College of Health Sciences

School of Laboratory Medicine and Medical Sciences

TITLE:

CYTOTOXIC AND ANTI-PROLIFERATIVE EFFECTS OF *MORINGA OLEIFERA LAM.* ON HELA CELLS

KRISHNAMBAL GOVENDER

For a thesis in fulfilment of the requirements of the degree of

Masters in Medical Science, in the School of Health Sciences

Supervisor: Prof. Raveen Parboosing

Co-supervisor: Prof. Indres Moodley

DECLARATION

I, Mrs Krishnambal Govender, declare as follows:

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    - Review of literature and formulation of research questions and hypotheses
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    - Application for funding from UKZN
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    - Acquiring new skills to enable the success of the practical research work
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    - Data analysis and interpretation
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4. Signed:  Date: 27th April 2021

Mrs. Krishnambal Govender (student)

Signed:  Digitally signed by Raveen Parboosing Date: 2021.05.14 14:17:02 +02'00'

Prof. Raveen Parboosing (Supervisor) Date: 27th April 2021

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Krishnambal Govender 30/11/2020

I. DEDICATION

This thesis is dedicated to my late father, Mr. Kisten Govender (1941 – 2016).

My heartfelt gratitude goes to God Almighty Whose Grace is always sufficient.

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B. List of Acronyms

APC	adenomatous polyposis coli
BRCA1	breast cancer gene 1
BRCA2	breast cancer gene 2
CAD	caspase-activated kinase
Cdk	cyclin-dependent kinase
cDNA	complementary deoxyribonucleic acid
CIN	cervical intraepithelial lesion
CIS	carcinoma-insitu
CV	coefficient of variation
DDR	DNA death receptor
DMSO	dimethyl-sulphoxide
DNA	deoxyribonucleic acid
E6AP	E6-associated protein
EGFR	epidermal growth factor receptor
FCS	foetal calf serum
G1	gap phase
HPV	Human papillomavirus
HIV	Human Immunodeficiency Virus
hTERT	human telomerase reverse transcriptase
HSIL	high grade squamous intraepithelial lesion
ICAD	inhibitors of caspase-activated DNase

MDM2	mouse double minute 2
M phase	mitotic phase
MTT	3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide
PARP	poly ADP (Adenosine Diphosphate)-Ribose Polymerase
PCR	polymerase chain reaction
PE	plating efficiency
pRb	retinoblastoma protein
RB1	retinoblastoma protein
RDS	relative standard deviation
RNA	ribonucleic acid
RT	room temperature
RT PCR	reverse transcriptase polymerase chain reaction
SIL	squamous intraepithelial lesion
SF	surviving fraction
TP53	tumour protein 53

IV. ABSTRACT

Moringa oleifera Lam., known to most as the 'drumstick tree', is a non-fastidious botanical that is native to India, and is cultivated on a global scale as a sustainable crop, for sustenance, medicinal and beauty applications, amongst others. The antitumour, antibacterial and antifungal effects of *M. oleifera* are well-documented, however, its specific effects on human papillomavirus (HPV)-induced malignancy have not been established.

High-risk HPV subtypes 16 and 18 are implicated in the carcinogenesis of more than 90% of cervical cancers. Despite well-established national cervical screening programmes, cervical cancer still remains the most common cancer affecting females in South Africa. This may partly be attributed to the high incidence of HIV infection in South Africa.

Some of the hallmarks of cancer are, the up-regulation of telomerase, over-expression of E2F1 transcription factor, and over-expression of cyclin E and cyclin B1. The aim of this current study was to establish whether 24-hour treatment with hexane and ethanol leaf extracts of *M. oleifera* modulate telomerase, E2F1, cyclin E, and cyclin B1. The apoptotic pathway and phase of cell cycle arrest were also investigated. The HeLa cell line, an aggressive cervical cancer cell line in which high-risk HPV-18 viral strands have been identified, was used in this study

A novel effect of *M. oleifera* leaf extract was evident in the inactivation of telomerase. The inactivation of telomerase implies that p53 function was restored by the repression of E6 gene expression. Another novel outcome of the study is that *M. oleifera* down-regulates E2F1, accounting for the dose-dependent antiproliferative effects seen. The inactivation of telomerase was demonstrated by caspase-3 and caspase-7 activation, which confirmed intrinsic apoptosis. The down-regulation of E2F1 possibly occurs through the repression of the E6 oncoprotein and the activation of p53. The quantitative assessment of cyclin E and cyclin B1, showed an overall down-regulation, and G2-M cell cycle arrest.

Taken together, this study provides convincing evidence that *M. oleifera* hexane and ethanol leaf fractions have potential antitumour effects, by targeting multiple abnormally elevated markers for down-regulation.

Other *M. oleifera* fractions investigated in a parallel study, and were excluded due to p values being greater than 0.05 and inconclusive findings, in dichloromethane and aqueous fractions.

V. Chapter 1

Introduction

The vast antitumour potential of *M. oleifera* is well-documented (Jung et al., 2015; Roy et al., 2014; Tiloke et al., 2013; Tiloke et al., 2016; Purwal et al., 2010). An unexplored area of research, however, is *M. oleifera's* specific effects on Human papillomavirus (HPV) induced malignancy, the key tumour being cervical cancer.

Cervical cancer is the most common female cancer in women aged 15 to 44 years in South Africa (SA HPV Centre, 2019). It is, however, a highly curable disease if detected and treated during its pre-malignant phase (Lindeque, 2005).The positive cytological and histologic diagnosis and removal of these abnormal cells, and subsequent regular follow-up of such patients, is highly effective in reducing the cervical cancer burden by about 80% in high resource settings (SA Cancer Association, 2017).

Cervical cancer has been studied extensively, generating a significant body of research that supports persistent concomitant high–risk human papillomavirus (HPV) infection of the cervix, as the main aetiological agent involved in its pathogenesis (Bharti et al., 2009; Chow et al., 2010). Persistent HPV infection leads to cervical cancer precursors that are graded according to their nuclear and cytoplasmic morphology as moderate or severe. Cervical lesions are reported as low-grade squamous intraepithelial lesions (LSIL) and high-grade squamous intra-epithelial lesions (HSIL), according to the Bethesda Classification (Reagan et al., 1953).

HPV is sexually transmitted, and the virus gains access to the host through mechanical abrasion of infected mucosal surfaces of the cervix and vagina. HPV DNA testing is an accurate means of detecting and typing HPV subtypes and sites that may be tested include vulva, vagina, cervical, oral, and anal tissues in females, and on the penis, glans, foreskin, scrotum, buccal cavity, and anus of males (Popescu et al, 1990). HPV infection in both sexes is mostly asymptomatic, transient and of minimal morbidity. HPV lesions often regress and become undetectable while others may remain the same. However, a minor subset of HPV infec-

tions, mainly the high-risk subtypes, will progress to severe dysplasia, carcinoma-in-situ (CIS) and ultimately to invasive cervical cancer. The prevalence of increased incidence of dysplasia and cervical cancer in immunocompromised patients, may imply that this could be a confounding factor in HPV pathogenesis (Frisch et al., 2000).

Since there is still no known effective treatment for HPV infection, the only means of reducing the cervical cancer burden is through prevention of HPV infection de novo. This may be through abstinence; mutual monogamy of virgins; or the use of condoms, which offers about 70% protection from viral transmission (Franco et al., 2001). The development of vaccines against high-risk HPV subtypes have become available in the last decade, enabling significant strides in the attempts at reducing cervical cancer statistics. The various pharmaceutical companies offer products that provide immunity to HPV 6, 11, 16 and 18. Immunity to these sub-types for a period of 6.5 years, have been tested in randomized trials for safety, immunogenicity, and efficacy. Immunogenicity is conferred by HPV subtype-specific L1 proteins that form virus-like entities upon injection into the host (Joura et al., 2007). There is compelling evidence from clinical trials to suggest that these vaccines are capable of preventing persistent infection with the specific subtypes represented in the vaccines. This research also suggests that protection is afforded from dysplastic lesions of the anogenital tract for those particular L1 types injected (Mariani et al., 2010). In view of the high prevalence rate of HPV globally, primarily through sexual transmission, it is suggested that for optimal prophylactic impact, girls and perhaps boys as well, should be immunized prior to initial sexual contact (Franco et al., 2001). The HPV vaccine is also recommended for sexually active females with normal pap smears or those who test negative for HPV-DNA.

Although there are reliable detection and prevention strategies for HPV infection, an effective treatment for the already infected millions, remains elusive, placing a tremendous strain on developing economies that are already over-burdened by their efforts to curb the HIV pandemic, and recent COVID-19 pandemic. Positive strides towards an effective treatment for high-risk HPV and cervical cancer, are desperately needed in order to reduce the global burden of pre-cancerous and cancerous lesions of the cervix.

The current study is an in-*vitro* investigation into the cytotoxic potential of *Moringa oleifera* (*M. oleifera*) crude leaf extracts against an aggressive cervical cancer cell line, HeLa. HeLa cells have an interesting history that goes all the way back to 1955, when unbeknown to her, or her family, cervical cancer cells from an aggressive tumour, was taken from a young black mother (Hendrietta Lacks) from the U.S.A, who soon succumbed to her disease. These cells have been propagated in vitro ever since, being the first cell line to thrive continuously in-vitro (Lucey et.al., 2009). This particular cell line was chosen for this study because of its strong link to the high-risk HPV-18 virus, which is reported to be integrated within the genome (Popescu et.al., 1987).

M. oleifera has shown antitumour effects against HeLa (Nair et al., 2011). The possible anti-HPV capabilities, apoptotic pathways, as well as the stage at which cell cycle arrest occurs with this particular botanic, are yet to be discerned.

The notion that *M. oleifera* is able to cause cell death through apoptosis or programmed cell death mechanisms was further explored as confirmation of the cytotoxic potential of the South African cultivar of *M. oleifera*. The apoptotic pathway of activation, whether intrinsic or extrinsic, is also of particular interest. More so since the high risk HPV E6 oncoproteins, when transfected into benign senescent cells have resulted in immortality of the transfected keratinocytes (Barbosa et.al., 1989). Other studies revealed that E6 is able to mediate ubiquitination of a key tumour suppressor p53 by binding to E6-associated protein (E6AP), initiating p53 degradation and subsequent progression of the cell cycle from G1 to S phase (Cooper et al., 2003). This in itself suggests that HPV, through the E6 oncoprotein, directly evades the intrinsic apoptosis pathway by degrading p53, preventing down-stream activities like cytochrome c release from mitochondria, and the activation of 'initiator' and 'executioner' caspases (caspase-8, -9, -10, -3, -6, and -7), responsible for the destruction of cells containing mutant DNA. In addition to p53 degradation, E6 activates the catalytic subunit of telomerase (hTERT), accounting for the high levels of telomerase activity in HeLa and other forms of cervical cancer (Veldman et. al., 2001). Telomerase, which is absent in somatic cells of the body, with the exception of stem cells, imparts immortality to cancer cells by preventing the natural aging and death of cancer cells through the avoidance of telomere shortening (Xu et al., 2013). Both of these oncogenic, anti-apoptotic, and proliferative effects of E6 occur in the G1-S phase, circumventing cell cycle arrest at this

vital checkpoint and thus allowing aberrant DNA to progress through the cell cycle (Howie et al., 2009). Other cancer promoting activities of high-risk HPV include that of E7, which is known to disrupt the E2F-Rb complex in the G1-S phase (Gonzalez et al., 2001). Unbound E2F promotes the transcription of essential proteins involved in cell cycle progression from G1 to S-phase. The inactivation of pRb by E7, and the subsequent separation of the complex, therefore results in the premature transcription of cell proliferation proteins, allowing abnormal S-phase entry along with viral and mutant DNA (DeGregori et al., 1997).

High-risk HPV E6 and E7 oncogenes synergistically enable the host cell to evade apoptosis, proliferate abnormally, and become immortal. It is therefore plausible to assume that if *M. oleifera* is able to induce apoptosis through caspase activation, then this implies E6 and E7 oncogene repression. A further assumption is that if telomerase and E2F1; expression are down-regulated, proliferative hindrance and reversal of immortality are imminent. Since abnormal telomerase activation and E2F1 expression are downstream effects of E6 and E7 oncogene activity, then it can be concluded that modulation of E2F1 and telomerase are attributed to the suppression of these oncogenes (Jeong Seo et al., 2004; Hwang et al., 2002). Repression of E6 and E7 oncogenes activate dormant p53 and Rb tumour suppressor pathways, leading to cell cycle arrest and apoptosis (Goodwin et al., 2000). The down-regulation of telomerase and E2F1 also implies p53 reactivation, triggering a 'stop' signal which halts cell division through p21arf1 expression (Sima et al., 2008).

VI. Chapter 2

2.1 Literature Review

2.1.1 The mitotic cell cycle

Introduction to cell division

Cell division is an intricate, carefully regulated process that occurs in defined phases that constitute the cell cycle. The role of the cell cycle is to produce duplicate copies of DNA strands and then compartmentalize them equally into 2 genetically identical daughter cells (Ehrenfeucht et al., 2004)

.

The cell cycle comprises 2 major phases which are identified by four stages namely: G1, S, G2, and M. The time lapse for S-phase or synthesis phase is approximately 10 – 12 hours, during which the duplication of DNA occurs and accounts for half of the time taken for a complete cycle in a typical mammalian cell. The M-phase (mitosis) follows the S-phase when chromosomes separate and split into two individual daughter cells occur. The M-phase takes about an hour to accomplish, within a complete cell cycle. The processes that occur during M-phase include: packaging of DNA, separation of chromosomes, and cytoplasmic pinching-off (cytokinesis). The M-phase is when a number of important cellular events transpire that are initiated by nuclear division, or mitosis. The first signs of mitosis begin with the condensation of chromosomes to form more compact counterparts in preparation for segregation. Intermittent to the S-phase and M-phase, is a resting phase known as the Gap phase (G1). The Gap phase sees the alignment of the chromosomes along the equator of the spindle in preparation for separation. The G1 to S phase is a check point in the cell cycle when the duplicated chromosomes are assessed for damaged, irreparable, and mutated DNA, prior to segregation into daughter cells. A second Gap phase exists between the S and M-phases, and final assessment of the duplicate DNA, as well as the packaging of DNA occurs during this phase. Cells may exit the cell cycle in G0 phase in the absence of signalling that initiates growth or pro-differentiating signals. The entire cell cycle is precisely regulated in a systematic fashion from one phase to the next by signal transduction molecules (Hartwell et al., 2009).

The role of the M-phase is to cause 2 identical strands of chromatin to separate to opposite poles of the cell undergoing mitosis. This is accomplished through condensation of chromatin in prophase, and through the re-organisation of microtubules in the cytoplasm in order to create a mitotic spindle (Chen, et al.1996).

At metaphase, each chromosome arranges itself toward the centre of the spindle. A number of actions precede this event, the first being the disintegration of the nuclear envelope, which then allows attached sister chromatids to adhere to the microtubules through attachment sites called kinetochores. The kinetochores have the ability to seize microtubules and are held tightly at areas called centromeres, located on sister chromatid pairs. The spindle pole duplicates to form 2 spindle poles which are on opposite poles and are each attached to one of a pair of kinetochores present on each of the sister chromatids. Opposing forces are exerted on each sister chromatin causing each chromosome to arrange themselves towards the centre of the spindle, which is the hallmark of the metaphase stage of cell division (Sprague et al., 2003).

