THE EFFECT OF *Commelina benghalensis* (COMMELINACEAE) ON MICRORNA EXPRESSION IN CERVICAL CANCER CELLS

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Abstract

Cervical cancer is a health burden in many countries worldwide and is the most prevalent cancer in women across the African continent. Even with the current treatment strategies such as radiotherapy, chemotherapy and surgery, cervical cancer still causes high rates of morbidity and mortality. Novel treatment strategies for this cancer are urgently needed. Medicinal plants are gaining a lot of interest as sources of bioactive compounds for the treatment of different diseases, including cancer. *Commelina benghalensis* extracts have been shown to have anti-cancer properties against Jurkat T cells and alveolar epithelial cells (AEC). However, there has been no report on the activity of this plant against cervical cancer cells. Additionally, microRNA (miRs) have shown to regulate carcinogenesis during the development of cervical cancer cells, but the effects of *C. benghalensis* extracts on miRs have not previously been investigated. The influence of *C. bengalensis* acetone extract on cell metabolism, apoptosis and miRNA expression in HeLa cervical cells were tested in this study. The stem and leaf extracts of *C. bengalensis* decreased the metabolic activity in HeLa cells as shown by MTT assay and caused cell apoptosis as demonstrated with Annexin V flow cytometry. Twelve miRs were identified that are involved in the apoptosis pathway and selected for further studies. HeLa cells were treated with 400 and 500 µg/ml *C. benghalensis* leaf and stem extracts for 24 hours. Six miRs were significantly upregulated by the different extracts and concentrations, namely miR-7, miR-20a, miR-34a, miR-182, miR-143 and miR-200a. Six of the miRs were not significantly affected by the *C. bengalensis* extracts, specifically miR-21, miR-27b, miR-29a miR-99a, miR-99b and miR-497. None of the miRs were down regulated by the *C. benghalensis* extracts. The 400 µg/ml stem and leaf extracts, as well as the 500 µg/ml leaf extract upregulated some miRs that are usually already upregulated in cervical cancer cells, thereby potentiating carcinogenesis. Following this observation, it can be concluded that these extracts may not be good candidates for the treatment of cervical cancer. The 500 µg/ml stem extract was the best candidate for potential cancer treatment as it significantly upregulated only miR-34a which is usually downregulated in cervical cancer. This concentration can be further investigated for the treatment of cervical cancer.

Abstrak

Wêreldwyd het sevikale kanker ‘n hoë siektelas en is die algemeenste kanker onder vrouens in Afrika. Selfs met die huidige behandeling vir servikale kanker met chemoterapie, radioterapie en chirugie het diè kanker steeds ‘n hoë mortaliteit en morbiditeit insidensie. Nuwe behandelingsmodaliteite word dringend vir servikale kanker benodig. Plant ekstrakte van *Commelina benghalensis* het in Jurkat T-selle en EAC selle teen-kanker einskappe getoon. MikroRNAs het ‘n effeck op die karsinogenese van servikale kanker, maar die effek van *C. benghalensis* op mikroRNAs in servikale kanker is nog nie getoets nie. In die navorsingsprojek word die effek van *C. benghalensis* asetoon plant ekstrakte op sel metabolisme, apoptose en die microRNA uitdrukking in HeLa selle ondersoek. Daar is gevind dat die stam en blaar ekstrakte van *C. benghalenis* die metabolisme in HeLa selle verlaag met die gebruik van MTT-toets, asook om apoptose te induseeer wat bewys is met Annexin V vloeisitometrie. Twaalf mikroRNAs wat die apoptose pad beinvloed is geidentifiseer vir verdere studie. HeLa selle is vir 24 uur met 400 µg/ml en 500µg/ml stam en blaar esktrakte behandel, waarna die mikroRNA uitdrukking bepaal is. Ses mikroRNA was opgereguleer, naamlik: miR-7, miR-20a, miR-34a, miR-182, miR-143 en miR-200a. Die ander ses mikroRNA, naamlik miR-21, miR-27b, miR-29 miR-99a, miR-99b, en miR-497 was nie betekenisvol geafekkteer deur die *C. benghalensis* blaar en stam ekstrakte nie. Geen van die mikroRNAs was afgereguleer deur die *C. benghalensis* ekstrakte nie. Die 400 µg/ml stam en blaar ekstrakte sowel as die 500µg/ml blaar ekstrakt het microRNAs opgereguleer, wat reeds in servikale kanker ook opgereguleer is en kan dus verder karsinogenese ondersteun. In die lig hiervan is diè konsentrasies nie goeie kandidate vir servikale kanker behandeling nie. Die beste kandidaat vir potensiële servikale kanker behandeling was die 500µg/ml stam ekstrak wat slegs miR-34 opgereguleer wat gewoonlik verlaag is in servikale kanker.

Abstract

Umdlavuza womlomo wesibeletho ungumthwalo wezempilo emazweni amaningi emhlabeni jikelele futhi uwumdlavuza odlange kakhulu kwabesifazane ezwenikazi lonke lase-Afrika. Ngisho namasu amanje okwelapha afana neradiotherapy, chemotherapy kanye nokuhlinzwa, umdlavuza womlomo wesibeletho usabangela izinga eliphezulu lokugula nokufa. Amasu amasha okwelapha lo mdlavuza adingeka ngokushesha. Izitshalo zokwelapha zithola isithakazelo esikhulu njengemithombo ye-bioactive compounds yokwelapha izifo

ezihlukahlukene, kuhlanganise nomdlavuza. Ukukhishwa kwe-*Commelina benghalensis* kukhonjiswe ukuthi kunezinto zokulwa nomdlavuza ngokumelene namaseli e-Jurkat T namaseli e-alveolar epithelial (AEC). Kodwa-ke, awukho umbiko mayelana nokusebenza kwalesi sitshalo ngokumelene namaseli omdlavuza womlomo wesibeletho. Ukwengeza, i-MicroRNA (miRs) ibonise ukulawula i-carcinogenesis ngesikhathi sokuthuthukiswa kwamangqamuzana omdlavuza womlomo wesibeletho, kodwa imiphumela ye-C. benghalensis extracts kuma-miRs ayizange iphenywe ngaphambilini. Umthelela we-C. bengalensis acetone extract on cell metabolism, apoptosis kanye nenkulumo ye-miRNA kumaseli omlomo wesibeletho we-HeLa ahlolwe kulolu cwaningo. Isiqu nesiqephu seqabunga se-C. bengalensis sehlise umsebenzi we-metabolic kumaseli e-HeLa njengoba kuboniswe ukuhlolwa kwe-MTT futhi kwabangela i-apoptosis yeseli njengoba kuboniswe nge-Annexin V Flow Cytometry. Ama-miR ayishumi nambili ahlonziwe abandakanyeka kumzila we-apoptosis futhi akhethelwa ukuqhubeka nezifundo. Amaseli e-HeLa aphathwe nge-400 kanye ne-500 µg/ml C. Iqabunga le-Benghalensis ne-stem extract amahora angu-24. Ama-miR ayisithupha aye alawulwa kakhulu izingcaphuno ezihlukene nokugxiliswa kuzo, okuyi-miR-7, miR-20a, miR-34a, miR-182, miR-143 kanye ne-miR-200a. Ama-miR ayisithupha awazange athintwe kakhulu izingcaphuno ze-C. bengalensis, ikakhulukazi i-miR-21, miR-27b, miR-29a miR-99a, miR-99b ne-miR-497. Awekho ama-miR aye phansi alawulwa yi-C. benghalensis extracts. I-400 µg/ml isiqu kanye nezikhishiwe zeqabunga, kanye ne-500 µg/ml yeqabunga elikhishwe kulawula amanye ama-miR ngokuvamile asevele elawulwa kumaseli omdlavuza womlomo wesibeletho, ngaleyo ndlela enze i-carcinogenesis. Ukulandela lokhu kubheka, kungaphethwa ngokuthi lezi zingcaphuno zingase zingabi abantu abafanelekile ekwelapheni umdlavuza womlomo wesibeletho. Ukukhishwa kwesiqu okungu-500 µg/ml bekuyikhandidethi elingcono kakhulu lokwelapha umdlavuza okungenzeka ukuthi lilawule kakhulu i-miR-34a kuphela evamise ukwehliswa kumdlavuza womlomo wesibeletho. Lokhu kugxilwa kungaphenywa kabanzi ekwelapheni umdlavuza womlomo

Keywords

Apoptosis; Cervical cancer; MicroRNAs; HeLa; HPV; *Commelina benghalensis*

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Abbreviations:

A

Akt: Protein kinase B (PKB)

ALCAM: Activated leukocyte cell adhesion molecule

ATG2: Autophagy-related protein 2

B

BANF1: Barrier to autointegration factor 1

BAK: Bcl-2-antagonist/killer 1

BAX: Bcl-2-assosiated X protein

BOK: Bcl-2 related ovarian killer

BCL-2: B-cell Lymphoma 2

BCL-B: B-cell Lymphoma Homolog to Boo protein

BCL-W: Bcl-2 like protein 2, also called BCL2L2

BCL-X B-cell Lymphoma extra large

B5P: Bisulfite genomic sequencing

BIM: BCL-2-like protein 11

BIRC5: Baculocviral IAP repeat containing 5

C

CCBRA: Bisulfite restriction analysis

CCL20: C-C chemokine ligand 20

CCNE1: Cyclin E 1

CCR5: C-C chemokine receptor type 5

CDC 25: Cell division cycle

CDH 11: Cadhedrin 11

CDK6: Cyclin dependant kinase 6

CDKN2A: Cyclin kinase inhibitor 2A

cDNA: Complementary deoxyribonucleic acid

CHL1: Close homolog of L1 (Nearal cell adhesion molecule L-1 like protein)

CIN: Cervical intraepithelial neoplasia

C-Met: tyrosine protein kinase mesenchymal epithelial transition factor

C-MYC: Myc proto-oncogene protein

C-MYB: Myb proto-oncogene protein

CpG: 5'-C-phosphate-G-3'

D

DCUN1D1: Defective in cullin neddylation 1 domain containing 1

DGCR8: DiGeorge syndrome critical region 8

DISC: Death signalling complex

DMEM: Dulbecco's modified eagle medium

DMSO: Dimethyl sulfoxide

DNA: Deoxyribonucleic acid

DNMT: DNA methyltransferases

E

ELK: ETS like-1 protein

EPG: Ectopic P-granules

EXOC5: Exocyst complex 5

F

FACS: Fluorescence- activated cell sorting

FBS: Fetal bovine solution

FBXW11: F-box and WD repeat domain

FFPE: Formalin-fixed, paraffin-embedded (tissue)

FIGO: International Federation of Gynaecology and Obstetrics

FOXO1: Forkhead box

FSTL1: Follistatin-related protein 1

FUS: Fused in sarcoma

G

G-Phase: Gap phase / growth phase

H

HIF1a: Hypoxia induced factor 1 alpha

HSP 47: Heat shock protein 47

HTERT: Human telomerase reverse transcriptase

HIV: Human immunodeficiency virus

HPV: Human papilloma virus

I

IGF: Insulin like growth factor 1

INK 4: Inhibition of cyclin dependant kinase 4

ISCN: International System for Human Cytogenic Nomenclature

J

JAGGED1: Protein jag 1 precursor

L

LATS1: Large tumour suppressor kinase 1

LAMB3: Laminin subunit beta 3

LDHA: Lactate dehydrogenase

LncRNA: Long non-coding RNA

M

MEG3: Maternally expressed gene 3

MCL1: Induced myeloid leukaemia cell differentiation

MiRs: MicroRNAs

mL: Millilitre (μl)

MMP2: Matrix metalloproteinase-2

MMP9: Matix metalloproteinase-9

M-Phase: Mitotic phase

MRna: Messenger ribonucleic acid

MS1-2: Mushasi 2

MSP: Methylation specific PCR

MTT: (3-(4, 5 – dimethlthiazol-2-yl-) – 2, 5- diphenyl tetrazolium bromide

mTOR: Mammalian target of rapamycin

N

NOTCH1: Neurogenic locus notch homolog protein 1 precursor

NOXA: PMAIP1: Phorbol-12-myristate-13-acetate-induced protein 1

O

OIPS-AS1: Opa interacting protein s antisense transcript 1

P

p: Petit (short chromosomal arm)

Pap smear: Papanicolaou smear

p53: Protein 53 (tumour protein 53)

PBS: Phosphate buffered saline

PCGEM 1: Prostate cancer gene expression marker 1

PCR: Polymerase chain reaction

PDCD4: Programmed cell death protein 4

PIK3CD: Phosphatidylinositol-4,5- bisphosphate 3-kinase catalytic subunit Delat

PLK2: Polo-like kinase 2

Pre-MiRNA: Precursor microRNA

Pri-MiRNA: Primary microRNA

PPARγ: Peroxixome proliferator activated receptor gamma

pRb: Retinoblastoma protein

PTEN: Phosphate and tensin homolog

PTTG1: Pituitary tumour-transforming gene 1

PUMA: Bcl-2-binding component 3 (BBC3)

Q

q: Queue (Long chromosomal arm)

R

RANGTP: Ras related nuclear protein guanosine triphosphate

RECK: Reversion-inducing-cysteine-rich protein

RNA: Ribonucleic acid

RT-PCR: Real time polymerase chain reaction

S

S-phase: Synthesis of DNA

STAT3: Signal transducer and activator of transcription 3

T

TGFB2: Transforming growth factor beta 2

THBS2: Thrombospodin 2

TIMP1: Tissue inhibitor of the metallprotenases 1

TMP: Tumour-associated membrane protein

TNKS2: Tankyrase-2

TOB1-AS 1: Human transducer of ERBB2.1 antisense RNA 1

TPM1: Tropomyosin alpha-1

TRF 1: Telomeric repeat factor 1

TRIB2: Tribbles homolog 2

V

VEGFA: Vascular endothelial growth factor A

vtRNA: Vault RNA

X

XIAP: X-linked inhibitor of apoptosis protein

XIST: X-inactive specific transcript

Y

YY1: Yin Yang 1

Z

ZEB1: Zinc finger E-box -binding homeobox 1

ZNF183: Zinc finger protein 183

All ten of my charges have failed the scalpel and fire drill. Their tumours continue to grow. The crabs continue to devour them." Edwin Smith Papyri, 3000BC (Breasted J.H., editor 1984).

"*It is crystal clear that microRNAs as either onco – or tumour suppressor genes can alter biological processes fundamentally to tumour initiation and progression. The connection between microRNA and oncogenesis are wide-spread enough to hold miRs as potential therapeutic targets and novel biomarkers. In this regard identifying the expression signature of miRs provides exciting opportunities for the diagnosis, prognosis and therapy of cancer*." (Babashah, 2014).

1 CHAPTER ONE: INTRODUCTION

2 BACKGROUND

The first description of cancer was found in an ancient Egyptian manuscript on surgery and cancer from 3000 BC, which was translated by Edwin Smith in 1930 (Breasted., editor, 1984). The word "cancer" is credited to Greek physician, Hippocrates (460-370 BC), meaning crab in Greek. It refers to the finger-like protrusion of a tumour spreading, resembling a crab. Even from the ancient times, cancer was a known disease, which was mostly treated by surgery (Adams, 1886). Current treatment strategies include chemotherapy, radiotherapy and surgery. All these strategies are effective to a certain degree and are linked to many side effects, such as nausea, vomiting, hair loss, loss of immunity, blood clots, tiredness and many more (American Society of Clinical Oncology). No foolproof treatment has yet been found that cures all cancer (Ferlay et al., 2020).

Cervical cancer is the most prevalent cancer in women in the sub-Sahara Africa region and the 4th most common cancer in the world among women. It is the leading cause of cancer deaths in Southern Africa (Sung et al., 2020). The high rate of this cancer has been exacerbated by high prevalence of the rampant human immune deficiency virus or acquired immune deficiency syndrome (HIV/AIDS) epidemic (Palefsky, 2017), which has also been shown to contribute to high Human papilloma virus (HPV) infection rates **(**Walboomers et al., 1999, Palefsky, 2017). Human papilloma virus infection is found in up to 90% of cervical cancer cases (Muñoz et al., 2006). Yearly, millions of women still die due to cervical cancer even though there are preventative and early screening/diagnostic measures such as HPV immunisation and Papanicolaou test, respectively (Sung et al., 2021). Affordable and effective treatment interventions are needed to give those affected a better morbidity and mortality outcome. Yi et al. (2003) stated that a successful anticancer drug should eliminate or incapacitate cancer cells without causing damage to normal cells. The current treatment strategies, such chemotherapy, are non-specific and target both cancer and noncancerous cells, thus, more research efforts are seeking novel specific and effective therapeutic drugs.

Studying microRNAs (miRs) has attracted a lot of attention in determining new cancer therapeutic targets. Understanding the role of miRs has expanded the understanding of the

oncogenesis process (Calin et al., 2002; Calin et al., 2004; He et al., 2005; Lu et al., 2005). MicroRNAs can be either oncomirs or tumour suppressors (Zhang et al., 2007; Banno et al., 2014). There is a growing list of miRs that are implicated in cancer development (Chan et al., 2005; Peng et al., 2016). Medicinal plants have been extensively researched as potential source of anticancer drugs (Greenwell et al., 2016), however their effect on the expression and function of miRs remains less understood and understanding their regulation may yield anticancer drug targets.

In cervical cancer, miRs regulate gene expression post- transcriptionally and have been implicated in pathogenic mechanisms involved in cervical carcinogenesis (Lu et al., 2005; Gomez-Gomez et al., 2013; Bano et al., 2014). Reverting microRNA expression in cervical cancer to precancer cell expression is strategy in understanding and treating cervical cancer (Wilting et al., 2013). Certain microRNAs are either up or downregulated in cervical cancer and can potentially be used to screen for cervical cancer, determine stage of cancer and for personalised treatment planning (Hu et al., 2010; Sharma et al., 2014). Some miRs in cervical cancer are also related to apoptosis and alteration of the cell cycle in cervical cells (Lui et al., 2013; Tang et al., 2013; Lui et al., 2015). The effect of plant extracts, such as *Commelina benghalensis*, on these miRs expression is yet to be determined.

Plants have many healing properties and have proven useful for the treatment of various diseases. Many modern medicines are derived from plant sources, such as morphine from opium of the dried seed pods of *Papaver somniferum* (poppy flower) (Bernáth, 1998), Niprisan from the powdered extract of *Pfaffia paniculata* used in sickle cell anaemia (Oniyngi et al., 2020), Paxitaxol chemo-therapy treatment for cancer derived from the tree bark of *Taxus brevifolia* (American Society of Pharmacognosy, 2003*)* and many more. Traditional healers also use plants to treat a number of illnesses, such as *Acunthus montanus* stems and twigs to treat syphilis, cough, vaginal discharge; *Combretum guandiflorum* leaves for jaundice; *Gloriosa superba* leaves and tubers for gonorrhoea, head lice and an antipyretic and many others (Abd El-Ghani, 2016). Some of these medicinal plants have been scientifically tested and some have shown anti-cancer properties (King et al., 2015; Kurrupupu et al., 2019). To find plants that have potential properties to cure cancer, more research needs to be done on these plants. *Commelina benghalensis* is one of the medicinal plants that have shown therapeutic potential. Its methanolic extract has shown to have apoptosis-inducing properties against Jurkat T leukaemia cells and EAC mice cells (Mbazimba et al., 2007; Lebogo et al., 2014; Karan et al., 2018). The effect of *C.*

benghalensis on cervical cancer specific miRs has not yet been investigated. In this study, the apoptosis-inducing potential of *C. benghalensis* extract against cervical cancer cells and the possible effect on the dysregulated miRs involved in the apoptosis pathway were investigated for future cancer treatment purposes.

2.1 PROBLEM STATEMENT

There is a high incidence of cervical cancer in South Africa and new therapeutic strategies are required. Currently radiation, chemotherapy and surgery are used but they do not lead to a cure for every patient, as not all patients survive cervical cancer. Over decades medicinal plants have been extensively used as drug sources worldwide, including South Africa. Their anticancer molecular pathways are not fully understood. Three studies have reported on the anti-cancer properties of *C. benghalensis* in human Jurkat cancer cells (Mbazima et al., 2008; Lebogo et al., 2014) and EAC cells in mice (Karan et al., 2018).

MicroRNAs belong to a class of biomolecules that have been reported as biomarkers for different cancers (Calin et al., 2004; Lu et al., 2005). Importantly, miRs are also involved in many biological processes and pathways such as apoptosis. Understanding the expression of miRs in cancer is important to understand the molecular mechanisms in carcinogenesis. Lebogo et al. (2014) demonstrated anti-cancer activities of *C. benghalensis* against leukaemia cells. There is no report on the effect of *C. benghalensis* on the expression of miRs in cervical cancer cells.

2.2 RESEARCH QUESTION

Does *Commerina benghalensis* acetone extract provide anticancer properties in selected miRs in Hela cells?

Currently, there is no data available on how the anti-cancer properties of *Commelina benghalensis* extracts work and whether they have an apoptotic effect on cervical cancer cells. Some genes responsible for apoptosis are under the control of miRs.

Therefore this study focuses on the effect of *C. benghalensis* extracts on apoptosis induction and the regulation of twelve MiRs in cervical cancer HeLa cells.

2.3 AIM AND OBJECTIVES OF THE STUDY

The study was aimed at determining the effect of *Commelina benghalensis* (Commelinacae) extract on cervical cancer cells metabolism and apoptosis (anti cancer properties) as well as the expression of miRs involved in apoptosis in cervical cancer cells after treatment with the extract. Twelve miRs that play a role in apoptosis in cervical cancer cells were identified from the literature.

2.3.1 OBJECTIVES OF THE STUDY

To address the aims of this study, the following objectives were pursued:

- To determine the potential anticancer activities of the *C. benghalensis* extracts against cervical cancer cells, plant phytochemicals from root, leaf and stems were extracted using acetone
- To investigate the potential effect of the root, stem and leaf *C. benghalensis* extracts, the viability of the treated HeLa cells was assessed using the 2,5-diphenyl-2H-tetrazolium bromide (MTT) assay.
- To establish the mechanisms involved in the effect of *C. benghalensis* plants extracts on HeLa cells via flow Cytometry was used to determine the effect of the active extracts on apoptosis in HeLa cell line.
- To further investigate the molecular mechanisms induced by the *C. benghalensis* stem and leaf extracts, the expression of twelve MiRs in HeLa cell line was determined using quantitative Real time PCR, employing the relative quantification delta-delta C_T value method.

2.4 STRUCTURE OF THE DISSERTATION

2.4.1 CHAPTER ONE

This chapter includes the background for the research and the aims and objectives that were set out to be achieved.

2.4.2 CHAPTER TWO

In this chapter the background information and literature regarding cervical cancer, miRs and *Commelina benghalesis* are discussed. This chapter also elaborates on how and which of the twelve miRs were identified for use in this experiment.

2.4.3 CHAPTER THREE

The materials and methods of the experiments are discussed in this chapter. The experiments described include the MTT assay to determine cessation of metabolism in HeLa cells after treatment with the *C. benghalensis* extracts. Next, flow cytometry is discussed to determine whether apoptosis took place after treatment with the *C. benghalensis* extracts. Lastly, reverse transcriptase real time PCR experiment procedures for determining if there was upregulation or downregulation of the twelve identified microRNAs are described.

2.4.4 CHAPTER FOUR

The results from the MTT for cell metabolism, annexin V flow cytometry for apoptosis and the miRs expression after treatment with *C. benghalensis* extracts is discussed in this chapter.

2.4.5 CHAPTER FIVE

This is the last chapter that concludes with a summary and the conclusion of the experiments, as well as shortcomings of the research project and future research opportunities.

3 CHAPTER TWO: LITERATURE REVIEW

3.1 INTRODUCTION: APOPTOSIS AND THE CELL CYCLE IN CANCER

Uncontrolled cell growth, coupled with the failure of cells to undergo apoptosis are among the hallmarks of cancer. The basic characteristics of cancerous cells were described by Hannahan and Weinberg (2011) in a review outlining that cancer cells act independently from external growth signals and have insensitivity to external anti-growth signals. These cells can also avoid apoptosis and thereby replicate and multiply indefinitely. Additionally, the cancer cells can vascularise and trigger angiogenesis that enables them to invade local and distant tissues as well as to metastasise to form secondary tumours. During the multi-step carcinogenesis process, the proto-oncogenes that promote normal cell proliferation are changed by either mutation, gene amplification or chromosome rearrangement into oncogenes and disruption of the cell cycle (Pierotti et al., 2003). Furthermore, tumour suppressor genes are inhibited, by either point mutations or small deletion and can thus no longer slow down cell division (Chial et al., 2008). In the normal cell cycle, there are many tumour suppressors of which the following three have been well studies, namely retinoblastoma (pRb), p53 and cyclin kinase inhibitor 2A (CDKN2A) (Hannahan et al., 2011; Strachan et al., 2014). Retroviruses can also lead to cancer development through mutations of oncogenic genes of a host when integrating their viral DNA into the chromosomal DNA, such as the human papilloma virus (HPV) that was linked to the development of cervical cancer a few decades ago (Dyson et al., 1989; Schefner et al., 1990).

