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Biological efects of South African plant extracts on RBBp6 as anti-cancer therapy in lung cancer

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Biological effects of South African plant extracts on RBBp6 as anti-cancer therapy in lung cancer

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A dissertation submitted to the Faculty of Science, University of the Witwatersrand, Johannesburg, in fulfilment of the requirements for the degree of Master in Science.

Johannesburg, February 2012

Declaration

I declare that **"Biological effects of South African plant extracts on RBBp6 as anti-cancer therapy in lung cancer**" is my own work that has not been submitted for any degree or examination in any other university and that all the sources I have used or quoted have been indicated and acknowledged.



Signature **Date: 28th February 2012**

Abstract

Plant extracts contain compounds, which are anti-cancer agents, that possess anti-tumour activity. Most of these compounds are known to hinder the activation of cell cycle and induce cell death. During various types of stress in a normal cell, p53 and Rb enhance programmed cell death to prevent human tumour cells from forming. However, programmed cell death is not enhanced in the presence of RBBp6 or Mdm2. Thus, cell cycle will be enhanced, resulting to abnormal cells proliferating and causing cancerous cells or tumours. RBBp6 is a p53-associated cellular protein and retinoblastoma binding protein containing a conserved N-terminal ring-finger domain, which takes part in p53 degradation. RBBp6 is highly expressed in lung cancer and may be a promising target for treating cancer. The aim is to use plant extracts to regulate the expression of RBBp6, switching it off from being pro-carcinogenic to becoming an anti-carcinogenic protein in lung cancer cells. Two cancer cell lines (Lsqr1 and A549) were cultured and treated with *Euphorbia mauritanica* and *Kedrostis hirtella* extracts. A MTT assay was used to determine 50% cell death, which was induced by 5 μg/ml and 10 μg/ml of the *Euphorbia mauritanica* and *Kedrostis hirtella* extracts after 22 hrs and 24 hrs, respectively. Molecular analysis using gel DNA fragmentation showed smeared DNA due to necrosis and a fragmented DNA induced by apoptosis. The quantity of cells undergoing apoptosis and necrosis was measured by flow cytometry, which showed a higher percentage of necrotic cells compared to apoptotic cells. Real-time PCR was used in order to analyse the RBBp6 expression in lung cancer cells treated with the plant extract. The results of the present study showed no change or an insignificant decreased in the expression of RBBp6 in lung cancer cells treated with the extracts. Therefore, plant extracts used in this research have no effect on RBBp6 as anti-cancer therapy.

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"I can do all things through God who gives me strength. I am able"

"My grace is sufficient for you, for my strength is made perfect in weakness"

List of Abbreviations

A549 cell line	Adenocarcinoma cell line
Amp	Ampicillin
BLAST	Basic Local Alignment Search Tool
bp	base pair
CAD	caspase-activated DNase
cdk	cyclin-D dependent kinase
cDNA	complementary DNA
DMEM	Dulbecco's modified medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotides
EDTA	Ethylene diamine tetra acetic acid
FCS	Foetal calf serum
GAPDH	Glyceraldehyde phosphate dehydrogenase
ICAD	inhibitor of CAD
Kb	Kilobase
kDa	kilo Dalton
l	Litre
Lqr1	Lung sqaumous cell line
mRNA	messenger RNA
MW	Molecular weight

NCI	National Cancer Institute
NSCLC	non-small cell lung cancer
p53	protein 53 (tumour protein 53)
PBS	Phosphate buffered saline
PCR	polymerase chain reaction
pRb	Retinoblastoma protein
PS	phosphatidylserine
RBBp6	Retinoblastoma binding protein 6
RNA	Ribonucleic acid
SCLC	small Cell Lung Cancer
Sec	seconds

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Chapter 1

1 Introduction

1.1 Overview of cancer

Cancer is a public health problem in the world. Diagnoses based on the updated incidences, mortality, new cancer cases and survival rate show that the major problem is in economically developing countries (low and medium resource countries) (Parkin *et al*, 1993). This global shift is influenced by factors such as genetic instability (pre-existing disease and family history of a cancer syndrome), chronic infections and entrenchment of modifiable risk factors, for example, environmental pollutants such as carcinogens from smoking (Parkin *et al*, 1993; Pauk *et al.*, 2005).

Smoking is a central pollutant that increases the progression of cancer more rapidly. It commonly affects the population age of 20-65 year olds in both smokers and non-smokers. Exposure to tobacco smoke may increase the risk of lung cancer causing over 500000 deaths each year (Massion and Carbone, 2003; Bradshaw *et al.,* 2003). Moreover, smoking can partly contribute to other types of cancer such as oral cavity, oesophagus, stomach, kidney and acute myeloid leukaemia. These cancers account for 1.4 million cancer deaths that occur worldwide especially in developing countries of which one-fifth is estimated to be lung cancer (Parkin *et al*, 1993; Pisani *et al*., 1999; Pauk *et al.*, 2005).

1.2 Lung cancer

Lung cancer has been a common cancer in the world that is often connected to smoking (Parkin *et al.,* 1993; Pisani *et al.,* 1999; Zang *et al*., 2000; Ries *et al.,* 2005). Lung cancer is divided into two types: the small cell lung cancer (SCLC) and non-small cell lung cancer (NSCLC).

1.2.1 Small cell lung cancer

Small cell lung cancer is a highly malignant, aggressive neoplasmic form of lung cancer, which has the ability to form central endobronchial lesions in chronic cigarette smokers (Fong *et al.,* 2003). It is frequently associated with distant metastates and has the poorest prognosis of all lung cancers. It can be subdivided into three types; namely, the small cell carcinoma (oat cell cancer), intermediate cell type and combined small cell carcinoma, which are capable of multiplying and spreading rapidly (Girard *et al.,* 2000). Small cell lung cancer cells normally lose alleles in chromosomes, triggering the early stage of lung cancer (Motadi *et al.,* 2007).

1.2.2 Non-small cell lung cancer

Non-small cell lung cancer is categorised into squamous cell carcinoma, adenocarcinoma and large cell carcinoma. Large cell carcinoma and adenocarcinoma tumours arise from damaged epithelial tissues and are transformed into cancer cells (Terasaki *et al.,* 2003; Motadi *et al.,* 2007). Squamous cell carcinoma grows on the epithelial surface of a bronchus resulting in uncontrolled multiplication of malignant cells (Franklin *et al.,* 2003). Squamous cell carcinoma accounts for 30% of lung cancer in men compared to women . Squamous cell tumours and large

cell carcinoma tumours are also associated with smoking and exposure to arsenic (Pauk *et al*., 2005).

Adenocarcinoma is responsible for 40% of lung cancer occurrence in women and often affects non-smokers. The chance of getting adenocarcinoma depends on how frequently one smokes. Other secondary risk factors that may lead to this type of lung cancer are age, family history, and being passive smoker, mineral and metal dust, asbestos and radon. Hence, adenocarcinoma is the most common lung cancer. Adenocarcinoma lung cancer grows slowly and its symptoms develop slowly. The symptoms are vaguely diagnosed by coughing, shortness of breath, wheezing, chest pain, and bloody sputum (Fauquier *et al*., 2003)

1.2.3 The cause of genetic mutation leading to lung cancer

Carcinogens of tobacco smoke have a benzo (a)-pyrene diol epoxide that causes genetic lesions in the respiratory epithelium. Genetic lesions lead to several chromosomal abnormalities and structural cytogenetic abnormalities (Hecht, 1999; Massion and Carbone, 2003; Bradshaw *et al.,* 2003; Pauk *et al.,* 2005). About 85% of mutations are due to chromosomal and structural cytogenetic abnormalities, which are enhanced by carcinogens. Carcinogens covalently bind to deoxyribonucleic acid (DNA), forming a DNA adduct that causes severe genetic mutations, which lead to defective structure and function of tumour suppressor proteins (p53 and Rb) involved in apoptosis and cell cycle (Wang *et al*, 2003; Wang *et al*, 2004; Weinstein and Joe, 2006).

The most commonly seen adduct formation occurs at the codon 157 hot spot of p53. The mutation pattern is the G→T transitions resulting from DNA 5-methylcytosine binding on the tobacco carcinogens at the CpG dinucleotide (Dennissenko *et al*., 1997; Lewis and Perry, 2004). This mutation pattern leads to a dysfunctional p53, which fails to delay or terminate the cell cycle when the DNA is damaged. Failure to terminate the cell cycle in such a case results in growth advantages of progressive and invasive cells that might lead to cancer (Evan and Vousden, 2001; Lowe *et al*., 1993; Wang, *et al*, 2003; Hemann and Narita, 2007).

1.3 Tumour suppressor proteins involved in apoptosis and cell cycle

1.3.1 p53

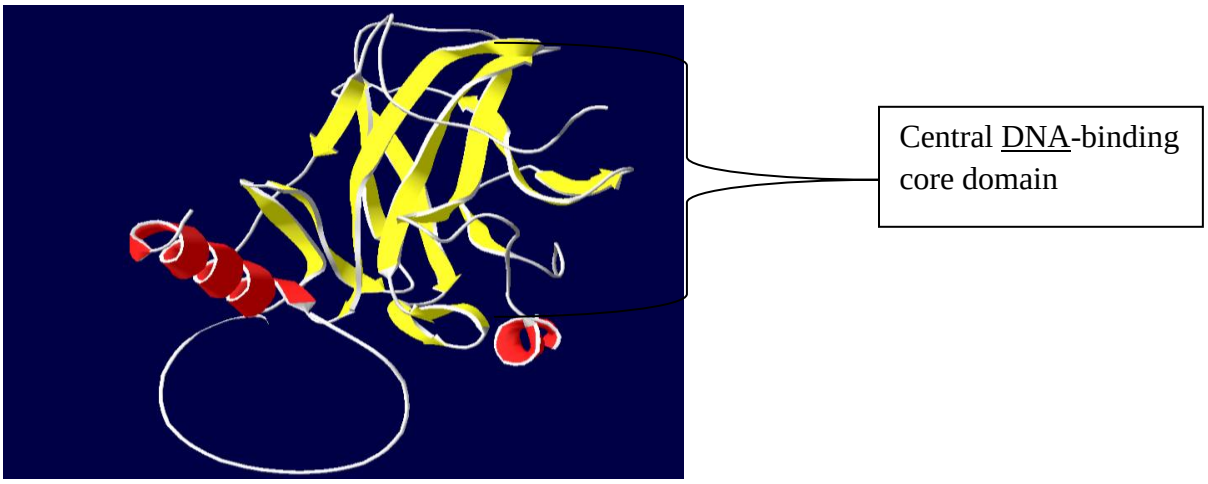


Figure 1:1 Ribbon representation of p53 tumour suppressor protein. The structure was generated using Swiss-pdb viewer (Perez-Canadillas et al., 2006). The PDB code used to generate this structure is 2FEJ**.**

p53 is a nuclear phosphoprotein and genes encoding for this protein are found at the short arm of chromosome 17. It consists of 393 amino acids with a molecular weight of ~53 kDa (Lane *et al.,* 1979; Levine et al., 1997). It is is a tetramer protein, with identical subunits. Each subunit contains seven domains. It contains a nuclear localization signaling domain, a DNA-binding core domain (p53DBD) which is essential for binding the p53 co-repressor. The N-terminal transcription domain activates transcription factors. It has an activation domain, which is important for apoptotic activity, and a tetramerization domain essential for the activity of p53 *in vivo*. C-terminal regulatory domain is involved in the down-regulation of DNA binding of the central domain. However, the main focus of the p53 structure (Figure 1:1) is on the DNA-binding core domain (p53DBD) (Ho et al., 2006; Perez-Canadillas et al., 2006)

1.3.2 Retinoblastoma (Rb1)

pRb is a pocket protein, genes encoding for pRB are located in human chromosome 13 and codes for a 110 kDa nuclear phosphoprotein. pRb is an important tumour suppressor protein for controlling the G_1 progression point and is involved at the first checkpoint of G_1/S metaphase, before the cell enters S phase for DNA replication. It regulates the cell cycle by interacting with G_1 cyclin and cyclin dependent kinases (Nigg, 1995; Taya, 1997).

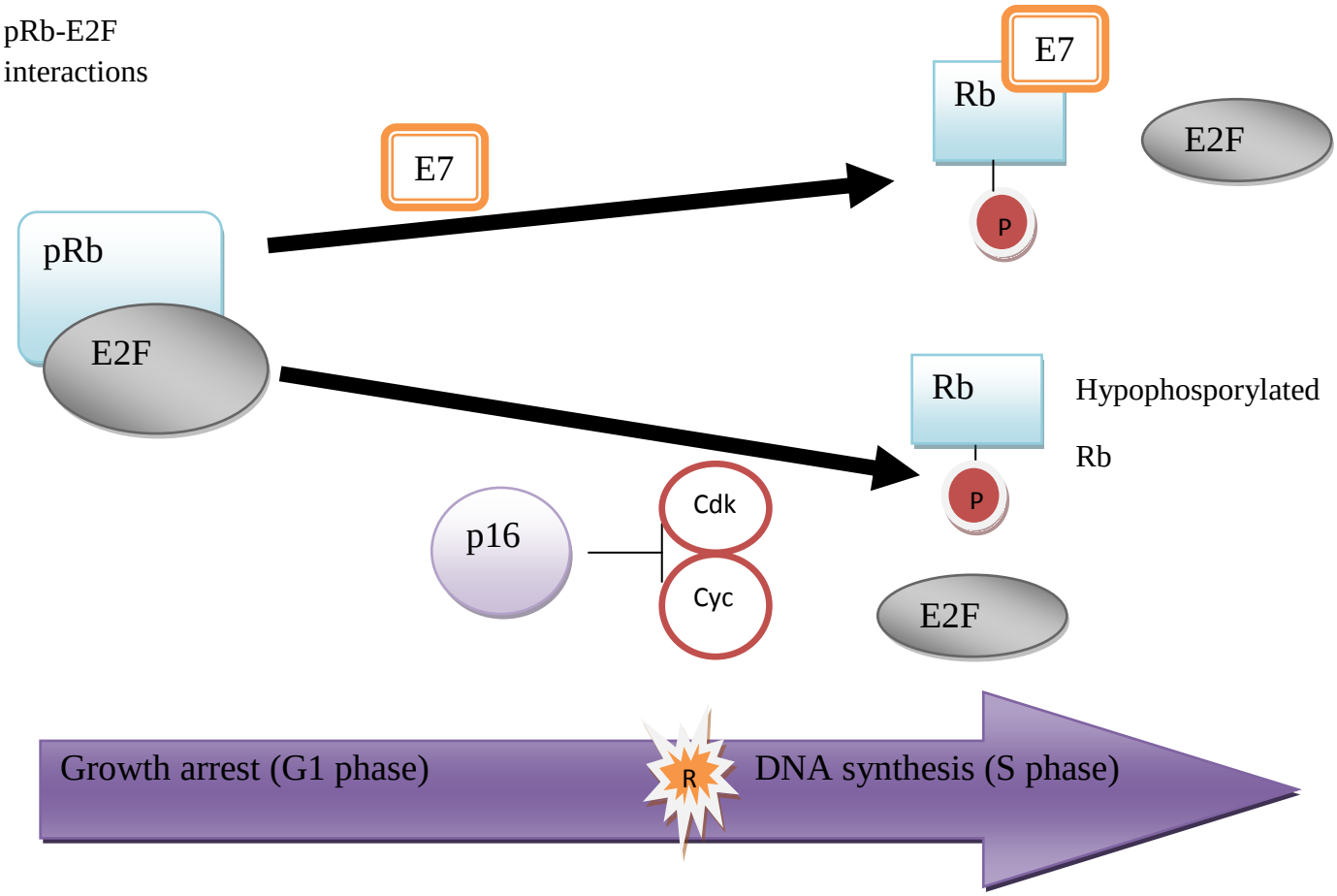


Figure 1:2 The dissociation of RB-E2F complex enhanced by kinase complex (Cdk-Cyc) which hypophosphorylates Rb, thus, promoting the release of E2F transcription factor resulting to cell progression.

pRB-E2F complex in Figure 1:2 promotes regulated cell growth or apoptosis (Hickman *et al*., 2002). (Figure 1:2) Rb is hypophosphorylated by kinase complex (Cdk-Cyc) at the Cdk consensus sites and dissociates Rb-E2F interactions. Hypophosphorylated Rb promotes cell progression to S-phase with activation and release of E2F transcription factor (Harbour *et al*.,

1999; Harbour and Dean, 2000) (Figure 1:2). When E2F transcription factor is functionally active, it will improve expressing transcriptional genes necessary for cellular replication functions during the S phase (Harbour and Dean, 2000). The cell will progress from G_1 into the S phase, and then cell division will occur at the S phase if cells are normal (Figure 1:2). Once cells have divided, Rb becomes dephosphorylated and begins to associates with E2F; this suppresses the G_1 to S phase transition. Hence, it prevents the cell from passing through the starting point of G_1 to S-phase by shutting off the transcription of genes needed for cell division (Harbour *et al.*, 1999; Harbour and Dean, 2000; DeGregori, 2005). Therefore, when the Rb gene has missing functional copies, it results in a mutant Rb gene product which is always phosphorylated and cannot be regulated by E2F. Thus, the control of cell division at the S phase does not occur and normal cells become cancerous (Harbour *et al.*, 1999; Harbour and Dean, 2000; Mani *et al.*, 2000).

