/Bax ratio. Even though there was down regulation of RBBP6 after treatment with TV and AG there was little impact on the p53 level to suggest that cell death induced was due to increased p53 level. However, more work need to be done in order to further understand the mechanism of TV and AG in cancer cell apoptosis induction.

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APPENDICES

APPENDIX A: TISSUE CULTURE

1. Growth Media

- Eagle's minimum essential media (MEM with L-glutamine)/ Dulbecco's Modified Eagles Medium (DMEM)
- 10 % Fetal Bovine Serum (FBS)
- 1% Penicillin streptomycin (pen/strep) (Whitehead scientific (Pty) LTD)

Make up to a total volume of 500ml and store at 4^{o}C

2. Phosphate buffer saline (1x)

136.9 mM Sodium chloride

2.68 mM Potassium chloride

10.1 mM Disodium hydrogen phosphate dodecahydrate

1.76 mM Potassium dihydrogen phosphate

Adjust pH 7.2 to 7.3

Make up to final volume with dH2O

Sterilize for 20 min at 151 lbq

Store at 4 °C

3. Trypsin: Ethylenediaminetetra-acetic acid (EDTA) solution

- **Trypsin solution**
  0.01 % Trypsin

  Make up to final volume with PBS

- **EDTA solution**
  0.004 % EDTA

Make up to final volume with PBS

MIX:

50 % Trypsin solution

50 % EDTA solution

Store at 4 °C

4. Freezing media

5mL Dimethyl sulfoxide (DMSO)

35mL MEM/ DMEM

10mL FBS

Store at 4 °C

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5. MTT Assay

MTT (3-(4, 5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide), Sigma- Aldrich, Saint louis, Missouri, USA

0.15g MTT

30mL of 1X PBS

To make 5mg/mL

Mix well and filter through the 0.45µm and store at -20^{o}C

6. FITC Annexin V Apoptosis Detection Kit I

BD Pharmingen™ Protocol:

http://www.bdbiosciences.com/external_files/pm/doc/tds/cell_bio/live/web_enabled/6900KK_559763.pdf

APPENDIX B: SOLUTIONS

1. Electrophoresis

10x TBE

0.9M Tris base

0.89M Boric Acid

25mM EDTA, pH 8.3

This stock solution was diluted 1X for the electrophoresis of agarose

2. 1% Agarose gel

1g Agarose (Sigma-Aldrich, Saint Louis, Missouri, USA)

100mL 1X TBE

Mix well and dissolve agarose by microwaving for a few minutes

Cool and add 2µL ethidium bromide (EtBr)

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3. 10X MOPS

0.4 MOPS, pH 7.0

0.1M Sodium Acetate

0.01M EDTA

Make up to 1L with DEPC water

0.1% DEPC (diethylpyrocarbonate)

4. SDS-PAGE sample buffer (2X sample buffer)

3.55ml Demonized water

1.25mL 0.5M Tris-HCl, pH 6.8

2.5mL Glycerol

2.0mL 10% (w/v) SDS

0.2mL 0.5% (w/v) bromophenol blue

Add 50µL of β-Mercaptoethanol to 950µl of sample buffer before use

5. RIPA extraction buffer (Thermo Scientific, Rockford, USA)

See suppliers manual online:

http://www.piercenet.com/instructions/2161782.pdf

6. Protein Estimation

Pierce® BCA Protein Assay Kit, Thermo Scientific, Rockford, USA

See: http://www.piercenet.com/instructions/2161296.pdf

Example of standard curve (protein estimation)