Anaphase is marked by the complete segregation of the two sister chromatids to either poles of the cell. This outcome is brought about by the antagonistic forces of the microtubules on each sister chromatid (Amon, 1999).

Subsequent to the polar separation of sister chromatids, the daughter cells are stimulated to physically separate through the pinching-off of the plasma membrane or cytokinesis (Rappaport, 1986).

Special regulatory systems have existed for billions of years, and function to intricately steer the cell through the various stages of the cell cycle. Similar regulatory systems exist today in unicellular eukaryotes like yeast cells and human cells (Novák, 2002).

Cdks are specific core activator proteins that are formed and activated at precise times during the cell cycle, and co-ordinate movement of the cell cycle through the various phases by selectively activating and switching off proteins (Harper et al., 2001). The systematic switching on and off of proteins, are carried out by phosphorylating these proteins at specific times.

Each Cdk contains two subunits which are the kinase subunit and the cyclin subunit. For a Cdk to possess enzymatic activity, it must be associated with a cyclin protein which acts as an activator (Graña et al., 1995).

Cyclins function as crucial regulators of the cell cycle and have a short period of existence, after which they are degraded, while kinase subunits remain throughout the cell cycle. Numerous types of Cdks and cyclins dominate at various phases of the cell cycle, and are systematically degraded at specific times in the cell cycle. As a particular cyclin is synthesized, it selectively binds to a kinase, and in so doing results in its activation (Johnson et al., 1999).

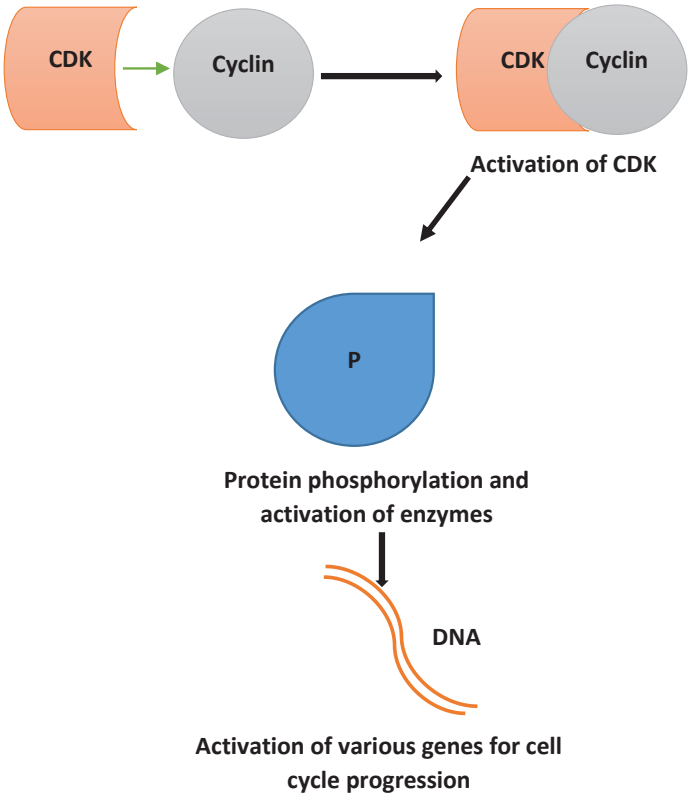

Figure 1: *Shows the specific binding of cyclins to Cdks causing the activation of that Cdk during various stages of mitosis. Activated Cdks promotes phosphorylation of specific proteins (P) and enzymes which are responsible for the activation of genes that enable cell cycle progression.*

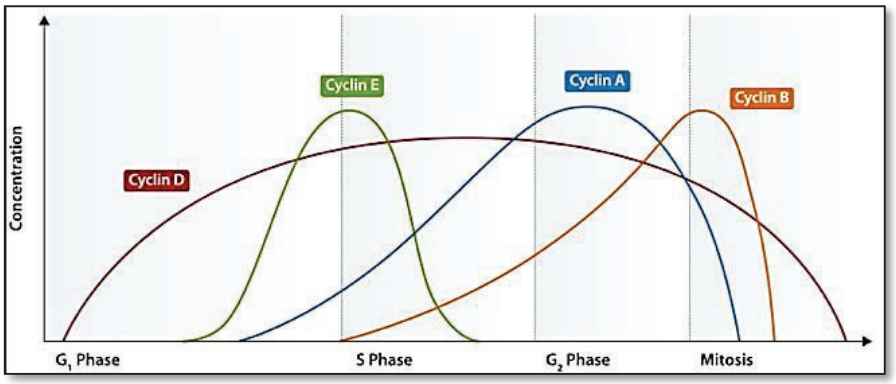

Figure 2: *Illustrates graphically the expression of the various cyclins during the cell cycle*

The G1 phase of the cell cycle is the only phase when cells are sensitive to external environmental signals (Duensing et al., 2004). Cyclin dependent kinases regulate transition of the cell at various stages during the cell cycle. The early and late G1 are closely regulated by cyclin dependent kinases and in so doing control the progression of the cell cycle. The G1-S checkpoint is commonly de-regulated in cancer cells (Ortega et al., 2002).

Cyclin E synthesis, which requires E2F1 transcription factor, is necessary for transition from early to late GI (Johnson et al., 1999). In non-proliferating and early G1 cells, the E2F transcription factor is bound to the cyclin E gene promoter. E2F1 function is modulated by its binding to the retinoblastoma protein (pRb), a nuclear phosphoprotein that is directly involved in cell cycle regulation. pRb is either active and under-phosphorylated or inactive and hyper-phosphorylated (Dyson, 1998). The active form of pRb prevents G1 to S phase transition. G1-Cdk increases at mid-G1, causing the phosphorylation and activation of the pRb protein. E2F1 release from inactive phosphorylated pRb exposes the E2F1 transcription domain. This allows for cyclin E gene transcription. The cyclin E protein binds to the Cdk2 kinase to form G1/S - Cdk. Other genes transcribed by E2F1 crucial for S-phase entry include the genes for DNA polymerase and thymidylate synthase (Ohtsubo et al., 1995; Horikawa et al., 2003).

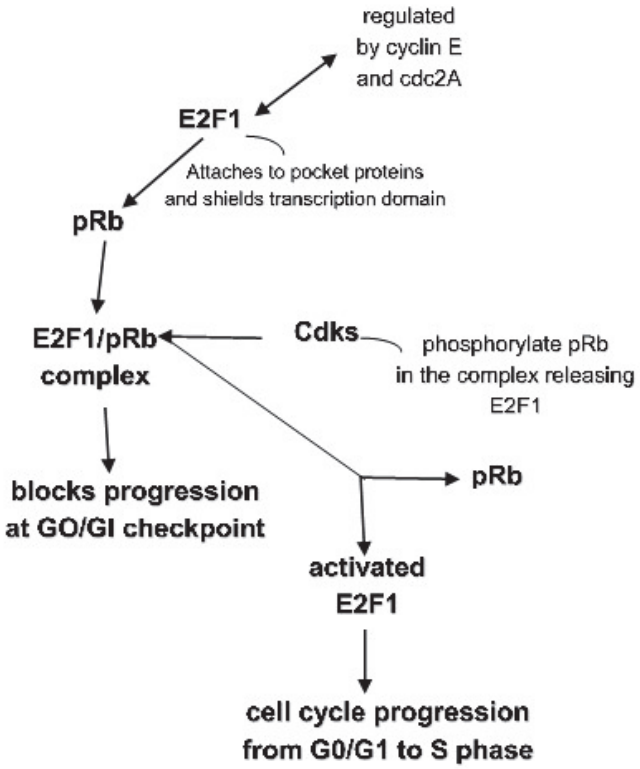

Figure 3: *E2F1 transcription factor, regulated by cyclin E, attaches to pRb pocket proteins forming a complex that shields the E2F1 transcription domain. Formation of the pRb/E2F1 complex blocks progression at the G0-G1 checkpoint. Cdks phosphorylate pRb within the complex, causing the release of E2F1 resulting in the exposure and activation of the E2F1 transcription domain. E2F1 promotes the transcription of proteins that drive S-phase cell cycle progression.*

The crucial event of Cdk2-cyclin E activation causes the cell to undergo irreversible entry into S-phase. This occurs due to a switch from mitogen-dependent to mitogen independent transition. No further growth factor stimulation is required once cells enter S-phase. Cells that contain genetic anomalies that do not require mitogen-dependent signals will bypass this key checkpoint.

The pRb protein functions as a 'brake' or inhibitor on the cell cycle, by effectively inhibiting the E2F transcription factor, and in so doing prevents the transcription of the cyclin E gene. p16, p21, and p27 act in a similar manner by binding to Cdk-cyclin complexes, inhibiting their protein kinase activity (Sherr & McCormick, 2002).

There are essentially two classes of Cdk inhibitors. The Ink4 family of proteins which includes p16, bind selectively to, and inhibit the G1 Cdks and Cdk4/6-cylin D. The other class is the Cip/Kip family of Cdk inhibitors which include p21 and p27, that bind to a variety of Cdk-cyclin complexes, preventing the progression of the cell cycle at numerous points. Cancer cells exhibit abnormal functioning of these Cdk inhibitors and are therefore able to bypass crucial points of cell cycle arrest (Vidal & Koff, 2000).

pRb plays a crucial role in inhibiting G1 to S-phase cell cycle progression, and represses DNA synthesis through the selective binding and inhibition of E2F1 transcription factors. Inhibition of E2F1 is brought about by the binding of E2F1 transactivation domain to the pRb pocket A–B interface, thereby repressing its transcription activity. The pRb pocket may contain mutations, that prevent binding of E2F1 and result in unchecked cell cycle progression into S-phase. The C-terminal region of pRb also binds E2F in a similar way, causing inhibition of transcription factors. This inhibitory role by pRb is overcome by its phosphorylation by G1 Cdks. E2F1 is only active in its unbound state and promotes various proteins that are actively involved in cell cycle progression like CDC6, dihydrofolate reductase, thymidine kinase, and DNA polymerase α. In addition, E2F1 promotes the transcription of cyclin E. Cyclin E forms an active complex with Cdk2 and in turn further activates E2F1 by increasing pRb phosphorylation.

E2F plays a vital role in the regulation of cell proliferation as well as anti-proliferative activity like apoptosis and senescence.

2.1.2 Overview of cancer

Each gene, of which there are about 25000 per human cell, is composed of a substance called DNA (deoxyribonucleic acid). The function of a specific gene is informed by a specific DNA sequence that encodes specific protein and enzymes for that particular cell's function/ s, while other genes may regulate the rate of synthesis of proteins produced by genes (Hyland, 2008).

The 'switching on' and 'switching off' of selective genes at precise points in time, results in the specialization of cells to perform a function/ group of functions called functional differentiation. Genes

required for the synthesis of proteins required for basic cell functioning, may remain switched on indefinitely e.g. cellular respiratory proteins and enzymes (Tyson et al., 2002).

Genetic anomalies are the root cause of cancer pathogenesis, as some genetic mutations are directly responsible for the development of cancer and other genes meant for cancer surveillance become aberrant (Ignatenko et al., 2003).

2.1.3 Pre-malignant transformation of the cervix

Immature squamous metaplastic cells within the cervical transformation zone of the ectocervix, is most at risk for abnormal transformation. Cervical dysplasia is atypical alteration that occurs at the narrow neck of the uterus called the cervix. LSIL, may persist, regress spontaneously, or progress to high-grade lesions. On the other hand, HSIL may persist indefinitely or progress to carcinoma in-situ (CIS) and invasive squamous carcinoma. It has been established that the HPV viral genome integrates with that of the host genome, and could be the reason for HSIL persisting rather than regressing (Melnokow et al. 1998).

CIS is virtually indistinguishable from invasive carcinoma cells on a pap smears, and requires histologic diagnosis to confirm that affected cells have not invaded through the basement membrane. CIS is also regarded as a 'stepping stone' to invasive carcinoma, as they are most likely to progress to invasive carcinoma if left untreated for a period of time. Human papillomavirus is concomitant with most cervical HSILs and cervical cancers, making it the prime aetiological agent in the pathogenesis of cervical cancer (Reagan et al., 1953).

2.1.4 Genetic mutations

2.1.4.1 General

DNA, contained within genes are constructs of specifically arranged building blocks called bases. The gene specificity and function is brought about by the defined arrangement of genes. The DNA of genes have a high propensity to become altered or mutated. These mutated genes can occur due to translocations, deletions, and other anomalies, where even a single change in the arrangement of bases can have adverse down-stream effects (Cox, 1995).

Some of the abnormal effects of mutations of particular genes may be the absence of synthesis of a key protein, or the synthesis of non-functional proteins. A mutation may also cause a gene to be abnormally switched on, producing aberrant, increased transcription of that protein. Other types of mutations may not have a significant effect on the cell's functioning ("Oncogenes, Tumour Suppressor Genes, and Cancer," 2011).

2.1.4.2 Hereditary mutations

Hereditary mutations are passed on from parent to child and may also be termed germ-line mutations. Such mutations are carried into all chromosomes in the body and may be passed on to future generations.

A hereditary mutation accounts for approximately 10% of all cancers diagnosed, and places carriers of these hereditary mutant genes at an increased risk of developing those particular types of cancers e.g. BRCA1 and BRCA2 in breast cancer (Ignatenko et al., 2003).

2.1.4.3 Acquired mutations

Most cancers are initiated by DNA mutations that occur during the lifetime of an individual. Such mutations are known as acquire, sporadic, or somatic mutations. The trigger for an acquired mutation may be an external carcinogen such as radiation exposure, cigarette-smoking, or accumulation of other toxins. In most cases, the aetiological agent is largely unknown (Abeloff's Clinical Oncology, 2008)

Acquired mutations are initiated in a single cell and may be seen only in the colonies of that particular cell, while hereditary mutations are present in all cells of the body. The latter cannot be passed on to offspring since they are not present in the reproductive cells. Mutations are thought to occur regularly within cells of the body, however, the cell is usually able to detect the change and repair it. If the gene is irreparable, the cell will receive a signal causing it to spontaneously die in a process called apoptosis. If an irreparable mutant gene escapes cell death, it may become malignant if the affected gene is directly or indirectly involved in cell division or if the gene is a tumour suppressor gene (Hanahan et.al., 2011).

More than one gene mutation, likely several, is thought to be responsible for cancer development. Since there are two genes present (one from each chromosome in a pair), this implies that even when a person has inherited one mutant gene, at least one more mutation in the same gene pair must occur to "knock out" the good copy of that gene, in order for that gene to lose complete functionality. This implies that for a hereditary cancer to develop, an acquired mutation is necessary. An individual born without any gene mutations, requires two mutations in corresponding gene pairs in order to ‘knock out’ that particular gene (Human & Project, 2001).