1.4 Cell cycle

Cell cycle is an important mechanism in the cell; it controls the normal cell from becoming cancerous by either proceeding to the next stage of cell division or induces apoptosis (Smith and Martin, 1973; Garrett, 2001). Cell cycle is strictly controlled from one phase to the next, and these are called checkpoints. Checkpoints can be considered as safety measures for the normal cell cycle by preventing the cell from entering another cycle before any damages are properly repaired and these depend on the production of tumour suppressor proteins such as p53 and Rb (Figure 1:3).

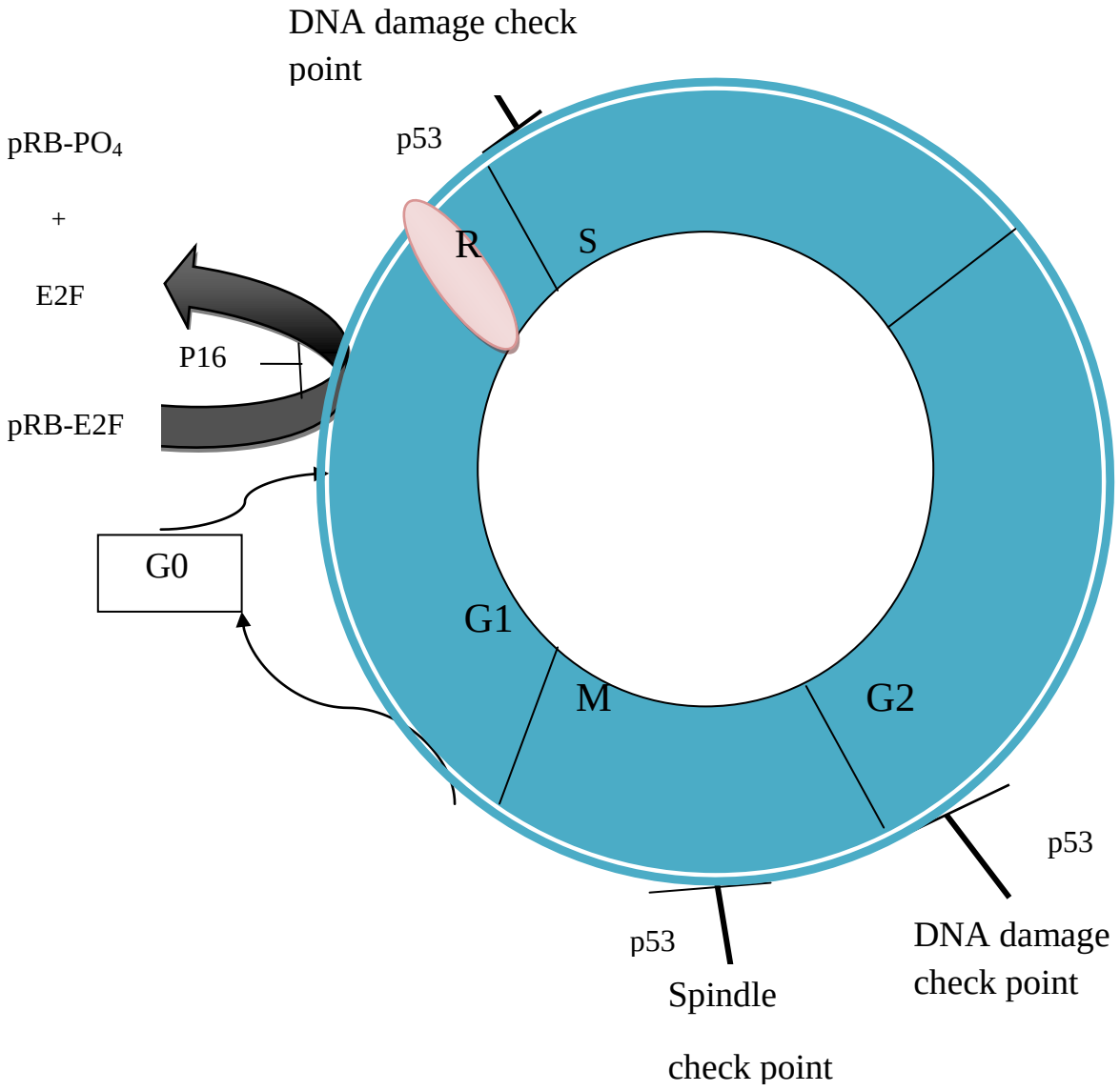


Figure 1:3 Cell cycle diagram showing different phases of which p53 functions to check and repair the damaged DNA at all check points and pRb activity is at the restriction point (R). Activation is indicated with → and inhibition (⊣). The S-phase is the synthesis phase, mitotic (M-phase), G2 interphase, before the S-phase and G2 is the interphase after the S-phase. The diagram was modified from Grana and Reddy (1995).

1.5 Apoptosis pathway

Pathological ways, which reduce tumour cells during intense stressful abnormal growth lead to apoptosis or programmed cell death. The suicide of tumour cells can be approached by the use of different drugs that affect genes, which mediate apoptosis (Kerr *et al*., 1972). However, damage to DNA or to other critical molecules can stimulate some inducers, which respond to the cellular stress. These inducers may have an intense impact on apoptosis pathway (Debatin, 1997; Kaufmann and Earnshaw, 2000; Herr and Debatin, 2001; Rich *et al*., 2000).

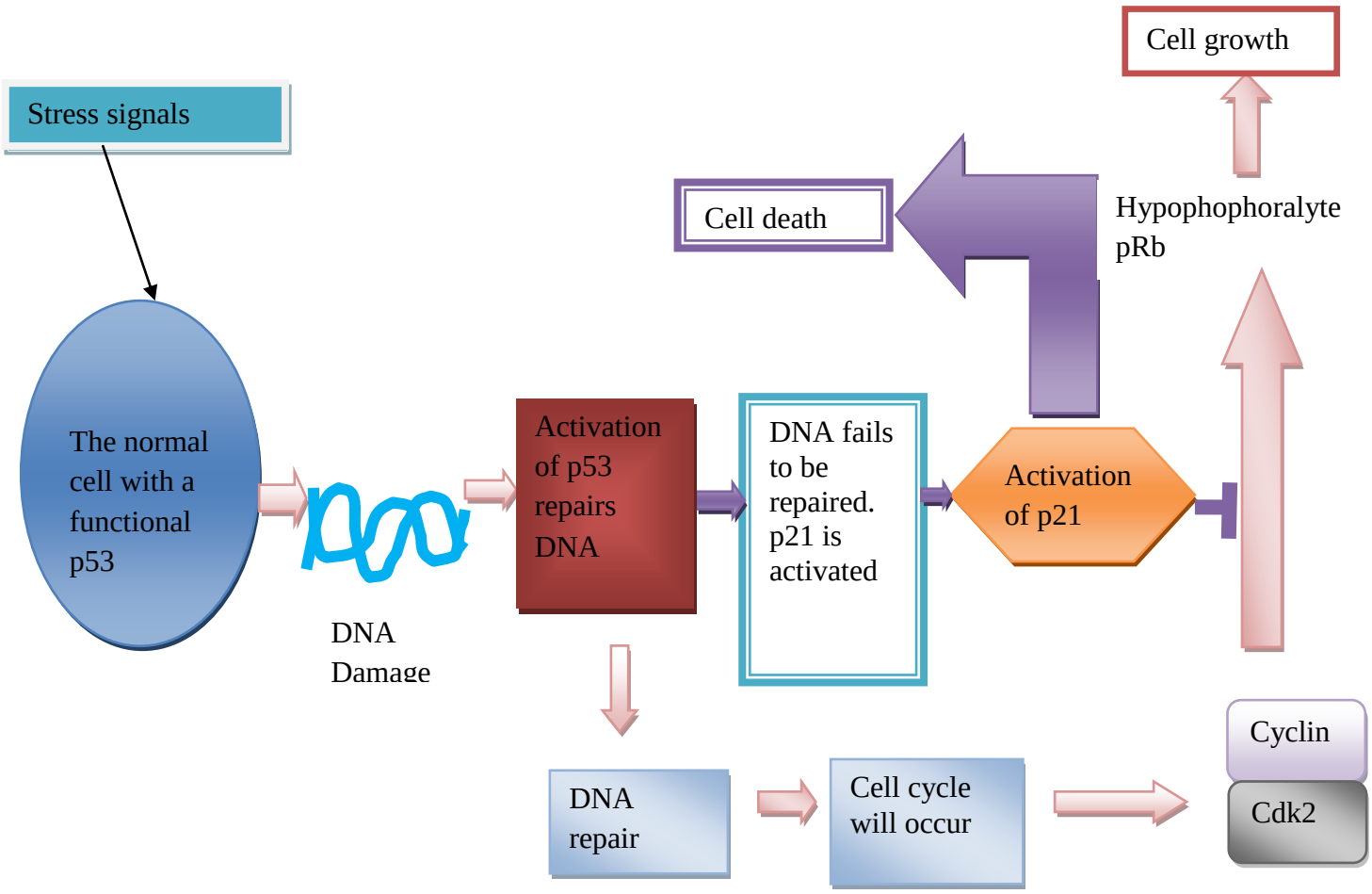


Figure 1:4 p53 as a mediator in Cell cycle and apoptosis pathway. The pink arrows represent cell cycle, and this is occurs when the DNA is repair. However, should the DNA fail to be repair cell

death will be induced this is indicated by the purple arrows. Activation of p21 will induce cell death as well as inhibit (⊣) cell cycle.

Figure 1:4 shows that stress signals or damaged DNA activates the build-up of p53 and a transcriptional expression of genes that are involved in cell cycle arrest or apoptosis (Figure 1:4). p53 is one of the inducers that induces intrinsic apoptosis pathways and it does so by acting as a receiver of stress signals that include damaged DNA. It is involved in multiple stress signals of a diverse antiproliferative series of responses (Rodin and Rodin, 2000; Hickman *et al*., 2002, Hussein *et al*., 2003). When p53 is functional, it repairs damaged DNA through the activation of p21 thus result in caspase activation which will induce intrinsic apoptosis (Levine et al., 1997; Wu *et al*., 2004). If damaged DNA fails to be repaired, pRb induces apoptosis by binding to E2F to form a complex that inhibits cell cycle, promoting cell death.

Ineffective DNA repair (Figure 1:3 and Figure 1:4) results to p53 activating p21, cdk2 inhibitors. These will regulate cell cycle event by stopping cell progression at G1/S and G2/M phases and apoptosis will take place (Garrett, 2001; Hussein *et al*., 2002). Defective p53 abolishes its capacity to activate p21 reporter (Levine *et al.*, 1997; Czajkowski *et al.*, 2002). In so doing, it prevents an intrinsic apoptosis pathway from occurring by enhancing the cell cycle of unsuccessful checkpoints and the completion of mitosis. Therefore, aberrant daughter cells will be produced resulting in uncontrolled growth (Hartwell & Weinert, 1989; Lowe *et al*., 1993; Stephen, 1996), altering other genes (p16, p14) involved in the Rb and p53 pathways (Murakami *et al*., 1991; Lowe *et al*., 1993; Hickman *et al.*, 2002).

1.5.1 RBBp6 (A p53 and Rb binding protein)

RBBp6 (Retinoblastoma binding protein 6) is a 250 kDa splicing-associated protein found in eukaryotes (Simon *et al.*, 1997). It is usually expressed in heart, lung, liver skeletal muscle and testes (Witte and Scot, 1997).

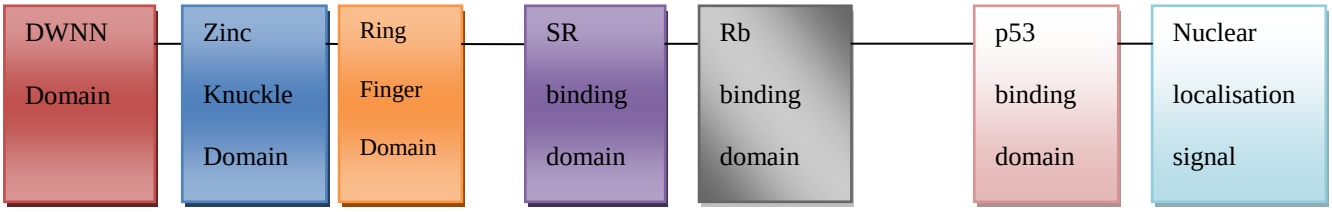


Figure 1:5 The structure of the RBBP6 proteins. The diagram is modified from Pugh et al. (2006)

Figure 1:5 shows that RBBp6 is a multi-domain protein made up of the DWNN domain, a zinc knuckle and a ring-finger domain. Highly conserved RBBp6 N-terminal has the DWNN domain and ring-finger domain. RBBp6 can also interact with 19 multifunctional Protein YB-1 through its ring-finger domain resulting in the ubiquitination of YB-1 and proteosomal degradation in the proteosome (Sakai *et al.*, 1995; Mather *et al.*, 2005). The DWNN domain has an ubiquitin-like fold which regulates pre-mRNA processing protein (Mather *et al*. 2005; Pugh *et al.*, 2006). Humans RBBp6 has C-terminal extension containing p53 and Rb-interaction domains. p53 binding domain interacts with p53 and suppresses the binding of p53 to DNA. The Rb domain binds to Rb and blocks the binding of E2F transcription factors (Figure 1:5) (Yoshitake *et al.*, 2004; Pugh *et al.*, 2006).

p53 has an excision repair system which is crucial for maintaining the genome integrity. RBBp6 protein associates with p53-cellular protein and stimulates ubiquination and degradation of p53. This will interfere with p53 binding to damaged DNA. As a result apoptosis will be inhibited (Wang *et al*., 2003). However, cell proliferation will be enhanced because Rb is prevented from binding to the transcription factors. Thus, in the presence of the functionally active transcription factors, expression transcriptional genes are necessary for cellular replication functions, and the cells with damaged DNA will replicate abnormally (Sakai *et al.*, 1995; Mather *et al.*, 2005).

Moreover, Li *et al.*, 2007 report that RBBp6 does not itself ubiquinate p53. Speculation says that it may play a role in scaffolding on the interactions that take place between p53 and Mdm2, and promote p53 degradation by enhancing the Mdm2 activity (Li *et al.*, 2007). Mdm2 has a highly conserved ring-finger domain which is similar to that of RBBp6, and has a similar function by interacting with tumour suppressor protein identified as p53 and Rb. In doing so, Mdm2 promotes cell cycle progression on normal cells, thus preventing these cells from programmed cell death. However, should cells be cancerous, Mdm2 still performs its function by preventing cell death and advancing cell cycle progression. Hence, most cancer cells have overexpression of Mdm2 and RBBp6 protein (Jones *et al*., 1995, Luna *et al*., 1995).

To prevent cancer, research was done to produce inhibitors that prevent p53 from interacting with Mdm2 (Justin and Murray, 2007), whereas others focused on creating anti-sense oligonucleatides that inhibit expression of Mdm2. Other ways were to alter the genes of this protein by alternative splicing and produce a spliced variant such as Mdm2b isoform, which has eight exons missing resulting in a structure without a p53-binding domain. For that reason, it is

clear that research done on Mdm2 was of benefit to inhibit Mdm2 from enhancing cancer cells (Wang *et al*., 2004).

1.6 Plant extracts as anti-cancer agents

Plant secondary metabolites are the source of pharmaceutical drugs and many medicinal derivatives (Harvey, 1999; van Wyk et al., 1997). Over the years, the system of drug discovery have developed useful chemotherapeutic drugs for various types of cancer, which are successful in most human cancer treatment, but do not guarantee long-life span. Tumours in adults have shown resistance to some antineoplastic drugs because they have a limited anti-solid tumour activity (Harvey, 1999; Mans *et al.*, 2000; Mann, 2002). Moreover, inadequacy of current chemotherapeutic agents for the treatment of advanced solid malignancies led to consideration of traditional drugs as anti-cancer agents, thus, improving their efficiency by critically assessing discovery methodologies of using plant-derived compounds for cancer therapy (Chabner, 1991; Harvey, 1999).