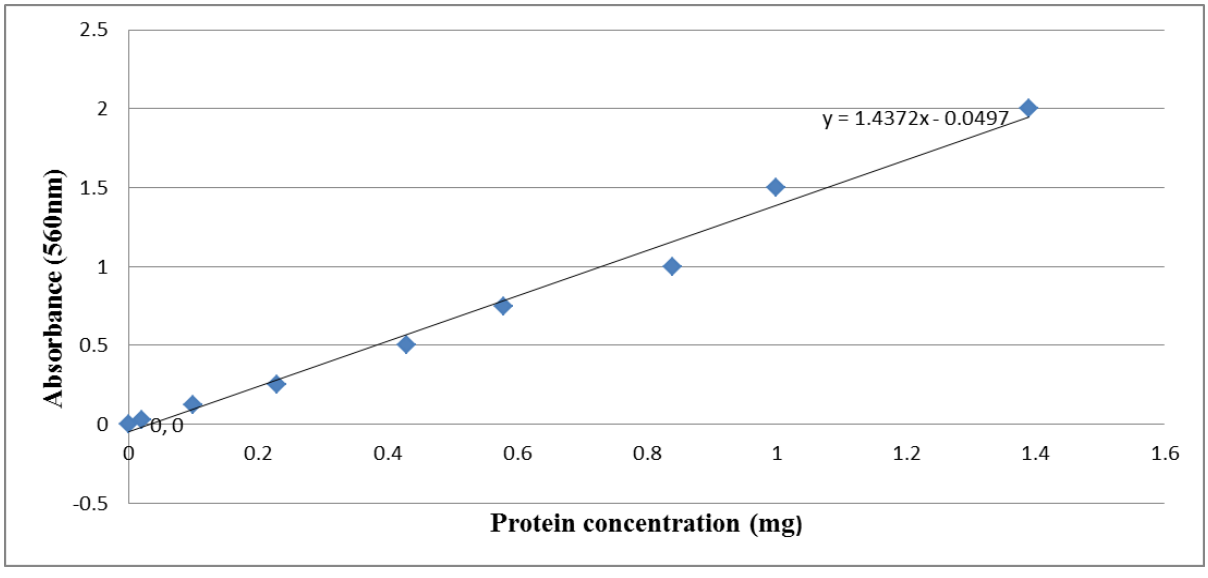


Figure 1.13: Showing an example of a protein estimation curve. The specific BSA standards, at varying concentration (20-2000µg/mL), x- axis and their respective absorbance value at 560nm (y-axis) were used to calculate the unknown protein concentration in a given sample.

7. SDS-PAGE Running Buffer (10X)

30.0g tris base

144.0 Glycin

10.0g SDS

Dissolve in deionized water (dsH_2O) and fill to 1000mL. Store 4^oC

8. Ammonium persulphate (APS)

A 10 % stock solution was prepared in deionised water

This solution was stored at 4 oC or prepared fresh at all times

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9. Separating gel

2mL 30% Acrylamide/ 1% Bis (Bio-Rad)

1.25mL 0.5M Tris-HCl, pH 8.8

50µL 10 % SDS

50µL 10% Ammonium persulphate (APS)

2µL 0.25 % N,N,N‟N‟-tetramethylenediamine (TEMED)

1.65mL dsH_2O

10. Stacking gel

0.5mL 30 % Acrylamide/ 1% Bis (Bio-rad)

190µL 1.5M Tris-HCl, pH 6.8

15µL 10% SDS

15µL 105 Ammonium persulphate (APS)

1.05mL dsH_2O

1.5µL 0.25 % N,N,N‟N‟-tetramethylenediamine (TEMED)

11. Coomassie Staining Solution

0.25% Coomassie Brilliant Blue R 250

45% Methanol

5% Acetic acid

12. De-staining solution

12 % Glacial acetic acid

10 % Methanol

Make up to final volume with dH_2O

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13. Western Blot

10X SDS-PAGE Transfer Buffer (Western Blotting)

25mM Tris-Base

192mM Glycin

20% Methanol

pH 8.3

Bring to 1L with deionized water

Make 0.2M CAPS (Sigma-Aldrich, Saint Louis, Missouri, USA)

88.52g CAPS in 2L deionised water

14. 1X Transfer Buffer

100mL 0.2M CAPS

200mL 10X transfer buffer

200mL Methanol (MeOH)

1500mL dsH_2O

Store at 4^oC for further use

15. Blocking Solution

5 % non-fat milk powder

Make up to final volume with 1X PBS or TBS + 0.01% Tween 20

Store at 4 °C

16. SuperSignal West Pico Chemiluminescent Substrate kit (Thermo scientific, Rockford, USA)

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Before use MIX:

50 % Luminol/Enhancer solution

50 % Stable peroxide buffer

Store in the dark

View membrane using ChemiDoc XRS image Analyse (Biorad laboratories Inc, USA)

Table 1.1: RNA extraction protocol

Step 1	Cells were harvested(10^6) and re-suspended in 200 µl PBS
Step 2	400 µl Lysis/Binding buffer was added, contents were mixed well, then added to upper reservoir of the High Pure Filter Tube assembly
Step 3	These were centrifuged for 15seconds at 8000 x g, flow through was discarded.

