Biological activities of Ficus Carica latex for potential therapeutics in Human Papillomavirus (HPV) related cervical cancers

Arshia Ghanbari

November 2019

This thesis is submitted to Kingston University in accordance with the requirements for degree of Doctor of Philosophy in the Faculty of Science, Engineering and Computing

Acknowledgements

I am wholeheartedly grateful to my supervisor Dr. G. Hossein Ashrafi for his outstanding guidance, constant support and help in so many ways and so many times during this project.

I would like to extend my gratitude to Dr. Adam Le Gresely for all his great support and expertise in medicinal chemistry wherever needed in this project. Many thanks to Dr. Siamak Soltani for sharing his expertise in this project. I would also like to thank Dr. Farshad Heidari and Dr. Nadia Aziz Salman for their help and support. Thanks to all friends and staff of Kingston University London for their kindly help.

Last but not least, I would like to acknowledge my parents and my sister for putting up with me and supporting me during my PhD study. They have been a source of encouragement and inspiration to me throughout my life and I am in debt for all the endless support and motivation they provided during my studies.

Table of Contents

List of figures

Chapter four

Chapter five

List of Tables

Page

Abbreviations

ADV	Aleutian Disease Virus
BPV	Bovine Papilloma Virus
BRCA	Breast cancer association gene
BSA	Bovine Serum Albumin
CaSki	Human Caucasian cervical epidermoid carcinoma
CAFs	Carcinoma associated fibroblasts
CDK	Cyclin-dependent kinase
CIN	Cervical intraepithelial neoplasia
Cm	Centimeter
CTL	Cytotoxic T lymphocyte
DAPI	4-6-diamidino-2-phenylindole
D2O	Deuterium oxide
DCM	Dichloromethane
DMEM	Dulbecco`s Modified Eagles medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOSY	Diffusion-ordered spectroscopy
ECM	Extra cellular matrix
ECV	Echo virus
EDTA	Trypsine-ethylenediaminetetraacetic acid

EGF	Epidermal Growth Factor
ESI	Electrospray ionization
EMEM	Eagle`s Minimum Essential Medium
ER	Estrogen
ER	Endoplasmic reticulum
FCS	Foetal calf serum
FGF	Fibroblast growth factor
FITC	Fluorescein isothiocyanate
G	Gram
GA	Golgi apparatus
HaCaT	Human Immortal Keratinocyte Cell
HeLa	Cervical Cancer cell originated from patient Henrietta Lacks
HER2	Human epidermal growth factor receptor 2
HPLC	High Performance Liquid Chromatography
HPV	Human Papillomavirus
HR-HPV	High risk Human Papillomavirus
Hr	Hours
HSV	Herpes simplex virus
HZ	Hertz
HUVEC	Human Umbilical Vein Endothelial cells
Ig	Immunoglobulin
kD	KiloDalton
Kg	Kilogram

kV	Kilovolts
L	Liter
LCR	Long control region
LR-HPV	Low risk Human PapillomaVirus
mAb	Monoclonal antibody
M	Molar
MDSCs	Myeloid derived suppressor cells
MeCN	Acetonitrile
MHC	Major histocompatibility complex
mg	Milligram
min	Minute
ml	milliliter
mM	Millmolar
m/z	mass-to-charge ratio
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
NMR	Nuclear magnetic resonance spectroscopy
ORF	open reading frame
PBS	Phosphate buffered salin
PH	power of hydrogen
ppm	parts per million
PI	Propidium Iodide
PR	Progestrone
Rb	Retinoblastoma

RNA	Ribonucelic acid
RNase	Ribonulease
RPMI-1640	Roswell Park Memorial Institute medium
rpm	revolution per minute
SCC	Squamous cell carcinoma
SIL	Squamous intraepithelial lesions
SW	spectral width
TBS	tris-buffered saline
Tween 20	Poluoxyethylene sorbitan monolaurate
TOCSY	Total Correlation Spectroscopy
TNF- α	tumour necrosis factor
TAMs	tumor associated macrophages
VEGF	Vascular endothelial growth factor
v/v	Volume per unit volume
v/w	Weight per unit volume
µl	Micro liter
µg	Micro gram

Single letter DNA Nitrogen base

A	Adenine
C	Cytosine
G	Guanine
T	Thymine

Abstract

Cancer is a major public health concern and is one of the leading causes of death worldwide. Cervical cancer is one of the most common types of cancer, affecting women on a global scale. Infection caused by high-risk human papillomaviruses (HPVs), especially type 16 and 18 are implicated in the aetiology of most cervical cancers. Coupled with their involvement in cancer, these viruses can cause life-long debilitating diseases that may be accompanied by a significant negative impact on quality of life. High- risk HPV infections interfere with the molecular pathways that are responsible for regulating epithelial differentiation as well as cell proliferation.

HPV onco-proteins E6 and E7 contribute towards cellular changes in HPV infected cells. These facilitate the persistence of infection that might allow the progression of the lesions towards cancer. E6 interacts physically with tumour suppressor protein p53 and prevents its function; this activity will ultimately impede apoptosis. On the other hand, E7 binds to retinoblastoma (Rb) protein and prevents the interaction of Rb with its natural target, namely transcription factor E2F. Consequently the checkpoint that controls G1/S transition becomes distorted, causing uncontrolled proliferative lesions. Once proliferative lesions persist they can progress to high-grade ones and become an invasive form of cervical cancer . It has been demonstrated that the presence of even minimal amounts of HPV DNA are associated with an increased risk in the development of cervical cancer. Given the importance of cervical cancer, to date, there has been no satisfactory medical treatment for human papillomavirus related cervical cancer as most of the developed treatments (e.g., surgical excision, chemotherapy, and cryotherapy) are eventually accompanied by excessive tissue injury. Therefore, there is a continuing demand for development of new strategies for treatment, which avoids tissue injury.

Herbal medicinal and biological studies have revealed that public interest in utilising traditional remedies has greatly increased. Among various medically relevant plants, the

fig latex (Ficus carica), when applied to low risk human papillomavirus (HPV) related skin warts and bovine papillomavirus (BPV) related warts, has shown potential as a possible cure for the virus without inevitable tissue injury and remedial complications. Fig latex also reportedly offers various therapeutic effects such as anti-Herpes Simplex Virus (HSV)-1, anti-bacterial activity and anthelmintic. As a consequence we investigated the biological activity of the Ficus carica latex on high risk HPV related cervical cancer. Herein, we show that Ficus carica latex effectively inhibits growth of HPV positive cervical cancer cells (CaSki and HeLa), without a cytotoxic effect on HPV and cancer-free human immortalised keratinocyte (HaCaT) cell line. The latex presents anti-cancer effects by various mechanisms, including induction of apoptosis and inhibition of cell transformation; colony formation, cell proliferation, migration and invasion. In addition to its potent anti-cancer effects, the results obtained indicate that Fig latex has profound influence on the deregulation of HPV oncoproteins (E6 and E7) and HPV diagnostic marker protein (p16) and initiates the reactivation of Rb and p53 tumor suppressor proteins. These findings provide insight into new therapeutic avenues against HPV- associated cervical cancers.

Chapter One
Introduction

1. Introduction

1.1 Cancer

Despite considerable progress, cancer is still one of the leading causes of death on global scale. It is recognised as being a complex, multistage, genetic disease in which the regulation of growth and maturity of normal cells is disrupted (WHO, 2018). Normal cells are persistently regulated with signals for cell division, differentiation into another cell or cell death. Cancer cells pose autonomy from these regulatory signals, resulting in uncontrolled growth and proliferation. In other word, cancer is defined as diseases associated with alteration or mutation in cell genome. Alterations in genomic materials (DNA) generate proteins that interfere with the balance between cell division and cell arrest (silence), leading to carcinogenesis (Ruttkay-Nedecky et al., 2013; Ramakrishnan et al., 2015).

Carcinogenesis is a process through which normal cells are transformed into cancerous cells. Unregulated proliferation of cancerous cells accompanied by further mutation resulting to the mildly aberrant stage. Consecutive mutation and expansion of these abnormal cells initiate the formation of a tumour mass (Figure1). Tumour growth and progression can break the basal membrane barrier surrounding tissues and invades or migrates to other parts of the body (metastasis). Majority of cancer-related deaths are due to metastasis (Munoz et al., 1992; Bosch et al., 2002; Woodman et al., 2011).

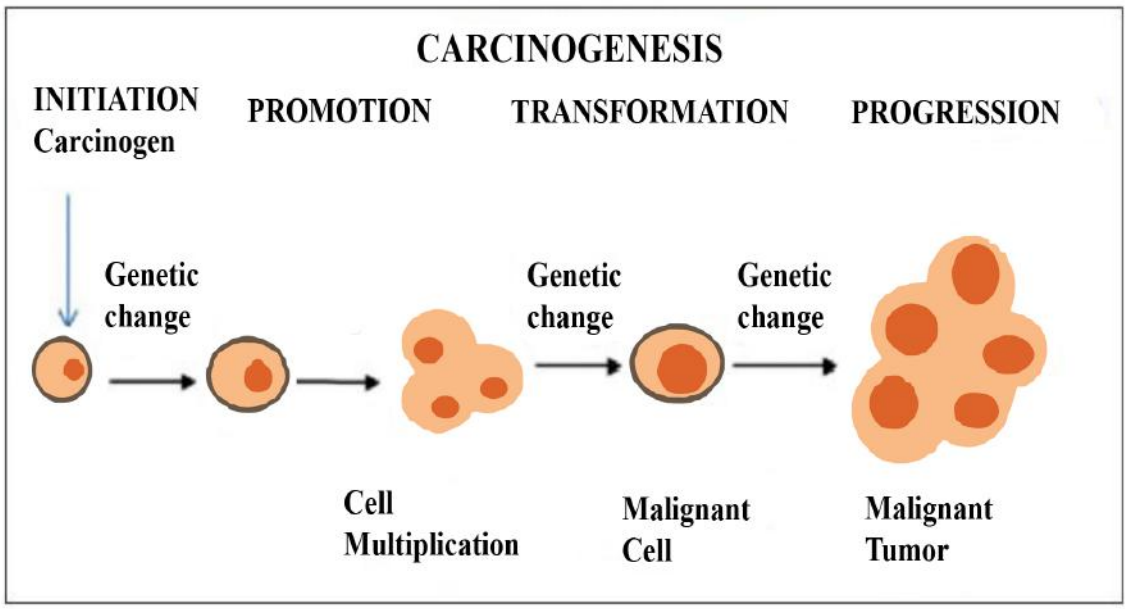

Figure1: Different stages of carcinogenesis. The multistep process of carcinogenesis, as the abnormal cells undergo further uncontrolled growth the proliferate and differentiate into malignant cells (Adapted from journal of cancer research and therapeutics)

1.2 Epidemiology of cancer

Cancer is a major public health concern and accounted as the second leading cause of death worldwide. Cancer was responsible for nearly 8.8 million deaths in 2015 on global scale meaning that approximately 1 in 6 death was due to cancer (WHO, 2018). World health organisation (WHO) also reported that almost 70% of deaths that are due to cancer, taking place in low- and middle-income countries. Data collected from most recent studies shows that Tobacco consumption is the major risk factor for cancer development (WHO, 2018). Studies showed that cancer-causing infections are also responsible for great proportion of cancer incidents especially in low- and middle-income countries. Only in the UK 359,960 new cases of cancer were identified in 2015 and 163,444 cancer related deaths were reported in 2016. Every four minutes a patient dies in the UK due to cancer. Cancer causes more than one in four of all deaths in the UK (CRUK, 2018).

Studies on the estimated numbers of cases and death due to cancer in both genders globally indicate that lung cancer is the most common diagnosed cancer that was accounted for 1.69 million deaths in 2015 alone (WHO, 2018). Cervical cancer was contributed to 3126 new cases of cancer in 2015 and 854 deaths in 2016 in the UK (CRUK, 2018). According to the research data by American association of cancer the rate of cancer incidence will experience an increasing trend and it is estimated to cause approximately 24 million deaths in 2035 (Figure2). These figures show the importance of research into the etiology, prevention and cure of this global killer.

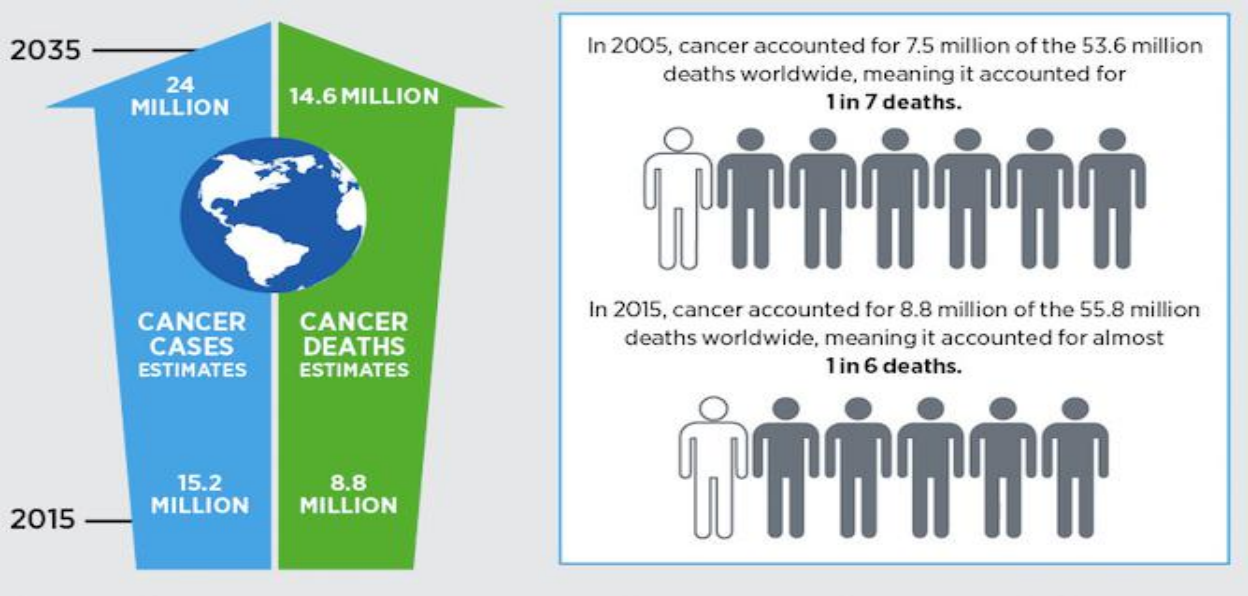

Figure 2: Estimated number of death due to cancer in 2035. Cancer is expected to become more prevalent in coming years (American Association for Cancer Research, 2017).

1.3 The hallmarks of cancer

The complexity of the cancer is not merely restricted to the cellular and molecular elements but also several regulatory affairs should be impressed to initiate carcinogenesis (Malmberg, 2004). These regulatory factors can be intrinsic or extrinsic. Alterations in genomic materials such as DNA mutation influence on these regulatory affairs of a cell cycle, leading to disruption of normal cell proliferation process and cell transformation (Lou et al., 2014). Overall, six common alterations in cell physiology introduced by Douglas Hanahan and Robert Weinberg in 2000 that eventually accelerate carcinogenesis (Hanahan and Weinberg 2000). These hallmarks were upgraded to 10 (figure 3) (Hanahan and Weinburg 2011; Weinberg, 2014; Wlibur et al., 2017). The role of each hallmarks in avoiding normal cell cycle pathway and disruption of cellular homeostasis is discussed below.

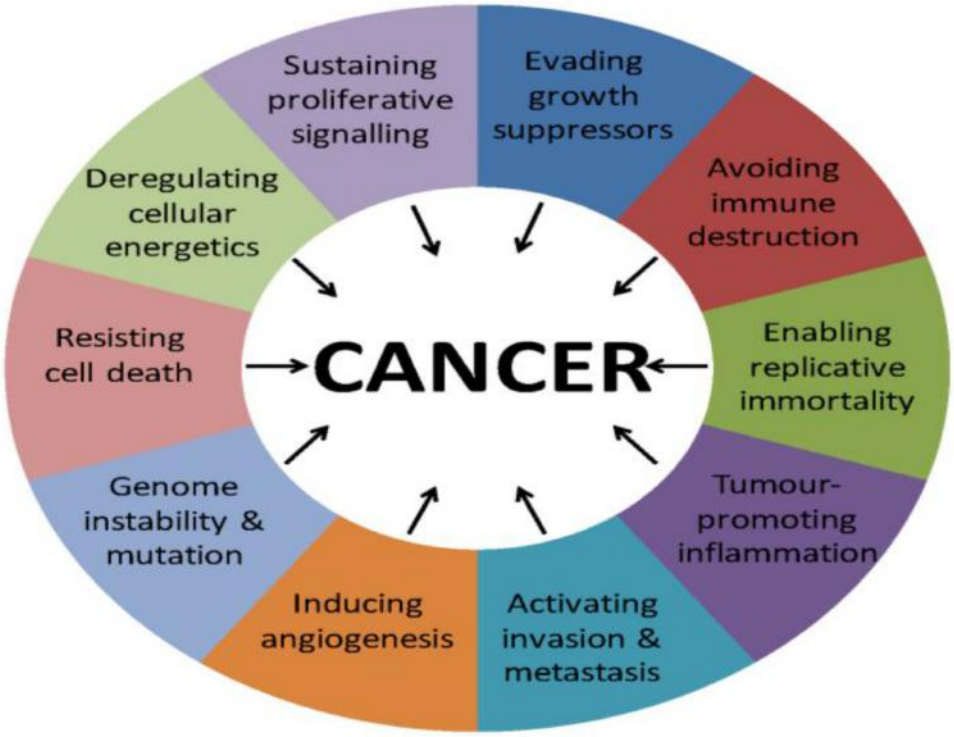

Figure 3: Hallmarks of Cancer. Adapted from (Hanahan and Weinburg, 2011)

1.3.1 Enabling replicative Immortality

Organisms pose a certain size and shape at the both tissue and cellular levels. Mammalian cells have many regulatory procedures to control cell growth and cell division to comply with standard size and shape. Cellular regulatory affairs are also essential to maintain the integrity of the both cell and the tissue. Interference and disruption in the innate cellular regulatory affairs promote the expansion of cell size and tumour formation (Llambi and Green, 2011).

Studies revealed that normal mammalian cells grown *in vitro* (in a flask) display a limited number of cell divisions and stop further proliferation once they reach to confluency. Once these normal cells are transferred to a new flask, they continue to divide frequently until they reach to certain number, when the rate of cell division slows down and ultimately stops. This procedure is called passaging (Shay and Wright, 2000).

Cancer cells in contrast to normal cells represent the capability to exceed natural dividing restrictions to almost indefinite levels. HeLa cell is a well-known example of this type of cells that was originated from a cervical adenocarcinoma of a cancer patient called Henrietta Lacks in 1951. These cells proliferate and grow uncontrollably due to disruption in controlling the replicative potential (Rahbari and Sheahan, 2009). Studies discovered that the ticking counter for regulating the finite cell division locates at the end of all human chromosomes called the telomeres. Telomeres are hexanucleotide sequences of DNA and each end of a linear chromosome contains thousands of copies of these repeats that are called junk. Telomeres do not encodes any proteins but they protect linear chromosomes through preventing end-end fusion and shield the DNA from degradation via nucleases (Blasco, 2005). DNA replicates during the S phase of the cell cycle, doubling the number of chromosomes. During the mitosis these chromosomes are dividing between daughter cells. Following each round of DNA replication of a sequence of the telomerase is missing from the ends of each chromosome that results in shortening the chromosomes. This shortening procedure is attributed to the inability of DNA polymerase for replication the 50-100 base pairs on the 3’ ends of the DNA (Gunes and Rudolph, 2013; Shay, 2016).

Each chromosome has a certain number of telomere repeats. A consecutive cycle of replication is associated with a constant ablation of the telomeres till they eventually cause genetic related alterations, chromosomal end-end fusions and cell death (Figure 4). Therefore, each normal cell completes a finite lifespan that is attributed to their length of telomeres (Ozturk et al., 2017).

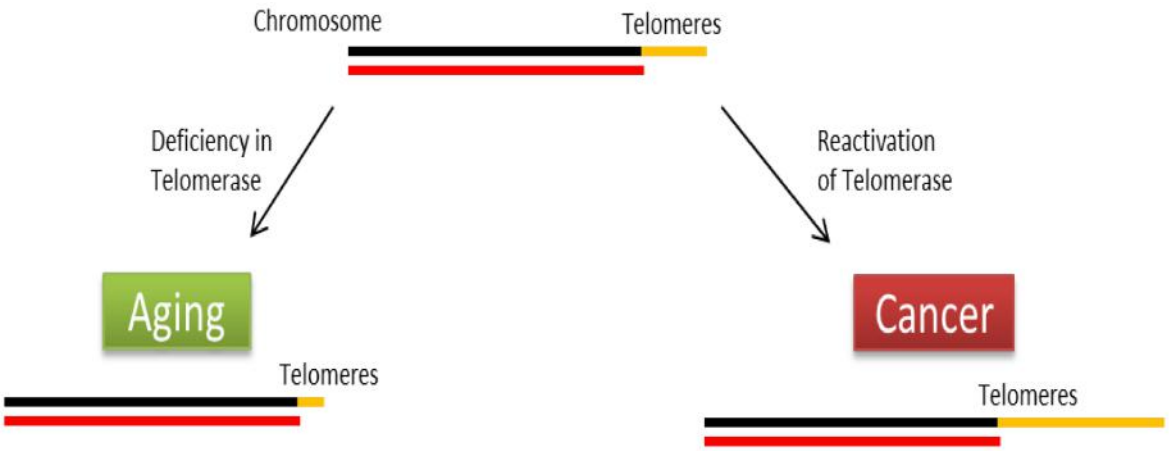

Figure 4: The role of telomerase in cancer development. Reactivation of telomerase in cancer cells leads to immortalization whereas deficiency in telomerase cause aging and induce cell death (Ozturk et al., 2017).

In contrast majority of cancer cells maintain their telomere lengths without loss of DNA base pairs through activation an enzyme called telomerase (Figure 4). Telomerase induces its function by adding a non-coding, hexanucleptide repeats to the ends of telomeric DNA. As a result, the necessary lengths will be retained, ablation will be prevented and replication will be permitted without any limitation (Campisi, 2013). Migration and invasion of cancer cells to surrounding tissues can be associated with the high levels expression of telomerase. Normal cells pose far lower levels of telomerase that is undetectable in most of the cases. (Maertinez and Blasco, 2011). Therefore immortality can be consider a principal feature of cancer cells as they can grow beyond the normal boundaries.

It can be proposed that cancer cells can be derived from normal cells such as; somatic cells and represent the capacity of unlimited proliferation. Biological aging or senescence

is a natural mechanism exists in cells to enter G0 phase of cell cycle and stop further proliferation. It should be noted that many of the therapeutic approaches now are targeting telomerase activity that help to kill tumor cells without certain cytotoxic impact on normal cells (Kim et al., 1994; Gunes and Rudolph, 2013; Shay, 2016; Ozturk et al., 2017).

1.3.2 Sustained growth signal

Cells are part of community that create a tissue or organ therefore, they are not able to survive and proliferate alone. Cells are producing growth signals that affect the surrounding cells proliferation and growth rate (Sharma et al., 2003; Vander et al., 2010). Growth factor signaling is a cell-signaling pathway that regulates the development of an organism. As shown in figure 5, secreted growth factors bind to trans-membrane growth factor receptors to stimulate cell-signaling cascades that promote proliferation, apoptosis and differentiation (Karnoub and Weinburg, 2008; Vander et al., 2009). Alteration or mutation in the number or/and structure of these receptors can transfer normal cells into cancerous cells. Receptors form tyrosine kinase families including; Epidermal Growth Factor (EGF), Fibroblast Growth Factor (FGF) and platelet-derived growth factor-like (PDGF) are the most frequent receptors implicated in cell transformation (Han et al., 2006; Boudreau et al., 2012). Growth factor receptors are usually overexpressed in variety of cancers and therefore cancer cells can become more sensitive to them. Breast cancer is a well-known example of such a condition which is associated with the over expression of various growth factor such as; Human epidermal growth factor receptor 2 (HER2), estrogen (ER) or progesterone (PR). (Klemke et al., 1994; Ning et al., 2005; Rexer et al., 2014).

In contrast to normal cells, cancer cells generate oncogenic proteins that mimic these normal growth signals. Oncogenes are derivate from normal cellular genes called proto-oncogenes. They encode proteins capable of transforming of normal cells into cancerous ones (Deberardinis et al., 2008; Vogelstein et al., 2013). Many factors such as; mutation and chromosomal alterations, genes amplification or viral infections can transform proto-oncogenes into oncogenes. As a result tumor cells lose their sensitivity to signaling

factors and can proliferate without these growth factors (Malumbres and Barbacid, 2009; Witsch et al., 2010).

On the other hand, alteration in extracellular growth signals can promote an increase in cell mass by stimulating the synthesis and inhibiting the degradation of various macromolecules. For example, cells infected with a viral oncoprotein v-sis can release massive amount of PDGF oncoprotein. The v-sis oncogene from a monkey cancer virus known as simian sarcoma retrovirus (SSV) comes from a gene that encodes platelet-derived growth factor, which stimulates growth of different cell types (Yang et al., 2008; Weissmueller et al., 2014).

In addition, alterations in intracellular signaling components such as; cell cycle related kinase and Ras oncogene are responsible for the initiation and progression of many tumours (Assoian and Klein, 2008; Pylayeva et al., 2009).

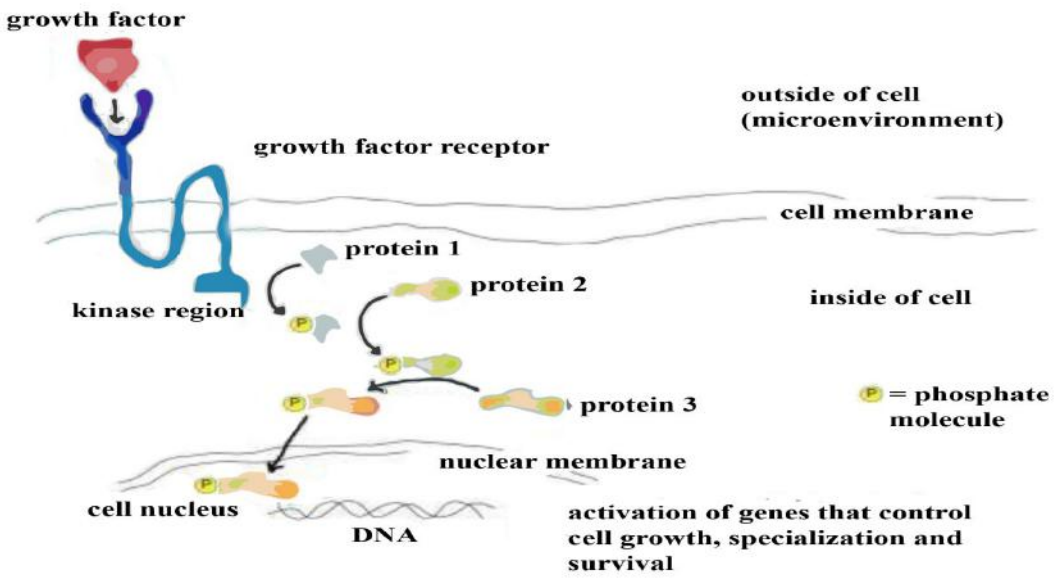

Figure 5: The growth factor and receptors in induction of cell proliferation. Growth factor binds to specific growth factor receptor on cell surface. This activates kinase region and adds phosphate to proteins and activates more proteins down stream. These proteins eventually move through the nuclear membrane into nucleus, bind to DNA and activate genes (Image obtained from Buddhini Samarasinghe `s website).

1.3.3 Evading growth suppressors

The balance between cell proliferation and quiescence is a vital phenomenon for cell life. There is a regulatory balance between cell proliferation and silence that is controlled by series of internal events. This consists of two opposite signaling pathways; proliferate and anti-proliferative signaling. Generally, normal cells and tissues are subjected to anti-proliferative signals that are responsible for cell silence. These signals act as negative regulators of cell division against proliferation signals and can include growth inhibitors and inhibitors on Extra cellular matrix (ECM) and cell surface (Pylaveya et al., 2011; Lopez and Tait 2015).

Suppressor signals for regulating the cell division are frequently received by cell surface receptors and are transferred through intracellular signaling pathways. Genes that control and block the normal cells divisions are called tumor suppressor genes (Kessenbrock et al., 2010). Their function is in close correlation with cell cycle clock to regulate cell progression via mitosis. Numerous interstice and extrinsic elements monitor cell conditions to rectify if further cell proliferation and division is necessary or cells should undergo silence and enter the post-mitotic phase (Malumbers and Barbacid 2009; Campisi, 2013). For instance, existence of proteins involved in DNA replication at the end of the G1 phase of cell cycle shift the cell into the S phase. There are few checkpoints in cell cycle to control cell progression and division at an appropriate time. Anti-growth signals push dividing cells into the G0 phase of the cell cycle distribution until a distribution or change in proliferative capacity and growth rate of cells in each phase. Some cells such as mature erythrocytes might push into a post-mitotic phase due to internal or external factors. Cancer cells overcome these anti-growth signals that enable them for further indefinite growth and proliferation (Labib and Piccoli 2011; Cross et al., 2011; Lou et al., 2014). Therefore a regulatory balance between cell proliferation and apoptosis is essential to prevent cancer initiation.

1.3.4 Inducing Angiogenesis

Cells and tissues require oxygen and nutrients to maintain their function, survive and grow. In the most cases cells that are located in the capillaries (endothelial cells) do not grow and divide. However, under particular circumstances like wound healing endothelial cell starts to divide, grow and form new capillaries. This process is termed angiogenesis or neovascularisation. Interestingly, tumours are able to accelerate angiogenesis (Carmeliet 2000). Indeed, angiogenesis is a key factor for expansion and transformation of cells into a malignant mass and spreading/invading to surrounding areas. Technically, the transformation may delay for considerable time unless angiogenesis is activated. Understanding the process of neovascularization in tumours is an important method for development of new therapeutic agents (Risau, 1997). Angiogenesis depends on a delicate balance between positive and negative signals that can stimulate or even block neovascularization (Koch, 2007).

Tumor cells activate endothelial cells via releasing pro-angiogenic factors such as vascular endothelial growth factor (VEGF). These factors infiltrate into surrounding tissues and bind to the receptors on endothelial cells (Figure 6). This interaction leads to the secretion of proteolytic enzymes like metalloproteins, causing degradation of basement membrane and ECM (Koch 2007; Musa and Davis 2016). Due to this degradation, endothelial cells are activated and start to proliferate and migrate towards the tumor. Integrins speed up the formation of new blood vessel. Afterwards, endothelial cells form a new basement membrane and secrete PDGF to stabilise the new vessel. Various mechanisms can interfere with angiogenesis process (Finetti et al., 2008; Rajabi et al., 2016). For example P53 regulates angiogenic inhibitor thrombospondin-1. Thrombospondin-1 is an adhesive glycoprotein that mediates cell to cell and cell to matrix interactions. Thus any loss of p53 function (human tumors) can cause thrombospondin-1 levels to decrease, resulting in removal of the inhibitory barrier for endothelial cells growth (Vousden and Lu 2002). Overall, tumor angiogenesis propose a principal medicinal target for management of many solid tumors. So far, several anti-angiogenic drugs have been developed and promised remarkable effect for cancer therapeutics. Studies also revealed that various types of tumor cells employ different molecular strategies to activate the angiogenic mechanism. Therefore, a single

antiangiogenic therapeutic modality is not capable to overcome and treat all cancers and tumors (Hanahan and Folkman 1996; Egeblad and Werb 2002; Kalluri 2003; Jain, 2005).

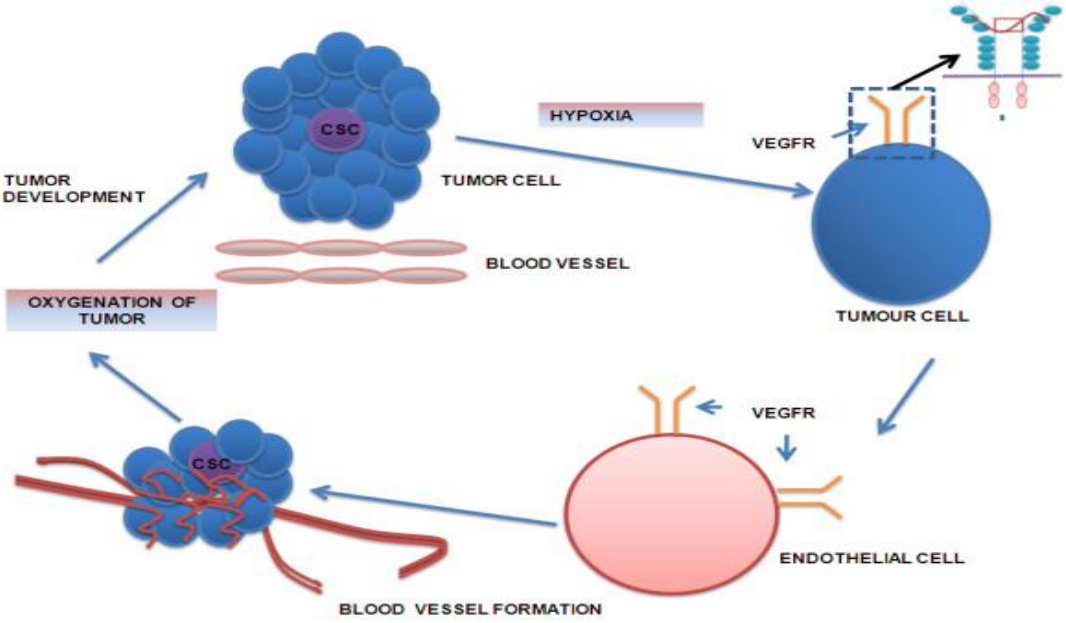

Figure 6: Pathways regulating tumour growth. Cancer cells trigger tumour growth in response to hypoxic condition and releases the signal for vascular endothelial growth factor (VEGF). The VEGF initiates the endothelial cell to proliferate and form blood vessels to vascularize the growing tumour. As soon as the tumour gets oxygenated, the tumour grows further in size (Ambasha et al., 2011).

1.3.5 Resisting cell death

As discussed in section 1.3.3 cells continuously maintain the integrity of its genomes via the regulatory pathways to balance their internal status. Tissue homeostasis is a balance between cell division and cell death, wherein the number of cells is relatively constant. If this equilibrium is disturbed, the cells will either divide faster, resulting in cancer development or die faster, resulting in tissue atrophy (Fulda, 2009). In terminally differentiated cells such as neurons, the induction of apoptosis can have fatal consequences, as seen in neurodegenerative conditions such as Alzheimer's disease. Deregulation of the tissue homeostasis complex has been implicated in formation of many cancers (Galluzzi et al., 2012). Detection of damages or alteration to this internal balance stimulates regulatory checking system to arrest cell growth and induce repairing pathways. Apoptosis is a normal part of this procedure (Lockshin and Zakeri, 2007; Falschlehner et al., 2009). Apoptosis is generally induced by either loss of positive signals or induction of negative signals. Lack of growth stimulating factors is an example of positive signals for induction of apoptosis. External molecules such as death ligands or internal signals can trigger the intracellular apoptotic signaling pathways. This scenario is mostly attributed to either viral infections or persisting a stressful circumstances and imbalance in oxygen and nutrient equiliibrium (Ashkenazi, 2008). However, both external and internal signals can activate apoptotic response via a very particular and well-planned manner. Upon activation of apoptosis, the cells are disassembled systematically and their membrane will be subjected to blebbing. Afterward, cell chromosome is fragmented and separated into apoptotic bodies along with the cytosol. Neighboring cells (phagocytes) engulf the remaining germs and utilize them for their own maintenance. This procedure is called phagocytosis and resulting in minimal inflammatory response (Finnberg et al., 2008; Falschlehner et al., 2009).

Apoptotic responses are generally divided into two consecutive pathways; sensing the apoptotic signals and executing the apoptosis. Sensing signals can continuously check both internal and external conditions that affect cell appreciation. External signals such as exposure to toxins, drugs and hormones will be transduced internally either via the receptors or transporters on the plasma membrane (Miller et al., 2006; Ashkenazi, 2008). External apoptotic signaling is regulated via cell surface receptors. They bind either to

death or survival factors and induce their effects. For instance, death signals are activated once tumor necrosis factor TNF-α binds to TNF receptor 1 (TNFR1) (Nagane et al., 2000). Internal signal monitor response to stress or DNA damage due to hypoxia, viral infections, growth factor insufficiency or even increase in the level of cellular calcium. Internal signals transfer apoptotic signals on the mitochondria. Some cytosolic proteins target mitochondria and induce swelling of the organelle and allow release of particular apoptotic effector proteins into the cytosol (Nagane et al., 2000; Ashkenazi, 2008). Generally, both extrinsic and intrinsic pathways merge via common effector pathways inside the cell (Miller et al., 2006; Fulda, 2013).

Cancer cells overcome apoptotic pathways via various mechanisms. For example, inactivation of p53, tumor suppressor gene, is one the most common causes of the loss of pro-apoptotic regulation. Majority of squamous cell carcinomas and also human cancers are attributed to mutation of p53 genes. As mentioned before p53 acts as DNA damage sensor and it activates either DNA repair pathways or apoptosis in response to DNA damage (Li et al., 2002; Janic et al., 2018). Therefore, targeting the factors that cause inactivation or mutation in tumor suppressor proteins can be suitable strategy for preventing and avoiding cancer progression.

1.3.6 Activating invasion & metastasis

As described before cancer cells act independently, and ignore the anti-proliferative signals. They start to grow, proliferate and transform via series of events. Once the proliferation rate of cells increases, organs and tissues become enlarged. This stage is termed hyperplasia (Emberton et al., 2003). The second stage is termed dysplasia and occurs when the cell growth persists and accompanied by abnormal alterations to the cells (Katz and Kaestner, 2002). The next stage is called anaplasia and occurs when cells loss their function. Generally, anaplastic cells are poorly differentiated or undifferentiated with manifestation of advanced cellular pleomorphism. The final stage takes place when the cells in tumor start metastasise and are able to migrate and invade to neighboring tissues and organs (Ellison, 2002).

Normal tissues requires proper adhesion with basement membrane and neighboring cells to create signals and transfer to each other for maintenance of their hemostasis. In contrast cancer cells demonstrate diminished cellular adhesion, allowing them to be motile and migrate to new sites (Lee et al., 2017). Indeed, Tumor cells employ their migratory and invasiveness properties to infiltrate to surrounding extra cellular stroma and enter into blood vessels (Wolf et al., 2013). They enter circulation and interact with each other or other blood factors forming intravascular tumor emboli. After formation of emboli they exit the circulation and migrate into surrounding parenchyma. This adaptation to new location leads to sustained growth. Metastasis progression genes form the basis for this growth and metastasis (Figure 7). Preinvasive tumor growth is accompanied by induction of hypoxia to the site. Following a disruption in tissue hemostasis an inflammatory response can initiate. This leads to secretion of tumor associated macrophages (TAMs), carcinoma associated fibroblasts (CAFs) and myeloid derived suppressor cells (MDSCs). These cells produce angiogenic elements and promote vascularization and growth factors that ultimately stimulate tumor invasion and motility (Dembo et al., 1988; Serhan and Savill, 2005; Simon and Green, 2005; Bonakdar et al., 2012).

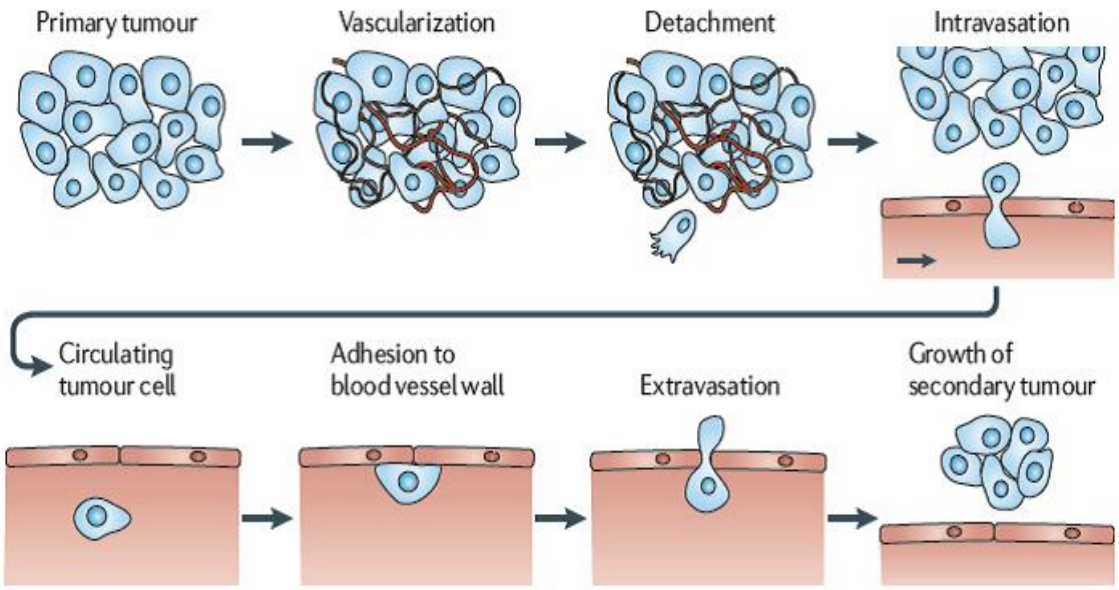

Figure 7. The steps of tumour metastasis. During metastasis, tumor cells exit their primary site of growth that is followed by local invasion and intravasation. Afterward, they get translocated systematically that is associated with survival in the circulation, arrest at a distant organ site and extravasation. Once they adapt to survive and thrive in the foreign microenvironments of distant tissues, micro metastasis stats forming and metastatic colonization occurs (Adapted from Medium.com).

1.3.7 Reprogramming Energy Metabolism

Cells alter their metabolism in response to various stimulation factors. Normal cells produce the required energy via oxidative phosphorylation whereas cancer cells produce their energy through glycosis (Warburg et al., 1924). Adjustment of energy metabolism for providing fuel for cell growth and division is a unique feature of cancer cells. Interestingly, cancer cells are able to reprogram glucose metabolism that is called aerobic glycolysis. Glycolytic fueling is in close correlation with activation of oncogenes in cancer cells that accelerates cell proliferation and exit from apoptosis. As mentioned before hypoxic condition operates in tumor cells that increase the glucose transport to cancer cells. Otto Warburg (1956) reported that cancer cells convert the glucose to lactate without presence of oxygen. Lactate production allows glycolysis to further continue. Warburg also noticed that tumors prefer glycolysis even in the presence of oxygen. Coupled with this, an increase in glycolysis facilitates the formation of biosynthetic macromolecules and organelles necessary for assembling new cells (Jones and Thompson, 2009; Hanahan and Weinberg, 2011).

1.3.8 Tumor promoting inflammation

It is well established that pathogen induced inflammation is a great risk factor for carcinogenesis. During lives, immune system will encounter with abnormalities and formation of cancerous cells. Immune system can detect and remove infection and cancer cells from body. However, sometimes these germs can escape from immune system and cause chronic inflammation. Normal inflammation is a self-limiting procedure as the production of anti-inflammatory cytokines follows the pro-inflammatory ones. In contrast, chronic inflammation seems to be related to the persistence of the initiating factors or a failure of mechanisms required for resolving the inflammatory response (Alicka and Marycz, 2018). In such a case, inflammatory cells of immune system fight against infections via production of free radicals. These free radicals cause oxidative damage and increase the risk of DNA mutations. Thus inflammation can induce oncogenic mutations and genomic instability leading to angiogenesis and early tumor development (Chang et al., 2002; Mantovani et al., 2008). In addition to that, inflammatory cells produce numbers of biochemical such as; growth factors, survival

factors and matrix-modification enzymes that accelerate cell proliferation and tumor growth. (Gasche et al., 2001; Samper et al., 2003; Borrello et al., 2005).

1.3.9 Avoiding Immune destruction

Cells and tissues are continuously monitored by immune system, and therefore immune surveillance is responsible for recognising and eliminating the cancer cells and tumors. However, solid tumors might manage to avoid detection by the immune system and restrict the extension of immunological killing, thereby evading eradication. There are various mechanism and factors that contribute to tumorgenesis despite having a normal function of immune system. Immune editing process such as inhibition of tumor antigen presentation is one of the key factor that help tumors evade surveillance, causing the tumors to lie dormant in patients for many years through senescence before reemerging (Messerschmidt et al., 2018). Down regulation of the antigen processing, especially the MHC class I pathway is a great example of such mechanism. For example, individuals with persistence HPV infection are at greater risk of development of cervical cancer that can be attributed to the role of E5 oncoprotein of HPVs (Ashrafi et al., 2006). E5 oncoprotein is contributed to down regulation of MHC class I (Ashrafi et al., 2005). This will be discussed in section 1.9.1. Else, tumor immune evasion is related to the mechanisms by which tumors induce antigen specific T cell tolerance and resistance to killing by the immune cells (Zamarron and Chen, 2011). Other factors such as production of immune suppressive cytokines, either by the cancer cells or even non cancerous cells can avoid immune destruction.