Traditional medicines derived from plant sources have been used as anti-cancer agents from the 1950s and were discovered in tropical and sub-tropical regions of the world. During 1960-1982, about 3500 plant samples were tested against leukemia cell lines. In 1985, the NCI (National Cancer Institute) started a new programme using plant extracts to test against human cancer cell lines. Recently, about 60% of anti-cancer drugs have been derived from natural sources such as plant extracts (Cragg and Newman, 2000; Mann, 2002; Newman *et al.*, 2000; Newman *et al.*, 2002). The extracts of plant species contain compounds which are anti-cancer agents that

possess anti-tumour activity. The mechanism of action of these anti-cancer agents from plant extracts remains unclear. However, the structure and mechanism of action of some anti-cancer agents have been elucidated and are used as drugs in chemotherapy (Nair *et al.,* 1991; Perry, 1992; Salomi *et al.,* 1992). The first anti-cancer agents to be used clinically were vinblastine and vincristine isolated from *Catharanthus roseus*. Vinblastine and vincristine isolate were used to test for cytotoxicity activity against cancer cell lines and normal tissue grown either *in vitro* or used *in vivo* models. Vinblastine and vincristine inhibits mitosis (cell division) in metaphase by preventing the cell from moving its chromosomes around as it divides; vinblastine also interferes with glutamic acid metabolism in order to fight cancer. Thus, vinblastine is useful against advanced testicular cancer, breast cancer, Kaposi's sarcoma and leukemia (Stokoe *et al.,* 2001; Groninger *et al.,* 2005).

Moreover, studies done by Wirger *et al.,* (2005) reveal that the flavopiridol compound isolated from leaves or stems of *Amoora rohituka* inhibit 50% proliferation in bladder cancer cells when used as cytotoxicity anti-cancer agents. Flavopiridol is involved in the mechanism of cyclin-dependent kinases by hindering activation and blocking cell cycle progression at G_1 or G_2 (Killand, 2000; Wirger *et al.,* 2005). Etoposide is an epipodophyllotoxin, derived from the mandrake plant *Podophyllum peltatum* and the wild chervil *Podophyllum emodi* also has significant activity against small-cell lung carcinoma (StÄhelin *et al,* 1973). Therefore, plant extracts have significant impact by interacting with genes that trigger cell death and control cell cycling in cancer cells. Hence, plant extracts were used in this research to target specific genes in lung cancer cells that might induce cell death.

Plant extracts used for this research were *Euphorbia mauritanica* and *Kedrosis Hirtella. Euphorbia mauritanica* is a spineless succulent bush plant. It's found in the Northern Cape, Western Cape Eastern Cape Kwazulu Natal and Free state. It is known to cause diarrhoea, fever and fatal disorder in sheep (Mitch, 1984) yet traditional healers use it to treat cancer. *Kedrostis hirtella* is green, and has velvet leaves and stem. It is found in Gauteng and Western Cape. The roots of this plant are used to make beer for tswana people but it fruit is posionus. Plant is also used for medicinal purpose in Transkei and has fatal poison (Watt and Beyer-Brandwijk, 1962; Van Wyk and Gericke, 2000). Although these plants are poisonous, traditional healers boil the leaves and roots and use the extracts to treat cancer. When boiling the plant some compounds become inactive and this might be the poisonous or harmful compounds. Therefore this study will prove whether the plant extracts are useful as cancer treatment.

1.7 Aim

The main aim of this research was to test for anti-cancer activity on RBBp6, when lung cancer cells are treated with *Euphorbia mauritanica* and *Kedrostis hirtella* extracts.

1.7.1 Objectives

To achieve the main Aim, the objectives were as follows:

- Determine whether lung cancer cells treated with Staurosporine, extracts of *Euphorbia mauritanica* and *Kedrostis hirtella* reach a net saturation level of 50% cell death
- Determine the percentages of viable cells, cells undergoing apoptosis and necrosis in cells treated with Staurosporine, extracts of *Euphorbia mauritanica* and *Kedrostis hirtella*

- Determine the expression level of RBBp6 and p53 in cells treated with Staurosporine, extracts of *Euphorbia mauritanica* and *Kedrostis hirtella.*

Chapter 2

2 Materials and Methods

2.1 Materials

The following reagents used were obtained from Sigma-Aldrich (USA): 10 mg/ml penicillin, 10 mg/ml streptomycin, trypsin; DMEM, FBS, 0.1 M phosphate buffered solution (pH 6.0), MTT, DMSO, Magnesuim chloride and SYBr green. RNA extraction kit, DNA ladder and the DNA loading dye were purchased from Fermentas (South Africa). The reverse transcriptase kit was obtained from Promega (South Africa). Proteinase K, SDS, phenol, chloroform, sodium acetate, 100% isopropanol, alcohol, Tris-borate, EDTA and agarose powder and ethidium bromide are products from Merck (South Africa). Annexin-V-FITC dye was purchase from Abcam. Primers were synthesised by Inqaba biotech (Pretoria, South Africa).

2.1.1 Ethics Approval

The University of the Witwatersrand Human Ethics Committee was applied to for an ethic clearance and it was granted.

2.1.2 Cancer cell lines

Normal primary human fibroblasts, A549 and Lqr1 cell lines were purchased from the Japan Health foundation. Normal primary human fibroblasts have structural framework (stroma) for animal tissues and they synthesize the extracellular matrix and collagen. A549 cells are adenocarcinomic human alveolar basal epithelial cells . Lsqr1 are squamous cell carcinoma and

theygrow on the epithelial surface of a bronchus resulting in uncontrolled multiplication of malignant cells. A549 and Lsqr1 grow as monolayer cells adhering or attaching to the culture flask when cultured in vitro.

2.2 Methods

2.2.1 Preparation of aqueous extracts

Fresh South African plants, roots and leaves (*Euphorbia mauritanica* and *Kedrostis hirtella*) were thoroughly washed with distilled water, and dried in a ventilation oven for 72 hrs at 35 °C. Dried leaves were ground to a fine powder and 10 g of powder was extracted in 1 litre of boiling water and allowed to cool prior to centrifugation at 1000 × g. Extracts of boiled leaves were passed through a 850 μm pore sieve to obtain the supernatant. Stocks of 40 mg/ml were freshly prepared and stored at −20 °C until needed.

2.2.2 Cell culturing

Two human lung cancer cell lines A549 (Adenocarcinoma) and Lsqr1 (Lung squamous) were used to carry out the plant extracts trial research work. Cells were thawed in a 37 ºC water bath and centrifuged for 2 min. The supernatant was discarded, and the pellet was resuspended in a tissue-culturing flask containing Dulbecco's minimum essential media (DMEM) supplemented with 10% Fetal Calf Serum (FCS) for medium growth and 1% pen/strep to prevent contamination. The cultured cell lines were grown at 37 °C in a humidified atmosphere of 5% (v/v) CO_2 until they were confluent. Adherent confluent cells were washed three times with PBS and trypsinised for 2 min to detach them from the surface of the flask. After cells had detached, 15 ml DMEM (supplemented with 10% FCS and 1% pen/strep) was added into the flask and the

flask was gently shaken to distribute media evenly, resuspend detached cells and deactivate trypsin. Cells were resuspended into a 15 ml tube, centrifuged at 3000 x g for 4 min and the supernatant was discarded. Cells were either supplemented into three flasks containing DMEM (supplemented with 10% FCS and 1% pen/strep) or frozen into cryovia. The process of freezing cells was as follows: cells were suspended in enough freezing media (DMEM containing 20% FCS and 10% DMSO) and pipetted up and down to ensure even mixture, followed by liquoring of 1 ml into storage vials. Vials were immediately kept in cotton wool and transferred to -70 °C.

2.2.3 Preparing cells (A549 and Lsqr 1) for treatment with plant extracts

Before conducting any experiment, cells were grown in a culture flask until they were confluent. Confluent cells were washed three times with PBS and trypsinised for 2 min in order for them to detach. After cells had detached, 10 ml DMEM (supplemented with 10% FCS and 1% pen/strep) was added in a cultured flask. Cells were seeded in a 96-well tissue culture plate at a concentration of $2.5x10^4$ cells per well and incubated at 37 °C overnight. Thereafter, cells were ready for testing with plant extracts and Staurosporine. Staurosporine was used as a positive control as it induces the desired cytotoxicity. Staurosporine has derivatives with an ability to block the cell cycle of cancer cells, but have a reversible protective effect of G_1 arrest on normal cells, which makes these potent agents in cancer treatment (Lin *et al.,* 1999; Gescher *et al.,* 1998; Bunch *et al.,* 1996, Chen *et al.,*1999; Mack *et al.,* 1999).

2.2.4 Cell cytotoxicity and viability assay

Cell cytotoxicity and viability assays measure cell death and the viability of untreated or treated cells. The assay measures the activity of enzymes that reduce MTT to formazan dyes resulting in a purple colour that allow cells to be recovered if desired. The MTT dye has tetrazolium salt which is used as a quantitative assessor for mammalian cell survival and proliferation. It detects living cells according to the degree of the signal generated in activated cells (Mossman, 1983). MTT can also be used to determine anti-tumour activity or cytotoxicity of potential anti-cancer drugs, which stimulate or inhibit cell viability and growth. Thus, the assay examines drug sensitivity profiles for patients with hematological malignancies and screen potential chemotherapeutic drugs (Alley *et al.,* 1982; Mossman, 1983). However, the assay does not measure dead cells because they are unable to reduce MTT within 30 min of lysis (Mossman, 1983).

Moreover, the MTT assay has high degree of precision because there is no washing step after adding MTT dye. MTT dye has a tetrazolium ring, which is cleaved by mitochondrial dehydrogenases of viable cells. Cleaving the tetrazolium ring forms purple MTT formazan crystals that are insoluble in an aqueous solution, but dissolved in acidified isopropanol or DMSO. When DMSO is added, it solubilises formazan crystals into a purple colour; this can be used to detect viable cells spectrophotometrically at 570 nm. In order to prepare for the experiment, MTT solution (1 mg/ml in PBS) was prepared, filtered through a 0.2 µm sterile filter.

Cells were seeded in five 96-well plates for treatment with the extracts (*Euphorbia mauritanica* and *Kedrostis hirtella*) and 25 nM Staurosporine. They were incubated overnight at 37 ^{o}C to allow them to adhere to the surface. Thereafter, seeded cells were divided in triplicates of untreated cells (negative control) and cells treated with 5 µg or 10 µg of plant extracts (*Euphorbia mauritanica* and *Kedrostis hirtella*) and 25 nM Staurosporine. Wells without cells were used as blank. Five cultured plates containing treated and untreated cells were incubated for 4 hrs, 8 hrs, 16 hrs, 22 hrs and 24 hrs at 37 ^{o}C, respectively. After each time interval, the media were discarded and 10 µl MTT plus 90 µl of DMEM were added into each well, including the cell-free (blank) wells. Plates were incubated for 4 hrs at 37 °C with 5% CO_2. After incubation, MTT formed formazan crystals of viable cells. The MTT solution with DMEM was removed and 100 µl of DMSO or isopropanol was added to solubilise the formazan crystals. The plates were further incubated for 5 min at room temperature and the optical density (OD) of the wells was determined using a plate reader. The plate reader used a light wavelength of 570 nm and subtracted background absorbance measured at 690 nm. Percentage cytotoxicity was calculated as follows:

$$\text{Cell viability \%} = \frac{\text{Absorbance of treated cells} - \text{Absorbance blank}}{\text{Absorbance of negative control} - \text{Absorbance blank}}$$

$$\text{\% Cell death} = 100\% - \text{\% Cell viability}$$

2.2.5 DNA fragmentation gel electrophoresis

Biochemical properties of cell death induced by a variety of agents may result in apoptosis or necrosis. A necrotic cell has a physical injury of the cell membrane ensuing from cell swelling due to the influx of water and sodium ions (Kroemer et al., 1997). Influx of water and sodium

ions is caused by loss of regulation of ion homeostasis, which results from a passive process with no energy requirement. An important distinction between necrosis and apoptosis is the facts that cells that have undergone apoptosis, form apoptotic vesicles that are phagocytosed by macrophages or neighboring cells preventing inflammation, whereas necrotic cells result in inflammation (Ren and Savill, 1998; Studzinski, 1999). In 1980, Wyllie A. reported apoptotic cells to have degraded nuclear DNA that is cleaved into internucleosomal DNA pattern.

DNA fragmentation is a characteristic of apoptosis, though the biological function of apoptotic DNA fragmentation is unclear. Nonetheless, it is speculated that during apoptosis specific DNase such as caspase-activated DNase (CAD) cleaves chromosomal DNA in a caspase-dependent manner (Glucksmann, 1951; Williason, 1970; Wyllie, 1980; Nagata, 2000). CAD attacks chromatin to yield 3-hydroxyl and 5-phosphate, creating 50- to 300-kb cleavage products which are oligonucleosomal fragmentation. Cancer cells, however, have DFF45 existing as a heterodimer in the nucleus. DFF45 is often called an inhibitor of CAD (ICAD). It is a 45-kDa chaperone, which has an inhibitory subunit (Widlak *et al*.,2005).

CAD is synthesised with the assistance of ICAD (inhibitor of CAD) working as a specific chaperone for CAD. CAD often forms a complex with ICAD, which will result in proliferating cells. However, dissociation between CAD:ICAD complex cells will enhance apoptosis (Williams et al.,1974). Thus, the aim of this experiment is to see if there is proliferation, necrosis or fragmentation in treated and untreated lung cancer cells.

2.2.5.1 DNA Fragmentation Assays for Apoptosis Protocol

DNA was extracted from treated and untreated A549 and Lsqr1 cell lines. The A549 cell line were treated for 22 hrs while the Lsqr1 cell line were treated for 24 hrs with 5 µg and 10 µg of plant extracts (*Euphorbia mauritanica* and *Kedrostis hirtella*), respectively, and 25 nM Staurosporine. The cells were harvested into a sterile 15 ml tube and centrifuged for 10 min at 10 °C (1200 x g). The supernatant was discarded and the cell pellet was resuspended in 1X PBS. The pellet was washed twice with 10 ml 1X PBS and centrifuged between the washes. The pellet was resuspended in 10 ml DNA buffer and centrifuged for 10 min at 10 °C (1200 x g) to remove the supernatant. DNA buffer (3 ml), 125 µl of Proteinase K and 400 µl of SDS were added to the pellet and gently shaken, then incubated overnight at 45 °C. After incubation, 36 µl of phenol was added and the tube was shaken for 10 min at room temperature and centrifuged for 10 min at 10 °C (3000 x g).

The supernatant was transferred into a new tube and 1.8 ml of phenol and chloroform were added. The solution was shaken by hand at room temperature and centrifuged for 10 min at 10 °C (3000 x g). The supernatant was transferred into a new tube; 1000 µl of sodium acetate buffer (pH 5.2) and 100% isopropanol were added and shaken until DNA precipitated. The precipitated DNA was transferred into a tube and washed with 5 ml alcohol. DNA was transferred into a sterile eppendorf tube, spin down, decant, and resuspend the precipitates in 20ul of deionized water-RNase solution (0.4ml water + 5ul of RNase) and 5ul of loading buffer for 30 minutes at 37°C. Also insert 2ul of Hindi III marker (12ul of Stock IV) on the outer lanes. Run the 1.2% gel at 5V for 5min before increasing to 100 V. Gel electrophoresis was used as a

visualisation technique, to investigate whether extracted DNA of treated or untreated cells are either fragmented, smeared or had a single band.

2.2.6 APOpercentage™ assay (Ascertaining whether cytotoxicity is induced by apoptosis or necrosis)

Flow cytometry was used to quantify apoptosis. It is a rapid, quantitative measurement of apoptotic cells. During apoptosis the cells shrink, resulting in the condensation of chromatin and DNA fragments because of endonuclease. Flow cytometry is used to measure apoptotic changes in cells by staining them with various DNA dyes (Darzynkiewiez *et al.,* 1992). The majority of staining methods are used on unfixed cells because of the fragility of cells undergoing programmed cell death and the importance of maintaining the cells in their natural state for reliable results (Dive and Hickman, 1991; Gougeon *et al.,* 1992; Ormerod *et al.,* 1992; Schmid *et al.,* 1994).