During the cell cycle cells are usually programmed to either progress through the cell cycle, withdraw from the cell cycle as non-dividing cells, or undergo cell death (apoptosis) in response to stimuli such as DNA damage. The cell cycle progression is controlled by cyclins and cyclin dependant kinases, which in turn, are regulated by certain check points. In cancer cells, these check points are defective, leading to replication of cells that have damaged DNA. Consequently, most cancer cells either have instability in the chromosome such as abnormal karyotypes, or rearrangements and missing chromosomes or microsatellite instability - MSI-changes of DNA bases in a microsatellite comparison with inherited microsatellite (Strachan et al., 2014).

3.2 APOPTOSIS AND ITS ROLE IN CANCER

The word “apoptosis” is derived from ancient Greek meaning “falling off” (Kerr et al., 1972). Apoptosis is defined as programmed cell death that does not lead to inflammation and is needed to maintain cellular homeostasis (Wyllie et al., 1980). In cancer cell the ability of a cell to undergo apoptosis is lost (Hannahan et al., 2011). Necrosis, on the other hand, is uncontrolled cell death due to cell damage, usually associated with inflammation (Manjo, 1964). In one treatment strategy for cancer, apoptosis of the malignant cells needs to be achieved for treatment success (Brown et al., 2005).

As mentioned earlier, p53 is a tumour suppressor gene activated when there is DNA damage in a target cell. If DNA damage is irreversible, apoptosis is activated (Wallace-Brodeur, 1999). Apoptosis takes place via various mechanisms and is associated with several hallmarks such as DNA degradation, nucleus separation into chromosome bodies and activation of the caspase family of proteases. Caspase action leads to loss of membrane integrity through phospholipid asymmetry and phosphatidylserine externalization. Thereafter, the cell shrinks causing membrane bulging, the disappearance of the microvilli on the cell membrane, fragmentation of chromosomal DNA and the formation of apoptotic bodies. The dead fragments are digested by phagocytes. Apoptosis takes place either via an intrinsic or an external pathway (Raychaudhuri et al., 2010). The intrinsic pathway is activated when damage in the cell leads to the release of proteins from the intermembrane space of the mitochondria. The extrinsic pathway, on the other hand, is due to signals from other cells, causing a death signalling complex (DISC) formation when external ligands bind to cell membrane death receptors (Fabisiak et al., 1998). The problem comes in when apoptosis does not occur in response to damaged DNA, causing the formation of defective daughter cells that can become carcinogenic (John et al., 2001).

The B-cell lymphoma-2 (BCL-2) protein family is the best-defined apoptosis regulating group of all the proteins. The BCL family consists of anti-apoptotic proteins, namely, BCL-2, BCL-X, BCL-W, MCL-1, BCL-B, and the pro-apoptotic proteins which are divided in two groups (Figure 2.1). These two pro-apoptotic groups are the multi-domain effectors BAK, BAX, BOK and the BH-3 only activators BIM, NOXA, PUMA, BAX, BAK, BOK (Oltiva et al., 1993). For the purposes of this study the well-researched BCL-2 and BAX are discussed. Apoptosis is regulated by p53, which directly or indirectly modulates the expression of BCL-2 and BAX. BCL-2 suppresses apoptosis by regulating the mitochondrial membrane

permeability and strictly controlling the intrinsic pathway (Letai et al., 2002; Hofseth et al., 2004). BCL-2 is found in the membranes of mitochondria and the endoplasmic reticulum, as well as the nuclear envelope. It stabilises the integrity of the mitochondrial membrane, leading to inhibition of pore formation and the release of cytochrome *C* (Tsujimoto et al., 1998; Vander et al., 1999). BCL-2 associated X protein (BAX), on the other hand, is a pro-apoptotic protein found in both the cytoplasm and mitochondria. It forms heterodimeric complexes with other members of the pro-apoptotic BCL-2 family leading to the formation of channels and pores in the mitochondria. This allows cytochrome *C* and apoptosis inducing factors to be released from the mitochondria into the cytosol, creating a cascade of events that lead to apoptosis through the activation of the caspases. The pore formation leads to loss of the mitochondrial selective ion permeability. This process can be blocked by and overexpression of Bcl-2 (Tsujimoto et al., 1998; Israels et al., 1999). However, besides the Bcl- family apoptosis regulators, there are other pathways that play a big role in tumour genesis, and these include the PI3K pathway.

The phosphatidylinositol-3- kinase (PI3K)/Akt/mTOR (rapamysin) pathway is also implicated in regulating apoptosis in cancer (Vivanco et al., 2002). This pathway plays a role in cell cycle progression, growth modulation and glucose metabolism. The pathway is also crucial for the survival of cells under pressure; it is highly activated in tumour cells, and therefore plays a pivotal role in keeping the tumour cells alive (Datta et al., 1999). Activation of the pathway in oncogenesis causes apoptosis inhibition, genetic instability, angiogenesis and therapy resistance (Zang et al., 2015). Akt/PBK (protein kinase B) can inactivate proapoptotic factors, specifically Bad and Procaspase-9 (Pawson et al., 2000).

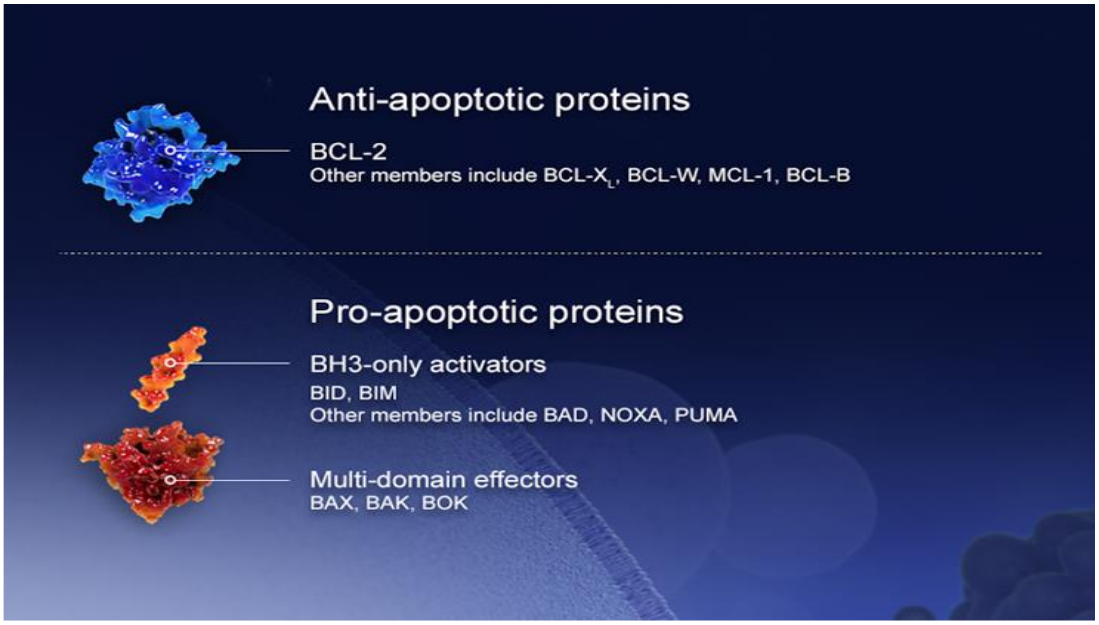

Figure 2.1: The BCL-family (Gentech oncology, 2022).

The cell cycle is divided into four phases, namely the mitosis (M), synthesis (S), G1 and G2 phases. Mitosis and cytoplasmic division (cytokinesis) take place in the M phase. Furthermore, DNA synthesis occurs in the S phase. The M and S phases are separated by two gap phases, G1 and G2. Cells spend most of their time in the G1 phase, where cell growth and centromere replication transpire. In the G2 phase, protein synthesis, accelerated cell growth and mitosis preparation take place. A cell can also enter the dormant G0 phase, creating a non-dividing cell state. The progression from one phase to the next is controlled by cyclins and cyclin dependant kinases as well as cell cycle check points. The three main check points are mentioned here. Firstly the G1/S phase is controlled by cyclin dependant kinase 2 (Cdk2)/cyclin E. Crucial to this study is the progression from G1 to S phase, under the control of p21 (CKD1), as deregulation promotes oncogenesis. Secondly the G2/M checkpoint is controlled by activation of Cdk1/cyclin B, leading to entry of cells into mitosis. Lastly the mitotic checkpoint is triggered by the anaphase-promoter complex. In a cancer cell, all these check points are usually faulty (Strachan et al., 2014). This is illustrated in Figure 2.2, and displayed in Section 2.3.

3.3 CERVICAL CANCER

Cervical cancer is the 4th most prevalent cancer in women worldwide (Sung et al., 2020). Invasive cervical cancer is the leading cause of cancer related deaths in sub-Saharan Africa. The low and middle-income countries carry almost 70% of the cervical cancer disease burden. In South Africa, cervical cancer is most prevalent in the age group between 15-44

years. It is the second most prevalent cancer in Southern African women. Cervical cancer is treated with surgery, chemotherapy and radiotherapy (Ferlay et al., 2013; Sung et al., 2020). Even with these available treatment strategies, the mortality and morbidity rates associated with cervical cancer are still high. In the words of Dr Rengaswamy Sankaranarayanan, lead investigator for the International Agency for Research on Cancer (IARC) in India on rural cervical cancer screening, "Cervical cancer can have devastating effects with a very high human, social, and economic cost, affecting women in their prime. But this disease should not be a death sentence, even in poor countries." (Press Release, WHO, 2013). Therefore, novel treatments for cervical cancer need to be investigated.

Risk factors predisposing to the development of cervical cancer include human immune deficiency virus (HIV) co-infection, multiple sexual partners, young age at first coitus and smoking (Muñoz et al., 2006). Women with HIV infection have a 5 times higher risk of developing cervical cancer, a poorer prognosis and a more aggressive cancer progression that can lead to mortality. Only 2% to 3% of HIV negative women with human papilloma virus (HPV) infection develop cervical cancer (UNAID, 2016). The progression of cervical cancer in HIV positive patients is through the microsatellite instability pathway, whereas in HIV negative patients it is due to the loss of heterozygosity. Cervical cancer is one of the AIDS defining diseases regardless of the CD4 count of the patient (Maiman et al., 1992; Clarke et al., 2002).

Up to 99% of cervical cancer is seen in association with long term HPV infection, of which HPV16 and 18 are the most prevalent (Bosch et al., 1995; Walboomers et al., 1999). HPV16 accounts for 50% of cervical cancer and HPV 18 for 20% (Walboomers et al., 1999). The rest of the cervical cancer is due to 13 other high risk HPV subtypes namely, 31, 33, 35, 39, 45, 51, 56, 58, 59, 68, 73 and 82 (Munzos et al., 2006; Bouvard et al., 2009; Schiffman et al., 2009; Ghittoni et al., 2015).

The HPV genome consists of 3 regions, namely the non-coding long control region (LRC), the early region encoding non-structural proteins (E) and the late region (L) encoding the 2 capsid proteins. The L1 and L2 capsids are the protein shell of HPV. E1 and E2 are responsible for the replication of the viral genes. In carcinogenesis, the loss of E2 is critical, as this increases cervical cancer cell growth (Thierry et al., 1987). E4 is associated with the intermediate keratin filaments, affecting the stability of the keratin network and thereby leading to release of viral particles. E5 encodes the major transforming viral protein and is

lost with integration of the HPV into the host chromosome (Muñoz et al., 2003; Muñoz et al., 2006). E6 and E7 are the important oncogenes in the development of cervical cancer. E6 inactivates p53 and therefore interrupts its tumour suppressive role associated with growth regulation and the induction of apoptosis (Werness et al., 1990). E7 inhibits retinoblastoma proteins (pRb), leading to deregulation of the cell cycle as seen in Figure 2.2 (Boyer et al., 1996). E6 and E7 suppression are potential targets for treatment of cervical cancer.

Besides inhibiting pRb and p53; E5, E6 and E7 also aberrantly activate the PI3K/Akt/mTOR pathway that inhibits apoptosis as previously mentioned in the introduction (Pim et al., 2005; Menges et al., 2006). PI3K usually modulates signals to prevent apoptosis and is amplified in HPV-induced cervical cancers (Lee et al., 2006). Akt also plays a role in apoptosis as well as in cellular processes. Mutations of AKT in cervical cancer may account for the activation of the PI3K/Akt pathway, which drives tumour genesis (Bertelsen et al., 2006; Henken et al., 2011). On the other hand mTOR integrates cellular signals from different pathways; for instance insulin growth factor (IGF), availability of nutrients and cell energy status. MTOR activation is frequently seen in cervical squamous cell carcinoma (Surviladze et al., 2013). In turn, the PI3K/Akt/mTOR pathway activates cellular functions that are necessary for HPV carcinogenesis to take place (Veeraraghavalu et al., 2005). MicroRNAs are other role players that influence carcinogenesis. The effect of HPV E5, E6 and E7 on miRs is discussed in section 2.3.6.

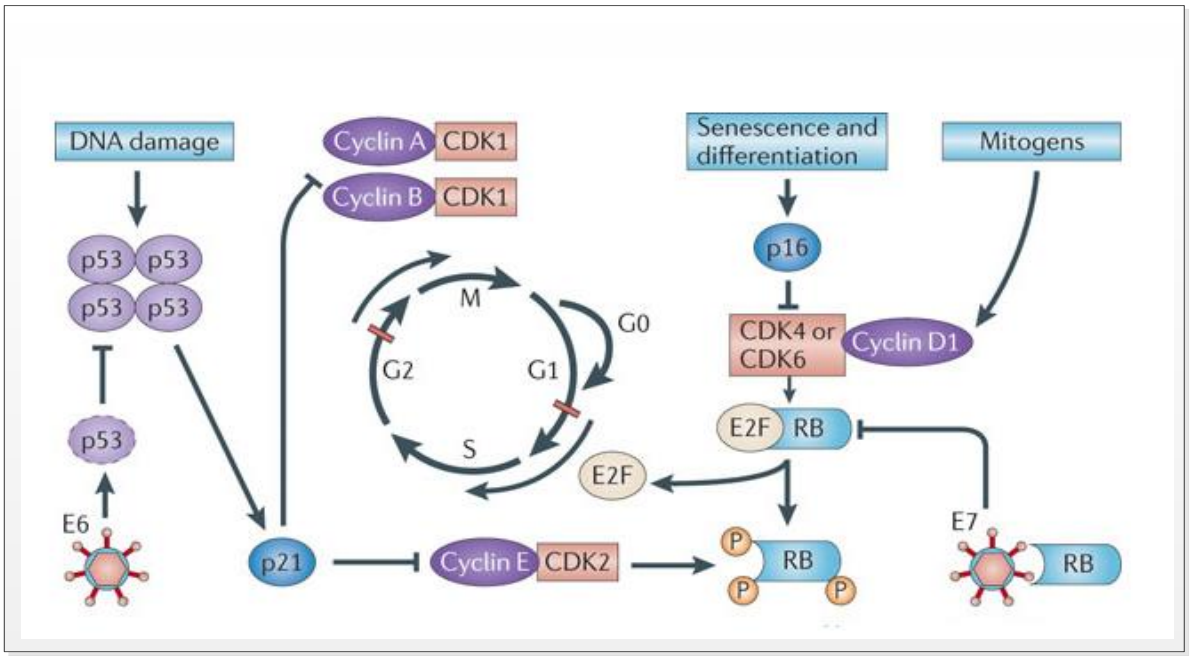

Figure 2.2: The cell cycle deregulated by HPV (Leemans et al., 2011).

3.4 MICRORNA DISCOVERY AND BIOGENESIS

MiRs form part of the small RNA group and are non-coding RNA species regulating other genes in the human genome. Many cell functions, such as growth, invasion, proliferation, apoptosis and migration are controlled by miRs and a single miR can control one or many cellular processes (Lu et al., 2005). MiRs regulate messenger RNA (mRNA) post transcriptionally and consist of 12-20 nucleotides (Lee et al., 1993). The discovery of the lin-4 miR in 1993 has led to a large body of research on this topic, especially in its relation to carcinogenesis (Lee et al., 1993). Currently, there are 38589 microRNA (miRs) listed on the miRbase (miRbase.org - accessed 20 November 2021). MiRs investigation has shed new light on the oncogenic process and research is ongoing in this field. One of the factors seen is that miRs can act either as oncogenes or tumour suppressors in cancer cells (Zhang B. et al., 2007). MiRs expression is altered in cervical cancer and these alterations often lead to progression of cancer.

The biogenesis of miRs starts in the cell nucleolus as seen in Figure 2.3. In the nucleolus a primary RNA (pri-RNA), of more than 1 kilobase (kb), is transcribed by the enzyme RNA polymerase II. MiRs are encoded on untranslated miR genes of either introns or exons (Borchert et al., 2006). The pri-RNA consists of a hairpin containing the mature miR structure. The hairpin is excised from the pri-RNA by DGGR8 (a nuclear RNAse III enzyme) and Drosha (an endonuclease) to form pre-RNA. DGGR8 is assisted by Drosha to identify the pri-RNA and double-stranded junction to cleave approximately 11 nucleotides from the stem. As a result the hairpin is released from the pri-RNA. The resulting premature RNA (pre-RNA) consists of a 5’-phosphate and a 3’deoxyribose overhang. Once pre-RNA has formed, exportin-5 is activated and through Ran guanine exchange. Ran-GTP transports the pre-RNA through the nucleolus into the cytoplasm. There, dicer (RNAse III enzyme) cuts off the terminal loop, creating a double-stranded RNA, comprised of a mature miR strand and a passenger miR strand (miR: miR* duplex). The passenger miR is released into the single strand mature miR that is specific to its complementary mRNA. The mechanism by which this happens is unclear.

There have been possible mechanisms proposed which include RNA helicase A involvement (Lee et al., 1999), dicer cleavage (Zhang et al., 2002), Ago2 cleavage (Cifuentes et al., 2010), as well as possible uncharacterized proteins being involved (Robb et al., 2007). A single pri-RNA can either produce a single mature miR or clusters of miRs (Lee et al., 2004; Borchert

et al., 2006; Rodrigues et al., 2004; Woods et al., 2007; Yi et al., 2003; Kin, 2005). The mature miR is incorporated into the ribonucleoprotein effector complex (RISC – RNAi-induced silencing complex). Together they are called miRISC and act as a guide to translational silencing of the target mRNA (Gregory et al., 2005). The complex typically contains a dicer enzyme, Argonaute 2 family protein (Ago2) and protein cofactors namely PACT (Lee et al., 2006) and TRB (Macrae et al., 2008; Hammond et al., 2001; Chendrimada et al., 2001). Gene expression is repressed by miR through the degradation and translation inhibition of mRNA. Degradation of mRNA is accomplished by either deadenylation and exonucleolytic attack (most prevalent in mammals), or by direct Ago2 catalysed endonucleolytic cleavage (Lui et al., 2004; Bracken et al., 2011).

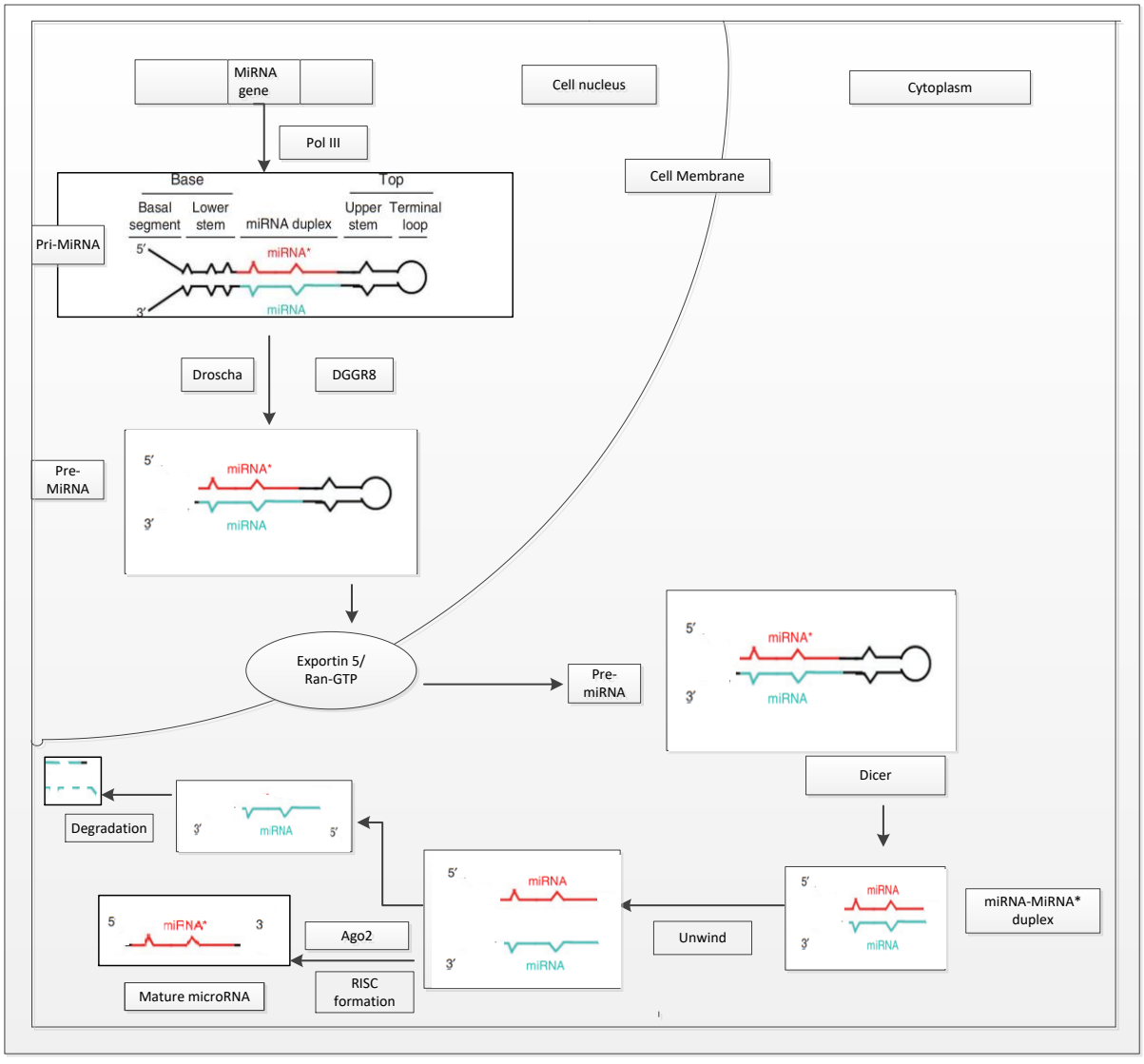

Figure 2.3: The biogensis of microRNA

3.4.1 MiRs IN OTHER CANCERS

As previously mentioned, miRs are often deregulated in cancer cells. MiRs are also often found at fragile sites of the chromosomes, influencing their changed expression in oncogenesis. More than half of the known miRs are found at these chromosomal fragile sites (Calin et al., 2004). Figure 2.4 depicts the fragile sites where miRs are located. The miR-21 gene, for example, is located on chromosome 17q23.2, in the common fragile site FRA17B (Volinia et al., 2006; Thorland et al., 2003), as seen in Figure 2.4. MiR-21 is upregulated in cervical cancer cells in comparison to normal cells (Lui et al., 2007). This region is amplified in many solid tumours (Yao et al., 2012) such as glioblastoma multiforme (Chan et al., 2005), breast cancer (Yan, 2008), hepatic (Meng et al., 2007), colorectal (Slaby et al., 2007), gastric (Zhang, 2008) and oesophageal cancer (Subramaniam et al., 2012). Inhibition of miR-21 leads to cell cycle arrest and increases chemotherapy sensitivity in these cancers (Wang et al., 2012).