2.1.4.4 Oncogenes

a. Inherited mutations of oncogenes

Proto-oncogenes are characteristically useful genes that often are mutated and become ‘bad’ genes called oncogenes. A proto-oncogene in turn may be described as the "good" genes that normally controls cell differentiation and the rate of cell division. Such mutant proto-oncogenes or oncogenes, become "bad" genes that may become abnormally and permanently activated causing cells to grow uncontrollably, which may lead to tumour development (Javier et al., 2008).

b. Acquired mutations of oncogenes

Inherited mutations of oncogenes are caused by inherited mutations of proto-oncogenes resulting in the activation of the oncogene that leads to cancer syndromes (Hussain, Hofseth, & Harris, 2003).

c. Tumour suppressor genes

The Majority of the cancer-causing mutations that are related to oncogenes are of an acquired nature. These oncogenes may arise as a direct consequence of chromosome rearrangement/ translocation, gene duplication, or mutations ("Oncogenes, Tumour Suppressor Genes, and Cancer," 2011).

Tumour suppressor genes function to slow down the rate of cell division, repair DNA aberrations, or initiate cell death through the activation of apoptosis pathways. Tumour suppressor genes malfunction, causing cells to divide uncontrollably, initiating carcinogenesis. Examples of tumour suppressor genes include tumour protein 53 (TP5) or p53, breast cancer gene 1 (BRCA1), breast cancer gene 2 (BRCA2), adenomatous polyposis coli (APC), and retinoblastoma protein (RB1). The main distinction between oncogenes and tumour suppressor genes is that the former causes malignant transformation by upregulation of proto-oncogenes, while tumour suppressor genes cause cancer when they are inactivated (Miyashita et al., 1994).

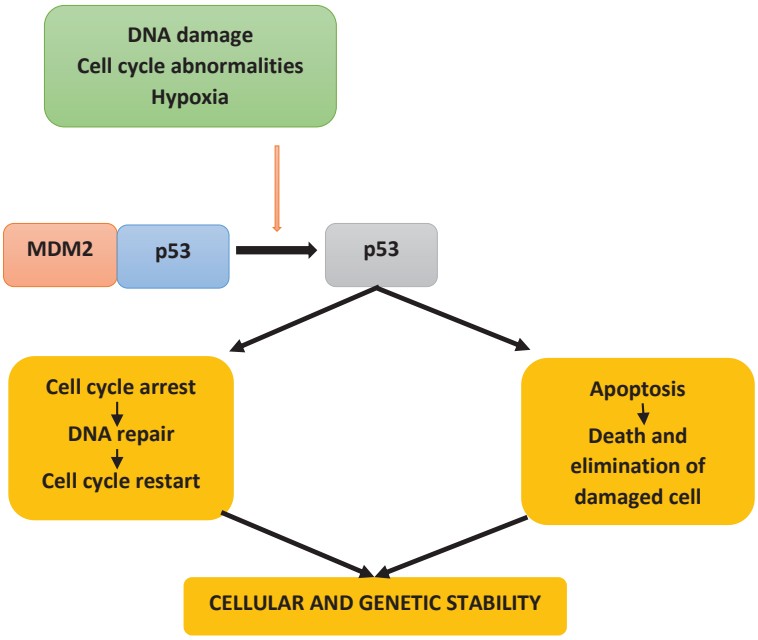

Figure 4: *Mdm2 acts as a regulator of p53 function. p53 is activated by DNA damage and stress induced conditions, leading to the separation of mdm2 from p53, allowing for repair of the damaged chromosome or apoptosis to discard the damaged cell.*

Most tumour suppressor gene mutations are acquired, and are pivotal events that precede carcinogenesis. An example of such a common acquired tumour suppressor gene mutation is that of the of the TP53 gene (which codes for the p53 protein), which is responsible for more than half of all human cancers. As stated earlier on, the p53 protein is involved in pathway in which cells with irreparable gene mutations undergo apoptosis (Figure 4). It stands to reason that if TP53 is mutated then cells with damaged DNA will continue to proliferate, resulting in increased numbers of cells with abnormal DNA (Greenblatt et al., 1994).

2.1.5 Human *papillomavirus* (HPV) and cervical carcinogenesis

2.1.5.1 HPV infection

Human papillomavirus has an increased cellular tropism for squamous epithelial cells or keratinocytes and is the main aetiological factor for cervical cancer development (Frisch et al., 2000). The concomitant nature between HPV infection, SIL, and cervical cancer was first made by Harold zer Hausen in the early 1970's. There is, however, no anti-HPV therapeutics available to treat millions of infected women.

More than 200 different HPV subtypes have now been documented, and new subtypes continue to be added regularly (de Villiers et al., 1981). These subtypes may be seen throughout the animal kingdom and have a propensity for squamous epithelia, causing condylomata or 'flat' wart formation. Wart formation was long suspected as a consequence of infection and this theory was eventually confirmed in the 19th century (Meisels et al., 1980). Subtypes of HPV are classified as high risk HPV i.e. (HPV 16,18, 31, 35, 39, 45, 51, 52, 56, 59, 66, 68, 69, and 73) (HR-HPV) and low risk HPV i.e. HPV 6, 11, 40, 42, 43, 44, 54, 61, 70, 72 and 81 (LR-HPV), according to their presence in cervical cancer. HPV-16 and HPV-18 are the most oncogenic/ pathogenic amongst the high-risk HPV subtypes, and collectively, have been isolated from approximately 80% of the total cervical cancer lesions (Bharti et al.,

2009). High-risk HPV has been isolated in 90 - 98% of all high grade squamous intraepithelial neoplasia (Narisawa-Saito et al., 2007), making high-risk HPV the main mediator in malignant transformation of the uterine cervix. Of these, HPV-16 accounts for approximately 50% all cervical carcinogenesis while HPV-18 is implicated in about 10 - 20%. Other malignant tumours where HPV has also been positively isolated include: the anus, vulva, vagina and penis.

a. High-risk HPV-18

Although HPV-18 statistically is the second most prevalent HPV subtype present in invasive cervical cancers, it is seldom found in pre-cancerous lesions (5%) (Cox, 1995). HPV-18 has been identified mainly in adenocarcinomas, and its prevalence may be equal to, and possibly greater than, HPV 16 in these tumours (Duggan et al., 1993). The high incidence of HPV-18 in adenocarcinomas also suggests that it may have a preference for endocervical cells. The low detection rate of HPV-18 in pre-malignant lesions may be in part due to the rapid progression of pre-cancerous lesions, so that they are not detected between pap smear screening intervals (Cox, 1995).

b. Molecular events leading to cancer progression

Human papillomavirus disrupts the normal programme of epithelial cell differentiation, which results in the formation of condylomata near the vagina and external genitalia, dysplastic lesions, or invasive cervical cancer. The replication of HPV occur at the cervical and vaginal mucosal surface and is dependent on the expression of viral oncogene products during the cell cycle. In HSIL, the viral genome amplification occurs close to the epithelial surface. These molecular events are not fully understood but may be influenced by the extent of viral oncoprotein expression following viral genome integration into the host chromosome in HSIL (Pett et al., 2007). Integration of HPV DNA into host genomes, is a critical event in cervical cancer carcinogenesis and is found in E6 transfected immortalized cells, most invasive cancers, and HSIL lesions. Viral genome integration occurs during time loss or disruption of viral sequences encoding E1, E2 and E4 occur. These proteins have an inhibitory effect on cell proliferation, are capable of causing cell-cycle arrest at the G2 phase (E2), and can inhibit mitosis by disrupting cyclin B/ Cdk1 (E4 of HPV-16) (Münger et al., 2004). The HPV virus therefore circumvents these

key tumour-inhibiting regulatory proteins and is able to induce immortality and progression to invasive cervical cancer. A key marker pointing to elevated HPV E7 oncogene expression is p16INK4A expression. P16INK4A is detected in cases of LSIL; and a larger proportion of HSIL lesions that exhibit viral genome integration. The importance of E6 and E7 oncoproteins in malignant transformation has also been demonstrated by introducing E2 to cancer cell lines like HeLa. Interestingly, E2, which binds to upstream regulatory regions, suppresses the expression of E6 and E7, resulting in apoptosis of HeLa cells.

c. Telomerase activation by E6 oncogene

Human telomerase is a ribonucleoprotein complex composed of a reverse catalytic transcriptase (hTERT) and an RNA component (hTR) (Feng et al., 1995). It is expressed in immortal cells e.g. germline cells, stem cells and cancerous cells (Guilleret et al., 2003). hTERT expression in normal cells stimulates telomerase activity, causing cells to circumvent cell death mechanisms and consequently suppress senescence (Masutomi et al., 2003). There is therefore a direct relationship between hTERT expression and telomerase activation. The high-risk HPV E6 oncoprotein stimulates telomerase activity allowing cells to maintain telomere repeat sequences, thus initiating immortality in epithelial cells (Oh et al., 2001). Masutomi et al. showed that E6 and Myc interaction activates the telomerase reverse transcriptase promoter (Masutomi et al., 2003). During E6 expression, the repressor complex of TERT promoter is supplanted by Myc through the degradation of NFX1-91 (cellular repressor of hTERT promoter) (Yago et al., 2002), thereby increasing TERT transcription and a corresponding increase in telomerase activation (Yago et al., 2002).

E7 is a small nuclear phosphoprotein made up of three distinct regions CR1, CR2 and CR3. The E7 oncoprotein elicits its viral pathogenicity by binding to the pRb (retinoblastoma tumour suppressor gene). Other proteins from the pRb family are also capable of being bound to E7 like p107 and p130,

in its CR2 region (Liu et al., 2006). Hyper-phosphorylated pRb and its family members, bind to transcription factors such as E2F, thus repressing the DNA synthesis and cell-cycle progression. Progression of the cell cycle into S-phase is induced by G1-Cdk's ability to phosphorylate pRb, thereby releasing E2F from the pRb-E2F complex. E7 on the other hand can bind to dephosphorylated pRb causing the abnormal dissociation of the pRb-E2F complex and resulting in atypical S-phase entry (Hwang et al., 2002). p16INK4a is a useful biomarker for assessing the pathogenicity of HPV in cervico-vaginal lesions. It acts by inhibiting the phosphorylation of pRb, and is typically up-regulated when E7 inactivates pRb. Up-regulation of p16INK4a in the absence of E7 would usually cause cell cycle arrest, but this function is nullified when E7 is expressed (Giarrè et al., 2001)

High-risk E6 is known to escape p53 mediated cell cycle arrest, thereby allowing aberrant DNA to accumulate, advancing cervical carcinogenesis by binding to p53 and E6AP (E6-associated protein). This leads to the ubiquitination of p53, and its degradation.

A key means of enabling malignant transformation by high-risk E6 is through the deliberate activation of the catalytic subunit of telomerase called human telomerase reverse transcriptase (hTERT). This is brought about by systematically joining hexamer repeat sequences to the telomere ends of chromosomes, which results in the loss of senescence and the manifestation of the immortal nature of invasive cancer cells.

The E6 and E7 high-risk HPV are able to transform keratinocytes in-vitro from senescent to immortal, by inducing telomerase activity. However, it is not clear whether E6 alone is responsible for the maintenance of telomerase activation in cervical cancer cells. E7 expression may play a secondary role in activating telomerase and promoting immortality, in the presence of increased p53 expression. There is evidence to suggest that both E6 and E7 contribute to the activation of hTERT and increased telomerase activity in particularly cervical cancer cells, including HeLa cells.

E6 expression is crucial for the maintenance of telomerase in cervical cancer cells, which is only possible through the inhibition of p53, since p53 plays a role in tumour suppression by inhibiting the

hTERT promoter. This correlates with the idea that E2-transduced cervical cancer cells cause reduced hTERT expression through a possible elevation in p53 levels. Studies show that the inhibitory effect of p53 may be dormant, and that for hTERT activity to be reduced, E6 must first be repressed.

2.1.6 Antitumour medicinal plants

Cancer is a dreaded disease that is the second leading cause of death worldwide. Treatment programmes consists of various combinations of surgery, radiation therapy, and chemotherapy. Despite these aggressive therapeutic options, cancer remains associated with high mortality rate (Redd et al., 2001). Most conventional cancer treatment modalities cause adverse side effects, and may not completely cure the disease but serves to extend the patient's lifespan. Due to the complex nature of the disease, a 'one size fits all' treatment for cancer remains elusive. Modern researchers may therefore benefit from exploring botanicals as a potential source of alternative therapy, or as an adjunct to conventional chemotherapeutics. Natural remedies, mainly derived from plants, have been used in folk medicine for the effective treatment of a wide variety of ailments for thousands of years (Fink, 2002). Native plants have been used historically for their medicinal value in Africa, Egypt, China, India, and Greece, and modern pharmaceutical drugs have done well to develop drugs from this ancient knowledge base. Phytochemicals derived from plant matter are widely used to offer protection against a variety of chronic diseases such as diabetes, and cancer (Wall et al., 1966). There has been a surge of interest in plant-based cancer studies in the last few decades, aimed at exploring novel chemotherapeutic approaches for the treatment of cancer. The outcomes of this interest phytochemical remedies are epidemiological and experimental studies highlighting compounds that may be useful in the prevention and treatment of various types of cancer. In the past two decades, the pharmaceutical industry has seen an increase in approved plant-derived drugs (>25%), and synthesized plant compounds (25%). Yet only a small proportional of botanicals have been studied for bioactive compounds against cancer. The clear advantage of using such compounds for cancer treatment is their relative non-toxicity to normal cells and their wide availability as supplements (Roja et al., 2000).

The properties of an ideal phytochemical, is one that possesses antitumour properties with minimal toxicity and has a defined mechanism of action. As greater discovery of the effects of plant derived-compounds on specific signalling pathways emerge, novel therapeutic approaches in reducing the global burden of cancer, can be expected (Fabricant et al., 2001).

Vinblastine (Velban) and Vincristine (Oncovin) are indole alkaloids, widely used in the treatment of Hodgkin's lymphoma, whose origins can be traced back to the Madagascar periwinkle plant (*Catharanthus roseus*). Biosynthesis of these alkaloids, although met with certain challenges, would be advantageous in meeting the ever increasing demand for these pharmaceutically valuable natural products.