1.3.10 Genome Instability and Mutation

One of the molecular hallmarks of cancer is genetic instability that is the consequence of deletion and/or mutational inactivation of genome guardians such as p53 (Hanel and Moll, 2012). In majority of hereditary cancers, genomic instability characterised by mutations in DNA repair genes, leading to carcinogenesis and cancer development. In non-hereditary cancers the etiology and molecular basis alterations for causing genomic instability is still unclear. Mutation is defined as an alteration in DNA sequence. The nucleotide letters A, T, C and G that make up DNA can be deleted or

substituted, consequently single or double stranded breaks may take place in the DNA molecule (Luijsterburg et al., 2007; Janavicius, 2010). Indeed mutation is an inevitable event that can occur during cell division and as the result the imperfect DNA replication process can introduce errors into genomic material. (Chan and Amon, 2009). In addition to the thousands of mutational events that can occur during tumorgenesis, epigenetic changes as a consequence of hyper- and hypo-methylation may alter expression levels of hundreds of genes (Ehrlich, 2009). While these epigenetic changes do not formally create tumor-specific neoantigens, they raise the concentration of encoded proteins, thereby dramatically affecting antigenicity (Grivennikov et al., 2010). It is essential to mention that new antigens are constantly being generated in tumors as a consequence of genetic instability once the carcinogenic process develops and progresses. This does not occur in normal, non transformed tissues, which maintain a very stable antigenic profile. These changes can occur spontaneously or form due to exposure to mutation promoters such as harmful chemicals, radiation or viruses (Fang and Zhang, 2011). Typically, body employs a repair mechanism that involves cutting out and re-synthesizing the damaged section of the DNA. Many proteins such as Breast cancer association gene I and II (BRCA1, BRCA2) play critical role in DNA repair and alteration to their expression level may consider as a precursor for cancer development. For instance, women with an abnormal BRCA1 or BRCA2 gene pose extremely high risk of developing breast and ovarian cancer (Hall et al., 1990; Domchek et al., 2010; Ludwig et al., 2016). Genetic changes in the progression of cancer typically affect two different types of genes, oncogenes and tumor suppressor genes (discussed in section 1.4). Both of the oncogenes and tumor suppressor genes encode many kinds of proteins that play a key role in cancer induction. These genes control cell growth and proliferation and mutation in these genes can contribute to the development of cancer (Yokota, 2000; Balmain et al., 2003; Yokota and Kohno, 2004).

1.4 Oncogenes and Tumor suppressor genes

Oncogenes are activated cellular proto-oncogenes because these genes code for proteins that are directly involved in regulation of cell growth and proliferation. The oncogenes

are abnormally expressed or mutated forms of the corresponding proto-oncogene. As a consequence of such alterations, the oncogenes induce abnormal cell proliferation and tumour development. The viral oncogenes and cellular oncogenes (e.g. Ras and myc) have defined a large group that can contribute to the abnormal behavior of malignant cells (Downward, 2003; Benassi et al., 2006).

Tumor suppressor genes are group of genes that play key role in regulation of cell division and cell death. Once DNA damages has been detected in cell, these genes can stop cells from further proliferation until the damages is fixed. In case tumor suppressor genes do not function appropriately, the cells with damaged DNA continue to divide and accumulate more DNA damage that can ultimately cause carcinogenesis and formation of cancer cells. In such a way tumor suppressor genes act similar to oncogenes. Majority of cancers are associated with inactivation or mutation in one or two proteins that normally induce cell death in G1 phase of cell cycle distribution such as pRb, p53 and p16 (Weber et al., 2000; Liu et al., 2004; Kim and Sharpless, 2006; Riley et al., 2008). Virtually all of the human tumors have inactivating mutation in proteins like p53 that normally regulates cell cycle checkpoints once DNA damage has been occurred (Yokota, 2000; Li et al., 2002). The functions of tumor suppressor proteins such as; Rb, p53 and p16 are discussed below.

1.4.1 RetinoBlastoma

The first tumor suppressor gene was identified as genetic locus associated with the development of a retinoblastoma (Rb), a rare childhood eye tumor. Children with hereditary retinoblastoma inherit a defective copy of the Rb gene, sometimes seen as a small deletion on chromosome 13. They develop retinal tumors early in life and generally in both eyes. The realisation that it was a loss of function of Rb that was associated with disease established the tumor suppressor paradigm. The characterisation of Rb as a tumor suppressor gene served as the prototype for the identification of additional tumor suppressors genes that contribute to the development of many different human cancers. The Rb family consists of three members, Rb, p107 and p130 altogether knows as pocket proteins (Dyson et al., 1989; Dyson 1998). Pocket proteins refer to the binding

region via which these members bind to viral onco-proteins as well as cellular factors like E2F family of transcription factors. E2F family is functional transcriptional regulator of genes involved in cell cycle progression, DNA synthesis, apoptosis and other cellular processes. Rb binds E2F and inhibits transcription by blocking the E2F transcriptional activation domain. In cycling cells, Rb is inactivated in mid to late G1 through phosphorylation by several cyclin-cyclin-dependant kinase (CDK) complexes (figure 8). Hypersphosphorylated Rb no longer binds E2F; consequently, repression of E2F-dependent promoters is relieved (Stevaux and Dyson 2002). The expression patterns of Rb family memebrs differ during Go/G1/S phase transition, with p130 highly expressed during G0, p107 highly expressed in S-phase and pRb expressed at a fairly steady level throughout the cell cycle. In addition, Rb protein is in charge for controlling the G1 checkpoint of cell cycle distribution. It blocks progression of cell cycle to S phase and therefore arrest cell growth and induces cell cycle exit (Weinberg, 1995; Dyson, 1998). PRb also plays a significant role in differentiation process in various organs and tissues. The Rb gene is inactivated in the majority of human neoplasms (Liu et al., 2004). The pRb signaling pathway or the level of expression can be disrupted through various ways. In other word, Rb protein is a key target for the oncoproteins of many DNA tumor viruses such as; human papillomaviruses. For example viral oncoproteins E7 from Human Papillomaviruses can interact with Rb protein, resulting in loss of its function (Dyson, 1989). This will be discussed in section 1.10.3.

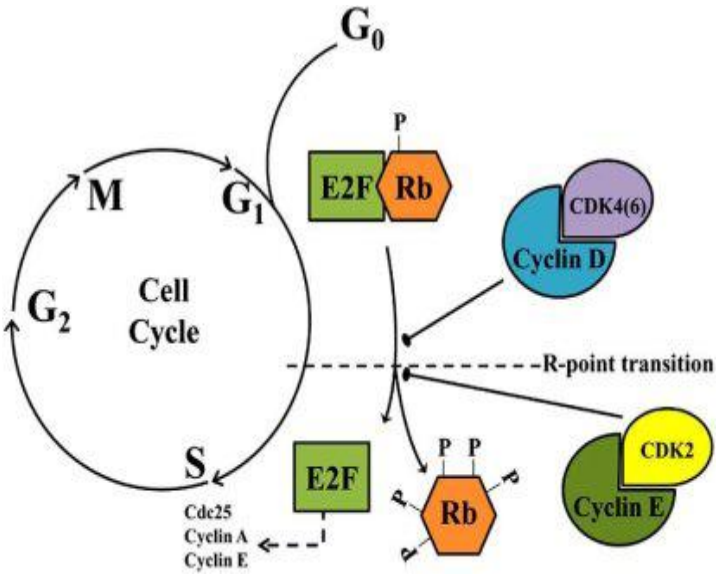

Figure 8: Representing the Rb-E2F pathway and effect on cell cycle progression. Conformational changes to the pRb structure due to its phosphorylation and release of E2F. The release of E2F is essential for the expression of S-phases genes (Biggar & Storey, 2009).

1.4.2 P53

P53 is one of the well-known suppressor proteins that plays an important role at level of cellular and sub cellular units. The p53 tumor suppressor gene encodes a nuclear phosphorprotein that functions as key regulator of DNA repair, cell cycle progression and apoptosis (Riley et al., 2008). P53 signaling pathways take place in response to a variety of intrinsic and extrinsic factors to the cells. The p53 protein is upregulated in response to a variety of cellular stress including DNA damages, hypoxia, oxidative stress and oncogene activation. Once these signals triggers an elevation in p53 proteins level, p53 blocks the progression of cell cycle and prevents replication of damaged DNA by inducing the temporary arrest of the cell cycle in the G1 or G2/M phase prior to mitosis for DNA repair (Li et al., 2002; Janic et al., 2018). This induction occurs either via mitochondrial pathways or activation of death receptor (Pietsch et al., 2008). However, the mechanism is still not fully understood. The regulation of p53 function occurs in transcription, translation, and structural alterations. Radiation, chemicals and viruses are major contributed factors to increase the risk of damages to p53 gene. Once P53 in inactivated, it allows indefinite cell proliferation and cell growth resulting in cancer. Following DNA damage, p53 holds the cell at checkpoints until the damage is repaired. If the damage is irreversible, apoptosis is triggered (figure 9). The normal p53 function can be inactivated by somatic and germ line mutations and by binding to different viral oncoproteins (human papillomavirus protein E6) (Dyson et al., 1998; Brooks et al., 2007; Uldrijan et al., 2007). Therefore targeting the inactivators of p53 could be considered for introducing the novel therapeutic modalities against wide range of cancers. The significance of this in virus associated cellular transformation will be discussed more fully later in this chapter.

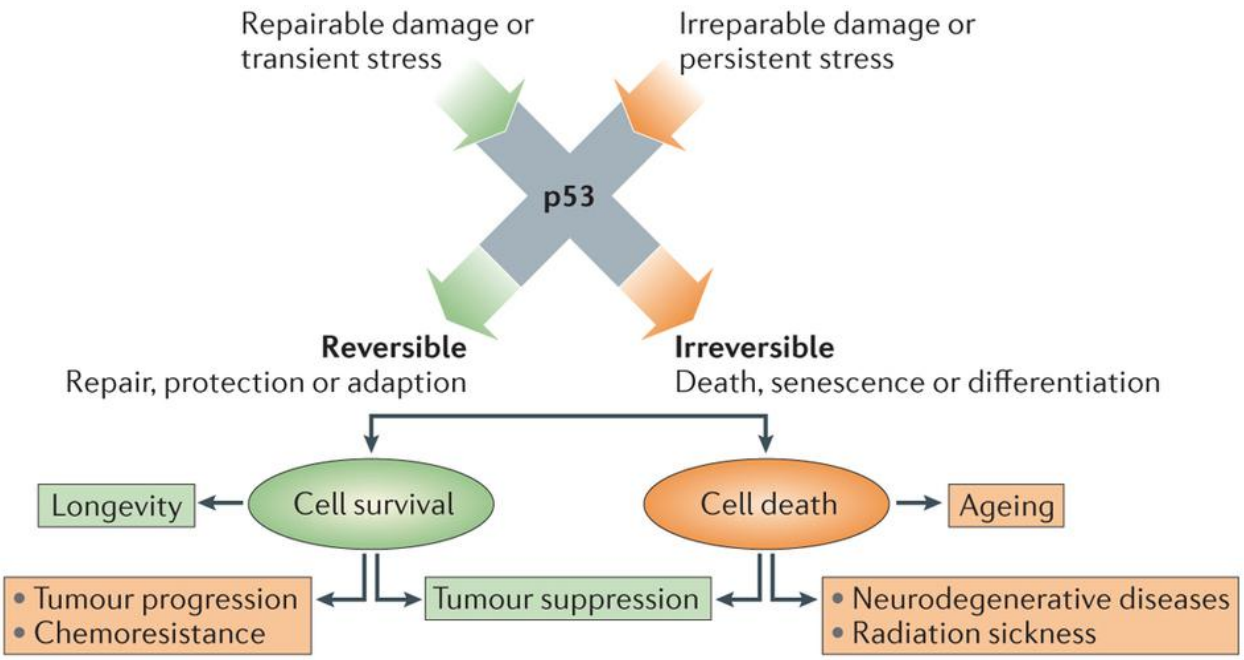

Figure 9. P53 pathway in cell survival and cell death. Following a damage or stress p53 will undergo either cell repair or induce cell death (Adapted and revised from Moll& Petrenko, 2003).

1.4.3 P16

P16 with molecular weight of 16 kDa is another tumor suppressor protein that is principal member of CDK inhibitors. Cycline D/CDK4 is a proliferation-stimulating factor. A gene localised on chromosome 9p21 within the INK4a/ARF locus codifies P16 (Serrano, 1997; Peipkorn, 2000; Kim and Sharplesss, 2006). P16 is a negative regulator of cell proliferation, consequently one of the main factors to suppress tumor formation. P16 suppresses the cell cycle by prohibiting progression of cells from G1 phase to S phase (Nam et al., 2006). P16 has also been implicated in other processes including apoptosis, cell invasion and angiogenesis (Li and Lu, 2010). In the absence of p16, CDK4 activity is elevated, leading to Rb phosphorylation and E2F accumulation. Therefore, the absence of p16 activity is functionally equivalent to loss of Rb (figure 10) (Slebos et al., 1994; Nam and Kim 2008). P16 inactivation is an early and critical event in tumor progression that may take place due to various molecular alterations. For instance, following a viral infection with HPV, the p16–Rb pathway is targeted by viral oncoproteins and is inactivated. The inactivation of pRb by E7 causes the p16 overexpression because p16 is regulated by negative feedback of pRb. Overexpression of p16 is a predictive marker for HPV infection (Dyson et al., 1989; Nam et al., 2007).

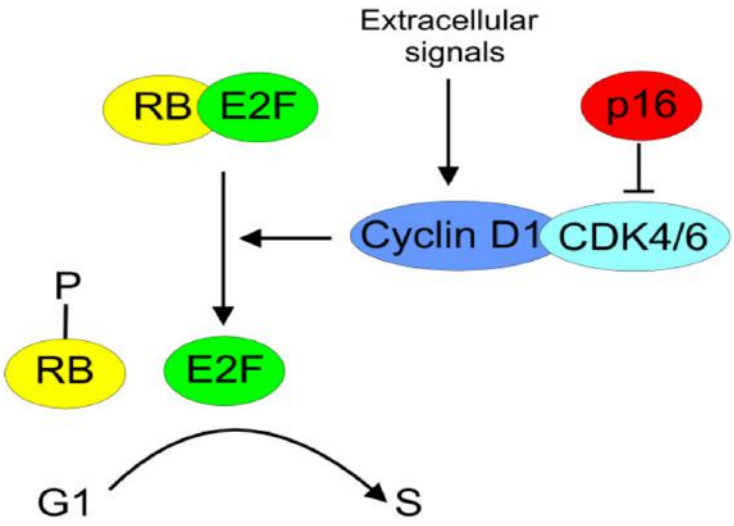

Figure 10. The regulation of cell cycle by P16. CDK4/6 binds to cyclin D forms an active protein complex leading to phosphorylation of pRb. This phosphorylation leads to dissociation of Rb from E2F and the release of transcription factor E2F. The release of E2F results in G1/S transition (Peurala et al., 2013). Expression of p16 inhibits CDK4/6 and suppresses cell cycle progression.

1.5 Etiology of cancer

Cancer is a multifactorial disease and many risk factors are contributed to its development. Initiation of carcinogenesis process can arise from either external (environmental) elements such as; tobacco, chemicals, radiation and infectious organisms or internal factors including; inherited mutations, hormones and immune conditions (Hill, 1965). These factors can act in combination or in sequence and accelerate cancer progression and uncontrolled cell division. Consequently, cell mass increases and invades to neighboring tissues. It takes months and years for these alterations to accumulate and form a detectable cancer (Evans and Muller, 1990). Cancer incidence rates vary significantly across the world. Several factors can considerably increase cancer incidences among some populations. Hereditary and environmental factors are contributed to these differences. Evidently certain populations host greater amount of cancer-susceptibility genes or it is presumed that the environment in which people live predominantly contribute to the higher cancer incidence rates (Hahn and Weinberg, 2002; Fitzmaurice et al., 2017).

1.5.1 Genetic factors

Genes are basic functional unit of hereditary and are made up of DNA. Even a small alteration in one base of DNA and genomic materials can eventually manifest as a major defect. A gene mutation can cause affects cell through various pathways. Gene mutations can be classified into; hereditary mutations or Acquired (somatic) mutations. Hereditary mutations are inherited from a parent while acquired mutations occur at some time during a person's life and can cause by environmental factors (Visscher et al., 2008; Loweve and Hill, 2010).

Mutations can stop protein production and change the protein structure, consequently it cannot act properly any longer (Risch, 2001; Couzin, 2015). These mutations are often associated with initiation of carcinogenesis process and may target different elements that control cell function and regulation such as oncogenes and tumor suppressor genes (Tenesa and Haley, 2013; Mucci et al., 2016).

1.5.2 Environmental factors

A number of environmental factors can affect and increase the probability of acquiring cancer. These factors are recognized as carcinogenic agents and there is a close correlation between exposure to such an agent and the occurrence of a certain kind of cancer (Purohit et al., 2005; Smith et al., 2016). As shown in figure 11 environmental factors are implicated in the etiology of majority of cancers. However, many of these cancers are preventable via avoiding persistence exposure and contact to the source of carcinogenic agents (Noble et al., 2016). Important examples of these carcinogens factors include; X-rays, UV light, viruses, tobacco products, pollutants and many other chemicals. X-rays is potent mutagens and induces carcinogenesis. For instance Marie Curie who discovered radium, died due to cancer as consequence of radiation exposure (Frias and Barness, 2008). UV light is implicated in etiology of skin cancers e.g. melanoma (Tomasetti and Vogelstein, 2015). Industrial chemicals e.g. benzene, arsenic and solvents are very toxic and carcinogenic, they can accelerate cell transformation (Grosse et al., 2016). It is also important to mention that the role of environmental agents is not independent of genetic factors. Sunlight can alter the tumor suppressor genes in skin cells and increase the chance of acquiring cancer. Smoking Cigarette is contributed to genomic alterations in lung cells that make them more sensitive to carcinogenic compounds in smoke (Farukh, 2013). These environmental agents act directly or indirectly on the genes that are important for cell transformation. Infectious agents like viruses can also cause major genetic mutation in the host genomic materials that contributes to cancer development (Parkin, 2006). This will be discussed in the following section.

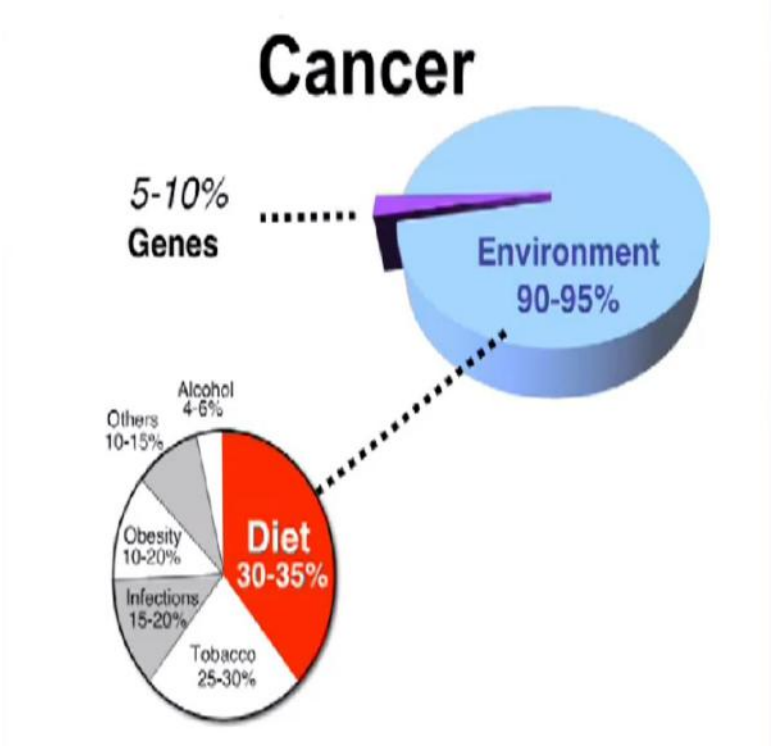

Figure 11. The role of genes and environment in the development and etiology of cancer. Adapted from Texas Chiropractic Association (chirotexas.org).

1.6 Viruses and Cancer

Viruses are categorised into two main groups, either having DNA or RNA as their genomic material. Epidemiological studies supported by clinical and molecular biological investigations of certain human cancers indicate that viruses contribute to one of several events that cause these malignancies. These include both DNA and RNA viruses (representing retro viruses) (Butel et al., 2000; Moore and Chneg 2010). Many of these viruses have mutagenic activity and can affect cellular behaviors. Examples of viruses associated with cancer are included but not limited to human papillomavirus (genital carcinomas), hepatitis B (liver carcinoma), Epstein-Barr virus (Burkitt's lymphoma and nasopharyngeal carcinoma) and human T-cell leukemia virus (T-cell lymphoma) (Liao, 2006; Anand et al., 2008). It is evident that viruses can either directly or indirectly interfere with normal cellular function thorough various pathways that are contributed to cancer development (Holems, 2014). They can directly interfere with host genomic materials i.e. DNA and cause irreversible damages. Viruses can disrupt the regulatory affairs in cells via inserting their genomes into the DNA of the host cell and cause mutation. Also certain genes of viruses may directly interact and disrupt the regulatory affairs of cells leading to rapid production of viruses (Osborne et al., 2004). Other viruses can pick the genes from their host and act as a carrier and transfect new cell. (Swale et al., 2000; Solan and Donald, 2010). Although many viruses examined for causing infection in humans but only a few of them are reported to promote human cancer. This chapter will focus on the role of human papillomaviruses in etiology of cancers which is directly associated with the work of this thesis.

1.7 Human Papillomaviruses (HPVs)

Papillomaviruses are small DNA virus from the family papovavirdae, without membrane but contain an icosahedral capside that is composed of 72 capsomeres (Antonsson et al., 2011). HPV`s DNA is circular, non-enveloped, double stranded, and consists of around 8000 base pairs (Scheurer et al., 2005; Pahud and Ault, 2015). Papillomaviruses are categorized based on their viral genome sequences and sub-genomic segments. However, the overall genome organization of papillomaviruses is similar. A specific characteristic feature of papillomavirus genomes is that all major open reading frames (ORFs), located on the same DNA strand. The genomes of HPVs consists of approximately eight open reading frames (ORF) and they are all transcribed from a single DNA strand. (Glaunsinger et al., 2000). The Human papillomaviruses (HPV) ORF is divided into three functional compartments; the early (E) region encoding E1-E7 proteins for viral replication and the late region encoding L1-L2 structural proteins for capsidisation virion assembly (Hebner and Laimins, 2006; Hafner et al., 2008; Ganguly and Parihar, 2009). Moreover, there is a largely non-coding compartment called the long control region (LRC) containing cis element for replication and transcription of viral DNA. More than 150 types (HPVs) are investigated and that are targeting the epithelial cells in mucosa and skin (Doorbar et al., 2015). They infect mucosal and cutaneous epithelia in a variety of higher vertebrates in a species-specific manner and resulting in uncontrolled cellular growth and proliferation (Schmitt et al., 2012). Based on their oncogenecity, HPV are classified as low-risk HPV (LR-HPV) or high-risk HPV (HR-HPV) types. HR-HPVs such as; 16, 18, 31-35, 51-52, 56, 58, 68, 70, 73 (type 16 and 18 are counted as the most common types so far) are mostly associated with cervical cancers and other cancers like anogenital and oropharyngeal cancers. The low risk HPV types such as; 6, 11, 40, 42-45, 53-55, 57, 59 are commonly cause benign warts and low grade premalignant lesion that regress and do not progress to cancer (Ault, 2006; Pahud and Ault, 2015; Nowinska et al., 2017).

1.8 HPV genome and life cycle

As explained in section 1.7 the genome of the HPV is divided into three regions; the long control region (LCR), the region that encodes the early (E) genes and the region that encodes the late (L) genes. The E1 ORF of HPVs encodes a nuclear phosphoprotein essential for viral DNA replication. The E2 ORF of HPVs encodes a nuclear phosphoprotein that regulates both viral DNA replication and transcription. Interaction of E2 with E1 is essential for replication of the viral genome. E2 binds to DNA binding site that flank the E1 binding site and recruits E1 to the origin of replication (Titilo et al., 2000; Hebner and Laimins, 2006). E4 is mostly involved during late stages of the life cycle of the virus. The expression of E4 is usually coincided with the initiation of viral replication amplification. It interferes with normal keratinocyte differentiation to favour the release of viral particles (Lee et al., 2000). E5 acts during both early and late phases and play key role in immune evasion (Ashrafi et al., 2006). The two early genes, E7 and E6 as the major transforming protein of HPV, play crucial role in tumor formation. They interact with couples of negative cellular regulators of cell cycle, pRb and p53, respectively (Brandsma et al., 2007; Shai et al., 2010). The L1 and L2 proteins assemble in capsomers, forming icosahedral capsids around the viral genome that is vital for formation of virions (figure 12 and Table 1) (Harper et al., 2006).

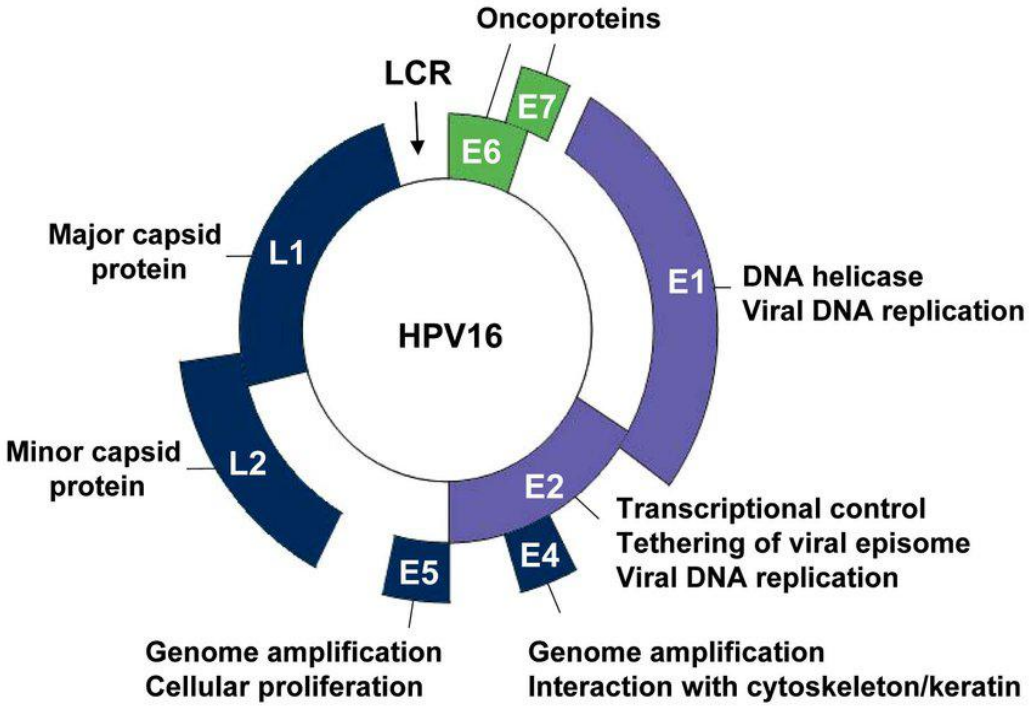

Figure 12. The structure of HPV. Adapted from Abramo and Archambault (2010).

Table1: HPV proteins and their function. (Ashrafi et al., 2006; Tristram and Fiander, 2007; Abramo and Archambault, 2011)

Protein	**Function**
E1	Viral replication and maintenance
E2	Regulates viral transcription and replication, induce cell transformation, inhibits apoptosis, controls transcription of HPV and cause immortalization,
E4	Interacts with cytoskeletal proteins, disturbs structural framework of keratin, plays role in viral releases
E5	Down regulates the expression of MHC class 1, prevents infected cells to be recognised by immune system.
E6	Binds to p53 tumor suppressor gene and avoids apoptosis and DNA repair, deregulates cell arrest and promotes cell proliferation.
E7	Binds to Rb tumor suppressor protein and disrupts the Rb - EF2-1 complex resulting in liberation of E2F-1, promotes gene transcription and cell cycle production.
L1	Major structural protein, encodes viral capsid
L2	Minor structural protein, encapsulates viral DNA

1.9 HPV Transmission

HPVs can infect human epithelial tissues thorough direct skin to skin contact or sexual activities. HPV can transmit even when an infected individual shows no clinical symptoms and signs of infection (Bryan and Brown, 2001). Various clinical manifestations such as; genital warts and dysplastic lesions in cervix can appear due to HPV infection. However, it may take several years following infection to develop symptoms. HPV is prevalent in all sexually active populations and some studies reported that at least half of sexually active individuals would acquire this virus at some point during their lives. The prevalence of HPV infections will increase in certain population and is in close correlation with gender, age and sexual activities of a person (Ault et al., 2006). Following an exposure to an infected person, virus can enter body via minor skin abrasion or trauma. Afterwards the virus interacts and binds to certain cellular receptors including; heparan sulphate proteoglycans and laminine and alpha-6 integrin. This interaction is associated with structural alteration in viral capsid, cleavage of L2 protein and attachment of a secondary receptor through clathrin-dependent endocytosis to the basal keratinocyte (Doorbar, 2005; Doorbar et al., 2010). This binding facilitates the delivery of the genomic material of the virus (DNA) to the nucleolus, enabling replication. Then early oncoprotein E6 inactivates p53, causing DNA damage, cell proliferation and cancer development. E7 disrupts the function of Rb, displaces E2F causing cell transformation and uncontrolled cellular proliferation. E5 retains MHC complex in Golgi apparatus, preventing it from reaching to the cell surface (Ashrafi et al., 2006). This reduces antigen presentation and prevents antigen recognition by cytotoxic T lymphocytes (Ashrafi et al., 2005). Consequently, HPV infections is established and infected cells escape from immune system responses. Once the infection persists additional lesions can arise that may ultimately form malignant tumors (Tristam and Fiander, 2007).

1.10 Viral transforming proteins

High risk HPVs such HPV-16 and HPV-18 encode three transforming protein E5, E6 and E7 which are mainly implicated in cell transformation (Munger and Howley, 2002). While E6 and E7 are mainly in charge for transforming properties, E5 has less

transforming activity (DiMaio and Matton, 2001).

1.10.1 HPV E5 Protein

E5 is a small hydrophobic protein located in the membranes of the endoplasmic reticulum (ER) and Golgi apparatus (GA) of the transformed cells. The expression of E5 is mostly attributed to establishment of HPV infection and initiation into cell transformation that is gradually decreased with the progression of cell transformation towards malignancy (Venuti et al., 2011). E5 downregulates the expression of cell surface major histocompatibility complex (MHC) class I by retaining it in the GA (Ashrafi et al., 2006). MHC class I present antigenic peptides to CTL, allowing recognition and killing of infected cells by immune system (Seifert et al., 2003). The down regulation of MHC class I by HPV E5 accelerates the establishment and persistence of virus infection, by allowing the infected cell to evade killing by CTL and thus increasing the probability of recurrent genital warts and cancer (Zhang et al., 2003; Ashrafi and Perumal., 2015).

1.10.2 HPV E6 protein

HPV replication like others viruses depend on the cellular DNA synthesis and requires stimulation of S-phase progression for the replication of its genome. The role of E6 is essential during a productive infection (Fehrmann & Laimins, 2003). E6 interacts with several cell proteins and this function can alter the cell. These proteins are included: transcriptional co-activators, proteins involved in cell polarity and motility, tumor suppressors (inducers of apoptosis), DNA replicators and repair factors (Thomas et al., 1999). Studies demonstrated that E6 protein interacts and degrades the tumor-suppressor protein p53 using the protein ligase, E6-associated protein (E6-AP) (Zur Hausen, 2002). In other word the main activity of E6 protein is the stimulation of the ubiquitin mediated degradation of p53 tumor suppressor protein. Both the binding of E6 to p53 and the ubiquitination are dependent on the cellular E6-AP (figure 13). The increase or overexpression of p53 inhibits viral replication (Thomas et al., 1999; Massimi et al., 2004). This interaction eventually inhibits the transcriptional activity of p53 and the abolition of p53-induced apoptosis (Fehrmann & Laimins, 2003). Degradation of p53 is

associated with the cell cycle arrest at the G1/S and G2/M checkpoints and is the initial reason for chromosomal instability. (Thomas et al., 1999; Zur Hausen, 2000). E6 also activates telomerase that is responsible for replicating telomeric DNA at the ends of chromosomes (Zur Hausen, 2002; Fehrmann & Laimins, 2003). Therefore the interaction of E6 and telomerase cause cell immortalisation.

1.10.3 HPV E7 Protein

E7 protein is one of the major oncoprotein of high-risk HPVs accounted for immortalisation of primary human cells and cervical carcinogenesis. As shown in figure 13, E7 is located in the nucleus of cells, where they interact with retinoblastoma gene product (pRb) to accelerate cell cycle progression into the S-phase. This occurs thorough stimulation of the S-phase genes of cell cycle such as; cyclin E and cyclin A (Zur Hausen, 2002). As discussed in section 1.4.1 the retinoblastoma protein family members play an important role in the regulation of the cell cycle. As cell cycle progress from Go through G1 and into S phase, Rb family members become hyperphosphorylated by G1 CDKs, consequently release the transcription factor E2F, which in turn activates genes involved in DNA synthesis and cell cycle progression. E7 interacts with hypophosphorylated Rb and therefore prevents its binding with E2F (Fehrmann & Laimins, 2003; Woodman et al., 2007). Hypophosphorylation of pRb occurs in G1 phase of cell cycle in normal cells and then it bounds to E2F transcription factor. Following on Rb hypophosphorylation in G1 phase and attachment to E2F transcription factor, it forms a complex and acts as transcriptional repressor. Once they separated, E2F acts as a transcriptional activator. This results in promoting cell cycle progression. E7 can also interact with two other members of the pRb family, p107 and p130, which can elevate the negative feedback on E2F transcription. (Zur Hausen, 2002; Fehrmann & Laimins 2003; Molijn et al., 2005).

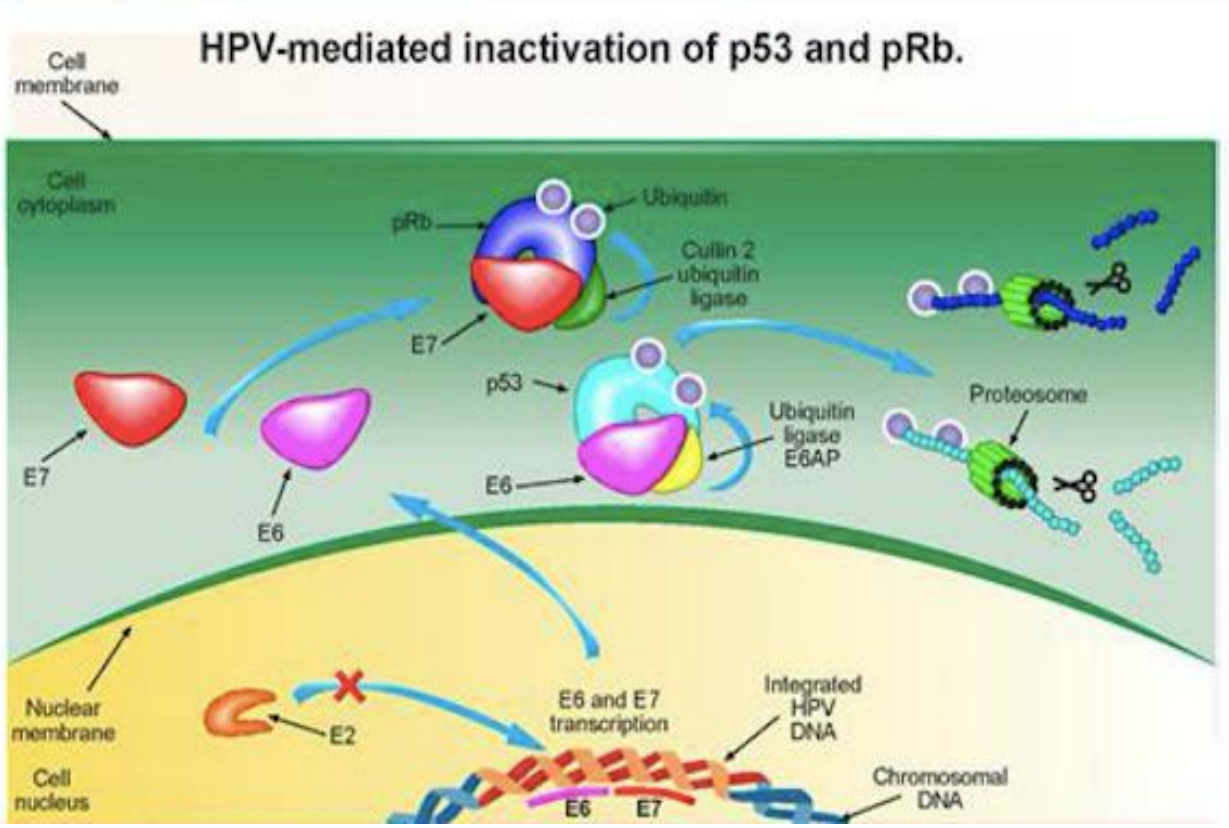

Figure 13. **Inactivation of p53 and pRb by HPV oncoproteins E6 and E7.** E6 binds to p53 and this binding promotes the degradation of p53 via the ubiquitin pathway. Binding of E6 to p53 and the ubiquitination pathway are dependent on the cellular associated protein E6-AP. This interaction ultimately prohibits the activity of tumor suppressor protein, p53.
E7 targets Rb for proteasomal degradation. Interaction between Rb and E7, prevents the binding of Rb and E2F resulting in uncontrolled cell cycle progression. (Adapted form immunopaedia.org. 2010).

1.11 Co-expression of E6 and E7

E6 and E7 are independently able to immortalise human cells, but as shown in figure 14 their co-expression leads to a complementary and synergistic effect and is associated with increased transforming efficiency. The complementary functions of E6 and E7 oncogenes leads to higher grades of immortalisation and an increase in invasiveness of cancers (Fehrmann & Laimins, 2003; Boulet et al., 2007; Yu et al., 2016). Thus, these two oncoproteins are considered to be excellent targets for therapeutic intervention and understanding the molecular mechanisms underlying their respective functions is critical for developing such antiviral therapies (Morris, 2005).

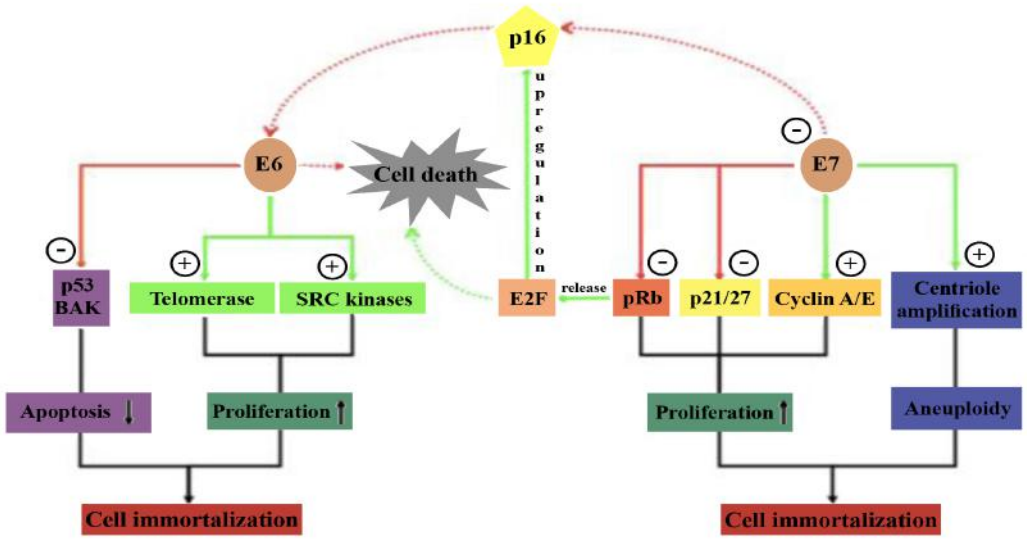

Figure 14. **Co-expression of E6 ad E7.** Cellular interactions of E6 and E7 oncoproteins and their synergy in induction of cell immortalization. E6 and E7 act synergistically to immortalize and transform the infected cells thorough their ability to degrade p53 and Rb respectively. The conjoined effects of E6 and E7 oncoproteins leads to uncontrolled cell proliferation, evasion of apoptosis and immune system allowing progression of infected cells with HPV toward malignancy. These alterations in cellular and sub cellular elements might accompanied by up regulation and/or down regulation of certain proteins/mediators (Boulet et al., 2007).

1.12 HPV and Cancer

Infections with certain types of HPV (mostly HR-HPV) can cause HPV-related cancers and diseases in both males and females. Based on the epidemiological studies approximately above 95% all cases of cervical cancer are caused by HPV (figure 15). Generally HPV is expected to be responsible for majority of anal and cervical cancers, which is followed by vaginal, vulvar and penile cancers. Interestingly, in male population oro-pharyngeal represents the majority of cases of cancers due to HPV infection (Braaten et al., 2008; Hoots et al., 2009). Since the main type of cancer linked to HPV infection is cervical cancer, the main focus of this study is on characteristics and therapeutic approaches for cervical cancer.

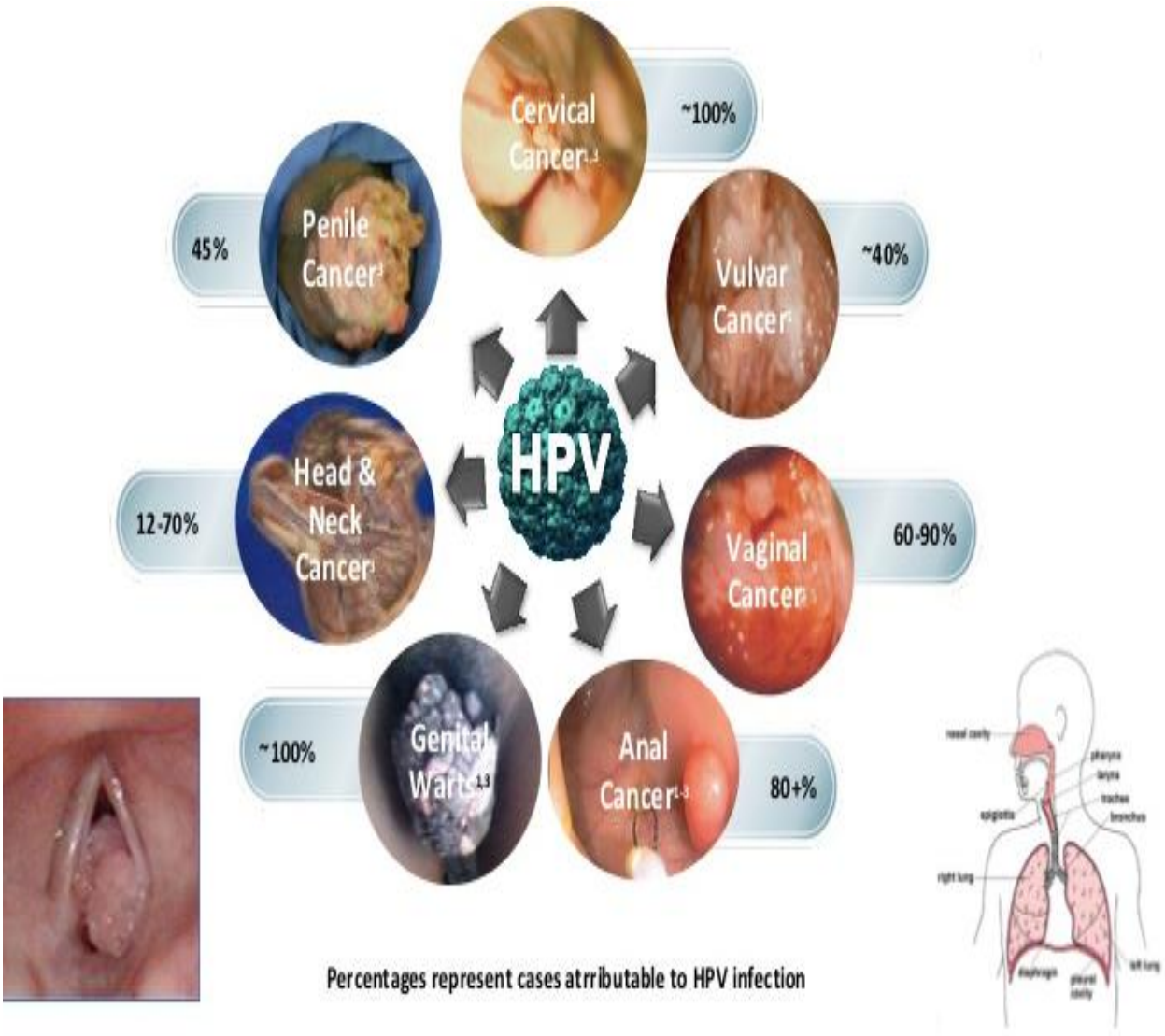

Figure 15. Various types of cancer due to HPV infection (Adapted from Hoots et al., 2009).