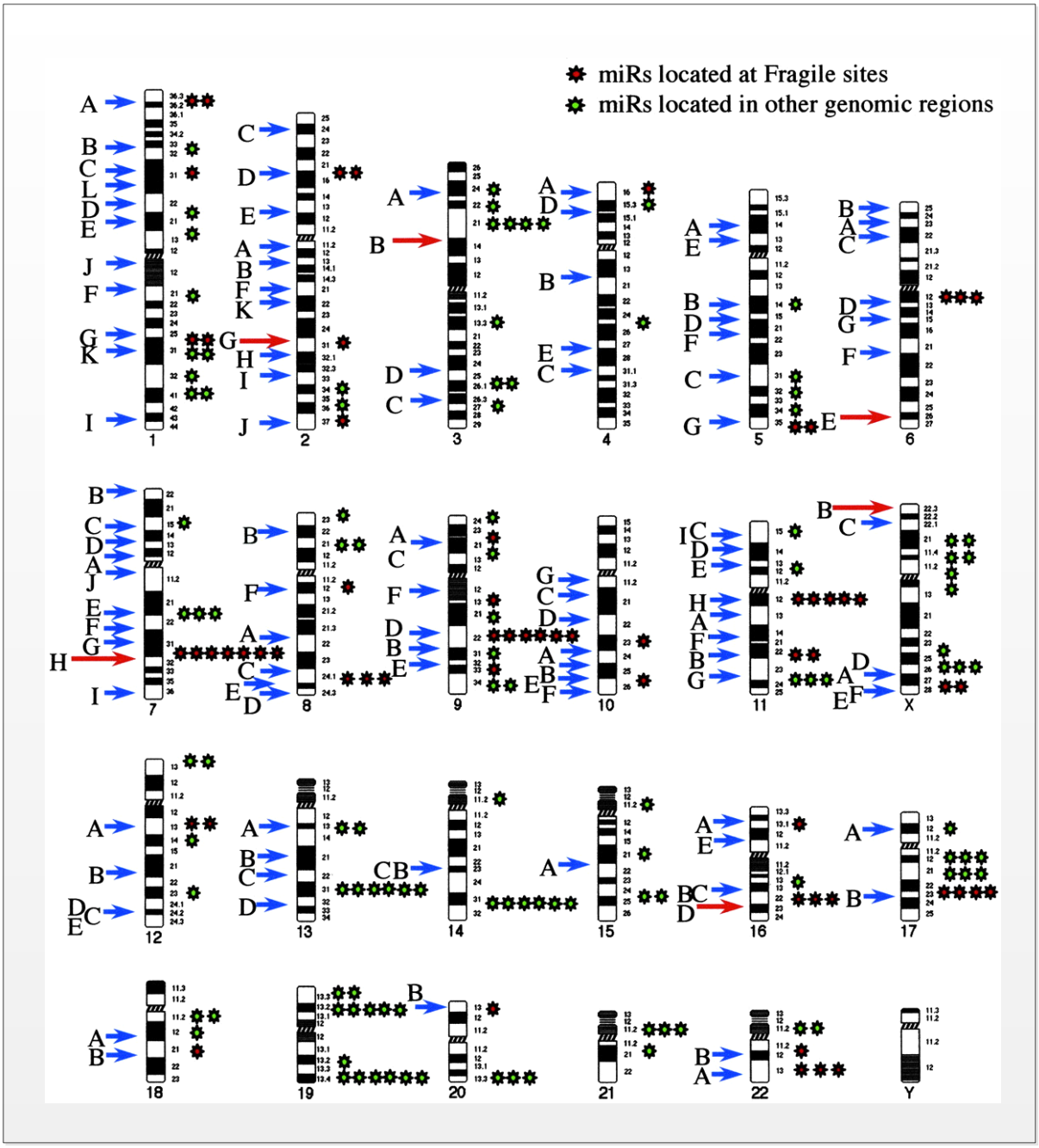

Figure 2.4: Karyotype showing MiRs located at fragile sites. (Calin et al., 2004).

186 miRNAs are presented in this figure of which 113 are located at fragile sites. The red arrows indicate fragile sites that are often observed. For instance, miR-21 often seen in cancer, is at chromosome 17q23.2.

Another example is the loss of miR-15a and miR-116-1 in chronic lymphocytic leukaemia (CLL), mapped to the fragile site of chromosome 13q14, that is deleted in up to 70% of CLL (Calin et al., 2002). Some miRs are tumour suppressive (pro-apoptotic) while others assist in tumour development and are called oncomiRs (anti-apoptotic) (Zhang et al., 2007; Banno et al., 2014). Gain of chromosome regions 1q, 3q, 5q, 8q, Xq, 20q and loss of 2q, 3p, 4p, 5q, 6q, 11q, 13q and 18q are also involved in carcinogenesis of the cervix (Yang, 2011; Wilting et

al., 2006; Cottage et al., 2001; Steenbergen et al., 1998). MiR expression changes are partly associated with chromosomal alterations in cervical cancer (Wilting et al., 2013).

MiR-203 is downregulated in hepatocellular cancer (Furata et al., 2010), colon adenocarcinoma (Schetter et al., 2008), bladder (Goottardo et al., 2007) and ovarian cancer (Iorio et al., 2007). In normal tissue, miR-203 has a suppressive role. Correction of the expression of miR-203 is a possible area to be investigated for treatment of these cancers. MiR-194 inhibits metastasis of small cell lung cancer (Wu et al., 2014). MiR-146a is another example of an upregulated miR in cancer is, such as in cervical cancer, breast, pancreatic and prostate cancers (Volinia et al., 2006).

MiRs can possibly be used for the classification and prognosis of cancers (Hu et al., 2010), although an algorithm for this is yet to be developed. In colon and rectal cancer, down-regulated miR-139 is indicative of advanced stage of carcinogenesis and a poor prognosis (Guo et al., 2012). Whereas in colon and pancreas cancer patients the overexpression of miR-203 correlates with a better survival (Greither et al., 2010). All these reports show that miRs play an important role in carcinogenesis and are promising targets as diagnostic markers and for anticancer drugs development.

Some medicinal plants have shown anticancer properties by regulating apoptosis (Bungu et al., 2006; Mbazimba et al., 2008; Lebogo et al., 2014). Understanding how these medicinal plants regulate apoptosis and involvement of miR regulation may lead to better understanding on how these natural remedies function. This will be discussed further in section 2.5.

3.4.2 MiRs in cervical cancer diagnosis and therapies

Screening and diagnosing cervical cancer are done by Papanicolaou (Pap) smear, that can be accompanied by HPV testing, a biopsy of the cervix and a colposcopy. Pap smears are not foolproof as it needs a well-trained clinician and pathologist with access to good equipment to interpret the smear, which is not always available. Many women omit doing a pap smear due to the discomfort of the screening test with a speculum. A gynaecologist usually needs to do a colposcopy and a cervical biopsy, which are not always available. New ways to diagnose cervical cancer to replace this old-fashioned method are needed. The presence of HPV in cervical cells is eliminated in 90% of women after 2 years and although HPV presence identifies higher risk cases, being diagnosed with positive HPV could cause a lot of unnecessary concern for the patient (Castle et al., 2009).

Another option is to use miRs expression in cervical cancer cells as a screening method. MiRs are deregulated in several cells and have been shown to play a role in the carcinogenesis process. In cervical cells, there are a growing number of miRs identified that are either downregulation or upregulated during the development of cervical cancer. Tian et al. (2014) showed that miR-424, miR-375 and miR-282 were downregulated in cervical cancer tissue. MiR-424 or miR-375 or a combination of miR-424/miR-372/miR-282 in exfoliated cervical cell with HPV presence has a superior screening performance over a Pap smear (Tian et al., 2014). Upregulation of either miR-21, mir-34a, miR-125b-5p, miR-205, miR-886-5p or miR-1246 in cervical cancer cells compared to normal cervical cells, could be a good diagnostic indicator of cervical cancer (Banno et al., 2015; Ribeiro et al., 2015; Xie et al., 2012; Li et al., 2011). Downregulation of miR-218 can also be used for diagnostic screening of cervical cancer (Martinez et al., 2008).

MiRs can also be found in granular vesicles and exosomes in the blood of a cervical cancer patient and can potentially be used as a diagnostic marker for cervical cancer. MiR-21, miR-27, miR-34, miR-34a, miR-126, miR-146, miR-155, miR-196a, miR-203 and miR-221 have been found circulating in normal serum of healthy women. Whereas miR-21, miR-27a, miR-34, miR-34a and miR-146a are expressed higher in the serum of squamous cell carcinomas patients (Mitchell et al., 2008; Liu et al., 2015; Gocze et al., 2013). In future, these miRs can be studied further as potential cervical cancer markers.

MiRs in serum and cervical cancer cells can also be used as markers for lymph node metastasis, which is an important indicator of cervical cancer prognosis. In the diagnosis and treatment of cervical cancer the FIGO (International Federation of Gynaecology and Obstetrics) staging system is used which, describes the areas involved in cervical cancer and metastasis (Appendix A). In cervical cancer stages 1B and 2A, the FIGO system does not consider pelvic lymph node metastasis into account. Patients with pelvic lymph nodes have a poorer prognosis than those without (Huang et al., 2012). Zhoa et al. (2013) showed that miR-20a is overexpressed in serum of cervical cancer patients with lymph node metastasis. The downregulation of miR-20a can lead to smaller tumour size, lower histological grade and less lymph node metastasis (Zhoa et al., 2013; Zhoa et al., 2015). MiR-203 expression is higher in sera of patients with cervical cancer than in normal serum. The presence of miR-203 in serum of cervical cancer patients was associated with an increase in lymph node metastasis (Lee et al., 2008). MiR-203 is also upregulated in cervical cancer cells and increases VEGF (Vascular endothelial growth factor) expression (Zhu et al., 2013). In early

cervical cancer, VEGF polymorphs affect the survival of cervical cancer patients through angiogenesis. VEGF also influences lymph angiogenesis, lymph node metastasis, tumour vascularisation (angiogenesis) and metastasis in cervical cancer. The VEGF expression is higher in adenocarcinomas than in squamous carcinomas. The micro density vessels (MVD) in adenocarcinoma are also higher than in squamous cell carcinoma. MVD is a marker for angiogenesis and a prognostic factor. This higher MVD indicates a poorer cancer prognosis in adenocarcinoma (Krill et al., 2015; Kim et al., 2010).

Downregulated let-7c, miR-143, miR-145, miR-199a, miR-125 decreases the risk of lymph node metastasis in squamous cell cancer (Huang et al., 2012). MiR-10 can be used as a marker for lymph node metastasis. The more upregulated miR-10a is the more likely lymph node metastasis is (Zeng et al., 2014). Higher expression of miR-224 and miR-1246 in cervical cancer cells has a poorer prognosis, more advanced FIGO stage, and increased lymph node metastasis (Shen et al., 2013; Chen et al., 2014).

Cancer metastases are also under the control of some miRs (Lu et al., 2005; Farazi et al., 2013; Pencheva et al., 2013). Upregulation of miR-1 could potentially lead to decreased metastasis in cervical cancer (He et al., 2015). Close homolog of L1 (CLH1) overexpression decreases the effect of miR-10 and can also block cervical cancer metastasis (Long et al., 2012). MiR-200 can possibly be a master suppressor for cervical cancer metastasis. MiR-200a predicts the metastatic potential of the cervical cancer whereas suppression of miR-200b can inhibit cancer cell growth. MiR-200 simultaneously targets multiple genes, such as regulation of cell adhesion that plays an important role in the metastatic potential of cervical cancer. Cervical cancer mortality is significantly reduced in with increased expression of miR-200 in the cervical cancer tissue (Hu et al., 2010; Wilting et al., 2013; Chen et al., 2014; Zeng et al., 2016). Increased miR-21, miR-125 and miR-127 expression correlates with increased metastasis and poorer prognosis (Lee et al., 2008; Fan et al., 2015; Ogechukwu et al., 2015).

The formation of new blood vessels (angiogenesis) to supply a tumour is needed for metastasis to take place. The downregulated expression of miR-99, miR-196, miR-203 and miR-205 in cervical cancer cells leads to increased angiogenesis and therefore metastasis (How et al., 2013; Xie et al., 2012; Zhu et al., 2013). Not all miRs have only an oncomir or tumour suppressor function. MiR-494 can act as either a tumour suppressor or an oncomir in cervical cancer, where it can be upregulated or downregulated. The PTTG1 gene is inversely

affected by miR-494 expression. When miR-494 is upregulated, downregulation of PTEN occurs, indicative of poor survival and prognosis (Yang et al., 2015).

Treatment of cervical cancer is devised by staging the cancer according to the FIGO staging, as mentioned earlier. The staging system is based on the local cervical infiltration, further spread to the uterus, then local and far metastasis. MiR expression can be used to improve the choice of treatment for a patient regardless of the FIGO stage. Deregulation of normal miR expression is characteristic of cancer progression and deregulates the cell cycle, proliferation, migration, invasion and apoptosis. If the miR levels can be restored to normal levels as it was pre-cancer, then normal cellular activity will be restored (Kumar et al., 2007; Lu et al., 2005). MiRs can also be used to inhibit oncomirs or to supplement tumour suppressive miRs. Synthetic nucleic acid-based therapy can be used for this therapy and potentially be delivered by antelocollagen - the protein that forms the terminal region of collagen 1 (Takeshita et al., 2006). The expression of miR-9 and miR-200 in cervical cancer cells has a predictive score of how radiosensitive the cervical cancer is (Hu et al., 2010). MiR-181 can also serve as a marker for increase cell sensitivity to radiotherapy and assist in sensitizing the cells to radiotherapy (Ke et al., 2013). Decreased miR-18a-5p or increased expression of either miR-21 or miR-375, or the presence of miR-218 increase the sensitivity to radiotherapy of cervical cancer (Yuang et al., 2014; Liu et al., 2015; Song et al., 2015). The higher the expression of miR-1246 in cervical cancer the more resistant the cancer cells are to radiotherapy (Zhang et al., 2013). Zhang et al. (2013) found that a signature overexpression of miR-630, miR-1246, miR-1290 and miR-3138 promotes 5-fold radio resistance in cervical cancer cells. MiR-106 and miR-125 can be used as possible biomarkers for treatment prognosis (Ribeiro et al., 2015; Cheng et al., 2016).

Increased levels of miR-126 enhance cervical cancer cells sensitivity to bleomycin (Selcuklu et al., 2009; Yu Q. et al., 2014). MiR-155 upregulation reverses epithelial mesenchymal transitions and increases the cervical cell sensitivity to cisplatin (Lei et al., 2012). Increased levels of miR-224 sensitize SIHA cervical cells to paclitaxel whereas miR-27 or miR-375 overexpression decreases paclitaxel-induced apoptosis in cervical cancer cells (Shen et al., 2013; Lin et al., 2015; Liu et al., 2016). MiR-27 can be used for miR-27b antisense nucleic acid therapy (Lui et al., 2016). The miR expression is important in choosing chemotherapy. Further research for cervical cancer chemotherapy and miRs mentioned needs to be done.

Another area of possible cervical cancer treatment is on the telomeres and their relation to miRs. Telomeres are found at the chromosomal ends with the code TTAGGG and keep chromosomes ends intact during cell division. As cell division takes place the telomeres get shorter over time and this has a role in carcinogenesis. The telomere length is regulated by the enzyme telomerase, which is controlled by miRs. The activity of telomerase in turn, is dependent on hTERT (human telomerase reverse transcriptase). Furthermore, hTERT is regulated by miR-138 and this reduces telomerase activity. In cervical cancer cells, miR-138 is downregulated leading to increased telomerase activity and carcinogenesis through the lengthening of the telomeres and causing cell immortalization. Increasing miR-138 expression can be used in treatment of cervical cancer as it suppresses cell growth (Chakrabarti et al., 2013; Wang et al., 2016).

One more potential novel therapeutic in cervical cancer is to target CCR5 through miR-107 (Che et al., 2015). CCR5 is a C-C 5 chemoreceptor oncogene which stimulates tumour growth, is upregulated in cervical cancer cells in comparison to normal cervical cells and under the control of MiR-107. CCR5 is also one of the co-receptors to which gp20 of the HIV viral envelop binds, together with CD4. MiR-107 directly targets and negatively regulates CCR5. CCR5 downregulation leads to suppression of cervical cell growth and proliferation.

As seen above, miR expression in cervical cancer can give an indication of whether the cancer cells will respond well to chemo- and radiotherapy. Using this information, cervical cancer treatment can be adapted to be personalised and not protocol-based, using miR expression levels. This is an area where more research is required. The expression patterns of these miRs can also potentially be used to classify cervical cancer sub-types.

3.4.3 DIFFERENCES BETWEEN MIRS IN ADENO- AND SQUAMOUS CERVICAL CANCER

Squamous cell carcinoma is more prevalent (at 75% - 80%) than adeno cancer (20% - 25%), although adeno cancer's prevalence is increasing due to more Pap smears screening (Mathew et al., 2009). Many women diagnosed with adeno cancer are young and often have skip lesions making diagnosis more difficult (Liu S et al., 2001). Adeno cancer is usually histologically less differentiated, has a lower tissue integrity, is more prone to distant metastasis and has a poorer prognosis than squamous cervical cancer (Galic et al., 2012).

Treatment outcome also differs between the two histological types. The treatment regimen is based on FIGO staging and this is less effective for adeno than squamous cancer (Williams et

al., 2015). MiR expression also differs between the two histological types. In a study done by Gocze (2013), there were high levels of miR-21, miR-27a, miR-34a, miR-196 and miR-221 in HPV positive squamous cell cancer, but not in adeno cancer. It will therefore be beneficial to also determine miR expression in these histological types to improve treatment outcome.

3.4.4 DOWNREGULATED AND UPREGULATED MIRS IN CERVICAL CANCER

In cervical cancer, the expression of some miRs is altered when compared to healthy cervical cells, as previously discussed. The expression of miRs associated with cervical cancer cells can also vary between population groups. This is influenced by various factors in population groups, such as their geographical area, genetic heritage, histological cancer type, the preparation and storage of the tissue and method used to determine miR expression. Ongechkwu et al. (2015) additionally indicated that the miR expression in cervical cancer can be influenced by stages of the cancer, the cell collection site, the oncoproteins present and the type of causative viral agent present. According to Metsdagh (2008), reverse transcriptase polymerase chain reaction (RT-PCR) is the most accurate way to determine miRs expression. Likewise, as mentioned previously, miR expression differs between cervical squamous and adenocarcinoma (Wilting et al., 2013). The next section discusses the diverging expressions of miRs in cervical cancer reported by different studies.

Ding et al. (2014) collected 10 squamous cervical cancer specimens from the Department of Gynaecology at the Xiangya hospital, China. None of the patients had received any chemo or radiotherapy yet. The specimens were frozen in liquid nitrogen and stored at -80 degrees celsius. MiR microarray analysis found that thirty-nine miRs were differentialy expressed, of which 22 were upregulated and 17 were downregulated, compared to non-cancerous cervical cells. The results were confirmed by RT-PCR (Ding et al., 2014). Ma et al. (2012), likewise, collected 8 cervical cancer specimens from the Peking Hospital's Obstetrics and Gynaecological ward (China). These specimens were, in like manner, frozen in liquid nitrogen and stored at -80 degrees celsius. In this study, 13 miRs were upregulated and 16 down regulated (Ma et al., 2012). Ogechukwu et al. (2015) did a study on the discordant reports of cervical cancer miR expression from different research articles found on PubMed, Google Scholar and Research gate. It was found that out of the 66 dysregulated miRs, 9 were controversially regulated between reports (thus they could be up or down regulated depending on the report), 20 were uncontroversial (thus having the same results in all the

reports and 37 were not yet fully validated whether up or down regulated by the studies (Ogechukwu et al., 2015).

How et al. (2015) tried to establish a candidate prognostic micro-RNA signature of 9 miRs for survival prognosis previously found altered in cervical cancer. These researchers could not successfully validate the 9 miRs, irrespective of the method used (RT-PCR, TLDA and NanoString). The reasons considered were, the formalin-fixed paraffin-embedded (FFPE) specimens lacked concordance to frozen specimens, tumour heterogeneity and the platform used to select the micro-RNAs (How et al., 2015). Storing cervical tissue as FFPE, as used in most hospitals, leads to degradation of RNA in the tumour cells (Wang et al., 2009). This study is an indication that miRs are differently expressed in patients' cervical cancer and that the storage method is also important. The survival of cervical cancer patients also varies even though their clinical stages are the same. This could also indicate different miR expression between different patient's cervical cancers.

A few authors have also tried to establish a miR profile for cervical cancer in different reviews. They all used the data available with various indicators. But even then, a common miR profile could not be established (He et al., 2014; He et al., 2016; Grandoz Lopez et al., 2014). He et al. (2016) performed a systemic study on current literature using PubMed, Cochrane, Gene expression Omnibus and array express. Only altered miRs being reported in two or more independent studies were included. There were many different expressions of miRs in the studies used. The conclusion reached was that, in cervical cancer, 21 miRs are down regulated and 42 upregulated (He et al., 2016).

In a review done by Granados Lopez (2014) compilations of miRs of different studies were also done and only dysregulated miR in 2 or more studies were included. Twenty miRs were found upregulated, namely miR-9, miR-10a, miR-15a, miR15b, miR-19a/b, miR-20a, miR-20b, miR-21, miR-31, miR-92a, miR-93, miR-106b, miR-133a, miR-133b, miR-146a, miR-146b-5p, miR-155, miR-200a, miR-200a*, miR-224. Fourteen miRs were down regulated namely miR-1, miR-23b, miR-26a, miR-27b, miR-99a, miR-99b, miR100, miR-125b, miR-195, miR-196b, miR-218, miR-375, miR-424, miR-497. Interestingly, some miRs were upregulated in one study and downregulated in another study, using the same method of testing i.e., PCR or different methods. Eighteen miRs fell into this category namely: miR-7, miR-10b, miR16, miR-17-5p, miR-27a, miR-29a, miR-34a, miR-125a-5p, miR-143, miR-145, miR-191, miR-193b, miR-199a, miR-200c, miR-203, miR-205, miR-210, miR-214

(Grandoz-Lopez et al., 2014). Reviews of Grandoz Lopez (2014) and He (2015) indicate that the miR expression in cervical cancer tissue varies and a more personal approach to miR expression in cervical cancer is needed. A summary of the miRs of these two reviews are in Table 2.1, and it is noted that there are differences of how miRs present in different cervical cancer cells are expressed. It is therefore important to note that each cervical cancer patient has a unique miRs expression signature, which influences the progression, metastasis and treatment outcome.

Table 2.1: Summery of dysregulated miRs in cervical cancer (Grandoz Lopez et al., 2014, He et al. 2015).

Upregulated miR He et al., 2015)	**Upregulated miR (Grandoz Lopez et al., 2014)**	**Downregulated miR (He et al., 2015)**	**Downregulated miR (Grandoz Lopez et al., 2014)**	**Varied regulation (Grandoz Lopez et al., 2014)**
Let-7d-5p		miR-1	miR-1,	miR-7,
Let-7f-5p		miR-29a	miR-23b,	miR-10b,
miR-9-5p	miR-9,	miR-34a	miR-26a,	miR16,
miR-10a-5p	miR-10a,	miR-99a-5p	miR-27b,	miR-17-5p,
miR-15a-5p	miR-15a,	miR-99b-5p	miR-99a,	miR-27a,
miR-15b-5p	miR15b,	miR-100-5p	miR-99b,	miR-29a,
miR-16-5p	miR-19a/b,	miR-125b-5p	miR100,	miR-34a,
miR-18a-5p		miR-126-3p	miR-125b,	miR-125a-5p,
miR-20a-5p	miR-20a,	miR-140-5p		miR-143,
miR-20b-5p	miR-20b,	miR-149-5p		miR-145,
miR-21-5p	miR-21,	miR-195-5p		miR-191,
miR-25-5p		miR-196b-5p	miR-195,	miR-193b,
miR-31-5p	miR-31,	miR-199a-3p	miR-196b,	miR-199a,
miR-34c-5p		miR-203		miR-200c,
miR-92a-3p	miR-92a,	miR-218-5p		miR-203,
miR-93-5p	miR-93,	miR-375	miR-218,	miR-205,

miR-96-5p		miR-376a-3p	miR-375,	miR-210,
miR-106a-5p		miR-376c-3p		miR-214
miR-106b-5p	miR-106b,	miR-494-3p		
miR-130b-3p		miR-497	miR-424,	
miR-133a-3p	miR-133a,	miR-617	miR-497	
miR133b-5p	miR-133b,			
miR-135b-5p				
miR-142-5p				
miR-146a-5p	miR-146a,			
miR-146b-5p	miR-146b-5p,			
miR-155-5p	miR-155,			
miR-181-5p				
miR-185-5p				
miR-189-5p				
miR-192-5p				
miR-196a-5p				
miR-200a-3p	miR-200a,			
miR-200b	miR-200a*,			
miR-200c-3p				
miR-224-5p	miR-224			
miR-338-3p				

miR-339-5p miR-425-5p miR-944 miR-1246				

3.4.5 INTERPLAY BETWEEN MIRS AND HPV IN CERVICAL CANCER

The interplay between miRs, HPV and its oncogenes is still elusive, although some miRs are reportedly directly influenced by HPV oncoproteins. E6 regulates a functional loop of Let-7a, STAT3 and miR-21 (Shishodia et al., 2014). E6 also activates miR-9 in a p53-dependant manner, leading to increased cell motility (Lui et al., 2014). E6 also downregulates miR-34 and thereby increasing the Warburg effect thus promoting tumour growth and invasion. The Warburg effect is a metabolic phenotype that allows cancer cells to utilize glucose under aerobic conditions. MiR-34 has a suppressive function on the Warburg effect (Zhang et al., 2016). E6 also downregulates miR-218 expression in HPV-16 infected cervical cancer cells (Martinez et al., 2008). In HPV-16-induced cervical cancer, E6 downregulates miR-1246 expression in cervical cancer (Yang et al., 2015).