Step 4	Each sample was incubated with a mixture of 90 µl DNase I Incubation Buffer and 10 µl DNase I for 15 minutes at 25°C.
Step 5	Wash Buffer 1(500 µl) was added to each sample, these were centrifuged for 15seconds at 8000 x g. Flowthrough was discarded.
Step 6	Wash Buffer II (500 µl) was added to each sample, these were centrifuged for 15seconds at 8000 x g. Flowthrough was discarded.
Step 7	Wash Buffer II (200 µl) was added to each sample, these were centrifuged for 2 minutes at 13000 x g. Flowthrough was discarded through collection tube.
Step 8	50 µl of Elution Buffer was added then centrifuged for 1minute at 8000 x g

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Table 1.2: The cocktail for cDNA synthesis

Reagent	**Volume**
10X reaction buffer	2.0 µl
25 mM Magnesium Chloride	3.0 µl
Deoxyribonucleotide mix	2.0 µl
Specific primers	2.5 µl
RNase inhibitor	1.0 µl
AMV reverse transcriptase	1.0 µl
sdH_2O	7.5 µl

RNA sample	1.0 µl
Total volume	20 µl

Table 1.3: Reverse transcription conditions

Step	**Temperature**	**Time (minutes)**
Step 1	25 °C	10
Step 2	42 °C	60
Step 3	70 °C	15
Step 4	4 °C	∞

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Table 1.4: Real-time PCR cocktail

Reagent	**Volume**
SyBr Green	10 µl
Forward primer	1 µl
Reverse primer	1 µl
H_2O	7 µl
1µl of the respective cDNAs were added lastly to the capillaries.	
Total	20 µl

Table 1.5: Real Time PCR parameters

Analysis	Cycle	Segment	Temperature in °C	Hold time
Enzyme activation	1	1	95	2 minutes
Quantification	35- 45	<u>Amplification</u> Annealing Extension	95 57 72	45 seconds 45 seconds 45 seconds
None	1	Final extension	72	10 minutes
Melting	1	<u>Melting curve</u> Denaturation Annealing Extension	95 65 95	10 seconds 15 seconds 0.1°C/Sec
None	1	Cooling	40	30 seconds

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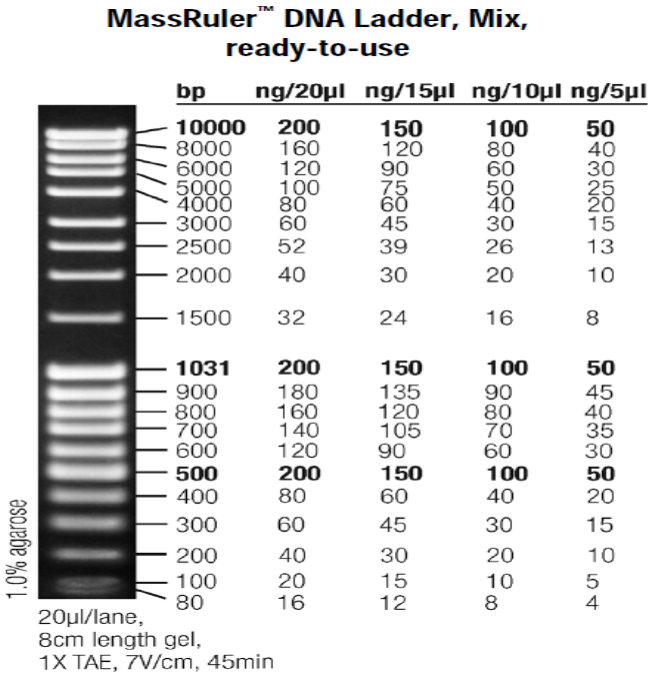


Figure 1.14: DNA Ladder, Mix from Fermentas, Thermo Fisher scientific, Rokford, USA, used in gel electrophoresis.