The APOpercentage™ detection assay is used to measure the occurrence of apoptosis and necrosis during a vitro culture. Annexin-V-FITC dye is used to select cells that are predominantly undergoing apoptosis. During apoptosis, phosphatidylserine (PS) is translocated from the cytoplasmics face of the plasma membrane to the cell surface. Annexin-V-FITC binds to the phosphatidylserine residue that appears on the surface of a cell as an early indication or parameter of apoptosis. Annexin-V-FITC has a strong Ca^{2+}- dependent affinity for PS. It can be used as a probe for detecting apoptosis by analyses of flow cytometry (Gougen, 1997).

Flow cytometry has staining methods that uses Annexin-V for detecting phosphatidylserine, which serves as a marker of apoptosis on the cell surface. During early apoptosis, the cell morphology changes, expressing phospatidylserine on the outer cell membrane. The expressed phospatidylserine binds to Annexin-V at the early stage of apoptosis and is used as a probe for detecting apoptosis (Vermes *et al.,* 1995). For further analysis of cell cycle and late apoptosis/necrosis was detected with propidium which is a fluorescent dye that binds to the DNA. When excited by 488nm laser light, it can be detected with 562-588nm band pass filter.

Propidium iodide or 7-amino-actinomycin D stain is added to discriminate dyed dead cells that are at late apoptosis or the necrosis stage, distinguishing them from early apoptotic cells (Vermes *et al.,* 1995; Telford *et al.,* 1992). However, viable cells have normal cell membrane phospholipid symmetry, which is why they will not bind to Annexin-V-FITC and propidium iodide. Therefore, necrotic, viable cells and apoptotic cells can be classified on the basis of a double labelling of annex V and PI analysed by flow cytometry (Ormerod, 1992; Schmid, 1994; Lecoeur and Gougen, 1997).

Cells seeded in a 48-well tissue culture plate were treated with plant extracts (24 wells), and Staurosporine (12 wells), and other wells (12 wells) contained untreated cells. Wells were washed three times with 1 ml PBS to remove plant extracts (*Euphorbia mauritanica* and *Kedrostis hirtella*) and Staurosporine. Cells were then detached from the surface with 10 µl trypsin in each well. Detached cells were aliquoted into four 15 ml centrifuged tubes (Staurosporine, plant extracts (*Euphorbia mauritanica* and *Kedrostis hirtella*) and untreated cells). Each tube was centrifuged at 500 × g for 5 min and the remaining media were discarded.

Cells were resuspended in 500 µl of 1× binding buffer, and then 5 µl of Annexin-V-FITC and 5 µl of propidium iodide were added. Samples were incubated for 15 min in a dark place at room temperature. Each sample was analysed using flow cytometry (FASCan instrument equipped with Ex= 488 nm and Em = 530 nm argon laser as a light source was used to analyse the samples). The Annexin-V-FITC binding was measured using FITC signal detector (FL1 channel) and PI staining by a phycoerythrin signal detector (FL2 channel).

2.2.7 Total ribonucleic acid (RNA) extraction

RNA was extracted from three different flasks that had either transfected cells, treated cells or untreated cells. Each flask was washed three times with PBS and 800 µl of trypsin was added and incubated (room temperature) for 2 min in order for cells to detach. The cells were resuspended in 500 µl PBS and transferred into sterile 1.5 ml centrifuge tubes. Samples were centrifuged at 16,000 × g for 15 minutes. The supernatant was discarded. Pellets were washed twice with 500 µl of PBS. PBS (200 µl) and lysis-binding buffer (800 µl) were added to samples. Samples were vortexed for 15 sec and centrifuged for 15 sec at 8000 x g at room temperature.

The supernatant was transferred into high filter tubes and centrifuged for 15 seconds. Diluted DNase I (90 µl Dnase incubation buffer mixed with 10µl Dnase 1) was added to each sample, followed by 15 min incubations at room temperature. Samples were washed with 500 µl of washing buffer and centrifuged for 15 sec at 8000 x g. The flowthrough was discarded and 500 µl of buffer II was added, samples were centrifuged and flowthrough was discarded. This was done twice. The filter tubes were inserted into sterile 1.5 ml centrifuge tubes. Elution buffer

of 20 µl was added to the upper reservoirs of the filter tubes to elute RNA during centrifugation at 8000 x g speed. The quantity of RNA was measured using a nano-drop.

2.2.8 cDNA synthesis

cDNA was synthesised from untreated RNA, transfected siRNA, treated with plant extracts and Staurosporine RNA. This was done using Promega reverse-transcription kit. The following components were set up in a sterile nuclease free 1.5 ml Eppendorf tube: 2 µl master mix, 3 µl Magnesium Chloride (25mM), 2 µl deoxyribonucleotide mix, 2.5 µl oligo-p$(dT)_{15}$, 1 µl RNase inhibitor, 1 µl RNA samples (either untreated and treated cells), 1 µl reverse transcriptase and 7.5 µl nuclease free water. Thereafter, the reverse transcription programme was set, using 2700 (Gene Amp) AB applied biosystem. The samples were placed in the machine and the process of the machine was set as follows: Denaturation of samples at 95 °C for 5 min, incubation at 25 ºC for 10 min to allow primers to anneal at mRNA sites, followed by incubation at 42 ºC for an hour for cDNA synthesis. After the cDNA synthesis, reverse transcriptase was inactivated by incubation at 95 ºC for 10 min and samples were cooled on ice.

2.2.9 Primer design

Specific primers for the genes of interest (p53, and RBBp6) and a housekeeping gene Glyceraldehyde phosphate dehydrogenase (GAPDH) were designed using nucleotide sequences from NCBI and primer-plus design software. GAPDH acts as an internal control that is amplified together with the cDNA. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is commonly used as housekeeping genes and its expression remains constant in the cells or tissues under

investigation. In this study it was used to normalise the amount of RNA present in other test samples. GADPH is of value in fully characterized systems because it's used in comparisons of gene expression data. Primers were optimised using PCR to avoid or minimise the formation of an artifactual product known as primer-dimers or primer-oligomers. The 3'-terminal sequence of the primer molecule is critical for the specificity and sensitivity of PCR. A run of 3 or more G or C bases at this position should be avoided since it may stabilize nonspecific annealing of the primer.

p53 primers

Primer F -gttccgagagctgaatgagg

Primer R-tctgagtcaggcccttctgt

RBBP6 primers

Primer_F- acagcctagaccctcagcaa

Primer_R-ctcctggagcgttttcactc

GAPDH primers

Primer F- ctccccatttatcagctcca

Primer R-atgcatcgttatgtcgtc

2.2.10 PCR

The PCR process is widely used in a tremendous variety of experimental applications to produce high yields of specific DNA target sequences known as cDNA. PCR was used in this experiment to observe if designed primers can produce products with high yields, but without primer dimers. Primer optimisation was carried out as follows: a cocktail mix containing 10 µl of master mix, 1 µl of forward and reverse primer, 2 µl MgCl2 and 7 µl of nuclease-free water. The sample was carried out, using AB applied biosystem PCR system 2700 (gene amp). Agarose gel electrophoresis was used to confirm product formation and if there were no primer dimers.

2.2.11 Quantitative Real Time PCR

Real-time polymerase chain reaction is a technique based on the polymerase chain reaction. A typical workflow of qPCR for gene expression measurement involves RNA isolation, reverse transcription, qPCR assay development, qPCR experiment and data analysis. Special attention is needed for preventing RNA degradation. For reliable quantification, always include samples for standard curves and measure proper reference genes, of which the expression is constant in your experimental conditions, as an endogenous control**.**

Real-time quantitative polymerase chain reaction (qPCR) differs from normal PCR because of the fluorescent reporter molecules in the reaction. The reporter molecule is used to detect and simultaneously quantify a specific sequence of p53, RBBp6 and GAPDH in a cDNA sample. The amplified cDNA is quantified as it accumulates in the reaction in real time after each amplification cycle. Thus, reaction of fluorescent reporter molecules increases proportionally

with the increase of DNA amplification in the thermocycler. A specialised thermocycler with fluorescence detection modules is necessary for qPCR because it monitors and records the fluorescence in real-time as amplification occurs (Pfaffl, 2001; Bustin, 2002).

Experimental procedure

Preparing qPCR components acquired caution to prevent plasmid DNA contamination. qPCR reaction for each sample was done in duplicates, these are technically replicates required to monitor machine error. To minimize variation in pipetting a master mixture containing everything except for the template was prepared, and then dispense to individual tubes. The templates were added at the final step; the capillary tubes were sealed and covered firmly. all liquid was collected at the bottom by brief centrifugation. A typical qPCR reaction tube using SYBR Green I chemistry has components in 30 µl of volume as in the following table. Note that SYBR green mixture has optimized amount of DNA polymerase, dNTP, reaction buffer and dyes.

Table 2:1 Preparation of qPCR components for a Real-time PCR reaction

Components Concentration	Final concentration
SYBR Green I mixture	15 µl
Primer Forward 10 µM	0.5 µl
Primer Revers 10 µM	0.5 µl
ddH_2O	9 µl
cDNA Template	5 µl

Table 2:2: Quantitative analysis of cDNA carried out using the Roche lightcycler. The parameters on the machine were set as follows:

Analysis	Cycle	Segment	Temperature (°C)	Hold time
Enzyme activation	1	1	95	2 minutes
Quantification	40	Amplification		
		Denaturation	95	45 seconds
		Annealing	Primer dependent	45 seconds
		Extension	72	45 seconds
None	1	Final extension	72	10 minutes
Melting	1	Melting curve		
		Denaturation	95	10 seconds
		Annealing	65	15 seconds
		Extension	95	0.1°C/Sec
None	1	Cooling	40	30 seconds

Chapter 3

3 Results

3.1 Cell viability and cytotoxicity assay

Cell cytotoxicity is a test tool to measure cell death after treating the cells with extracts of *Euphorbia mauritanica* and *Kedrostis hirtella* and Staurosporine. The aim of this experiment is to reach a net saturation level of 50% cytotoxicity on treated cells. Staurosporine was used as a positive control as it induces the desired cytotoxicity. Untreated cells were a point of reference to show the effectiveness of the *Euphorbia mauritanica* and *Kedrostis hirtella* extracts.

Cell viability and cytotoxicity percentages of the A549 cell line

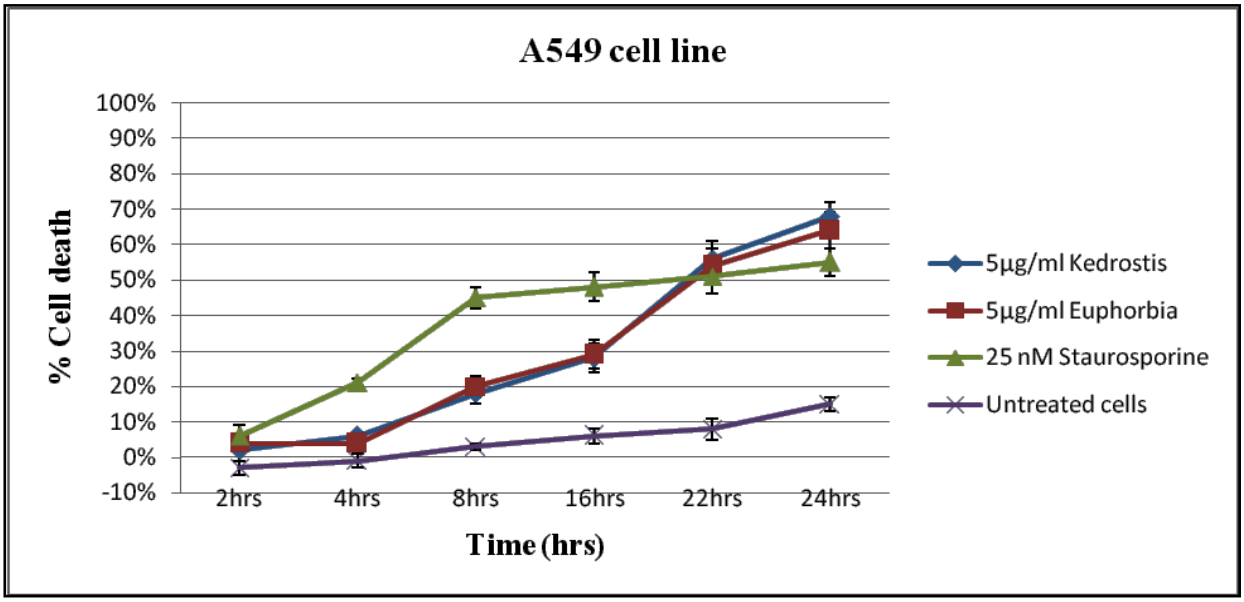


Figure 3:1 Percentage cell death of untreated and treated A549 cells. Samples of cells treated with 5 µg/ml of *Kedrostis hirtella* and *Euphorbia mauritanica* extracts, and 25 nM Staurosporine were taken at different time intervals.

After 2 to 4 hrs of treating cells with 5 µg/ml extracts of *Euphorbia mauritanica* and *Kedrostis hirtella* Figure 3:1 points out an insignificant cell death of 1% to 2%, whereas cells treated with Staurosporine exhibit 4% cell death. However, Staurosporine became reactive with a high cell death of 20% at 4 hrs. There is no cell death of untreated cells at this point although after 8 hrs they show an insignificant cell death of 4%. Cells treated with 5 µg/ml *Euphorbia mauritanica* and *Kedrostis hirtella* have a constructive response with cell death improved to approximately 19%.

Evaluating the trend of a positive control (25 nM Staurosporine); extracts demonstrate that, with enough time exposure, the target of 50% cell death will be reached. Hence, an estimated 50% cell death was achieved at 22 hrs. IC_{50} is decribed as the half maximal inhibitory concentration which is a measure of the effectiveness of a compound in inhibiting biological and biochemical function. This quantitative measure indicates how much of a particular drug or substance is required to inhibit a given biological process. Thus, concentration of the extract at 5 µg/ml is satisfactory at 22 hrs to cause the desired cell death because the negative control has trivial cell death of approximately 7%. On the contrary, at 24 hrs, there is an undesirable cell death of nearly 60% for cells treated with extracts. Though 25 nM Staurosporine (positive control) maintains the approximately accepted 55%, cell death of 15% for untreated cells makes it unacceptable to treat cells with 25 nM Staurosporine at 24 hrs.

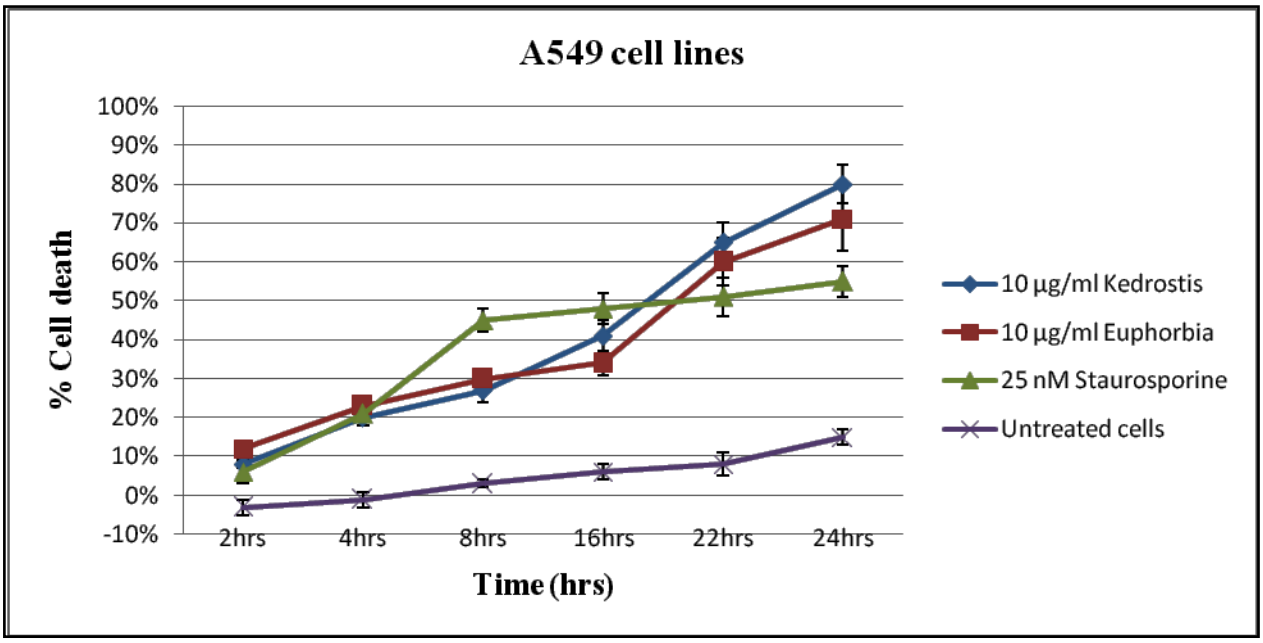


Figure 3:2 Percentage cell death of A549 untreated cells and treated cells. Samples of cells treated with 10 µg/ml of *Kedrostis hirtella* and *Euphorbia mauritanica* extracts, and 25 nM Staurosporine were taken at different time intervals.