1.13 Cervical Cancer

Cervical cancer is one of the major causes of death among women on global scale with an estimated 530000 new cases and 270000 deaths in 2016. Although majority of cervical cancers occurs in developing countries but developed countries are also facing a huge amount of incidents (Martel et al., 2017). Cervical cancers are mainly divided into two sub categories; squamous cell carcinoma (SCC) and adenocarcinomas. Majority of cases are SCC whereas adenocarcinomas are rare (Gadducci et al., 2007). Cervical squamous cell carcinomas progress slightly from intra epithelial neoplasias (CIN) or squamous intraepithelial lesions (SIL). Dysplastic lesions on the surface of cervix are known as cervical intraepithelial neoplasia (Winer et al., 2005; Koutsky et al., 1992). CIN are more frequently take place on the cervix but they might occur in other anatomical structures of urogenital system. Cervical intraepithelial neoplasia is a precursor of cervical cancer, is classified histologically into three classes (CIN I, CIN II and CIN III), based on its severity (figure 16) (Cuschieri and Wentzensen, 2008). The level of cellular alterations is determined by histopathology and microscopy techniques. Infections of the genital tract by HPVs can initially result in low-grade lesions termed dysplasia or CIN I. These lesions manifest just mildly altered patterns of differentiation and immune system can clear many of them in couple of months. Some of these lesions are not removed by immune system and may persist for long periods of time. Persistence of infection by high risk HPVs is the major risk factor for development of genital malignancies. CIN-II may progress into cancer and invade into normal tissue once remained untreated. CIN-III is mostly attributed to the infection with high-risk human papillomaviruses and is categorized as an initial stage of cervical carcinoma (Bosch et al., 2002; Woodman et al., 2011). Certain strains of HPV are associated with different risk levels of the transformation into cervical tumors. High risk HPV type 16 and 18 are associated with moderate to severe cases of cervical dysplasia and are found with the highest frequencies in cervical cancer (Doorbar et al., 2015).

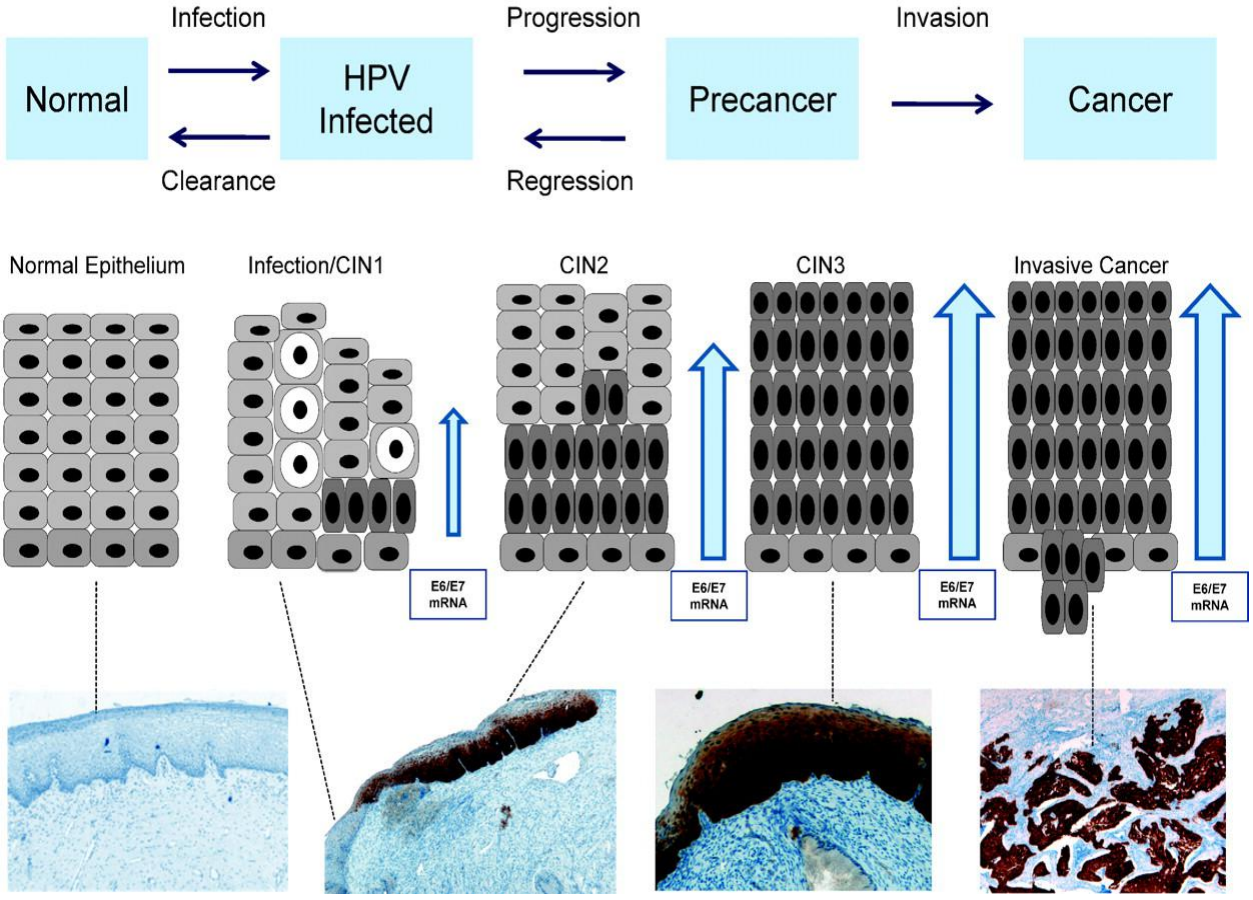

Figure 16. Progression scheme depicting the stages leading to the development cervical cancer from HPV infection to invasive diseases. Persistent infection with HPV is a necessary cause for development of cervical cancer. Following on infection with HPV low grade squamous intraepithelial lesion might manifest that represent high rate of regression back to normal cells (CIN 1). In case these lesion progresses to CIN 2 moderate dysplastic lesion might appear and confined to basal of the epithelial cells. CIN 3 is associated with severe dysplastic lesions and undifferentiated neoplastic cells. At this stage cells are mostly transformed into cancerous cells and carcinogenesis is taken place (Adapted form Cuschieri and Wentzensen, 2008).

1.13.1 Etiology

Many factors are determined in formation and development of cervical cancer. Some of these factors are not sufficient enough to induce cell transformation and initiates carcinogenesis but they can play notable role in development of lesions into cancer malignancies. Sexually transmitted infectious agents such as; syphilis, herpes simplex virus and gonorrhea as well as smoking cigarrete, multiple pregnancies, oral contraceptive and existence of other immune related disorders are numbers of the predisposing factor (Vikki et al., 1998; Rigoni, 2004; Shapley et al., 2006). However, Zur Hausen (2002) introduced the strong link between human papillomaviruses infections and initiation of cervical cancers. Later on high risk HPV especially type 16 and 18 were isolated from cervical cancers and it has been established that infection with HPVs was implicated as a main factor in the etiology of cervical cancer (Stapley and Hamilton, 2011).

1.13.2 Treatment

Cervical cancer can cause impact on patient's sexual life and the ability to have children. It is of vital importance to consider all available and possible treatment options, including their aims and probable adverse effects prior to make any decisions. Treatment for cervical cancer depends on the TNM system scoring (Boeker et al., 2016). The letter "T "stands for the size and location of the tumor. Higher number indicates (0-4) the larger tumors and invasion to surrounding tissues. The letter "N" explains whether a tumor growth into the lymph nodes. It shows the number of lymph nodes that are affected by cancer (0-3). The letter "M" represents whether the tumor has metastasized or has been spread to the other site in body (Boeker et al., 2016; Lim et al., 2018). Currently, there are few types of treatment available for cervical cancers and each option has pros and cons. Based on the type and stage of cervical cancer individuals may need more than one type of treatment or even combination of therapeutic methods (Saito et al., 2010). During the early stages of cervical cancer, either surgery or radiation combined with chemo may be employed. During later stages, radiation combined with chemotherapy is often recommended as the therapeutic modality. Chemotherapy is mostly employed to treat

advanced types of cervical cancer (Lopez et al., 2005). The most available therapeutic approaches towards cervical cancers are discussed below e.g. surgery, radiotherapy and chemotherapy.

1.13.3 Surgery

There are many surgical procedures available for management of cervical cancers and each strategy is accompanied by advantages and disadvantages.

1.13.3.1 Cryosurgery

Cryosurgery is mostly used to remove external genital warts via freezing the cells and sometimes used to stop cell proliferation towards carcinogenesis. However, usage of cryosurgery is associated with causing blisters, damage to nearby tissues and vessels and the long-term effectiveness is questionable (Ferly et al., 2012).

1.13.3.2 Hysterectomy

Hysterectomy is based on removing cancerous tissue and is routinely employed for management of cervical cancer. Currently, laparoscopic hysterectomy is performed using video camera to minimize the surgical incision to for removing the tissues. Although hysterectomy has developed more than a decade ago and many surgeon and gynecologist may still offer it to patients but it can cause infertility and chance of damage to urinary and intestinal system is considerable (Zatonski et al., 1996; Zola et al., 2007).

1.13.3.3 Pelvic exenteration

This method is employed to treat recurrent and hugely metastatic cervical cancer. In addition, depending on the affected areas, bladder, vagina, rectum and parts of colon may be removed. This surgery may cause severe and long term implications on the patient quality of life and takes months or years for patient to recover (Margina, 1990; Marjina et al., 1997).

1.13.4 Radiation Therapy for cervical cancer

Radiation therapy is based on using high energy x-rays and radioactive particles to kill cancer cells. Radiation therapy is usually employed as a part of the main treatment

regimen for some stages of cervical cancer based on TMN scoring system. It is also used to treat recurrent cancer after treatment or those are spread to other tissue and organs. However, there are possible adverse effects of radiotherapy such as; menstrual changes, low blood count, nausea and vomiting, weakened bones and swelling of legs. (Windschall et al., 2005; Mundt et al., 2003).

1.13.5 Chemotherapy

Chemotherapy is based on administration of anti-cancer drugs that are injected into a vein or given orally. This strategy is useful to target the cancer cells in most parts of the body. Chomo is usually administrated in cycles therefore each period of treatment is followed by a recovery period. Chemotherapy is used as a part of the main regimen for treatment of cervical cancer or might be used to manage cancers that spread to other tissue and organs. Chemotherapy can support radiation therapy to work more effectively. Chemotherapy can kill cancer cells at the same time cause damages on normal cells. These adverse effects are generally depends on duration, type and dose of drug and may include loss of appetite, loss of hair, mouth sours, increased chance of infection, shortage of blood platelets and white blood cells, neuropathy, menstrual changes and increased risk of leukemia due to bone marrow damages (Hogg and Friedlander, 2003; Lopez et al., 2005; Jain et al., 2007).

1.14 Development of new modality for treatment

Given the importance of cervical cancer, to date, there has been no satisfactory medical treatment for human papillomavirus related cervical cancer as most of the developed treatments (e.g., surgical excision, chemical ablation, and cryotherapy) are eventually accompanied by excessive tissue injury (As stated before). Therefore, there is a continuing demand for development of new strategies for treatment that must be biocompatible to tissue as well as in the prevention of transmission of infection.

1.14.1 Herbal Medicine

Medicinal plants have long played a critical role in the development of potent therapeutic modalities against wide range classes of diseases. Studies estimate that great proportion of population in developing countries still relies on traditional remedies including various species of plants for their primary health care. More interestingly Herbal medicine is at the center of attention and demand currently and their popularity is increasing day by day (Hosseinzadeh et al., 2015; Zhang, 2015). In a wider concept herbal remedy refers to use of therapeutic herbs to prevent and treat diseases and common ailments and support health and healing process. Many drugs and medications are basically originated or prepared from plants and they presented valuable effects on health related issues. Herbal medicine is the oldest form of health care on a global scale. Studies reported that around 500 plants with medicinal properties were used in ancient literature and approximately 800 plants have been employed in indigenous systems of medicine. Herbal drugs refer to utilisation of the whole plants, parts of plants or even herbals materials to treat diseases and manage illnesses (Gunjan et al., 2015). World health organization (WHO) introduced herbal remedies as proper and labeled medicinal agents that have vigorous ingredients (Chan et al., 2011). They can be originated from secretive parts of the plants, non-secretive parts or combinations. Food and drug administration (FDA) and WHO have implemented precise and comprehensive guidelines for the evaluation of the safety, efficacy, and quality of herbal medicines (Lam et al., 2010). Generally, herbs are safe compounds because they are derivate from natural sources. In addition to their safety, administration and consumption of herbal remedies is cost effective (Marks et al., 2002). Many of the synthetic medication and drugs are responsible for wide range of toxicity and adverse effects on body organs. This leads to rapid demand and increases in the number of herbal drug manufacturers (Abdullah et al., 2003). Ease of application and consumption with no need for prescription make them more popular for customers. Herbal remedies were investigated for the probable toxic effects and some of them have been discontinued while others have been modified or combined with additional herbs to counterbalance side effects (Gerber et al., 2006). Some natural products have even been considered as important sources of potential anticancer agents. Common examples that

have been associated with anti-cancer activity include ginger (Karna et al, 2012), garlic (Vaidya et al, 2008), Curcumin (Abusnina et al, 2015) and dandelion (Ovadje et al, 2011; Chatterjee et al, 2011). Among all the natural plants a traditional method for treatment of human papillomatosis (wart) in some rural areas of developing countries is the use of the fig tree (Ficus carica) latex as a localised treatment (Zargari, 2000). Ficus carica and its potential biological activities were investigated in this research project and are discussed in detail in the section below.

1.14.2 Ficus Carica

Ficus carica is a well-known member of the genue Ficus and commonly referred to "fig". Fig tree is one of the first plants cultivated by human and originated from Middle East and eastern Mediterranean and south Asia. Globally, Fig fruit itself is in use in both fresh and dried forms. Its common edible part is fleshy and hollow. The dried form is the source of vitamins, minerals, phenolic and poly phenolic compounds, carbohydrates, sugars and organic acid. Some studies reported that the overall amount of the phenolic compounds in fig is higher than wine and tea that are introduced as the great sources of these compounds (Bandelji et al., 2007). In addition to its fruit, fig root and leaves are also promised some therapeutic properties in management of common ailments including anti-inflammatory and antispasmodic remedies and treatment of gastrointestinal and respiratory diseases. Fig latex presents as viscous in the plant in extremely small quantities. Fig latex (Ficus carica), when applied to low risk human papillomavirus (HPV) related skin warts has shown potential for cure without inevitable tissue injury and remedial complications (Ghazanfar, 1994). Despite the fact that other parts of fig fruit like latex have also been reported to have little known pharmacological properties. The first scientific study regarding the possible therapeutic properties of fig latex was performed in 1940s (Ulman et al., 1945). High dose of injected fig latex to rats was toxic and lethal to them whereas lower doses showed inhibitory effect on the growth of tumor however this study was very basic and reported limited results. Latex from fig tree was used in Iranian folklore medicine (figure 17) for treatment of papilomatosis. Hemmatzadeh et al., (2003) showed the therapeutic effects of fig latex on bovine papillomatosis caused by bovine papillomaviruses. Fig latex also showed cytotoxicity

activity against Raji melanoma cells and it was contributed to the inhibitory properties of 6-O-acyl-beta-D-glucosyl-beta-sitosterols (Rubnov et al., 2001). Hexanic extract of fig latex also inhibited viral activity of Herpes simplex viruses (HSV), Echovirus (ECV-11) and Aleutian disease virus (ADV) and suggest the capabilities in controlling the infections (Lazreg Aref et al., 2011). Mostafaie et al (2010) showed that fig latex have anti-angiogenesis properties by using human umbilical vein endothelial cells (HUVEC). Fig latex also offers various therapeutic effects such as anti- bacterial activity (Al sabawi et al., 2008), anthelmintic (Stepek et al., 2005), destroyed cutaneous forms of avian pox (Abid and Khotam 2013), and alleviated invasiveness of glioblastoma multiformis (Tezecan et al., 2015). These therapeutic properties might be due to the presence of various enzymes and phenolic compounds in fig tree latex (Mahmoud et al, 2010; Tezcan et al., 2015).

Figure 17. Application of fig fruit latex on warts for therapeutic purpose (Adapted from www.123RF.com).

Aims of Ph.D. project

As discussed in section 1.12.1 infections by high risk HPVs especially type 16 and 18 are implicated as causative agent in cervical cancer. To date, there has been no satisfactory treatment for cervical cancer as most of the current treatments (e.g., surgical excision, chemotherapy and cryotherapy) are eventually accompanied by excessive tissue injury. Therefore, there is a continuing demand for development of safer and effective strategies towards treatment. Natural products like Ficus carica when applied on human HPV related skin warts and in bovine papilloma caused by bovine papillomaviruses, shown potential as a therapeutic modality. Based on the above rationale, the aim of this PhD study was to explore the *in vitro* biological activities of fig latex and elucidates its possible mechanisms of action on cervical cancer cell lines CaSki and HeLa positive for HPV type 16 and 18, respectively.

Specific objectives were included:

To characterise the chemical constituents of fig latex that may contribute to the exhibition of therapeutic activities.

To investigate in vitro biological activity of fig latex on transforming properties of cervical cancer cells such as cell growth, cell proliferation, cell invasion, cell migration and apoptosis.

To explore in vitro biological activity of fig latex on HPV oncoproteins (E6 and E7) and their target tumor suppressor proteins p53 and Rb respectively as well as HPV diagnostic marker protein (p16 tumor suppressor proteins).

Chapter Two
Materials and Methods

2. Materials and Methods

2.1 Materials

2.1.1 Antibodies

-Abcam (Cambridge, UK)

Anti-ki67 antibody (cat: ab92742)

Gout Anti-Rabbit (cat: ab6717)

Anti-HPV16 E6 + HPV18 E6 [C1P5] (cat: ab70)

Anti-HPV16 E7 (cat: ab30731)

Anti-HPV18 E7 (cat: ab100953)

Anti P53 antibody [PAb 1801] (cat: ab28)

Anti Rb Antibody [Rb1 1F8] (cat: ab24)

-LI-COR Bioscience (Cambridge, UK)

IRDye 800CW donkey anti-, mouse IgG (cat: 925 68022)

Chameleon duo pre-stained protein ladder

-Santa Cruz (Heidelberg, Germnay)

B-Actin Antibody (cat: sc-47778)

-Sigma (Suffolk, UK)

Anti p16 antibody (cat: MAB4133)

2.1.2 Cell Lines

-American type culture collection, ATCC

HeLa; expressing HPV type 18 **(England, UK)**

CaSki; expressing HPV type 16 **(England, UK)**

- Kind gift from Dr Ashrafi, Glasgow University

Immortalised human keratinocyte cells (HaCaT)

2.1.3 Cell culture materials

-Kingston University London

Sterile phosphate buffered saline (PBS)

-American type culture collection, ATCC

Eagle`s Minimum Essential Medium (EMEM)

-Becton Dickinson Labware (Oxford, UK)

Falcon 2098 polupropylene tubes

-Life Technologies (UK)

Prolong diamond antifade mounyant with 4-6-diamidino-2-phenylindole (DAPI)

-Bibby Sterilin Ltd (Staffordshire, UK)

Sterile plastic universal containers

-Costar Corporation (Bucks, UK)

6 well plates

96 well plates

-Elkay International (Basingstoke, UK)

Microcentrifuge tubes

-Invitrogen (UK)

Dulbecco`s Modified Eagles medium (DMEM), high glucose

Bolt™ 4–12% Bis-Tris gel

Extra cellular matrix (ECM)

-Sigma (Suffolk, UK)

Roswell Park Memorial Institute medium (RPMI-1640)

Penicillin (100 U/ml) and streptomycin (100 μg /ml)

Foetal calf serum (FCS)

Trypsine-ethylenediaminetetraacetic acid (EDTA) solution 0.25%

BSA (bovine serum albumin) solution

Whatman cellulose filter

Disposable filter membrane 5 μm

Bright-Line hemocytometer

MTT (3-(4,5-Dimethylthiazol-2-Yl)-2,5-Diphenyltetrazolium Bromide) reagent (cat: M6555-100MG)

Crystal violet

24-well Transwell chamber with pore size 8 μm

-Nune (Hereford, UK)

Cryotubes

-Fisher Scientific (Loughborough, UK)

Filter Whatman No. 1

Tissue culture flask sterilized filter cap 75 cm

X960 ST RACK GRADUATED 1250 TIP sterile

- VWR (Van Waters and Nat Rogers) (Leicestershire, UK)

Slides with 90 ground edges, super frost white

Round cover slips, German glass

-Merck (Darmstadt, Germany)

Ethanol gradient grade

2.1.4 Chemical and Reagents

-Carl Roth (Karlsruhe, Germany)

Isopropanol (Rotisolv® HPLC grade)

Acetonitrile (Rotisolv® HPLC ultra gradient grade)

Chloroform (Rotisolv® HPLC grade)

Tetra-dodecylammonium bromid

Ammonium formate

-Kingston University

Ethanol

- VWR (Van Waters and Nat Rogers) (Leicestershire, UK)

Dimethyl sulfoxide (DMSO)

-Fisher Scientific (Loughborough, UK)

Resolving buffer (Tris(hydroxymethyl)aminomethan [Tris] / SDS [sodium dodecyl sulphatel)

Poluoxyethylene sorbitan monolaurate (tween 20)

Resolving buffer (Tris/SDS)

Tris base

-Sigma (Suffolk, UK)

Triton™ X-100

Bradford assay reagent

Formic acid (puriss., ≥ 98% (T) for mass spectrometry),

Acetic acid

Formaldehyde

Acetonitrile (MeCN)

Dichloromethane (DCM)

Trichloromethane

Petroleum ether

Sterile dimethyl sulphoxide (DMSO)

Sodium formate solution

-Invitrogen (Loughborough, UK)

PI/RNase Staining Solution (100 mL)

RIPA buffer

Bolt® Transfer Buffer (20X)

20X Bolt® MES SDS Running Buffer

2.1.5 Materials for chemical analysis

- Goss Scientific (Cheshire, UK)

3mm Wilmad tubes

NMR Tubes (527-PP-7)

-Sigma (Suffolk, UK)

Deuterium oxide (D2O)

-GPE Scientific (Leighton Buzzard)

Standard Series Tube HT 7" Economy High Throughput, 5mm

-Smith Scientific (Edenbridge, UK)

Hi-Vac Greaseless Stopcock, Right Angled arm with plain end, Fine Thread, 8mm bore,

B24 cone with tip, 80mm from bore to cone tip (RA-FI-8/B24/SP1)

2.2 Methods

2.2.1 Collection of fig latex

Fig fruit latex was collected drop-by-drop without squeezing over summer months from unripe fruits of fig trees in Solughan (Tehran-Iran) (Figure 2.1.).

Figure 2.1. Collection of fig fruit latex drop by drop during summer time from Solughan, (Iran).

2.2.2 Preparation of latex

The collected fig latex was initially filtered using Whatman No. 1 to remove any impurities. Afterwards, a part of collected fig latex was lyophilized using freeze-drying technique. The freeze-dried latex was used for chemical analysis of fig latex.

As fig latex consists of two phases; polymeric gum and aqueous phase, a part of the collected fig latex was centrifuged at 4 °C and x 1000-rpm in order to separate the polymeric gum from the aqueous phase properly.

Therefore three different samples including; lyophilised fig latex, polymeric gum of fig latex and aqueous part were obtained and were preserved at – 20 °C to be used for experiments.

Additionally, in order to narrow the chemical compounds in fig latex, the above separated aqueous part of fig latex (centrifuge) was extracted using various chemical compounds. A

number of chemical agents such as; MeCN (Acetonitrile), DCM (Dichloromethane), Trichloromethane and petroleum ether were employed in order to extract the fig latex.

However, as the petroleum ether extraction showed the better response from biological perspectives the aqueous phase of fig latex was extracted three times with petroleum ether at 65 °C with stirring for 30 minutes and the residue was preserved at -20 for further investigation. Serial dilutions technique was used to prepare range of concentrations for biological studies (Figure 2.2).

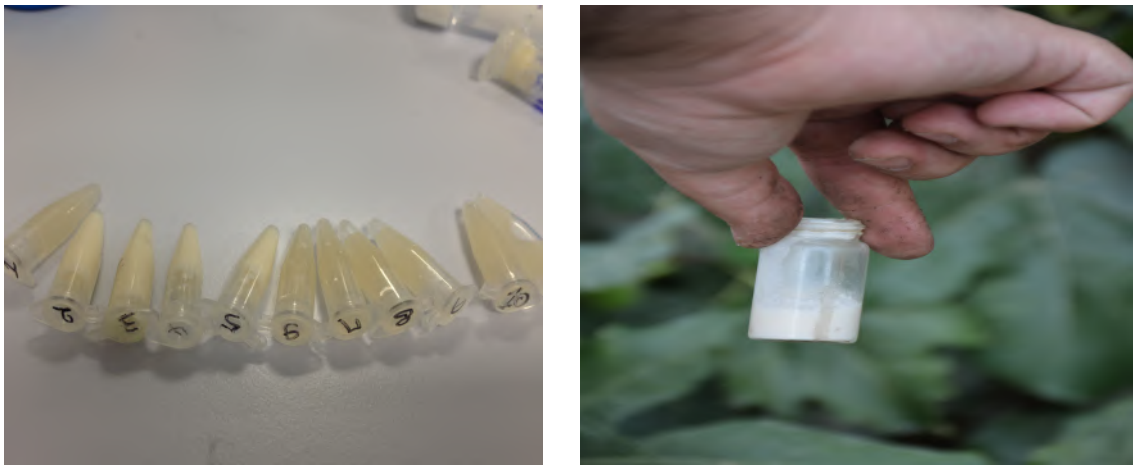

Figure 2.2. Collected fig latex was then filtered, centrifuged and was aliquoted for further analysis.

2.2.3 Chemical analysis

Chemical analysis of fig latex extract (sections 2.2.3.1 - 2.2.3.4) was kindly carried by Dr Adam le Gresley and his collaborators (Department of Life Sciences & Chemistry Faculty of Health, Jacobs University Bremen, Germany).

2.2.3.1 NMR analysis

A Bruker Avance III 600MHz NMR spectrometer with 5mm TXI Probe and temperature control unit was used for all ^{1}H NMR experiments. Both 5mm Bruker Single Use NMR tubes (biofluid and DOSY) and 3mm Wilmad tubes (Pellet) obtained from (335-pp-7) were used. All spectra were acquired on Topspin 3.1 (Bruker, Germany). All NMR experiments were carried out at 25°C. The Pulsecal routine was run prior to all experiments and the pulse angles adjusted accordingly for total correlated spectroscopy (TOCSY) and diffusion ordered spectroscopy (DOSY) experiments. For NMR Experiment gummy compartment, lyophilized latex and aqueous phase were used.

^{1}H spectra for fig latex samples were acquired using 65 536 complex data points over a sweep width of 20.57 ppm using a pre-saturation of the water signal at 4.7ppm and one spoil gradient. Serum samples were buffered to pH 7.0 (+/- 0.1) and a 700ul aliquot was diluted with 300uL D_2O. ^{1}H spectra were acquired using a 60° pulse angle and 32768 data points were collected over a SW of 11.9878 ppm with a relaxation delay of 10s. Pellet samples were prepared by dissolving the pellet in 200µL water (dd) with 20µL D_2O added for lock signal. ^{1}H pellet data was processed using an exponential windows function with LB=1 with 5th order polynomial baseline correction. ^{1}H biofluid data was processed using an exponential windows function with LB=1 with 3rd order polynomial baseline correction.

2D TOCSY data was collected using a phase sensitive homonuclear Hartman-Hahn transfer DIPSI2 sequence with water suppression using excitation sculpting with gradients (DIPSI2ESGPPH). 2048 data points were collected over a SW of 10.212 using 8 scans. Relaxation delay was set to 1.5s and a TOCSY mixing time of 60ms. The homospoil pulse length is 1000µs with a recovery delay of 0.2µs. 2D TOCSY data was processed using a QSINE window function phase shifted by 90° (LB = 1) using a 5th order polynomial baseline correction.

Diffusion spectra were obtained over 64K data points (SW 10.3112ppm) using Longitudinal Eddy current Delay bipolar pulsed field gradient with 2 spoil gradients and pre-saturation sequence (LEDBPGPPR2s). This was used to obtain the diffusion series with δ = 4.6 ms and Δ = 125ms. The relaxation delay was set to 4s and the diffusion ramp consisted of 64 linear gradient steps, from 2-95% gradient intensity, each consisting of 16 scans.

Diffusion data was processed using a sine bell shaped window function phase over all data points prior to Fourier transformation (16 384 points) using Topspin 3.0 (Bruker, Germany). Diffusion data was processed using DOSY Toolbox, created by Mathias Nilsson, Manchester University. Individual peaks were fitted exponentially after a 2nd order polynomial baseline correction was employed. Errors in diffusion coefficient were calculated based on the Standard Deviation for each diffusion curve and are in line with the estimated error as reported for a similar mixture of *ca* 0.1 $\times 10^{-10}$ m^2s^{-1} . The Residual Sum of Squares for each of the was processed using DOSY Toolbox, created by Mathias Nilsson, Manchester University and a sine bell shaped window function phase over all data points prior to Fourier transformation (16 384 points) by Topspin 3.0 (Bruker, Germany).

2.2.3.2 HPLC chromatographic conditions

Lipid molecular species were separated using an HPLC equipment (Agilent 1100 series, Waldbronn, Germany). For HPLC analysis, a concentration of 0.045 mg/mL in chloroform/ethanol (50/50) of ficus oil extract (Petroleum ether) was prepared. The column used in this study was a Pursuit XRs C18 (250 mm × 3 mm i.d., 5 μm particles). The temperature of the column oven was set to 35 °C. 3 μL of sample were injected. Solvent A consisted of acetonitrile with 0.01% formic acid and solvent B consisted of ethanol with 10 mM/L ammonium formate and 0.01% formic acid. The mobile phase was pumped through the column at a flow rate of 0.6 mL/min. The gradient elution program consisted of holding solvent steady A (100) for 5 min; followed by a linear gradient to solvent A/B (70/30) for another 5; then by a linear gradient to solvent B (100) for 90 min, and ending with isocratic elution at solvent B (100) for 10 min. The column was equilibrated at 100 solvent A for 5 min before reuse.

2.2.3.3 High-resolution mass spectrometry conditions

High-resolution mass values were acquired using a time of flight MicrOTOF Focus mass spectrometer (Bruker Daltonics UHT Ultra, Bremen, Germany) fitted with an ESI source used as the detector with the following parameter settings: capillary voltage of 4.5 kV; nebulizing gas

pressure of 2 Ba; drying gas flow rate of 10 L/min; drying gas temperature of 220 °C. ESI mass spectra were measured in the range of m/z 200–1200 in the positive ion mode. Internal calibration was achieved with 10 mL of 0.1 M sodium formate solution injected through a six-port valve prior to each chromatographic run. Calibration was carried out using the enhanced quadratic mode. For this experiment petroleum ether extraction of fig latex was used.

A number of chemicals such as Ethanol gradient, isopropanol (Rotisolv® HPLC grade), acetonitrile (Rotisolv® HPLC ultra gradient grade), chloroform (Rotisolv® HPLC grade) , Tetra-dodecylammonium, ammonium formate LC-MS Ultra and formic acid (puriss., ≥98% (T) for mass spectrometry), and acetic acid were used for chemical analysis and adjustment of methods and equipment. Ethanol was subjected to distillation prior use.

2.2.3.4 Tandem Mass spectrometry conditions

LC-tandem MS was carried out using an Ion-Trap detector in positive ion mode equipped with an ESI source (Bruker Daltonics UHT Ultra, Bremen, Germany). The full scan mass spectra were recorded in the range m/z 200-1200 operating in positive ion mode. Capillary temperature was set to 350 °C, drying gas flow rate of 10 L/min and nebulizer pressure of 10 psi. Tandem mass spectra were acquired in Auto MSn (smart fragmentation) using a ramping of the collision energy. For this experiment petroleum ether extraction of fig latex was used.

2.2.4 Cell lines and culture

Non-cancerous human immortalised keratinocytes cell line (HaCaT), cervical cancer cell lines CaSki and HeLa expressing HPV type 16 and 18, respectively, were used for this study. CaSki cells were maintained in Roswell Park Memorial Institute medium (RPMI-1640), HeLa cells in Eagle`s Minimum Essential Medium (EMEM) and HaCaT cells in Dulbecco`s Modified Eagle`s Medium (DMEM). All medias were then supplemented with 10% Fatal Calf serum (FCS) and penicillin (100 U/ml) and streptomycin (100 µg /ml). All cells were incubated in a 5% CO_2 incubator at 37 °C.

2.2.4.1 Cell culture

The whole cell culture work was performed considering strict aseptic methods/techniques inside laminar flow hood. Cells in flaks, plates and other materials were incubated in a dry atmosphere at 37 °C containing 5% CO2.

2.2.4.2 Maintenance of the cells

Cells well grown and cultured in the appropriate media as suggested by resource companies. Cell were fed three times weekly, during which old medium was removed from subconfluence culture flask and then fresh medium which had been warmed at 37 °C in water bath, was added. Once cells were reached to confluence of around 90%, trypsinisation was performed to prepare a new flask. Trypsine is a proteolytic enzyme that breaks down proteins and helps to dissociate adherent cells from the flaks or plates in which they are being cultured. Initially, the medium was removed and cells were washed twice with sterile PBS. Then the PBS was aspirated and trypsine-EDTA solution 0.25% was added to the cell. Flasks were incubated for 3-5 minutes until cells had detached properly form the flask. The cell suspension was added to two volumes of an appropriate media to deactivate/neutralize the effect of trypsin on cells. Cells were pelleted using centrifuge at 1000 rpm for 5 minutes. The cell palette was re-suspended in a fresh media and appropriate amount of the cells was added to the flask/plate.

2.2.4.3 Cell counting

Cell counting was an essential step in both molecular and functional studies to ensure same amount of the cells were added for each experiments. Cell counting was performed using trypan blue, which stains dead cell in dark blue. Approximately 10 μl of cell suspension was added into haemocytometer with a coverslip. The total number of non-coloured cells in each of the 4 open squares was counted under the 10x objectives. Afterwards, the mean was calculated, multiplied by 10^4 (because the volume of each chamber is 10^{-4}) and dilution factor to obtain the number of cells per ml.

2.2.4.4 Long-term cell storage

For long term storage, cells were trypisnised and pelleted was collected as described in section 2.2.7.2. Cell counting was performed to obtain approximately $1x10^6$ cell/ml. A cell suspension including 50% fresh media and cells, 10% (v/v) sterile DMSO and 40% (v/v) FCS was prepared. DMSO was employed as a cryo-protective. Cell suspension was aliquoted into 1 ml nunc cryo-tubes. Tubes were labeled properly, placed in polystyrene box and stored at -80 °C overnight. The following day tubes were placed in liquid nitrogen for further practice. When required, tubes were taken from liquid nitrogen, immediately placed into water bath to increase the temperature to 37 °C. Once properly defrosted, cells were added to flask.

2.2.5 MTT assay

The effect of fig latex on cell growth was determined by cell cytotoxicity assay employing MTT powder (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide). MTT is a colorimetric assay for assessing cell metabolic activity. Cell suspensions (4000) were plated in 100 µl of media/well. After overnight incubation, supernatant was removed and 180 µl of fresh media was added into each well. Serial dilutions of samples were prepared by dissolving Fig latex in an appropriate media and various concentrations (0.125, 0.25, 0.5, and 1 µg/ml) of fig latex were added. The treatment was performed for 24, 48 and 72 hours. Then 20 μl of MTT solution 5mg/ml in phosphate buffer solution (PBS) was added into each well and plates were cultivated at 37 °C in 5% CO2 for 3-4 hours. As shown in figure 2.3 the color of the plate has changed after cultivation with MTT. Afterward, supernatant was removed, the remaining formazan crystals

were dissolved in 100 µl of DMSO and the absorbance was measured at 540nm with Epoch plate reader (BioTek, UK) considering wells without cells as blank. Obtained values were analysed with Gen5 software (BioTek, UK). The percent of viability in each treated cell line was normalised based on their control wells. All experiments were performed at least in triplicate (Figure 2.3).

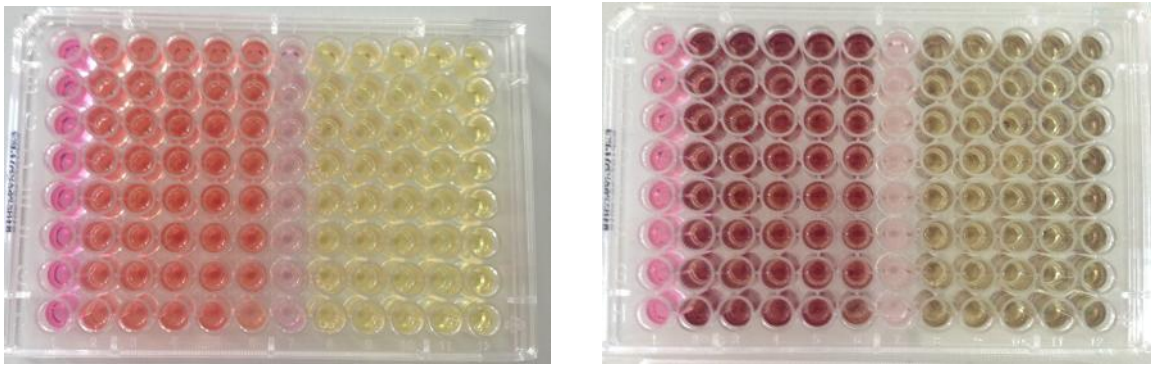

Figure 2.3. 96 well plate. Left plate shows the cells color before adding MTT. Right plat shows the color change after adding MTT solution to the cells.

2.2.6 Colony formation assay

Cervical cancer cells (CaSki and HeLa) and non-cancerous cell (HaCaT) were plated at approximately 600 cells/well in 2.5 mL media in 6-well corning plate and cultivated for 12 hrs to adhere. After that, cells were treated with appropriate concentrations of fig latex for period of 14 days. The cells then were washed 3 times with PBS, fixed in 3.7 % formaldehyde and stained with crystal violet. Crystal violet was prepared by dissolving 2 g of crystal violet in 20 ml of 95% ethyl alcohol. Visible colonies (~35-50 cells) were counted and presented as the ratio of the number of colonies in treated cells divided by the number of colonies in the control ones.

2.2.7 Cell migration and invasion assays using Transwell

Cell migration assay was performed using 24-well Transwell chamber with pore size 8 μm. In total, 250 μl of cell suspension (50,000) without FCS was added into upper chamber. Lower chamber was filled with medium containing 10% FCS as a chemoattractant. Fig latex with concentration of 0.125 μg/ml was added to upper chamber. Cells were then cultivated at 37 °C for 24 hours in order to migrate from upper chamber towards lower one. For cell invasion assay 250 μl of ECM was added to lower chamber. Cells from upper chamber were removed gently with cotton swap, washed 3 times in PBS and fixed with 3.7% formaldehyde for 30 min. The cells invading the lower chamber were stained with 0.2% crystal violet for 30 min. Crystal violet was prepared by dissolving 2 g of crystal violet in 20 ml of 95% ethyl alcohol. All experiments were repeated all least three times. Number of cells was counted in 5 fields randomly and was scored based on their untreated wells.

2.2.8 Analysis of cell cycle distribution by Fluorescence Activated Cell Sorting (FACS)

Cell cycle distribution was assessed by flow cytometry. Cells were seeded into T75 flasks; once cells were confluent they were treated with concentration of 0.125 μg of fig latex for 48 hrs. Approximately ($1x10^6$) cells including dead and alive ones were harvested from both untreated and treated flasks. Then cells were washed three times with cold PBS and fixed in 70% ice-cold ethanol for 1 hr. Cells were then washed two times in PBS and centrifuged at 1000 rpm to collect the pellet. The amount of 500 μl of PI/RNase staining solution was added to each sample for 20

minutes at 37 °C. Propidium Iodide (PI) is a well-known fluorescent vital dye that is able to stain DNA and RNA. PI binds to the both DNA and RNA. RNA will be removed thorough digestion with ribonucleases leading to optimal DNA resolution. Consequently, the content of DNA as determined by flow cytometry, can reveal useful information about the cell cycle. Stained cells were then excited at 488 nm using the FL-3 detector (620 nM) of a BD FACS Calibur flow cytometer (Becton-Dickinson). Acquired data was analysed using CellQuest software (Becton-Dikinson).

2.2.9 Detection of cell proliferation marker, Ki67, by Immunofluorescence microscopy

The Ki67 protein was visualised by immunofluorescence technique. HaCaT, CaSki and HeLa cells were grown in T75 flaks with an appropriate media until sub-confluent. After removal of the medium the cells were washed three times with PBS and detached from the flask with trypsin/EDTA and pelleted at 1000 rpm for 4 minutes at room temperature. For this assay, 150 µl of cell suspension (1000 cells) were seeded on the cover slips in 24 well plates and were incubated for 24 hour in order to attach. The following day media was removed and cells were treated with concentration of 0.125 µg of fig latex for additional 48 hours. After that, cells were activated in serum free media for 10 min in room temperature. Media was removed and cells were fixed in formaldehyde 3.7 % for 30 minutes. Cells were permiabilised by adding 250 µl of permeabilising solution, PBS containing 0.1% triton-100, for 5 times and 5-7 minutes per wash on rotator. Cells were then blocked by adding BSA/PBS 3% for 90 minutes at 37 °C on moderate plate shaker at room temperature. Afterwards cells were incubated with 250 µl of primary antibody, Anti-ki67 antibody for 2 hrs with gentle shaking at 37 °C. Antibody was diluted in BSA/PBS 1% with final dilution 1:200. Following that, cells were washed with 1x PBS plus 0.2 % tween 20 for 5 times each wash 5 minutes. Secondary antibody Gout Anti-Rabbit FITC (Fluorescein isothiocyanate) was diluted 1:250 in tween 0.2% and added to the cells and it was incubated for 90 minutes with gentle shaking at room temperature. Later on cells were washed 5 times with 1xPBS plus 0.1% Tween 20 for 5 minutes at 37 °C. After final wash the coverslips were mounted onto slides with 3-5 µl of DAPI (4′,6-diamidino-2-phenylindole) (for nucleus staining). The images were captured using LEICA TCS SP scanner microscope at 488nm

wavelength for FITC, 405 for DAPI. Images were analyzed by Leica Confocal Software (Heidelberg, Germany).

2.2.10 Protein quantification Bradford assay

To load same amount of the protein for all the treated and untreated cells with fig latex, Bradford assay was performed. To determine the actual concentration of a protein a standard curve was plotted using absorbance and a varying amount of some known protein. Bovine serum albumin (BSA, 1 mg/ml) was used as standard. The various amount of BSA (0, 2, 4, 6, 10, 15 and 20µl) were assigned into wells of a 96-well plate. Samples were reconstituted in 500 µl of protease inhibitors solution and then 20 µl of each samples were transferred in 96 well plates. Afterwards appropriate amount of Bradford reagent was added in all wells (Total volume to 200 µl) and was mixed gently using pipette. The absorbance of wells was measured with Epoch plate reader (BioTek, UK) at 570nm. A standard curve has been given by excel program based on the linear regression of the line. Therefore, the protein concentration in all samples was determined from BSA standard.

2.2.11 Detection of HPV onco-proteins and tumor suppressor proteins by Western Blotting

To investigate the effect of fig latex on HPV onco-protein (E6 & E7), tumor suppressor genes (P53 & Rb) and cervical cancer marker protein (P16), standard semi-dry western blotting technique was used. Cells were grown until sub-confluent in T75 flaks. For western blotting technique proteins were extracted using RIPA buffer. RIPA is a lysis buffer employed to lyse the cells for western blotting technique. Cells were then treated with concentration 0.125 µg/mL of fig latex for 48hrs. Afterwards, cells were detached and transferred to microfuge and re-suspended in 1 ml of PBS, centrifuged and pelleted. Supernatant was discarded and pellet was re-suspended in 0.25 ml of RIPA buffer. Later on samples were shaken gently on ice for 10 minutes. Following that samples were centrifuged at 14000 rpm for 15 minutes to pellet cell debris.

Proteins were quantified using Bradford assay as explained before and equal amounts (10µg) of protein lysate from each cell line were boiled at 95 °C for 5 minutes. Boiling ensured that samples were properly denatured. In addition, high temperature loosens up samples gummy from DNA and cellular debris, making the samples easier to load. Samples were then added into western blot gels. Each electrophoresis tank was filled with running buffer. Electrophoresis power supply was set up on 150 V for 45-50 minutes. Protein were electrophoresed using Bolt™ 4–12% Bis-Tris gel and transferred on to 0.2 µm Nitrocelulose membrane using MES running Buffer: 50 mM MES, 50 mM Tris-HCl, 0.1% (w/v) SDS, 1 mM EDTA, pH 7.3. Protein ladder was used to estimate the size of proteins resolved by gel electrophoresis. Following the migration of band towards the bottom of the gels, gels were removed from electrophoresis tank and proteins were transferred to nitrocellulose membrane using semidry blotting apparatus at 30V/150A for 2 hours. Before transferring to the membrane the gel sandwich was soaked with transfer buffer. The gel sandwich contains a pre-cut nitrocellulose membrane sandwiched between two filter paper. The forst sheet of filter paper soaked in transfer buffer and then was placed neatly on to the gel avoiding anu air bubbles. Then the nitro cellulose membrane was laid on the gel and then a further sheet of filter paper, all soaked in transfer buffer was added to the top. Next, the sandwich was rolled with a glass pipette to eliminate any air bubbles and place into the transfer machine and run at 30V for 2 hours. Once the transfer was completed the membranes were blocked with 5% (w/v) non-fat dry milk in TBS (Tris buffer saline)-T (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 1% (v/v) Tween20) at room temperature for 2 hours to reduce non-specific binding. The membrane was then incubated with the mAb Anti-HPV16 E6 + HPV18 E6 antibody (1:500), mAb Anti-HPV16 E7 (1:500) + HPV18 E7 (1:750) antibody, Anti P53 antibody (1:500), Anti Rb Antibody (1:1000) Anti p16 antibody (1:750) in 1% (w/v) non-fat milk in TBS-T at 4 °C overnight. Then primary antibody solution was removed and the membrane was washed with TBS-Tween buffer for 20 minutes 3 times and then incubated with IRDye secondary antibody Donkey anti-Mouse IgG (diluted at 1:10000-15000) (*Li-Cor*) at room temperature for 2 hours. Then it was incubated overnight with B-actin antibody (1:10000). B-actin was employed as a loading control to normalise the levels of protein detected and also confirm that protein loading is the same for all samples.Then secondary antibody (goat anti-mouse) was added (1 in 10,000-15,000) and incubated for 2 hours. The membrane was then visualised using OdysseyCLx Imaging System (Li-cor).