Etoposide and teniposide are compounds that are semi-synthetic derivatives of podophyllotoxin, a metabolite of the roots *Podophyllum peltatam* and show promising antimitotic effects. The Mayapple plant is popularly used as plant derived home remedies from ancient times. Etoposide is effective as a topoisomerase II inhibitor. This essential enzyme is known to participate in regulating levels of DNA supercoiling and therefore plays a crucial role in cell growth of eukaryotes. Etoposide was approved in the U.S.A for the treatment of lung cancer, choriocarcinoma, ovarian, testicular cancer, lymphoma, and acute myeloid leukaemia. Teniposide is used for the treatment of tumours of the central nervous system, malignant lymphoma, and bladder cancer. Taxol (paclitaxel), a diterpene alkaloid, is famous as an antitumour agent. It was initially discovered in plants but was later identified as a fungal metabolite. Taxol is produced by fungi such as *Taxomyces adreanae*, *Pestalotiopsis microspora*, Tubercularia sp. and *Phyllosticta citricarpa*. Originally isolated from the bark of the Pacific yew tree (*Taxus brevifolia*), Taxol showed antitumour activity. One of the major challenges was the minimal quantities that were available in nature, prompting its growth and production by plant cell culture or by semi-synthesis from taxoids made by Taxusspecies. Currently the preferred means of producing Taxol is through the use of the cell *Taxus chinensis*. Taxol is now sanctioned for the treatment of breast and ovarian cancer, being able to inhibit de-polymerization of micro-tubules. Moreover, Taxol enhances tubulin polymerization and inhibits rapid cell mitosis in mammalian cancer cells (Horwitz et al., 1993). Camptothecin is a modified monoterpene indole alkaloid which is produced by certain plants (angiosperms). It is used for

the treatment of recurrent colon cancer and exhibits unusual activity against lung, ovarian, and uterine cancer. Camptothecin is known commercially as Camptosar® and Campto®. Camptothecin's have 2 water-soluble derivatives, irinotecan and topotecan, that are also used clinically. The cellular target of camptothecin is type I DNA topoisomerase. In the event of patients becoming resistant to irenotecan, it may be combined with the monoclonal antibody Erbitux (Cetuximab) in order to expand its use. Erbitux also inhibits tumour proliferation and the together exhibits antitumour activity in metastatic colorectal cancers containing epidermal growth factor receptors (EGFR). EGFR protein is expressed in approximately 80% of such tumours. The drug combination is effectual in reducing tumour invasion metastasis (Wall et al., 1966).

2.1.7 Apoptosis

2.1.7.1 General

Programmed cell death, or apoptosis, is an ongoing homeostatic mechanism in which excess or damaged cells are effectively eliminated from the body. It serves to maintain balance between the quantity of new cells produced and dead cells, as well as an important mechanism by which cells with various gene mutations are eliminated. In order for enough abnormal cells to proliferate and cause cancer, alteration of both normal apoptotic mechanisms as well as normal cell proliferative mechanisms usually occur (Rastogi et al., 2009).

Apoptosis has a key role in developing tissue, and in mature tissue, through localized apoptosis. The process of apoptosis requires the careful monitoring and timely triggering of a class of proteases intracellularly known as caspases, which normally occur in an inactive form called procaspases (Tazawa et al., 2007).

Cell-surface death receptors are responsible for the activation of an extrinsic apoptotic pathway. Procaspase activation can occur extracellularly by inducing a signalling protein called Fas ligand, which

selectively binds to its Fas receptors on target cells. The binding of the Fas 'death receptor' triggers the binding of adapter proteins that connect the receptor to procaspase-8 molecules, stimulating it to cleave, thereby activating a proteolytic cascade culminating in apoptosis (Fulda et al., 2006).

The intrinsic apoptotic pathway directly involves the mitochondria. Certain events that cause cell injury such as stress or DNA damage can trigger apoptosis through pro-caspase activation, and cause mitochondria to release cytochrome-c into the cytoplasm, which binds and activates Apaf-1. The Apaf-1 forms an apoptosome which organizes the procaspase-9 molecules that in turn triggers the sequential caspase cascade and ultimately, apoptosis of the damaged cell (Harada et al., 2003; Fulda et al., 2006).

The Bcl-2 family of proteins act as gate-keepers, strictly regulating cytochrome-c release from the mitochondria into the cytosol. There are three subclasses of Bcl-2 proteins, and common to all 3 is the BH protein domain. The BH protein domain allows for direct binding between a single pro-apoptotic protein and a single anti-apoptotic protein, to form heterodimers. Bak and Bax are crucial BH123 family members, and function to create channels for the escape of cytochrome-c out of the mitochondria and into the cytosol (Ignatenko et al., 2003). The direct binding to the anti-apoptotic Bcl-2 proteins actively inhibit Bak and Bax, preventing apoptosis and promoting the cell to survive. A pro-apoptotic subclass called BH3 are able to bind and form heterodimers with various subsets of the anti-apoptotic Bcl-2 proteins. Interestingly, BH3 can indirectly induce apoptosis by binding to Bcl-2 protein inhibitors from Bak and Bax, permitting the formation of mitochondrial channels and inducing apoptosis through the cleavage of caspase-9 and the activation of the caspase cascade (Rastogi et al., 2009).

The signals that control which cells survive or die, are not clearly understood. However, what is known is that there exists an intricate and strict control between the actions of the anti-apoptotic Bcl-2 proteins and the two sub-lasses of pro-apoptotic proteins that lead to the triggering of the caspase cascade and ultimately apoptosis through the intrinsic pathway.

Cancer cells have an innate ability to circumvent apoptosis through the intrinsic pathway (due to DNA mutation), by altering the amount of pro-apoptotic and anti-apoptotic proteins (Zimmermann et al., 2001; Wlodkowic et al., 2011).

2.1.7.2 p53, the cell cycle and apoptosis

Cell cycle is carefully regulated by checkpoints, which are the quiescent stages of the cell cycle when cell cycle progression is halted due to excess DNA damage and possible mutations. The G1 DNA damage checkpoint is one such checkpoint where cell cycle arrest is initiated upon identification of irreparable damage to DNA. p53 is a key transcription factor that is recruited during DNA damage G1-S checkpoint. It's binding to Mdm2 leads to degradation and progression through the G1-S checkpoint, however, during DNA damage phosphorylation of p53 occurs blocking the binding to Mdm2, allowing for its accumulation. p53 then binds to the p21 promotor, a well-known Cdk inhibitor, causing p21 transcription and accumulation. p21 inhibits certain Cdks resulting in cell cycle arrest. The p53 pathway is crucial in preventing the replication of mutant or damaged DNA and initiating cell death mechanisms. Cancer cells are commonly known to possess defective p53 pathways that circumvent the cells ability to undergo cell death through apoptosis by allowing defective DNA to pass through the G1 checkpoint (Haupt et al., 2003).

2.1.7.4 Methods of detection

Several detection techniques are used to detect apoptosis. The techniques that can be used to study the morphology of apoptotic cells are: electron, light, and fluorescent microscopy (McCarthy et al., 1998). The most widely used techniques include reverse transcriptase-polymerase chain reaction (RT-PCR) and gel electrophoretic analysis of DNA amplicons (Chen et al., 2010). RT- PCR is a particularly important technique since modern biological investigations have indicated that the proliferation of some neoplastic cells are related to the prevention of apoptosis mechanisms (Chen et al., 1998). Recently, Chen et al. (1998) has grouped apoptotic genes into living or dead genes. The living genes include the cells proliferation and bcl-2 genes (Chen et al., 1998), while the death genes include suppressing cell proliferation (p53), and Bax genes (Cheng et al., 1997). Bax and Bcl-2 genes have recently been studied

extensively in gastric, lung and prostate cancer and have shown to be important in the regulation of the intrinsic pathway of apoptosis (Xue et al., 1999; Pillai et al., 2004).

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

RT-PCR is used to study the expression of genes, such as those involved in apoptotic signalling (Shiao, 2003). B-actin or GADPH are used as internal RT-PCR controls in apoptotic/ cell death studies, and its expression in cell lines is a useful indicator of cell viability at the time of performing the RT-PCR experiment (Shiao, 2003).

2.1.8 *Moringa oleifera* Lam.

2.1.8.1 General

Figure 5: *Photograph depicting a Moringa oleifera tree, captured in Kwa-Zulu Natal (South Africa), bearing typical 'drum-stick' fruit*

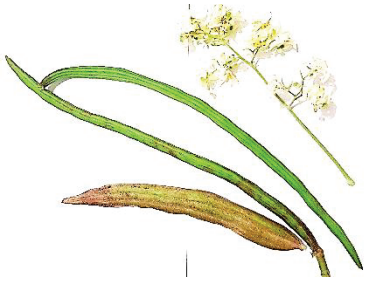

Figure 6: *Photograph taken of 'Drum-stick' fibrous mature and immature fruit, and flowers, captured in Kwa-Zulu Natal (South Africa). The actual tree from which it was harvested appears in Fig. 5.*

Figure 7: *A photograph taken of a typical mature Moringa oleifera stem with ovoid leaves, harvested from Kwa-Zulu Natal (tree as per Fig. 7), South Africa.*

Figure 8: *Picture of the ground mature leaves of Moringa oleifera used in the current study, harvested from Limpopo, South Africa*

Moringa oleifera is the most extensively cultivated species of the Moringaceae family, which has its origins in eastern countries like sub-Himalayan India, Pakistan, Bangladesh and Afghanistan. Other popular names of this plant species include: the horseradish tree, drumstick tree, benzolive tree, kelor, marango, mlonge, moonga, mulangay, ébéday, saijhan, sajna or Ben oil tree. Other ancient civilizations have reportedly used *M. oleifera* namely the Romans, Greeks and Egyptians (Anwar et al., 2007). *M. oleifera* is currently widely cultivatedF and is grown in many locations in the tropics (Türkiye et al., 1994). It is arguably the most useful tree on earth, with its application ranging from traditional medicinal, a source of food, water clarification, and animal fodder, to industrial uses (Anwar et al., 2007). *M. oleifera*, the 'Miracle Tree' has increased in popularity in the last decades, for its nutrient and medicinal value, and is hailed as a 'super food' which is cultivated as a sustainable food crop in various countries around the globe such as India, Ethiopia, the Philippines, Sudan, South Africa, tropical Asia, Latin America, the Caribbean, Florida and the Pacific Islands (Dubey et al., 2013).

2.1.8.2 Active compounds

M. oleifera is plentiful in compounds like rhamnose, which is a simple sugar, and it also boasts an abundance of compounds called glucosinolates and isothiocyanates (Gassenschmidt et. al, 1995). Certain agents of *M. oleifera* extracts show hypotensive potential (Faizi et al., 1995), anticancer (Jung, 2014), and antibacterial activity (Caceres et al., 1991). The active compounds include 4-(4'-O-acetyl-α-L-rhamnopyranosyloxy)benzyl sothiocyanate, 4-(α-L- rhamno-pyranosyloxy)benzyl isothiocyanate, niazimicin, pterygospermin, benzyl isothiocyanate, and 4-(α-L-rhamnopyranosyloxy) benzyl glucosinolate (Sharma et al., 2011). It comprises an abundance of a wide range of vitamins and minerals, as well as phytochemicals and carotenoids (including β-carotene or pro- vitamin A) (Becker et. al, 2003).

Inherent in *M. oleifera* leaves, is a wide array of antioxidant compounds like ascorbic acid, flavonoids, phenolics, and carotinoids (Goyal et al., 2007). It also contains a high concentration of oestrogenic

substances and β-sitostero, iron, calcium, phosphorus, copper, vitamins A, B and C, α-tocopherol, riboflavin, nicotinic acid, folic acid, pyridoxine, β-carotene, protein, and essential amino acids such as methionine, cysteine, tryptophan, and lysine (Fahey, 2005).

Moringa species has been widely used for decades as a traditional antitumour therapy. Active compounds from *M. oleifera* are potent inhibitors of phorbol ester (TPA)-induced Epstein-Barr virus early antigen activation in lymphoblastoid (Burkitt's lymphoma) cells (Juevara et al., 1999, Murakami et al., 1998). An isolated study also showed inhibition of tumour promotion in a mouse two-stage DMBA-TPA tumour model. Further to this, Bharali et al have investigated skin tumour prevention following ingestion *M. oleifera* seedpod extracts (Bharali et al., 2003). A significant reduction in skin papillomas was discovered during this investigation, which advocates for the traditional practice of the use of native plants in cancer prevention. The modern day practitioner has advanced from the traditional practitioners by isolating bioactive compounds from crude extracts.

In the current study, the anti-cancer and anti-HPV effects of HeLa cell line was investigated. The extraction solvents utilized were hexane and ethanol. It has been previously reported that climatic factors, as well as stages of maturity, could cause variation in distribution of active phytochemicals in leaves of *M. oleifera* (Nweze et al., 2014). Another key factor, is the choice of solvent/ s used in the extraction process, as different solvents have diverse extraction capabilities and solubility of phytoconstituents (Dai et al., 2010).

Various polar and non-polar solvents, and possibly combinations of the same are widely used for the extraction of phenols, some of which include water, methanol, ethanol, acetone, and ethyl acetate. The choice of solvent will dictate the quantity and rate of polyphenols extracted (Xu & Chang, 2007). Methanol is known to extract lower molecular weight polyphenols, while aqueous acetone has shown favourability for higher molecular weight flavanols (Benoît Labarbe et al., 1999). Ethanol is an ideal solvent for polyphenol extraction, and has an added advantage in that it is non-toxic and may be consumed. An acidified organic solvent like methanol and ethanol are commonly used in the preparation of anthocyanin-rich phenolic extracts. Its success is due to its ability to denature the cell membranes,

while also dissolving the anthocyanins, while stabilizing them. In addition, sulphured water may be used as an extraction solvent in order to minimise the use of organic solvents and limit costs.

2.1.8.3 Anti-cancer and anti-proliferative effects

M. oleifera leaf extract, has induced the apoptosis of human hepatocellular carcinoma cells (Jung et al, 2015). Ethanol extract of root bark is successful at inducing apoptosis on human myeloid leukaemia cells (Roy et al., 2014). Crude aqueous leaf extract of *M. oleifera* has shown anti-cancer effects on human oesophageal cancer cells (Tiloke et al., 2016), as well as on cancerous human alveolar epithelial cells (Tiloke et al., 2013). Leaves and fruits of *M. oleifera* have shown anti-cancer activity on mouse melanoma (Purwal et al., 2010). *M. oleifera* has successfully induced cell death through apoptosis in breast cancer cell lines and colorectal cancer cell lines (Al-Asmari et al., 2015). Leaf extracts have shown apoptotic activity against the HeLa cell line in a previous study (Nair et al., 2011), however, the apoptotic pathway was not established.

2.1.9 Plant extraction

2.1.9.1 General

Extraction is a necessary step performed by organic chemists and researchers, to aid in the separation of an organic compound from a mixture of compounds, usually from natural material or products of synthetic reactions. The medicinal properties of natural plant material are derived from the active compounds contained there-in. Extraction is one of the technique commonly used to separate the mixture of phytochemical compounds.

Extraction is carried out as a crucial first step in the investigation of medicinal plants for therapeutic value, since the crude material needs to be removed from the plant for further characterization. Prior to

extraction, plant material is washed, completely air-dried, or freeze dried and ground to a fine powder which increases the surface for solvent extraction. Procedures performed during extraction should allow for optimal preservation of active components of the plant material. Polar, non-polar or a combination of the types of solvents may be used in the extraction of plant material, but depends largely on the active compounds being extracted. A wide variety of solvents are available possessing varied properties such as their boiling points, polarity, as well as the compounds they extract. Once the solvent contains extracted plant compounds, it is known as the menstruum.

2.1.9.2 Properties of extraction solvents

The solvent should be non-toxic and dissolve only the desirable compound, with a minimum amount of inert material. The boiling point of a solvent is a vital characteristic and determines the rate of evaporation of the solvent. Solvents that are of low boiling point are able to spontaneously evaporate in minutes (small volume) at room temperature, while their counterparts that possess high boiling points, such as water, require heat, airflow, and/the use of vacuum to enable faster evaporation. Knowledge of the boiling point of the solution being evaporated is required, prior to setting the rotary evaporator for the evaporation of the solvent-compound mixture (Dai et al., 2010).