Untreated cells and cells treated with Staurosporine in Figure 3:2 display a line graph similar to that of Figure 3:1. Figure 3:2 versus Figure 3:1 illustrates that a higher concentration of *Euphorbia mauritanica* and *Kedrostis hirtella* extracts (10 µg/ml) has a notable change in cell death of nearly 5% at 2 hrs. Figure 3:2 displays gradual increase of about 10% cell death parallel to that of Staurosporine (from 2 to 8 hrs) which plateaus after 8 hrs. Rapidly, between 16 hrs to 22 hrs, cells treated with *Euphorbia mauritanica* and *Kedrostis hirtella* extracts show 50% cell death at the intersections between Staurosporine and each plant extract. Thus, higher concentrations of the extracts reach 50% cell death faster when compared to Figure 3:2. On the other hand, Staurosporine reaches 50% at the exact time as Figure 3:1. Cell death percentage

after 24 hrs is over 70% and falls out of the desired range for cells treated with 10 µg/ml *Euphorbia mauritanica* and *Kedrostis hirtella*

Cell viability and cytotoxicity percentages of the Lsqr1 cell line

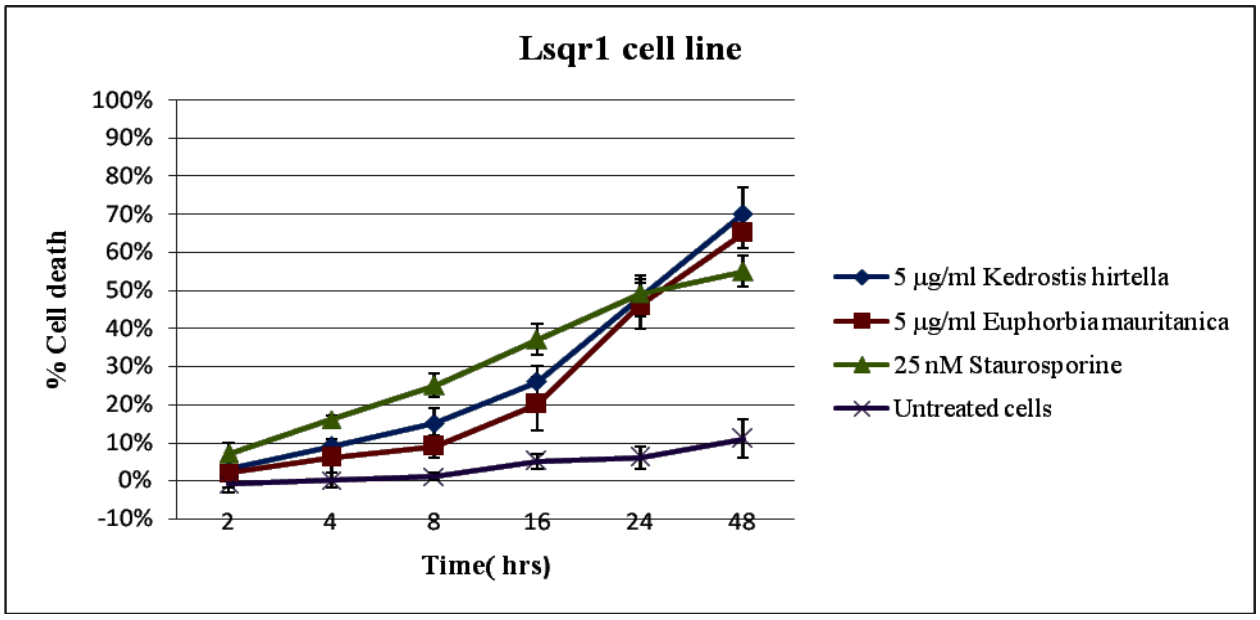


Figure 3:3 Cell death percentage of Lsqr1 untreated and treated cells. Samples of cells treated with 5 µg/ml of *Kedrostis hirtella* and *Euphorbia mauritanica* extracts, and 25 nM Staurosporine were taken at different time intervals.

Figure 3:3 highlights the following: untreated cells have cell death of less than 10% from 2 to 24 hrs. After 2 hrs, Lsqr1 cells treated with 5 µg/ml *Euphorbia mauritanica* and *Kedrostis hirtella* extracts show no cell death. After 4 hrs, cells treated with Staurosporine, *Euphorbia mauritanica* and *K. hirtellous* extracts have a responsive cell death of 17%, 5% and 9%, respectively. Over 8 hrs to 16 hrs, cells treated with *Euphorbia mauritanica* and *Kedrostis hirtella* extracts show a 5% increased cell death, whereas cells treated with Staurosporine show a 10% higher cell death. After 16 hrs, cells treated with 5 µg/ml *Euphorbia mauritanica* and *Kedrostis hirtella* extracts

are almost reaching 50% cell death at 24 hrs. However, cells treated with Staurosporine have reached 50% cell death at saturation point. Later, at 48 hrs, cells treated with extracts display a much higher cell death of over 65%, which falls out of the considered necessary range.

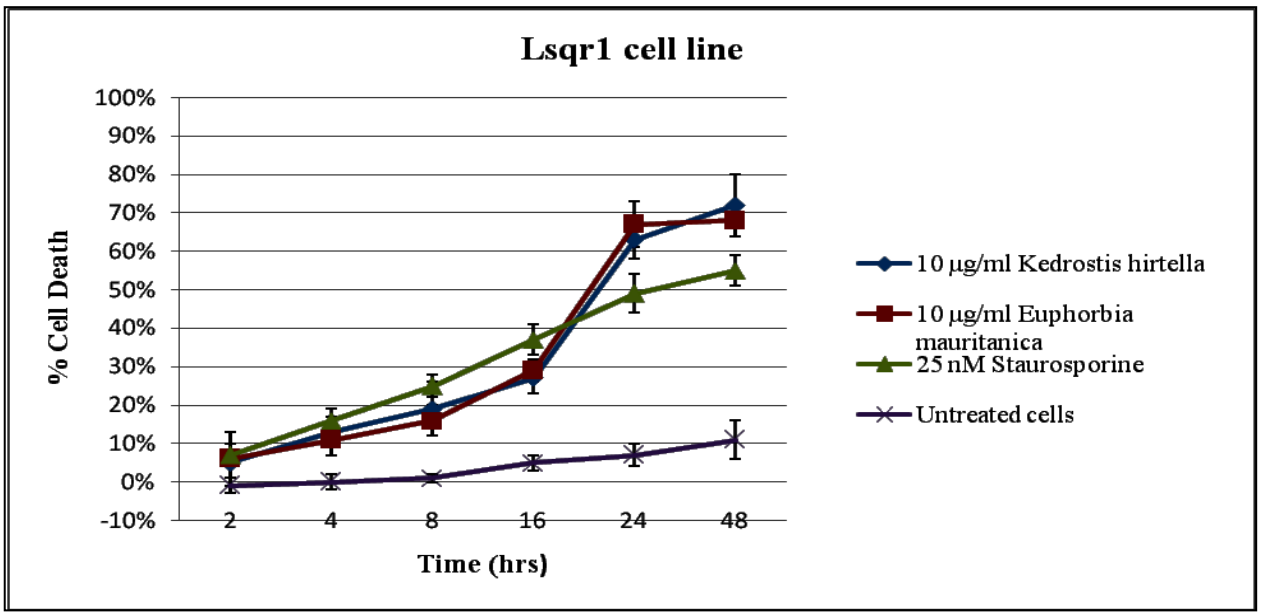


Figure 3:4 Percentage cell death of Lsqr1 untreated and treated cells. Samples of cells treated with 10µg/ml of *Kedrostis hirtella* and *Euphorbia mauritanica* extracts, and 25 nM Staurosporine were taken at different time intervals.

Figure 3:4 shows cell death of less than 8% for untreated Lsqr1 cells (from 2 to 24 hrs). Lsqr1 cells treated with 10 µg/ml of *Euphorbia mauritanica* and *Kedrostis hirtella* extracts display cell death of 5% at 2 hrs. The cell death percentage of Lsqr1 cells treated with the above extracts continues increasing by roughly 5% until 8 hrs. After 8 to 16 hrs, cells treated with 10 µg/ml of *Euphorbia mauritanica* and *Kedrostis hirtella* extracts as well as cells treated with 25 nM Staurosporine, showed a higher cell death of 10%. Cells treated with 25 nM Staurosporine, 10 µg/ml of *Euphorbia mauritanica* and *Kedrostis hirtella* extracts reached 50%, 55% and 57% cell death at 24 hrs, respectively. After 48 hrs, there is an undesirable cell death percentage of over 68% to 70% in cells treated with 10 µg/ml of *Euphorbia mauritanica* and *Kedrostis hirtella* extracts.

Cell viability and cytotoxicity percentages of normal primary human fibroblast cells

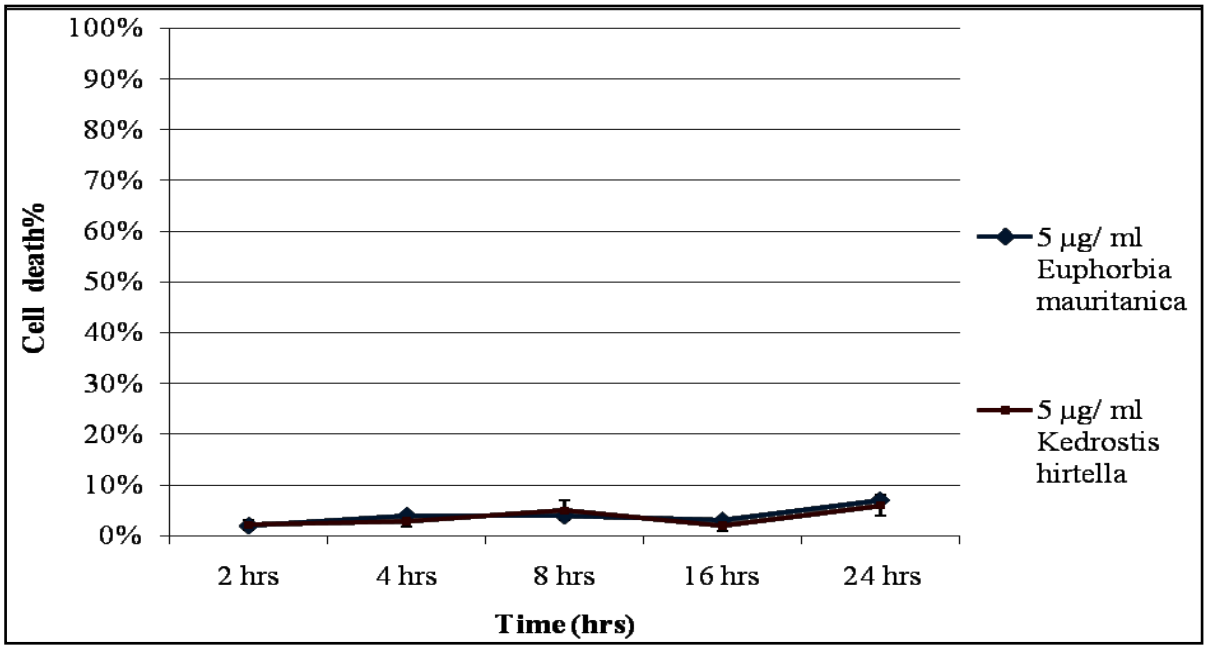


Figure 3:5 Percentage cell death of normal primary human fibroblast cells treated with 5 µg/ml of *Euphorbia mauritanica* and *Kedrostis hirtella* extracts. Samples were taken at different time intervals to observe cell death**.**

Figure 3: 5 shows that normal primary human cells are resistant to plant extract. The cell death percentage of these normal cells remains constant until 8 hrs and decreases insignificantly at 16 hrs. This graph shows no rapid increase in cell death percentage between 8 hrs to 24 hrs. Therefore, when normal cells are treated with 5 µg/ml *Euphorbia mauritanica* and *Kedrostis hirtella* extracts there is insignificant cell death percentage.

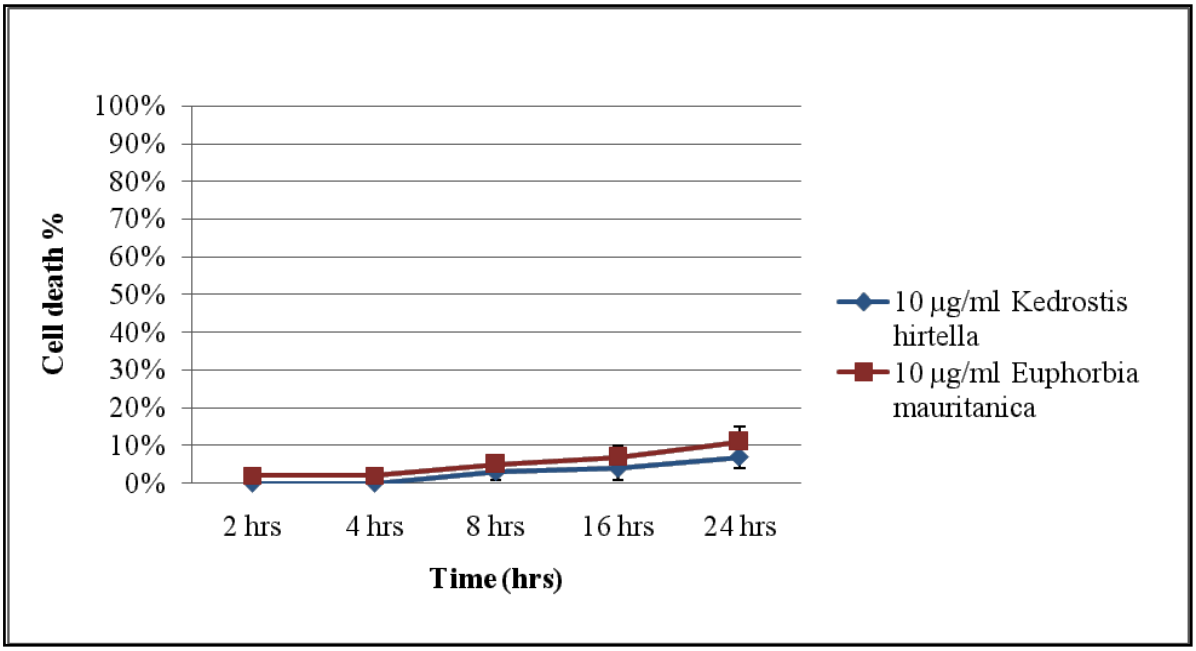
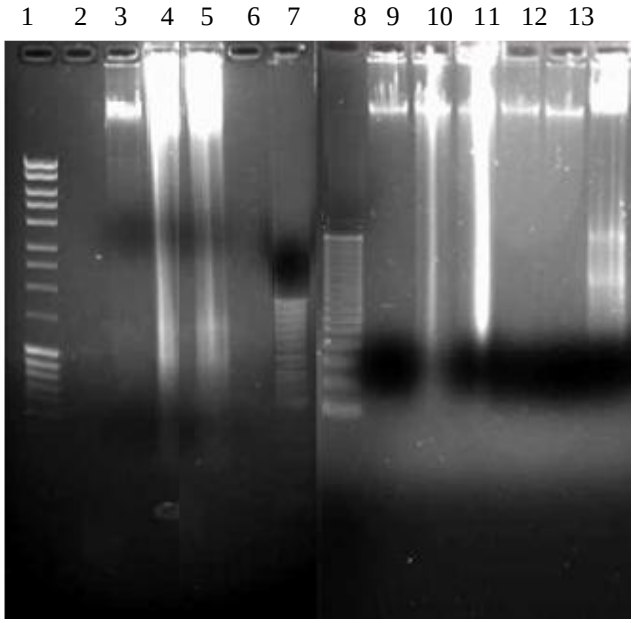


Figure 3:6 Percentage cell death of normal primary human fibroblast cells treated with 10 µg/ml of *Kedrostis hirtella* and *Euphorbia mauritanica* extracts. Samples were taken at different time intervals to observe cell death.

Figure 3:6 compared to Figure 3:5 exhibit no significant changes in cell death percentage, even if the concentration of *Kedrostis hirtella* and *Euphorbia mauritanica* extracts is increased to 10 µg/ml. Therefore, the plant extracts have no significant effect on normal cells.