2.2.12 Statistical analysis

Statistical analyses were performed using Microsoft Excel 2011 version (14.3.8). The correlations between cell viability and response to treatment were analysed using logarithmic regression of line. To calculate IC50 we obtained logarithmic regression of line ($y = a \ln(x)+b$), using Excel. Since we were looking for inhibitory concentration for 50% of the cells, y value (axis) was equaled to 50. A and b values were given by excel therefore; calculation of x value was performed. X value represents concentration. Values were presented as mean ± standard error of the mean (S.E.M.). Statistical analyses were performed employing an unpaired, two-tailed student t-test. $*P < 0.05$, $*p<0.01$, $*P<0.005$ were considered statistically significant, very significant and extremely significant.

Chapter 3

Results

Chemical analysis and cell viability assay

3. Results

3.1 Chemical Analysis and cell viability assay

3.1.1 Comparison of substances from crude, aqueous part and polymeric gum of fig latex

Plant derived natural products have received considerable attention in recent years due to their diverse pharmacological benefits. Although the medicinal properties of certain plants have been known for centuries, many of them are not determined (Gunjan et al., 2015). Fig latex is a milky fluid comprising aqueous medium and gum that makes the latex sticky and solidifies on exposure to air. Direct application of milky fluid on cells as well as preparation of serial dilution for molecular biology studies was limited due to the stickiness of latex. To overcome this restriction and determine the active biological compartment of fig latex for therapeutic approaches, three approaches were used. Initially part of fig latex was lyophilised and also aqueous part (supernatant) was separated form polymeric gum using centrifuge. A lyophilised sample of fig latex was prepared by freezing the product and lowering the pressure. Afterwards, chemical components of lyophilised, aqueous part and polymeric gum of fig latex were compared using Nuclear Magnetic Resonance (NMR) and Mass spectrometry (MS). NMR is an analytical chemistry technique used for determination the content and molecular structure of a compound. In analytical chemistry NMR is used to observe local magnetic fields around atomic nuclei. Mass spectrometry is a powerful analytical technique used to identify unknown compounds within a sample and to elucidate the structure and chemical properties of different molecules.

Crude NMR analysis (Figure 3.1) revealed significant differences between the chemical constituents of the lyophilised latex and the aqueous part of the latex. The lypholisied powder showed a mixture of saturated and unsaturated fatty acids/triglycerides and was not contained biologically active compound. The Polymeric gum did not present significant chemical components.

Therefore lyophilised powder and polymeric gum were not appropriate elements for determination of chemical constitutes of fig latex and investigation of biological activities of fig latex on cervical cancer cell lines.

Based on the data obtained from NMR, aqueous part of fig latex showed a broader spectrum of active compounds. Thus, the aqueous part of latex was compared to whole latex for biological activities on cancerous (HeLa and CaSki) and normal non-cancerous cells (HaCaT) and result from this study revealed that the aqueous part of latex was as effective as the whole latex on cells from biological activity perspectives. Coupled with this, the components in the aqueous part of the latex were easier to discern than in as the crude latex (Lyophilised latex powder). Therefore the aqueous part of the latex /supernatant was used and exclaimed as “latex” throughout this study. This phase was used for further biological and medicinal chemistry investigation.

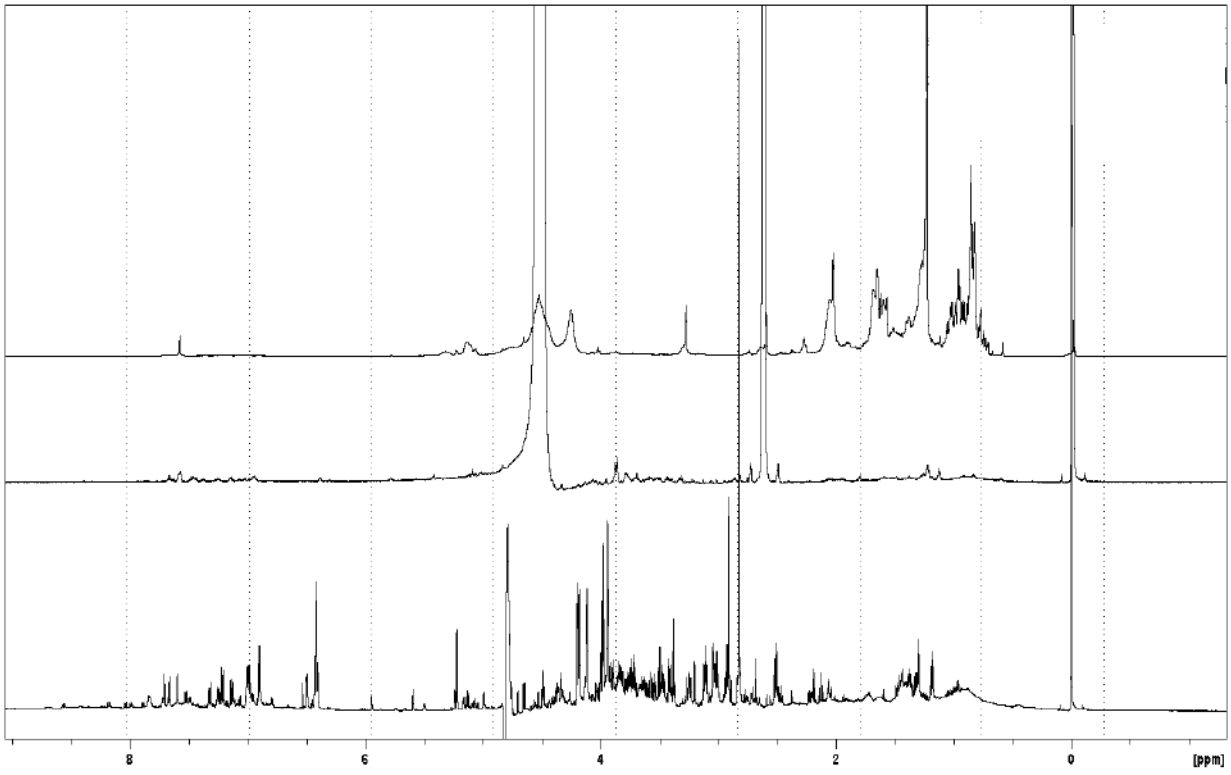

Figure 3.1. S1 ^{1}H NMR Spectra in D_2O/H_2O 90:10% of Fig latex extracts. Top – Fig Latex Gum without significant chemical components, Middle, Fig Latex Powder representing mixture of saturated and unsaturated fatty acids/triglycerides, Bottom, Fig Latex Supernatant showed more biologically active compounds.

3.1.2 Ficus carica Latex inhibits growth of cervical cancer cells

To carry out the investigation, the next stage was to determine an appropriate concentration of fig latex for biological studies. To find the best concentration of fig latex that could inhibit growth of cancer cells, cytotoxicity assay was employed. Cytotoxicity assay are widely used in in vitro studies to monitor the response and viability of cells in culture following treatment with different stimuli (Huyck et al., 2012). Various experimental methods can be used to compare the rate of proliferation of cancer cells with normal cells in the presence and absence of treatment during specific time frame. Among all, MTT assay is the most common used for detection of cytotoxicity and cell viability flowing an administration of treatment. MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) is a water-soluble tetrazolium salt that is converted to insoluble purple formazan within the mitochondria. Therefore, the formation of the purple color depends on the activity of mitochondria (Barltrop and Owen, 1991). MTT assay was used against many cell lines to explore cytotoxicity, proliferation and growth rate of cells (Mossmann, 1983).

To assess the cytotoxic activity of fig latex on cells, cervical cancer cells (HeLa and CaSki) expressing HPV type 18 and 16 respectively and HaCaT cells as control representatives of non-cancerous cells were used. In order to determine the optimal concentration of fig latex that are capable of preventing cancer cell growth, cells were treated with different concentrations (0.125, 0.25, 0.5, and 1 μg/mL) of aqueous part of fig latex for 24, 48 and 72 hours and the values were calculated as percentages of cell viability for each treatment.

Statistical analysis of MTT assay data showed a significant reduction (**$P < 0.01$, approximately 1.6-fold) of cell growth in CaSki and HeLa cells treated for 24, 48 and 72 hours with 0.125 μg/mL of fig latex while, importantly, no significant effect was found on non-cancerous control HaCaT cells. This finding suggests that, the inhibitory effect of latex on cell growth occurs in a dose-dependent but not significantly time-dependent manner. However, treatment with higher concentration of fig latex (0.25 μg/ ml, 0.5 μg/ml and 1 μg/ml) resulted in inhibition of the cell growth in all cell lines but to lesser

extent in control cells for example; 1.4-fold reduction of cell growth in control cells (HaCaT) and an approximately 2-fold decrease in cervical cancer cell lines was seen with 0.25 µg/ ml (Figure 3.2 a,b,c).

As shown in figure 3c, 0.125 µg/mL of fig latex did not show a noticeable cytotoxic effect on HaCaT cells (which was confirmed by statistical analysis) whereas it showed cytotoxic effect on HeLa and CaSki cells (Figure 2a,b). These findings suggests that the usage of 0.125 µg/mL of fig latex for 48 hrs would be best to suppress cervical cancer cell proliferation "without a toxicity" effect on the normal HaCaT cells. Therefore, this concentration was used to further investigate the anti-cancer activity of fig latex against cervical cancer cells. The aqueous part of latex was as effective as the whole latex on cells from a biological activity perspective. Therefore, the aqueous part of the latex (supernatant) was used and exclaimed as "latex" throughout of this study.

To confirm the above findings, this study further analysed the data extracted from MTT assay using IC50 assessment. IC50 values were determined using logarithmic regression of line. The half maximal inhibitory concentration (IC50) is a value of the potency of a substance or drug for inhibiting a certain biological or even biochemical function. This quantitative value represents how much of a certain drug or other compound is required to inhibit biological process by half. In other word IC50 shows the concentration of a drug that is needed for 50% inhibition in vitro (Caldwell et al., 2012). As shown in figure 3.2 (d), the IC50 values also validate that the inhibitory effect of fig latex on cancer cells growth is under dose dependent manner and 0.125 µg/mL of fig latex is the best concentration to treat the cells.

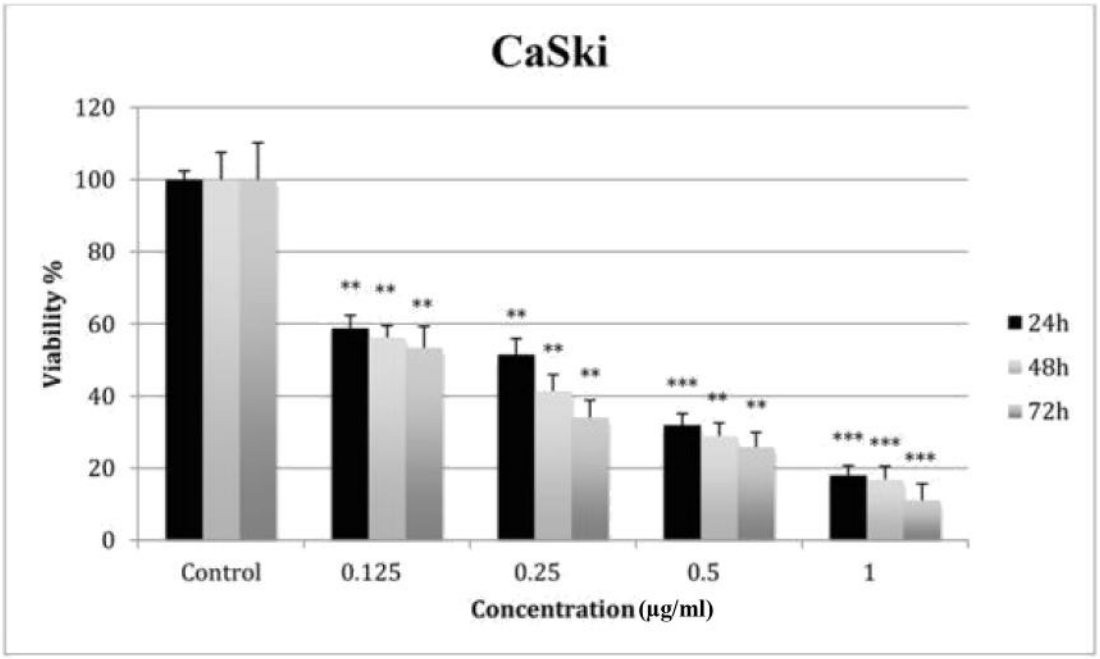

Figure 3.2(a). Fig Latex showed cytotoxicity effects on CaSki cells under dose dependent manner. Cell viability was assessed using MTT assay after 24, 48 and 72 hours of treatment with fig latex. Control represents treatment with PBS. Results represent the means of at least 3 independent experiments and were normalised to control wells. Error bars indicate SEM; *p < 0.05, **p < 0.01, ***p < 0.001 were considered statistically significant, very significant and extremely significant, respectively.

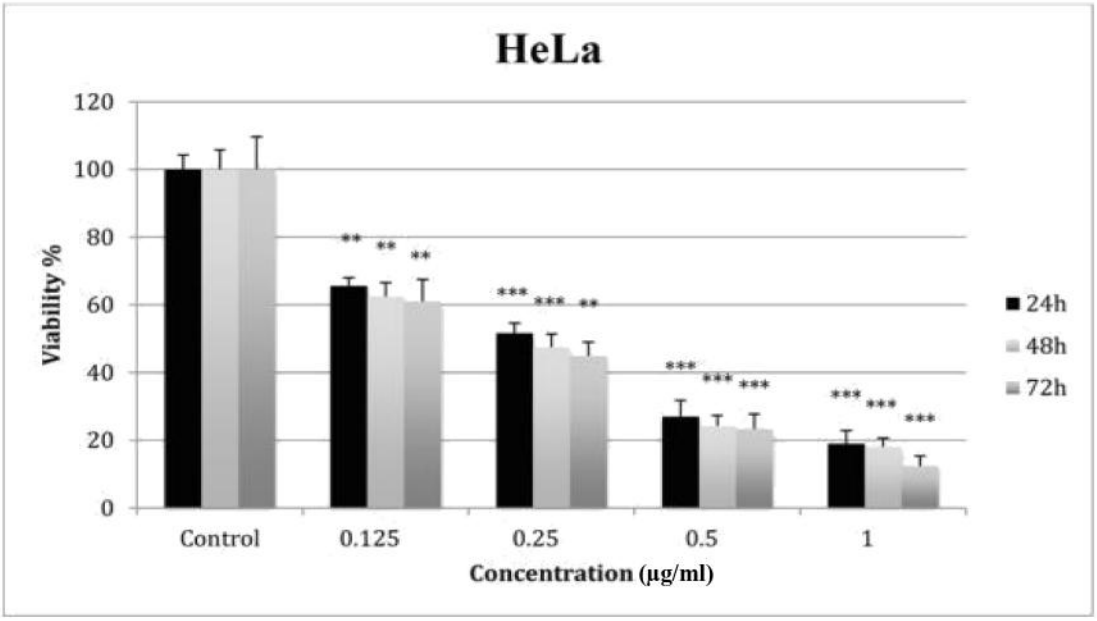

Figure 3.2(b). Fig Latex showed cytotoxicity effects on HeLa cells under dose dependent manner. Cell viability was assessed using MTT assay after 24, 48 and 72 hours of treatment with fig latex. Control represents treatment with PBS. Results represent the means of at least 3 independent experiments and were normalised to control wells. Error bars indicate SEM; *p < 0.05, **p < 0.01, ***p < 0.001 were considered statistically significant, very significant and extremely significant, respectively.

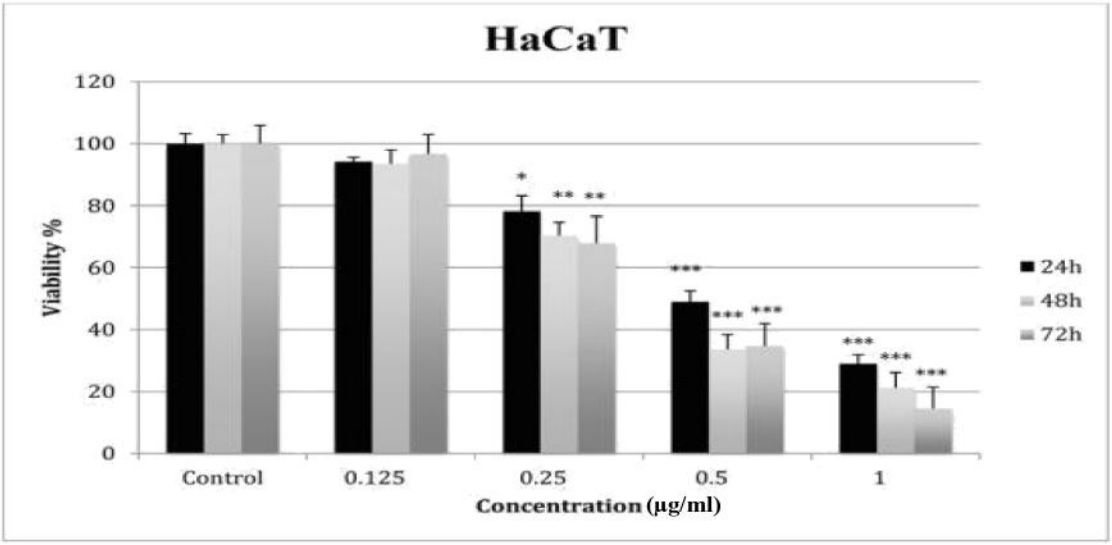

Figure 3.2(c). Fig latex showed cytotoxicity effects on HaCaT cells under dose dependent manner. 0.125 µg Fig Latex showed no cytotoxicity effects on HaCaT cells. Cell viability was assessed using MTT assay after 24, 48 and 72 hours of treatment with fig latex. Control represents treatment with PBS. Results represent the means of at least 3 independent experiments and were normalised to control wells. Error bars indicate SEM; *p < 0.05, **p < 0.01, ***p < 0.001 were considered statistically significant, very significant and extremely significant, respectively.

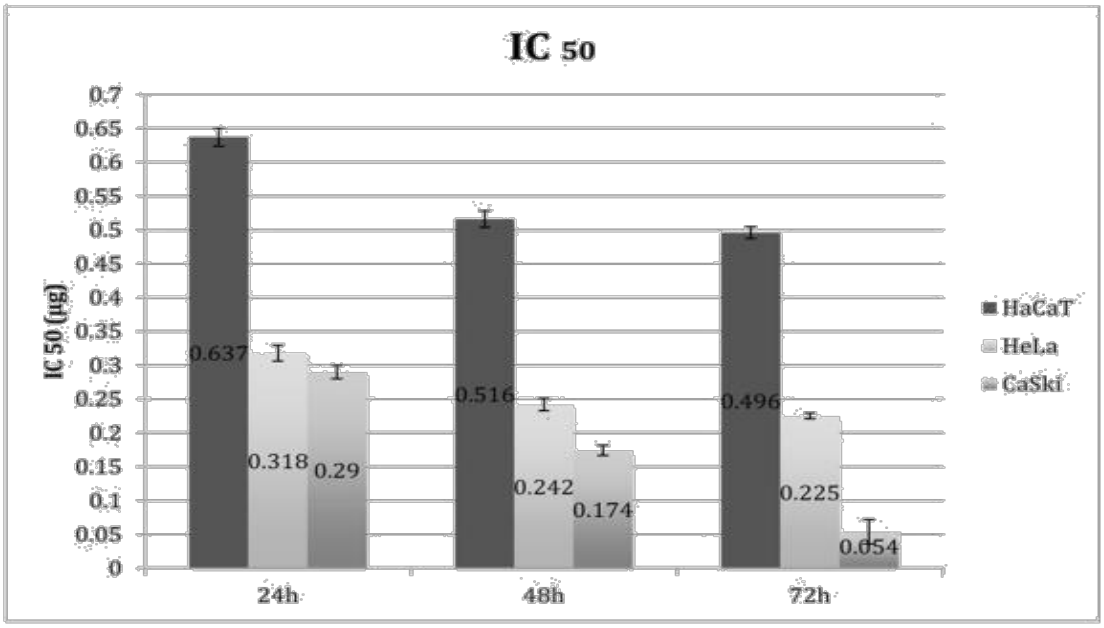

Figure 3.2(d). Determination of IC50 values following treatment with fig latex. IC50 values of HaCaT, HeLa and CaSki cells was calculated after 24, 48 and 72 hrs of treatment with fig latex. Results represent the mean of at least 3 independent experiments. Error bars indicate SEM.

3.1.3 Effects of various extractions of Ficus carica Latex on growth of cells

Data obtained from the MTT assay confirms that certain concentration (0.125ug) of the aqueous part of the fig latex is effective to prevent cervical cancer cell growth without a toxicity effect on the non-cancerous control cells. This findings provide evidence that the aqueous part of fig latex contains active chemical constitutes that may contribute to the exhibition of therapeutic activities. Hence this study aimed to identify the nature of the chemical species responsible for this activity. However, it was very difficult to investigate the chemical constitutes of the aqueous part of the latex without extraction procedures. Therefore, to overcome this problem, the aqueous part of fig latex (supernatant) was extracted using different chemical materials, such as, Acetonitrile (MeCN), dichloromethane (DCM), Chlroforme (Trichloromethane) and Petroleum ether. These extracts were tested for anti-cancer activity on cervical cancer cells expressing high risk HPV using MTT assay and were initially applied on CaSki cells. As shown in the figures 3.3 (a)-(b)-(c) and 3.4 (a)-(b)-(c) the MeCN, DCM and chloroform extractions of fig latex did not show cytotoxic activity on CaSki and HaCaT cells, indicating that these compounds were not an appropriate chemical to extract the fig latex and narrow down the compounds inside the fig latex for further chemical analysis. However, the petroleum ether extract of the fig latex supernatant showed the most potent anticancer activity (Figure 3.3.d).

Analysis of MTT assay data showed statistically very significant and extremely significant reduction (**$P < 0.01$, ***$P < 0.001$ approximately 1.7-fold) of cell growth in CaSki cells treated with 0.125 µg/mL of Petroleum ether extraction of fig latex whereas, no significant effect was found on CaSki cells following treatment with various concentration of MeCN, DCM and Triclоromethane extractions. The effect of MeCN, DCM, Triclоromethane and also petroleum ether extractions on CaSki cells was not under time dependent manner.

Overall, the 0.125 µg/ml of Petroleum ether extraction showed cytotoxic effect on CaSki cells and considering the higher concentration (0.25 µg/ ml, 0.5 µg/ml and 1 µg/ml) the inhibitory effect on cell viability was under dose dependent manner (Figure 3.3(d)).

All these extracts were applied on non-cancerous HaCaT cells as a control. Results from

this study showed that 0.125µg/ml of Petroleum ether extraction has no cytotoxicity effect on HaCaT cells and the effect was under dose dependent manner figure 3.4.(d).

Moreover, analysis of MTT assay data showed no significant effect on normal HaCaT cells growth treated with 0.125 µg/mL of petroleum ether extraction of fig latex. In contrast increasing the concentration to 0.25 µg/ ml, 0.5 µg/ml and 1 µg/ml decreased the cell viability.

These results were quite similar to the data obtained from application of the supernatant (without any extraction) of fig latex on cells and therefore petroleum ether extraction was used for further chemical analysis of fig latex.

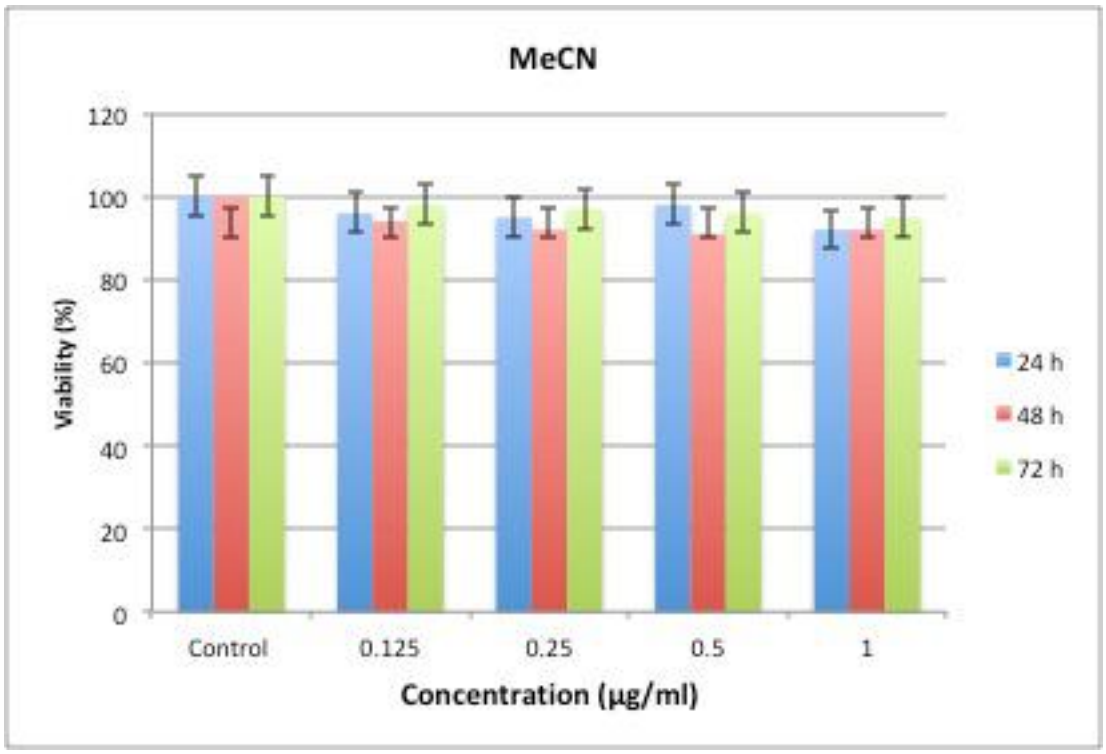

Figure 3.3(a). CaSki cells viability using MeCN extraction of fig latex. Cell viability was assessed using MTT assay after 24, 48 and 72 hours of treatment with various concentrations (0.125, 0.25, 0.5, and 1 µg/ml). Control represents treatment with MeCN. Results represent the means of at least 3 independent experiments and were normalised to control wells. Error bars indicate SEM.

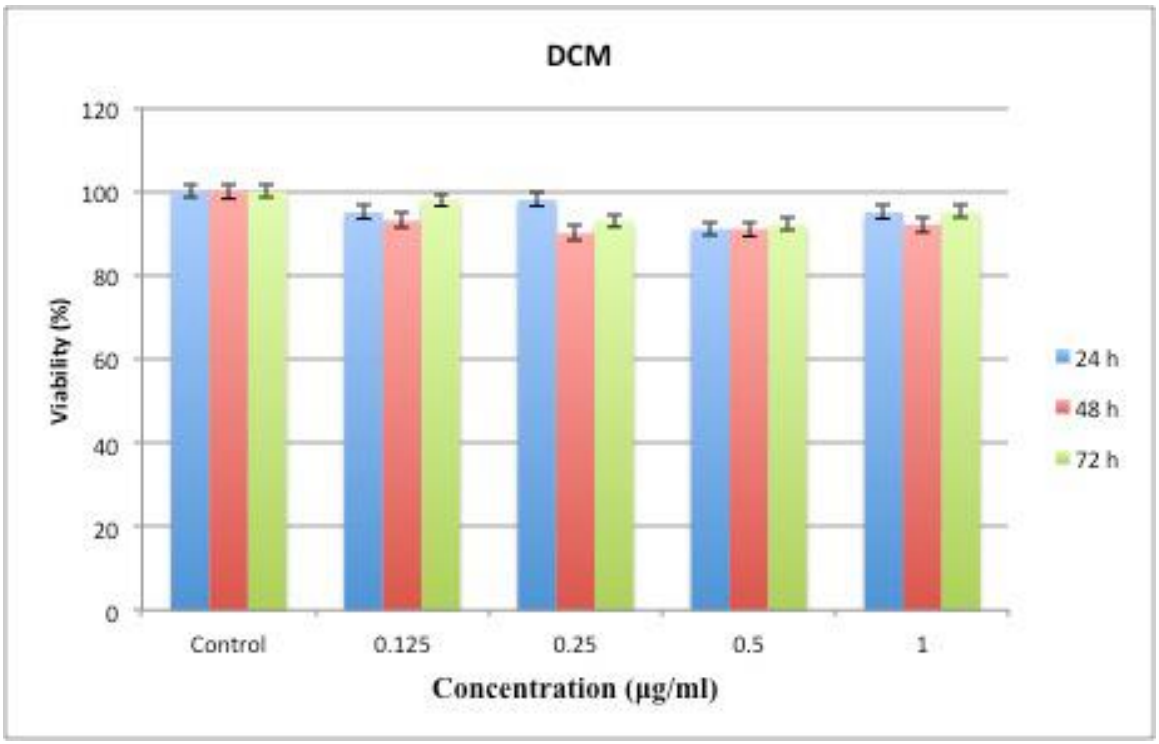

Figure 3.3(b). CaSki cells viability using DCM extraction of fig latex. Cell viability was assessed using MTT assay after 24, 48 and 72 hours of treatment with various concentrations (0.125, 0.25, 0.5, and 1 µg/ml). Control represents treatment with DCM. Results represent the means of at least 3 independent experiments and were normalised to control wells. Error bars indicate SEM.

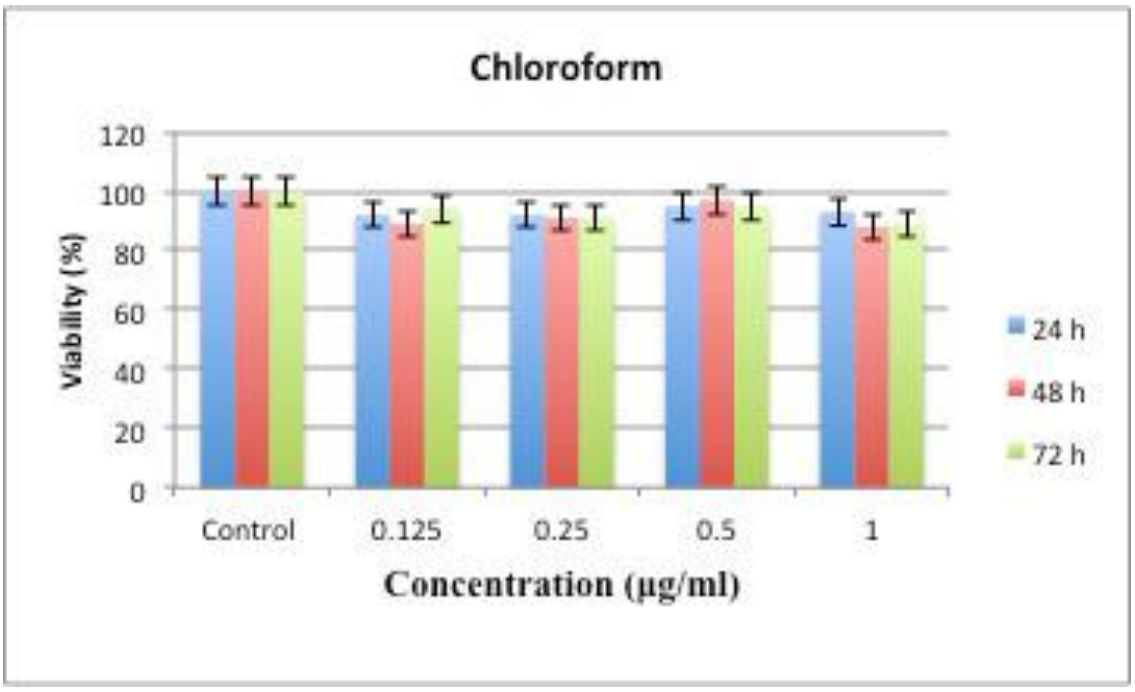

Figure 3.3(c). CaSki cells viability using Chloroform extraction of fig latex. Cell viability was assessed using MTT assay after 24, 48 and 72 hours of treatment with various concentrations (0.125, 0.25, 0.5, and 1 µg/ml). Control represents treatment with Chloroform. Results represent the means of at least 3 independent experiments and were normalised to control wells. Error bars indicate SEM.

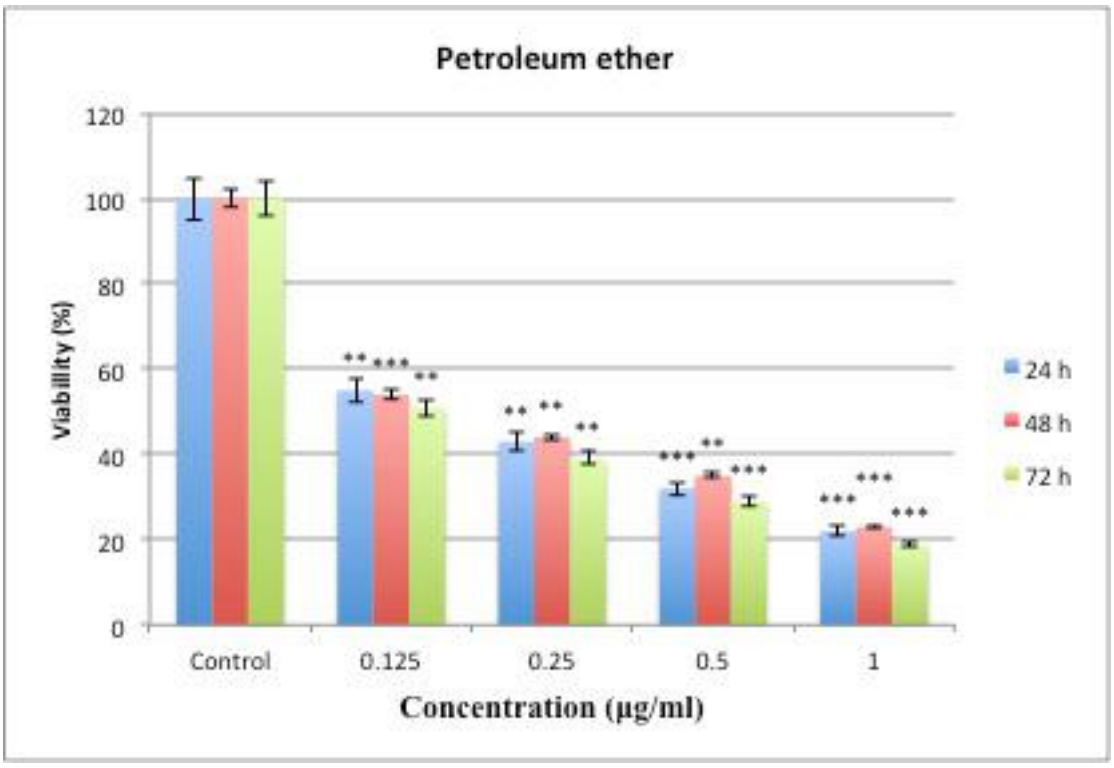

Figure 3.3(d). CaSki cells viability using Petroleum ether extraction of fig latex. Cell viability was assessed using MTT assay after 24, 48 and 72 hours of treatment with various concentrations (0.125, 0.25, 0.5, and 1 µg/ml). Control represents treatment with Petroleum ether. Results represent the means of at least 3 independent experiments and were normalised to control wells. Error bars indicate SEM; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ were considered statistically significant, very significant and extremely significant, respectively.

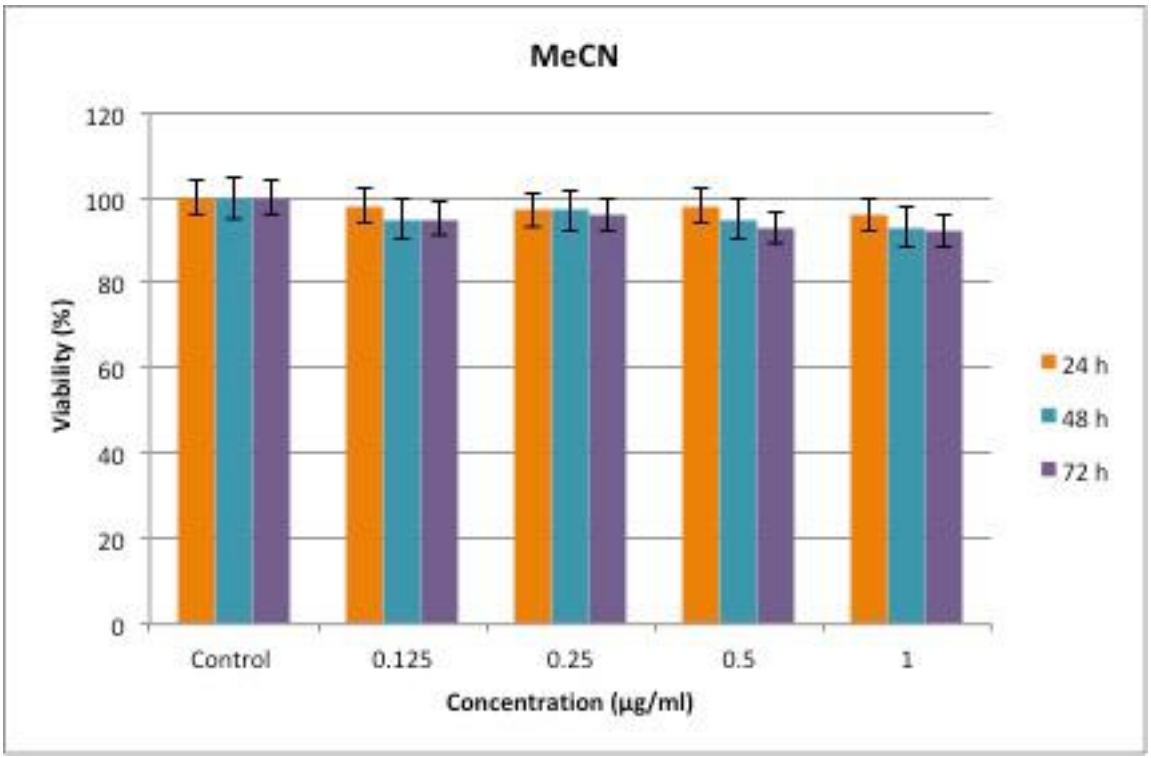

Figure 3.4(a). HaCaT cells viability using MeCN extraction. Cell viability was assessed using MTT assay after 24, 48 and 72 hours of treatment with various concentrations (0.125, 0.25, 0.5 and 1 µg/ml). Control represents treatment with MeCN. Results represent the means of at least 3 independent experiments and were normalised to control wells. Error bars indicate SEM.

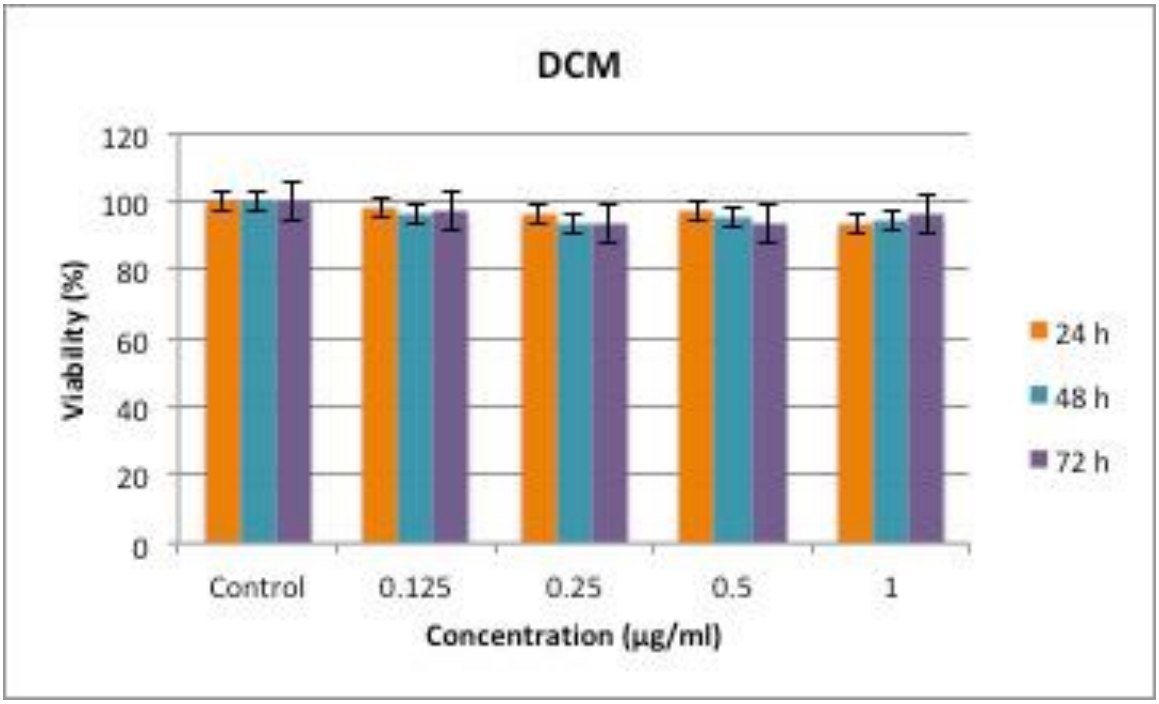

Figure 3.4 (b). HaCaT cells viability using DCM extraction. Cell viability was assessed using MTT assay after 24, 48 and 72 hours of treatment with various concentrations (0.125, 0.25 , 0.5 and 1 µg/ml). Control represents treatment with DCM. Results represent the means of at least 3 independent experiments and were normalised to control wells. Error bars indicate SEM.

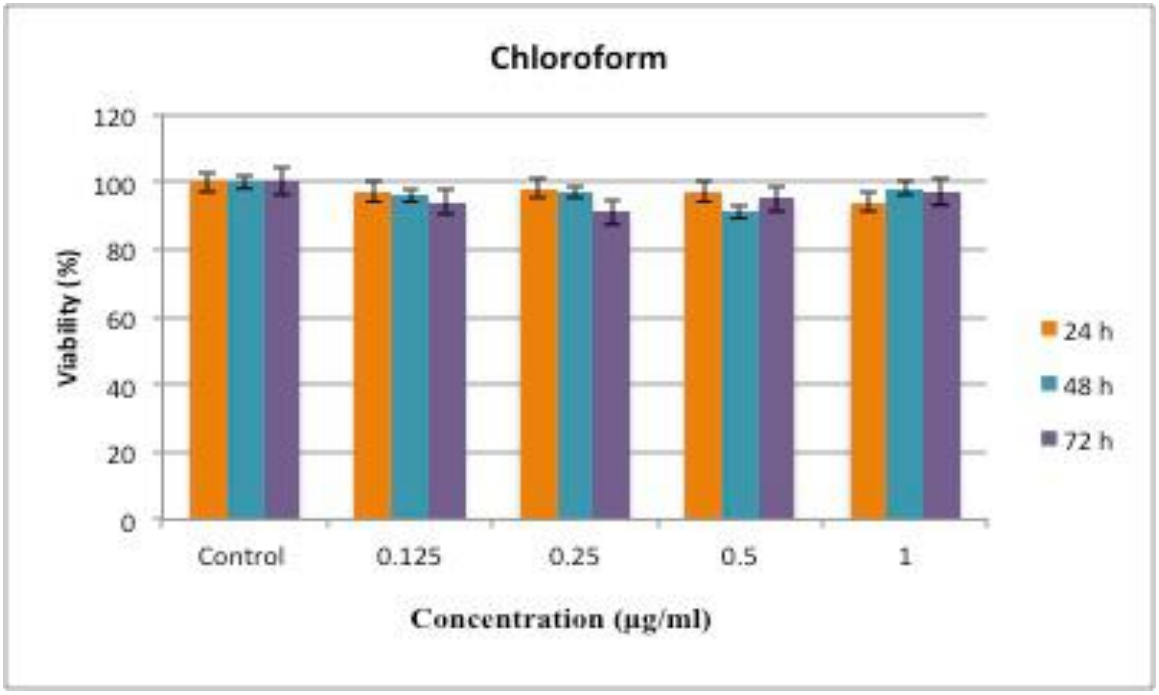

Figure 3.4 (c). HaCaT cells viability using Chloroform extraction. Cell viability was assessed using MTT assay after 24, 48 and 72 hours of treatment with various concentrations (0.125, 0.25 , 0.5 and 1 µg/ml). Control represents treatment with Chloroform. Results represent the means of at least 3 independent experiments and were normalised to control wells. Error bars indicate SEM.