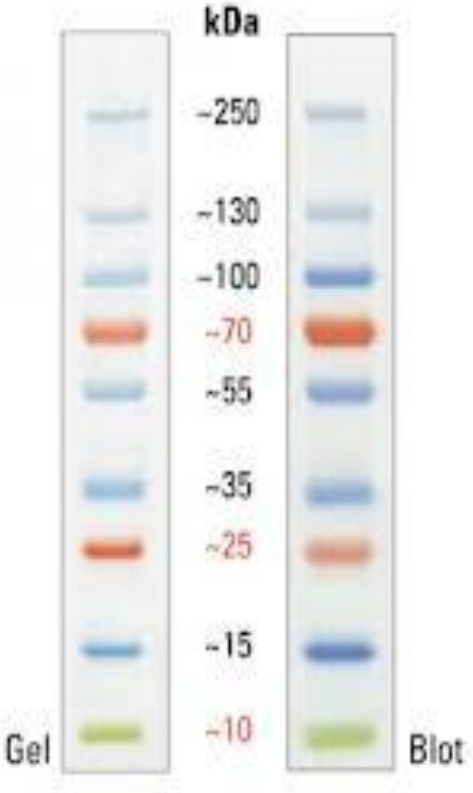
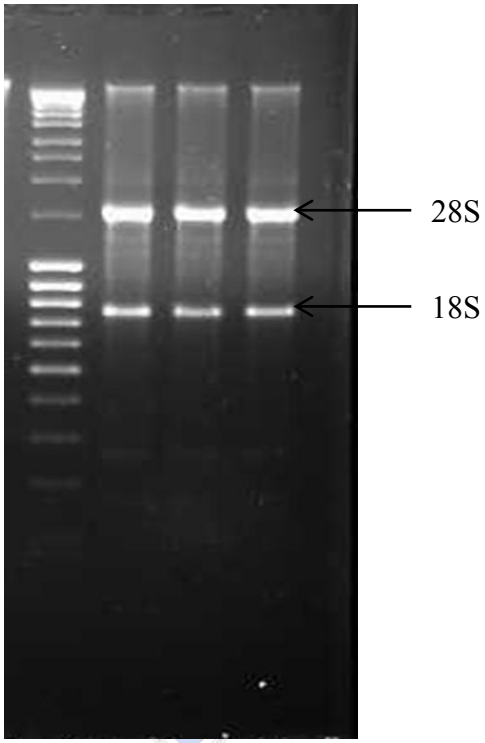


Figure 1.15: Showing the PageRuler™ Prestained Protein Ladder Plus, from Fermentas, Thermo Fisher Scientific, Rockford, USA

17. Total RNA and complementary DNA (cDNA) Synthesis

Total RNA isolated from KMST-6 human fibroblast and HeLa and ME180 cell lines was subjected to electrophoresis before it was used to synthesize cDNA by reverse transcription. The RNA was of good quality as demonstrated in figure 5.1 with distinct 18S and 28S rRNA bands as indicated the arrows.



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Figure 1.16: Gel electrophoresis indicating the integrity of the RNA extracted from the HeLa, ME-180 and KMST-6 cell lines, Lane-M represent the RNA molecular marker while lanes 1-3 represent HeLa, ME-180 and KMST-6 cell line RNAs respectively. The two bands indicated by the arrows as 28s and 18s, which is indicative of the great RNA integrity.