The polarity of the menstruum (solvent), as well as the choice of extraction method, are pivotal factors in the preservation and efficacy of biochemical activities.

The overall polarity of a molecule can be defined as a characteristic value, named the electric moment. The electric moment is the measure of the separation of positive and negative electrical charges within a molecule. As a general rule, polar substances are able to readily dissolve in polar substances owing to the thermodynamically favourable environment for the interaction of solvent-solute forces. The extent to which a substance is extracted, is dependent on the polarity of the solvent. Based on the electric moment of the solvent described earlier, solvents can be characterized into polar, semi-polar, and non-

polar. Typically, polar solvents can dissolve ionic and other polar compounds, whereas non-polar substances can dissolve non-polar substances (Azmir et al., 2013).

Interestingly, semi-polar solvents have the ability to confer polarity to some extent, in non-polar molecules, and thereby enhancing the miscibility of polar and non-polar solvents.

2.2 Core research problem and its significance

Cervical cancer is the second most common cancer amongst women in South Africa. High-risk Human papillomavirus (HPV) is the main causative agent implicated in the pathogenesis of 90% of cervical cancers (Wang et al., 2010). A natural remedy/drug that is effective against HPV infected cancer cells and elicits minimal/ no side-effects would be a significant breakthrough. Such a drug would reduce the ever increasing financial burden of developing countries in treating pre-malignant lesions, as well as the morbidity and mortality associated with cervical cancer.

2.3 Hypothesis

Moringa oleifera Lam. leaf extracts induce apoptosis mechanisms through the suppression of high-risk HPV E6 and E7 gene expression, the re-expression of dormant p53 and pRb tumour suppressor genes, and the subsequent inhibition of telomerase activation and E2F1-mediated apoptosis.

2.4 Aims of the study

- Assessment of cytotoxicity and apoptosis
    - — To establish whether cytotoxicity occurs in a dose-dependent manner
    - — To establish if *M. oleifera* leaf extracts initiate apoptosis cell death mechanisms through the mitochondrial pathway
    - — To establish the effects of *M. oleifera* leaf extracts on cell proliferation and colony formation
- *To determine the stages of the cell cycle at which cell cycle arrest possibly occurs, for each fraction
- *To determine whether *M. oleifera* leaf extracts inhibit telomerase activation thereby establishing E6 oncogene repression
- *To determine the effects of *M. oleifera* leaf extracts on E2F1 gene expression
- *To determine the effects of *M. oleifera* leaf extracts on Cyclin E gene expression
- *To determine the effects of *M. oleifera* leaf extracts on Cyclin B1 gene expression

**Represents novel aspects of the study*

2.5. References

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VII. Chapter 3

The research manuscript as titled below, was provisionally accepted for publication by Bentham Science Publishers (*The Natural Products Journal*, Submission no. BMS-NPJ-2020-218).

Research Article

Title: Cytotoxic and Anti-proliferative Effects of *Moringa oleifera Lam.* on HeLa Cells

Mrs. Krishnambal Govender[1] (B.Tech), Prof. Indres Moodley[2] (PhD), Prof. Raveen Parboosing[3] (PhD)

[1] Department of Biomedical Sciences, Faculty of Natural Sciences, Mangosuthu University, Durban, KZN, SA, [2] Department of Public Health, College of Health Science, University of Kwazulu-Natal, Durban, KZN, SA,

[3] Department of Virology, University of Kwazulu-Natal/National Health Laboratory Service, Durban, KZN, SA

Corresponding Author:

Govender Krishnambal, Mrs

Department of Biomedical Sciences

Mangosuthu University of Technology

511 Mangosuthu H/way

Umlazi, Durban, KZN

Tel : +27 82 892 8085

Email : govender@mut.ac.za

Running title: *Moringa oleifera* LAM downregulates key cell cycle proteins in HeLa cells

Abstract

Background:

The aim of the study was to determine the mechanism of *Moringa oleifera*-induced apoptosis in Hela cells. HeLa cells over-express cyclin E and cyclin B1, abrogate G0-G1 and G2-M cell cycle arrest, promoting tumorigenesis. Abnormal telomerase activation is common in all malignant tumours. Cyclin E, cyclin B1, E2F1 and telomerase expression, and caspase-3 and -7 activation was assessed, after 24-treatment with *M. oleifera* leaf fractions.

Material and methods:

Apoptosis through caspase-3 and caspase-7 activation was determined quantitatively by the FAM FLICA™ Caspase-3/7 assay. Cyclin E, cyclin B1 and E2F1 were quantified by flow cytometry.

Telomerase was evaluated by Telomeric repeat amplification protocol (TRAP reaction). The effects on colony formation was assessed by seeding treated cells in six-well plates for 7 days under culture conditions. The MTT assay was used to determine cell survival.

Results:

HeLa cells treated for 24 hours with *M. oleifera* leaf fractions showed dose-dependent cytotoxicity, activation of caspases-3 and -7, down-regulation of cyclin E, cyclin B1, E2F1 and inhibition of telomerase expression. Cell cycle analysis of the dead cell population showed G2-M cell-cycle arrest.

Conclusion:

M. oleifera leaf fractions triggered apoptosis through the mitochondrial pathway, and cell cycle arrest at G2-M phase in HeLa cells after 24-hour treatment, through down-regulation of cyclin E and cyclin B1 expression, and caspase-3 and -7 activation.

In addition, *M. oleifera* leaf extract induces senescence in HeLa cells through the down-regulation of telomerase. Colony formation and cell proliferation were inhibited in a dose-dependent manner corresponding with telomerase inhibition.

Key words: *Moringa oleifera*, cervical cancer, Human papillomavirus, apoptosis, cytotoxicity, E2F1, cyclin, telomerase

3.1 Introduction

M. oleifera, is commonly used for nutritional and medicinal purposes, and has been widely studied particularly for its anti-cancer potential. Extracts from this plant show anti-cancer effects against human hepatocellular carcinoma cell lines [1], oesophageal cancer cells [2], cancerous human alveolar epithelial cells [3], mouse melanoma [4], breast and colorectal cancer cell lines [5], human myeloid leukaemia cells [6], and HeLa human endocervical cell line [7].

This study focuses on specific effects on cell cycle check points, apoptotic mechanisms and antiproliferative mechanisms elicited by *M. oleifera* leaf extracts on the HeLa cervical cancer cell line. These represent novel aspects not previously described. Human papillomavirus (HPV), in particular the high-risk subtypes [16, 18], is implicated as the main aetiological agent in cervical carcinogenesis, a groundbreaking association made through the Nobel-prize winning work of Harold zer Hausen [8].

In this study, hexane and ethanol extracts from mature leaves of *M. oleifera* were assessed for effects on proliferation, colony formation, caspase activation, cyclin E, cyclin B1, E2F1, and telomerase expression in HeLa cell line. Cyclin E and cyclin B1 are involved in G1-S and G2-M cell cycle arrest respectively and are abnormally upregulated in most cancers including cervical cancer. The HPV oncoprotein E7 protein binds to pRB, resulting in upregulation of E2F1, a key transcription factor that drives in cell proliferation, apoptosis and senescence. E2F1 also shows increased expression in cancer cells. Telomerase is activated during carcinogenesis, inducing immortality in cancer cells through telomere extension.

3.2. Materials and Methods

3.2.1 Material

The HeLa human cervical carcinoma cell line was obtained from Cellonex™ (Gauteng, South Africa) Roswell Park Memorial Institute (RPMI) 1640 medium and foetal bovine serum (FBS), phosphate buffered saline (PBS), dimethyl sulfoxide (DMSO) and -(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) (MTT) was purchased from Sigma Aldrich, S.A. Hexane and ethanol were obtained from Merck, S.A. Vybrant™ FAM Caspase-3 and -7 kit (kit #V35118) was purchased from ThermoFisher Scientific, S.A. Propidium iodide (PI), anti-mouse primary antibody, secondary monoclonal mouse anti-cyclin B1 and mouse anti-cyclin E were purchased from BD Bioscience, S.A. Secondary anti-E2F1 secondary ant-mouse monoclonal antibody was purchased from ThermoFisher Scientific, S.A. The TeloTAGGG Telomerase PCR ELISA kit (kit #11854666910) was purchased from Abcam, S.A.

3.2.2 Collection of Plant Material

M. oleifera leaves were harvested in spring, from the province of Limpopo, South Africa (S.A). The plant species was confirmed at the Department of Nature Conservation, Mangosuthu University of Technology, S.A. *M. oleifera* is grown as a sustainable crop in S.A and is not listed as an endangered botanical species locally or globally.

Mature leaves were selected, washed and dried indoors, away from direct sunlight, for a period of 2 weeks before being milled to a fine powder.

3.2.3 Plant crude extraction

Hexane and ethanol extractions were performed in a Soxhlet apparatus [9]. A quantity of 200 g plant material was packed into filter paper, and extracted into 300 mL of solvent. The remaining solvent present following the extraction process was removed by rotary evaporation under vacuum. The crude

was weighed, frozen and stock solutions prepared. *M. oleifera* extracts were dissolved in DMSO and vortexed until dissolved.

3.2.4 Treatment with extract

Cells were treated with 50 µg/mL *M. oleifera* extracts and pre-mixed RPMI containing 10% FBS. The final DMSO concentration in the treatment solution was maintained below 0.1% of the total volume of the working solution.

3.2.5 Cell Culture

HeLa cells were grown (antibiotic-free) in RPMI 1640 medium supplemented with 10% FBS, in line with supplier recommendations. Cells were incubated at 37°C with 5% CO_2. A passage number under 20 was maintained and assays were carried out during the linear growth phase. Sub-confluent cells were trypsinized when viability was at least 85%.

3.2.6 Live and Dead cell count by Trypan Blue Dye Exclusion

The trypan blue exclusion assay was performed to assess cytotoxicity. After treatment with the various *M. oleifera* extracts for 24 h, the cells were stained with 0.4% trypan blue and the live and dead cells in 4 squares of the grid were counted using the hemocytometer. The viable cells appeared transparent, while the non-viable cells which consistently absorbs the trypan blue dye, appeared dark blue in colour.

3.2.7 Cell viability assay [10]

Cell viability was determined using the MTT assay. HeLa cells (10 x 10^3 cells/ well) were seeded into 96-well plates in a volume of 200 µL culture medium and treated with the indicated concentrations (0, 10, 25, 50, 100, 150 and 200 µg /mL) of *M. oleifera* for 24 hours at 37°C. A volume of 20 µL MTT (5

mg/ mL) solution was added to each well following incubation for 4 hours at 37°C. Once the medium was removed, 150 µL of DMSO was added to the formazan crystals and allowed to dissolve on a shaking table at 150 rpm for 5 minutes. The optical density was read at 560 nm using an enzyme-linked immunosorbent assay reader spectrophotometer (Tecan Sunrise) and background absorbance was subtracted at 670 nm. Cell viability was then expressed by the percent (%) of viable cells relative to the untreated cells (100%).

3.2.8 Assessment of colony formation [11]

Sub-confluent cells (> 85% viability) were trypsinized, and a total of 10 cells were plated per 6-well plate containing complete media. The number of cells plated were optimized to compensate for the mitotic rate of HeLa cells (especially those that have entered the M-phase of mitosis) and to prevent merging of colonies which may complicate counting. The plates were incubated for 6 hours and examined for attachment under a stereomicroscope. 50 µg/mL of each extract was added to the attached cells in each well. Untreated controls diluted in complete media were included. The media was carefully removed after 7 days and the cells fixed with 70% ethanol. Staining with 0.5% crystal violet solution followed. The 6-well plates were inverted onto absorbent paper and allowed to dry for a few days. Colonies containing 50 or more cells were counted using the Evos Cell Imaging System (ThermoFisher, South Africa).

3.2.9 Vybrant™ FAM Caspase-3 and -7 staining of cells grown on glass coverslips for qualitative caspase-3 and caspase-7 analysis [12]

A count of 1 x 10^6 cells was grown in complete medium overnight in sterile glass petri dishes containing glass coverslips. 50 µg/mL of each extract was added to each petri-dish, carefully mixed by tilting and reverse pipetting, followed by incubated (37^0C, 5% CO_2) for 24 hours. Media was pipetted off and coverslips rinsed thrice with PBS. FLICA working solution was flooded onto the coverslips, and petri dishes were covered with foil following an hour of incubation. Coverslips were then rinsed twice in

PBS to remove any excess or unbound FLICA reagent. Untreated HeLa cells were included as control. Images were captured using the EVOS Cell Imaging System (X100 magnification).

3.2.10 Flow Cytometry – Analysis of Apoptosis induction (FLICA)

As per kit protocol, sub-confluent cells were trypsinized, harvested, and re-suspended to a concentration of 1×10^6 cells/ mL in culture media. 300 µL of cell suspension was then transferred to flow cytometry tubes. 10 µL of 30x FLICA working solution was directly added to the 300 µL cell suspension, mixed by flicking and incubated at 60 minutes at 37ºC and 5% CO_2, protected from light. The suspension was agitated twice during incubation to minimize cell settling. A volume of 2 mL of 1x wash buffer was added to each tube after incubation. The cells were pelleted by centrifugation and the supernatant discarded and re-suspended in 1 mL buffer, and pelleted. Cells were re-suspended in 400 µL of 1x wash buffer. Cells were further stained with 20 mL propidium iodide for 20 minutes and analysed on the Facscanto (BD Bioscience) with 488 nm excitation and green emission.

3.2.11 Flow cytometry – Cyclin E [13]

Cells were harvested, counted, and pelleted following standard procedures, then centrifuged and the supernatant removed. The pellet was re-suspended in 0.5 –1 mL 1x PBS. Cells were fixed with formaldehyde (at a final concentration of 4%) for 10 minutes at 37°C. Tubes were chilled on ice for 1 minute followed by permeabilization by gradually adding ice-cold 100% methanol to pre-chilled cells, with gentle vortexing. Once a final concentration of 90% methanol was achieved, tubes were incubated for 30 minutes on ice. 0.5–1 x 10^6 cells were aliquoted into each assay tube (by volume). Cells were washed twice, pelleted and re-suspended in 100 µL of optimally diluted primary cyclin E Alexa flour 488 mouse antibody and incubated for 1 hour in the dark at room temperature. This was followed by further washing, pelleting and re-suspension in 3 mL incubation buffer. A volume of 100 µL anti-mouse secondary antibody was added to the suspension and incubated in the dark for a 30 minutes, washed with PBS, and followed by re-suspension in 0.5 mL PBS. A volume of 0.5 mL of propidium iodide was added and

allowed to incubate for 30 minutes away from direct light, at room temperature. Cell suspensions were analysed on the Facscanto (BD Bioscience, S.A).