MiR27-b is upregulated by E7 through DGGR8, while negatively regulating polo-like kinase 2 (PLK2). Upregulation of miR-27b promotes cell proliferation and invasion and thereby inhibiting paclitaxel-induced apoptosis (Liu et al., 2016). E5 potentiates the effect of E6 and E7. Between these three oncogenes, E5 is so far, the least studied. E5 upregulates miR-146a, supresses miR-234-5p and at late points supresses miR-203 (Greco et al., 2011).

MiR-375 is a master oncogene suppressor of HPV-associated cancer. It suppresses E6, E7 and E6AP, thereby securing tumour suppression of p53 and Rpb, as well as regulation of telomerase activity. Decreased expression of miR-375 in HPV-associated cancer promotes carcinogenesis (Jung et al., 2014). MiR-125b levels in the transformation zone are downregulated by trans-infection of HPV L2, which is the minor capsule of the HPV particle. MiR-125b levels are markedly decreased in cervical intraepithelial neoplasm stage 1 lesions (CIN 1). There is a strong connection between HPV L2 expression and miR-125b (Nuovo et al., 2010). The interaction between miRs and oncoproteins are summarised in Table 2.2.

Table 2.2: The role of miRs on the interaction of HPV oncoproteins with cellular oncoproteins.

MiRs and expression in cervix cancer that affect HPV	HPV oncoprotein affecting MiRs	Cellular oncoprotein	Cellular effect	References
Let -7a Downregulated	E6	STAT3	Let 7a negatively regulates STAT3 in cervical cancer.	Shishodia et al., 2014.
Let-7d-5p Downregulated	E6/E7		Suppress anti-proliferative p21 gene.	Honegger et al., 2015.
MiR-9 Upregulated	E6	VEGA, EPG, FSTL1, ALCAM P53 related manner	Increases cell motility.	Wilting et al., 2013; Lui et al., 2014; Lee et al., 2008.
MiR-15a-5p Upregulated	E7	c-Myc, c-Myb, PPAR	Controls cell proliferation, survival and invasion.	Zheng et al., 2011.
MiR-15b-5p Upregulated	E7		Recognises mismatched nucleotides before their repair and initiates eukaryotic genome replication.	Zheng et al., 2011; Myklebust et al., 2011.
MiR-16-5p Upregulated	E7	c-Myc, c-Myb, PPAR	Control of cell proliferation and invasion.	Zheng et al., 2011.
MiR-21-5p	E6	P53, PDCD4, RECK, TMP1, CCL20, MEG3, STAT3, LATS1,	Increase cell growth and decreases	Liu et al., 2015; Yao et al., 2009; Selcuklu et

Upregulated		ATG2, TIMP2 (Yao et al., 2009) MiR-21 negatively regulated p53 and Cdc25, involved in regulation of cell proliferation, RECK and TPM1 which suppress metastasis and PDCD4 and PTEN which induce apoptosis in malignant cells (Sekulu et al., 2009). PTEN (Ben et al., 2015) MEG3 (Zang et al., 2015) STAT3 is suppressed by mir-21(Shishodia et al., 2014) LATS1 suppressed in miR-21 overexpression (Lui S et al., 2015). ATG2 and TIMP2 leads to invasion and metastasis (Ogechukwu et al., 2015).	apoptosis.	al., 2009; Ben et al., 2015; Yoa et al., 2012; Zhang et al., 2015; Shishodia et al., 2014; Lui et al., 2015; Ogechukwu et al.,2015.
MiR-27a Downregulated	E6			Ben et al., 2015.
RiR-27b Downregulated	E7	PLK2, DGCR-8 mediates up regulation of miR 27b via HPV E7.	E7 upregulates miR-27b, that promotes cell proliferation and invasion inhibiting paclitaxel apoptosis.	Liu F et al., 2016.
MiR-29a Downregulated	E6/E7	P53 YY1, p53, CKD6, HSP47	Normal levels restrain cell progression and induce apoptosis.	Yamamoto et al., 2013; Li et al., 2011; Park et al., 2009.
MiR-34a Downregulated	E6 and E7	P53, p18Ink4c, NOTCH1, JAGGED1	Cell cycle progression, cell senescence and apoptosis.	Li et al., 2010; Wang et al., 2011; Pang et al., 2010; Li et al., 2020.

		Inverse correlation with WNT1.		
MiR-125b Downregulated	L2	PIK3CD	Inhibits apoptosis and increases tumour growth volume. Infleunces cell motility, invasion, glucose metabolism and chemo sensitivity.	Nuovo et al., 2010; Huang et al., 2012; Cui et al.2012.
MiR-146 Upregulated	E5	ZNF183	Cell adhesion and cell cycle.	Greco et al., 2011.
MiR-203 Downregulated	E5 E7	VEFD, BANF1	Infleunces cell junction, cell migration, cell motility as well as angiogenesis, lymph node and tumour metastasis.	Zhu et al., 2013; Zhoa et al., 2013; Moa et al., 2015.
MiR-218 Upregulated	E6	LAMBB3, BIRC5, DCUN1D1	Affects the AKT-mTOR pathway, Rictor protein, SFMBT1 protein and EMT process.	Martinez et al., 2008; Li et al., 2012; Yaun et al., 2014; Jiang et al., 2016; Zhou et al., 2010; Kogo et al., 2014
MiR-875 Upregulation	E6		Increases apoptosis, causes disruption of the HPV-host interaction and could potentially lead to the clearance of cervical cancer cells infected with HPV.	Lin et al., 2015.
MiR-1246 Upregulated	E6	THBS2	Affects cell proliferation, migration and invasion.	Chen et al., 2014.
MiR-3144	E6		Increases apoptosis, causes disruption of the HPV-host	Lin et al., 2015

Upregulation			interaction and could potentially lead to the clearance of cervical cancer cells infected with HPV.	

Figure 2.5 depicts the oncomirs of HPV, their role in cervical cancer, their gene targets and their influence on miRs. This figure is an amalgamation of information and diagrams from the following articles: Munger et al., 2004; Nuovo et al., 2010; Greco et al., 2011; Wang et al., 2009; Martinez et al., 2008 and Jung et al., 2014.

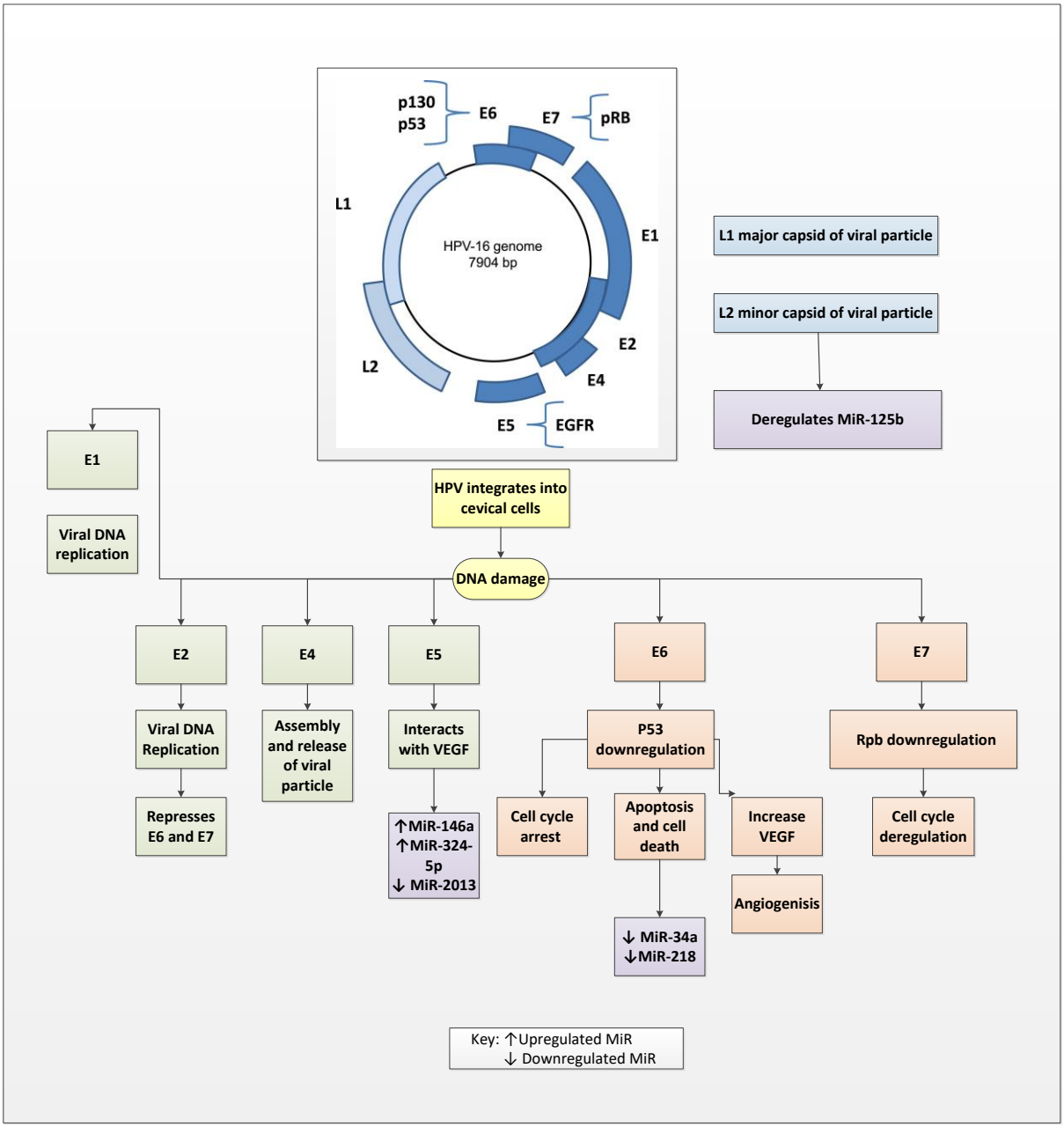

Figure 2.5: HPV oncoproteins role in cervical cancer.

Various plants have anti-cancer properties through apoptosis, and as some miRNAs play a role in apoptosis, further investigation into the effect of plant extracts on these miRs are warranted. This will be discussed in the next section.

3.5 PLANT MEDICINE AND COMMELINA BENGHALENSIS

Plants have been used from the earliest of times to treat sickness and diseases. The history of plant medicine dates back to long before modern time. The 'De Materia Medica', written by Pedanius Dioscroides dates to 50-70 AD and is one of the oldest sources of plant-based medicine, consisting of 5 volumes and referencing roughly 600 plant based substances that can be used as medicines. Seen as the precursor of modern pharmacopeia, David Sutton, a science historian, said 'De Materia Medica' is "one of the most enduring works of natural history ever written" and "it formed the basis of Western knowledge of medicine for the next 1500 years" (Sutton., 2007).

From there onwards, humans have used plants and their extracts in various forms. From traditional healing to chemotherapy, the ingredients have a plant basis today. Many people around the world still make use of traditional, complementary and alternative medicine for disease and cancer treatment (King et al., 2015). Medicinal plants are used by about 65-80% of the world's population as per the World Health Organization (Pan et al., 2014). In South Africa, herbal medicine obtained from 1020 different plant species and their derivatives are used (Dauskardt et al., 1990; Williams et al., 1996). Of the 1020 plants, 156 are used for gynaecological problems (Steenkamp, 2003). Cancer patients also use plant medicine, such as non-Hodgkin's lymphoma patients, where 89% had at some point during their cancer treatment used alternative medicine (Rauch Ossian et al., 2015). This is due to many factors, including the cost and side effects of chemotherapy, cultural beliefs, and cancer drug resistance to standard chemotherapy (Cragg et al., 2005).

There is a definite basis in the treatment usage of plants. Sixty nine percent of anti-cancer medication developed between 1980 and 2002 contained plant material (Newmann et al., 2007). Several anti-cancer drugs are derived from plant compounds such as, vinblastine, vincristine, camptothecin derivate, topotecan, irinoteca, etoposide (derived from epipodhyoctoxin) and paclitaxel (taxol) (Cragg et al., 2005). Vincristine and vinblastine are derived from leaves of *Catharanthus roseus*, usually called Madagascar periwinkle. These derivatives are, for instance, used for the treatment of leukaemia (Guerite el al., 2005; Drugdigest 2007) and Hodgkin's disease (Flora of China). The semi-synthetic derivatives of vinblastine and vinchristine, namely vinorelbine and vindesine, are also used as chemotherapy against leukaemia, lymphoma, breast, lung and testicular cancer. Paclitaxel,

derived from the bark of *Taxus brevifolia* nut called pacific yew, is used for chemotherapy in breast, ovarian and small cell lung cancer (Cragg et al., 2005).

Some other untested plants are also promising in the treatment of cancer. *Ganoderma lucidum (G. lucidium)* is a plant species used in traditional Chinese medicine for treatment of leukaemia and gastric cancer. The polysaccharide isolate from *G. lucidum* has anti-tumour and anti-oxidant properties (Patterson et al., 2006). In traditional Chinese medicine, various plants and their extracts are used to treat cancer.

Two South African plants, namely, *Tulbaghia violacea* (wild garlic) and *Cotyledon orbiculata* (Pigs ear), have also been shown to have anti-cancer activities (Cordell et al., 1991; Van Wyk et al., 2000). *Tulbaghia violacea* induced apoptotic features in cervical cancer HeLa cells (Bungu et al., 2006). Correlating with these results, a concentration of 150 µg/ml *T. violacea* induced 42% of HeLa cervical cancer cells to undergo apoptosis (Mthembu et al., 2014). Dichloromethane and methanol extracts from *Cotyledon orbiculata* had moderate anti-cancer activities (Fouche et al., 2006).

Up to this point other medicinal plants were mentioned, however in the next section *Commelina benghalensis* is introduced and formed the central focus of the plant extraction used throughout this research.

3.5.1 COMMELINA BENGHALENSIS

Commelina benghalensis, belonging to the Commelinaceae family, is a common weed from Asia and Africa, called benghal day flower, dew flower or tropical spiderwort and has shown anticancer properties. It is found abundantly is the northern parts of South Africa such as the Limpopo province. It is a 1 meter high weed with ovate leaves of 2.5-2.7 cm, a crawling or erect stem, purple flowers and rectangular seeds that appears once a year (Webster et al., 2005).

Traditionally *C. benghalensis* (Van Wyk et al., 2000) and *Commelina africana* (Watt et al., 1967) are used for infertility treatment. *C. benghalensis* is also traditionally used for pain, headcase, leprosy, constipation, fever, snakebites, jaundice (Kirtikar 1998; Ghani 2003; Hasan et al., 2010), epilepsy (Okello et al., 2007) and psychosis (Sumithra et al., 2017). Several studies have demonstrated the analgesic (Ghani, 2003; Hasan et al., 2010; Hossain et al., 2014), anti-inflammatory (Sambrekar et al., 2011; Tiwari et al., 2013; Alba et al., 2014),

anti-microbial properties (Kunle et al., 2009) and antioxidant (Anusuya et al., 2012; Prakash et al., 2014; Misra et al., 2016) effects of *C. benghalensis* as used in traditional medicines.

The chemical active substances of plants are where the medicinal value lies, of which alkaloids, tannin, flavonoid and phenolic compounds are the most important (Edeogal, 2005). The phytochemical screening of *C. benghalensis*, collected from Chaza village in Suleja, showed the following phytochemical groups: phlobatannins, carbohydrates, tannins, glycosides, volatile oils, resins, balsams, flavonoids and saponins. Whereas terpenes, sterols, anthorquinones and phenols were not present. Plant extractions were done with hexane, ethyl acetate and methanol (Chioma et al., 2010). The presence of tannis point to potential anti-viral and anti-bacterial effects. Important to this study is the presence of flavonoids and saponins indicate that C. *benghalensis* can have anti-cancer activities. (Kunle et al., 2009; Evans, 2002).

Using *n*-hexane fractionates and methanol extract of *C. benghalensis* and subjecting them to ^{1}H NMR (nuclear magnetic resonance) spectroscopy, *C. benghalensis* was found to have terpenoids and phytosteroids, which have antioxidant properties (Khatun et al., 2019). Karan et al. (2018) evaluated the effect of methanol leaf extract of *C. benghalensis* on mice with Ehrlich Ascites carcinoma and a decrease in EAC cells, inhibition of viable cells, reduced tumour volume, increased haemoglobin levels and increased lifespan of the treated mice were observed. This provided evidence of the anticancer activity of *C. benghalensis*.

Mbazima et al. (2008) used a crude methanolic stem extract from *C. benghalensis* in concentrations of: 0, 100, 250, 400 and 600 µg/ml on Jurkat T cells over a time of between 0 to 72 hours. At a higher concentration and longer exposure, cell proliferation and viability of the Jurkat T cells decreased. Non-cytotoxicity was proven on monkey Vero cells for methanolic extracts up to 500 µg/ml. Due to the fact that the viability of the Jurkat t cells decreased at higher concentreations, concentrations of 400, 500 and 600 µg/ml was selected for the study. The treatment with *C. benghalensis* increased *Bax* and p53 expression and decreased *Bcl-2,* therefore inducing apoptosis in a time- and dose-dependent manner. On the other hand, *C. benghalensis* acetone extracts of the plant stem, fractioned using *n*-hexane (F1) and dichloromethane (F2) also inhibited Jurkat T cell proliferation and viability in a time- and concentration-dependent manner (Lebogo et al., 2014). The longer the time (24 to 72 hours) and higher the concentration (40 to 90 µg/ml), the more Jurkat T cells growth was arrested. The extracts induced apoptosis and cell cycle arrest at G1/S interphase of the Jurkat T cells.

The effect of F1 and F2 fraction on cell division regulatory genes, *Bcl-2, bax, p21, cdc2* and *cyclin B1* were tested. The F1 treatment decreased *Bcl-2* levels and increased *bax, cdc2* and *cyclin B1* levels. The cellular concentrations of p53, p21, and cdc2 were undetectable at the protein level under the same experimental conditions. Contrastingly the F2 treatment led to an increase in mRNA of *bax, bcl2, cyclin B1* and *cdc2*. Protein expression of cdc2, bcl-2, cyclin B1 and p21 were also increased (Lebogo et al., 2014). *C. benghalensis* induced apoptosis in leukaemia Jurkat T cells and decreased cell viability in a dose and time dependent manner (Mbazima et al, 2008; Lebogo et al., 2014). Thereby, *C. benghalensis* addresses the main problems of uncontrolled cell growth in carcinogenesis and inhibition of apoptosis.

C. benghalensis thus showed anticancer properties in EAC cells (Karan et al., 2018) and Jurkat T cells (Mbazima et al, 2008; Lebogo et al., 2014) and therefore a candidate for research as an anticancer treatment. In the mentioned studies only leaves and or stems were used. There are currently no previous studies on the effect of *C. benghalensis* on cervical cancer cells or on their miR expression after being treated with *C. benghalensis* extract, this was explored in this research. Futhermore leaves, roots and stems were used as no previous research on *C. benghalenis* extracts have been done on HeLa cells.

3.6 CONCLUSION

In this research, understanding the role of miRs and cell cycle control are especially important for *C. benghalensis* induced apoptosis, as seen in Jurkat T-cells and EAC cells. As seen from Table 2.2 above, some apoptotic genes are directly or indirectly under the control of miRs in cervical cells. Genes that are involved in the regulation of apoptosis in cervical cancer and cell cycle related genes namely p53, Bcl2 and Bax, were tested by Mbazimba et al. (2008). There are increasing literature showing that miRs regulate cancer-, apoptosis- and cell cycle-related genes, for example, *Bcl-2* is a target of miR-34c and miR-143 (Chen et al., 2014; Cannel et al., 2010). Bcl-2 protein inhibits apoptosis and provides cell resistance to apoptosis stimulating factors (Wang et al., 2012). *Bcl-2* also regulates p53 gene activations and prevents cells from arresting in the G1 phase (Ryan et al., 1994). MiR-886-5p negatively regulates Bax and thereby inhibits apoptosis in cervical cancer cells (Li et al., 2011). As seen in Figure 2.5, miR-21 regulates p53 and caspase 3 through the tumour suppressor MEG3. Upregulation of miR-21 suppresses MEG3 and, in turn, suppresses caspase 3 and p53 (Zhang et al., 2015). Whereas normal miR-29 levels activate p53, miR-29 is downregulated in

cervical cancer, leading to suppression of p53 (Park et al., 2009). MiR-125a is a tumour suppressor which is downregulated in cervical cancer due to the suppressive effect of HPV on p53 (Fan et al., 2015). Cell cycle progression from G1/G0 phase to S phase is induced by upregulated miR-150 in cervical cancer, which also upregulates cyclinD1 (Li et al., 2015). Another gene that influences apoptosis and is under the control of miRs is miR-7 which downregulates XIAP. XIAP usually stops apoptotic cell death induced by viral infection such as HPV and apoptosis by overproduction of caspases (Liston et al., 1996; Duckette et al., 1998). Overexpression of miR-7 in cervical cancer downregulated *XIAP* and therefore enhances apoptosis (Liu et al., 2013). HPV, on the other hand, affects the cell cycle by affecting certain checkpoints in the cell cycle (Leemans et al., 2011).

The miRs selected to be used in this study affect the apoptosis pathway in one way or another. Since *C. benghalensis* caused apoptosis in Jurkat-T cells, the miRs that influence apoptosis are most likely to produce results.

2.6.1 THE MIRS USED IN THE EXPERIMENT THAT AFFECT APOPTOSIS PATHWAY IN CERVICAL CANCER: (Due to cost restrictions of the assay only 12 were selected)

1. miR-7
2. miR-20a
3. miR-21
4. miR-27b
5. miR-29a
6. miR-34a
7. miR-99a
8. miR-99b
9. miR-143
10. miR-182
11. miR-200a
12. miR-497

Table 2.3 is a summary of these twelve miRs that influence the apoptosis pathway in cervical cancer. The usual expression of these miRs in cervical cancer, the targeted genes and cellular process involved are reported. The table also gives information on the miRs location on the chromosome and, most importantly, how the information can be used in diagnosis, screening, treatment and the prediction of mortality and morbidity in cervical cancer. The types of

cervical cell lines used in the studies that were performed are also mentioned. Many of the miRs have the potential to be used as cancer treatments when their expression is changed in cancer cells and *C. benghalensis* could also play a role in this.

Table 2.3: Discussion of miRs involved in apoptosis

(Legend SCC-squamous cell carcinoma, AdeCC-adenocarcinoma).