3.2 Apoptosis and necrosis detection using DNA fragmentation gel

A DNA fragmentation gel electrophoresis is used to test if 50% cell death was by induced by 5 µg/ml and 10 µg/ml of *Euphorbia mauritanica* and *Kedrostis hirtella* on A549 and Lsqr1 cells, respectively.



1) Marker

3) Untreated Lsqr1 cells for 24 hrs

4) Lsqr1 cells treated with 10 µg/ml extracts of *Euphorbia mauritanica* for 24 hrs

5) Lsqr1 cells treated with 10 µg/ml extracts *Kedrostis hirtella* for 24 hrs

7) A549 cells treated with 25 nM *Staurosporine* for 22 hrs

8) Lsqr1 cells treated with 25 nM *Staurosporine* for 24 hrs

9) Untreated A459 cells for 22 hrs

10) A549 cells treated with 5 µg/ml extracts of *Euphorbia mauritanica* for 22 hrs

11) A549 cells treated with 5 µg/ml extracts of *Euphorbia mauritanica* for 22 hrs

12) Untreated A549 cells for 22 hrs

13) Untreated Lsqr1 cells for 24 hrs

Figure 3:7 DNA fragmentation gel characterising cells undergoing apoptosis, necrosis or cell proliferation when untreated and treated with extracts *Euphorbia mauritanica* and *Kedrostis hirtella* and Staurosporine.

Lane 1 (Figure 3:7) is a marker that is used to compare bands of the DNA extracted from untreated and treated Lsqr1 and A549 cells. Untreated Lsqr1 cells (Lane 3) and A549 cells

(Lanes 9, 12 and 3) exhibit a single DNA band, thus no necrosis or apoptosis is stimulated. On the other hand, smeared DNA (lanes 4 and 5) are observed when Lsqr1 cells are treated for 24 hrs with 10 µg/ml of *Euphorbia mauritanica* and *Kedrostis hirtella*. Yet again, A549 cells treated with 5 µg/ml extracts of *Euphorbia mauritanica* for 22 hrs (lanes 10- 11) show signs of smeared DNA. However, A549 cells are treated with 5 µg/ml *K hirtella* (lane 14) have a fragmented DNA. Fragmented DNA are also observed in lane 7 and 8 when Lsqr1 and A549 cells are treated with 25 nM of Staurosporine.

3.3 Measuring apoptosis percentages using flow cytometry

This technique substantiates results obtained from a DNA fragmentation gel electrophoresis as well as cell cytotoxicity assay. It quantifies the number of cells and the percentages of viable and dead cells that are apoptotic and necrotic. Lsqr1 cells treated with 10 µg/ml and A549 treated with 5 µg/ml of *Euphorbia mauritanica* and *Kedrostis hirtella* were incubated for 24 hrs and 22 hrs, respectively. Untreated cells were incubated for 24 hrs for Lsqr1 cells and 22 hrs for A549 cells.

3.3.1 Cells treated with *Kedrostis hirtella*

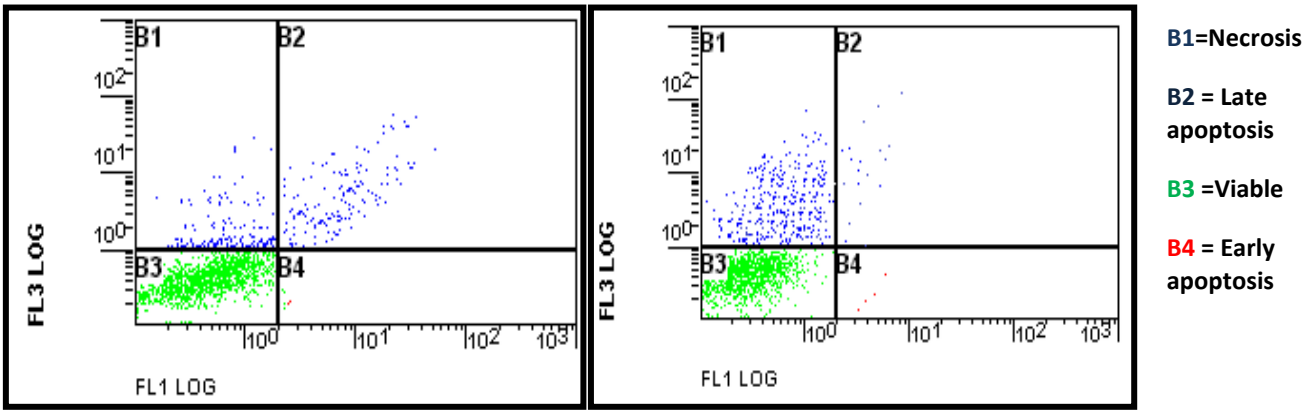
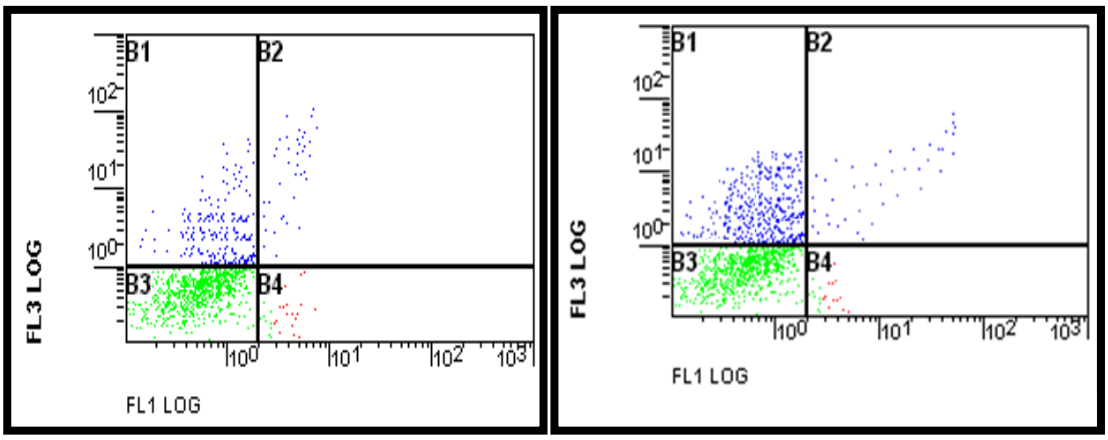


Figure 3:8 Cells (A549 on the left and Lsqr1 on the right) treated with *Kedrostis hirtella.* B1, B2, B3 and B4 represent a population percentage of cells that are viable and those undergoing necrosis, early, and late apoptosis.

Figure 3:8 show that cells treated with *Kedrostis hirtella* display a high viable population of 78.16% for A549 and a required 52.31% of viable Lsqr1 cells. A population of dead A549 cells

is distributed more on the late apoptosis stage by 4.56% in contrast to necrosis stage. However, Lsqr1 cells have a higher population of necrotic cells by 44.10% and a lower 3.09% of dead cells for late apoptosis. Therefore, A549 cells treated with *Kedrostis hirtella* are more apoptotic, whereas Lsqr1 is more necrotic.

3.3.2 Cells treated *Euphorbia mauritanica*



B1=Necrosis

B2 = Late apoptosis

B3=Viable

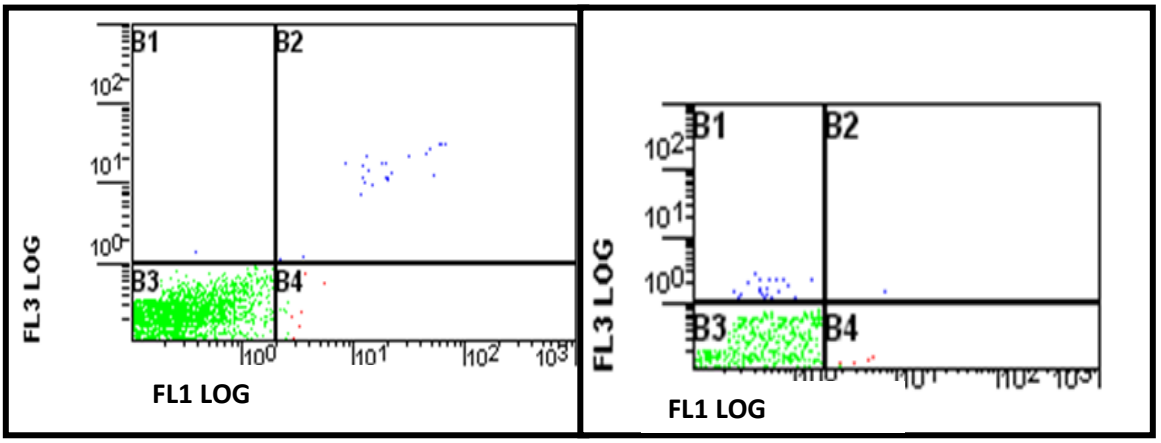
B4= Early apoptosis

Figure 3:9 Graphs displaying cells (A549 on the left and Lsqr1 on the right) treated with *Euphorbia mauritanica.* B1, B2, B3 and B4 represent a population percentage of cells that are viable and those undergoing necrosis, early, and late apoptosis.

Cells treated with *Euphorbia mauritanica* (Figure 3:9) show 68.8% and 58.8% viable population for A549 and Lsqr1 cells, respectively. *Euphorbia mauritanica* extract seems to induce necrosis with a cell death percentage of 20.51% for A549 and 30.50% for Lsqr1 cells. However, there is a low cell death percentage of about 11.7% for both A549 and Lsqr1 cells at early and late

apoptosis. Therefore, treating A549 and Lsqr1 cells with *Euphorbia mauritanica* largely induces necrosis.

3.3.3 Untreated A549 and Lqr1 cells



B1=Necrosis

B2 = Late apoptosis

B3=Viable

B4= Early apoptosis

Figure 3:10 untreated A549 (right) and Lqr 1 (left) cells. B1, B2, B3 and B4 represent a population percentage of cells that are viable and those undergoing necrosis, early, and late apoptosis.

Figure 3:10 shows a mainly viable cell population of 93% for Lsqr1 and A549 untreated. An insignificant cell death percentage of 1.15% for A549 and 0.26% for Lsqr1 cells were seen at an early stage of apoptosis. A late stage of apoptosis shows 0.77% and 0.13% cell death in A549 and Lsqr1, respectively, whereas necrotic cells showed an insignificant cell death percentage of 1.29% for Lsqr1 and 0.05% for A549 cells.

3.3.4 Cells treated with Staurosporine

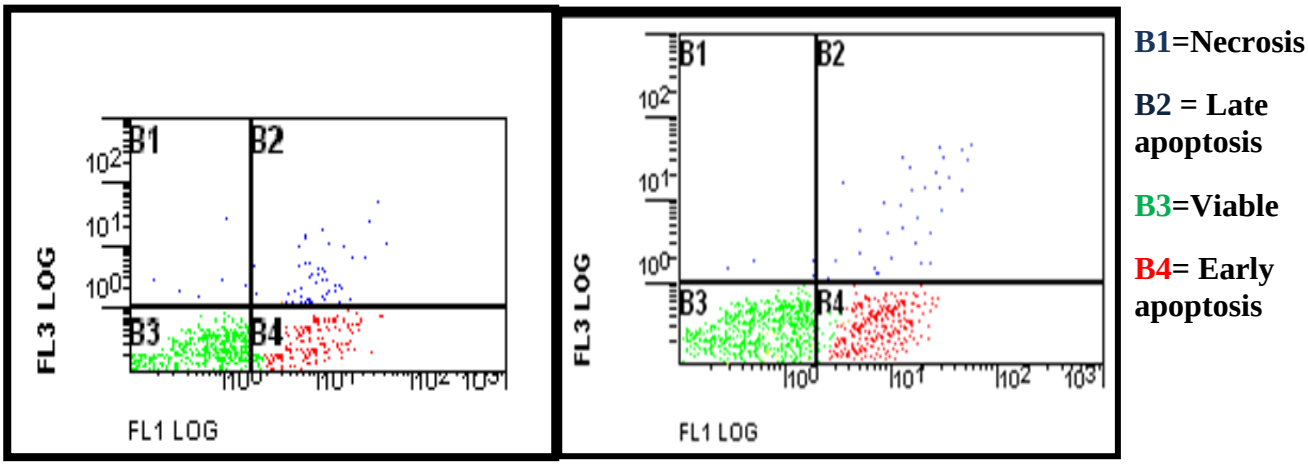


Figure 3:11 A594 cells on the left and Lsqr1 cells on the right treated with 25 nM Staurosporine. B1, B2, B3 and B4 represent a population percentage of cells that are viable and those undergoing necrosis, early, and late apoptosis.

Cells treated with 25 nM Staurosporine obtained a viable population of about 60.26% Lsqr1 cells and 57% A549 cells which resulted in population of 35.09% and 39.64% of A549 and Lsqr1 cells, respectively, undergoing early apoptosis. However, cells undergoing late apoptosis show a low cell death population of 4.11% for A549 cells and 2.77% for Lsqr1 cells. An insignificant 0.54% and 0.59% population of A549 and Lsqr1 dead cells population point out necrosis. Therefore, cells treated with Staurosporine have populations of cells predominating in early apoptosis.

3.3.5 Normal primary human fibroblast

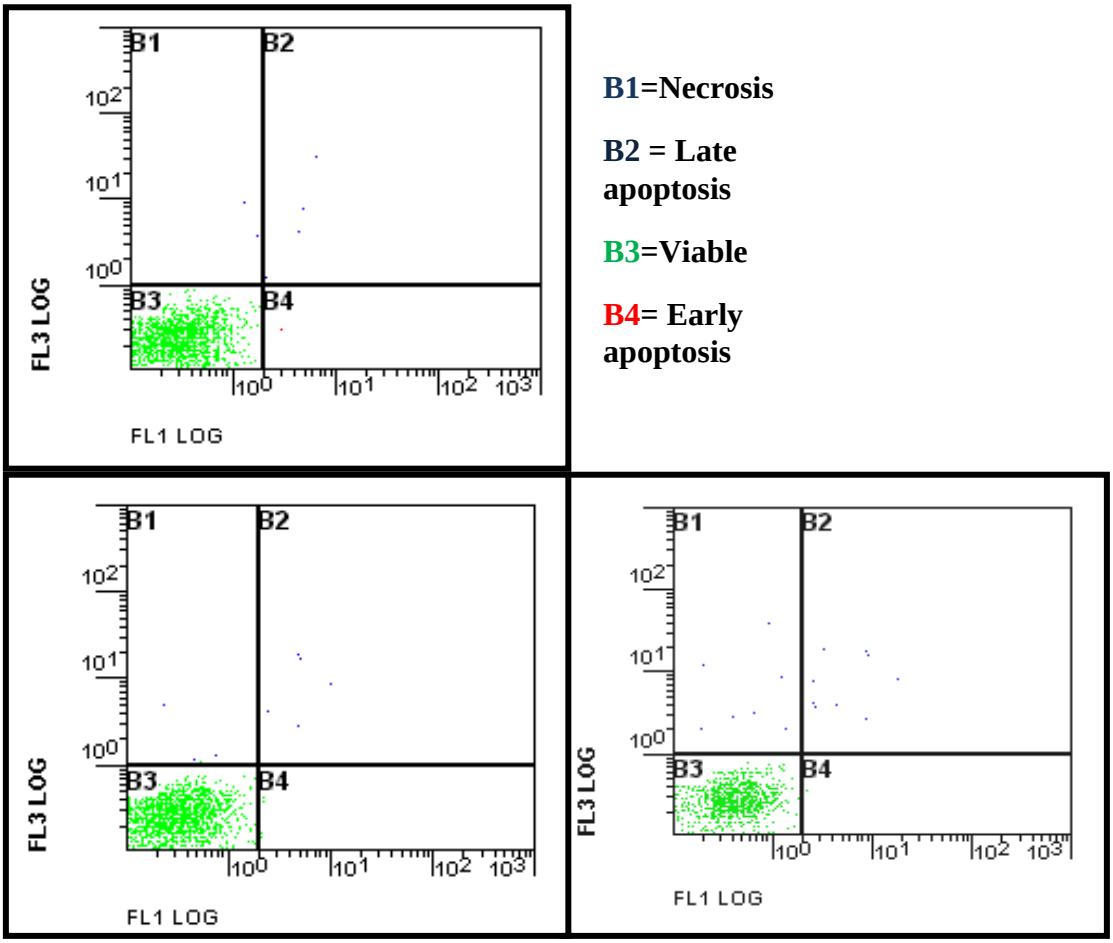


Figure 3:12 Untreated normal primary human fibroblast cells (top left) and normal primary human fibroblast cells treated with 10µg/ml of *Kedrostis hirtella* (bottom left) and *Euphorbia mauritanica* (bottom right) extracts for 24 hrs. B1, B2, B3 and B4 represent a population percentage of cells that are viable and those undergoing necrosis, early, and late apoptosis

Normal primary human fibroblast cells treated with 10µg/ml of *Kedrostis hirtella* and *Euphorbia mauritanica* extracts and untreated normal primary human fibroblast cells exhibit more viable cells. Therefore plants extracts have an insignificant cell death in normal primary human fibroblast cells.