18. Melt Curve and the standard curve for figure 1.12 C and D

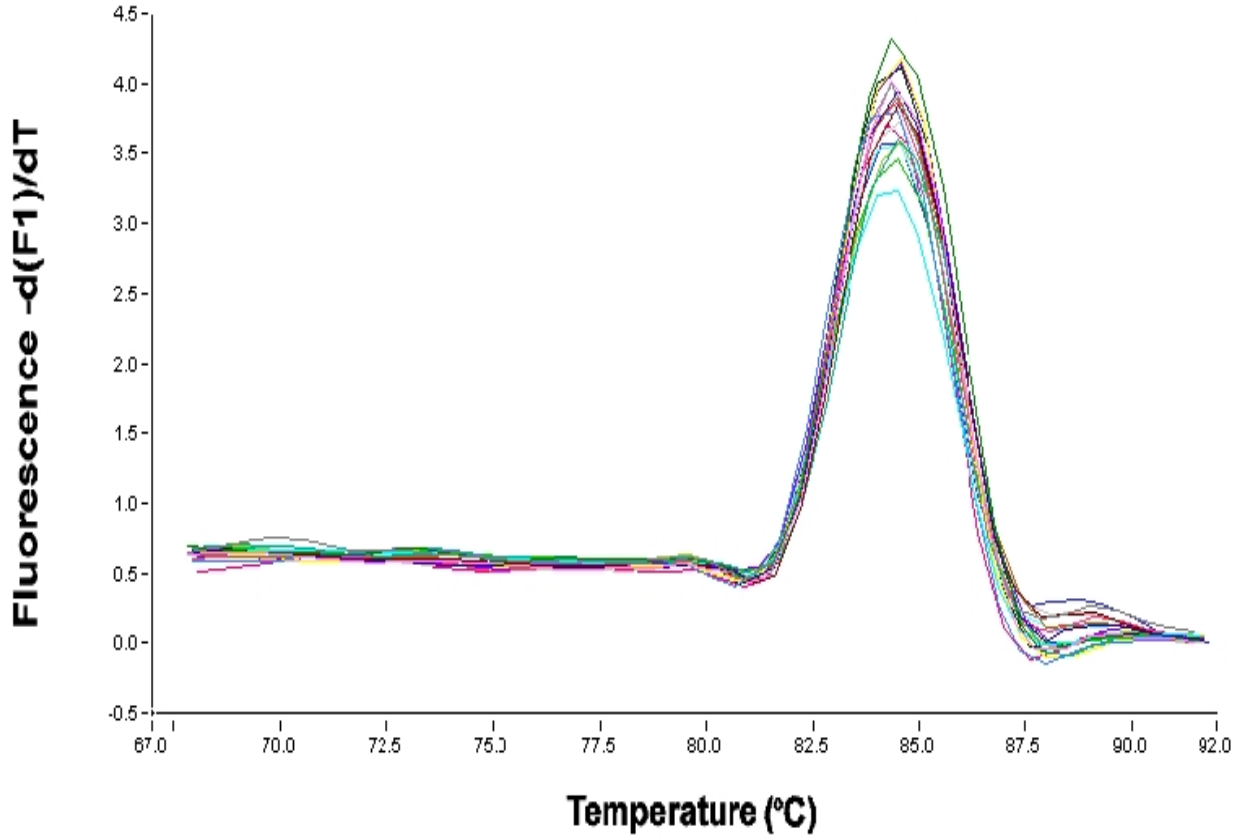


Figure 1.17: The above figure illustrates the Melting point with RBBP6 product at ~ 84 °C.

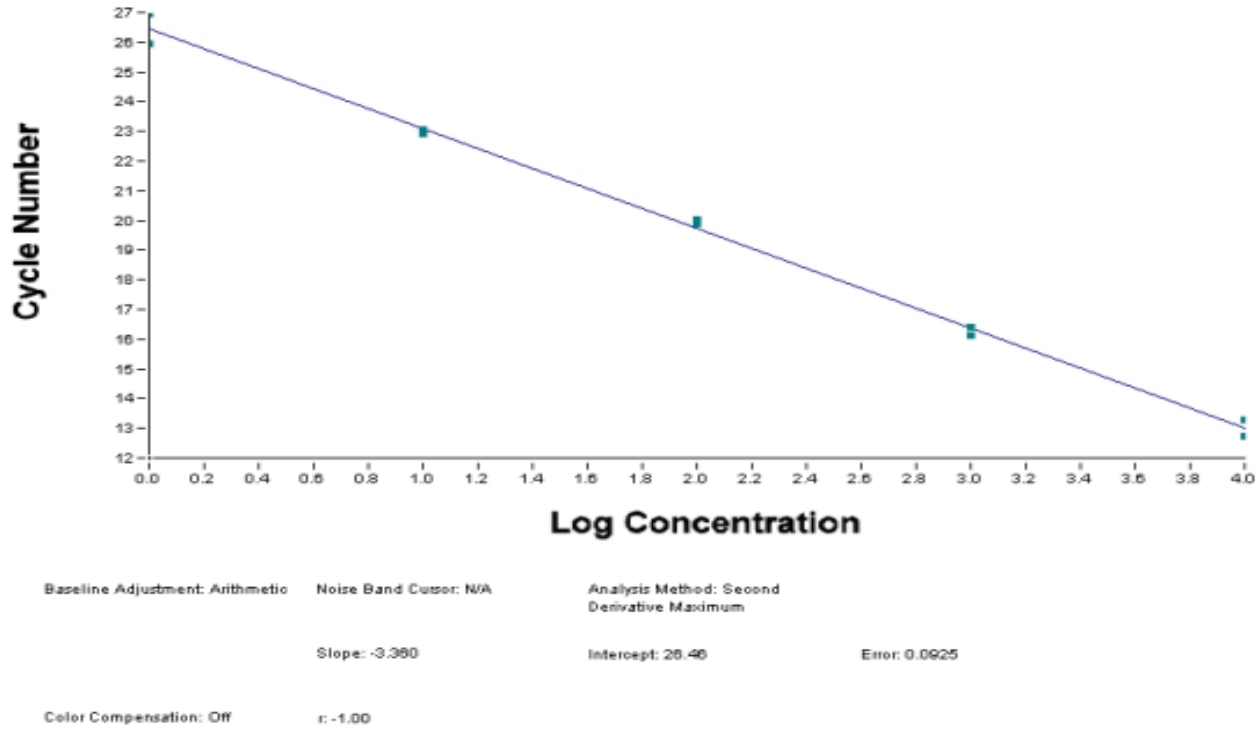


Figure 1.18: To access the efficiency of the primers, a standard curve was prepared based on serially dilution of HeLa cDNAs. The r value is equals to -1