MiRs expression in cervical cancer, histological type and gene location	**Cellular targets and pathways**	**Angiogenesis, metastasis and prognosis**	**Potential usage in cervical cancer treatment and screening**	**References**
miR-7 Upregulated HeLa and C33A cells 19q13.42	Overexpressed miR-7 downregulates *XIAP*, suppresses cell growth and promotes apoptosis in cervical cancer.		Use of *XIAP* as a new target involved in miR-7-mediated growth suppression and apoptosis for induction of cervical cancer treatment.	Liu et al., 2013.
miR-20a-5p SCC Upregulated 13q.31.3	MiR-20a leads to inhibition of *ATG7* and *TIMP2*, which are regulators of the autophagy and apoptosis pathway. *TNKS2* is affected by miR-20a that plays a role in the telomere extension in the telomerase pathway.	MiR-20a is marker for lymph node metastasis in early stages of squamous cell cancer. Downregulation of miR-20a can lead to smaller tumour size, lower histological grade and less lymph node metastasis.	Suppression of *TNKS2* leads to metastasis and suppression of invasion and could be used as a potential treatment.	Zhao et al., 2013; Zhao et al., 2015; Weidberg et al., 2011; Kang et al., 2012; Ingenuity Target explorer.
miR-21-5p Upregulated SCC, AdeCC	MiR-21 negatively regulates p53 and Cdc25 that is involved in regulation of cell proliferation and apoptosis. MiR-21 supresses *RECK* and	MEG3 is supressed in cervical cancer by miR-21-5p and is associated with increased tumour size, lymph node metastasis.	MiR-21 is a biomarker for cervical cancer and it modulates the radiotherapy response. The higher the expression of miR-21 the more resistant the cervical	Lui et al., 2015; Yao et al., 2009; Selcuklu et al., 2009; Ben et al., 2015;

17q23.2	*TPM1* which usually suppress metastasis. MiR-21 affects *PDCD4* and *PTEN* that induce apoptosis in malignant cells. MiR-21 overexpression increases *ATG2* and *TIMP2,* leading to invasion and metastasis.		cancer is to radiation by decreasing radiation induced G2/M block and increases S phase accumulation.	Shishodia et al., 2014; Zhang et al., 2015; Ogechukwu et al., 2015; Banno et al., 2014.
MiR-27b Upregulated CaSki and SiHa cells lines 9q22.32	Upregulated miR-27b downregulates Polo-like kianse2 *(PLK2)* gene. It also targets CDH11 and inversely TOB1-AS1and inhibits PPARγ.		Overexpression of miR-27b leads to increased cell invasion and inhibits paclitaxel induced apoptosis in cervical cancer cells. Reduced expression leads to restrained cell growth, hampers cell invasion and increased paclitaxel-induced cell apoptosis in cervical cell cancer. MiR-27b can be used in future to predict and use antisense nucleic acid for cervical cancer therapy.	Liu F. et al., 2016; Yoa et al 2018; Zang et al 2015.
miR-29a Downregulated SCC 7q32.2	YY1, CKD6 *HSP47* Normal miR-29 levels restrain cell cycle progression and induce apoptosis by activating p53 pathway.	Down regulation of miR 29a leads to angiogenesis and decreased apoptosis in cervical cancer.	Increasing miR-29 in cervical cancers cell leads to inhibition of cell migration and invasion through the upregulation of *HSP47*.	Yammamoto N., et al., 2013; Li et al., 2011; Park et al. 2009.
miR-34a Downregulated	p18Ink4c NOTCH1, JAGGED1, WNT1 MiR-34a is involved in P53	Can be used as a prognostic and survival predictor marker. Lower miR-34a expression has	MiR-34a/LDHA axis exhibits a tumour suppressive role in cervical cancer.	Li B., et al., 2010; Li et al., 2020; Wang X.,

CaSki and HeLa cells 1p36.22	dependant pathways namely cell cycle progression, cellular senescence and apoptosis.	more cancer metastasis, advanced cancer stage and shorter survival rate.		et al., 2011; Pang et al., 2010; Zhang R et al., 2016; Ribeiro et al., 2015; Chen et al., 2017.
miR-99a-5p Downregulated HeLa, SCC 21p21.1	Downregulated miR-99a leads to increase TRIB2 and increase in cervical cell proliferation. TRIB2 is part of the mitogen activated protein kinase pathway. MTOR is upregulated by downregulated levels of miR-99a and leading to increased cervical cell proliferation and invasion. Influencing the pI3K/AKT/mTOR pathway that is important in regulation the cell cycle, apoptosis and tissue development.	Downregulation of miR-99a increases angiogenesis and lymph node metastasis.		Xin J et al., 2013; Wang L. et al., 2014; Pereira et al., 2010.
miR-99b-5p Downregulated SCC 19p13.41	Downregulation of miR-99b increased mTOR leading to cell proliferation and inhibition. Therefore, miR-99 influences the pI3K/AKT/mTOR pathway that is important in regulation of the cell cycle.	MiR-99 usually inhibits cell proliferation and invasion. Downregulated miR-99 is negatively related to lymph node metastasis.		Wang L., et al, 2014.

miR-143 Downregulated HeLa, SCC 5q32	Bcl-2 is a direct target of miR-143 and decrease of miR-143 leads to increase in cell apoptosis and tumour formation. Other targets are OIP5-AS1, MSI- 2 and HIF 1α.	Downregulated miR-143 levels are associated with increased tumour size, lymph node metastasis and can be used as a reference of severity of cancer before surgery.	MiR-143 does not influence Taxol sensitivity. It can possibly be used for diagnosis for cervical cancer.	Liu L.et al., 2012; Huang et al., 2012 Chen Y., 2014; Banno et al., 2015. Lui et al., 2018; Song et al., 2020; Zhao et al., 2021.
miR-182 Upregulated HeLa, SiHa, C33A, ME180 CaSki, CC2, CC3 7q32.2	MiR-182 negatively regulates FOXO1 regulation and upregulated miR-182 leads to decrease in apoptosis.	Lnc RNA PCGEMI promotes cell proliferation migration and invasion by targeting the miR-182 /FBXW11 pathway.	Inhibition of miR-182 could lead to tumour growth recession.	Tang et al., 2013, Javudi et al., 2018, Zang et al., 2019.
miR-200a-3p Upregulated SCC, AdeCC 1p36.33	Upregulation of miR-200a leads to over-expression of MMP-2, MMP-9 and suppression of RECK gene (Angiogenesis pathway, tumour invasion and metastasis). TIMP1 is a target of miR-200(Apoptotic pathway). ZEB1 and ZEB2 are downregulated by miR-200a.	MiR-200a is associated with metastasis and invasion of cervical cancer and can therefore act as a master suppressor for cervical cancer metastasis, predict metastasis and patient prognosis.		Wang L. et al., 2013; Hu et al., 2010; Zhu et al., 2018.

	TGFB2 and EXOC5 are targeted by miR-200a (Apoptosis pathway). F4T competitively binds with miR200a, which is upregulated by LNCRNA XIST, accelerating cancer progression.			
miR-497 Downregulated Cervical cells, HeLa, CaSki, SiHa 17p13.1	IGF-1R is negatively regulated by miR-497(Apoptotic pathway). MiR-497 downregulation increases CCNE1 (Cell cycle).	Low miR-497 levels in patients have a poorer survival level, higher FIGO stage and lymph node metastasis.	Overexpression of miR-497 could suppress CCNE1 and lead to cervical cell proliferation suppression as well as decreases growth of cervical cancer cells by inducing Capese-3-dependant apoptosis and reduces invasion.	Luo et al., 2013; Han et al., 2014.

As evident from the literature review, there is no data available on how the anti-cancer properties of *C. benghalensis* extracts work and whether they have an apoptotic effect on cervical cancer cells. From the discussion above it is deduced that *C. benghalensis* influences apoptosis in leukaemia Jurkat cells and EAC cells. Some genes responsible for apoptosis are under the control of miRs. As defined in the research question in section 1.3, can *C. benghalensis* acetone extracts have anticancer activities against HeLa cells as well and whether this plant regulates miRs related to cervical cancer?

4 CHAPTER THREE: MATERIALS AND METHODS

4.1 INTRODUCTION

This chapter covers the materials and the methods used to achieve the aim and objectives of this study. The materials reported include the plant material (section 3.2.1), the cells used namely HeLa cell line (section 3.2.2) and the biochemical consumables (3.2.3) utilized in the study. Lastly, the chapter explains the methods (section 3.3) that were performed in this study.

4.2 RESEARCH MATERIAL

4.2.1 Plant material

In this study, a complete *C. benghalensis* plant was collected and authenticated from Polokwane, Limpopo province by Prof Zukile Mbita, February 2017. It was identified at the University of Limpopo's herbarium (voucher number: UNIN121047). The leaves, stems and roots were used.

4.2.2 CERVICAL CANCER CELL LINE

The cervical cancer cell line, HeLa S3 (ATCC nr: CCL-2.2), 20-40 μm in size, was donated by Dr A. Skepu from Mintek, Biolabels Unit, Johannesburg, South Africa. HeLa cells were originally derived in 1952 from a 31-year-old patient with adenocarcinoma cervical cancer, Henrietta Lacks, hence the name. HeLa cells, which were the first cell line to be immortalized, were established by George Otto Grey, and were characterised as containing HPV 18 (Gey et al., 1952; Jones et al., 1971). The HPV 18 genome is primarily integrated on chromosome 8q23 to q 24 as well as chromosome 9q31 to q34 and abnormal chromosomes 5 and 22q12 to q13. These sites are at or in proximity of chromosomal fragile sites (Popescu and dePaolo, 1987).

4.2.3 SOLUTIONS AND KITS

The solutions and kits that were used to perform the experiments can be found in Appendix B and C.

4.3 METHODS

In this section the methods of the acetone *C. benghalensis* plant extraction, HeLa cell culturing, maintenance, sub culturing and seeding are discussed. Furthermore, the treatment of the HeLa cells with the *C. benghalensis* plant extracts after which thiazolyl blue tetrazoium bromide (MTT) assay for metabolism is described. Next flow cytometry for apoptosis analysis is explored after HeLa cell treatment with *C. benghalensis* extracts. Lastly the effect of the *C. benghalensis* extracts on the miRs expression are examined by real time quantitative reverse transcriptase.

4.3.1 PHYTOCHEMICAL EXTRACTION

C. benghalensis roots, stems and leaves were separately dried in an oven for two weeks. The dried roots, stems and leaves were separately ground to fine powder using a Knifetec 1095 sample mill (Foss, Sweden) and stored in induvidual bags until further processing.

Powder (1 g) from each plant part (either leaves or stem or roots) was extracted separately using 10 ml absolute acetone as an extraction solvent, in multiples of the formulation 1 g plant powder to 10 ml acetone. The mixtures were then shaken in a Scientific Incubator Shaker 353 (Scientific Systems Co Inc, USA) at 150 rpm for 24 hours. The extracted material was filtered through Whatman No. 3 filter paper and centrifuged at 1960 x g for 20 minutes. After the first centrifugation, only the supernatant was retained. The combined supernatant and solvents were left to dry in vials under a fume hood. The dried extracts were then reconstituted with 1:10 dilution of DMSO in DMEM (10% DMSO in DMEM) to a concentration of 10 mg/ml.

4.3.2 CELL CULTURING AND MAINTENANCE

HeLa cells were used as a cervical cancer model cell *in vitro* model in this study to determine the potential anticancer activity of *C. benghalensis* plant extracts. The growth media for the HeLa cells were prepared from 445 ml Hyclone Dulbecco's modified eagles medium, 5% (50 ml) Hyclone research grade fetal bovine serum (FBS) and 1% (5 ml) penicillin streptomycin and made up to total of 500 ml, stored at 4 ^{o}C. The HeLa cells were cultured in this media and maintained at 37^{o}C in conditions of 5% CO_2. The cells were grown in 25 cm^3 corning flasks, T-25, and viewed under the BF inverted microscope (Olympus, USA), to confirm viability and observe their morphology.

4.3.3 SUBCULTURING

When the cells reached 80% confluency, as confirmed under the BF inverted microscope (Olympus, USA), the cells were sub cultured into 75 cm^3, (T75) corning flasks. Trypsin-EDTA (diluted to 1X using PBS) was pipetted into the flask containing cells. The flasks were gently swirled and incubated at 37 ^oC for 5 minutes till the cells started to float. If cells were still adherent to the flask after 5 minutes of incubation, the flask was gently tapped against the palm of the hand to loosen them. Once the cells were no longer adherent, 5 ml complete growth medium was added to deactivate trypsin. Cells were again viewed under the BF inverted microscope to confirm that trypsinization had ceased.

The trypsinized cells were pipetted into a 15 ml centrifuge tubes and centrifuged for 5 minutes at 560 x *g*. The supernatants were decanted, and the pellets were resuspended in complete growth media. The cell suspensions were diluted 1:4 in complete media and plated in T-25 cell culture flasks and allowed to grow in CO_2-containing humidified incubator at 37 $^o C$.

4.3.4 CELL SEEDING

Prior to seeding (at 100 x 10^5), cell viability was determined using the trypan blue exclusion method. Ten microliters (10 µl) of the cells were dislodged using a cell scraper and mixed with 10 µl of trypan blue. The trypan blue-cell mixture (10 µl) was used to count the cells, utilizing the haemocytometer under the microscope. Trypan blue excludes dead cells from live cells. Live cells are colourless and bright while the dead cells membranes stain blue and are non-refractive. The cell populations in 4 outer quadrant and middle block on the haemocytometer were counted (Absher, 1975). The total cells per ml were determined using the following equation:

$$Total\ per\ cells\ ml = \frac{total\ cells\ counted\ x\ dilution\ factor}{total\ squares\ counted}\ x\ 10\ 000\ cells$$

4.3.5 TREATMENT OF THE HELA CELLS

HeLa cells in DMEM media were seeded into two 96 well plates for 3-(4,5-dimethyltriazol-2-yl)2,5-diphenyl-2H-tetrazolium bromide (MTT) assay. About 100 x 10^5 HeLa cells were seeded into each well. The plates were then incubated for 24 hours, after which the plant extracts were added as well as the positive control (hydrogen peroxide). All experiments were performed in triplicates. The concentrations of 400 µg/ml (4%), 500 µg/ml (5%) and 600

µg/ml (6%) were used for the root, stem and leaf extracts as per the previous experiment done by Mbazimba (2008). In the experiment by Mbazimba, monkey Vero cells showed no toxicity up to the 500 µg/ml of extract of *C. benghalensis*, as mentioned in section 2.4.1. Hydrogen peroxide (5%) and DMSO (5%) were used as positive and negative control, respectively.

4.3.6 THIAZOLYL BLUE TETRAZOLIUM BROMIDE (MTT) ASSAY FOR CELL METABOLISM DETERMINATION

All plates were incubated for either 24, 48 or 72 hours, after which MTT assay was performed. Exactly 20 µl of 5 mg/ml MTT in PBS was added to each well and then left to incubate for 3 hours at 37°C until formazan crystals were formed, which were viewed under the inverted light microscope. The formazan crystals were dissolved by adding 100 µl DMSO into wells, followed by a further 30 minutes incubation. The absorbance of the dissolved formazan was read at 570 nm using a spectrophotometer (Thermo Scientific Varioskan Flash, USA).

The readings were then used in the following calculation:

$$\%Cell\ viability = \left(\frac{A_{treated\ cells} - A_{Blank}}{A_{control} - A_{blank}}\right) x\ 100$$

A – $Absorbance_{570nm}$

See appendix D for these readings and calculations.

4.3.7 FLOW CYTOMETRY FOR APOPTOSIS ANALYSIS

Flow cytometry testing was done with annexin V and propidium iodine to determine whether apoptosis of the HeLa cells occurred after treatment with *C. benghalensis* extracts. This is performed by annexin V binding to phosphatidylserine that is released from the cytoplasmic surface of the cell when the cell membrane breaks down in apoptosis. Annexin V is a calcium-dependant phospholipid binding protein that has a high affinity for phosphatidylserine (PS). Annexin V is labelled with a fluorophore that is activated when bound to PS and detected by the flow cytometer. Propidium iodide (PI) stains dead cells with red fluorescence but is unable to penetrate live and apoptotic cell. Therefore, annexin V show

green fluorescence in apoptosis and dead cells show red and green fluorescence (Fried et al., 1975; Vermes et al,, 1995).

Extracts of *C. benghalensis* at 400 and 500 µg/ml, were used to study the induction of apoptosis HeLa cells. These concentrations led to the greatest cessation of metabolism in HeLa cells with MTT without toxicity to the cells. At the concentration of 600 µg/ml *C. benghalensis* had toxic effect on monkey Vero cells and was therefore not considered for further experiments (Mbazimba, 2008). Additionally, with flow cytometry, it was also determined whether there were live cells, early and late cell apoptosis as well as cell necrosis.

4.3.8 FLOW CYTOMETRY METHOD FOR APOPTOSIS IN HELA CELLS

Apoptosis induction in treated HeLa cells was analysed using annexin V/PI kit (Biotium, USA) using the BD FACS Aria II flow cytometer (BD Lifesiences, USA). Manufacturer's instructions were used. The binding buffer was prepared by diluting 1 ml of 5X annexin V binding buffer with 4 ml distilled water. Propidium iodide was made by diluting 2 µl of PI in 18 µl of the prepared binding buffer.

Cells were seeded in two 6 well plates as per protocol. Each well contained 3 ml media with cells at a density of 1 x 10^5 cells/well, determined by haemocytometer cell counting, as discussed elsewhere (Section 3.2.3).

The volume was worked out with the following formula:

V1C1=V2C2

Cells were treated with 400 µg/ml and 500 µg/ml extracts. The concentration of DMSO in each plant extract was less than 300 µl/ml (3%) and should have no effect on the biological assay (Houghton and Raman, 1998), therefore separate testing on DSMO was not done.

After treatment with the stem and leaf extracts, the cells were incubated for 24 hours at 37 ^{o}C. Subsequently, the cells were scraped off the bottom of the wells using a cell scraper and then transferred into small Eppendorfs. This was then centrifuged for 5 minutes at 560 x *g* and the supernatant discarded. The cell pellets were washed with PBS and resuspended in 100 µl 1X PBS with 5 µl annexin V and 1 µl PI into tubes. Thereafter, this was incubated for 30 minutes

in the dark at room temperature. Afterwards, 400 µl 1X binding buffer was added to each tube.

The cells were kept on ice until the flow cytometry was performed with the BD FACS Aria II machine (BD Biosciences, USA), with fluorescence emission of 530 nm, cell sorter nozzle of 85 µl, as HeLa cells are between 20 and 40 µm in diameter. Furthermore, a FSC ND (neutral density) 2 filter was used as it relays a set percentage of light and reduces the intensity transmitted equally by all wavelengths (BD FACS Aria II User guide, USA). The fluorochrome lasers, CF Alexa 488 (Blue) was used to detect annexin V and PE-CF594 (orange) was used to observe PI. Alexa Fluor 488 has a maximum excitation of 495 nm and maximum emission of 519 nm, whereas PE-CFS594 has maximum excitation at 535 nm and maximum emission of 612 nm. Live cells show low level fluorescence, apoptotic cells show green fluorescence and dead cells should show both green and red fluorescence (Biotium, CF 488A annexin V and PI Apoptosis kit).

4.3.9 REAL TIME QUANTITATIVE REVERSE TRANSCRIPTASE PCR

In order to determine if the *C. benghalensis* extracts influenced the expression of the twelve apoptosis related miRs identified elsewhere (section 2.5), real time quantitative reverse transcription PCR was performed after reverse transcriptase of miRNA to cDNA was done.

I. TOTAL RNA EXTRACTION

HeLa cells were grown in 6 well plates till 80% confluency was reached. The cells were treated with 400 µg/ml and 500 µg/ml leaf and stem extract for 24 hours. These concentrations were used as they led to cytotoxicity, confirmed by the MTT assay, as well as increased proportion of apoptotic cells, confirmed by flow cytometry. At these concentrations the extracts were not toxic to Vero cells (Mbazimba et al., 2008).

II. RNA PURIFICATION

Isolation and purification of miRs were done using a RNA isolation protocol (Pattern, abcam.com), following the method developed by Chomczynki et al. (1995). For reverse transcriptase, the negative control and HeLa cells treated with the *C. benghalensis* extract were harvested at 1 x 10^5 from each of the wells and labelled by scraping the cells off and then pipetting into 2 ml Eppendorfs. There were 5 tubes in all, namely the: untreated HeLa cells, HeLa cells treated with 400 µg/ml stem extract, HeLa cells treated with 500 µg/ml stem

extract, HeLa cells treated with 400 µg/ml leaf extract and HeLa cells treated with 500 µg/ml leaf extract.

The medium was aspirated off and the cells were washed with cold PBS. The cell pellets were then spun at 300 x *g* for 5 minutes. The media was removed, and the cells resuspended in ice cold PBS. Subsequently the cells were pelleted by spinning them at 300 x g for another 5 minutes. 1 ml of TRizol reagent (Qiagen, USA) was added to each tube (1 ml TRizol was used per 1 x 10^5 of cells). The cells were lysed by repetitive pipetting. The homogenized samples were incubated for 5 minutes at room temperature for complete nucleoprotein complex dissociation. After the incubation period the homogenates were centrifuged at 300 x *g* to remove cell debris and the supernatant transferred to new 2 ml tubes.

0.2 ml chloroform per 1 ml of TRizol reagent was added to each tube. The contents were vortexed for 15 seconds and incubated at room temperature for 5 minutes, after which the tubes were centrifuged for 5 minutes at 12000 x *g* at 4 ^{o}C. After the centrifugation, the mixture was separated into a lower red, phenolchloroform phase, an interphase and a colourless upper aqueous phase. The RNA was exclusively contained in the upper phase. This was carefully pipetted and transferred into 1.5 ml tubes. The aqueous phase volume was 60 percent of the volume of the TRIzol reagent added (about 600 µl).

The RNA was precipitated from the aqueous phase by adding 500 µl isopropyl alcohol (per 1 ml of TRIzol reagent used earlier). The samples were then incubated at room temperature for 10 minutes and thereafter centrifuged at 12 000 x *g* for 10 minutes at 4 ^{o}C. The supernatant was completely removed, and the RNA pellet was washed using 1 ml 75% ethanol. The samples were mixed by vortexing and centrifuging at 7500 x *g* for 5 minutes at 4 ^{o}C. All the ethanol was then removed by pipetting. This procedure was repeated once, and all remaining ethanol pipetted out. The RNA pellet was left to dry for 10 minutes and dissolved in 15 µl RNAse free water. Spectrophotometry and RNA electrophoresis were done to determine the integrity of the RNA for the PCR reaction.

III. QUANTITATION OF RNA

The RNA was then quantified by the BioDrop unit (BioChrom, UK), which uses low volume spectrometry. The spectrophotometer measures the amount of ultraviolet radiation absorbed by the nucleotide bases. The BioDrop microlite pathway was used and thus only 0.5 µl of each of the samples were tested.

IV. REVERSE TRANSCRIPTION

Reverse transcription is the process whereby mRNA, or in the case of this study microRNA, is a template for synthesis of a complementary (cDNA) by a reverse transcriptase. For reverse transcription, the following were needed, namely: parent miRs, primer, dNTPs, reverse transcriptase and DNA polymerases.

For the reverse transcription of the RNA, the miScript II RT kit was used. The RNA template was thawed on ice. The 10 x miScript Nucleics mix and the 5 X miScript HiFlex buffer were mixed by flicking the tubes. The tubes were briefly centrifuged to collect all residual liquid and stored on ice. The reverse transcriptase reaction mix was prepared on ice as tabulated in table 3.1:

Table 3.1: Reverse transcriptase mixture.

Component	**Volume**
5 x miScript Hiflex buffer	4 µl (20%)
10 x miScript Nucleics mix	2 µl (10%)
RNase free water	10 µl (50%)
miScript Reverse Transcriptase mix	2 µl (10%)
Template RNA	2 µl (10%)
Total volume	**20 µl (100%)**

Once all the components were added and lastly the template RNA, the tubes were briefly centrifuged and placed on ice. Thereafter, the tubes were incubated for 60 minutes at 37 ^{o}C and the miScript reverse transcriptase was inactivated by incubating the mixture for 5 minutes at 95 ^{o}C. Then the tubes were frozen at -20 ^{o}C, to do real time PCR, the next day.

V. REAL TIME QUANTITATIVE REVERSE TRANSCRIPTASE PCR

As noted in the literature review, the expression patterns of twelve miRs (section 2.5.1) were investigated as they are related to apoptosis. The miR primers were procured from Qiagen, USA and custom made to specifications (See Appendix F for miR sequences and primers).

The following precursor miRs were targeted:

1. miR-7
2. miR-20a
3. miR-21
4. miR-27b
5. miR-29a
6. miR-34a
7. miR-99a
8. miR-99b
9. miR-143
10. miR-182
11. miR-200a

MiR-497 was not available as a precursor of the miR from Qiagen, therefore a mature miRNA was used. For accurate and reproducible results for the real time PCR of the miRNA, the amount of target miRNA needed to be normalized by an endogenous reference RNA. In this experiment the housekeeping gene glycerakdehyde 3-phosphate dehydrogenase (GAPDH) [New England Biolabs, USA] was used as indicated by the protocol of miScript PCR kit.