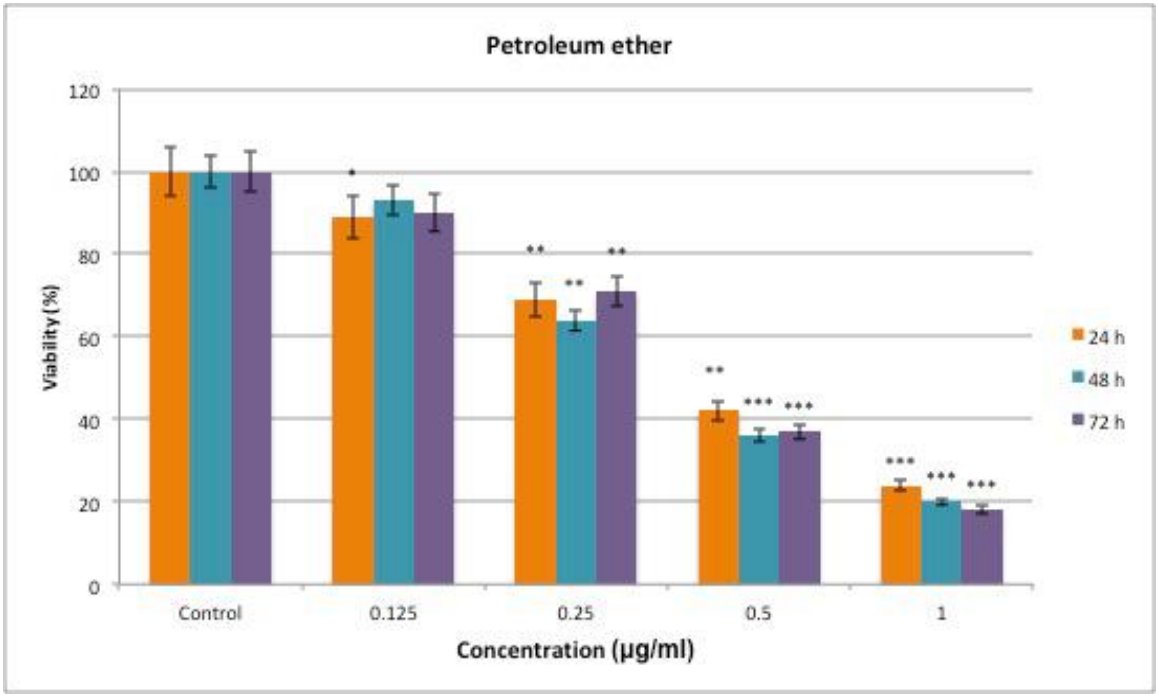

Figure 3.4(d). HaCaT cells viability using Petroleum ether extraction of fig latex. Cell viability was assessed using MTT assay after 24, 48 and 72 hours of treatment with various concentrations (0.125, 0.25, 0.5, and 1 µg/ml). Control represents treatment with Petroleum ether. Results represent the means of at least 3 independent experiments and were normalised to control wells. Error bars indicate SEM; *p < 0.05, **p < 0.01, ***p < 0.001 were considered statistically significant, very significant and extremely significant respectively.

3.1.5 Determination of chemical constitutes of Ficus Carica Latex

Following the determination of an appropriate extract a more detailed analysis was carried out to characterise the active chemical constituents of fig latex that may contribute to the inhibition of properties that are associated with cervical cancer transformed cells. To this end, fig latex extract went through chemical analysis discussed in materials and methods section (2.2.3.2 to 2.2.3.4).

As shown in figure 3.5 Initial LC-MS data indicated that there were many isomers present with a mass of 409, molecular formula being $C_{30}H_{48.}$ The tandem MS was consistent with a cyclic terpene.

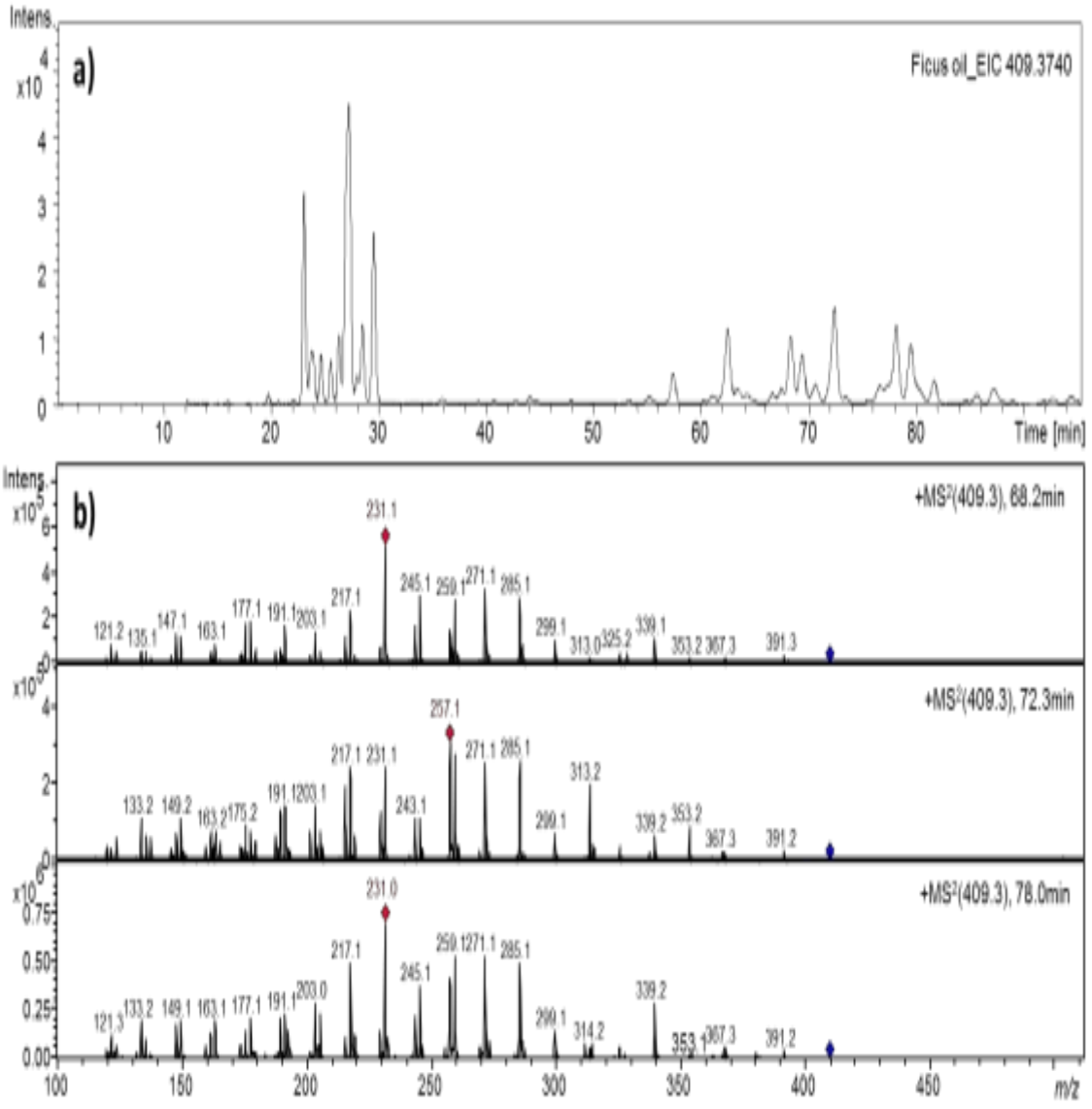

Figure 3.5. (a) Extracted ion chromatogram in positive ion mode of *m/z* 409.3740 and (b) tandem mass spectrum showing the fragmentation of three isomers of *m/z* 409.3740 in Ficus oil extract.

Furthermore, 2D NMR analysis supports the MS, and also suggests that there are steroidal compounds present in the petroleum ether extraction of the fig latex supernatant. Specifically, there is also evidence for the related phytosterol fatty acid glycosides - 6-O-acyl-β-D-glucosyl-β-sitosterols. There is a precedent for this as reported by Rubnov et al., 2000. What is also worthy of note is the presence of phenolic components, which in the Pet. ether fraction are easy to visualise via NMR in the aromatic region (Figure 3.6).

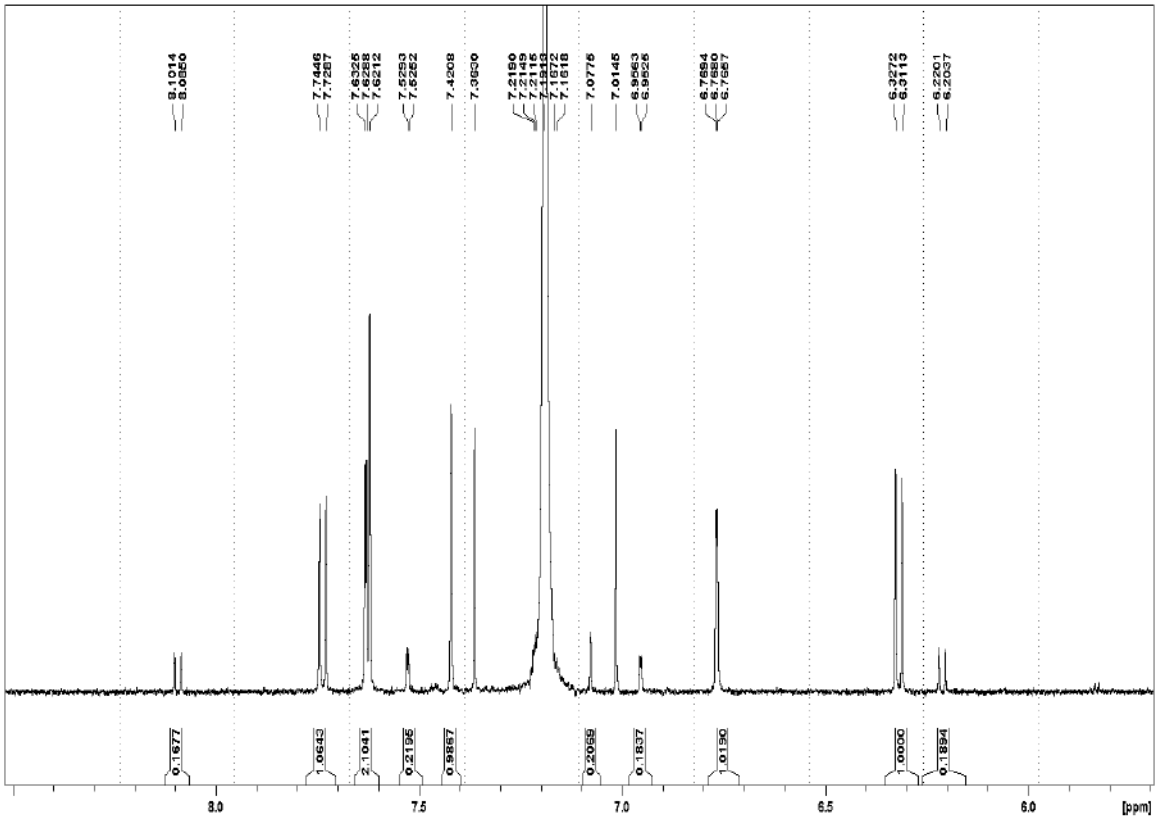

Figure 3.6. S2 ^{1}H NMR Aromatic region of petroleum ether extract of fig latex supernatant showing at least two aromatic components, suggested as ferrulic, caffeic or chlorogenic acids.

Whilst the analysis is preliminary and further, more demanding, purification steps will be required to narrow down the many components to that one active, there is evidence of either two ferulic/caffeic/chlorogenic acid components in the pet ether fraction. In isolation these would be unlikely to partition well into the Pet. ether fraction from the fig latex supernatant, owing to their hydrophilicity, but if covalently attached to a sterol or cyclic terpene, these derivatives, whilst anticipated, have not yet been reported in fig latex as being responsible for any of the observed biological activity. LC-ESI-MS chromatogram in positive ion mode showed that the most abundant lipid compounds present in the oil have the masses between 300 and 450 m/z ratio and elute at very low R_t (Figure. 3.7 (a) and (b)). The low R_t indicates that the most abundant compounds have a polar nature. Besides, the same chromatograms showed the occurrence of few triacylglycerol molecular species and the presence of a compound with m/z value of 409.3840 as a mixture of various isomers eluting at different R_t covering a wide range between 19.5 min and 100 min (Figure. 3.7 (a)). High-resolution ESI-TOF mass spectrometer operating in the positive ion mode lead to the assignment of molecular formulae of $C_{30}H_{49}$ with an error lower than 2 ppm. Tandem MS of the different isomers has shown similar fragmentation pattern resembling the tandem MS of a steroid compound. This supported the idea of oflanosteroltriene or oleandiene. This is supported by the NMR data. It was also observed that there were a few fatty acid glycosides of phytosterols e.g. stigmasterol, present in the supernatant (Figure 3.8). The compounds eluting between 20 min and 30 min have shown the presence of molecular ion of m/z 469.4047, which will correspond to the $[M+H]^+$ of the isomers of triterpenoid phytosterol Lupeol acetate already described in literature (Mawa et al., 2013 Barolo et al., 2014).

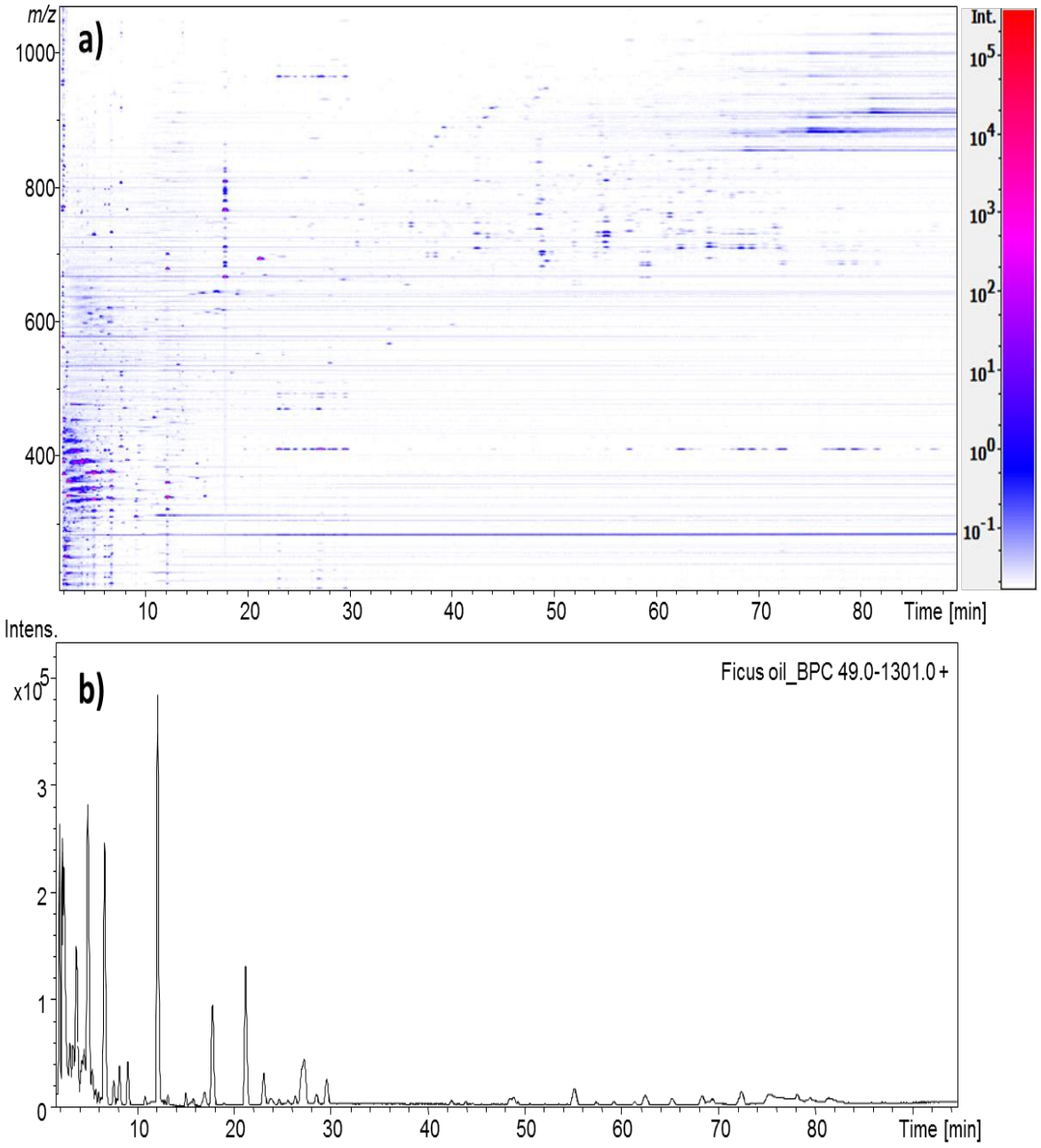

Figure 3.7.(a) Two-dimensional (2D) map of lipid of Ficus oil, by reversed-phase high resolution HPLC/ESI-MicroOTOF MS analysis. Colour represents the intensity of the signals (peaks) with red being the highest and blue the lowest and (b) HPLC-MS profile of Ficus oil in positive ionization mode.

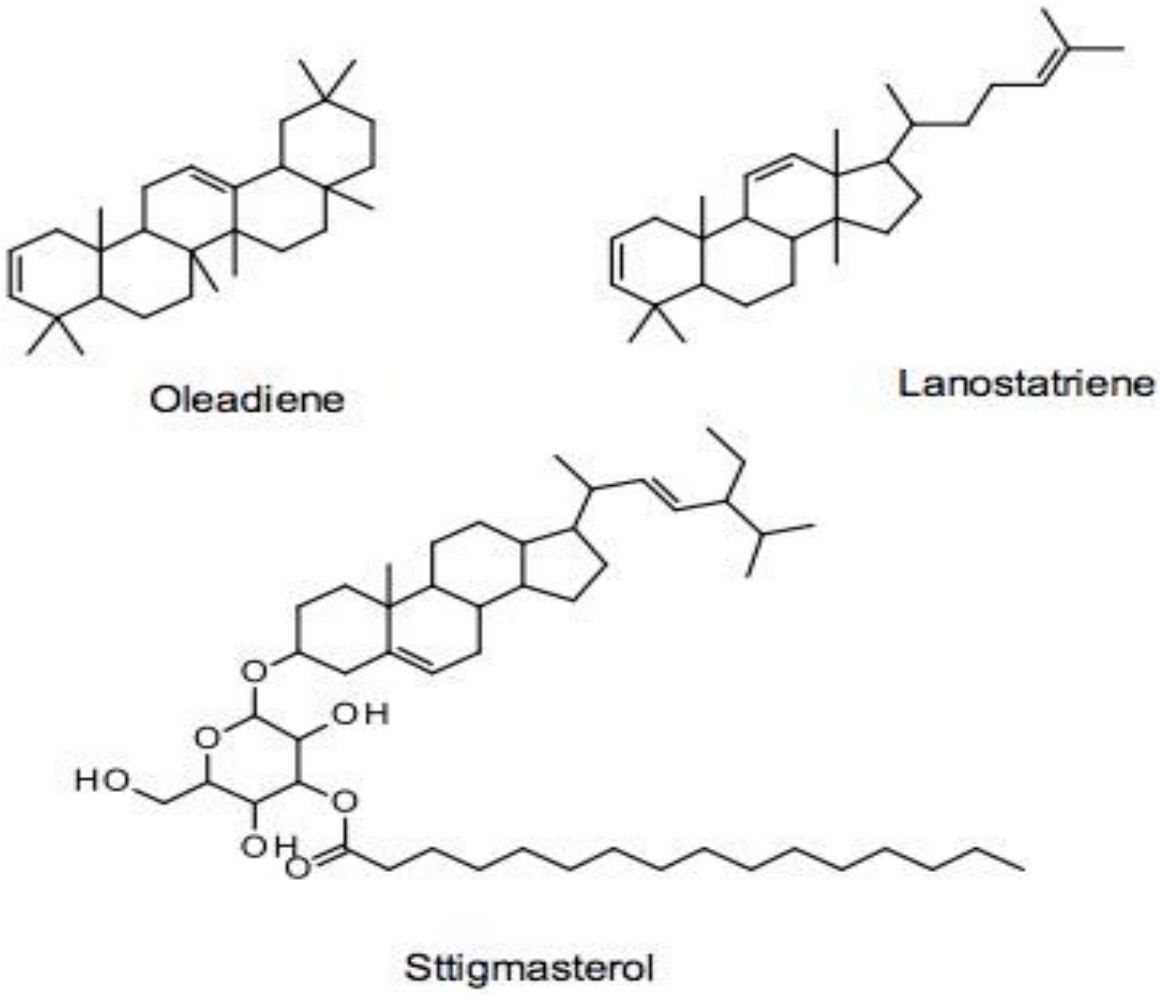

Figure 3.8. MS determined structures of main chemical components of pet ether fraction of fig latex supernatant.

3.2 Conclusion

Based on the results obtained from cytotoxicity study, it is envisaged that fig latex may possesses anticancer activity by selectively inhibiting the growth of cancerous cells but not normal cells at certain concentration of 0.125 μg/ml. This certain concentration has been used for further biological assessment in this study.

Prior to this study, no report of chemical analysis on Polymeric gum, lyophilised latex and the aqueous part of the latex was observed that revealed aqueous part of fig latex contains broader spectrum of active compounds and was as effective as whole latex from biological activity perspectives. Elucidation of chemical compounds responsible for the anti-cancer activity of fig latex leads to observation of ferulic/caffeic/chlorogenic acid components in the petroleum ether fraction that are likely attached to sterol derivate or cyclic terpene compounds. Hence, more purification and characterisation of bioactive compounds responsible for the observed anti-cancer activity/anti-growth will be required. Some of these constitutes of fig latex might serve as a novel and potent anti-cancer agents.

Chapter 4

Results

Effect of Fig latex on specific properties of HPV related transformed cells

4.1 Functional Studies

4.1.1 *Ficus carica* latex suppresses clonogenic ability (contact inhibition) of cervical cancer cells

The term contact inhibition describes two different cellular characteristics: contact inhibition of locomotion (CIL) and contact inhibition of proliferation (CIP). CIL occurs once fibroblast cells retract and change their direction to avoid collision with another cells. Contact inhibition regulates the growth rate of cells and formation of a monolayer of cells (Carmona et al., 2008; McClatchy and Yap, 2012). Normal cells continue to proliferate and move around until they occupy the whole surrounding area. Once motile cells gets confluence in culture area, their mobility and proliferation rate reduce. Studied revealed that CIL is a necessary mechanism for occurrence of CIP (Harris and Tepass, 2010; Batson et al., 2013). Mechanical contact and mechanical stress between cells acts as an inhibitory factor for cell proliferation. When collision is unavoidable, a different phenomenon occurs whereby growth of the cells of the culture itself eventually stops in a cell-density dependent manner. Both types of contact inhibition are well-known properties of normal cells and contribute to the regulation of proper tissue growth, differentiation, and development (Zhao et al., 2007; Halder and Johnson, 2011). It is worth noting that both types of regulation are normally negated and overcome during organogenesis during embryonic development and tissue and wound healing. However, contact inhibition of locomotion and proliferation are both aberrantly absent in cancer cells, and the absence of this regulation contributes to tumorigenesis (Heckman, 2009). Therefore a major characteristic of normal cells such as non cancerous HaCaT is density dependent of growth; once a group of cells grow to completely cover the plate/flask and cells are in contact, cell division cease (contact inhibition). To investigate whether the cervical cancer cells loss contact inhibition when they are treated with fig latex their anti clonogenic ability was assessed using colony formation assay. Cervical cancer cells (CaSki and HeLa) and non-cancerous HaCaT cells were treated with fig latex 14 days and number of colonies they formed was counted. As expected non cancerous HaCaT cells did not form any colonies to be affected by fig latex. However, CaSki and HeLa cells formed colonies in the absence of fig latex and as shown in figure 4.1 following the

treatment with fig latex noticeably decreased the number of colonies.

Statistical analysis of colony formation assay data on cervical cancer cells (HeLa and CasKi) treated with fig latex showed significant reduction in colony numbers. In Hela cells the reduction was approximately 3-fold with 0.125 μg/mL of fig latex and 4.5-fold with 0.25 μg/mL of fig latex). This reduction was slightly higher in CaSki cells treated with fig latex (approximately 4.5-fold with 0.125 μg/mL of fig latex and 9-fold with 0.25 μg/mL of fig latex) (Figure 4.2)

Overall, results from colony formation assay and data from statistical analysis suggest that fig latex is able to prevent colony formation through ceasing of contact inhibition property driven by cervical cancer.

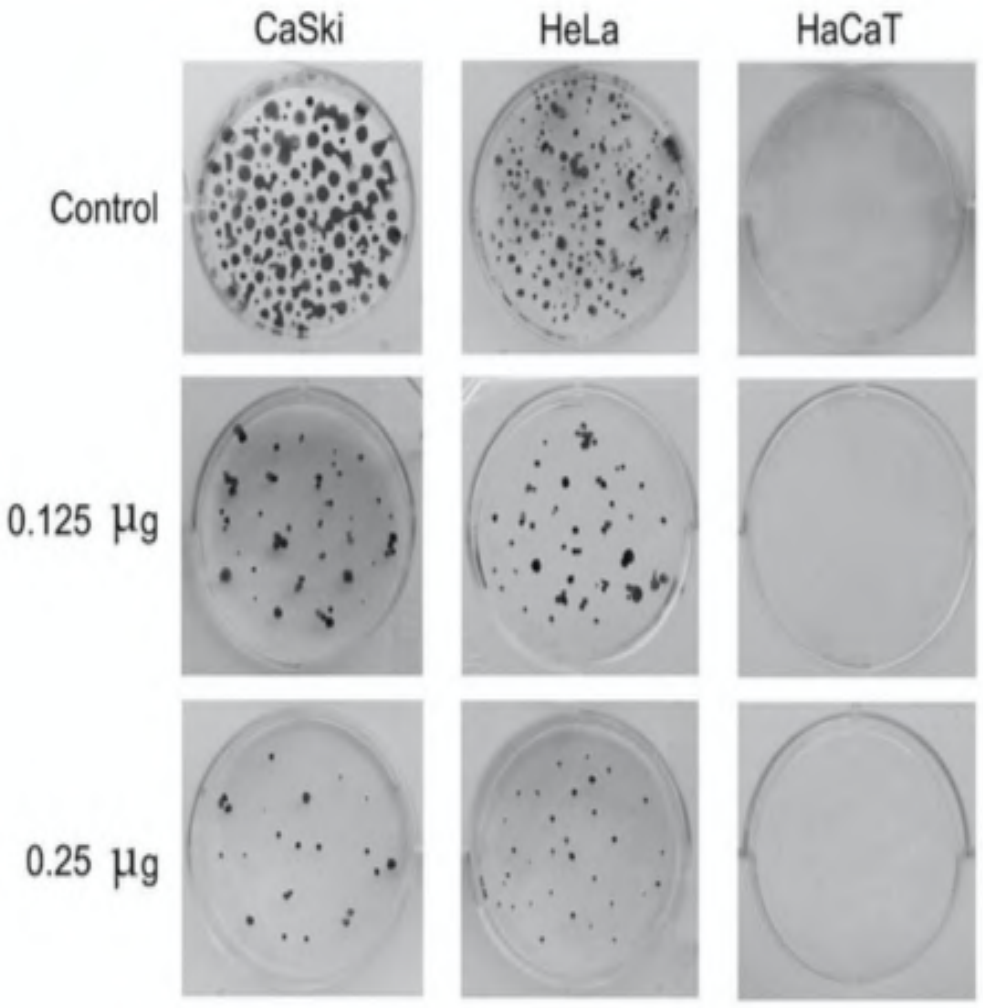

Figure 4.1. Representative colony formation images of CaSki, HeLa and HaCaT cells with and without treatment with Fig latex. Fig latex treatment inhibits colony formation in cervical cancer cell, CaSki and HeLa noticeably. As expected HaCaT cells did not form any colonies to be affected by fig latex. Cells were stained with crystal violet and the results are representative of three independent experiments.

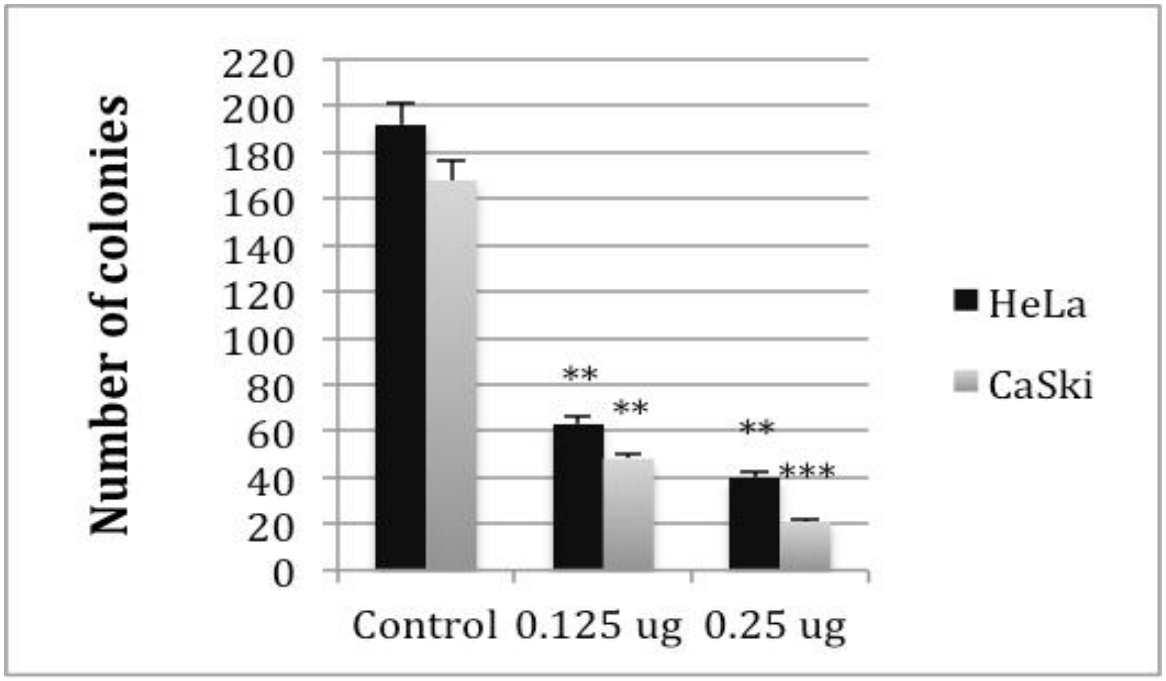

Figure 4.2. Graph represents the statistical analysis of inhibitory effects of fig latex on cervical cancer cells by colony counting. Following treatment with 0.125 µg/ml of fig latex statistically very significant and extremely significant reduction was observed in the number of colonies formed by CaSki and Hela cells. Error bars show SEM; Experiments, n=3, average of three independent experiments; *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ were considered statistically significant, very significant and extremely significant, respectively.

4.1.2 Ficus carica latex affects the presence/expression of cellular proliferation marker, Ki67 protein in cervical cancer cells

Cell proliferation is a principal feature in cell growth and division. In cancerous cells, cell proliferation is accompanied by deregulation in balance between cell growth and cell death, leading to over growth of cells. Proliferation represents pivotal role in cancer development and carcinogenesis. It is assumed that deregulation of cell proliferation is an important and critical property of potential neoplastic cells. Cell proliferation is also used to determine the response to drugs and toxic substances. Evaluation of proliferation proteins such as ki67 protein is one of the most common methods to mark and score cell proliferation. Ki67 is an antigen recognized with monoclonal antibody by immunizing mice with nuclei of Hodgkin lymphoma cells (Gerdes et al., 1983). It binds to the per-chromosoma layer in mitosis of cell cycle. Ki67 is a valuable marker for examination of proliferation rate in cells and assessment of the response to tumor therapy (Dowsett et al., 2011). Ki67 is involved in the organization of nuclear chromatin during interphase in cell cycle division. Formation of prechromosomal layer to prevent aggregation of mitotic chromosomes is an essential event in cell division. Ki67 is expressed during all phase of cell cycle except Go (Gerdes et al., 1984; Bruno et al., 1992). Ki67 is also is a labeling index for determination of survival rate in patients (Niazi et al., 2013; Tian et al., 2016).

To investigate whether the fig latex influences cell proliferation in cervical cancer by targeting the Ki67 protein, control and cervical cancer cell lines were treated with a non-cytotoxic concentration (0.125 μg/mL) of fig latex for 48 hours. The presence/expression of the Ki67 was investigated by immunofluorescence (IF) following incubation with Anti-ki67 antibody and DAPI to visualise cell's nucleus. Data obtained from this study shows the presence/expression of Ki67 protein in the nucleolus of untreated cancerous (HeLa and CaSki) and non-cancerous cells (HaCaT), as expected (figure 4.3).

Treatment of non-cancerous HaCaT cells with 0.125 μg/mL of fig latex did not affect the presence/expression of the Ki67 in the nucleolus, as Ki67 was almost exclusively located in the nucleolus of treated HaCaT cells, similar to untreated HaCaT cells. However, interestingly, in cervical cancer cell lines (HeLa and CaSki) treated with 0.125 μg/mL of fig latex, Ki67 protein was depleted almost exclusively in the nucleolus (Figure 4.4 and 4.5).

Figure 4.6 shows the summary of findings from inhibitory effects of fig latex on Ki67 proliferation protein and confirms the MTT findings that 0.125 μg/mL of fig latex does not affect normal cells growth properties.

Finding from this study strongly suggests that fig latex may potentially target the expression of Ki67 in cervical cancer cells to prevent cell proliferation that could ultimately play a role in the inhibition of cancer progression.

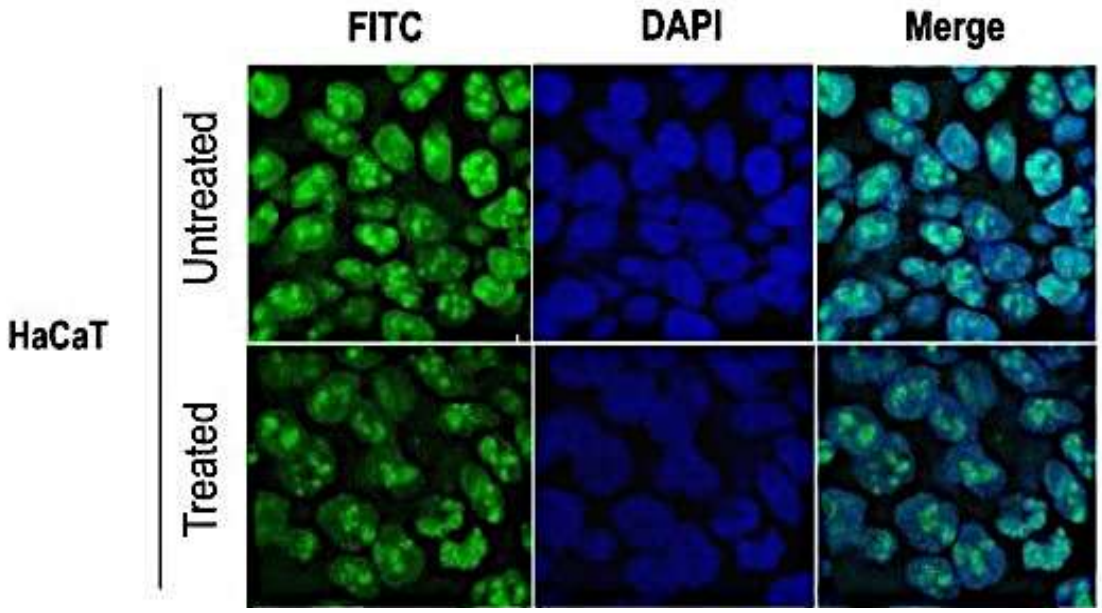

Figure 4.3. Inhibitory effects of fig latex on Ki67 proliferation protein marker in HaCaT cells. Following treatment with 0.125 µg/ml of latex for 48 hours, the level of ki67 protein expression in HaCaT cells did not change noticeably. Images were acquired using Leica Confocal Microscope to demonstrate visualisation of the Ki67 protein expression in cells. Results are representative of three independed experiments (N=3).

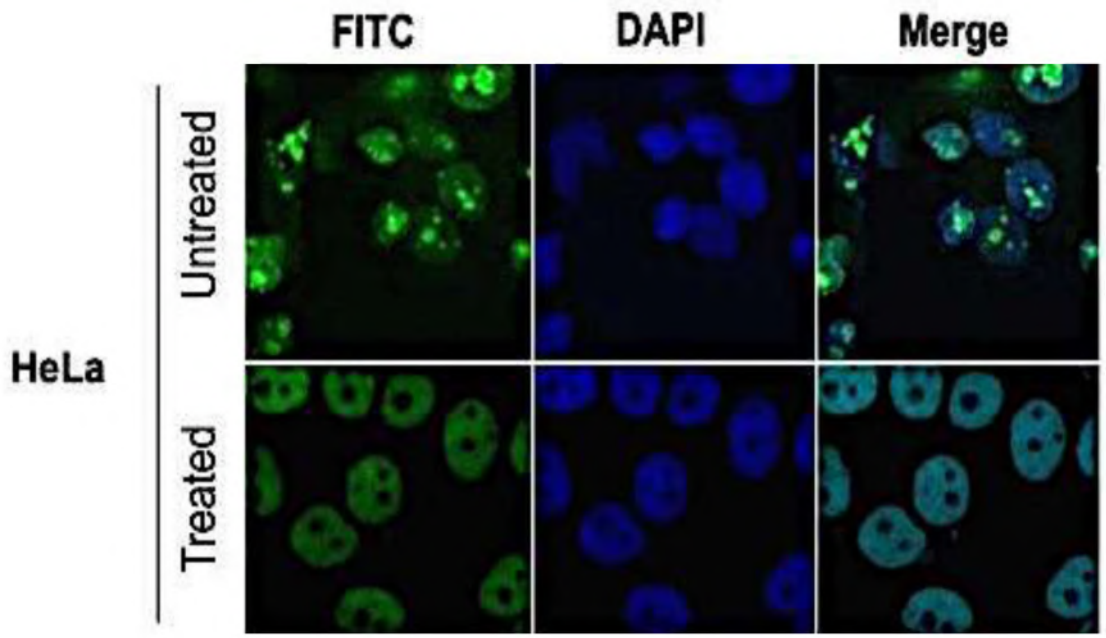

Figure 4.4. Inhibitory effects of fig latex on Ki67 proliferation protein marker in Hela cells. Following treatment with 0.125 µg/ml of latex for 48 hours, the level of ki67 protein expression in Hela cells was downregulated. Images were acquired using Leica Confocal Microscope to demonstrate visualisation of the Ki67 protein expression in cells. Results are representative of three independed experiments (N=3).

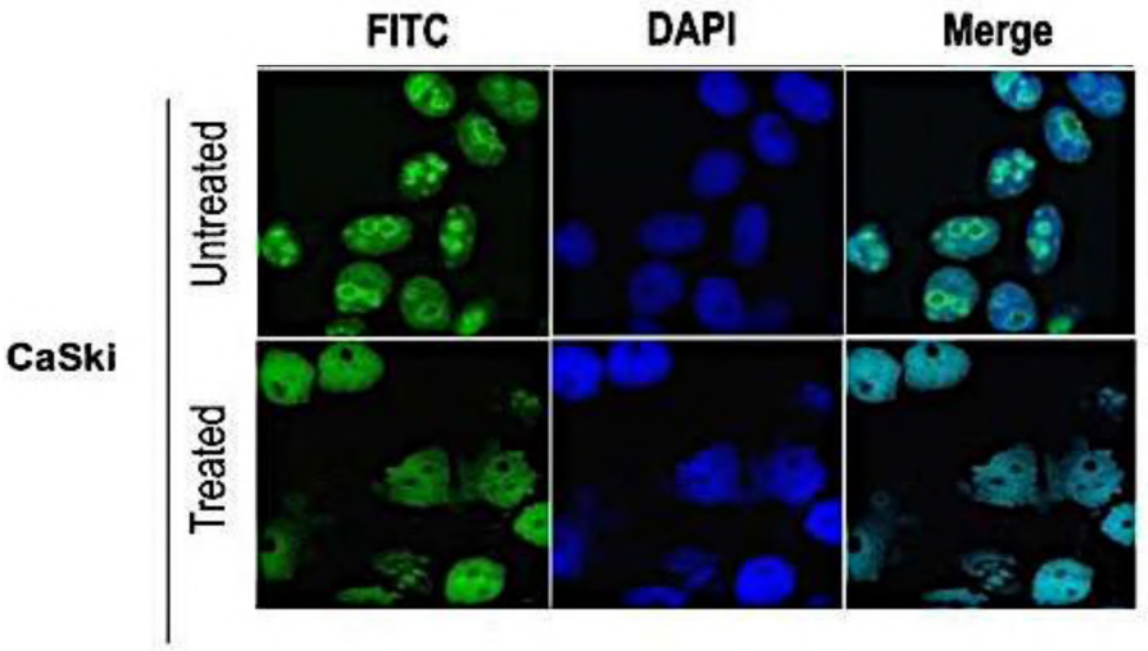

Figure 4.5. Inhibitory effects of fig latex on Ki67 proliferation protein marker in CaSki cells. Following treatment with 0.125 µg/ml of latex for 48 hours, the level of ki67 protein expression in CaSki cells was downregulated. Images were acquired using Leica Confocal Microscope to demonstrate visualisation of the Ki67 protein expression in cells. Results are representative of three independed experiments (N=3).

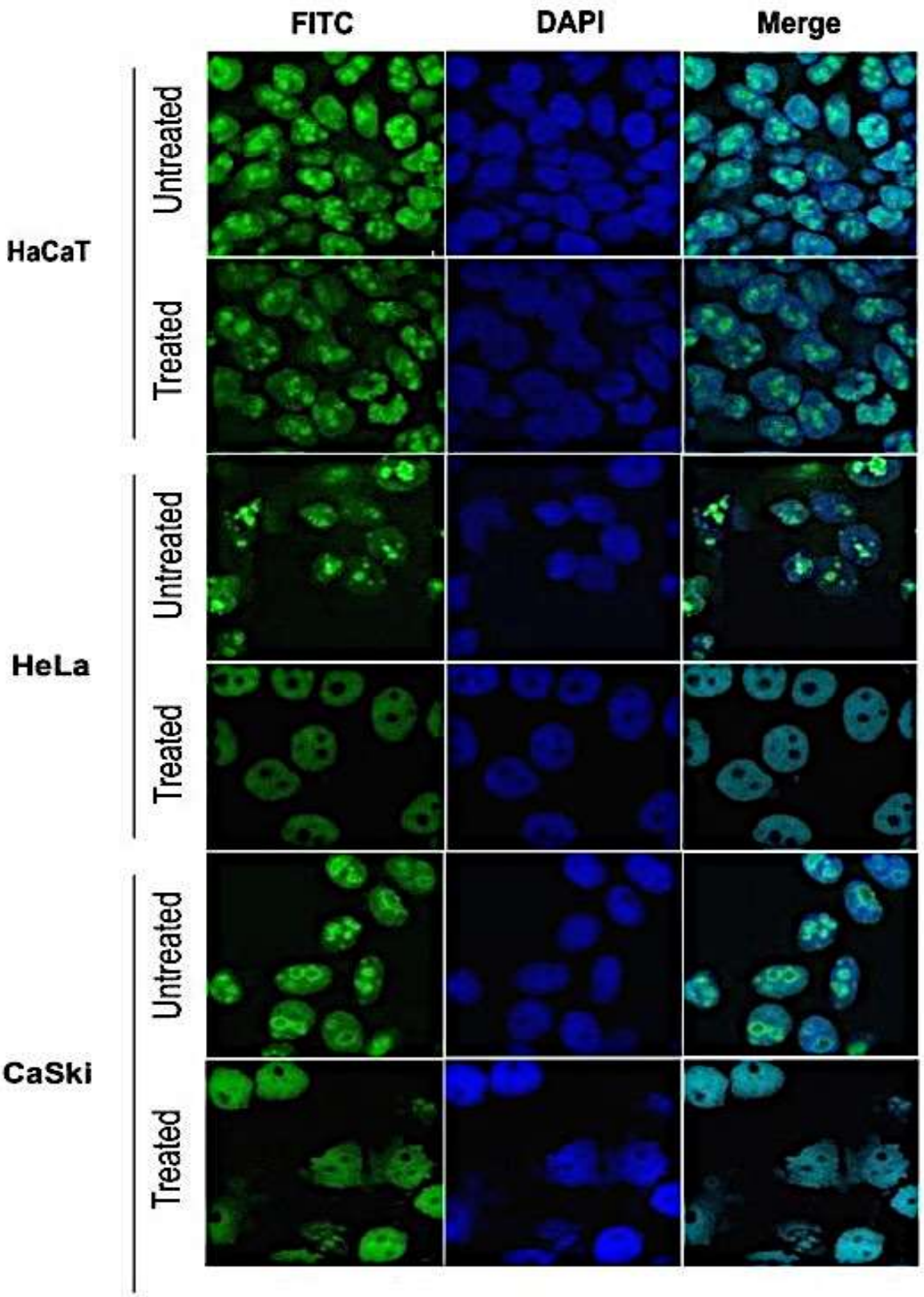
FITC
DAPI
Merge
HaCaT
Untreated
Treated
HeLa
Untreated
Treated
CaSki
Untreated
Treated

Figure 4.6. Summary of inhibitory effects of fig latex on Ki67 proliferation protein marker on cervical cancer cells and non-cancerous cells using immnoflourcent. Following treatment with 0.125 μg/ml of latex for 48 hours, the level of ki67 protein expression in HaCaT cells did not change noticeably. In contrast, level of ki67 expression was downregulated noticeably in treated cervical cancer cell lines. This suggests the selective inhibitory effect of fig latex on expression of Ki67 in HPV positive cells. Images were acquired using Leica Confocal Microscope to demonstrate visualisation of the Ki67 protein expression in cells. Results are representative of three independed experiments (N=3).