3.2.12 Flow Cytometry – Cyclin B1 [14]

Cells were harvested, counted, and pelleted following standard procedures followed by washing, and re-suspension of the pellet in 30 mL of wash buffer. The tubes were centrifuged at 200 x g for 10 minute and the supernatant aspirated. To fix cells, 10 mL cold 75% ethanol was added to the cell pellet, drop by drop, while vortexing. Fixed cells were incubated for at -20 ^{0}C for at least 2 hours before subsequent steps. Just prior to staining, the ethanol was remove by centrifugation at 200 x g for 10 min and washed with wash buffer. The cell suspension was pelleted and a volume of 5 ml cold 0.25% Triton X-100 in wash buffer was added to the cell pellet, vortexed and incubated for a further 5 minutes on ice. Cells were washed, pelleted and re-suspended in wash buffer. Thereafter, 20 µL of anti-cyclin B1 Alexa flour 488 mouse antibody at optimal working dilution was added to tubes and incubated in the dark for 30 minutes at room temperature. This was followed by washing of cells and addition of 20 µL of Alexa flour-conjugated goat anti-mouse antibody at optimal working dilution. Further incubation in the dark for a period of 30 minutes at room temperature was performed. Washing and pelleting of cells preceded re-suspension in 0.5 mL PI solution for 20 minutes, for parallel flow cytometric DNA analysis DNA content and cyclin B1.

3.2.13 Flow Cytometry – E2F1 [15]

To permeabilize cells, ice-cold 100% methanol was added slowly to the pre-chilled cells, with gentle vortexing, to a final concentration of 90% methanol. Cells were incubated for 30 minutes on ice. As part of the staining procedure, 1 x 10^2 cells were aliquoted into each assay tube (by volume) and 3 mL incubation buffer was added to each tube and washed twice by centrifugation. Cells were re-suspended in 100 µL of diluted primary anti-mouse antibody (appropriately diluted in incubation buffer) followed by incubation for 1 hour at room temperature. Cells were washed by centrifugation in 2 mL incubation

buffer and the deposit re-suspended in 0.5 mL PBS. This was followed by further treatment with diluted anti-mouse E2F1 secondary antibody, half an hour of incubation, washing and re-suspension in PBS. For PI staining, the cells were re-suspended in 0.5 mL of PI and incubated for a period of 30 minutes at room temperature, followed by acquisition in DNA staining solution on a Facs Canto (BD Biosciences, South Africa) flow cytometer.

3.2.14 TeloTAGGG Telomerase PCR ELISA kit protocol

This assay was performed as per kit protocol. Lyophilized cell extract was reconstituted with 20 µL autoclaved double distilled water and mixed thoroughly. The concentration was about 1×10^3 cell equivalents per microliter. The solution was dispensed, on ice, into suitable aliquots (2 µL per reaction). To prepare cell extracts, the cells were harvested and counted, and 2×10^5 cells were transferred per single reaction into a fresh Eppendorf tube. The cells were pelleted at 3000 × g for 10 minutes in a refrigerated centrifuge at 2 - 8°C. The supernatant was carefully removed, the cells re-suspended in phosphate buffered saline (PBS), and the centrifugation steps repeated. The supernatant was carefully removed, and the re-suspended cells were pelleted in 200 µL lysis reagent (solution 1), pre-cooled on ice by reverse pipetting at least 3 times and then incubated on ice for 30 minutes. The lysate was centrifuged at 16 000 × g for 20 minutes at 2 - 8°C. The supernatant was then carefully removed and transferred to a fresh tube.

3.2.15 Telomeric repeat amplification kit protocol (TRAP reaction)

For each sample tested and the controls, 25 µL Reaction mixture (solution 2) was transferred into a tube suitable for PCR amplification. Samples consisted of 3 µL cell extract per tube (3×10^3 cells per tube) and negative control, 3 µL (3×10^3 cells per tube) of the corresponding RNase treated or heat-treated cell controls extract. For a positive control 3ul of the reconstituted solution as per kit contents (corresponding to 1×10^3 control cell equivalents) was used. Sterile water was added to obtain a final volume of 50 µL. The tubes were transferred to tubes and then to a thermal cycler where a combined primer

elongation/ amplification reaction was performed as per kit protocol, followed by hybridization protocol. Absorbance values are reported as the A450 nm reading less the absorbance of the reference wave length of A690 nm.

Calculation of results

The difference in absorbance readings of the negative controls and that of samples were calculated. Sample readings of greater than 0.2 (A450 nm – A690 nm units) were regarded as positive.

Gating strategy and cell cycle analysis:

Cell debris was excluded by gating of forward scatter and side scatter, exclusion of clumps and doublets by forward scatter width (FSC-W) and height (FSC-H). Cell cycle analysis was conducted through addition of regions at G1, S, and G2-M on PI histograms (DNA content).

3.3 Statistical Analysis

Data was presented as the mean ± standard deviation (SD). Analysis was performed in triplicate. One-factor analysis of variance (ANOVA) test was used to determine whether there are any statistically significant differences between the means of independent groups and controls. The PAST version 3.25 statistical software (www.softpedia.com/get/Science-CAD/PAST.shtml) was used for statistical analysis and ModFit LT v3.3 application software (Verity Software House Inc., Topsham, ME, USA) for flow cytometric data analysis. $p < 0.05$ was deemed as a statistically significant value. The CV and RDS values of less than < 5% and < 2% respectively, were deemed acceptable.

3.4 Results

3.4.1 Cell viability assay

Treatment with *M. oleifera* decreased the cell viability of cervical cells in a dose-dependent manner compared with the untreated cells (Fig. 9A). The Ec50 value for hexane fraction was 224.8 µg/L and ethanol fraction was 219.5 µg/L (Fig. 9B).

3.4.2 Assessment of colony formation

Cells treated with *M. oleifera* leaf fractions for 24 hours after attachment, showed significant reduction in colony forming ability in both hexane (Fig. 10B, Fig. 10C) and ethanol (Fig. 11B, Fig. 11C) fractions. Groups viewed under a stereomicroscope consisting of 50 or more cells were considered a positive colony. Reduction in colony formation is observed in a dose-dependent manner in both fractions.

3.4.3 Qualitative analysis of apoptosis through mitochondrial pathway

After 24-hour treatment, HeLa cells showed bright green cytoplasmic immunofluorescence in both hexane and ethanol fractions, clearly indicating caspase-3 and caspase-7 activation (Fig.12).

3.4.4 Analysis of apoptosis through caspase-3 and caspase-7 cleavage

After 24-hour treatment with *M. oleifera* hexane and ethanol fractions, a highly fluorescent FLICA population emerged (Fig. 13 B, C, E and F), indicating caspase-3 and caspase-7 cleavage. Further analysis of the gated regions showed an accumulation of cells in the G2 phase for both hexane and ethanol fractions.

3.4.5 Quantitative assessment of cyclin E

Cyclin E expression was reduced in cells treated with both hexane and ethanol fractions when analysed against the untreated control (Fig. 14). The suppression of cyclin E expression was more pronounced in the HeLa cells treated with the ethanol fraction than the hexane fraction (Fig. 14).

3.4.6 Quantitative assessment of cyclin B1

Both hexane and ethanol fractions showed a reduction in mean fluorescence as compared to the un-treated control (Fig. 15A). Analysis of the dead cell population (Fig. 15 E, F) showed accumulation of cells in the G2-M region (Fig. 15 G, H)

3.4.7 Quantitative and cell cycle analysis of E2F1 expression after 24-hour treatment with extracts

Data analysis and interpretation of the graph (Fig. 16), after 24-hour treatment of *M. oleifera* leaf, reveal that E2F1 expression was up-regulated with both hexane, and ethanol fractions of *M. oleifera*. Ethanol induced the largest percentage increase in E2F1 gene expression as compared to untreated control cells.

3.4.8 M. oleifera induces G0-G1 arrest by down-regulating E2F1 transcription factor

To determine the effect of *M. oleifera* on cell cycle arrest in HeLa cells, the cell cycle distribution was investigated using flow cytometry (Fig. 17 - histograms). Cell cycle analysis of E2F1 cells after 24-hour treatment with hexane (Fig. 18 B) and ethanol (Fig. 18 C) leaf extracts reveals a decrease in the cells (Fig. 18 B and C) expressing E2F1 in the G0-G1 phase as compared to the untreated control (Fig. 10 A), and an increase in the number of cells (Fig. 18 B and C) expressing E2F1 in the S-phase.

3.4.9 Quantitative assessment of telomerase (TRAP)

The percentage of telomerase inhibition was calculated against untreated HeLa cells. The results showed that both fractions of *M. oleifera* caused down-regulation of telomerase after 24-hour treatment (Fig. 19). Of the two fractions, hexane showed the largest percentage inhibition (28.52%) than ethanol (16.37%).

3.5 Discussion

This study showed that *Moringa oleifera* leaf extract elicits its antiproliferative effects through the repression of cyclin B1 expression. The outcome of the latter is evident of apoptosis and G2-M cell cycle arrest. *Moringa oleifera* leaf extract also elicits its antiproliferative effect by repressing cyclin E activity and curbing S-phase entry.

The finding of this study shows that after 24-hour treatment, *M. oleifera* leaf extracts down-regulate E2F1 expression in the hexane, and ethanol fractions, while increasing the accumulation of cells expressing E2F1 in the S-phase. This may imply the re-binding of unphosphorylated pRb to E2F1, forming a complex which restricts transcription of mitotic factors thereby inhibiting S-phase transition and enforcing G1-S cell cycle arrest. The anti-proliferative effects of *M. oleifera* can be attributed to the downregulation of E2F1.

Telomerase activity was significantly inhibited in HeLa cells after 24-hour treatment with extracts of 50 µg/mL of *M. oleifera* leaf extract. The largest percentage inhibition in telomerase activity was seen in the hexane fraction. The repression of telomerase activity can only manifest if E6 expression is first downregulated, since it is directly involved in stimulating hTERT and telomerase activity. Furthermore, E6 repression has a direct impact on p53 by causing p53 activation from its dormant state [16]. This scenario was previously proven where once dormant p53 was reactivated in cervical cancer cells in the presence of E6 specific siRNA. Moreover, the activation of p53, further down-regulates hTERT and telomerase expression through mediation of p21waf1 gene expression [17].

One possible antiproliferative mechanism of *M. oleifera* leaf extract is through repression of the E7 oncoprotein, prompting the release of pRb, and the binding of E2F1 to pRb pocket proteins. E2F1 transcriptional activity is regulated by pRb/E2F1 complex triggering G1/S phase cell cycle arrest. *M. oleifera's* pro-apoptotic ability involving E2F1 mediation, could indeed be initiated by the repression of E6 oncoprotein. This would in turn inactivate E6AP and prevent further ubiquitination and degradation of p53. Over-expression of E2F1 causes p53 phosphorylation and accumulation of p53, triggering apoptosis. This was confirmed through effector caspase-3 and -7 activation. The anti-proliferative effects of *M. oleifera* can be attributed to the down-regulation of E2F1, which inhibits the transcription of key mitotic factors involved in cell cycle progression from G1 to S-phase.

However, for unphosphorylated pRb to bind to E2F1 implies it's release from the E7 oncoprotein, which would in turn only be possible if *M. oleifera* leaf extract is successful in repressing the E7 oncoprotein present in the high-risk HPV-18 infected Hela cells.

Over-expression of cyclin B1 could play a crucial role in chromosomal instability in cancer cells through spindle checkpoint interference, thereby promoting tumorigenesis [18]. Suppression of the kinase activity of mitogen promoting factor (MPF) could possibly be a worthy target for proliferative inhibition of tumour cells. Therapeutic drugs that down-regulate cyclin B1, resulting in the inhibition of proliferation, are also able to target and suppress Cdc2 activity [19].

There is convincing evidence that cyclin B1 depletion inhibits cellular proliferation in cervical cancer, breast cancer, osteosarcoma, and prostate cancer cell lines [20], [21]. In one study, treatment of HeLa cells with small interfering ribonucleic acid (siRNA) resulted in arrest in G2-M phase. Of particular interest, was that Cdc2/ cyclin B1 activity was almost totally nullified [19].

Like cyclin B1, other key regulators of cell cycle checkpoint, specifically that of G1-S, are Cyclin E and p21. Cyclin E, a positive phosphorylates Rb, which is necessary for progression from G1 into the S-phase of the cell cycle. A gate-keeper role of p21 is to negatively regulate Cdk2 activity. Cyclin E levels vary during the cell cycle, and peak at G1-S transition. Transcriptional regulation or cyclin E is an important function of the E2F1 transcription factor. Dysregulation of cyclin E promotes premature entry into S-phase, genomic instability, and tumorigenesis [22]. The regulation of cyclin E protein levels

has recently been an area of focus of cancer researchers. Specifically targeting cyclin E repression, inhibits S-phase entry and arrests the cell at G1-S phase.

The high-risk HPV E7 oncoprotein is known to bind to unphosphorylated pRb of the pRb-E2F1 complex in the G1 phase of the cell cycle, causing dissociation of the complex, and inactivation of pRb. The resultant effects of the disruption of the complex is dysregulation of E2F1 transcription, and the progression of the cell cycle to S-phase, promoting viral genome replication [23].

In addition to E2F1's role in controlling cell cycle progression by transcribing key cell proliferation proteins, E2F1 is also known to induce apoptosis through p53-dependent and p53-independent mechanisms. p53–dependent cell cycle arrest takes place at G1-S checkpoint in response to DNA damage and a variety of stresses, however, cell cycle arrest at G2-M can be induced by p53 in response to irreparable DNA damage.

There is compelling evidence to suggest the over-expression of E2F1 as a major proponent of tumour development. E2F expression is regulated by p53 by the elimination of those cells deemed to be expressing E2F1 abnormally [24]. HeLa cells, being a cervical adenocarcinoma cell line, over-expresses E2F1 while p53 is silenced. Moreover, transgenic p53 null mice developed skin tumours while simultaneously induced by E2F1 over-expression, suggesting that p53 is required to modulate E2F1 transcriptional activities, while another study showed that overexpression of E2F1 in p53 competent cells induced mass apoptosis [25]. This is most likely the case in HeLa cells that are known to over-express E2F1, while p53 is depleted as a result of ubiquitination and degradation induced by E6AP activation by the encoding of the E6 oncogene. Repression of HPV E6 and E7 oncogenes results in the systematic activation of dormant p53 and Rb tumour suppressor pathways, inducing cell cycle arrest and triggering apoptosis [26]. Up-regulation of E2F1 promotes apoptosis in in-vitro models. One study reported that when E2F1 expression was induced in fibroblasts, S-phase entry progressed, leading to death through apoptosis in an E2F1 dose-dependent manner [27].

Telomerase activation is demonstrated in the HeLa cervical cancer cell line. The high-risk E6 HPV oncoprotein activates hTERT and induces immortality in senescent cervical cells by adding hexamer repeats at the 3' end of chromosomes, thereby abrogating DNA death receptor activation [21].

The activation of telomerase is strictly modulated by the tumour suppressor p53 [22]. The E6 oncoprotein degrades p53 by ubiquitination, through proteasomal degradation, and inhibition of phosphorylation. This in turn allows telomerase activity to continue permitting immortalization [23]. There is intriguing evidence indicating that E6 suppression (by specific siRNA) activates dormant p53, and inhibits hTERT. This finding proposes E6 suppression as a novel target for drug development against high-risk HPV, as well as cervical cancer.

Telomerase may be activated by the direct activation of hTERT by E6, or through the suppression of p53 which allows for activation of the promotor. p53 also interacts with p21WAF1 downstream, preventing G1 cell cycle transit by abrogating certain Cdk activities, and promoting cell cycle arrest at the G1-S checkpoint.