The precusrsor and mature miRs were used for real time quantitative PCR and this was performed using the miScript SYBR Green PCR kit using the StepOnePlus Real time PCR system (Applied Biosystems, USA).

The miScript precursor assays and miScript mature assay were shipped lyophilized and reconstituted in 550 µl TE. All the reagents (2 x QuantiTEct SyberGreen PCR master mix, 10 x miScript universal primer and RNase free water) and the cDNA were thawed on ice. A 96 well PCR plate was used (The plate set up can be seen in Appendix G).

All the miRs are precursors, except for miR-497 which is a mature miRs and has a different mix.

> For the pre-miRs (miR-7, miR-20(a), miR-21, miR-27b, miR-29a, miR-34a, miR-99a, miR-99b, miR-143, miR-182, miR-200a), the mixture per 96 well was prepared as tabulated in Table 3.2:

Table 3.2: Precursor miRs mixture.

Component for precursor miRs mix	**Volume per well**
2 x QuantiTEct SyberGreen PCR master mix	12.5 µl (50%)
10 x miScript universal prime	2.5 µl (10%)
RNase free water	7.5 µl (30%)
Template c DNA (added last)	2.5 µl (10%)
Total volume	***25 µl (100%)***

1. For miR-497, the reaction mixture for the 96 well plate was prepared as tabulated in table 3.3:

Table 3.3: Mature miR mixture.

Component for mature miRs	**Volume per 96 well**
2 x QuantiTEct SyberGreen PCR master mix	12.5 µl (50%)
10 x miScript universal prime	2.5 µl (10%)
RNase free water	5 µl (20%)
10 x miScript Primer assay (miR 497)	2.5 µl (10%)
Template cDNA (added last)	2.5 µl (10%)
Total volume	***25 µl (100%)***

A master mix reaction was prepared and pipetted into wells by a multichannel pipette. Then 2,5 µl template cDNA was added to each well. The PCR plate was then tightly sealed with the film and centrifuged for 1 min at 1000 x *g* at room temperature. Thereafter, the PCR plate was subjected to respective thermal cycles using the RT-qPCR machine, namely the Light Cycler 480 (Roche, Switzerland). Ramp temperature was set to 1 ^{o}C. To activate the process

of the PCR, denaturation for 15 minutes at 95 °C was done. Thereafter, a 3-step cycle was done for 45 cycles: 15 seconds at 94 °C to denature the DNA, 30 seconds at 55 °C for annealing of primers and 30 seeconds at 70 °C to extend DNA.

VI. REVERSE TRANSCRIPTION REAL TIME PCR CALCULATION METHOD

The RT-qPCR data was analysed by relative quantification using the ddCT method in excel (Livak et al., 2001). This method was used to determine the fold change of the miRs levels in treated cells against untreated HeLa cells (See appendix G for the calculations as done in excel from the raw data).

The average values of the expression levels were used. The cycle threshold (CT) normalization of the target gene was done against GAPDH [2ˆ (CT (GAPDH – CT (miRs)]. After this, normalization of ddCT of the miRs in treated cells was done against the miRs in untreated HeLa cells, using the equation below:

HeLa miR control expression = $\frac{Untreated\ HeLa\ cells\ MiRs}{Untreated\ HeLa\ cells\ miR}$

MiR treated expression = $\frac{Treated\ HeLa\ cells\ miRs}{Untreated\ Hela\ cells\ miR}$

Lastly, the fold change was determined with the ddCT method, using the equation below:

ddCt = dCt treated HeLa MiRs – dct untreated HeLa MiRs.

The fold change was then determined by miR relative to untreated Hela cells by using the equation:

= 2^ -ddct.

(Livak et al., 2001)

5 CHAPTER FOUR: ANTICANCER ACTIVITIES OF *COMMELINA BENGHALENSIS* AGAINST HELA CELLS

5.1 INTRODUCTION

The purpose of this study was to determine whether the *Commelina benghalensis* root, stem and leaf acetone extracts influence the metabolic activity of HeLa cells or cause apoptosis. Furthermore, the effects of the *C. benghalensis* extracts on the expression of 12 microRNAs involved in apoptosis in HeLa cells were studied. In this chapter, the metabolic cessation, the apoptosis analysis and the miRS expression in *C. benghalensis* extracts-treated HeLa cells are discussed. The MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] was used to determine the metabolic activity of the HeLa cells after treatment with different concentrations of various extracts of the leaves, stems and roots of *C. benghalensis*. The root extract had no influence on metabolic activity of HeLa cells. However, the stem and leaf extracts exhibited cytotoxic effects against the HeLa cells and were thus selected for subsequent experiments. Annexin V/propidium iodide flow cytometry testing was used to confirm the induction of apoptosis in HeLa cells after treatment with *C. benghalensis* stem and leaf extracts. Lastly, reverse transcription real time PCR was used to investigate the effect of the *C. benghalensis* stem and leaf extracts on the 12 selected miRS.

5.2 PLANT EXTRACT YIELD

The dried plant extracts of *C. benghalensis* plant segments yielded different amounts of extracts, as described in Table 4.1. The percentages were calculated by dividing the amount of dried extract by the total plant extract used (method as per section 3.2.1). The average percentage of each acetone extract was calculated, with the leaves yielding the most extract at 1.55%, the stems yielding second most at 1.27% and the roots yielding the least at 0.8%.

Table 4.1: Acetone extract yield amounts of C.bengalensis roots, leaves and stems and yield percentages

(Extraction done on different occasions as the need for plant extracts arose. Therefore, in later experiments no further roots extracts were needed, as only the stems and leafs were used).

	100 g to 1000 ml	**%**	**5 g to 50 ml**	**%**	**20 g to 200 ml**	**%**	**5 g to 50 ml**	**%**	**5 g to 50 ml**	**%**	**100 g to 1000 ml**	**%**	**Average %**
Roots	0.77	0.77	0.0419	0.83	-	-	-	-	-	-	-	-	0.8
Stems	1.14	1.14	0.0813	1.62	0.2606	1.30	0.0579	1.16	0.060	1.20	1.171	1.17	1.27
Leafs	1.39	1.39	0.074	1.48	0.2571	1.29	0.074	1.49	0.75	1.5	1.162	1.16	1.55

5.3 *COMMELINA BENGHALENSIS* LEAF AND STEM EXTRACTS REDUCE THE VIABILITY OF HELA CELLS

The MTT assay was conducted on *C. benghalensis* extracts-treated HeLa cells after 24, 48 and 72 hours. The results were analysed as per the formula described in section 3.5 and the calculations were done in excel (Appendix D). The viability of untreated control cells, treated cells and the DMSO control HeLa cells were compared to determine the effect of the various *C. benghalensis* extracts. The results are presented as mean and standard deviation (± SD) of three independent triplicate experiments. Dimethylsulfoxide (DMSO) 1% was used as a solvent control and, as expected, did not have any effect on the metabolic rate of the HeLa cells, as depicted in Figure 4.1, 4.2 and 4.3. Hydrogen peroxide was used as positive control. HeLa cells were used as negative control at 100%, and not depicted in the figures.

In Figure 4.1, after 24 hours of treatment, the root extract of *C. benghalesis* induced the least reduction of metabolic rate of HeLa cells. Treatment with this extract at 400, 500 and 600 µg/ml resulted in metabolic rate of 56.7, 54.7 and 55.04%, respectively. The stem and leaf extracts, at 400 µg/ml, decreased metabolic rates to lowly 38 and 33%, respectively. As shown in Figure 4.1, the leaf extract had the best cytotoxic effect on cells, with cell viability of 32.8, 41.7 and 45% recorded for cells treated with 400, 500 and 600 µg/ml, respectively.

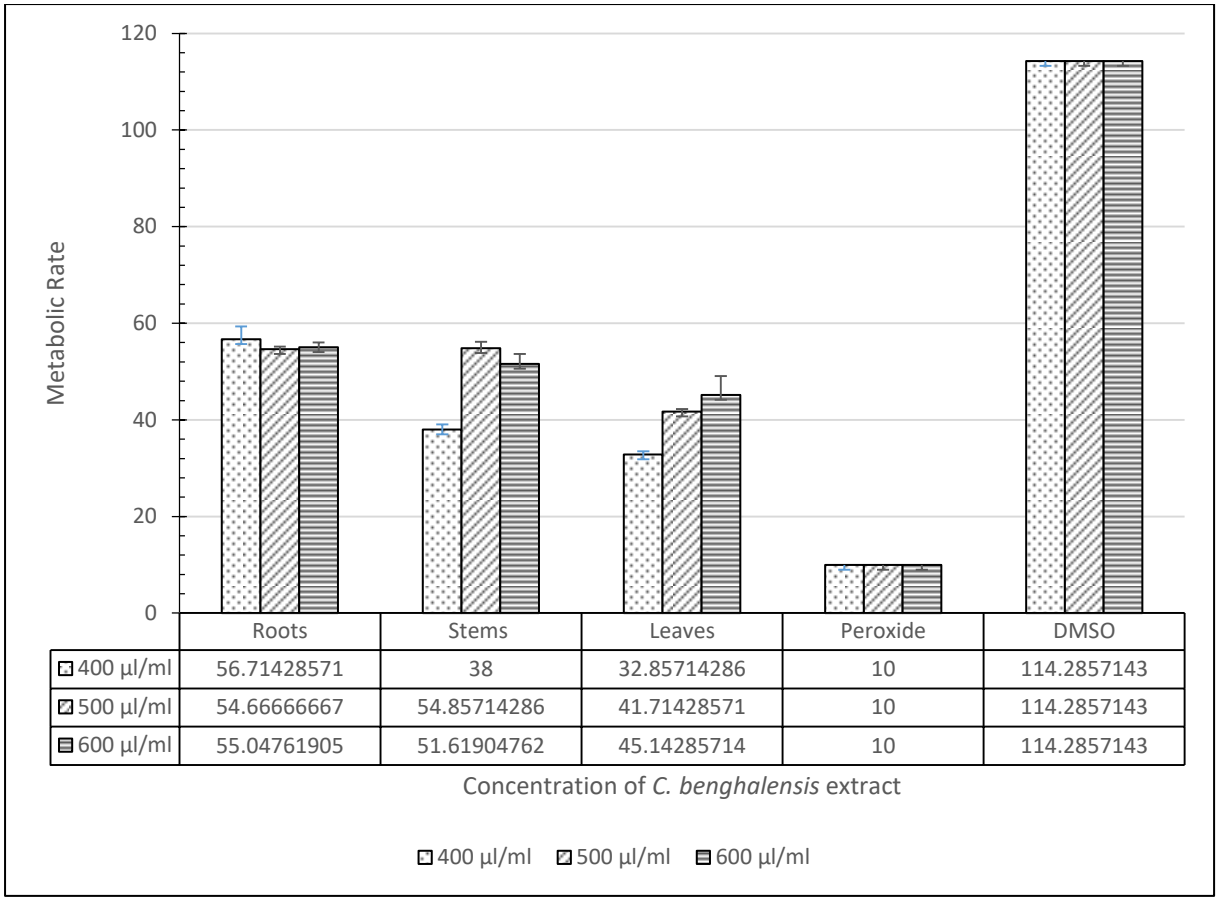

Figure 4.1: Cell viability of HeLa cells treated for 24 hours with 400 µg/ml, 500 µg/ml and 600 µg/ml C. benghalensis extract.

In Figure 4.2, 48 hour post-treatment, the *C. benghalensis* root extract increased the metabolic rate to 395, 502 and 199 % at concentrations of 400, 500 and 600 µg/ml, respectively. The stem extract maintained metabolic rate at about 65.32 % for 400 µg/ml, 57.49 % for 500 µg/ml and 54.75 % for the 600 µg/ml treatment. The leaf extract decreased metabolism rate of the treated HeLa cells to 53.60 % , 58.35 % and 54.97 % after a 48 hour treatment with 400, 500 and 600 µg/ml, respectively.

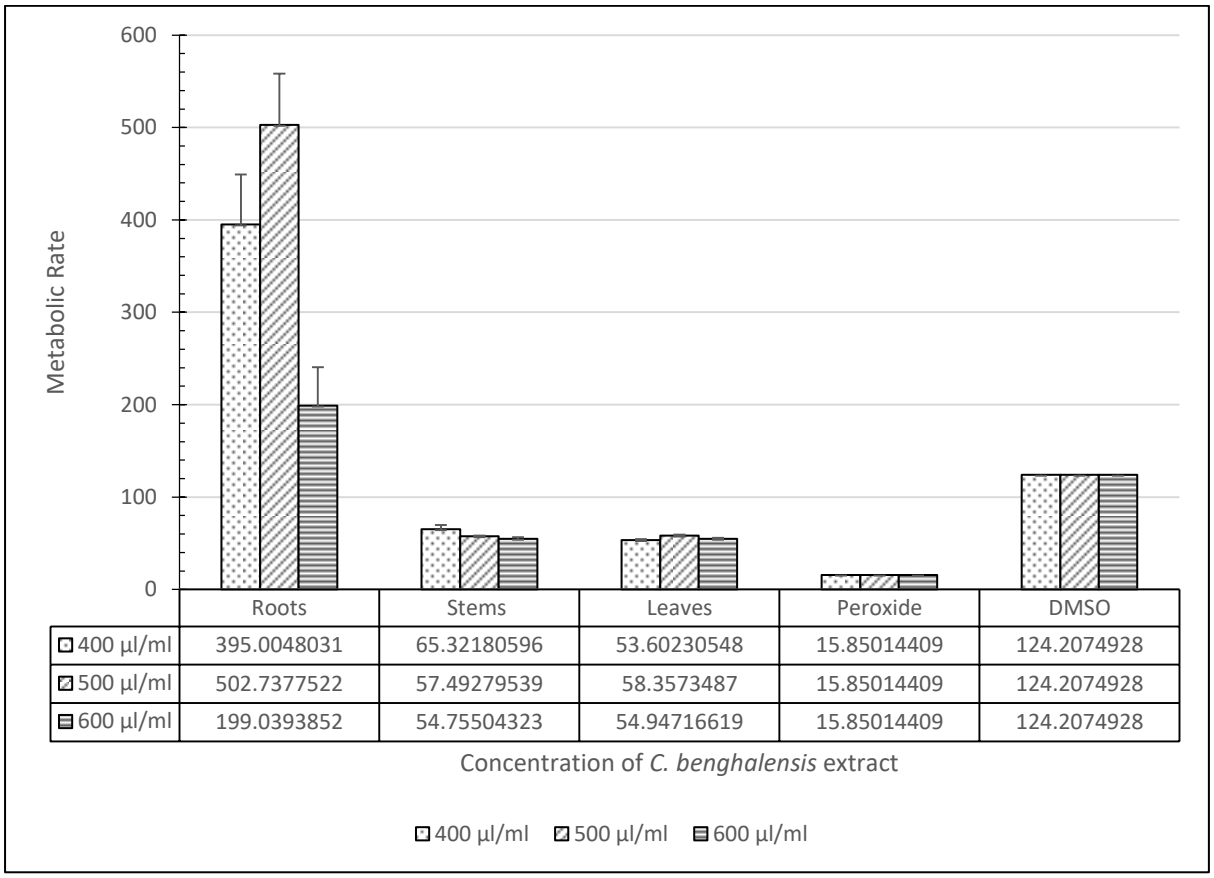

Figure 4.2: Viability of HeLa cells treated for 48 hours with 400 µg/ml, 500 µg/ml and 600 µg/ml C. benghalensis extract.

In Figure 4.3, 72 hours after treatment of HeLa cells with root extract of *C. benghalensis* resulted in a marked increase in metabolic rate, reaching levels as high as 259.19, 138.16 and 87.76 % at 400, 500, and 600 µg/ml, respectively, compared to control cells treated with DMSO. The stem extract decreased the metabolic rate to the levels of 36.63, 39.59 and 40 % at 400, 500 and 600 µg/ml, respectively. The leaf extract led to a decrease in metabolic rate to 41.46, 50.54 and 40.58 % at 400, 500 and 600 µg/ml, respectively.

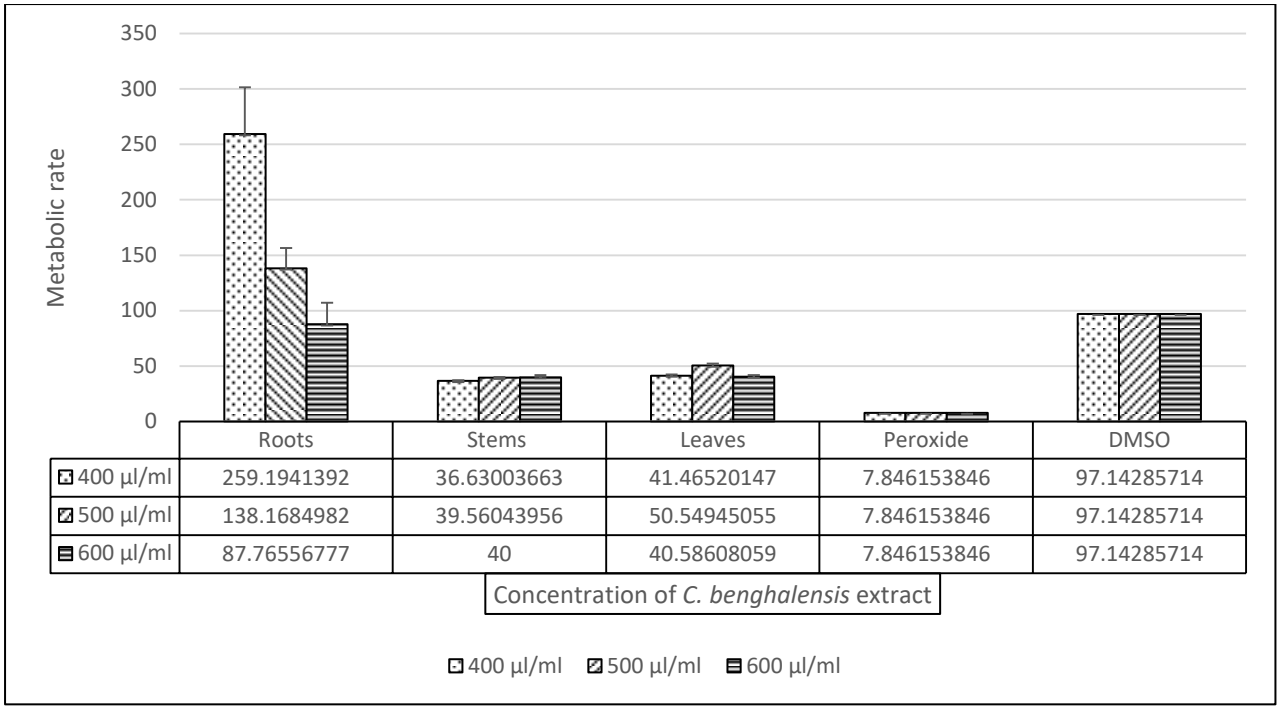

Figure 4.3: Percentage cell viability of HeLa cells treated for 72 hours with 400 µg/ml, 500 µg/ml and 600 µg/ml C. benghalensis extracts.

After 24 hours treatment, all treatments decreased the viability of the HeLa cells. However, after treatment for 48 and 72 hour, only the stem and leaf extracts decreased metabolic activity, whereas the root extract instead increased the metabolic acivity of the treated HeLa cells. Thus, futher anticancer activity of *C. benghalensis* was only investigated using the stem and leaf extracts. These are also the plant parts of the plants used by Mbazima et al. (2008); Lebogo et al., (2014) and Karan et al., (2018 that had anticancer properties. After 24 hour incubation period, the metabolic levels of cells treated with the stem and leaf extracts were in fact less than at 48 hours of treatment. Consequently, 24 hour treatment seemed more informative and was used henceforth.

5.4 *COMMELINA BENGHALENSIS* LEAF AND STEM EXTRACTS TRIGGER APOPTOSIS IN HELA CELLS

Flow cytometry was performed to determine the mechanism behind the reduction in cell viability of the treated HeLa cells and annexin V and propidium assay demonstrated that the *C. benghalensis* extracts induced apoptosis. The HeLa cells were treated with 400 and 500 µg/ml leaf and stem extracts of *C. benghalensis* for 24 hours. The 24 hour treatment was used from here onwards as there was significant metabolic cessation after this period, as was seen in the previous section (Figure 4.1). The selection of the concentration of the extract to use in assays was guided by previous work performed using *C. benghalensis* by Mbazima et al.

(2008). As previously reported (Section 3.3.7), concentrations up to 500 µg/ml had no cytotoxic effect on the monkey Vero cells and had anticancer properties in Jurkat T-cells at this concentration. For this reason concentrations above 500 µg/ml were not considered in subsequent experiments.

The annexin Alexa vs PI plots from the gated cells show the populations corresponding with viable and non-apoptotic cells (Annexin V-/PI-), early apoptosis (Annexin V+/PI-) and late apoptosis (Annexin V+/ PI+). Figure 4.4 includes the dot plot and histograms of each experimental group namely: [A] HeLa treated with 400 µg/ml stem extract, [B] HeLa treated with 500 µg/ml stem extract, [C] HeLa treated with 400 µg/ml leaf extract, [D] HeLa treated with 500 µg/ml leaf extract, [E] HeLa treated with Peroxide and [F] untreated HeLa cells. Gates are used to define a subset of data as in this case the live cells as P1 to see them in the density plot. The histograms present single parameter data, either Alexa or PI. The horizontal axis presents the increasing signal intensity of the parameter and the vertical axis represents the number of events.

Figures 4.4 and 4.5 show HeLa cells treated with either 400 µg/ml or 500 µg/ml stem or leaf extract for 24 hours and stained with annexin V/PI stain, respectively. In Figure 4.4A, the treatment of HeLa cells with 400 µg/ml stem extract resulted in 18.4% and 12% of the cells in late and early apoptotic stages, respectively. Treatment of HeLa cells with 500 µg/ml stem extract for 24 hours induced apoptosis in 29.6% of the cell population, with 11.2% in early apoptosis and 18.4% in late apoptosis (Figure 4.4B). Incubation of HeLa cells with 400 µg/ml leaf extract for 24 hours induced apoptosis in 36% of the cell population, with 13.8% of the cells in early apoptosis and 22.2% in late apoptosis (Figure 4.4 C). Figure 4.4D shows that 31.4% of HeLa cells treated with 500 µg/ml leaf extract for 24 hours underwent apoptosis (early apoptosis in 14.3% and late apoptosis in 27.1%). Treatment of cells with 400µ g/ml hydrogen peroxide, a positive control, for 24 hours caused necrosis in 79.4% of the cells and early and late apoptosis at 0.6% and 13.5%, respectively (Figure 4.4C). Lastly, as expected, 99.8% of the untreated HeLa cells (negative control) were alive and 0,1% were undergoing early apoptosis (Figure 4.4F). The raw data can be seen in Appendix E. In Figure 4.5, the data is presented as percentage of viable cells, cells undergoing early or late apoptosis or percentage of cells undergoing necrosis.