4.1.3 Ficus carica latex suppresses cervical cancers migratory ability and alleviates invasiveness

As stated in section 1.3.6 metastasis is a hallmark of cancer and is considered as a leading cause of death among cancer patients. Metastasis requires cancer cell dissemination to various organs from the primary tumor site (Sleeman et al., 2011). Dissemination is defined as a complex cell motility phenomenon that requires series of molecular coordination such as invasion and contractility activities of tumor cells to achieve directed cell migration (Song et al., 2012). As discussed before (1.3.6) metastasis begins with migration of cancer cells from primary tumor location that is termed tumor invasion. Cell migration and invasion are essential factors in various physiological events such angiogenesis, wound healing and inflammation (Nurden et al., 2008). In addition, the migratory and invasiveness capability are also implicated in the etiology of wide range of disease especially in carcinogenesis (Sleeman et al., 2011). Indeed these unique properties of cancer cells stand up on the road of malignancy. Generally, migration begins due to the cell response to external signals, leading to the movement of cells (Gumbiner, 2005). Cancer cells then interact with the ECM that is accompanied by the function of adhesion receptors of the integrin family and proteolytic mechanisms to overcome tissue barriers (Cho and Klemke, 2000; Adams, 2001). These cells traverse the stromal connective tissue and then cross the basement membranes of blood or lymphatic vessels to disperse in these vessels. This process is called intravasation. The invaded cells cross back via the vascular basement into a new tissue that is called extravasation and therefore they create metastasis (Shin et al., 2011; Rybinski et al., 2014).

In order to investigate whether fig latex could also mediate with other transforming properties of cancer cells such as migration and invasion abilities of cervical cancer cells, cell migration and invasion assays were performed using transwells. For these assays cervical cancer cells (CaSki and HeLa) and non-cancerous cells (HaCaT cells) were treated with 0.125 μg/mL of fig latex for 48 hrs and the numbers of migrated and invaded cells towards lower chambers of transwells were counted accordingly.

As shown in figure 4.7 cancer cells were able to migrate towards lower chambers of transwells without adding treatment (fig latex). However, following treatment with 0.125 μg/mL of fig latex for 48 hrs the numbers of migrated cells has decreased noticeably. As expected noncancerous HaCaT cells did not migrate through transwells chambers both before and after treatment.

Analysis of cell migration assay also showed statistically extremely significant reduction (***P < 0.001 approximately 2.5-fold with 0.125 μg/mL of fig latex in numbers of migrated CaSki and Hela cells towards lower chambers (Figure 4.8).

Data obtained from cell invasion assay (figure 4.9) show that cancer cells were able to invade towards lower chambers of transwells without adding treatment (fig latex). However, following treatment with 0.125 μg/mL of fig latex for 48 hrs the numbers of invaded cells has decreased noticeably. As expected and same as cell migration assay, noncancerous HaCaT cells did not invaded through transwells chambers both before and after treatment. Data from this experiment show that fig latex can prevent cancer cells migration and invasion through the transwells.

Statistically analysis of cell invasion assay also showed extremely significant reduction (***P < 0.001 approximately 2.8-fold with 0.125 μg/mL of fig latex in numbers of invaded CaSki and Hela cells towards lower chambers (figure 4.10).

Taken together, findings from this experiment show that fig latex may play a significant role on the suppression of migratory capabilities of cervical cancer cells and could alleviate their invasiveness.

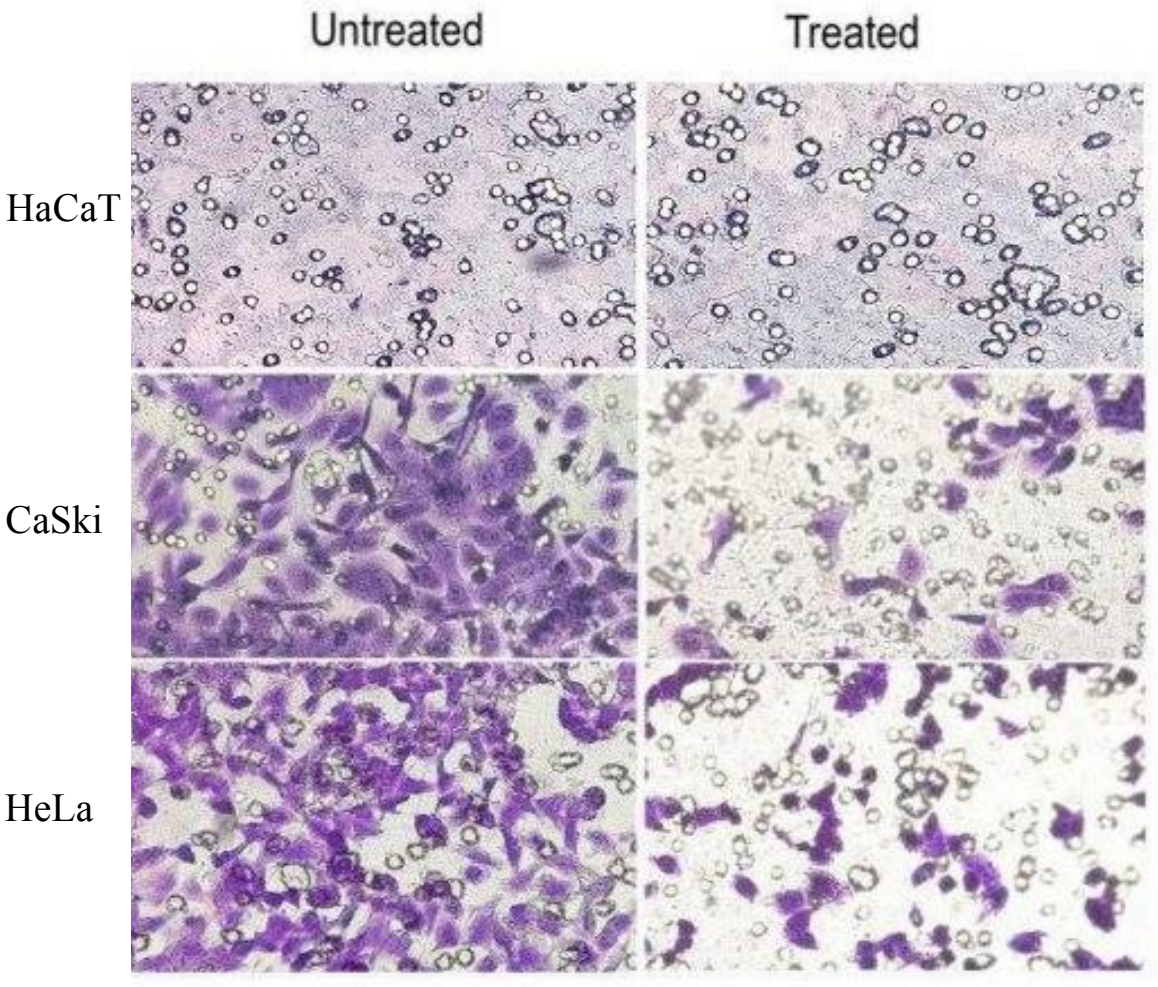

Figure 4.7. Suppression of cell migratory ability of cervical cancer cells. Images are representative of Transwell migration assays without and with treatment by 0.125 μg/ml of fig latex. Untreated images show the migratory ability of HeLa and CaSki cells through chambers. Treated images represent the inhibitory effect of *Ficus carica* latex on the number of migrated cervical cancer cells. Following treatment with fig latex the number of migrated cancer cells towards lower chambers has decreased noticeably. HaCaT cells did not migrate through chambers before and after adding treatment. Images are representative of six independent experiments.

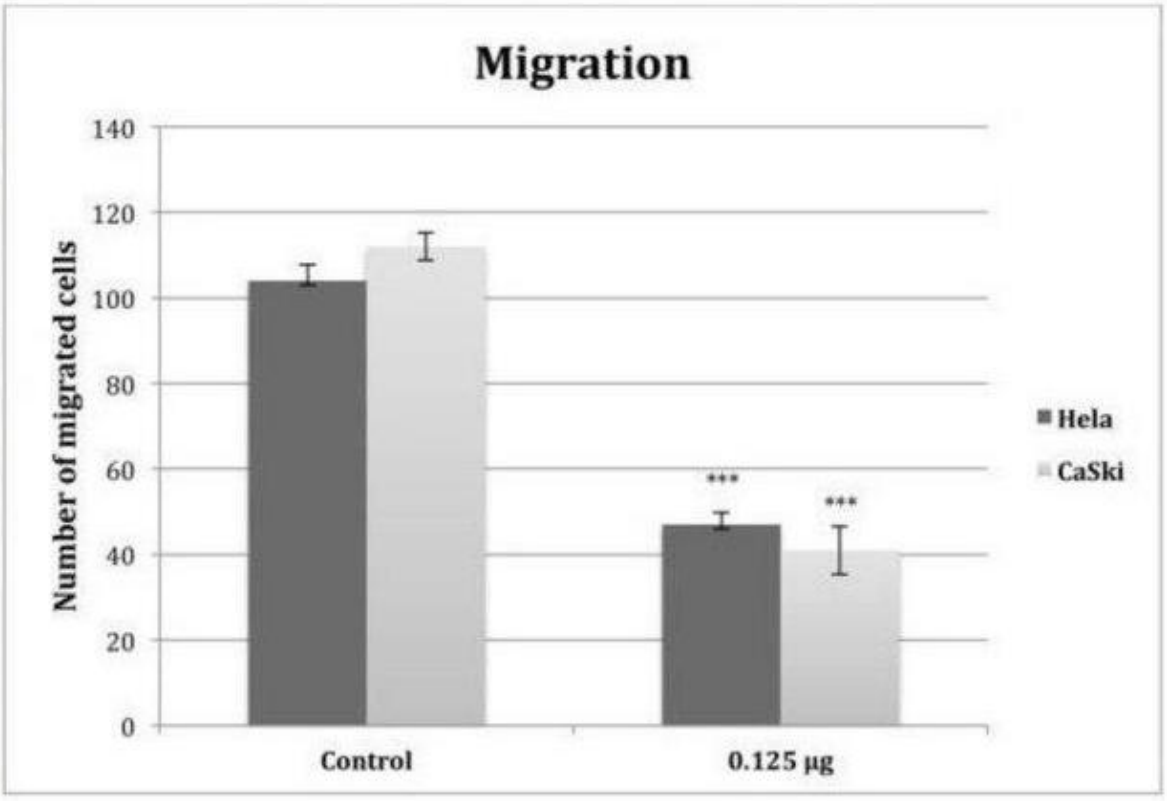

Figure 4.8. Graph represents statistical analysis of the inhibitory effects of Ficus Carica latex on the number of migrated cervical cancer cells. Following treatment with 0.125 µg/ml of fig latex an extremely significant reduction was observed in the numbers of migrated CaSki and Hela cells towards lower chambers. Error bars show SEM; Experiments, n=3, average of three independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001 were considered statistically significant, very significant and extremely significant, respectively.

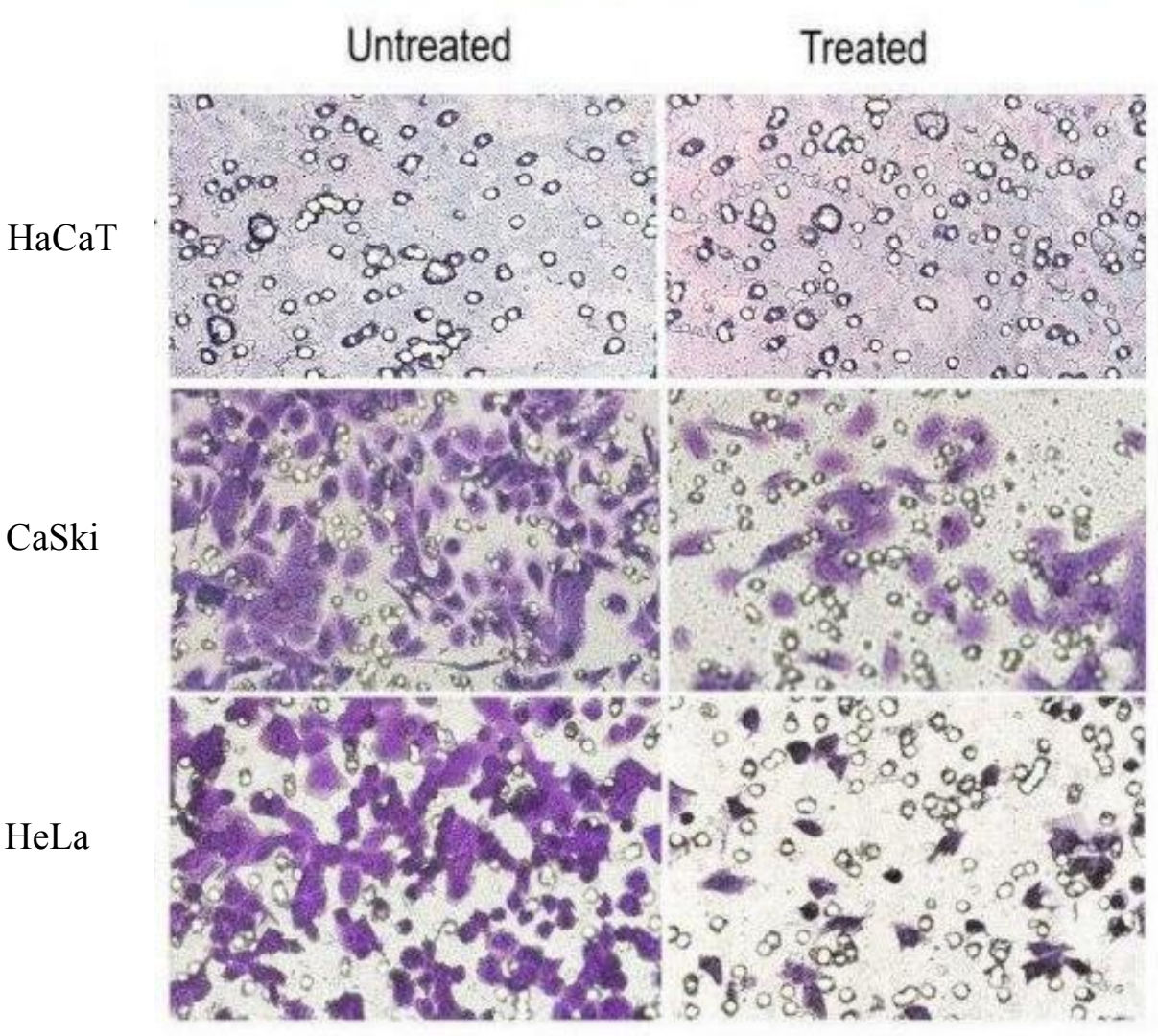

Figure 4.9. Suppression of cell invasion of cervical cancer cells. Images are representative of Transwell invasion assays without and with treatment with 0.125 µg/ml of fig latex. Untreated images show the invasiveness of HeLa and CaSki cells through chambers. Treated images represent the inhibitory effect of *Ficus carica* latex on the number of invaded cervical cancer cells. Following treatment with fig latex the number of invaded cancer cells towards lower chambers has decreased noticeably. HaCaT cells did not invaded through chambers before and after adding treatment. Images are representative of six independent experiments.

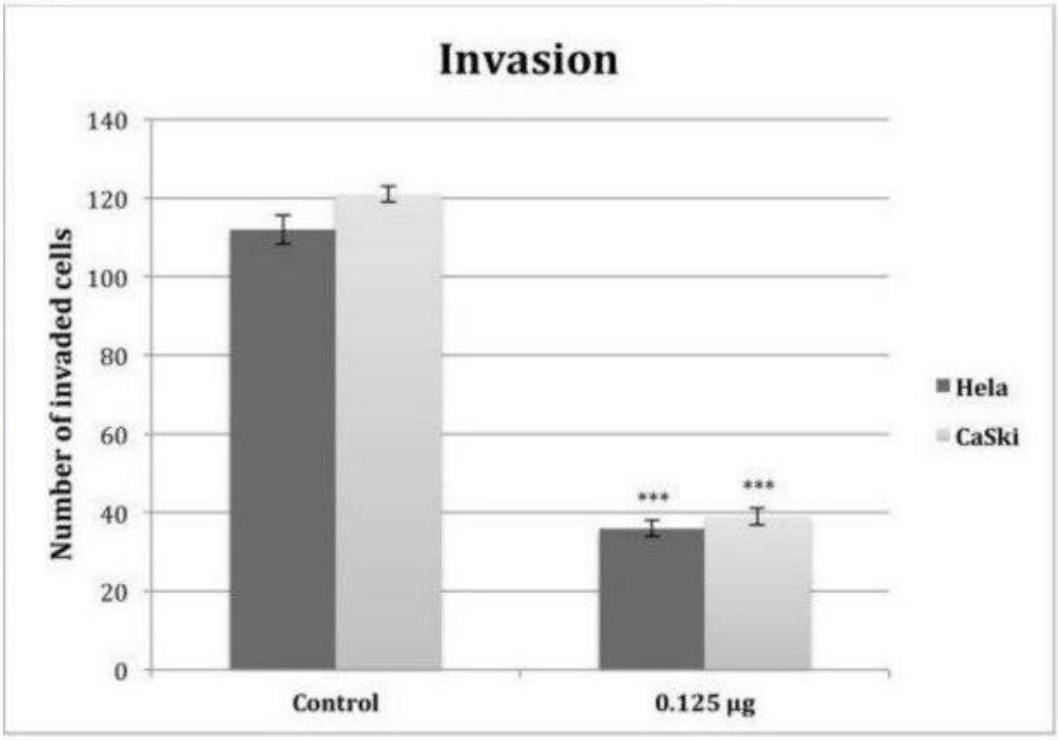

Figure 4.10. Graph represents statistical analysis of the inhibitory effects of Ficus Carica latex on the number of invaded cervical cancer cells. Following treatment with 0.125 µg/ml of fig latex an extremely significant reduction was observed in the numbers of invaded CaSki and Hela cells towards lower chambers. Error bars show SEM; Experiments, n=3, average of three independent experiments; *p < 0.05, **p < 0.01, ***p < 0.001 were considered statistically significant, very significant and extremely significant, respectively.

.

4.1.4 Ficus carica latex induces cell cycle arrest in Sub G1 phase

The cell cycle consists of series of events that occurs in cells life span and begins from one cell division towards the production of the next generation of cells. Cell cycle span varies based on function and type of cells. For Example, skin and bone cells those require repair, growth, or replacement will more quickly and constantly repeat the cycle. Generally an eukaryotic cell cycle is divided into two fundamental phases; interphase and mitosis. Majority time of the cell cycle span is spent in a growth and development phase that is called interphase (Biggar and Storey, 2009). However, some cells such as nerve cells do not require further division and growth and they are remained interphase. Interphase represents the phase between two successive M phases prior to cell division. The interphase is divided into three phases; G1, S (synthesis) and G2 phase (Weinberg, 2013). During G1 phase cells are metabolically active and constantly grow without any DNA replication. Indeed G1 correlates with the interval time between mitosis and DNA replication. During S phase, DNA replication initiates and therefore the amount of DNA in each cell gets doubled. G2 phase corresponds to protein synthesis and preparation of mitosis. Sometimes cells stops from further growth or proliferation. These cells exit the G1 phase of cell cycle and enter to the inactivate phase called quiescent (Go) phase (Morgan, 2007; Alberts and Johnston 2014).

The M Phase of cell cycle represents the real cell division. During a period of cell cycle that requires 24 hours, a cell division procedure completes in an hour. The interphase lasts more than 95% of the duration of cell cycle. During mitosis the nucleus gets divided and forms two identical nuclei. Each new nucleus is identical to the one it originated from (Gregan, 2007). Mitosis is described as a series of continuing phases. These stages of mitosis include; prophase, metaphase, anaphase and telophase. The mitosis begins with the nuclear division, continues with the separation of daughter chromosomes (karyokinesis) and ends with division of cytoplasm (cytokinesis). Many proteins play role in cell cycle distribution especially during mitosis phase (Cullen et al., 2005; Nasmyth and Hearing 2009; Lancaster 2010).

So far this study showed that fig latex inhibits properties that are associated with HPV-positive cervical cancer transformed cells such as rapid growth and invasion. As

mentioned in section 1.3.3 cancer is the result of unchecked cell division caused by a breakdown of the mechanisms that regulate the cell cycle and therefore the distribution of cells in each phase can be affected.

To ascertain whether the growth-inhibitory effect of fig latex on CaSki and HeLa cells is related to the cell cycle arrest, cells were treated with 0.125 µg/mL of fig latex for 48h and the distribution of cells in Sub G1, G1, S and G2/M phases was determined and compared before and after treatment with fig latex using FACS analysis.

As shown in figure 4.11, majority of the cells (HaCaT, CaSki and HeLa) were accumulated in G1 phase of cell cycle before treatment with fig latex. However, after treatment with 0.125 µg/mL of fig latex for 48h, HaCaT cells population in Sub G1 and G2/M phases slightly increased from 5.39 and 9.4 in untreated samples to 9.80 and 13.5 in treated samples respectively. This increase was accompanied by a slight decrease in G1 phase population from 78.04 in untreated samples to 69.04 in treated cells. Following treatment the population of cells in S phase has very slightly elevated from 6.08 to 7.44 in treated samples (figure 4.11).

Interestingly, following treatment with 0.125 µg/mL of fig latex for 48h, CaSki and HeLa cells population in Sub G1 phase was increased with noticeable amount from 4.89 and 4.19 to 36.87 and 23.42 respectively. The G1 phase population in CaSki and HeLa cells decreased from 75.83 and 71.53 in untreated samples to 43.94 and 59.46 in treated samples respectively. A slight decrease from 11.8 to 8.11 in CaSki and 11.74 to 9.18 in HeLa cells was observed in G2/M phase (figure 4.12 and 4.13). Moreover, CaSki cells showed a light elevation in S phase population from 7.44 to 10.63. HeLa cells demonstrate the same ratio for S phase population by a negligible elevation following treatment (figure 4.13).

Overall, data from this experiment strongly suggest that fig latex induces cell death in Sub G1 and accumulates G1 phase population in CaSki and HeLa cells. In contrast, HaCaT cells treated with fig latex showed approximately same ratio in all phases of the cell cycle (Figure 4.12 and 4.13).

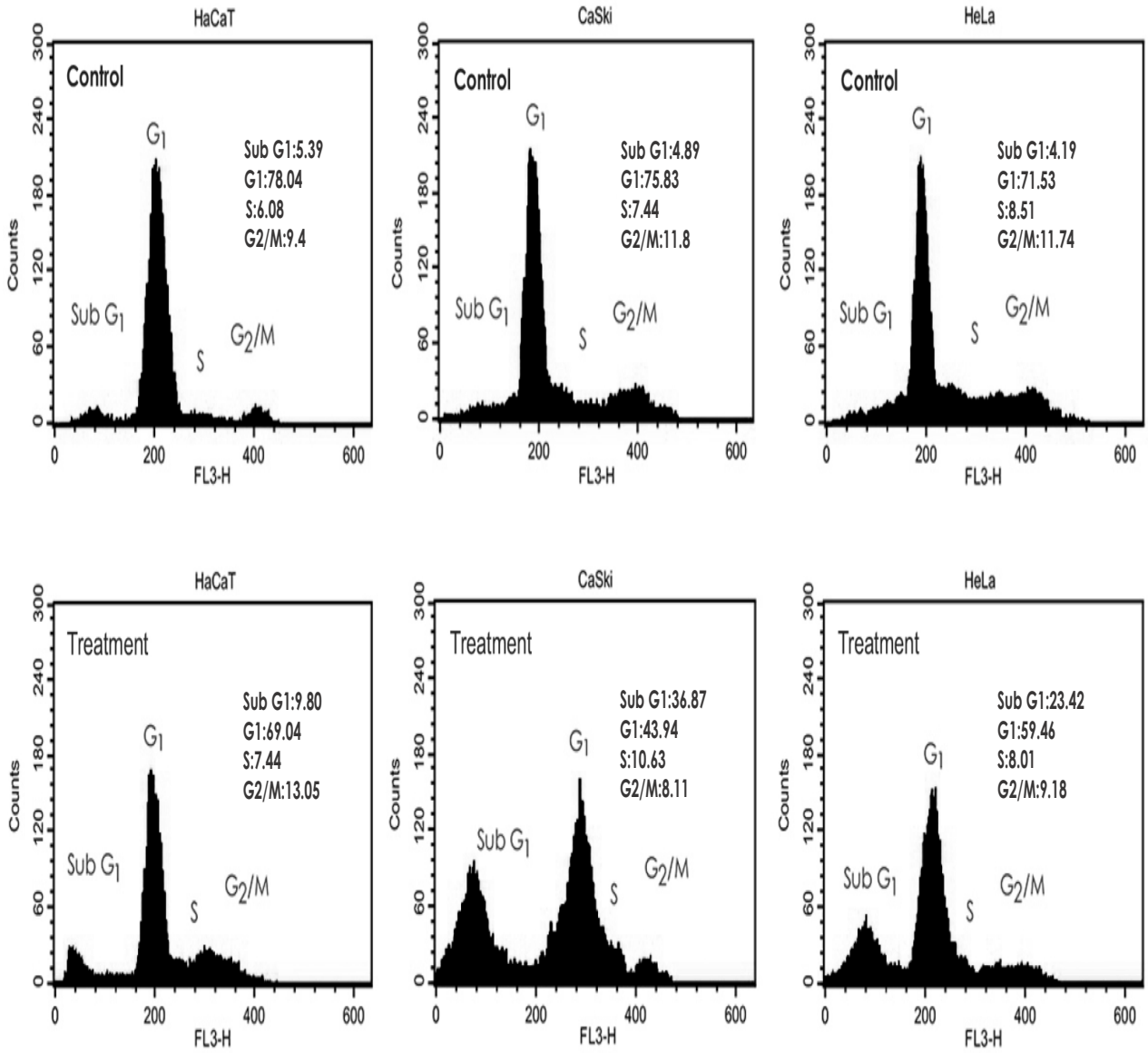

Figure 4.11. Effect of *Ficus carica* latex on cell cycle distribution of HaCaT, CaSki and HeLa cells. Control represents the untreated cells with fig latex. Treated ells with 0.125 µg/mL of fig latex for 48h were labeled as a treatment. HaCaT cells showed approximately same ratio in all phases before and after treatment with fig latex. Fig latex induces cell death in Sub G1 and accumulates G1 phase population in CaSki and HeLa cells. Results are representative as mean (n = 3) ± SD of at least three independed experiments (N=3).

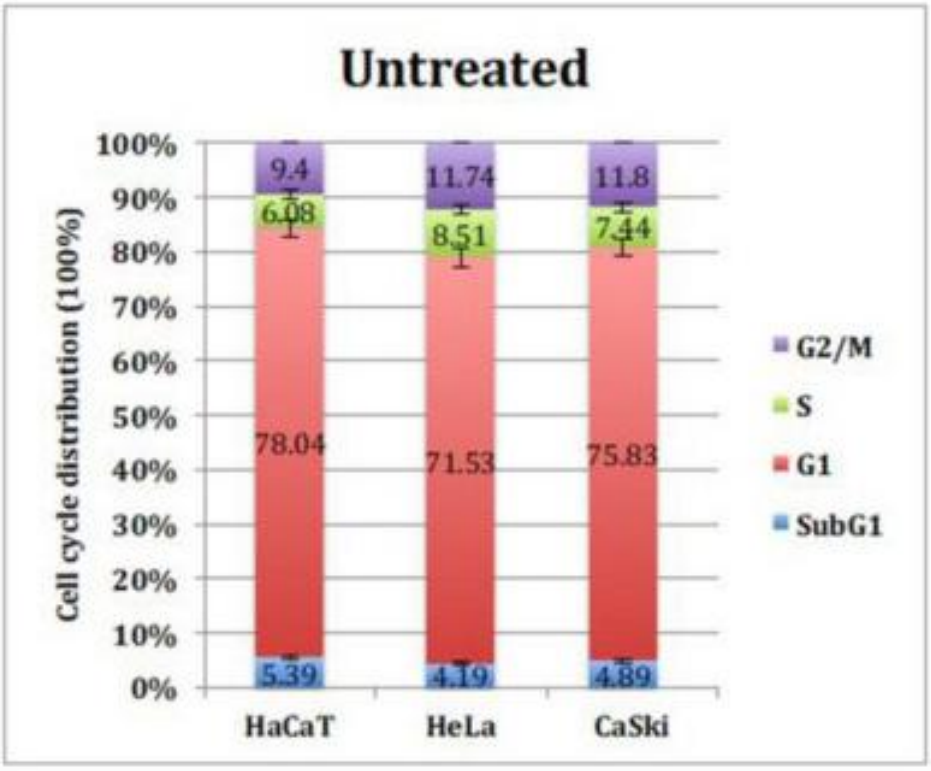

Figure 4.12. Representative cell cycle analysis in untreated cells. Summarised cell cycle distribution data in untreated cells. Untreated cervical cancer cells, CaSki and HeLa, and non-cancerous HaCaT cells approximately showed same percentage of populations in all phases of cell cycle. Results were presented as mean (n = 3) ± SD of at least three independent experiments.

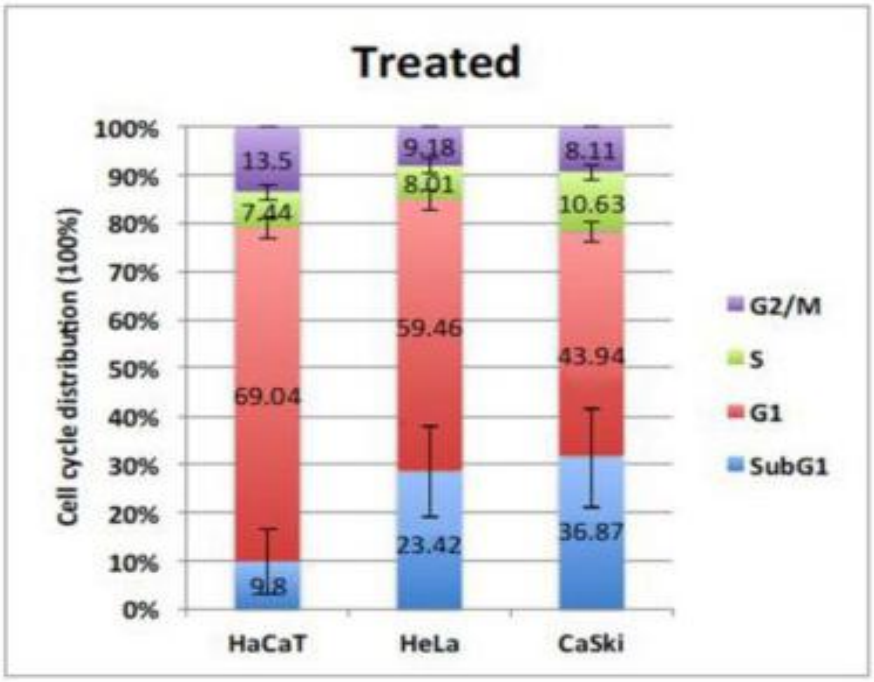

Figure 4.13. Representative cell cycle analysis in treated cells. Summarised cell cycle distribution data in treated cells. Following treatment with 0.125 µg/mL of fig latex for 48h the population of cervical cancer cells (CaSki and HeLa) in Sub G1 phase has incaresed considerably. The incarsee is followed y a decrease in the G1 phase. Whereas following treatment with with 0.125 µg/mL of fig latex for 48h, HaCaT cells approximately maintained the same ratio like untreated samples. Results were presented as mean (n = 3) ± SD of at least three independent experiments.

4.2 Conclusion

Findings from the present study demonstrated that Ficus carica latex inhibits certain properties that are associated with HPV-positive cervical cancer transformed cell (CaSki and HeLa). This was further supported by assessing anti clonogenic activity of fig latex wherein a very significant decrease in the number of colonies was observed in both the cervical cancer cell lines. Interestingly, fig latex downregulated the presence/expression of cellular proliferation marker, Ki67 protein in both the cervical cancer cell lines. In contrast, non-cancerous HaCaT cells expressed Ki67 both after and before treatment with fig latex. Moreover, Ficus carica latex reduced the migration as well as invasion capability of the cervical cancer cell lines. Fig latex was also able to block cell cycle progression at sub G1 phase in HeLa and CaSki cells. It was concluded that fig latex induce cell death in Sub G1 phase and accumulates G1 phase copulation in cervical cancer cell lines. Thus, these findings provide a strong basis for further exploration of Ficus carica as a potent therapeutic modality against cervical cancer.

Chapter 5

Results

Effect of Fig latex on expression of HPV oncoproteins and their target tumor suppressor proteins

5.1 Ficus carica latex downregulates the expression of HPV onco-proteins

So far this study has clearly shown that Fig latex confers contact inhibition, cell cycle arrest, reduction in invasiveness, and reduction in cell proliferation in cervical cancer cells lines. Our data also showed that the inhibition of cell growth by fig latex is specific for cancer cells not immortalised cancer free cells (HaCaT). As discussed in the introduction, HPV infection is implicated in the etiology of cervical cancer. Using its transforming proteins (E6 and E7) this oncogenic virus can persist in the infected cells and cause the cell transformation/ cancer progression (Boulet et al., 2007; Doorbar et al., 2015; Yu et al., 2016).

The viral E6 and E7 oncoproteins are the main mediators of carcinogenesis that is attributed to their interactions with various cellular targets. The oncogenic properties of E6 are associated with cell immortalization, p53-degradation, telomerase activation and anti-apoptotic effect (Li et al., 2002; Brooks et al., 2007). The oncogenic properties of E7 are associated with cell immortalization; interaction with pRb and transactivation of E2F-dependent promoters (Dyson, 1989; Stevaux and Dyson, 2002). Therefore E6 and E7 are the primary targets in the development of new modality for HPV related cervical cancers.

To investigate whether anti-transforming properties effect of fig latex observed on HPV positive cervical cancer cells lines was due to its impact on the expression of HPV oncoproteins, this study analysed the expression of E6 and E7 protein in cervical cancer cells (CaSki; positive for HPV 16 and HeLa positive for HPV 18) treated and untreated with 0.125 µg/mL of fig latex for 48 hrs by western blotting.

As discussed in section 2.2.11 anti- HPV16 &18 E7 and Anti-HPV16 &18 E6 antibodies were used to detect the E6 and E7 oncoproteins. In the entire western blotting experiments, Beta-Actin (42 kDa) is used as a loading and internal control to validate that similar amount of protein was applied for each samples.

As shown in figure 5.1, 5.2, 5.3 untreated cervical cancer cells (CaSki and HeLa) expressed appropriate amount of E6 and E7 oncoproteins (figure 5.2 and 5.3). Interestingly, there appeared to be a noticeable reduction/downregulation on the expression level of E6 and E7 in cervical cancer cells (CaSki and HeLa) treated with fig latex. This experiment determined that same amount of protein has been applied for each

samples and the fig latex treatment has no effect on the overall level of Beta-Actin (internal) protein (Figure 5.1 and 5.2 and 5.3).

Findings from this study confirms that fig latex affect the expression of HPV transforming proteins (E6 and E7) in cervical cancer cells and this down-regulation may alter the expression of their target tumor suppressor proteins preventing the cell transformation properties in cervical cancer cells.

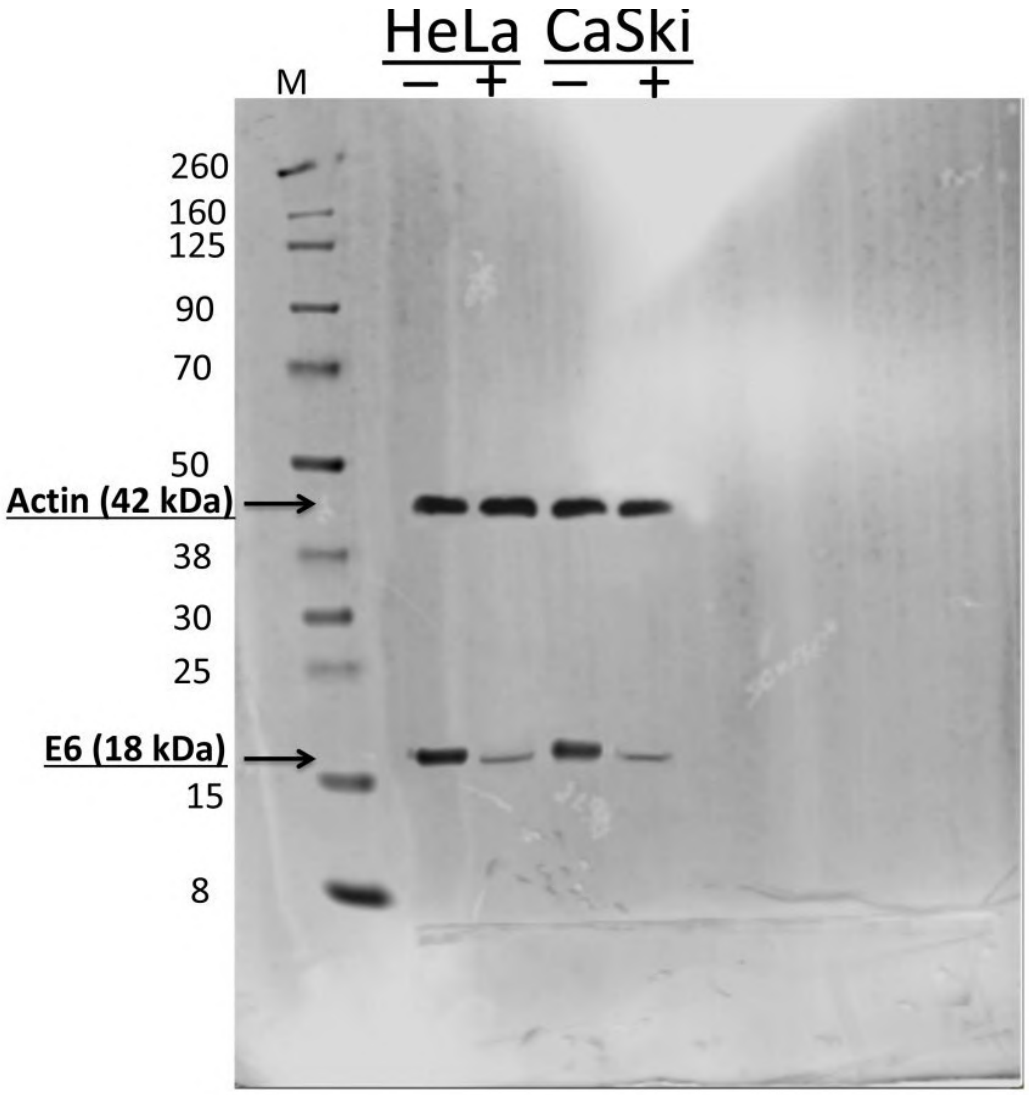

Figure 5.1. Effect of fig latex on E6 oncoprotein expression. Equal amounts (10μg) of protein lysate from each cell line were incubated with appropriate antibodies. E6 oncoprotein (18 kDa) was expressed strongly in untreated HeLa and CaSki cells. Following on treatment with fig latex the expression level of E6 oncoprotein downregulated in the both HeLa and CaSki cells. Actin expression served as a loading control (42 kDa) and it did not vary before and after treatment with fig latex. Results were presented of at least three independent experiments.

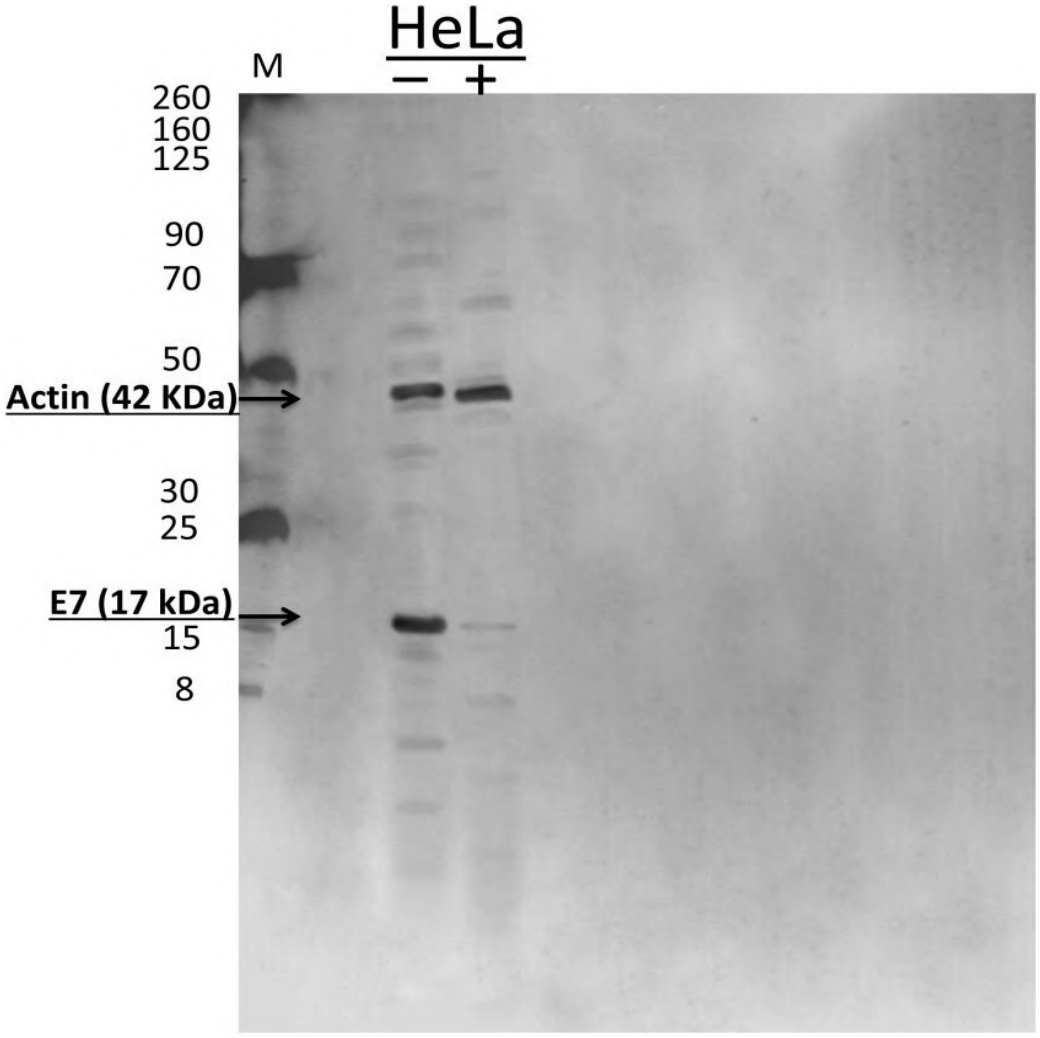

Figure 5.2. Effect of fig latex on E7 oncoprotein expression. Equal amounts (10µg) of protein lysate were incubated with appropriate antibodies. E7 oncoprotein (17 kDa) was expressed strongly in untreated HeLa and CaSki cells. Following on treatment with fig latex the expression level of E7 oncoprotein downregulated in HeLa cells. Actin expression served as a loading control (42 kDa) and it did not vary before and after treatment with fig latex. Results were presented of at least three independent experiments.

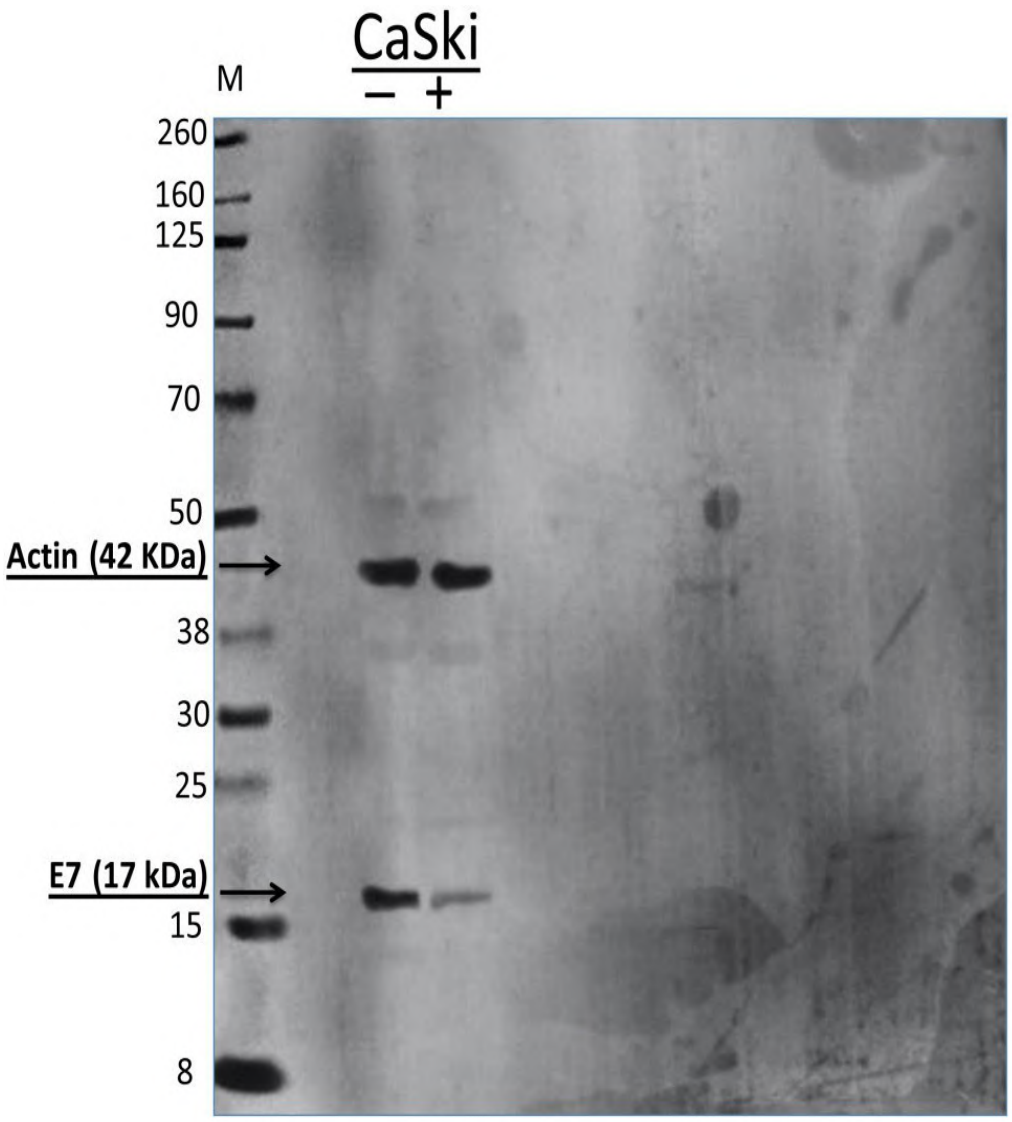

Figure 5.3. Effect of fig latex on E7 oncoprotein expression. Equal amounts (10µg) of protein lysate were incubated with appropriate antibodies. E7 oncoprotein (17 kDa) was expressed strongly in untreated HeLa and CaSki cells. Following on treatment with fig latex the expression level of E7 oncoprotein downregulated in CaSki cells. Actin expression served as a loading control (42 kDa) and it did not vary before and after treatment with fig latex. Results were presented of at least three independent experiments.