3.6 Conclusion

M. oleifera leaf extract is well known for its putative anti-cancer properties. In this paper, we describe multiple mechanisms by which this extract exerts its anti-proliferative effects in the HeLa cervical cancer cell line. The hexane and ethanol leaf fractions of *M. oleifera* have dose-dependent anti-proliferative effects, triggers apoptosis through caspase-3 and -7 cleavage, and down-regulates cyclin E, cyclin B1 and E2F1 gene expression.

Furthermore, the study results suggest that *M. oleifera* leaf extract induces senescence in HeLa cells through E6 suppression, the possible activation of dormant p53 tumour suppressor gene and the down-regulation of telomerase activity.

Similar to its other natural counterparts like curcumin, *M. oleifera* has potential as a potent multi-targeting anti-cancer therapeutic agent.

Further to the current study, it would be beneficial to assess the effects of treatment of HeLa cells with *M. oleifera* leaf fractions for possible reactivation of dormant p53 and pRb tumour suppressor proteins.

The quantitative analysis of E6AP is also worth exploring in order to assess potential E6 oncoprotein suppression.

3.7 List of abbreviations

Cdk	cyclin-dependent kinase
CIN	cervical intraepithelial lesion
CIS	carcinoma-insitu
CV	coefficient of variation
DMSO	dimethyl-sulphoxide
DNA	deoxyribonucleic acid
E6AP	E6-associated protein
FCS	foetal calf serum
G1	gap phase
HPV	Human papillomavirus
hTERT	human telomerase reverse transcriptase
HSIL	high grade squamous intraepithelial lesion
ICAD	inhibitors of caspase-activated DNase
M phase	mitotic phase
MTT	3-(4,5-dimethythiazol- 2-yl)-2,5-diphenyl tetrazolium bromide
PARP	poly ADP (Adenosine Diphosphate)-Ribose Polymerase
PCR	polymerase chain reaction

PE	plating efficiency
pRb	retinoblastoma protein
RB1	retinoblastoma protein
RDS	relative standard deviation
RNA	ribonucleic acid
RT	room temperature
RT PCR	reverse transcriptase polymerase chain reaction
SIL	squamous intraepithelial lesion
SF	surviving fraction
TP53	tumour protein 53

3.8 Ethics

Ethics approval was obtained from University of Kwazulu-Natal Biomedical Research Committee (BREC/00001021/2020).

3.9 Consent for publication

Not applicable

3.10 Availability of Data and Materials

All data sets used/ analysed during the current study are available from the corresponding author on reasonable request.

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RP provided laboratory space, equipment, consumables, technical support, assistance with data analysis and editing of manuscript. IM contributed towards conceptualization, research design and editing of the manuscript.

All authors have read and approved the final manuscript.

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3.13 Figures and Legends

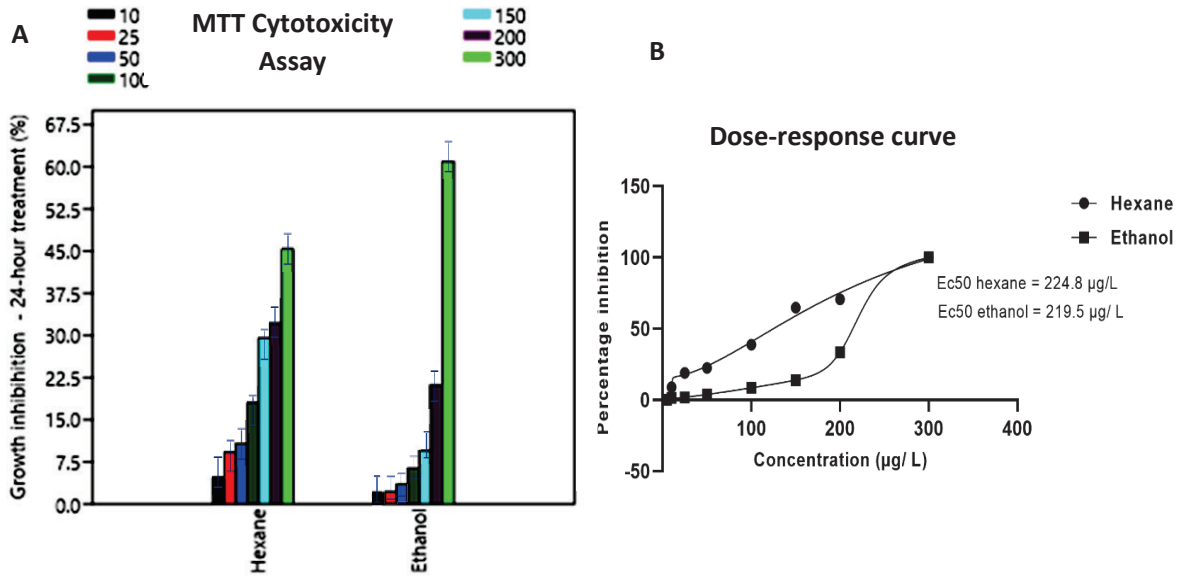

Figure 9: *MTT assay (Fig. 9A) was performed in triplicate on sub-confluent cells of at least 85% viability after 24-hour treatment with ethanol and hexane fractions of M. oleifera leaf fractions of concentrations ranging from 10 µg/mL to 300 µg/mL. The percentage inhibition was calculated as the average absorbance of untreated controls, after the average background absorbance was subtracted ($p < 0.02$). The optical density was read at 560 nm using an enzyme-linked immunosorbent multi-well plate reader. Fig. 9B shows the Dose-response curves and Ec50 values for both fractions.*

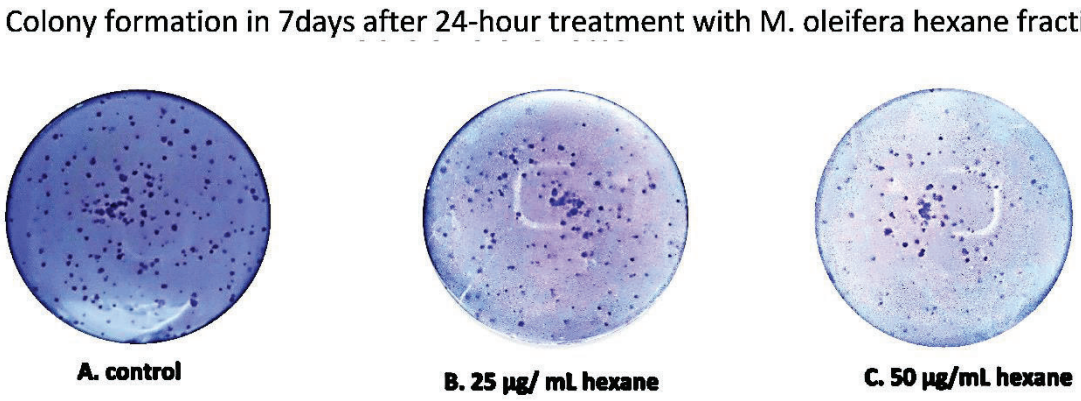

Figure 10: *24-hour treatment of HeLa cells with 25 µg/mL and 50 µg/mL of hexane fractions of M. oleifera leaf extracts was evaluated for possible effects on colony formation. Seeded cells were allowed to attach for 6 hours to 6-well plates before treatment with extracts diluted in complete media. The media was carefully removed and replaced with fresh complete media after 24 hours and allowed to incubate for 7 days. Plates were fixed and stained with crystal violet as per protocol. Treated HeLa cells showed dose-dependent inhibition of colony formation (B and C). A cluster containing 50 or more cells constituted a single colony.*

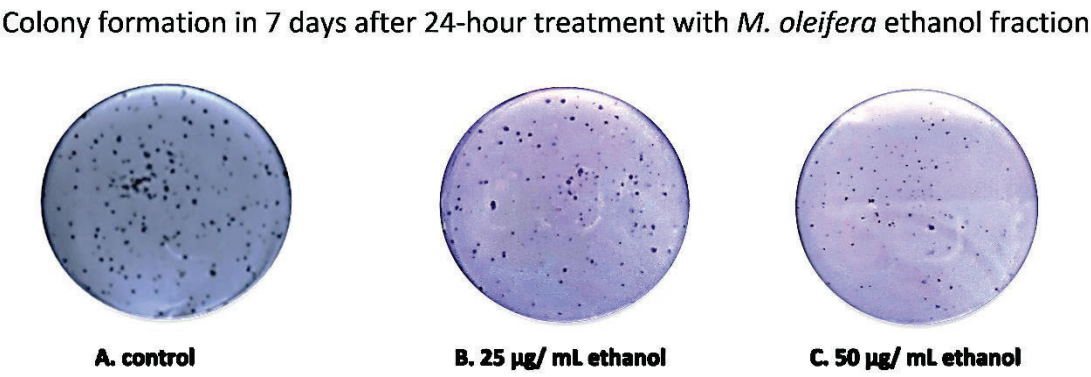

Figure 11: *HeLa cells were treated with 25 µg/mL (B) and 50 µg/mL (C) of M. oleifera leaf fraction as per protocol described in Fig. 10. Colony inhibition was observed in a dose-dependent manner.*

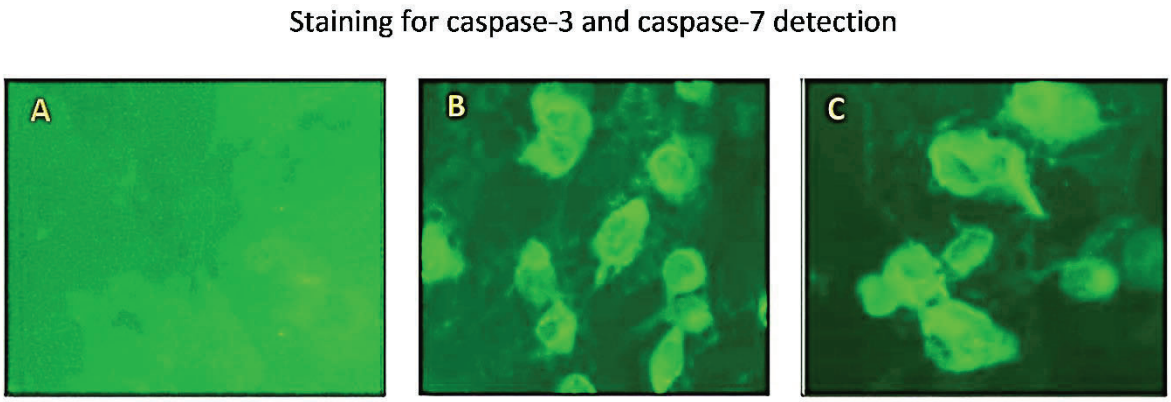

Figure 12: *Untreated control and HeLa cells treated for 24 hours with 50 µg/mL hexane and ethanol leaf extracts of M. oleifera, stained with FLICA FAM-VAD-FMK, and examined by fluorescent microscopy (x100) for caspase-3 and caspase-7 activation and morphologic assessment of apoptotic changes. Untreated control cells remain unstained indicating caspase negativity (A), HeLa cells treated with hexane extract show FLICA FAM-VAD-FMK retention, and caspase activation. Cells morphologically appear to be undergoing early apoptotic alterations affecting the cytoplasm (B). HeLa cells treated with methanol extract for 24 hours shows FLICA FAM-VAD-FMK uptake, caspase-3 and -7 cleavage and moderate apoptotic alterations.*

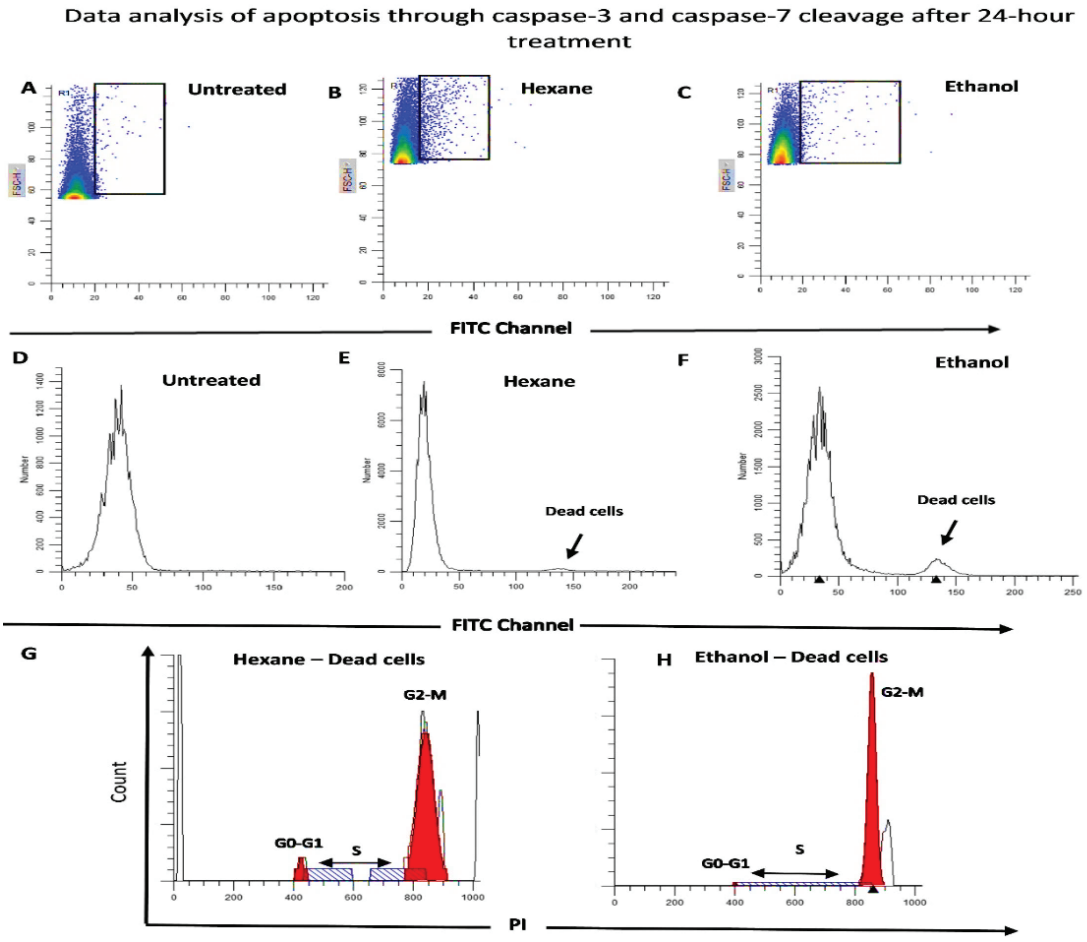

Figure 13: *HeLa cells were treated for 24 hours with hexane and ethanol crude fractions and quantitatively assessed for apoptosis by staining with FLICA reagent and flow cytometric (Facscanto® – BD Bioscience) acquisition and analysis. Cells were simultaneously stained with Hoescht for assessment of DNA content. A clearly defined, highly fluorescent dead cell population emerged in both fractions (B, C, E and F). The DNA cell cycle analysis of the apoptotic population shows G2-M accumulation and cell cycle arrest (CV = < 4%, RCS < 2.5)*