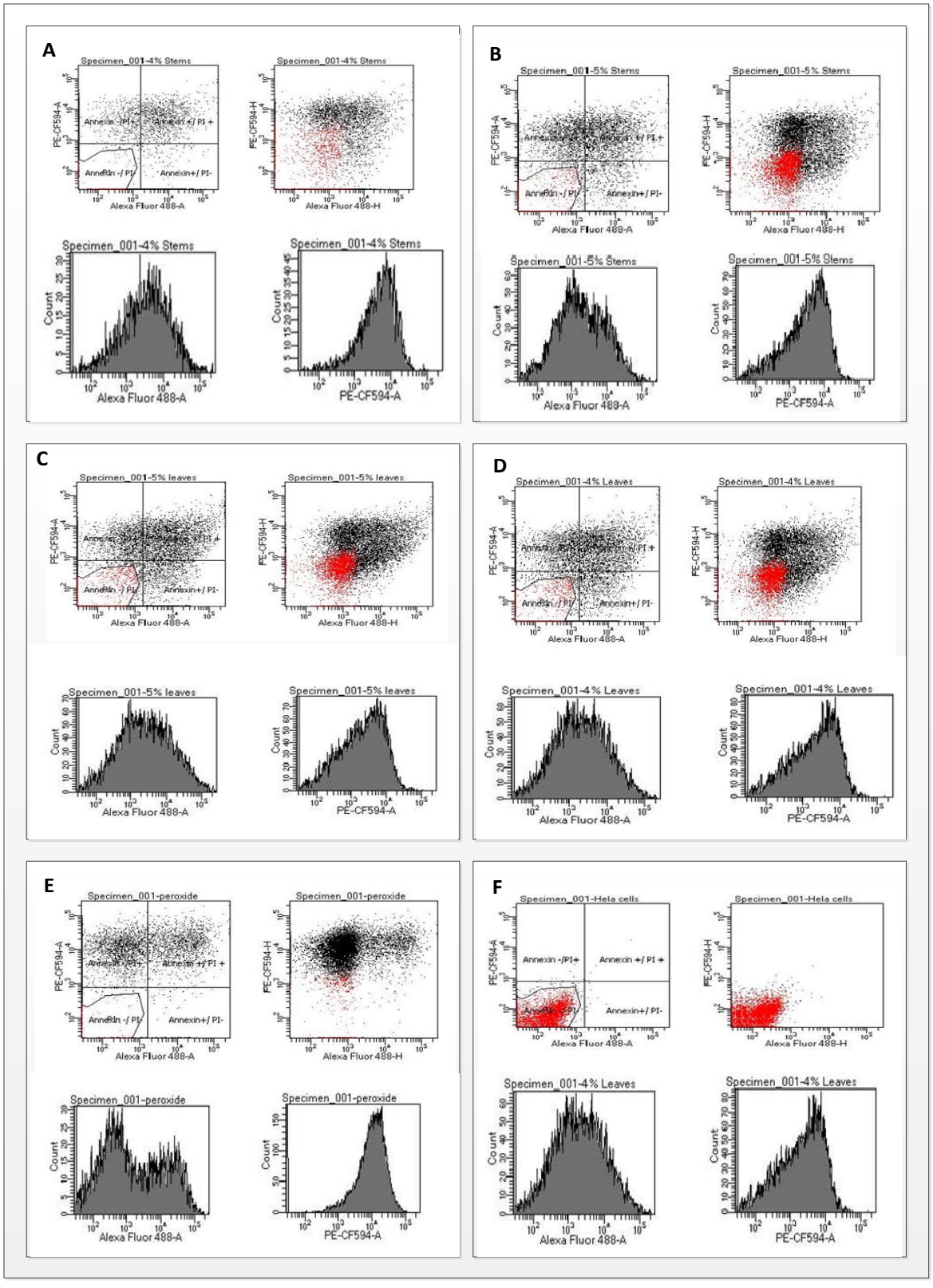

Figure 4.4: Scatter plot and histogram of Flow cytometry results for HeLa cells after 24 hours treatment with C. benghalensis.

[A4] HeLa treated with 400 µg/ml stem extract, [B] HeLa treated with 500 µg/ml stem extract, [C] HeLa treated with 400µ g/ml leaf extract, [D] HeLa treated with 500 µg/ml leaf extract, [E] HeLa treated with Peroxide, [F] untreated HeLa cells.

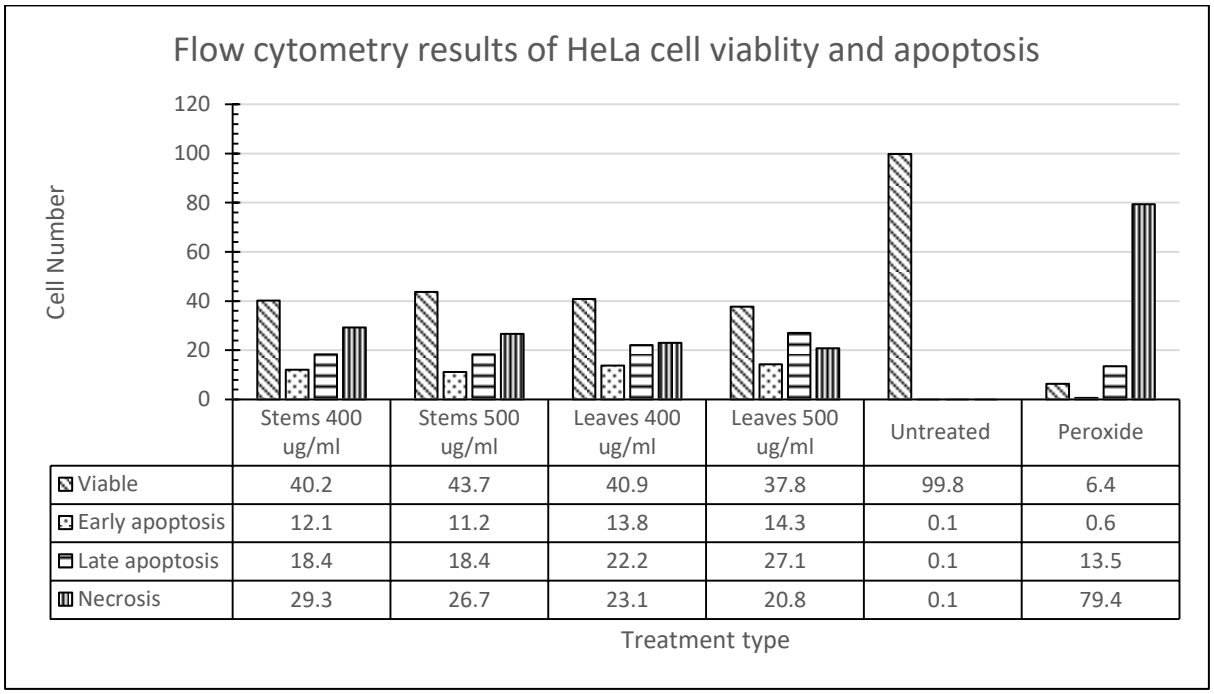

Figure 4.5: Flow cytometry results showing viable cells, cells with early apoptosis, late apoptosis and necrosis after 24-hour treatment with 400 µg/ml or 500 µg/ml C. benghalensis stem and leaf extract, peroxide and untreated HeLa cells.

Of all the extracts and concentrations used, as demonstrated in Figure 4.5, the 500 µg/ml leaf extract led to the highest concentration of apoptosis at 41.4%, with early and late apoptosis at 14.3% and 27.1%, respectively. This was followed by the 400 µg/ml leaf extract, which led to 36% apoptosis, with 13.8% of the cells in early apoptosis and 22.2% in late apoptosis. Treatment of cells with 400 µg/ml stem extract resulted in 18.4% of the cell population in late apoptosis and 12.1% in early apoptosis. Treatment of cells with a higher concentration of the stem extract (500 µg/ml) induced apoptosis in 11.2% of the population. On the other hand, the 500 µg/ml leaf extract induced the least necrosis 20.8%, followed by the 400 µg/ml leaf extract at 23.1%. Interestingly, the leaf extract had a higher apoptosis-inducing activity and possible anticancer properties than the stem extract. This could be investigated in future studies. Only the stem extracts were tested in the studies done by Lebogo (2014) and Mbazima (2008) on Jurkat T cells and only the leaf extracts used for study done on EAC cells by Karan et al. (2018).

As noted in section 2.5.1, certain miRs influence the apoptosis pathway through various mechanisms as seen in Table 2.3.

5.5 STEM AND LEAF EXTRACT REGULATE APOPTOTIC AND CELL CYCLE RELATED MIRS

HeLa cells were treated with 400 µg/ml and 500 µg/ml stem and leaf extract respectively for 24 hours as discussed in section 3.5. Two plates were analysed on real-time quantitative polymerase chain reaction (RT-qPCR) on two separate occasions. As mentioned in section 3.5.6, the RT-qPCR data was analysed by relative quantification using the ddCT method in excel. This method determines the fold change of the miRs levels in treated cells against untreated HeLa cells (See Appendix F for the calculations as done in excel). The figures below (Figures 4.6 to 4.9) depict the fold change of the treated HeLa miRs against untreated HeLa MiRs. A fold change of at least 2 was taken as significant (Dalman et al., 2012) for when looking at smaller fold changes very clean samples, biological replicates and the use of a positive sample of a known fold change are needed (IRIC, 2017). Consequently, only a fold change of more than two is remarkable. The results are presented as mean standard deviation (± SD) of two independent duplicate experiments.

5.5.1 MIR EXPRESSION IN HELA CELLS TREATED WITH 400 µG/ML STEM EXTRACT

As shown in Figure 4.6 the 400 µg/ml stem extract highly upregulated miR-143 by 18.2 fold, miR-34a by 14.5 fold, miR-182 by 14 fold and to a lesser extent miR-20a by 4.7 fold when compared to the untreated HeLa cells (Figure 4.6). As seen in Table 2.3, miR-143 (Lui et al., 2012) and miR-34a (Li et al., 2010; Wang et al., 2018) are downregulated in HeLa cells and thus their upregulation can assist in inhibition of tumour growth. Whereas miR-20a (Zhoa, 2013) and miR-182 (Tang et al., 2013) are already upregulated in SCC, and further upregulation could lead to increased tumour growth. Hence, the 400 µg/ml stem extract was not a good candidate to be used as a treatment for cervical cancer.

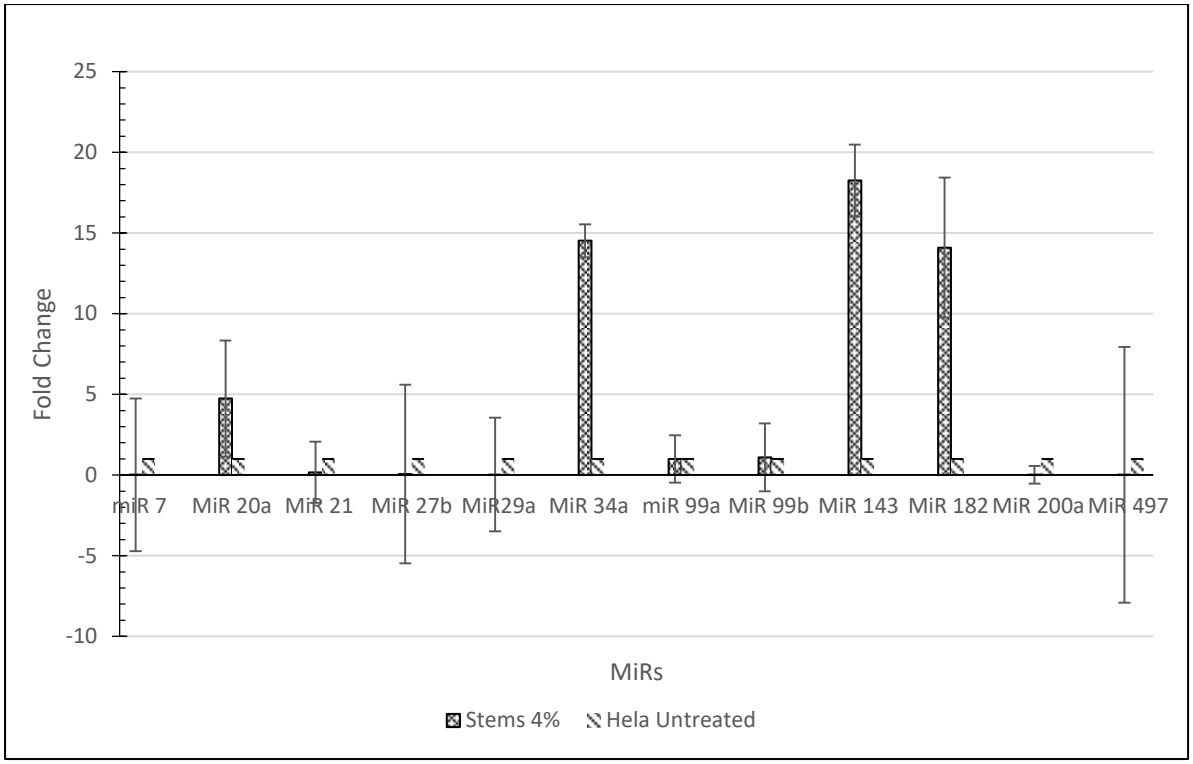

Figure 4.6: MiRs expression tested with qRT- PCR after the HeLa cells were treated with 400 µg/ml C. benghalensis stem extract for 24 hours.

5.5.2 MiR EXPRESSION OF HeLa CELLS AFTER TREATMENT WITH 500 µG/ML STEM EXTRACT

After treating HeLa cells with the 5% stem extract, only miR-34 was highly upregulated, and its expression was increased by 32 fold when compared to the untreated cells (Figure 4.7). As it is usually downregulated in HeLa cells (Li et al., 2010; Wang et al., 2013), the treatment with 500 µg/ml stem extract can be a potential treatment for cervical cancer. It also seems that, at the 500 µg/ml concentration, it also eliminates the upregulation of miR-182 and miR-20a, as was seen in with treatment of the 400 µg/ml stem extract.

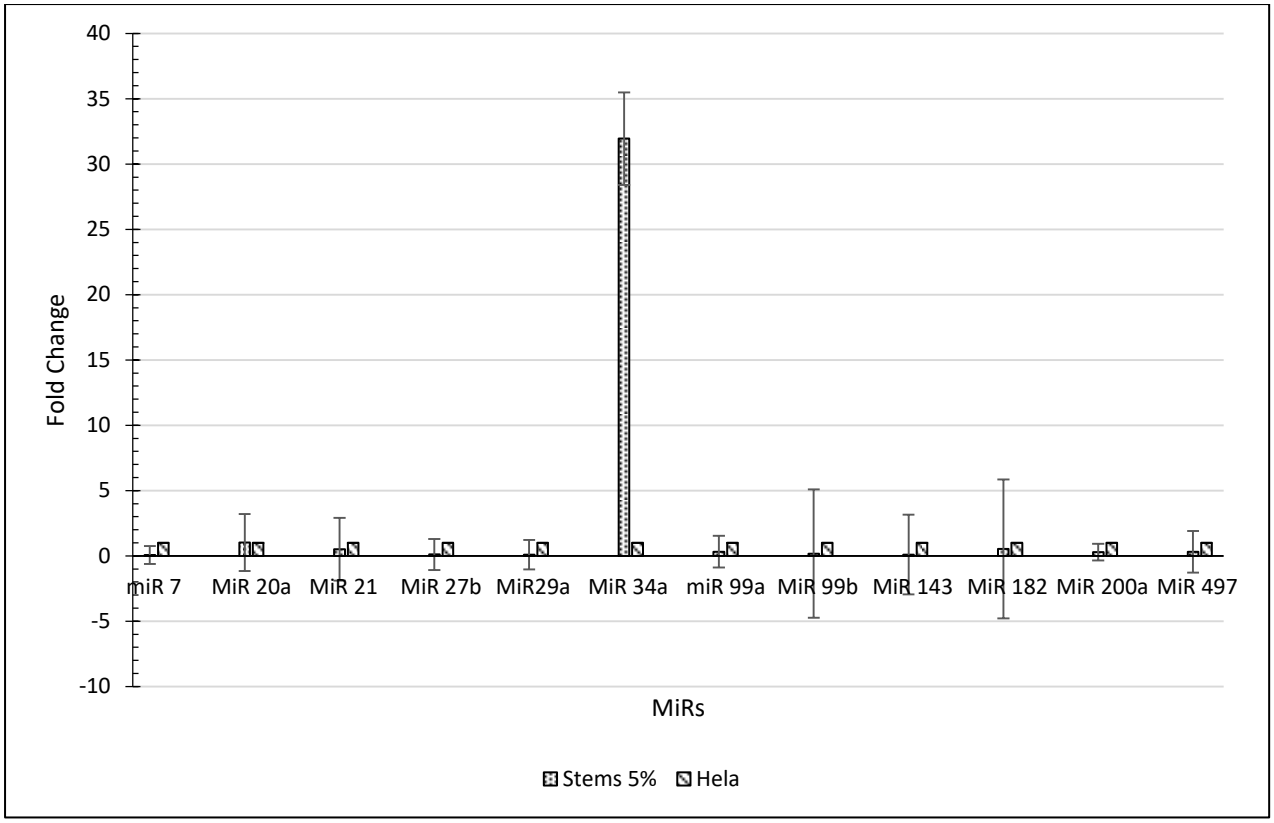

Figure 4.7: MiRs expression tested with qRT- PCR after HeLa was cells treated with 500 µg/ml C. benghalensis stem extract for 24 hours.

5.5.3 MiR EXPRESSION OF HeLa CELLS AFTER TREATMENT WITH 400 µG/ML LEAF EXTRACT

When the HeLa cells were treated with 400 µg/ml leaf extract, miR-34a was the most upregulated miR with a fold change of 4.87, followed by miR-200a, with a fold change 2.3. MiR-27b was upregulated to less than 2 with a fold change of 1.67 (Figure 4.8). The expression of miR-7, miR-99a, miR-143 and miR-182 in treated HeLa cells was less than the expression in untreated HeLa cells, but not more than a fold change of 2.

MiR-34a is usually downregulated in HeLa cells as mentioned before (Li et al., 2010; Wang et al., 2018) and miR-200a is upregulated in SCC and adenoCC (Wang et al., 2013; Hu et al., 2010). Since the 400 µg/ml leaf extract upregulated miR-200a, which is already upregulated in cervical cancer cells, this might negate the arrested growth of the cancer caused by the miR-34a upregulation.

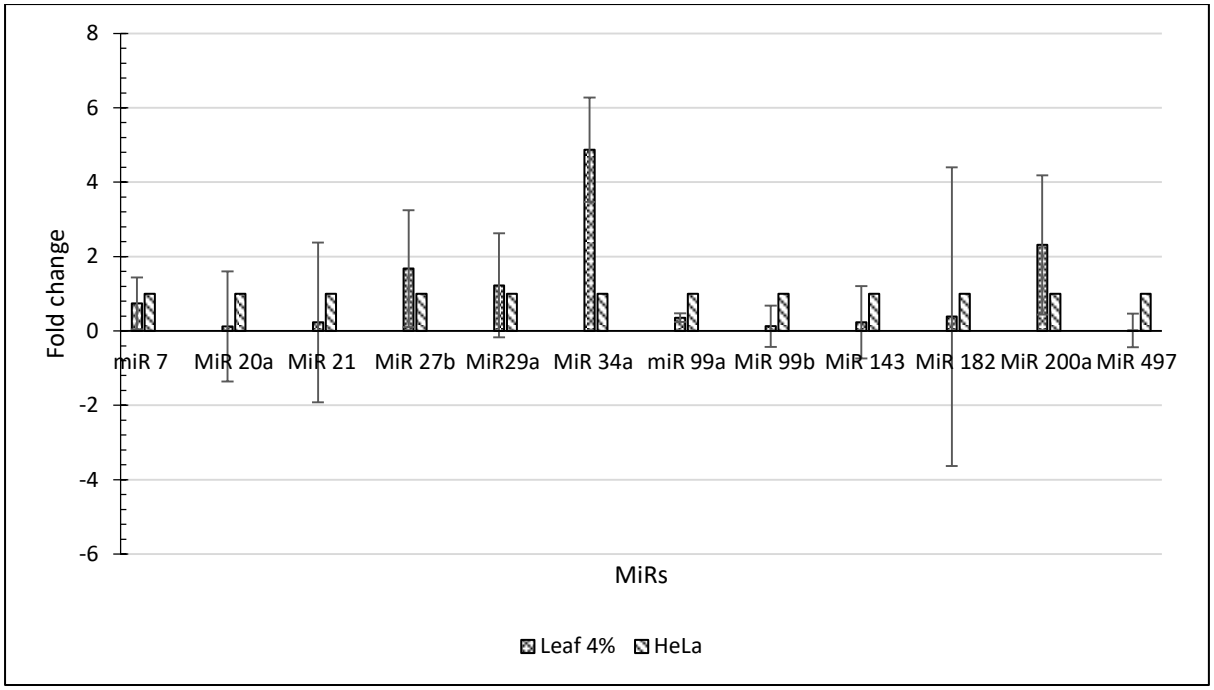

Figure 4.8: MiRs expression tested with qRT- PCR after HeLa was cells treated with 400 µg/ml C. benghalensis leaf extract for 24 hours.

5.5.4 MiR EXPRESSION OF HeLa CELLS AFTER TREATMENT WITH 500 µG/ML LEAF EXTRACT

For the HeLa cells treated with 500 µg/ml leaf extract, as seen in Figure 4.9, the results interestingly showed slightly less expression of the miR-34a, miR-27b and miR-34a than in the untreated HeLa cells. The expression of miR-7, with a fold change of 5.01, was the most upregulated, followed by miR-20a with a fold change of 2.20, and lastly miR-200a with a fold change of 2.11. (For all the miRs and their expression in cervical cancer see Table 2.3). MiR-21 was upregulated by a fold change of less than 2 at 1.9. All the miRs that were upregulated by the 500 µg/ml leaf extract was also upregulated in cervical cancer cells, thus this would lead to further upregulation and increase tumour growth and apoptosis suppression. Interestingly none of the extracts led to down regulation of any miRs.

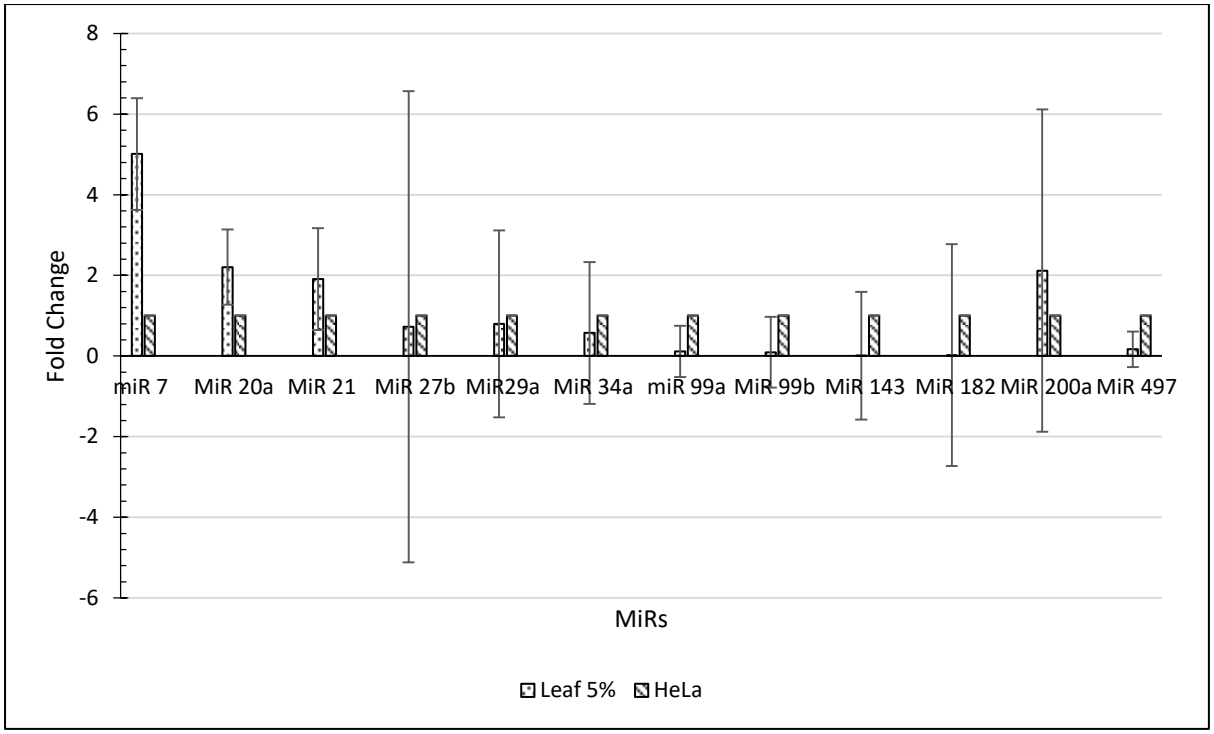

Figure 4.9: MiRs expression tested with qRT- PCR after HeLa was cells treated with 500 µg/ml C. benghalensis leaf extract for 24 hours.

5.5.5 SUMMARY OF DEREGULATED MIRS AFTER TREATMENT WITH STEM OR LEAF EXTRACT

Table 4.2 gives a summary of all the miRs that were deregulated after treatment with extracts of *C. benghalensis*. Only miRs with a fold change of 2 or more were seen as significant, as mentioned in section 4.5. The expression of these miRs in cervical cancer cells supports carcinogenesis. The different *C. benghalensis* extracts had an upregulating effect on miR-7, miR-20a, miR-34, miR-143, miR-182 and miR-200a, but had no significant effect (a fold change of more than2) on miR-21, miR27b, mir-29a miR-99a, miR-99b and miR-497. Up regulation of some miRs would suppress cancer progression, but upregulation of other would promote the progression of cervical cancer.

Table 4.2: Summery of the expression of miRs after treatment with C. benghalensis leaf and stem extracts.

(Only miRs with a fold change of 2 or more were seen as significant, as mentioned in section 4.5, and depicted in the table).