5.2. Ficus carica latex reactivates the expression of tumor suppressor proteins

Up to this point, our data from western blotting verifies that treatment with fig latex causes the downregulation of E6 and E7 expression and as a consequence it may perhaps upregulate the expression of their target tumor suppressor proteins P53 and pRb, respectively.

As discussed in the introduction, P53 plays a critical role in cell cycle regulation and induction of apoptosis and therefore defective p53 can allow abnormal cell to proliferate out of control, resulting in cancer development.

Rb protein prevents excessive cell growth by inhibiting cell cycle progression. Inactivation/downregulation of the RB protein is one of the most fundamental events in development of cancer and tumorgenesis. Many cancers can impair pRb function via causing alteration in the expression of Rb regulators such as; cyclin D, CDK4 and also their principal inhibitor, p16.

The disruption of the E6 and E7 proteins in cervical cancer cell lines by fig latex may restore the expression of their target proteins, p53 and Rb, and initiate the activation of the apoptosis mechanisms, arresting the cell cycle and also other transforming properties of cervical cancer cells. To investigate whether the inhibitor effects of fig latex on cell transformation observed in this study is due to the reestablishment of the tumour suppressor proteins target for E7 and E7, the overall expression level of these proteins in cervical cancer cells (HeLa and CaSki) treated and untreated with fig latex were analysed by western blotting using anti-p53 and anti-pRb proteins.

As expected untreated cervical cancer cells (CaSki and HeLa) have expressed very low level of p53 and pRb protein however, as shown in figure 5.4 and 5.5 the expression of these protein noticeably increased in cervical cancer cells treated with fig latex. Despite the upregulation of the p53 and Rb expression, there appear to be no changes in the expression level of Beta-Actin protein in cells treated and untreated with the fig latex. These findings suggests that upregulation of the tumor suppressor proteins p53 and pRb may be due to the ability of fig latex in downregulating the expression of HPV E6 and E7 proteins. This may cause the inhibition of transformation properties of cervical cancer cells.

As stated before P16 is a negative regulator of proliferation in cells via downregulating the activity of (CDK) 4 once the pRB has been inactivated. Following infection with HPV, its oncoproteins integrate into the host genome. This disruption is associated with buildup of p16 level and its overexpression in transformed cervical cells. Therefore p16 is promising marker in identifying the HPV related cervical cancer development.

Based on this rational, this study also aimed to explore the effect of Ficus carica latex on HPV diagnostic marker P16 expression level in cervical cancer HeLa and CaSki cells treated and untreated with fig latex was analysed by western blotting using anti-p16 antibody. In the entire western blotting experiments, Beta-Actin (42 kDa) is used as a loading control to show that very similar amounts of protein were loaded in each lane.

As shown in figure 5.6, p16 is expressed in the untreated cervical cancer cells (CaSki and HeLa) while fig latex treatment downregulated the expression P16 in both HeLa and CaSki cells. The results show the fig latex treatment has no effect on the expression level of internal control, Beta-Actin.

These promising results show that fig latex can be used to target HPV oncoproteins and interfere with the transformed phenotypes of HPV related cervical cancer cells. However, this needs to be validated by further experiments discussed in the future works.

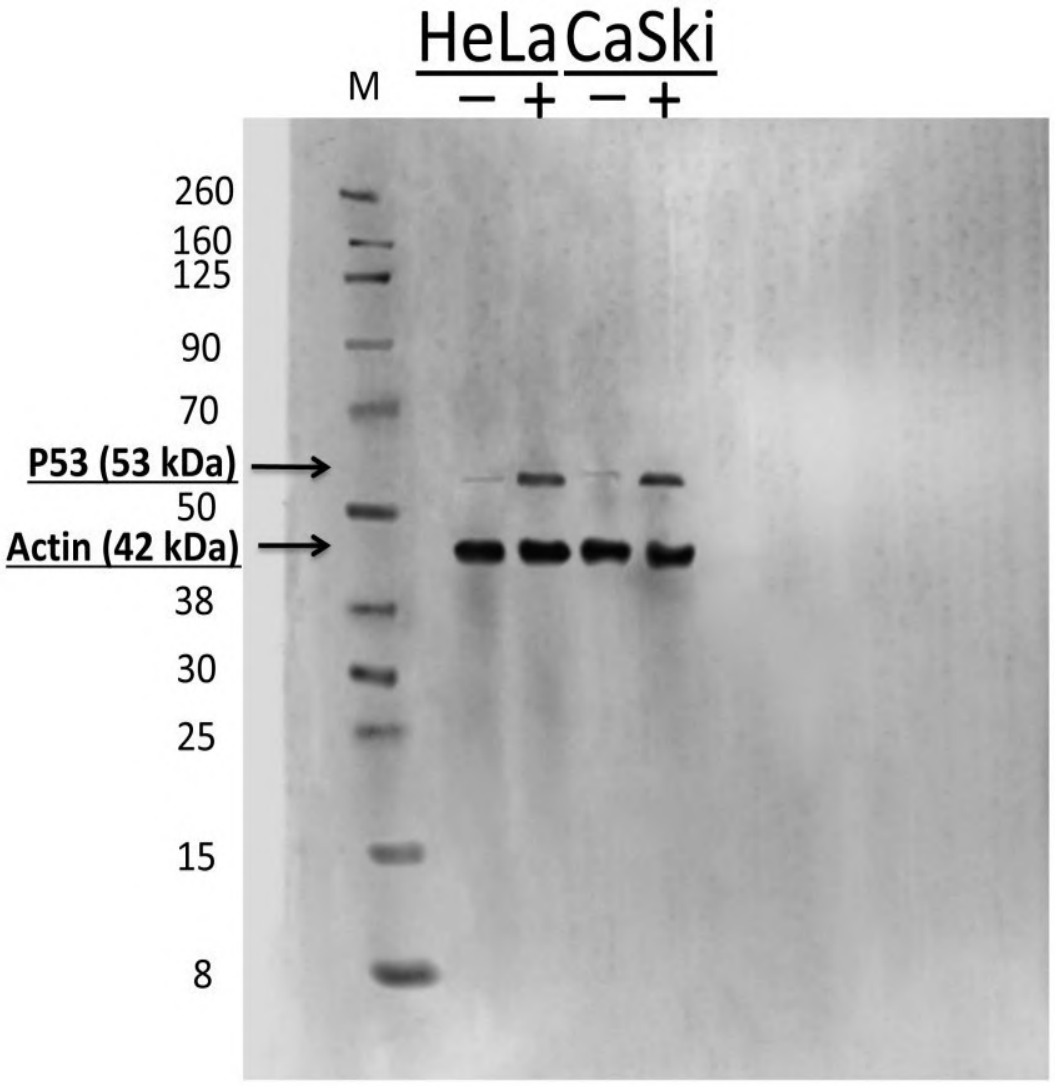

Figure 5.4. Effect of fig latex on P53 tumour suppressor protein expression. Equal amounts (10µg) of protein lysate from each cell line were incubated with appropriate antibodies. The p53 tumour suppressor protein (53 kDa) was expressed at relatively lower level in untreated HeLa and CaSki cells. Following on treatment with fig latex the expression level of P53 tumour suppressor protein upregulated in the both HeLa and CaSki cells. Actin expression served as a loading control (42 kDa) and it did not vary before and after treatment with fig latex. Results were presented of at least three independent experiments.

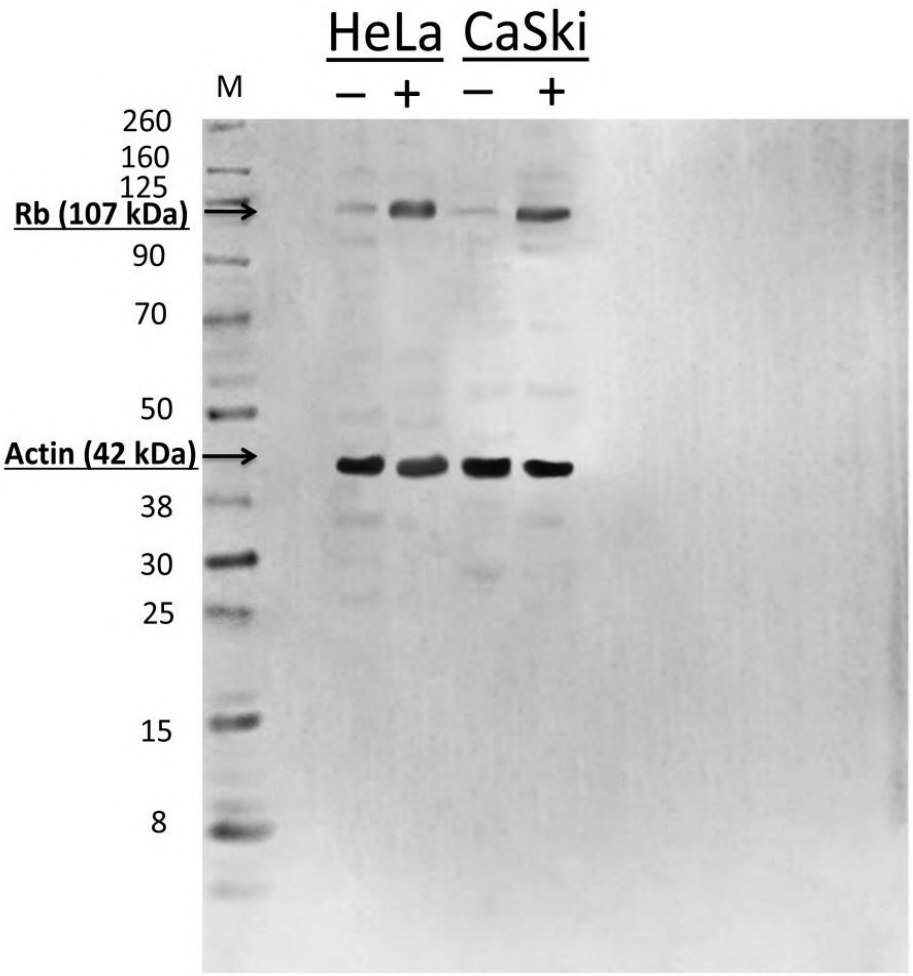

Figure 5.5. Effect of fig latex on P53 tumour suppressor protein expression. Equal amounts (10µg) of protein lysate from each cell line were incubated with appropriate antibodies. The pRb tumour suppressor protein (107 kDa) was expressed at relatively lower level in untreated HeLa and CaSki cells. Following on treatment with fig latex the expression level of pRb tumour suppressor protein upregulated in the both HeLa and CaSki cells. Actin expression served as a loading control (42 kDa) and it did not vary before and after treatment with fig latex. Results were presented of at least three independent experiments.

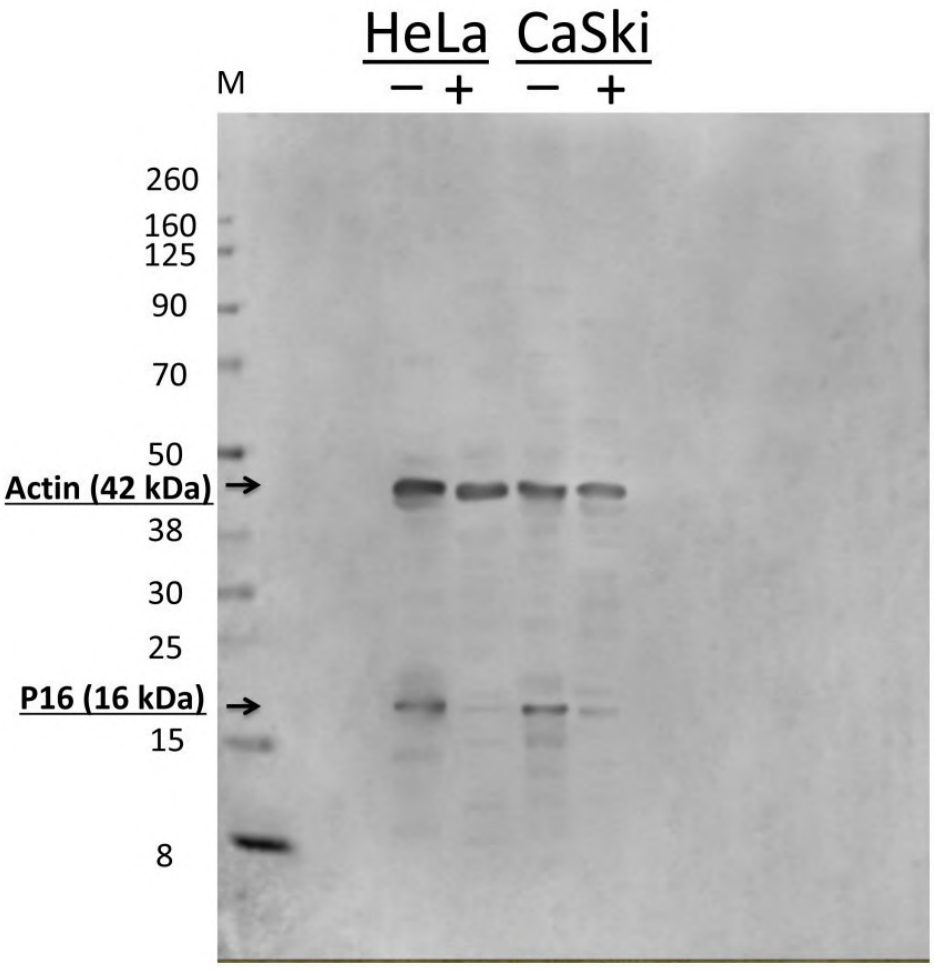

Figure 5.6. Effect of fig latex on HPV diagnostic marker P16 expression. Equal amounts (10µg) of protein lysate from each cell line were incubated with appropriate antibodies. The p16 tumour suppressor protein (16 kDa) was expressed at relatively higher level in untreated HeLa and CaSki cells. Following on treatment with fig latex the expression level of P16 downregulated in the both HeLa and CaSki cells. Actin expression served as a loading control (42 kDa) and it did not vary before and after treatment with fig latex. Results were presented of at least three independent experiments.

5.3 Conclusion

In the view of the findings from functional studies that displayed potent anti-cancer activity of Ficus Carica latex on both HPV16 (CaSki) and HPV18 (HeLa) positive cell lines, the expression of the viral oncoproteins (E6 and E7) and also tumor suppressor protein (p53, Rb and P16) in the treated and untreated cells with fig latex were examined. It was observed that the expression level of E6 and E7 oncoproteins in both treated CaSki and HeLa cells reduced, which was accompanied by upregulation of P53 and Rb protein expression indicated in the summary figure 5.7. These observations suggest that upregulation of the tumor suppressor proteins p53 and pRb may be attributed to ability of fig latex in downregulating the expression of HPV E6 and E7 proteins. Fig latex also downregulated the expression of HPV diagnostic marker P16 in both cell lines. In conclusion, anti-cancer activity of fig latex on cervical cancer cells might be correlated with its anti HPV activity that needs further investigation.

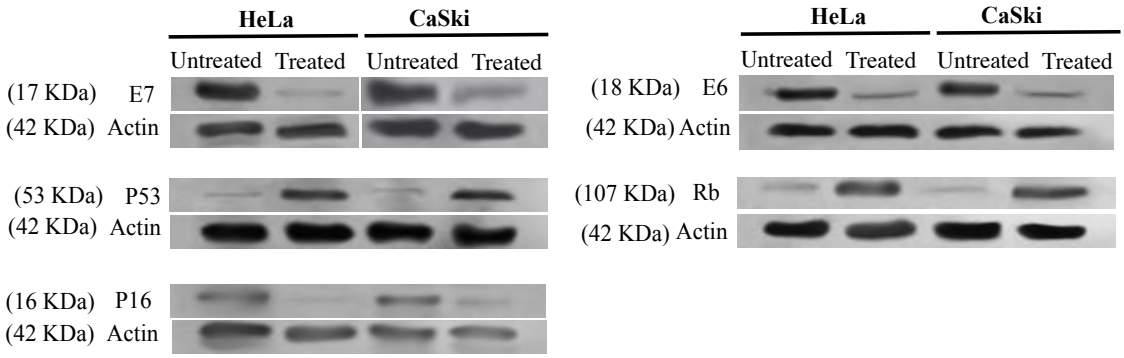

Figure 5.7. Cropped blots representing summary of the effect of *Ficus carica* latex on the expression of HPV oncoproteins (E6 and E7), tumor suppressor proteins (P53 and Rb) and HPV diagnostic marker (P16). Equal amounts (10µg) of protein lysate from each cell line listed were analyzed by immunoblotting with appropriate antibodies. Actin expression served as a loading control (42 kDa). *Ficus carica* latex down-regulated the expression of p16 and HPV oncoproteins but upregulated the expression of tumor suppressor proteins. No significant differences were seen in the expression of actin.

Chapter 6
Discussion

6. Discussion

6.1 Introduction

Despite considerable developments in medical sciences, cancer is still a major cause of death globally (WHO, 2018). A major challenge for the development of an appropriate anticancer drug is the complexity of this disease (Woodman et al., 2011). The function of the majority of cancer therapies is directed towards a limited number of well-established targets (hallmarks of cancers) (Malmberg, 2004). This reflects the difficulty in development of new modality for targeting the cancer cells. Generally the cancer complexity is categorised into ten hallmarks; enabling replicative immortality, sustained growth signal, evading growth suppressors, inducing angiogenesis, resisting cell death, activating invasion and metastasis, reprogramming energy metabolism, tumor promoting inflammation, avoiding immune destruction and genome instability (mutation) (Hanahan and Weinberg 2011; Weinberg, 2014). These hallmarks provide an organisational framework of cellular properties involved in cell transformation. It is essential to take into account that the aforementioned characteristics can act as complementary promoters in cell transformation, carcinogenesis and progression of malignancy (Wlibur et al., 2017). The sustained growth signals and evading growth suppressors` features of cancer cells propose a potential of self- sufficiency for survival and growth. These features are attributed to the role of telomerase and can associate with alterations in oncogenes or by the inactivation of cellular tumor suppressor genes (Rexer et al., 2014). Enabling replicative immortality and resisting apoptosis leads to uncontrolled cell division and proliferation, contributing to cancer progression and formation of tumor mass (Fulda, 2009). Angiogenesis facilitates and provides the essential elements for tumor invasion and metastasis (Ashkenazi, 2008; Rajabi et al., 2016). Cancer cells can alter pathways for their energy metabolism (Warburg et al., 1924) enabling to reprogram the sources for their growth. Although cancer cells facing with immune responses, but they can escape immune surveillance (Zamaron and Chen, 2011). Coupled with this, there is a link

between chronic response of immune system (inflammation) and development of cancer (Lou et al., 2014).

It is well known that multiple risk factors are associated with the development of cancer and many studies have been conducted on the causative factors responsible for the initiation of cancer (Hahn and Weinberg, 2002). Among all, infectious agents have been considerably implicated, either as direct carcinogens or as promoters for development of cancer. Viral infections, in particular, human papillomaviruses (HPVs) are recognised as carcinogenic agents in humans, and are responsible for a significant share of the global cancer burden (Ault et al., 2006). HPVs can infect epithelial surface such as skin and genital causing benign lesions (warts). Persistence of high risk (HR) HPVs infection in the host cells can eventually cause progression of infected lesion to malignant disease. Notably infection in the cervix with HR-HPV types 16 and 18 has been reported in initiation of cervical cancer (Stapley and Hamilton, 2011). HPV onco-proteins E6 and E7 are responsible for cellular alterations in HPV infected cells. E6 interacts with tumor suppressor protein p53 and prevents its function; this activity can eventually impede apoptosis (Brooks et al., 2007). On the other hand, E7 binds to retinoblastoma (Rb) protein and prevents the interaction of Rb with its natural target, namely transcription factor E2F (Dyson, 1989). As a result the checkpoint that controls G1/S transition becomes distorted, causing uncontrolled proliferative lesions (Liu et al., 2004). These proliferative lesions can progress to high-grade lesions and form an invasive type of cervical cancer (Winer et al., 2005). Although numbers of cancer treatment modalities has grown over the past decade. To date, there has been no satisfactory medical treatment for human papillomavirus related cervical cancer as most of the developed treatments (e.g., surgical excision, chemotherapy, and cryotherapy) are eventually accompanied by excessive tissue injury (National Cancer Institute, 2018). Thus development of a safer and more efficient strategy is highly demanding.

The use of herbal medicine and plant derived remedies has continuously increased over the past decades (Hosseinzadeh et al., 2015; Zhang et al., 2015; Gunjan et al., 2015). Among the such medically relevant plants, the fig latex (*Ficus carica)*, when applied to low risk human papillomavirus (HPV) related skin warts and bovine papillomavirus (BPV) related warts, has shown potential as a possible cure for the viral related lesions

without inevitable tissue injury and remedial complications (Ghazanfar., 1994; Hemmatzadeh, et al., 2003; Bohlooli et al., 2007). Fig latex also reportedly offers various therapeutic effects such as anti-Herpes Simplex Virus (HSV)-1 (Wang *et al*., 2004), anti-bacterial activity (Al-Sabawi., 2010), anthelmintic (Stepek et al., 2005).

Although a few studies consider the distinctive medicinal features of fig latex, the mechanisms of action of the biologically active components of the Ficus carica species remains poorly understood. This study provided a deeper and extended insight into the mechanisms of action and biological activities of Ficus carica latex on human cervical cancer cell lines expressing high risk HPVs type 16 and 18. The main findings of this PhD study are discussed below.

6.1 Chemical Analysis and cell viability assay

Fig latex comprises aqueous medium and gummy compartment that makes the latex sticky and restricts *in vitro* biological studies. To overcome these restrictions, one optimal procedure in this study was to determine the biologically active compound in the gummy compartment of fig latex. To address this question, the chemical components of aqueous part, gummy part and whole latex were compared from biological perspectives. This study for the first time reported some of the chemical constituents of crude fig latex, aqueous supernatant and polymeric gum of fig latex. Findings from chemical analysis with novel observation illustrated that polymeric gum did not have significant chemical components and consequently it has been excluded from this study. The lyophilised fig latex (powder) represented mixture of saturated and unsaturated fatty acids/triglycerides while fig latex Supernatant (aqueous compartment) showed more biologically active compounds.

To address these observations more precisely, the aqueous part of latex was compared to whole latex for biological activities on cancerous (HeLa and CaSki) and normal non - cancerous cells (HaCaT) using MTT assay. Data obtained from biological analysis confirmed that the aqueous part of the latex was as effective as the crude latex and was suitable to carry out further functional studies. Therefore the aqueous part of the latex (supernatant) was used and exclaimed as “latex” throughout this study. Subsequently, using cell viability assay this study has ascertained that the cytotoxic effect of latex on

cell growth of cervical cancer cell line (CaSki and HeLa) as well as non-cancerous HaCaT cells is dose-dependent but not significantly time-dependent manner. Analysis of MTT assay data showed very significant reduction (approximately 1.6-fold) of cell growth in CaSki and HeLa cells treated with 0.125 μg/mL of fig latex while, importantly, no significant effect was found on control non-cancerous HaCaT cells. In addition, treatment with higher concentration of fig latex (0.25 μg/ ml, 0.5 μg/ml and 1 μg/ml) resulted in inhibition of the cell growth in all cell lines but to lesser extent in HaCaT cells. The IC50 values also confirm that the inhibitory effect of fig latex on cancer cells growth was under dose dependent manner but not noticeably time dependent manner and 0.125 μg/mL of fig latex was the most suitable concentration to treat the cells. Therefore a certain concentration 0.125 μg/ml (IC50) of fig latex was the most appropriate concentration to investigate the activities of latex on cervical cancer cells. This obviates any toxic effect on the control human immortalised keratinocyte (HaCaT) cells, hence preventing any confounding data.

These results corroborate the findings of previous studies by Rubnov et al (2001) on Raji cells and Hashemi and Abediankenari (2013) on esophageal cancer cell who also reported the dose dependency effect of fig latex on cancer cells.

Another study conducted by Wang et al., (2008) demonstrated that fig latex represents strong dose and also time dependent cytotoxicity activity against human glioma and human hepatocellular carcinoma (HHC) cells with minimal inhibitory effect on normal liver cells. However in contrast, this current study showed that effect of fig latex on cervical cancer cells is not time dependent. The most reasonable explanation for these findings is that the cytotoxic activity of fig latex in terms of time dependency might vary using different cancer cell lines. Nevertheless, further evidences by using various cancer cells could help to appraise the probable time dependent inhibitory effect of fig latex.

Further elucidation of biologically active compounds in fig latex was performed using chemical extraction such as; Acetonitrile (MeCN), Dichloromethane (DCM), Chloroform (Trichloromethane) and Petroleum ether. As fig latex was comprised of wide spectrum of chemical compounds the extraction procedure was an essential approach to narrow down the chemical constitutes of aqueous part of the fig latex (supernatant).

All the extracts were examined for cytotoxicity activity against cervical cancer cells and also non-cancerous HaCaT cells using MTT assay. Analysis of MTT assay data showed the 0.125 μg/ml of Petroleum ether extraction generates quite similar result to the data obtained from application of the unexctracted supernatant on cells and therefore petroleum ether extraction was used for further chemical analysis of fig latex.

Following the determination of an appropriate extract, a more detailed analysis MS/NMR data with the novel observation proposed that aromatic region of petroleum ether extract of fig latex supernatant showed at least two aromatic components, suggested as ferrulic, caffeic or chlorogenic acid. According to these findings the biologically active component in fig latex is likely to be lipophilic and possibly a chlorogenic/ferrulic/caffeic acid plant sterol derivative. Coupled with this, 2D NMR also suggested that there are steroidal compounds present in the pet ether extraction of the fig latex supernatant. Specifically, there was also evidence for the related phytosterol fatty acid glycosides - 6-O-acyl-β-D-glucosyl-β-sitosterols. These findings are in agreement with previous findings wherein they detected similar compound in fig latex (Rubnov et al., 2000).

Complementary to the MS/NMR findings/subsequently, by using HPLC-MS profile of fig latex the structures of main chemical components of petroleum ether fraction of fig latex supernatant were determined and are proposed to be; Oleadiene, Lanostatriene and Sttigmasterol. Whilst the analysis is preliminary and further, more demanding, purification steps will be required to narrow down the many components

Isolation/identification of the active compounds from the Petroleum ether fraction of the fig latex is now a priority, hampered by the limited amounts of fig latex that can be obtained from each plant. The collection process of fig latex was very complicated as each fruit provides maximum of one to two drops of latex and unripe fruit needs to be picked by mid of summer at the best. Other critical predicaments such as complication during transportation from origin to laboratory of Kingston University and preservation were very stressful and restricted the study. Therefore, further samples of fig latex are essential to continue the investigation more comprehensively.

6.2 Fig latex inhibits specific properties of HPV related transformed cells

Based on the findings from chemical analysis and cell viability experiments the investigation was extended on the functional activity of fig latex on cervical cancer cells to evaluate whether Ficus carica can inhibit specific properties of previously HPV related transformed cells. The influence of fig latex on each cancer cell transformation properties is discussed below.

6.2.1 Contact inhibition

One of the transforming properties of cancer cells is loss of contact inhibition or the ability of cancer cells to form colony. Contact inhibition regulates cell proliferation and arrest cell growth as a consequence of mechanical cell to cell interaction. This characteristic is lost when cells undergo malignant transformation, leading to uncontrolled proliferation and tumor formation.

Using a focus formation assay, as expected this study showed that only cervical cancer cells (CaSki and HeLa) were able to form colony whereas non-cancerous HaCaT cells were able to cease proliferation and growth when they become confluent. Interestingly, fig latex treatment has allowed cancer cells to exit contact inhibition. This has lead to a noticeable quantitative reduction of colony in treated cells compare with the numbers of colony formed in untreated ones. Taken together, results from colony formation assay suggest that fig latex is able to seize the inhibitory effect of contact inhibition driven by cervical cancer. Previous studies reported the anticancer activity of various natural products by reducing clonogenicity of cervical cancer cells such as; Curcumin on SiHa and CaSki (Zaman et al., 2016) and Livistona on CaSki and HeLa (Ming et al., 2016). Results from previous studies (Bai et al., 2015; Jia et al., 2018) are also in accordance with our findings and show that the inhibition of cell growth besides suppression of colony formation ability of cancer cells could be a target for development of new therapeutic modality against cervical cancer.

6.2.2 Cell proliferation

As discussed in 4.1.2, cell proliferation is a principal feature in cell growth and division and ki67 is one the most common methods for evaluation of proliferation. The expression of Ki-67 protein is highly correlated with the proliferative status of a cell. It is expressed in the nucleolus of proliferating cells but is absent in resting cells. Because proliferation plays key role in transformation of cancer cells, targeting ki67 propose a potential for development of anti-cancer modalities. The expression of ki67 has been evaluated in various cancers such as breast cancer (Kittaneh et al., 2013), soft tissue sarcomas (Sorbye et al., 2012), lung cancer (Ishihara et al., 2012) and prostate cancer (Josefsson et al., 2012).

Our data from cytotoxicity and cologenic assay showed that fig latex poses anti-proliferative characteristics against CaSki and HeLa cells. To address this more precisely, we investigated the effect of fig latex on presence/expression of the Ki67 using immunofluorescence. Ki67 was expressed in nucleolus of non-cancerous HaCaT cells and also cervical cancer cells (HeLa and CaSki). Interestingly, at non-cytotoxic dose, Ki67 protein was depleted almost exclusively in the nucleolus of CaSki and HeLa cells. But, conversely Ki67 was almost exclusively located in the nucleolus of treated HaCaT cells. These results validate the results form MTT assay that 0.125 μg/mL of fig latex does not affect normal cells growth properties. There is limited data available regarding depletion of Ki67 especially with regards to cervical cancer cells. Nonetheless, one possibility is that ki67 expression is disrupted due to alteration in cellular elements or the ki67 protein is degraded. Other possible explanation is the probability of dissociation and dislocation of this proliferation marker. However, further examination is required to figure out the effect of fig latex on Ki67.

Altogether, these finding strongly suggests that fig latex may target the expression of Ki67 in cervical cancer cells and plays a substantial role in preventing cell proliferation that could ultimately inhibit cancer progression.

6.2.3 Migration and invasion

Tumor metastasis in a variety of sites is one of the most common causes of cancer death. Therefore targeting the migratory and invasiveness capabilities of cancer cells via development of effective anti-cancer therapies can reduce cancer mortality. Studies have yielded evidences about the importance of cell migration and invasion in progression of numerous cancers such as; gastrointestinal cancers (Ren et al., 2019), breast caner (Huang et al., 2018), cervical cancer (CaSki) (Zhang et al., 2017) and glioblastoma (brain cancer) (Cornelison et al., 2018).

In the light of these findings and also the importance of metastasis in cancer development, this study showed the influence of fig latex treatment on other transforming properties of cancer cells (CaSki and HeLa) such as migration and invasion.

These findings represented that cancer cells were able to migrate and invade towards lower chambers of transwells without adding treatment whereas these migratory and invasiveness capabilities reduced noticeably after treatment with fig latex. Analysis of cell migration and invasions assay data also showed extremely significant reduction in numbers of migrated and invaded CaSki and Hela cells towards lower chambers after treatment. As expected non-cancerous HaCaT cells are unable to migrate and invade towards the chambers of the transwells without treatment.

A study conducted by Tezcan et al., (2014) reported that combination of fig latex and temozolomide (anti-cancer drug) synergistically reduced invasiveness of glioblastoma tumor. These data are partially in agreement with our findings and demonstrate fig latex could alleviate invasiveness ability of cancer cells. While results in this study showed that fig latex is capable of suppressing cancer cells migration and invasion, it would be of particular interest to employ other methods such as; 3D modeling, and would healing assay to elaborate our understanding about mechanism of action of fig latex.

Taken together, these data revealed that fig latex could prevent dissemination of cervical cancer cells into new areas and may barricade formation of metastatic tumors and reduce cancer related mortality.

6.2.4 Cell cycle Distribution

Findings obtained from this study up to this point lead to the hypothesis that inhibition of cell growth and anti proliferative effect of fig latex on cancer cells might be in correlation with deregulation in cell cycle distribution. Thus distribution of the cells in Sub G1, G1, S and G2/M phase of cell cycle were examined and compared before and after treatment with fig latex. Data obtained from this study suggested a correlation between inhibition of cell growth and induction of cell cycle arrest. Cells (HaCaT, CaSki and HeLa) were predominantly accumulated in G1 phase of cell cycle before adding treatment however, following treatment with 0.125 µg/mL of fig latex for 48h, CaSki and HeLa cells population in Sub G1 phase was increased considerably. Conversely, unlike cancer cells, when HaCaT cells were treated with fig latex they showed approximately same ratio in all phases of the cell cycle.

Treatment with fig latex has allowed the cell cycle exit by inducing the cell cycle arrest in Sub G1 phase in cervical cancer cell lines. The inhibition of cancer cell growth could occur due to induction of apoptosis and these findings concur with findings from previous studies that attributed the inhibition of cell growth to cell cycle arrest in various human cancers such as; cervical cancer, prostate cancer, oral cancer and osteosarcoma using a well-known plant-derived product (Quercetin) (Bishayee et al., 2013; Gokbulut et al., 2013; Sak, 2014). Also, data from a study conducted by Haddaad et al (2006) represented the critical role of natural products in induction of cell death by perturbing the cell cycle progression that complies with these findings.

Still further examinations are needed to strengthen these findings and determine the apoptosis pathways due to fig latex treatment. Evaluation of apoptosis mediators from Caspase family, especially Caspase 3, might shed some lights on way this happened.

Altogether, this finding demonstrates the ability of fig latex in preventing cell progression and proliferation thorough induction of apoptosis and shifting the cells population into Go phase. The cell cycle deregulation observed in this study may represent an efficient anti-cancer strategy in delaying cervical cancer development.

6.3 Fig latex downregulates the expression of HPV oncoproteins and reactivates the expression of tumor suppressor proteins

Until the present, data obtained from this study demonstrate a number of parameters of cell transformation (lack of contact inhibition, cell cycle arrest, invasiveness, and cell proliferation) are conferred by the Fig latex. As mentioned before, high risk HPVs, especially type 16 and 18, can infect cervical cells and cause lesions. These lesions can persist and progress to cervical cancer by preventing the expression of tumour suppressor proteins (P53 and Rb). Expression of E6 and E7 promotes cell invasion and metastasis, impedes apoptosis, and induces uncontrolled cellular growth and immortalization. E6 interacts with P53 that is substantially contributed to the regulation of cell cycle progression via apoptosis pathways, whereas E7 stimulates hyper proliferation of the infected cells through deactivation of Rb-dependent G1/S checkpoint activation. In addition, Rb regulates p16 via negative feedback system and therefore the Inhibition of Rb through E7 could increase the expression of p16. P16 acts as a negative regulator of proliferation in cells once the Rb has been inactivated. A considerable endeavors have been taken towards specific pharmacological targeting of HPV-mediated events during carcinogenesis. It has been shown that elevated levels of P53 was accompanied by the reduced levels of HPV E6 in CaSki and HeLa upon treatment with β-sitosterol (Cheng et al., 2015) leading to inhibition of cell proliferation. Munagala et al., (2011) reported that repression of HPV E6 and E7 oncogenes using natural product (Withaferin A), resulted in sequential reactivation of p53 tumor suppressor activity leading to growth inhibition of cervical cancer cells (CaSki and HeLa).

Therefore, it is of great interest to develop a more efficient and cost effective modality that is not merely affecting cancer cells transforming properties but also substantially target HPV oncoproteins.

To examine whether the effects of fig latex observed in this study were due to its impact on the expression of HPV oncoproteins, E6 and E7, the expression of these proteins in cervical cancer cells treated with the fig latex was analysed. Indeed, findings from this study showed that fig latex considerably downregulates the expression level of HPV oncoproteins, E6 and E7 as well as p16 (marker for HPV infection) that was accompanied by an increase in expression level of P53 and Rb.

These observations suggest that the *in vitro* effect of fig latex on the cervical cancer cell transformation might be due to remedial characteristics of the chemical constitute of fig latex on the expression of HPV oncoproteins.

In conclusion, our findings reveal that fig latex has a potential to play a role in preventing cervical cancer progression by inhibiting growth of cervical cancer cells through the upregulation of tumor suppressor proteins, p53 and pRb, which is most likely a consequence of the reduced levels of E6 and E7, respectively. The outputs of this study provide a strong incentive to advance research in delaying HPV related cancer progression.

6.4 Future Work

Data obtained from this PhD study represented the biological activities of Ficus Carica latex in cervical cancer cells. Chemical analysis indicated the presence of many isomers present with a mass of 409, molecular formula being C30H48. Obtained data from this study suggests that there are steroidal and phenolic compounds present in the pet ether extraction of the fig latex supernatant. The analysis is preliminary and further, more demanding, purification steps will be required to narrow down the many components to that one active. Therefore, characterisation of the chemical constituents in fig latex and exploration of functional activities of these specific isolated chemical substances that may contribute to the exhibition of therapeutic activities is of great interest. Another important notion to be made is that once biologically active compounds are combined together, they can either produce synergistic or antagonistic effect in the overall result of their therapeutically efficiency. With regards to the therapeutic effect of fig latex further in detailed investigations are essential to clarify this point.

Findings from this study also have shown that in HPV positive cervical cancer cell lines (HeLa and CaSki) treated with fig latex, Ki67 protein was depleted almost exclusively in the nucleolus. This strongly suggests that fig latex may potentially target the expression of Ki67 in cervical cancer cells to prevent cell proliferation that could ultimately play a role in the inhibition of cancer progression. Else, using western blotting technique the overall levels of Ki67 in the control non-cancerous cells (HaCaT), HPV positive cervical cancer cells (HeLa and CaSki) can be determined. The outcome of this study will indicate that whether that aberrant expression of Ki67 is either because of targeting the HPV oncoproteins or direct effect on cervical cancer cell line. Additionally we will examine the levels of additional proliferation markers such as cyclin A and PCNA will enrich the study.

The 2D *in vitro* findings suggests that the latex presents anti-cancer effects by various mechanisms, including inhibition of cell transformation. In addition to its potent anti-cancer effects, the results obtained also indicate that Fig latex has profound influence on

the deregulation of HPV oncoproteins (E6 and E7) and HPV diagnostic marker protein (p16) and initiates the reactivation of Rb and p53 tumor suppressor proteins.

Using more closely resemble in vivo study (3D culture) We are planning to validate the previous findings from our functional study (colony formation, cell proliferation, migration and invasion, and the expression of tumor suppressor proteins) between the non – cancerous, normal cervical keratinocytes, and HPV positive and negative cervical cancer cell line, as mentioned above.

Moreover, data from FACS analysis suggested that the latex presents anti-cancer effects by induction of apoptosis. An investigation on the levels of apoptotic proteins such as caspase 3 would help to determine cell's entry point into the apoptotic signaling pathway following on treatment with fig latex.

Ashrafi et al (2006) reported that E5 retains MHC class I in Golgi apparatus and therefore HPV infection can invade from immune system and get persisted. Consequently it would be of great interest to evaluate alteration of other HPV oncoproteins such as E5 and its target protein MHC class I. It would also be very interesting to determine whether fig latex can affect HPV protein that are involved in viral encapsulisation and facilitates viral entry to host cells such as L1 and L2.

Finally, to investigate whether fig latex has the potential to be used in the development of therapeutic modalities of other cancers (Not Just cervical cancer), this study will further investigate the role of fig latex on the breast cancer cell lines positive and negative for HPV.

References

Abdullah ASM, Lau Y, Chow LWC (2003). "Pattern of alternative medicine usage among the Chinese breast cancer patients: implication for service integration". The American Journal of Chinese Medicine; **31,** 649–658.

Abid T, and khotam A. (2013) Proteolytic versus surgical removal: the therapeutic effect of fig tree latex (Ficus carica L) on cutaneous and diphtheric forms of avian pox in pigeons (Columba domestica) Iraqi journal of veterinary medicine **28**(1) ; 49-53.

Abramo C.M., Archambault J. (2011). Small molecule Inhibitors of Human Papillomavirus Protein-Protein Interactions, The open Virology Journal, **5**, 80-95.

Abusnina A, et al., (2015) Tumour growth inhibition and anti-angiogenic effects using curcumin correspond to combined PDE2 and PDE4 inhibition, Thrombosis and Haemostasis .113(2). 221-435.

Alberts B, Johnson A (2014). Molecular Biology of the Cell. Garland Science. **7**, 4-9.

Alicka, M. and Marycz, K. (2018). The Effect of Chronic Inflammation and Oxidative and Endoplasmic Reticulum Stress in the Course of Metabolic Syndrome and Its Therapy. *Stem Cells International*, 2018, **41**. 1-13.

Al-Sabawi NA. (2010) " The Antibacterial Effect of Fig (Leaves Extract and Latex) on Enterococcus faecalis as Intracanal Medicament" .Al–Rafidain Dent J. 2010; **10**(1): 62 - 71.

American Asssociation Cancer Society. Cancer Facts & Figures (2017). Atlanta (GA): American Cancer Society; 2017.

Ambasta, Rashmi K, Archita Sharma & Pravir Kumar (2011). "Nanoparticle mediated targeting of VEGFR and cancer stem cells for cancer therapy" Vascular Cell, **3**:1-3.

Anand P, Kunnumakkara AB, Sundaram C, Harikuar KB, Tharakan ST, Lai OS, Sung B and Aggarwal BB (2008). "Cancer is a preventable disease that requires major life style changes". Pharmaceutical Research; **25**, 2097-2116.

Antonsson A, Spurr TP, Chen AC, Francis GD, McMillan NAJ, Saunders NA, Law M and Bennett IC (2011). "High Prevalence of human papillomaviruses in fresh frozen breast cancer samples". Journal of Medical Virology; **83**, 2157-2163.

Ashrafi GH, Haghshenas MR, Marchetti B, O'Brien P, Campo MS (2005). "The E5 protein of human papillomavirus type 16 selectively down-regulates surface HLA class I". International Journal of Cancer. **113**, 276–283.

Ashrafi GH, Tsirimonaki E, Marchetti B, O'Brien PM, Sibbett GJ, Andrew L, Campo MS (2002). "Down-regulation of MHC class I by papillomavirus E5 oncoproteins. Oncogene. **21**, 248–259.

Ashrafi GH, Briwn DR, Fife K and Campo M. (2006) Down-regulation of MHC class I is a property common to paiplommaviruses E5 proteins. Virus Research; **120**, 208-211.

Ashrafi GH, Pemural D (2015). Human Papillomavirus and Cancer-Immunological Consequences of MHC Class I Down-Regulation International biology biomed journal, **1**, 1-7.

Ashkenazi A. (2008). Targeting the extrinsic apoptosis pathway in cancer. Cytokine Growth Factor Rev. **19**, 325–331.

Assoian RK, Klein EA (2008). Growth control by intracellular tension and extracellular stiffness. Trends Cell Biol.; **18** , 347–352.

adams Jc. (2001). cell-matrix contact structures. *Cell Mol Life Sci,* **58**, 371-92.

Ault KA (2006). "Epidemiology and Natural History of Human Papillomavirus Infections in the Female Genital tract". Infectious Diseases in Obstetrics and Gynecology. **23**, 1–5.

Aziz Salman et al., (2017). "Association of High Risk Human Papillomavirus and Breast cancer: A UK based Study. *Scientific Reports* volume**7**, Article number: 43591

Balmain A, Gray J, Ponder B. (2003). The genetics and genomics of cancer. *Nat Genet Suppl* ; **33**: 238–44.

Bandelji, D., Javornik, B., & Jakse, J. (2007). Development of microsatellite markers in the common fig Ficus Carica L. Molecular Ecology Notes, **7**,1311-1314.

Barolo, M. I., Ruiz Mostacero, N. & López, S. N. Ficus carica L. (Moraceae): an ancient source of food and health. *Food Chem.* **164**, 119–127 (2014).

Barltrop J, Owen T. (1991). 5-(3-carboxymethoxyphenyl)-2-(4,5-dimethylthiazoly)-3-(4-sulfophenyl)tetrazolium, inner salt (MTS) and related analogs of 3-(4,5-dimethylthiazolyl)-2,5-diphenyltetrazolium bromide (MTT) reducing to purple water-soluble formazans as cell-viability indicators. Bioorg Med Chem Lett. **1**, 611–614.

Batson J, et al. (2013). "Regulation of contact inhibition of locomotion by Eph-ephrin signaling". J Microsc. **251**, 232–241.