3.4 Real-time PCR

PCR products are quantitated by generating a standard curve relative to a control gene. Real time standard curves utilise cDNA and an absolute concentration of each standard is known. The relative quantitation method measures the housekeeping or control genes to normalise expression of the target gene. Standard curves were used to quantify the values of unknown concentration of target genes expressed in lung cancer cells when treated with plant extracts.

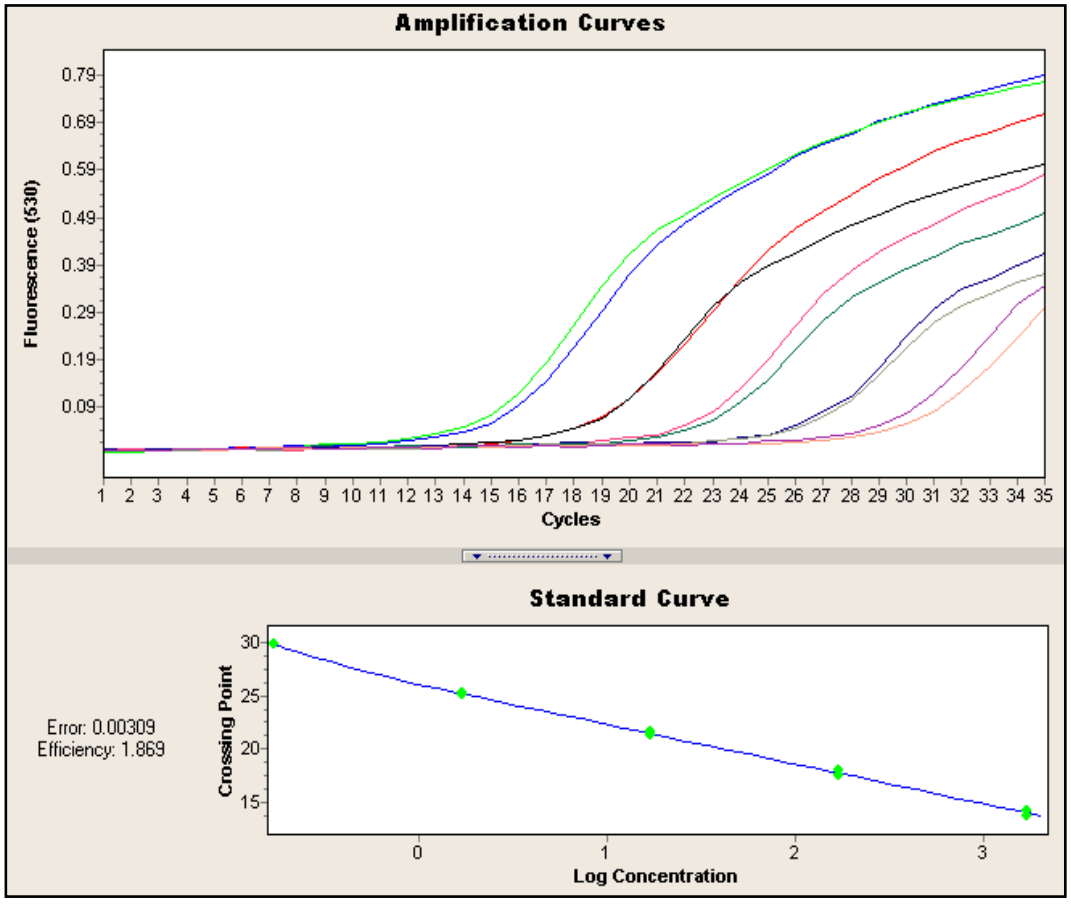


Figure 3:13 The efficiency of GAPDH primers for cDNA amplification were confirmed by a standard curve. GAPDH primers have an efficiency of 1.869 and an error of 0.00309.

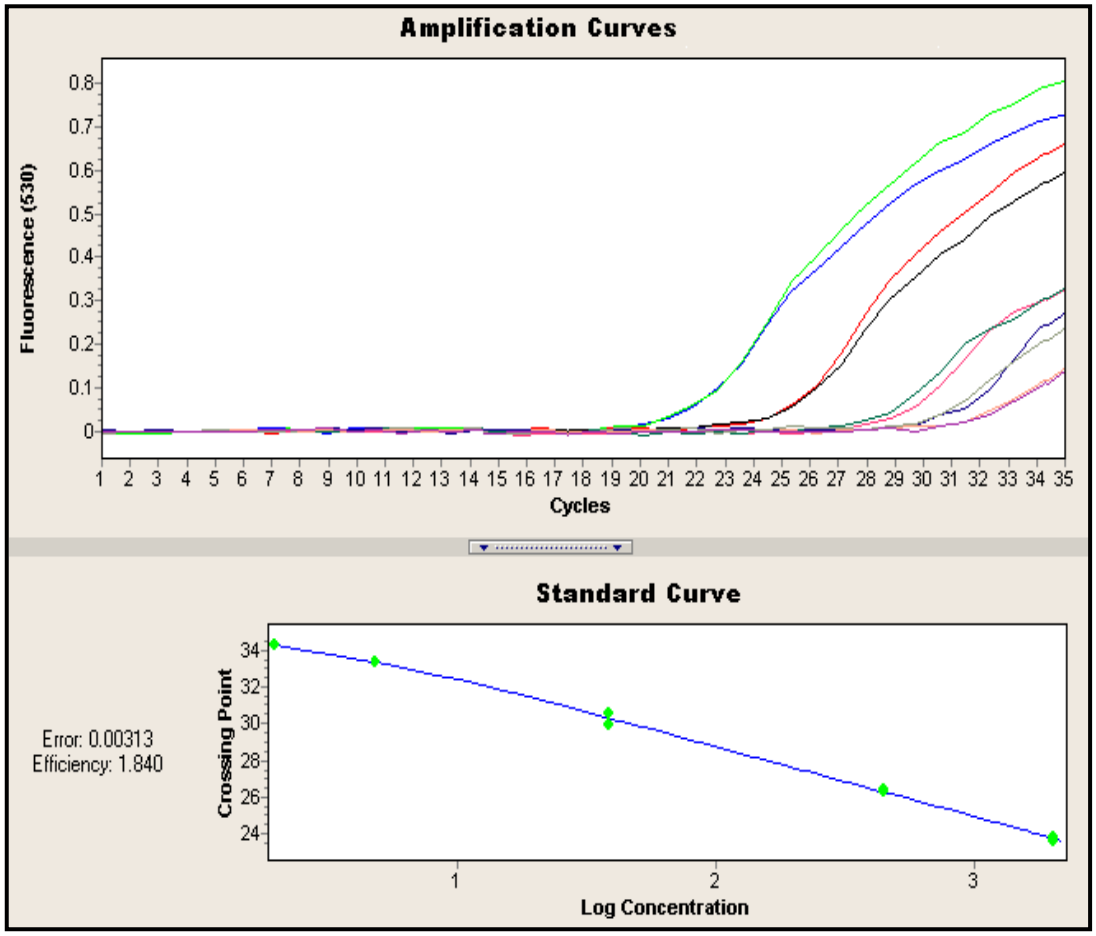


Figure 3:14 The competence of p53 primers in amplification of cDNA was confirmed by a standard curve. p53 primers had an efficiency of 1.840 and an error of 0.00313.

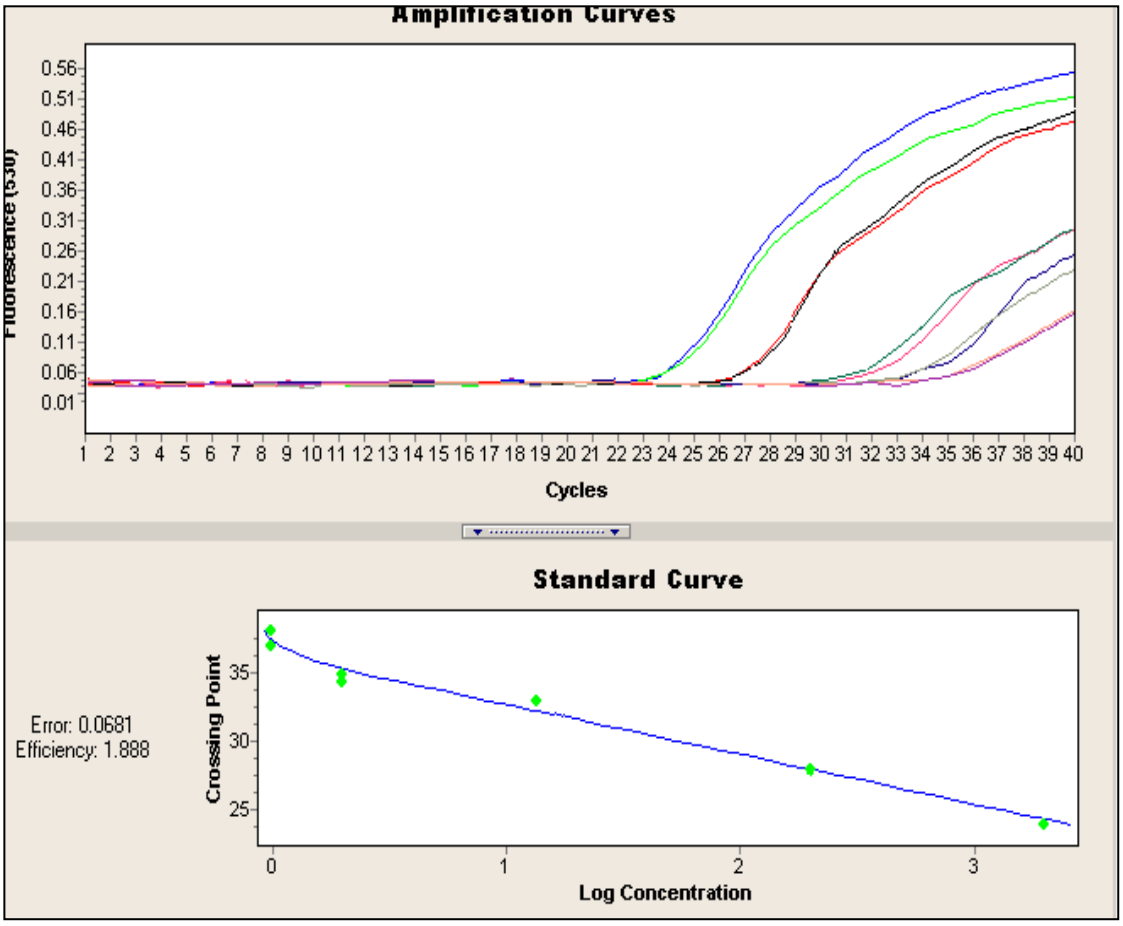


Figure 3:15 The effectiveness of RBBp6 primers in amplification of cDNA was proven by the standard curve. The standard curve efficiency is 1.888, with an error of 0.0681.

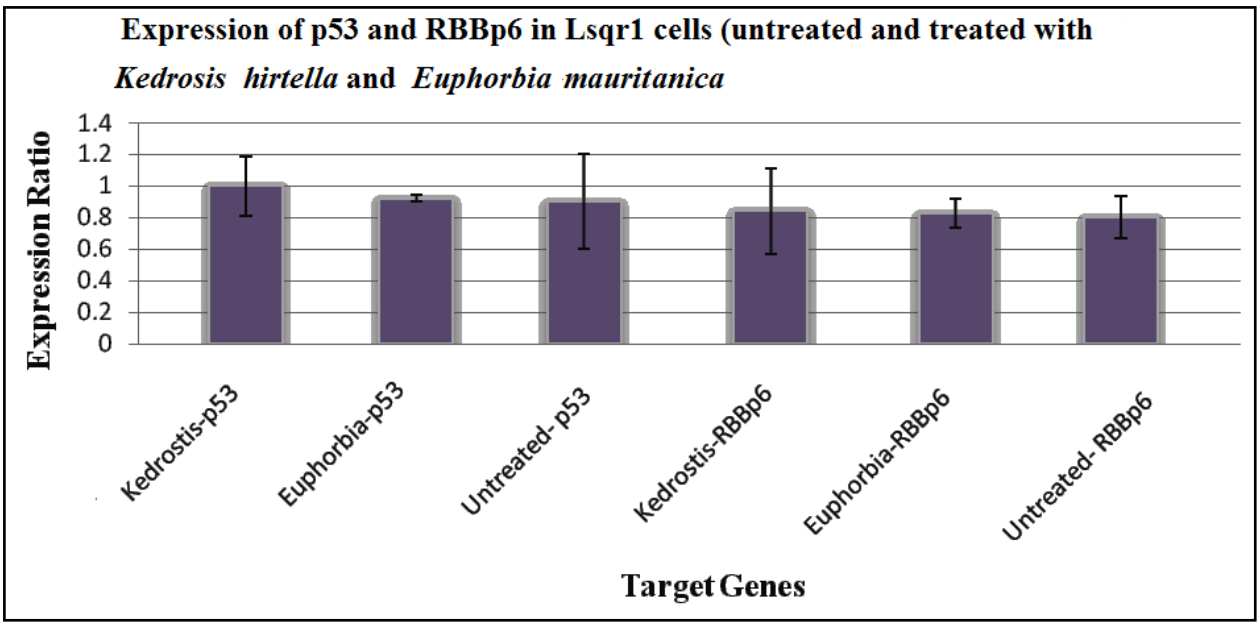


Figure 3:16 The expression comparison of RBBp6 and p53 cDNA for untreated Lsqr1 cells, including cells treated with 10 µg/ ml of the plant extract.

Figure 3:16 shows 0.9 p53 expression fold of Lsqr1 cells treated with *Euphorbia mauritanica* and untreated Lsqr1 cells treated with *Kedrostis hirtella* have a is slightly higher by 0.1 fold. On the other hand, RBBp6 expression is the same within 0.8 fold for Lsqr1 cells treated with *Euphorbia mauritanica* and *Kedrostis hirtella,* and untreated Lsqr1 cells. Moreover, observation of p53 expression versus RBBp6 expression shows 0.1 fold expression ratio difference for Lsqr1 untreated cells; this is equivalent to Lqr cells treated with *E. mauritanica*. However, Lsqr1 cells treated with *Kedrostis hirtella* have a p53 expression level that is slightly higher by 0.2 fold, compared to RBBp6 expression level. Therefore, *Kedrostis hirtella* has an effect on p53 expression and no effect on RBBp6 expression when compared to untreated Lsqr1 cells. On the other hand, *Euphorbia mauritanica* has no effect on the expression of p53 and RBBp6 when weighed against untreated Lsqr1 cells.

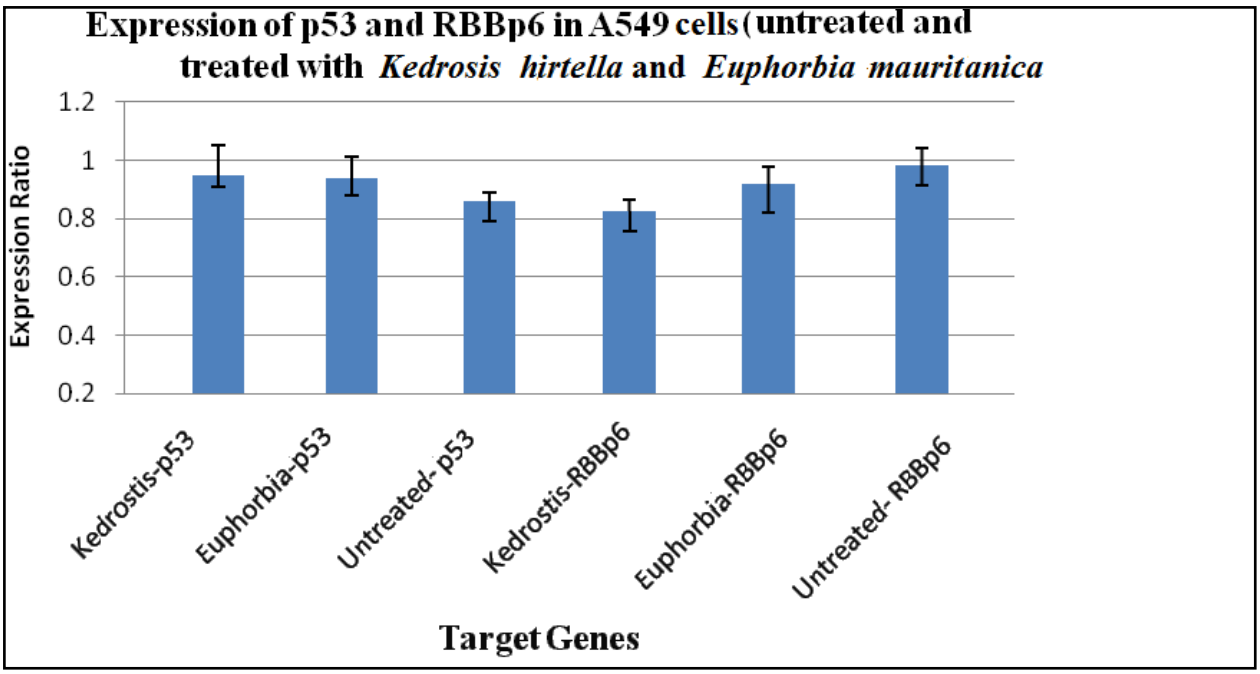


Figure 3:17: The expression comparison of RBBp6 and p53 cDNA in untreated A549 cells including those treated with 5 µg/ ml of the plant extract.