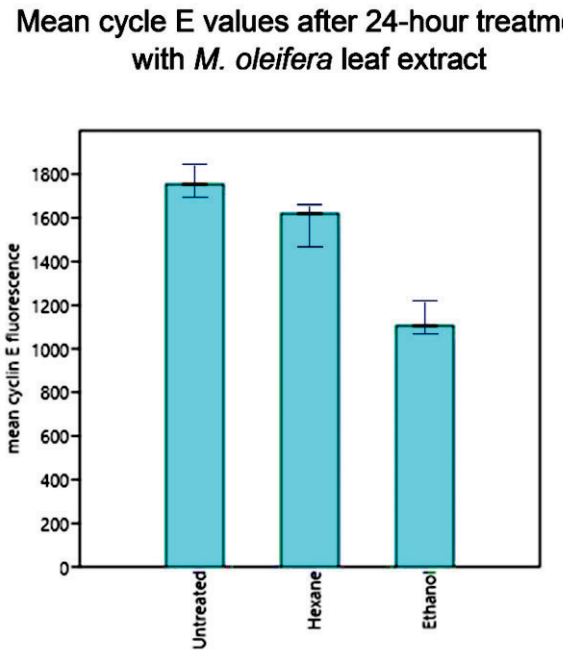

Figure 14: *HeLa cells were treated with 50 µg/mL hexane and ethanol crude fractions for 24 hours, and assessed for cyclin E expression by flow cytometry (Facscanto® – BD Bioscience, S.A). The bar graph represents the mean fluorescence for treated and untreated control cells. Both hexane and ethanol show a down regulation in cyclin E expression after 24-hour treatment.*

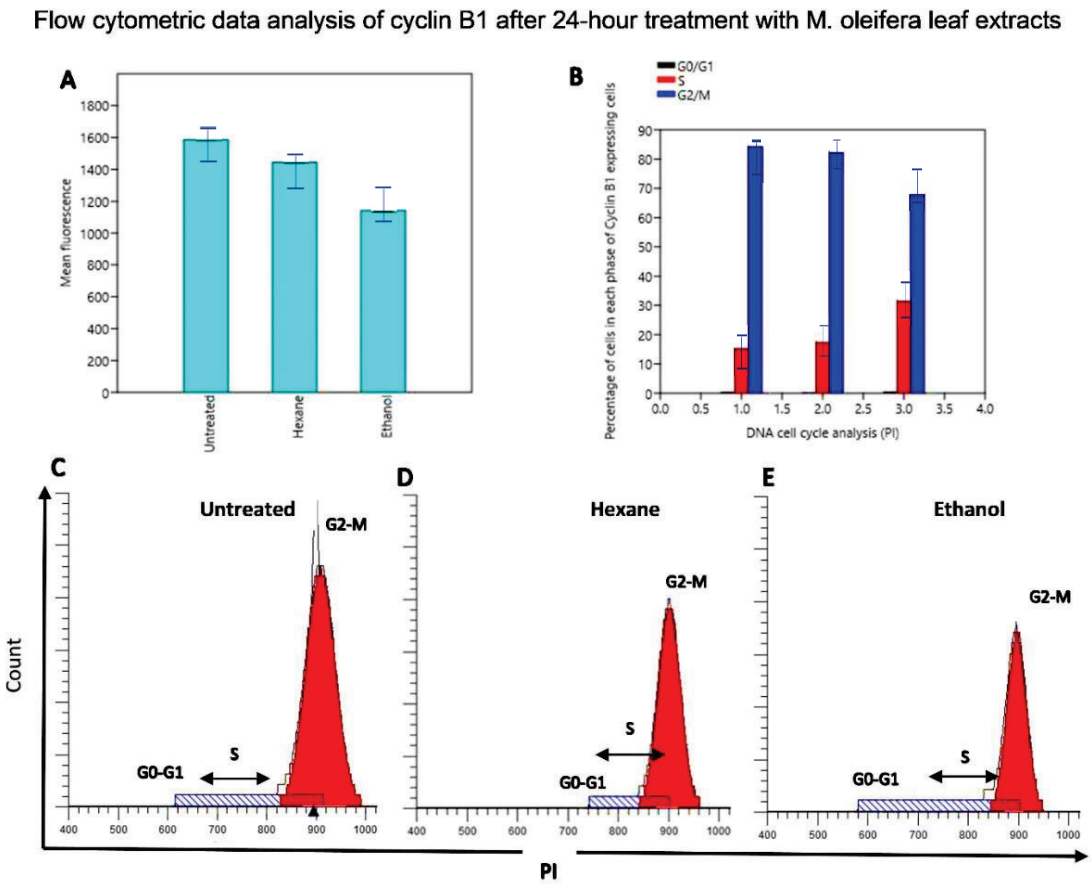

Figure 15: *HeLa cells were treated with 50 µg/mL hexane and ethanol crude fractions for a 24-hour period and assessed for cyclin B1 expression by flow cytometry (Facscanto® – BD Bioscience, S.A). The bar graph represents the mean fluorescence for treated and untreated cells. Both hexane and ethanol fractions show a down-regulation of cyclin B1 after 24-hour treatment (A). DNA cell cycle analysis of cyclin B1-expressing untreated HeLa cells confirms the over-expression of cyclin B1 at G2-M phase (B). Treated cells expressing cyclin B1 shows the down-regulation of cyclin B1 at G2-M phase (B, D and E).*

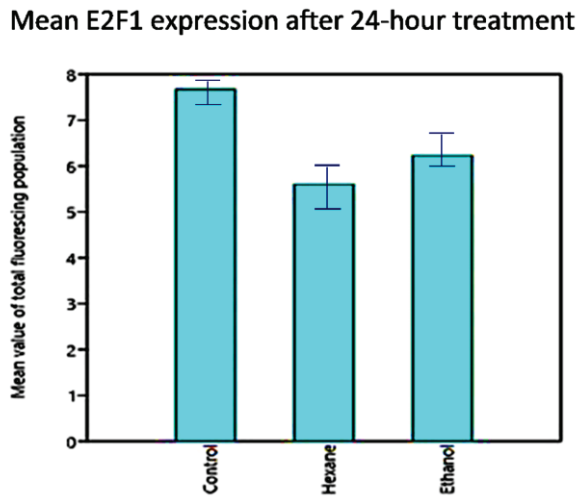

Figure 16: *Scattergrams in the Alexa flour 488 channel show E2F1 population, and histograms (cv < 0.71) show cell cycle analysis of E2F1 expressing population before (control) and after 24-hour treatment with 50 µg/mL M. oleifera leaf extracts.*

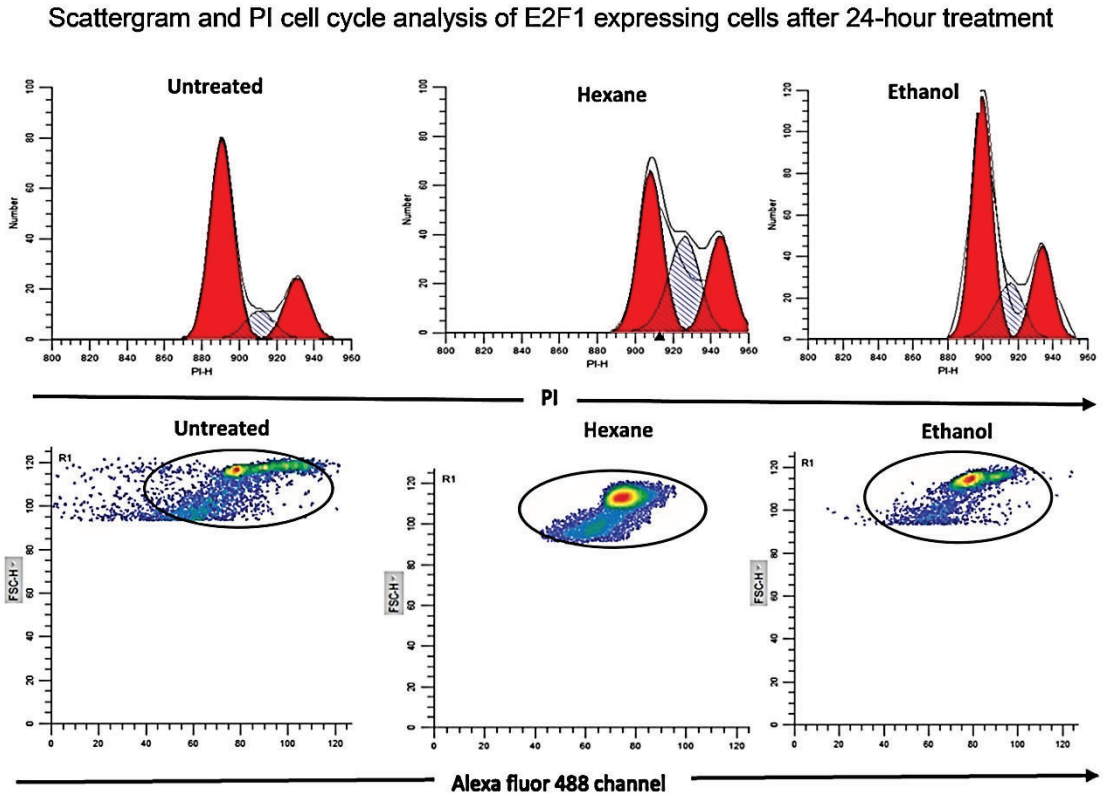

Figure 17: *Cell cycle analysis (propidium iodide) of E2F1 cells after 24-hour treatment with hexane (B) and ethanol (C) leaf extracts reveals a decrease in the cells (B and C) expressing E2F1 in the G0-G1 phase as compared to the untreated control (A), and an increase in the number of cells (B and C) expressing E2F1 in the S-phase ($p < 0.05$).*

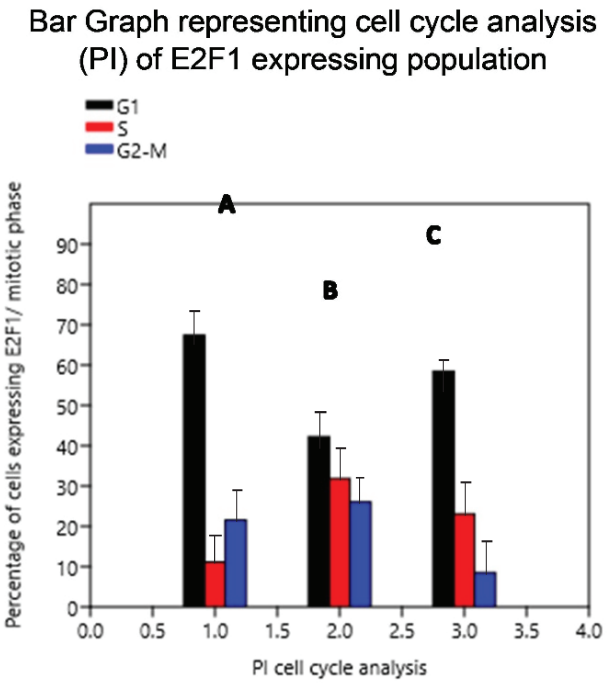

Figure 18: *Cell cycle analysis (propidium iodide) of E2F1 cells after 24-hour treatment with hexane (B) and ethanol (C) leaf extracts reveals a decrease in the cells (B and C) expressing E2F1 in the G0-G1 phase as compared to the untreated control (A), and an increase in the number of cells (B and C) expressing E2F1 in the S-phase ($p < 0.05$).*

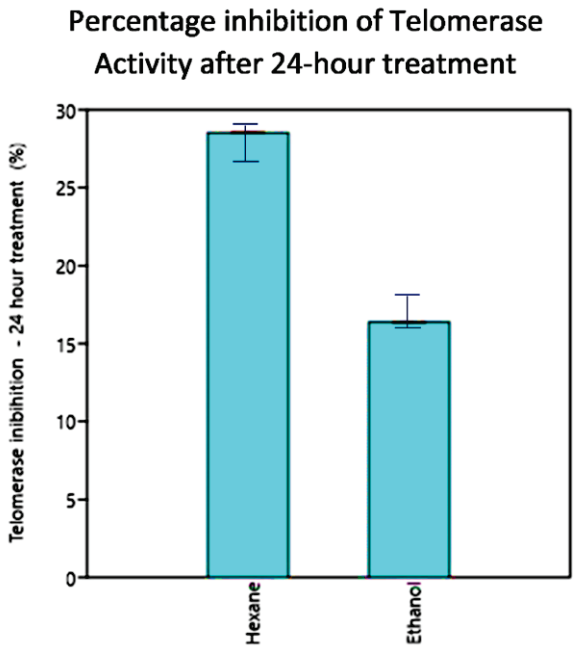

Figure 19: *HeLa cells treated with hexane and ethanol leaf extracts of M. oleifera for 24 hours and amplified using the Telomeric repeat amplification protocol (TRAP reaction). The absorbance readings of the negative controls were subtracted from those of the samples. Samples were regarded as telomerase-positive if the difference in absorbance is higher than 0.2 (A450 nm – A690 nm) units. The graph showed down-regulation of telomerase activity in both the hexane and ethanol fractions.*

VIII. Chapter 4

4.1 Conclusion

The anti-cancer properties of *M. oleifera* leaf extract are well described (Asima Shaban et al., 2012), while its antiproliferative mechanisms, particularly in the HeLa cervical cancer cell line, are less well understood. This study showed that hexane and ethanol leaf fractions of *M. oleifera* have dose-dependent anti-proliferative effects in the HeLa cervical cancer cell line, possibly by triggering apoptosis through caspase-3 and -7 cleavage and down-regulating cyclin E, cyclin B1 and E2F1 gene expression.

M. oleifera Lam. leaf extracts induce caspase-mediated apoptosis mechanisms through the suppression of high-risk HPV E6 and E7 gene expression, the re-expression of dormant p53 and pRb tumour suppressor genes, and the subsequent inhibition of telomerase activation and E2F1-mediated apoptosis.

Further studies are necessary to confirm the multi-targeting anti-cancer therapeutic potential of *M. oleifera,* as well as its ability to suppress the proliferation of pre-cancerous high-risk HPV lesions.

4.2 Recommendations

In light of the ability of *M. oleifera* to inhibit telomerase in HeLa cells, it would be valuable to characterize the active compounds inherent in this botanic, and perform similar assays individually for their effects on telomerase. The outcomes of such as study would positively identify the active compound/s responsible for the de-activation of telomerase and the induction of senescence in cervical cancer cells. This active compound would provide a major breakthrough in cervical cancer chemotherapeutics as well as in the majority of other cancer types that express high levels of telomerase activity. The charac-

terized anti-telomerase compound/s inherent in *M. oleifera* could possibly also be used as a potent ingredient in vaginal douches as anti-HPV prophylaxis and treatment, or as a telomerase inhibitor combined with conventional therapeutics, in an effort to reduce the global burden of cervical cancer worldwide.

IX. APPENDIX

Plagiarism Report - TURNITIN®

CYTOTOXIC AND ANTI-PROLIFERATIVE EFFECTS OF MORINGA OLEIFERA LAM. ON HELA CELLS

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