Benassi, B., Fanciulli, M., Fiorentino, F., Porrello, A., Chiorino, G., Loda, M., Zupi, G. and Biroccio, A. (2006). c-Myc Phosphorylation Is Required for Cellular Response to Oxidative Stress. *Molecular Cell*, **21**(4), 509-519.

Biggar K., Storey K. (2009). "Perspectives in Cell Cycle Regulation: Lessons from an Anoxic Vertebrate. Current Genomics, **10**, 8-12

Bishayee K, et al., (2013). Quercetin induces cytochrome-c release and ROS accumulation to promote apoptosis and arrest the cell cycle in G2/M, in cervical carcinoma: signal cascade and drug-DNA interaction. Cell Prolif. **46**:153–63.

Blasco MA. Telomeres and human disease: ageing, cancer and beyond (2005). Nat Rev Genet. ; **6** ,611–22.

Boeker, M., França, F., Bronsert, P. and Schulz, S. (2016). TNM-O: ontology support for staging of malignant tumours. *Journal of Biomedical Semantics*, **7**(1).

Borrello MG, et al (2005). Induction of a pro inflammatory program in normal human thyrocytes by the RET/PTC1 oncogene, Proc. Natl Acad. Sci., **102**, 14825-14830.

Bonakdar N., Butler J.P., Fabry B., Koch T.M., Mu S. (2012). 3D traction forces in cancer cell invasion. PLoS ONE; **7**: 33476.

Bosch FX, Lorincz A, Muñoz N, Meijer CJ and Shah KV. (2002). "The causal relation between human papillomavirus and cervical cancer." J Clin Pathol., **55**, 244–265.

Boudreau, L.J. van't Veer, M.J. Bissell (2012). An "elite hacker": breast tumors exploit the normal microenvironment program to instruct their progression and biological diversity Cell Adhes. Migr. **6**. 236-2489.

Boulet G. et al. (2007). The International Journal of Biochemistry & Cell Biology ,**39,** 2006–2011.

Brandsma JL, Shlyankevich M, Zelterman D, Su Y. (2007). TherapeuticVaccination of rabbits with a ubiquitin-fused papillomavirus E1, E2, E6 and E7 DNA vaccine. Vaccine; **25**, 6158-63.

Braaten KP et al., (2008). Human Papillomavirus (HPV), HPV-Related Disease, and the HPV Vaccine. Rev Obstet Gynecol. **1**, 2-10.

Brooks CL, Li M, Gu W (2007). Mechanistic studies of MDM2-mediated ubiquitination in p53 regulation. *J Biol Chem* ,**282**, 22804-15.

Bruno S, Darzynkiewicz Z (January 1992). "Cell cycle dependent expression and stability of the nuclear protein detected by Ki-67 antibody in HL-60 cells". *Cell Proliferation*. **25**, 3140.

Bryan JT. and Brown DR. (2001). Transmission of Human Papillomavirus Type 11 infection by Desquamated Cornified cells. Virology; **281**, 35-45.

Butel JS et al., (2000). Viral Carcinogenesis: Revelation or molecular mechanism and etiology of human disease. Carcinogenesis; **3**, 405-426.

Caldwell GW, Yan Z, Lang W, Masucci JA, (2012). The IC50 concept revisited. Curr Top Med Chem ,**11**, 1282-90.

Cancer Research UK. Worldwide cancer statistics. [online] Available at: http://www.cancerresearchuk.org/health-professional/cancer-statistics/worldwide-cancer [Accessed 30 May 2018].

Campisi J. (2013). Aging, cellular senescence, and cancer. Annu Rev Physiol, **75**, 685-705.

Carmeliet P (2000). Mechanisms of angiogenesis and arteriogenesis. Nat. Med. ,**6**, 389–395.

Carmona-Fontaine C, et al (2008). Contact inhibition of locomotion in vivo controls neural crest directional migration. Nature. **456** , 957–961.

Chan KKL, Yao TJ, Jones B, et al. (2011). The use of Chinese herbal medicine to improve quality of life in women undergoing chemotherapy for ovarian cancer: a double-blind placebo-controlled randomized trial with immunological monitoring. Annals of Oncology. **22** , 2241–2249.

Chan L.Y., Amon A (2009). The protein phosphatase 2A functions in the spindle position checkpoint by regulating the checkpoint kinase Kin4 Genes Dev, **23**, 1639-1649.

Chang CL, et al (2002). Oxidative stress inactivates the human DNA mismatch repair system, Am. J. Physiol. Cell Physiol. **283**, 148-154.

Chatterjee SJ, Ovadje P, Mousa M, Hamm C, Pandey S. (2011), The efficacy of dandelion root extract in inducing apoptosis in drug-resistant human melanoma cells, Evid Based Complement Alternat Med.

Cho SY, Klemke rL. (2000). Extracellular-regulated kinase activaion and caS/crk coupling regulate cell migration and suppress apoptosis during invasion of the extracellular matrix. *J Cell Biol,* **149**, 223-36.

Couzin-Frankel, J. (2015). The bad luck of cancer. *Science* ,**347**, 12–12.

Cross F. R., Buchler N. E., Skotheim J. M. (2011). Evolution of networks and sequences in eukaryotic cell cycle control. Phil. Trans. R. Soc. B ,**366**, 3532–3544.

Cullen CF, Brittle AL, Ito T, Ohkura H. (2005). The conserved kinase NHK-1 is essential for mitotic progression and unifying acentrosomal meiotic spindles in *Drosophila melanogaster*. J Cell Biol ,**171**, 593–602.

Cuschieri k and Wentzensen N., (2008) "Human Papillomavirus mRNA and p16 Detection as Biomarkers for the Improved Diagnosis of Cervical Neoplasia" Cancer epidemiology biomarkers and prevention. **10**, 1055-1065.

Deberardinis RJ, Lum JJ, Hatzivassiliou G, Thompson CB (2008). The biology of cancer: Metabolic reprogramming fuels cell growth and proliferation. Cell Metab ,**7**, 11–20.

Dembo M., Torney D., Saxman K., Hammer D (1998). The kinetics of membrane-to-surface adhesion and detachment. Proc. R. Soc. **234** ,55–83.

DiMaio D and Mattoon D. (2001). Mechanism of cell transformation by paiommavirus E5 proteins. Oncogen. **20**: 7866-73.

Domchek SM, Friebel TM, Singer CF, Evans DG, Lynch HT, Isaacs C, et al (2010). Association of risk-reducing surgery in BRCA1 or BRCA2 mutation carriers with cancer risk and mortality. JAMA. **304**, 967–75.

Doorbar, J. (2005). The papillomavirus life cycle. *Journal of Clinical Virology*, **32**, 7-15.

Doorbar et al., (2015). "Human papillomavirus molecular biology and disease association" Rev Med Virol . **125**, 2-23.

Downward, J. (2003). Targeting RAS signalling pathways in cancer therapy. *Nature Reviews Cancer*, **3**(1), 11-22.

Dowsett M, Nielsen TO, Ahem R, Bartlett J, Coombes RC, Cuzick J et al., (2011). Assesemnet of ki67 in breast cancer: recommendation from the International ki67 in Breast cancer working group. Journal of the national cancer institute ,**103**, 1656-1664.

Dyson N (1998). The regulation of E2F by pRB-family proteins. Genes Dev , **12** ,2245–2262.

Dyson N, Howley PM, Munger K, Harlow E (1989). The human papilloma virus-16 E7

oncoprotein is able to bind to the retinoblastoma gene product. Science , **243**, 934–937.

Egeblad M, Werb Z (2002). New functions for the matrix metalloproteinases in cancer progression, Nat Rev Cancer. **2**, 161-174.

Ehrlich, M. (2009). DNA hypomethylation in cancer cells. *Epigenomics*, **1**(2), 239-259.

Ellison D (2002). Classifying the medulloblastoma: Insights from morphology and molecular genetics. J Neuropath Appl Neuropathol. **28**, 257–82.

Emberton M, Andriole GL, de la Rosette J, et al. (2003). Benign prostatic hyperplasia: a progressive disease of aging men. Urology.**61**, 267–73.

Evans AS, Mueller NE (1990). Viruses and cancer causal associations. *Ann Epidemiol*, **1**, 71-92.

Falschlehner C., Schaefer U., Walczak H. (2009). Following TRAIL's path in the immune system. Immunology ,**127**, 145–154.

Fang X., Zhang R. (2011). Aneuploidy and tumourigenesis Semin Cell Dev Biol, **22** , 595-601.

Farukh M (2013). "Tobacco Smoking and lung cancer" Sultan Qaboos Univ Med J. **13**, 345-358.

Ferlay J, Soerjomataram I, Ervik M, et al. (2012). "Cancer Incidence and Mortality Worldwide: IARC" CancerBase. **11**, 10-18

Fehrmann, F., & Laimins, L. A. (2003). Human papillomaviruses: Tar- geting differentiating epithelial cells for malignant transformation. Oncogene, **22**, 5201–5207.

Finnberg N., Klein-Szanto A. J., El-Deiry W. S. (2008). TRAIL-R deficiency in mice promotes susceptibility to chronic inflammation and tumorigenesis. J. Clin. Invest. **118**, 111–123.

Finetti F., Solito R., Morbidelli L., Giachetti A., Ziche M., Donnini S. (2008). "Prostaglandin E2 regulates angiogenesis via activation of fibroblast growth factor receptor-1." J. Biol. Chem; **283**, 2139–2146.

Fitzmaurice C, Allen C, Barber RM, et al (2017). Global Burden of Disease Cancer Collaboration. Global, regional, and national cancer incidence, mortality, years of life lost, years lived with disability, and disability-adjusted life-years for 32 cancer groups, 1990 to 2015: a systematic analysis for the Global Burden of Disease Study. JAMA Oncol. **3**, 524-548.

Frias JL, Gilbert-Barness E (2008). Human Teratogens: Current Controversies. Advances in Pediatrics. **55**,171–211.

Fulda S. (2009). Tumor resistance to apoptosis. Int. J. Cancer. **124**, 511–515.

Fulda S. (2013). Targeting c-FLICE-like inhibitory protein in cancer. Expert Opin. Ther. Targets. **17**, 195–201

Gadducci A, Tana R, Fanucchi A, Genazzani AR (2007). Biochemical prognostic factors and risk of relapses in patients with cervical cancer. Gynecol Oncol. **107**, 23–26.

Galluzzi L., Vitale I., Abrams J. M., Alnemri E. S., Baehrecke E. H., Blagosklonny M. V., et al. (2012). Molecular definitions of cell death subroutines: recommendations of the Nomenclature Committee on Cell Death. Cell Death Differ. **19**, 107–120.

Ganguly N, Parihar SP (2009). "Human papillomavirus E6 and E7 oncoproteins as risk factors for tumorigenesis". Journal of Biosciences. **34**, 113–123.

Gasche C et al., (2001). Oxidative stress increases frameshift mutations in human colorectal cancer cells, Cancer Res., **61**, 7444-7448.

Gerber B, Scholz C, Reimer T, Briese V, Janni W (2006). Complementary and alternative therapeutic approaches in patients with early breast cancer: a systematic review. Breast Cancer Research and Treatment. **95**, 199–209.

Gerdes J, Schwab U, Lemke H, Stein H. (1983). Production of a mouse monocloncal antibody reactive with a human nuclear antigen associated with cell proliferation. Internationla journal of cancer. **31**, 13-20.

Gerdes J, Lemke H, Baisch H, Wacker H-H, Schwab U, Stein H (1984). Cell cycle analysis of a cell proliferation-associated human nuclear antigen defined by the monoclonal antibody Ki-67. J Immunol.**133**,1710–1715.

Ghazanfar SA. (1994). Handbook of Arabian Medicinal Plants,**1**, 148-156.

Glaunsinger BA, Lee SS, Thomas M, Banks L, Javier R. (2000). "Interactions of the PDZ-protein MAGI-1 with adenovirus E4-ORF1 and high-risk papillomavirus E6 oncoproteins". Oncogene; **19**, 5270-80.

Gokbulut AA, Apohan E, Baran Y. (2013). Resveratrol and quercetin-induced apoptosis of human 232B4 chronic lymphocytic leukemia cells by activation of caspase-3 and cell cycle arrest. Hematology; **18**: 144–150.

Gregan J, Riedel CG, Pidoux AL, Katou Y, Rumpf C, Schleiffer A, Kearsey SE, Shirahige K, Allshire RC, Nasmyth K. (2007). The kinetochore proteins Pcs1 and Mde4 and heterochromatin are required to prevent merotelic orientation. Curr Biol. **17**, 1190–1200.

Grivennikov S.I., Greten F.R., Karen M (2010). Immunity, inflammation, and cancer Cell, **140**, 883-899.

Grosse et al (2016). "Carcinogenicity of some industrial chemicals" *The lancet Oncology* ,**17**, 419-420.

Gumbiner BM (2005). regulation of cadherin-mediated adhesion in morphogenesis. *Nat Rev Mol Cell Biol,* **6**, 622-34.

Gunes C and Rudolph KL (2013). The role of telo- meres in stem cells and cancer. Cell. **152**, 390-393.

Gunjan ,M , Naing ,TW, Saini R, Ahmad, AB, Naidu J.R, Kumar I. (2015). " Marketing trends and future prospects of herbal medicine in the treatment of various disease" World Journal of Pharmaceutical Research. **4,** 132-155.

Haedicke J and Iftner T. (2013). Human papillomavirus and cancer. Radiotherapy and Oncology, **108**, 397-402.

Hafner N, Driesch C, Gajda M, et al (2008). Integration of the HPV16 genome does not invariably result in high levels of viral oncogene transcripts. Oncogene. **27**, 1610-17.

Hajdu SI (2006). A note from history: the link between koilocytes and human papillomaviruses. Ann Clin Lab Sci. **36**, 485–487.

Hahn C, Weinberg RA (2002). Rules for making human tumor cells. *N. Engl J Med*, **347***,* 1593-1603.

Hall J.M., Lee M.K., Newman B., Morrow J.E, Anderson L.A., Huey B., (1990). "Linkage of early-onset familial breast cancer to chromosome 17q21Science". **3**, 1684-1689.

Hanahan D, Weinberg RA (2000). The hallmarks of cancer. Cell. **100**, 57–70.

Hanahan, D., and Weinberg, R.A. (2011). Hallmarks of cancer: the next generation. Cell **144**, 646 674.

Hanahan D, Folkman J. (1996). Patterns and emerging mechanisms of the angiogenic switch during tumorigenesis, Cell , **86**, 353-364.

Han S, Khuri FR, Roman J. (2006). Fibronectin stimulates non-small cell lung carcinoma cell growth through activation of Akt/mammalian target of rapamycin/S6 kinase and inactivation of LKB1/AMP-activated protein kinase signal pathways. Cancer Res. **66**, 315–323.

Hanel, W. and Moll, U. (2012). Links between mutant p53 and genomic instability. *Journal of Cellular Biochemistry*, **113**(2), 433-439.

Harper DM, Franco EL, Wheeler CM, et al. (2006). Sustained efficacy up to 4.5 years of a bivalent L1 virus-like particle vaccine against human papillomavirus types 16 and 18: follow-up from a randomised control trial. Lancet, **367**, 1247-55.

Harris TJ, Tepass U (2010). Adherens junctions: From molecules to morphogenesis. Nat Rev Mol Cell Biol. **11**, 502–514.

Hashemi SA, Abediankenari S (2013). "Suppressive Effect of Fig (Ficus carica) Latex on Esophageal Cancer Cell Proliferation" Scientific Journal of the Faculty of Medicine in Niss ; **30** 93-96.

Hashemi SA, Abediankenari S, Ghasemi M, Azadbakht M, Yousefzadeh Y, Dehpour AA. (2011) The Effect of Fig Tree Latex (Ficus carica) on Stomach Cancer Line. Iran Red Crescent Med J. **13**, 272-5.

Hebner CM, Laimins LA. (2006) Human papillomaviruses: basic mechanisms of pathogenesis and oncogenicity. Rev Med Virol ,**16**, 83-97.

Heckman C.A. (2009). Contact inhibition revisited. J. Cell. Physiol. **220**, 574–575.

Hemmatzadeh, F., Fatemi, A. and Amini, F. (2003). 'Therapeutic effects of fig tree latex on bovine Papillomatosis', Journal of Veterinary Medicine Series B. **50**, 473–6.

Hill B. (1965). The environment and disease: association or causation?. *Proc R Soc Med*, **58**, 295-300.

Hosseinzadeh, S., Jafarikukhdan, A., Hosseini, A. and Armand, R. (2015). 'The application of medicinal plants in traditional and modern medicine: A review of Thymus vulgaris', International Journal of Clinical Medicine**, 06**, 635–642.

Hogg R, Friedlander M (2003). Role of systemic chemotherapy in metastatic cervical cancer. Expert Rev Anticancer Ther. **3**, 234–240.

Holmes D. (2014) The cancer-virus cures. Nature Medicine; **20**, 571-574.

Halder, G. and Johnson, R. L. (2011). Hippo signaling: growth control and beyond. Development **138**, 9-22.

Hoots BE et al., (2009). International Journal Cancer. **124**, 2375-2383

Huyck L, Ampe C, Van Troys M. (2012). "The XTT cell proliferation assay applied to cell layers embedded in three-dimensional matrix". Assay Drug Dev Tech; **10** ,382–392.

Jain P, Hunter RD, Livsey JE, Coyle C, Swindell R, Davidson SE (2007). Salvaging locoregional recurrence with radiotherapy after surgery in early cervical cancer. Clin Oncol, **19**:763–768.

Jain RK. (2005). Normalization of tumor vasculature: an emerging concept in antiangiogenic therapy, Science, **307**:58-62.

Janavicius R. (2010). Founder BRCA1/2 mutations in the Europe: implications for hereditary breast-ovarian cancer prevention and control. EPMA J. **1**, 397–412.

Janic, A. et al., (2018). DNA repair processes are critical mediators of p53-dependent tumor suppression. *Nature Medicine*, **24**(7), 947-953.

Jones RG, Thompson CB (2009). Tumor suppressors and cell metabolism: a recipe for cancer growth. Genes Dev, **23**:537-548.

Kalluri R (2003). Basement membranes: structure, assembly and role in tumour angiogenesis, Nat Rev Cancer, **3**: 422-433.

Karna P. et al., (2012) Benefits of whole ginger extract in prostate cancer. Br J Natur **107**(4):473-84.

Karnoub, AE and Weinberg, RA (2008). *Ras oncogenes: split personalities*. Nature Reviews Molecular Cell Biology. **9**, 517–31.

Katz, J.P. and Kaestner, K.H. (2002). Cellular and molecular mechanisms of carcinogenesis. *Gastroenterol Clin North Am*.31: 379–394.

Kessenbrock K, Plaks V and Werb Z (2010). Matrix metalloproteinases: regulators of the tumor microenvironment. Cell, **141**: 52-67.

Klemke RL, Yebra M, Bayna EM, Cheresh DA (1994). Receptor tyrosine kinase signaling required for integrin αvβ5-directed cell motility but not adhesion on vitronectin. J. Cell Biol.**127**, 859–866.

Kim NWet al., (1994). "Specific association of human telomerase activity with immortal cells and cancer". Science. **266**, 2011–5.

Kim, W. and Sharpless, N. (2006). The Regulation of INK4/ARF in Cancer and Aging. *Cell*, **127**(2), 265-275.

Koch A.E., Distler O. (2007). Vasculopathy and disordered angiogenesis in selected rheumatic diseases: Rheumatoid arthritis and systemic sclerosis. Arthritis Res. **9**, 1-3.

Koutsky LA, Holmes KK, Critchlow CW, et al. (1992). A cohort study of the risk of cervical intraepithelial neoplasia grade 2 or 3 in relation to papillomavirus infection, N Engl J Med , **18**, 1272-1278.

Labib K., De Piccoli G. (2011). Surviving chromosome replication: the many roles of the S-phase checkpoint pathway. Phil. Trans. R. Soc. **366**, 3554.

Lam W, Bussom S, Guan F, et al. (2010). Chemotherapy: the four-herb Chinese medicine PHY906 reduces chemotherapy-induced gastrointestinal toxicity. Science Translational Medicine. **2**: 45-59.

Lancaster OM, Breuer M, Cullen CF, Ito T, Ohkura H. (2010). The meiotic recombination checkpoint suppresses NHK-1 kinase to prevent reorganisation of the oocyte nucleus in *Drosophila*. PLoS Genet, **6**, 147-158.

Lazreg Aref H, Gaaliche B, Fekih A, Mars M, Aouni M, Pierre Chaumon J, Said K. (2011). In vitro cytotoxic and antiviral activities of Ficus carica latex extracts. Nat Prod Res. **25**, 310-9.

Lee, B., Konen, J., Wilkinson, S., Marcus, A. and Jiang, Y. (2017). Local alignment vectors reveal cancer cell-induced ECM fiber remodeling dynamics. *Scientific Reports*, **7** (1).

Lee SS, Glaunsinger B, Mantovani F, Banks L, Javier RT (2000). Multi- PDZ domain protein MUPP1 is a cellular target for both adenovirus E4-ORF1 and high-risk papillomavirus type 18 E6 oncoproteins. J Virol. **74**, 9680-93.

Li, L. and Lu, Y. (2010). Inhibition of Hypoxia-Induced Cell Motility by p16 in MDA-MB-231 Breast Cancer Cells. *Journal of Cancer*.126-135.

Liao JB. (2006). Viruses and Human cancer.Yale Journal of biology and medicine; **79**, pp: 115-122.

Lim W. et al., (2018). The 8th lung cancer TNM classification and clinical staging system: review of the changes and clinical implications. Quant Imaging Med Surg **8**: 709–718.

Liu H, Dibling B, Spike B, Dirlam A, Macleod K. (2004). Curr Opin Genet Dev **14**: 55-64.

Li M, Luo J, Brooks CL, Gu W. (2002). Acetylation of p53 inhibits its ubiquitination by Mdm2. *J Biol Chem* **277** :50607-11.

Llambi F and Green DR (2011). Apoptosis and oncogenesis: give and take in the BCL-2 family. Curr Opin Genet Dev. **21**: 12-20.

Lopez J and Tait SWG (2015). Mitochondrial apoptosis: killing cancer using the enemy within. Br J Cancer, **112**: 957-962.

Loewe L., Hill G. W. (2010) "The population genetics of mutations: good, bad and indifferent" Journal philosophical transaction **365**: 1153–1167.

Lockshin R. A., Zakeri Z. (2007). Cell death in health and disease. J. Cell. Mol. Med. **11**, 1214–1224.

Lopez-Graniel C, et al., (2005). Pre-exenterative chemotherapy, a novel therapeutic approach for patients with persistent or recurrent cervical cancer. BMC Cancer. **5**:118-119.

Lou X, Zhang J, Liu S, Lou X, Liao DJ. (2014). The other side of the coin: the tumor-suppressive aspect of oncogenes and the oncogenic aspect of tumor-suppressive genes, such as those along the CCND-CDK4/6-RB axis. Cell Cycle. ; **13**:1677–1693.

Luijsterburg M.S., Goedhart J., Moser J.,. Kool H, Geverts B., Houtsmuller A.B., et al. (2007). Dynamic in vivo interaction of DDB2 E3 ubiquitin ligase with UV-damaged DNA is independent of damage-recognition protein XPC J Cell Sci, **120**:2706-2716.

Ludwig KK, Neuner J, Butler A, Geurts JL, Kong AL (2016). Risk reduction and survival benefit of prophylactic surgery in BRCA mutation carriers, a systematic review. Am J Surg. **212** :660–9.

Magrina JF (1990). Types of pelvic exenterations: a reappraisal. Gynecol Oncol. **37**:363–366.

Magrina JF (1997). Stanhope CR, Weaver AL. Pelvic exenterations: supralevator, infralevator, and with vulvectomy. Gynecol Oncol. **64** :130–135.

Mahmoud SYM, Gad -Rab MFS, Hussein N and Shoreit A AM, (2010), Antiviral Activity of Latex from Ficus nitida Against Plant Viruses, Global Journal of Biotechnology & Biochemistry **5** (3): 198-205.

Marks LS, DiPaola RS, Nelson P, et al. (2002). PC-SPES: herbal formulation for prostate cancer. Urology. **60** :369–375.

Martel C., et al., (2017). "Worldwide burden of cancer attributable to HPV by site, country and HPV type" Int J Cancer **15**: 664-670.

Mantovani A, et al. (2008). Cancer-related inflammation, Nature, **454**: 436-444.

Massimi, P., Gammoh, N., Thomas, M., & Banks, L. (2004). HPV E6 specifically targets different cellular pools of its PDZ domain- containing tumour suppressor substrates for proteosome-mediated degradation. Oncogene, **23,** 8033–8039.

Martinez P and Blasco MA (2011). Telomeric and ex- tratelomeric roles for telomerase and the telo- mere-binding proteins. Nat Rev Cancer. **11**: 161-176

Malumbres M and Barbacid M (2009). Cell cycle, CDKs and cancer: a changing paradigm. Nat Rev Cancer ; **9**: 153-166.

Malmberg KJ (2004). Effective immunotherapy against cancer. Cancer Immunol Immunother. **53**:879–892.

Mawa, S., Husain, K. Jantan, I. (2013). Ficus carica L. (Moraceae): Phytochemistry, traditional uses and biological activities. *Evid. Based Complementary Altern. Med.* **2013**, 974256

McClatchey, A. I. and Yap, A. S. (2012). Contact inhibition (of proliferation) redux. Curr. Opin. Cell Biol. **24**, 685-694.

Messerschmidt, J., Prendergast, G. and Messerschmidt, G. (2016). How Cancers Escape Immune Destruction and Mechanisms of Action for the New Significantly Active Immune Therapies: Helping Nonimmunologists Decipher Recent Advances. *The Oncologist*, **21**(2), 233-243.

Miller M. A., Karacay B., Zhu X., O'Dorisio M. S., Sandler A. D. (2006). Caspase 8L, a novel inhibitory isoform of caspase 8, is associated with undifferentiated neuroblastoma. Apoptosis **11**, 15–24.

Moore PS and Chang Y (2010). Why do viruses cause cancer? Highlights of the first century of human tumor virology. Nature Reviews Cancer: **10**, 878-889.

Molijn, A., Kleter, B., Quint, W., & van Doorn, L. J. (2005). Molec- ular diagnosis of human papillomavirus (HPV) infections. J. Clin. Virol., **32**, 43–51.

Mostafaie, A, Mansouri K Norooznezhad, A, Mohammadi-Motlagh HA, (2011) Anti-Angiogenic Activity of Ficus carica Latex Extract on Human Umbilical Vein Endothelial Cells Cell Journal (Yakhteh) **12**; 525-8.

Moll E and Petrenko (2003). " The MDM2-p53 Interaction" Molecular Cancer research

Morgan DO (2007). The Cell Cycle: Principles of Control. London: New Science Press.

Morris, B. (2005). Cervical human papillomavirus screening by PCR: advantages of targeting the E6/E7 region. *Clinical Chemistry and Laboratory Medicine* , **43**(11).

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: Application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, **65**(1-2), pp.55-63.

Mousa S.A., Davis P.J. (2016) Angiogenesis and anti-angiogenesis strategies in cancer. 1st ed. Academic Press; Amsterdam, The Netherlands: 2016.

Mucci, L. A. et al (2016). Familial risk and heritability of cancer among twins in nordic countries. *JAMA* **315**, 68–76.

Munger K and Howley PM. (2002). Human Papillomavirus immortalization and transformation functions. Virus Res. **89**: 213-28.

Mundt AJ, Roeske JC, Lujan AE, Yamada SD, Waggoner SE, Fleming G, Rotmensch J (2003). Initial clinical experience with intensity-modulated whole-pelvis radiation therapy in women with gynecologic malignancies. Gynecol Oncol. **82**:456–463.

Munoz N, et al. (1992). The causal link between human papillomavirus and invasive cervical cancer: a population-based case-control study in Colombia and Spain. Int. J. Cancer **52**,743-749.

Nagane M., Pan G., Weddle J. J., Dixit V. M., Cavenee W. K., Huang H. J. (2000). Increased death receptor 5 expression by chemotherapeutic agents in human gliomas causes synergistic cytotoxicity with tumor necrosis factor-related apoptosis-inducing ligand *in vitro* and *in vivo*. Cancer Res. **60**, 847–853.

Nam EJ et al. (2007). The expressions of the Rb pathway in cervical intraepithelial neoplasia; predictive and prognostic significance. Gynecol Oncol. **104**:207–211.

Nam EJ, Kim YT. (2008). Alteration of cell-cycle regulation in epithelial ovarian cancer. Int J Gynecol Cancer.

Nam EJ, , et al (2006). Relationship between $p16^{INK4a}$, pRb and high risk HPV infection and recurrence. Korean J Obstet Gynecol. **49**:1437–1445

Nasmyth K, Haering CH. (2009). Cohesin: Its roles and mechanisms. Annu Rev Genet **43**: 525–558

National Cancer Institute. Cervical Cancer Treatment. online Available at, https://www.cancer.gov/types/cervical/patient/cervical-treatment-pdq, Accessed 30 May 2018 (2018).

Niazi MKK, et al., (2013). Entropy based quantification of Ki-67 positive cell images and its evaluation by a reader study. In: SPIE Medical Imaging: 2013. Bellingham: International Society for Optics and Photonics, p. 86760I.

Ning Y, et al. (2005). Down-regulation of integrin α2 surface expression by mutant epidermal growth factor receptor (EGFRvIII) induces aberrant cell spreading and focal adhesion formation. Cancer Res. **65**:9280–9286.

Nowinska, K; Ciesielska, U; Podhorska-Okołow, M; Dzięgiel, P (2017). "The role of human papillomavirus in oncogenic transformation and its contribution to the etiology of precancerous lesions and cancer of the larynx: A review". Advances in Clinical and Experimental Medicine: Official Organ Wroclaw Medical University. **26**: 539–547.

Noble, R. J., Kaltz, O., Nunney, L. & Hochberg, M. E. (2016). Overestimating the role of environment in cancers. *Cancer Prev. Res*. **9**, 773–776 .

Nurden AT, Nurden P, Sanchez M, Andia I & Eduardo A (2008). Platelets and wound healing. Front Biosci **2**, 3532–3548.

Osborne C, Wilson P and Tripathy D. (2004). Oncogenes and tumor suppressor genes in breast cancer : potential diagnostic and therapeutic applications; The oncologist, **9**, pp.361-377.

Ovadje P, et al., (2011), Selective induction of apoptosis through activation of caspase-8 in human leukemia cells (Jurkat) by dandelion root extract, JEthnopharmacol. **133**(1):86-91.

Ozturk, Mert, Yinghui Li & Vinay Tergaonkar. (2017). "Current Insights to Regulation and Role of Telomerase in Human Diseases" **6**:1-7.

Pahud, BA; Ault, KA (2015). "The Expanded Impact of Human Papillomavirus Vaccine". Infectious Disease Clinics of North America (Review). **29** : 715–24.

Parkin DM. (2006). The global health burden of infection-associated cancers in the year. Int J Cancer. **118**:3030–3044.

Peurala, Emmi et al. (2013). "The prognostic significance and value of cyclin D1, CDK4 and p16 in human breast cancer". **15**:1 Breast Cancer Research.

Piepkorn M., (2000) The expression of p16(INK4a), the product of a tumor suppressor gene for melanoma, is upregulated in human melanocytes by UVB irradiation J. Am. Acad. Dermatol., **42**, 741-745.

Pietsch EC, Sykes SM, McMahon SB, Murphy ME. (2008). The p53 family and programmed cell death. *Oncogene* **27** :6507-21.

Purohit V, Khalsa J, Serrano J. (2005). Mechanisms of alcohol-associated cancers: introduction and summary of the symposium. Alcohol. **35** :155–160.

Pylayeva-Gupta Y, Grabocka E and Bar-Sagi D. (2011). RAS oncogenes: weaving a tumorigenic web. Nat Rev Cancer. **11**: 761-774.

Pylayeva Y, et al. (2009). “Ras- and PI3K-dependent breast tumorigenesis in mice and humans requires focal adhesion kinase signaling”. J. Clin. Invest. **119**:252–266.

Rajabi M., Sudha T., Darwish N.H.E., Davis P.J., Mousa S.A. (2016). Synthesis of MR-49, a deiodinated analog of tetraiodothyroacetic acid (tetrac), as a novel pro-angiogenesis modulator. Bioorg. Med. Chem. Lett. **26**:4112–4116.

Rahbari R, Sheahan T, Modes V, Collier P, Macfarlane C, Badge RM (2009).” "A novel L1 retrotransposon marker for HeLa cell line identification. *BioTechniques*. **46** : 277-284.

Ramakrishnan, S., Partricia, S. & Mathan, G. (2015). Overview of high-risk HPV's 16 and 18 infected cervical cancer: Pathogenesis to prevention. Biomed. Pharmacother. **70**,103-110.

Rexer BN, Chanthaphaychith S, Dahlman K, Arteaga (2014). CL Direct inhibition of PI3K in combination with dual HER2 inhibitors is required for optimal antitumor activity in HER2+ breast cancer cells. Breast Cancer Res ; **16**:19.

Rigoni-Stern D (2004). ‘Fatti statistici alle mallattie cancerose che servirono di base alle poche cose dal dott’ [Italian] F Prog Patol Terap. **2**:499–517.

Risau W. (1997). Mechanisms of angiogenesis. Nature. **386**:671–674.

Risch, N. (2001). The genetic epidemiology of cancer. *Cancer Epidemiol. Biomarkers. Prev.* **10**, 733–741.

Riley T, Sontag E, Chen P, Levine A. (2008). Transcriptional control of human p53-regulated genes. *Nat Rev Mol Cell Biol* **9** :402-12.

Rubnov S, Kashman Y, Rabinowitz R, Schlesinger M, Mechoulam R. (2001) .“Suppressors of cancer cell proliferation from Fig (Ficus carica) Resin: Isolation and Structure Elucidation.” J. Nat. Prod.; **64**:993–996.

Ruttkay-Nedecky, B., et al. (2013). Relevance of infection with human papillomavirus: The role of the p53 tumor suppressor protein and E6/E7 zinc finger proteins. Int. J. Oncol. **76**, 1754-1762.

Rybinski B, Franco-Barraza J & Cukierman E (2014). The wound healing, chronic fibrosis, and cancer progression triad. Physiol Genomics **46,** 223–244.

Sagvolden G., Giaever I., Pettersen E.O., Feder J. (1999). Cell adhesion force microscopy. Proc. Natl. Acad. Sci. USA. **96**: 471–476

Sak K. (2014). Cytotoxicity of dietary flavonoids on different human cancer types. Pharmacog Rev; **8**:122–146.

Samper E, et al. (2003). Mitochondrial oxidative stress causes chromosomal instability of mouse embryonic fibroblasts, Aging Cell, **2** : 277-285.

Saito R. et al. (2010). Molecular targets for therapeutic interventions in human papillomavirus-related cancers. Oncol Rep. **24**:1419–1426.

Scheurer ME, Tortolero-Luna G, Adler-Storthz K (2005). "Human papillomavirus infection: biology, epidemiology, and prevention". International Journal of Gynecological Cancer. **15** : 727–746

Schmitt M. et al., (2012). "Prevalence and viral load of 51 genital human papillomavirus types and 3 subtypes". Int J Cancer. **132** : 2395–2403.

Seifert U, Marañón C, Shmueli A, Desoutter JF, et al (2003). An essential role for tripeptidyl peptidase in the generation of an MHC class I epitope. Nature Immunology, **4**, 375–379.

Serrano M. , A.W. Lin, M.E. McCurrach, D. Beach, S.W.(1997) LoweOncogenic ras provokes premature cell senescence associated with accumulation of p53 and p16INK4a, Cell; **88**, 593-602.

Serhan C.N., Savill J. (2005). Resolution of inflammation: The beginning programs the end. Nat. Immunol. **6** :1191–1197.

Sharma, SV, Bell, DW, Settleman, J and Haber, DA (2003). Epidermal growth factor receptor mutations in lung cancer. Nature Reviews Cancer **7**: 12-15.

Shapley M, Jordan J, Croft PR. (2006). A systematic review of postcoital bleeding and risk of cervical cancer. Br J Gen Pract. **56** :453–460.

Shai A, Pitot HC, Lambert PF. (2010). E6-associated protein is required for human papillomavirus type 16 E6 to cause cervical cancer in mice. Cancer Res, **70**: 5064-73.

Shay, JW; Wright, WE (2000). "Hayflick, his limit, and cellular ageing". *Nature Reviews. Molecular Cell Biology*. **1** : 72–6.

Shay JW. (2016). Role of telomeres and telomerase in aging and cancer. Cancer Discov, **6**: 584- 593.

Shin MK, Kim SK, Jung H. (2011). ” Integration of intra- and extravasation in one cell-based microfluidic chip for the study of cancer metastasis. Lab Chip **11**: 3880–3887.

Simon S., Green C.E. (2005). Molecular mechanics and dynamics of leukocyte recruitment during inflammation. Annu. Rev. Biol. **7**:151–185.

Slebos RJ. et al., (1994). p53-dependent G1 arrest involves pRB-related proteins and is disrupted by the human papillomavirus 16 E7 oncoprotein. Proc Natl Acad Sci U S A. **91**: 5320–5324.

Sleeman JP, Nazarenko I, Thiele W (2011). Do all roads lead to Rome? Routes to metastasis development. Int J Cancer **128**: 2511–2526.

Smith, G. D., Relton, C. L. & Brennan, P.V. (2016). Chance, choice and cause in cancer aetiology: individual and population perspectives. *Int. J. Epidemiol.* **45**, 605–613.

Solan, EJ and Donal EA. (2010). Myc, Mondo and Metabolism, Genes & Cancer, **1** : 587-596.

Song S, Ji B, Ramachandran V, Wang H, Hafley M, Logsdon C, Bresalier RS (2012). Overexpressed galectin-3 in pancreatic cancer induces cell proliferation and invasion by binding Ras and acti- vating Ras signaling. PLoS One; **7**: 117-128.

Stapley S, Hamilton W. (2011). Gynaecological symptoms reported by young women: examining the potential for earlier diagnosis of cervical cancer. Fam Pract. **28** :592–598.

Stepek G, Buttle DJ, Duce IR, Lowe A, Behneke JM. (2005) "Assessment of the anthelmintic effect of natural plant cysteine proteinases against the gastrointestinal nematode, Heligmosomoides polygyrus. In-vitroParasitol."**130**: 203–211.

Stevaux O, Dyson NJ (2002). A revised picture of the E2F transcriptional network and RB function. Curr Opin Cell Biol **14**:684–691.

Swale VJ and Quinn AJ. (2000). Tumour suppressor genes. Experimental Dermatology; **25,** 231-235.

Tenesa, A. Haley, C.S. (2013). The heritability of human disease: estimation, uses and abuses. *Nat. Rev. Genet.* **14**, 139–149.

Tezcan, G. et al., (2014) 'Ficus carica latex prevents invasion through induction of let-7d expression in GBM cell lines', Cellular and Molecular Neurobiology, **35**(2), 175–187.

Thomas, M., Pim, D., & Banks, L. (1999). The role of the E6–p53 interaction in the molecular pathogenesis of HPV. Oncogene, **18**, 7690–7700.

Tian Y.et al., (2016) "Clinicopathological and prognostic value of Ki-67 expression in bladder cancer: a systematic review and meta-analysis." PLoS One. **36**; 14-17.

Titolo S. et al. (2000). Identification of domains of the human papillomavirus type 11 E1 helicase involved in oligomerization and binding to the viral origin. J Virol. **74**: 7349-61.

Tomasetti, C. and Vogelstein, B. (2015). Cancer risk: role of environment—response. *Science* **347**, 729–731.

Tristram A and Fiander A. (2007) Human papillomavirus (Including vaccines). Obstetrics, Gynecology and Reproductive Medicine; **11** (17).

Uldrijan S, Pannekoek WJ, Vousden KH. (2007). An essential function of the extreme C-terminus of MDM2 can be provided by MDMX. *Embo J* **26** :102-12.

Ulman , S. B. (1945) .HAlberstaedter, L:Leibwitz J. Exp. Med. Surg. **3**, 11-23.

Vander H. MG, Cantley LC, Thompson CB (2009). Understanding the Warburg effect: The metabolic requirements of cell proliferation. Science **324**: 1029–1033.

Vaidya V, Ingold KU and Pratt DA. (2008) Garlic: Source of the Ultimate Antioxidants—Sulfenic Acids, Angewandte Chemie International Edition, **48**(1); 157-160

Vander H. et al., (2010). Evidence for an alternative glycolytic pathway in rapidly proliferating cells. Science **329**: 1492–1499.

Venuti A, et al., (2011). Papillomavirus E5: the smallest oncoprotein with many functions. Molecular Cancer. **10**, 140.

Visscher, P. M., Hill, W. G. & Wray, N. R. (2008). Heritability in the genomics era—concepts and misconceptions. *Nat. Rev. Genet.* **9**, 255–266.

Viikki M, Pukkala E, Hakama M. (1998). Bleeding symptoms and subsequent risk of gynecological and other cancers. Acta Obstet Gynecol Scand. **77** :564–569.

Vousden KH, Lu X. (2002). Live or let die: the cell's response to p53. Nat Rev. **2**:594–604.

Vogelstein B, et al., (2013). Cancer genome landscapes. Science. **339**: 1546- 1558.

Warburg O, et al., (1924); (German). Reprinted in English in the book on metabolism of tumors by O. Warburg, Publisher: Constable, London, 1930. **152**:319-344.

Warburg O. (1956). On the origin of cancer cells. Science. **123**:309-314.

Wang, J., et al., (2008). 'Cytotoxicity of fig fruit latex against human cancer cells', Food and Chemical Toxicology, **46**, 1025–1033.

Wang S, et al., (2004). Doxorubicin induces apoptosis in normal and tumor cells via distinctly different mechanisms intermediacy of H(2)O(2)- and p53-dependent pathways. J Biol Chem. **279** :25535–25543.

Weinberg RA (2013). The Biology of Cancer. 2nd ed Garland Science.

Weinberg RA. (2014). Coming full circle-from endless complexity to simplicity and back again. Cell. **157**:267–271.

Weinber RA (1995). "Cell and cellular features" Cell **81** :323-330.

Weissmueller S, et al. (2014). Mutant p53 drives pancreatic cancer metastasis through cell-autonomous PDGF receptor β signaling. Cell. **157** :382–394.

Winer RL. et al. (2005)., Development and duration of human papillomavirus lesions, after initial infection, J Infect Dis, **191** , 731-738.

Windschall A, Ott OJ, Sauer R, Strnad V. (2005). Radiation therapy and simultaneous chemotherapy for recurrent cervical carcinoma. Strahlenther Onkol. **181**:545–550.

Wilbur MA, Shih IM, Segars JH, Fader AN. (2017). Cancer implications for patients with endometriosis. Semin Reprod Med. **35** :110–116.

Witsch E, Sela M and Yarden Y. (2010). Roles for growth factors in cancer progression. Physiology **25**: 85-101.

Wolf, K. et al. (2013) Physical limits of cell migration: control by ECM space and nuclear deformation and tuning by proteolysis and traction force. *J. Cell. Biol.* **201**, 1069–1084.

Woodman, C. B. J., Collins, S. I., & Young, L. S. (2007). The natural history of cervical HPV infection: Unresolved issues. Nat. Rev. Cancer, **7**, 11–22.

Woodman CB. et., al (2011). "Natural history of cervical human papillomavirus infection in young women": a longitudinal cohort study. **357** ;1831-1836.

World Health Organization. (2018). Cancer. [online] Available at: http://www.who.int/news-room/fact-sheets/detail/cancer [Accessed 30 May 2018].

Yang W, Wetterskog D, Matsumoto Y, Funa K. (2008). Kinetics of repression by modified p53 on the PDGF beta-receptor promoter. Int J Cancer. **123** :2020–2030.

Yokota J. (2000). Tumor progression and metastasis. *Carcinogenesis*; **21**: 497–503.

Yokota, J. and Kohno, T. (2004). Molecular footprints of human lung cancer progression. *Cancer Science*, **95**(3), 197-204.

Yu L. et al., (2016) .p16/Ki-67 co-expression associates high risk human papillomavirus persistence and cervical histopathology: a 3-year cohort study in China. Oncotarget **7**: 64810–64819.

Zamarron B.F., Chen, W. (2010). Dual roles of immune cells and their factors in cancer development and progression Int J Biol Sci, **7** : 651-658.

Zatonski W, Tyczynski J. Zaklad B. (1996). "Cancer in Poland in 1993, a study by department of Epidemiology of Cancer Prevention". National Cancer Registry Journal. **4** . 22-41.

Zhang B. et al., (2003). The E5 protein of human papillomavirus type 16 perturbs MHC class II antigen maturation in human foreskin keratinocytes treated with interferon-gamma. Virology. **10**, 100–108.

Zhao, B., et al., (2007). Inactivation of YAP oncoprotein by the Hippo pathway is involved in cell contact inhibition and tissue growth control. Genes Dev. **21**, 2747-2761.

Zhang, J., Onakpoya, I.J., Posadzki, P. and Eddouks, M. (2015) 'The safety of herbal medicine: From prejudice to evidence', Evidence-Based Complementary and Alternative Medicine, **3**:1–3.

Zola P, et al., (2007). Could follow-up different modalities play a role in asymptomatic cervical cancer relapses diagnosis? An Italian multicenter retrospective analysis. Gynecol Oncol. **107** :150–154.

Zur Hausen, H. (2002). Papillomaviruses and cancer: From basic stud- ies to clinical application. Nat. Rev. Cancer, **2**, 342–350.

Zur Hausen, H. (2000). Papillomaviruses causing cancer: Evasion from host-cell control in early events in carcinogenesis. J. Natl. Cancer Inst., **92**, 690–698.