Figure 3:17, Untreated A549 cells show a high RBBp6 expression of 0.985 and a lower p53 expression of 0.83 when compared to other treated A549 cells. The expression of RBBp6 is reduced to 0.9 and 0.8 for A549 cells treated with *Euphorbia mauritanica* and *Kedrostis hirtella*, respectively. However, p53 expression has increased to 0.9 for A549 cells treated with *Euphorbia mauritanica* and *Kedrostis hirtella.* Therefore, plant extracts have an insignificant change in the expression of RBBp6 and p53 in A549

Chapter 4

4 Discussion

4.1 Introduction

The elucidation of molecular and cellular targets critical in cancer development and prevention is an area of intensive research and is driving the development of novel small-molecule compounds, which may prevent carcinogenesis, curtail its progression, or even cure the disease. On the other hand, in spite of significant advances over the last half century in our understanding of the genesis of lung cancer and the application of targeted drug therapy, lung cancer remains the leading cause of cancer deaths among men and the second leading cause of cancer deaths among women. New chemopreventative and chemotherapeutic approaches to the prevention and treatment of lung cancer are needed to reduce mortality since many patients with advanced lung cancer fail to respond to current treatment regimens. In this study we illustrate the use of two natural plant extracts as potential target.

4.2 Cell cytotoxicity

Cell viability and cytotoxicity assays are used for drug screening and cytotoxicity test of different chemical compound. In this study we used the MTT assay as our assay of choice to determine cell growth or inhibition activity of cancer cells aftertreatment with plant extracts are used as anticancer compounds (Cardellina *et al.,* 1993). Previous studies used the MTT assay to measure significant variations in the inhibition activity when multi-drugs were tested on leukaemia cell lines. Thus, it was rated the most accurate assay amongst other cell viability assays (Marks *et al.,* 1992). It has no limitations for testing more than variables such as drug

concentration, time of exposure to drug, length of assay, and cell density (Mosmann, 1983). MTT was used to measure 50% cell growth or inhibition induced by *Euphorbia mauritanica* and *Kedrostis hirtella* extracts on A549 cells and Lsqr1 cells. Untreated cells were used as control and cells treated with Staurosporine as a positive control. Figure 3:1-3:4 showed that A549 and Lsqr1 untreated cells had a trivial cell death. Thus, uncontrolled factors such as natural death or lack of nutrients are not the cause of attaining 50% cell death when cells are treated with the extracts. Therefore, this data is reliable.

The A549 cell line treated with 5 µg/ml of *Euphorbia mauritanica* and *Kedrostis hirtella* extracts attained 55%-56% growth inhibition at 22 hrs (Figure 3:1). The concentration of the extracts in Figure 3:2 was further increased to 10 µg/ml for *Euphorbia mauritanica* and *Kedrostis hirtella*. Inhibition of 50% growth was reached after short time exposure, between 16 to 22 hrs. Therefore, extracts showed a significant reduction of A549 cell growth in a dose and time-dependent manner. Similarly, Lsqr1 cells exhibit cell growth inhibition properties that are dependent on the dosage of the extracts and exposure time. Lsqr1 cells treated with 5 µg/ml *Euphorbia mauritanica* and *Kedrostis hirtella* extracts reached approximately 49% inhibition at 24 hrs. However, a high dose of 10 µg/ml *Euphorbia mauritanica* and *Kedrostis hirtella* extracts resulted in a 50-51% growth inhibition at 24 hrs incubation time (Figure 3:4). These results are similar to that of Creemers *et al.* (1996); in which they showed antitumour activity against colorectal and ovarian cancer tested with Nyssacea *Camptotheca accuminata* extracts. They further discovered that compounds such as Irinotecan and topotecan derived from Nyssacea *Camptotheca accuminata* are the main stimulates of antitumor activity against colorectal and ovarian cancer (Creemers *et al.,* 1996). However, Kummalue *et al.* (2010) compared inhibition

of two lung cancer cell lines and a breast cancer cell line when treated with extracts and they observed a significant growth inhibition in the dosage dependent manner, but only in the A549 cell line treated with 2.597 μg/ml *Sapindus rarax* water extract. Hence, the extract contains compounds, which inhibit human tumour cell growth. Thus, *Euphorbia mauritanica* and *Kedrostis hirtella* extracts might contain a wide range of compounds that induce antitumour activity at certain dosages with time. For example, phenolic compounds of *Terminalia chebula* show an inhibition of growth in human (MCF-7) and mouse (S115) breast cancer cell lines, a human osteosarcoma cell line (HOS-1), a human prostate cancer cell line (PC-3) and a non-tumorigenic, immortalized human prostate cell line (PNT1A) (Saleem *et al.,* 2002).

In addition, compounds derived from plant extracts are not the only ones with anti-proliferative activity. Compounds isolated from bacteria also exhibit anti-proliferative activity e.g. Staurosporine used as a positive control in this study. Staurosporine is compound isolated from *Streptomyces staurosco*pes bacterium and capable of inducing apoptosis (Omura *et al.,* 1977, Chae *et al.,* 2000) and results obtained (Figure 3:1-3:4) in this study shows that Staurosporine continue to induce cancer cell death in an apoptotic manner.

Previous studies have shown that Staurosporine decreases phosphorylation of pRB, meaning that it directly inhibits protein kinases that are important for phosphorylation of pRB (Bruno *et al.,* 1992). Inhibition of protein kinases promotes pRB-E2F association and dephosphorylation of pRB, which induces apoptosis and inhibits the progression of cancer cells (Hickman, 2002, Chae *et al.,* 2000). Therefore, Staurosporine is effective as a positive reference when considering previously studies (Bruno *et al.,* 1992). If they were to be considered for treatment against lung

cancer, plant extracts used in this experiment were expected to behave in a similar manner as Staurosporine, but they increase cell death with time exposure. This means that at high dosages there might be a possibility that the extracts may result in normal cells cytotoxicity. Therefore, investigation such as flow cytometry, DNA fragmentation and gene expression are needed to gain insight information into the mechanism of action these extract employ.

4.3 DNA fragmentation

DNA fragmentation is a key feature of programmed cell death and also occurs in certain stages of necrosis. Apoptosis is characterized by the activation of endogenous endonucleases with subsequent cleavage of chromatin DNA into internucleosomal fragments of 180BP. In this study we have used DNA fragmentation assay to determine whether cells treated with *Euphorbia mauritanica* and *Kedrostis hirtella* extracts have anti-proliferating effects.

A pattern of laddering is characteristically commonly associated with the apoptotic process, but not with other modes of cell death, like necrosis which is represented by a smeared DNA band pattern (Zhivotosky *et al*., 1999). Untreated Lsqr1 and A549 cell lines were used as controls and produced genomic DNA features of a single band as an indication of no cell death. These results are in line with several other reported lack of DNA fragmentation in both the untreated cancer cells and treated normal cells with are always selected as control (Zhivotosky *et al*., 1999).

Euphorbia mauritanica and *Kedrostis hirtella* extracts were used to treat Lsqr1 and A549 cells. Smeared DNA wells were observed when Lsqr1 cells were treated for 24 hrs with 10 µg/ml of *Euphorbia mauritanica* and *Kedrostis hirtella,* and A549 cells are treated with 5 µg/ml extracts

of *Euphorbia mauritanica* for 22 hrs. These results were similar to those observed by Chowdhury et al, 2009 inwhich they also observed a smearing DNA profiling after treating HeLa cells with some plant extract. It was observed that even though plant extracts failed to induce DNA fragmentation in HeLa cells, they were able to induce cell death, which meant that some toxic chemicals or compounds could induce cell death without DNA fragmentation (Chowdhury *et al.,* 2009). Smeared DNA might suggest that necrosis took place resulting in random digestion of the DNA (Wiliams *et al.,* 1974).

In this study using A549 cells treated with 5 µg/ml *Kedrostis hirtella,* we were able to observe a weak signal of nucleosomal DNA fragments. Similarly, Zhivotosky and Orrenius (2001) studies demonstrates pattern of laddering DNA, which was observed only in the treated samples. This suggests that plant extracts can induce apoptotic cells as result lead to oligonucleosomal DNA degradation. When cells were treated with Staurosporine a fragmented DNA pattern similar to that of a DNA ladder was observed on the gel. This clearly demonstrated that Staurosporine induced apoptosis as detected by apoptosis because of characteristics such as chromatin condensation and DNA fragmentation (Williamson *et al.,* 1970; Skalka *et al.,* 1976)

Belmokhtar *et al* (2001) performed a study on Staurosporine and discovered that it has a possibility of inducing at least two different signalling pathways of cell death. Their results correspond with findings of McCarthy *et al*. (1997), which proposed that apoptosis is triggered by an enzyme that requires a nuclear event. Hence, they were able to activate two unrelated endonucleases responsible for DNA fragmentation. Thus, DNA fragmentation of Lsqr1 and A549 cells treated with Staurosporine was in line with that by McCarthy *et al*., (1997). In order

to verify percentage apoptosis, Flow cytometry studies was applied to confirm if cell death was by way of apoptosis or necrosis.

4.4 Flow cytometry

Graphs of cytogram dot plots represent two measurement parameters, on the x- and y-axes. The x-axis of cytochrome dot plots displays FL1-FITC detection and the y axis represents FL2-PI. The FITC signal detector (FL1 channel) is used for measuring Annexin-V-FITC binding and the phycoerythrin signal detector (FL2 channel) measures PI staining (Nicoletti *et al.,* 1991; Lyons *et al.,* 1992; Lecoeur, 2001). For this purpose, the cytogram graphs show a labelling pattern of cell count height on a density gradient, which is represented in dots. Dot density fraction of the cells is interpreted as follows: a fraction of the cells dots on B3 are viable cells which are negative for both PI and Annexin-V. Untreated cells are most likely to have a higher viable population as a result of not binding to PI and Annexin-V (Gougen, 1997; Lecoeur, 2002). Untreated Lsqr1 and A549 show a 90% viable population. While those treated with Staurosporine and extracts presented 50% cell viability. These results confirmed our earlier results obtained in the cell cytotoxicity assay (Figure 3.1-3.6).

Mostly because cells treated with Staurosporine undergo apoptosis, they are likely to be distributed between B2 (late apoptosis) and the B4 (early apoptosis) stage (Bunch *et al.,* 1992; Gester, 1998). Staurosporine induces cell death predominately in early apoptosis, but reduced population of dead cells in late apoptosis. The density dots population on B4 of the cytogram represent early apoptotic cells that are PI negative and Annexin-V positive (Nicoletti *et al.,* 1991; Lyons *et al.,* 1992). The late apoptotic cells in B2 are PI positive and Annexin-V positive

(figure). This data suggests that Staurosporine is involved in the induction of early apoptosis events. Previous studies suggested that Staurosporine triggers both the p53-independent and dependent apoptotic pathways (Lin *et al.,* 1999, Chen *et al*., 1999, Belmokhtar *et al.,* 2001). Apoptosis specific caspase-3 cleavage products can be measured in blood of a patient receiving cytotoxic treatment. This can serve as a specific marker to detect improvement of a cancer patient under therapy.

A549 cells and Lqr1 cells treated with *Euphorbia mauritanica* and *Kedrostis hirtella* extracts resulted in predominantly necrotic cells (Figure 3.8-3.9). Plant extracts produce toxins that affect the external of the cell causing a disruption in the cell membrane leading to necrosis (Edinger *et al*., 2004; Zong *et al*., 2006). Necrosis is an unregulated process that disrupts the plasma membrane leading to the spillage of intracellular protein. The spillage of intracellular protein enhances or activates the host immune response to remove dead cells by tissue macrophages (Buzzai *et al*., 2005, Olofsson et al., 2007). However, necrotic cells are internalized by macropinocytotic mechanism. Hence, a part of these cells can be engulfed by phagocyte whereas other dead cells become localized in a certain area causing a build-up that leads to local inflammation (Edinger *et al*., 2004; Krysto *et al*., 2006). Furthermore, if therapeutic drugs or remedies result in necrosis, it becomes difficult to check if the patient is responding well to treatment. Necrosis has a very few specific methods of detection (Buzzai *et al*., 2005, Olofsson et al., 2007).

However, in Figure 3.8 there is some evidence of late apoptosis in the case of A549 cells treated with 5 µg/ml of *Kedrostis hirtella.* In a study by Chinkwo, (2005) on the *Sutherl*and*ia frutescens*

extract they confirmed that there are few compounds in the extracts that have anti-cancer properties that are well considered to activate the central death caspase-3.*Euphorbia mauritanica* and *Kedrostis hirtella* extracts might have compound which activate apoptosis. Therefore, gene expression of p53 (pro-apoptotic) and RBBp6 (pro-proliferation) were studied to check if there might be compound in the extracts that could inhibit or stimulate the expression of the two genes as they antagonise each other and play a role in both cell survival or cell death.

4.5 Real-time PCR

In most cases, cancer cells show overexpression of RBBp6 and has been characterised by mutations or malfunctioning of p53 (Motadi *et al.,* 2011). However, in normal cells p53, RBBp6 and Mdm2 expressions are controlled at moderate level, but during cellular stress and oncogenic warnings, p53 is highly expressed. Cellular stresses stimulate apoptosis and deactivate cell growth, but once the problem has been resolved, p53 levels are reduced via Mdm2/ RBBp6 mediated ubiquitination. Mdm2 is a cellular antagonist of p53 and acts to limit the p53 expression and suppresses its function in unstressed cells (Gao and Scott, 2003; Moll and Petrenko, 2003, Yoshitake *et al*., 2004). Mdm2 has a short lifespan, thus it will not eradicate p53 completely. As a result, p53 can continue with function in normal cells.

Cancer cells, however, cannot undergo cell cycle because it is not controlled and there will be abnormal growth of defective cells. Thus, there are drugs that prevent the interaction between Mdm2 and tumour suppressor protein (RB and p53). In so doing, programmed cell death is enhanced (Moll and Petrenko, 2003). Now, the question is: how do drugs targeted for RBBp6 in cancer cells inhibit the function of p53 degradation. RBBp6 arise from p53-associated cellular

protein (PACT) family and has conserved N-terminal which facilitates in regulation of RB and p53 (Yoshitake *et al*. 2004; Li *et al.,* 2007). However, RBBp6 enhances cell growth in cancer cells and normal cells. RBBp6 has p53 and Rb binding sites, but little is known about preventing an interaction between RBBp6 and tumour suppressor proteins (Rb and p53).

Studies show that a higher expression of RBBp6 is associated with poor prognosis and is useful for predicting the outcome of a cancer patient after surgery or treatment (Gao and Scott, 2003;Yoshitake *et al*., 2004; Motadi *et al.*, 2011). If RBBp6 is down regulated, the expression of p53 increases whereas p53 silencing results in RBBp6 accumulation (Motadi *et al.*, 2011). Lsqr1 and A549 cells were treated with *Euphorbia mauritanica* and *Kedrostis hirtella* extracts illustrated no significant effect in RBBp6 level expression.

4.6 Conclusion

Although, plant extracts are not significantly affecting the RBBp6 expression level, they areused for anti-cancer therapy, because they do not have an effect on normal cells and they induce necrosis and late apoptosis in lung cancer cells. However, further studies have to be done to illustrate how these plant extract are inducing late apoptosis and necrosis in lung cancer cells.

4.7 Future recommendation

Further studies such as Detection Fluorescence Microscopy should be done to verify results obtained from flow cytometry and detect cell morphology in treated lung cancer cells. RBBp6 and p53 protein expression level have to be checked using western blot.

Chapter 5

5 References

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