Plant extract	***Stem 400 µg/ml***	***MiR expression***	***Stem 500 µg/ml***	***MiR expression***	***Leaf 400µ g/ml***	***MiR expression***	***Leaf 500 µg/ml***	***MiR expression***
MiRs upregulated	20a 34a 143 182	Up Down Down Up	34a	Down	34a 200a	Down Up	7 20a 200a	Up Up Up
MiRs down regulated	None		None		None		None	

6 CHAPTER FIVE: DISCUSSION, CONCLUSION AND LIMITATIONS

6.1 INTRODUCTION

This study set out to determine the anticancer effect of *Commelina benghalensis* plant extracts on HeLa (cervical cancer). Cervical cancer is the prevailing cancer that causes death in women in Southern Africa, and the fourth most diagnosed cancer worldwide (Sung et al., 2021). The incidence of cervical cancer has been intensified by the high prevalence of high rate of HIV (Maiman, 1993) and HPV infections in this sub-Saharan Africa region (Ferlay, 2012). This disease still causes high rates of morbidity and mortality, even with the current screening methods, including Papanicolaou (Pap) smear and treatment strategies that include surgery, chemo- and radiotherapy. The treatment of cervical cancer with chemotherapy and radiotherapy also damages healthy cells. New treatment modalities that only affect the cancer cells will alleviate suffering associated with this disease. For a cancer treatment to be effective it should ideally increase apoptosis in cancer cells without harming healthy cells or inducing necrosis, which is associated with adverse side effects.

C. benghalensis extracts induced apoptosis in Jurkat T leukaemia cells and demonstrated anticancer properties (Mbazimba et al., 2008; Lebogo et al., 2014). Karan et al. (2018) showed that *C. benghalensis* possessed anticancer effect on EAC cells in mice. MicroRNAs are involved in gene regulation, expression and implicated in the carcinogenesis processes associated with cervical cancer (Lu et al., 2005). Some miRs are also involved in apoptosis, and thus alteration of their expression can assist in treatment of cervical cancer. The effect of *C. benghalensis* on cervical cell metabolism, apoptosis and miRs has not been previously investigated.

The objective of this study was to determine the effect of *C. benghalensis* extracts on HeLa cells. The acetone stem, leaf and root *C. benghalensis* extracts were used. Firstly, the effect on cellular metabolism of HeLa cells after 24, 48 and 72 hour treatment with *C. benghalensis* extracts using MTT was investigated. Secondly, it was studied whether treatment with *C. benghalensis* after 24 hours caused apoptosis in HeLa by using flow cytometry. Lastly, twelve miRs involved with the apoptosis pathway in cervical cancer were identified and their expression in HeLa cells were determined after 24 hours treatment with *C. benghalensis* with reverse transcription quantitative PCR.

6.2 VARIOUS *C. BENGHALENSIS* PLANT EXTRACTS HAD VARYING CYTOTOXIC EFFECTS AGAINST HELA CELLS

HeLa cells were treated with 400, 500 and 600 µg/ml stem, leaf and root extract for 24, 48 and 72 hours. This was then tested for metabolic activity with MTT. It was found that the stem and leave extracts had the most cessation of metabolic activity after 24 hours. After 48 hours only the leaf and stem extract showed decrease in metabolic activity although not as much as after 24 hours, whereas the roots showed increase metabolic rate. Hence forth the HeLa cells were treated with the stem and leaf extract for 24 hours for the rest of the experiments. As Mbazimba et al. (2008) showed that concentrations of *C. benghalensis* of up to 500 µg/ml were non-toxic; 400 µg/ml and 500 µg/ml extracts were used in subsequent experiments.

6.3 INDUCTION OF APOPTOSIS OF HELA CELLS BY *C. BENGHALENSIS* ACETONE EXTRACTS

The HeLa cells were treated with 400 and 500 µg/ml stem and leaf extracts and tested for apoptosis by Annexin V flow cytometry. The 500 µg/ml leaf extract followed by the 400 gµ/ml leaf extract had the highest rate of early and late apoptosis. The stem extract also led to apoptosis, although to a lesser extent than the leaf extract. Interesting to note is that the 500 µg/ml leaf extract induced the least cell necrosis followed by the 400 µg/ml leaf extract. These results are in keeping with the results of the study conducted by Lebogo et al. (2017) and Mbazima et al. (2008), where acetone and methanolic extracts of *C. benghalensis* stems led to apoptosis of Jurkat T cells.

6.4 THE EFFECT OF *C. BENGHALENSIS* ACETONE EXTRACTS ON THE EXPRESSION OF MIRS IN CERVICAL CANCER HELA CELLS

Twelve miRs involved in the apoptosis pathway of cervical cancer were identified and their expression was tested after 24 hour treatment with *C. benghalensis* stem and leaf extract by reverse transcriptase real time PCR. These miRs were selected as they are involved in apoptosis and as mentioned earlier *C. benghalensis* extract caused apoptosis in Jurkat T cells (Mbazimba et al., 2008) and EAC cells (Karan et al., 2018). No previous studies have been done on the expression of miRs after treatment with *C. benghalensis* extract. In this study apoptosis on HeLa cells was demonstrated as discussed in sections 4.4 and 5.3.

More than 50% of miRs are located near fragile sites of genes, and deregulated miRs at these sites are typically associated with cancer (Calin et al., 2004). In cervical cancer these fragile gene sites of the miRs are also where HPV integration takes place (Skukulu et al., 2009). For instance, miR-21 is located at the common fragile site FRA17B on chromosome 17q23.2 (Volinia et al., 2006; Thorland et al., 2003).

Six miRs were significantly upregulated, with a fold change of 2 or more by the *C. benghalensis* extracts, namely: miR-7, miR-20a, miR-34, miR-143, miR-182 and miR-200a. This is not beneficial for cervical cancer treatment in all the miRs, as some of them are already upregulated in cervical cancer, thereby potentially increasing the cervical cancer progression. Six of the miRs were not notably affected by the *C. benghalensis* extracts namely: miR-21, miR27b, miR-29a, miR-99a, miR-99b and miR-497. None of the treated miRs were down regulated.

6.5 DISCUSSION OF MIRS AFFECTED BY *C. BENGHALENSIS* LEAF AND STEM EXTRACTS AND THEIR APOPTOSIS PATHWAYS

The miRs affected by the *C. benghalensis* extracts treatment in this study are discussed in further detail. The upregulation of miR-7 in cervical cancer leads to downregulation of XIAP that suppresses cell growth and promotes apoptosis (Lui et al., 2013). When miR-7 is downregulated in cervical cancer cells, it leads to decreased metastasis (Hoa et al., 2015). MiR-7 was upregulated by the 500 µg/ml stem extract and therefore this can be unbeneficial if miR-7 is already upregulated in the cancer cells. MiR-20a is an oncomir, but when it is upregulated in cervical cancer it contributes to tumour development and lymph node metastasis (Zhoa et al., 2013). It promotes migration and invasion of cervical cancer cell by targeting TRF1 that interacts with TNKS2 - a direct target of miR-20a (Zhoa et al., 2015). The 400 µg/ml stem and 500 µg/ml leaf extract upregulated miR-20a, which will enhance its carcinogenic effect in cervical cancer.

MiR-34a is also tumour suppressive and prevents cell invasion by downregulating Notch 1 and Jagged (Pang et al., 2010), but it is usually down regulated in cervical cancer by E6 targeting p53 (Ribeiro et al., 2015). WNT1 is inversely targeted by miR-34a in the presence of HPV E6 and E7, thus downregulated miR-34a, which causes increased WNT1, which promotes cervical cell proliferation and invasion by the WNT1/ß catherdrin pathway that

induces the E-P catherin switch (Li et al., 2020). Furthermore, Bcl-2 and C-Met are also miR-34a targets that promote cervical cancer development. The decrease expression of miR-34a in cervical cancer is associated with more lymph node metastasis, advanced cancer stage and histological grade and shorter survival time (Chen et al., 2017). MiR-34 was upregulated by the 400 µg/ml, 500 µg/ml stem and 400 µg/ml leaf *C. benghalensis* extracts. This indicates that miR-34a is a potential usable target for cervical cell cancer treatment with *C. benghalensis*.

MiR-143 is a p53 induced tumour suppressive gene that is downregulated in cervical cancer where it is associated with increased tumour size, lower survival rate and HPV infection (Lui et al., 2012; Dang et al., 2017; Huang et al., 2012). Bcl-2 is a major target of miR-143, and Bcl-2 is also modulated by HOTAIR via miR-143 (Liu et al., 2018). Increase of Bcl-2 concentration leads to inhibition of apoptosis, uncontrolled cell proliferation and metastasis (Lui et al., 2012; Lui et al., 2018). Proliferation and apoptosis in cervical cancer is also regulated by the miR-143 target HIF1a (Zhoa et al., 2021) Furthermore, upregulated TCONS_0026907 in cervical cancer promotes its progression by inhibition of miR-143 and ELK 1 (Jin et al., 2017). MSI-2, an oncoprotein (Dang et al., 2017) and INCRAA OICP AS 1 also deregulates miR-143 in cervical cancer and thereby promoting cancer progression (Song et al., 2020). In this study, miR-143 was upregulated by the 400 µg/ml stem extract which would potentially assist in cervical cancer treatment.

MiR-182 is upregulated in cervical cancer by HPV E7 via the TGF-B smad u signalling pathway and disrupts cell proliferation (Chen et al., 2019). MiR-182 binds to FOXO1, which induces arrest in the G2/M cell cycle phase and also leads to overexpression of p21 CDK inhibitors (Javadi et al., 2018). PCGEM 1 (LIN C00173) is a direct target of miR-182 and targets FBXW11 that increases cell proliferation, migration and invasion in cervical cancer cells (Zang et al., 2019). When treated with *C. benghalensis* 400 µg/ml stem extract, miR-182 was significantly upregulated, thereby supporting its expression in cervical cell cancer. Lastly, miR-200a is upregulated in cervical cancer, which is associated with metastasis and invasion. Increased miR-200a increases TIMP1 and in turn increases the expression of MMP2 and MMP9 (Wang et al., 2013). MiR-200a also regulates TNKS2 (Chen et al., 2013) and LCNRNA XIST via the up regulation of Fus that competitively binds miR-200a (Zhu et al., 2018). MiR-200a was upregulated by the 400 µg/ml and 500 µg/ml leaf extract, which would potentially support tumour growth.

6.6 CONCLUSION

Treatment of HeLa cells with *C. benghalsensis* stem and leaf extracts caused metabolic cessetation, apoptosis and affected six of the treated miRs significantly. Therefore it was demonstrated that *C. benghalensis* stem and leave extracts have anticancer properties.

None of the tested miRs were downregulated by the stem or leaf extracts. MiR-34a was upregulated by the 400 and 500 µg/ml stem extracts and the 400 µg/ml leaf extract. The 500 µg/ml leaf extract did not show a significant increase of miR-34a, but of miR-7, miR-20a, and miR-200a. The 400 µg/ml stem extract also upregulated miR-20a, miR-143 and miR-182. MiR-20a (Zhoa, 2013) and miR-182 (Tang et al., 2013) are already upregulated in SCC, and further upregulation by the stem extract could lead to increased tumour growth. MiR-143 (Lui et al., 2012) and miR-34a (Li et al., 2010; Wang et al., 2018) are downregulated in HeLa cells and their upregulation could assist inhibition of tumour growth. These effects could thus negate each other and therefore, the 400 µg/ml stem extract was not a good candidate as a potential treatment for cervical cancer.

The 500 µg/ml leaf extract was not a good candidate for cervical cancer treatment either. Besides the upregulation of miR-34a, when treated with the 500 µg/ml leaf extract, miR-200a was also upregulated. The 500 µg/ml leaf extract led to lowered expression of miR-34a than in untreated HeLa cells. Furthermore miR-7, miR-20a, and miR-200a were upregulated. All these miRs are already upregulated in cervical cancer.

When looking at the results as discussed above, the 500 µg/ml stem extract seemed to be the best candidate for further investigation as a potential treatment in cervical cancer. It induced cessation of cell metabolism (section 4.1 and Figure 4.1) and led to apoptosis of the treated HeLa cells (section 4.3 and Figure 4.5). Additionaly, it upregulated only miR-34a, which is usually downregulated in cervical cancer (Li et al., 2010; Wang et al., 2018). Furthermore, of all the upregulated miRs, miR-34 had the most significant fold change of 34.9. No other miRs that are already upregulated in cervical cancer were further upregulated by the 500 µg/ml stem extract, as was seen with the 400 µg/ml stem, 400 µg/ml and 500 µg/ml leaf extracts. Moreover, miR-34a is also involved in the progression of the cell cycle, cellular senescence, and apoptosis through its effect on p53 (Zang et al., 2016). MiR-34 also influences the NOTCD1 /JAG 1 genes that in turn influence apoptosis, cell growth and maturation (Pang et al., 2010). It also influences miR-34a/LDHA axis that exhibits a tumour suppressive role in cervical cancer (Zhang R et al., 2016; Ribeiro et al., 2015; NCBI, 2020). WNT1 is a target of

miR-34a and its upregulation results in decreases WNT1 expression. WNT1 down regulation decreased cell proliferation and invasion of cervical cancer cells. MiR-34a also regulates the E-cadherin to P-cadherin switch that also inhibits cancer cell proliferation and tumorigenesis via the inactivation of the WNT1/ß-catenin pathway (Li et al., 2020). The E6 oncogene also influences miR-34a and influencing the cell cycle and apoptosis (Wang et al., 2011). Decreased expression of HPV-16 E6/E7 results in miR-34a upregulation and WTN1 downregulation (Li et al., 2020). Interesting to note is that miR-21, miR-27b, miR-29, miR-99a, miR-99b and miR-497 expression were not markedly affected by any of the *C. benghalensis* extracts.

6.7 LIMITATIONS AND FUTURE RESEARCH

A limitation to the study was that only two PCR plates could be run for the miRs, as there was no more stock available for more experiments due to budgetary constraints since miR analysis is costly. Additionally, for miR-497, no precursor miRs was available from Qiagen, thus mature miRNA primers were used. This could potentialy also influence the results. In future, precursor miRNA primers should be used for all experiments. Only twelve miRs expression could be tested. In future research more miRs that are dysregulated in cervical cancer can be tested for the effect of treatment by *C. benghalensis* extracts. Only HeLa cells were used in this study and in future research the effect of *C. benghalensis* on CaSki cells as well can be done.

In the experiment done by Mbazimba et al. (2008), treatment with *C. benghalensis* caused upregulation of Bax, p53, cdc2, cyclin B1 and p21 and downregulated Bcl-2 which induced apoptosis. Furthermore, in the study performed by Lebogo et al. (2014), the treatment of *C. benghalensis* extracts arrested the cell cycle in the G1/S interphase. MiR-182 is upregulate in cervical cancer and thereby arresting the cell cycle in the G2 M2 cell cycle phase and increasing p21 expression. This upregulation was also enhanced by the 400 µg/ml stem extract, which could further increase p21. MiR-143 is usually downregulated in cervical cancer which is induced p53 and increases Bcl-2. Treatment with *C. benghalensis* increased miR-43 expression, which in turn could decrease expression of p21. When miR-34a is downregulated in cervical cancer it decreases p53 and increases Bcl-2. Treatment with 400 µg/ml, 500 µg/ml and 400 µg/ml leaf extracts upregulated miR-34 and could potentially increase p53 and Bcl-2. This expression of genes under the influence of miRs after treatment with *C. benghalensis* expression could be investigated further in future.

Upregulation of miR-34a could help to depress the cancer pathways and assist in suppression of tumorigenesis. The 500 µg/ml stem extract could be tested for a potential treatment in cervical cell cancer. Ideally, a larger number of miRs should be used against the 500 µg/ml stem extract in future research.

Even though the stem and leaf extracts induced apoptosis, it is important for future research into this plant extract's potential as treatment to make sure that it does not enhance tumorigenesis due to its effect on certain miRs. It is important to note that not all miRs are expressed in the same way in all cervical cancer types, therefore before using a treatment that targets a specific miR, a screening of the miR expression should be done. This is an area where more research can be done. The fact that only upregulation of certain miRs occurred poses the question of whether *C. benghalensis* extracts only causes upregulation or no effect on certain miRs in cervical cancer cells. This is an area that can be investigated in future.

Another question is why miR-21, miR-27b, miR-29a, miR-99a, miR-99b and miR-497 were not significantly affected by the *C. benghalensis* extracts. Could this be that since the cells are not under the influence of HPV E6 and E7 or perhaps because the miRs themselves are not affected by *C. benghalensis*, but their targets such as Bcl-2 and Bax are? Is it because these miRs are not affected by Bax and Bcl-2 and thus not altered by *C. benghalensis*? Therefore, does *C. benghalensis* affect the miRs or their targets? Does *C. benghalensis* influence XIAP, MTCR or IGF-R, which are targeted by miR-7, miR-99a, miR-99b and miR-497 targets? This can also be researched in future.

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8 APPENDIXES:

8.1 APPENDIX A: FIGO STAGING CERVICAL CARCINOMAS.

Stage I: Stage I is carcinoma strictly confined to the cervix; extension to the uterine corpus should be disregarded. The diagnosis of both Stages IA1 and IA2 should be based on microscopic examination of removed tissue, preferably a cone, which must include the entire lesion.

Stage IA: Invasive cancer identified only microscopically. Invasion is limited to measured stromal invasion with a maximum depth of 5 mm and no wider than 7 mm.

Stage IA1: Stage IA1: Measured invasion of the stroma no greater than 3 mm in depth and no wider than 7 mm diameter.

Stage IA2: Stage IA2: Measured invasion of stroma greater than 3 mm but no greater than 5 mm in depth and no wider than 7 mm in diameter.

Stage IB: Stage IB: Clinical lesions confined to the cervix or preclinical lesions greater than Stage IA. All gross lesions even with superficial invasion are Stage IB cancers.

Stage IB1: Stage IB1: Clinical lesions no greater than 4 cm in size.

Stage IB2: Stage IB2: Clinical lesions greater than 4 cm in size.

Stage II: Stage II is carcinoma that extends beyond the cervix, but does not extend into the pelvic wall. The carcinoma involves the vagina, but not as far as the lower third.

Stage IIA: No obvious parametrial involvement. Involvement of up to the upper two-thirds of the vagina.

Stage IAB: Obvious parametrial involvement, but not into the pelvic sidewall.

Stage III: Crcinoma that has extended into the pelvic sidewall. On rectal examination, there is no cancer-free space between the tumour and the pelvic sidewall. The tumour involves the lower third of the vagina. All cases with hydronephrosis or a non-functioning kidney are Stage III cancers.

Stage IIIA: No extension into the pelvic sidewall but involvement of the lower third of the vagina.

Stage IIIB: Extension into the pelvic sidewall or hydronephrosis or non-functioning kidney.

Stage IV: Stage IV is carcinoma that has extended beyond the true pelvis or has clinically involved the mucosa of the bladder and/or rectum.

Stage IVA: Spread of the tumour into adjacent pelvic organs.

Stage IVB: Spread to distant organs.

Source: TNM Classification of malignant tumours. L. Sobin and C.H. Wittekind (eds.), UICC International Union against Cancer, Geneva, Switzerland.
pp155-157; 6th ed. 2002

8.2 APPENDIX B: THE SOLUTIONS AND THEIR RECIPES USED IN THIS STUDY

Solutions	**Preparation Method**	**Company and Country**
Growth medium	445 ml Hyclone Dulbecco's modified eagles medium 50 ml Hyclone research grade fetal bovine serum (FBS) 5 ml Penicillin streptomycin(pen/strep) Made up to total of 500 ml and stored at 4^{o}C	Hyclone Laboratories, USA Biowest, France
Trypsin	0.01% made to up to final volume with Hyclone phosphate buffered saline (PBS) 1 ml trypsin-EDTA 10X and 9 ml	Hyclone Laboratories, USA Biowest, France

	PBS	
MTT	0.15g Invitrogen MTT mixed with 30 ml 1 x Hyclone Phosphate-buffered saline (PBS) to form 5mg/ml PBS Mixed well, filtered and stored at -20^{o} C	Invitrogen, USA Hyclone Laboratories, USA

8.3 APPENDIX C: KITS AND SOLUTIONS USED IN EXPERIMENT.

- Invitrogen MTT (3-(4-,5-dimetylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) [USA]
- Biotium CF 488A annexin V an PI apoptosis kit (USA)
- GAPDH New England Biolabs (USA)
- TRIZol Qiagen (USA)
- Qiagen miScript II RT Kit (USA)
- Qiagen miScript precursor assay (USA)
- Qiagen miScript primer assay (USA)
- Qiagen SYBER green PCR kit (USA) used with miScript primer assay or miScript precursor assay

8.4 APPENDIX D: MTT CALCULATIONS IS EXCEL

	Hela cells treated with *C. benghalensis* for 24 hours								
	400 µl/ml	**400 µl/ml**	**400 µl/ml**	**500 µl/ml**	**500 µl/ml**	**500 µl/ml**	**600 µl/ml**	**600 µl/ml**	**600 µl/ml**
Roots	0.109	0.161	0.127	0.101	0.094	0.092	0.104	0.085	0.1
Stems	0.101	0.085	0.08	0.101	0.081	0.106	0.101	0.103	0.067
Leafs	0.07	0.077	0.083	0.076	0.067	0.076	0.081	0.117	0.039
			Peroxide			**DMSO**			**Cells**
			24			**24**			**24**
400 µl/ml			0.071			0.25			0.241
500 µl/ml			0.072			0.15			0.109
Average			0.0715			0.2			0.175

Averages	**400 µl/ml**	**500 µl/ml**	**600 µl/ml**	**Calculations**	**400 µl/ml**	**500 µl/ml**	**600 µl/ml**
Roots	0.09925	0.095667	0.096333	**Roots**	56.71429	54.66667	55.04762
Stems	0.0665	0.096	0.090333	**Stems**	38	54.85714	51.61905
Leafs	0.0575	0.073	0.079	**Leafs**	32.85714	41.71429	45.14286
Peroxide	0.0175	0.0175	0.0175	**Peroxide**	10	10	10
DMSO	0.2	0.2	0.2	**DMSO**	114.2857	114.2857	114.2857
Negative	0.175	0.175	0.175				

	Hela cells treated with *C. benghalensis* extract for 48 Hours							
	400 µl/ml	**400 µl/ml**	**400 µl/ml**	**500 µl/ml**	**500 µl/ml**	**500 µl/ml**	**600 µl/ml**	**600 µl/ml**
Roots	0.089	0.825	1.142	0.162	1.082	1.163	0.145	0.822
Stems	0.085	0.09	0.165	0.099	0.094	0.112	0.113	0.078
Leafs	0.09	0.087	0.102	0.113	0.095	0.102	0.104	0.085
		Peroxide			**DMSO**			**Cells**
		48			**48**			**48**
400 µl/ml		0.0285			0.256			0.182
500 µl/ml		0.0265			0.175			0.165
Average		0.0275			0.2155			0.1735

Averages	**400 µl/ml**	**500 µl/ml**	**600 µl/ml**	**Calculations**	**400 µl/ml**	**500 µl/ml**	**600 µl/ml**
Roots	0.685333	0.87225	0.345333	**Roots**	395.0048	502.7378	199.0394
Stems	0.113333	0.09975	0.095	**Stems**	65.32181	57.4928	54.75504
Leafs	0.093	0.10125	0.095333	**Leafs**	53.60231	58.35735	54.94717
Peroxide	0.0275	0.0275	0.0275	**Peroxide**	15.85014	15.85014	15.85014
DMSO	0.2155	0.2155	0.2155	**DMSO**	124.2075	124.2075	124.2075
Negative	0.1735	0.1735	0.1735				

	Hela cells treated with *C. benghalensis* extract for 72 hours								
	400µg/ml	**400µg/ml**	**400µg/ml**	**500µg/ml**	**500µg/ml**	**500µg/ml**	**600µg/ml**	**600µg/ml**	**600µg/ml**
Roots	0.61	1.002	0.157	0.404	0.437	0.102	0.423	0.079	0.097
Stems	0.083	0.078	0.089	0.085	0.087	0.098	0.112	0.081	0.08
Leafs	0.096	0.083	0.104	0.128	0.095	0.122	0.104	0.093	0.08
	Peroxide			**DMSO**			**Cells**		
	72			**72**			**72**		
400µg/ml	0.0151			0.21			0.358		
500µg/ml	0.0206			0.232			0.097		
Average	0.01785			0.221			0.2275		
Averages	**400µg/ml**	**500µg/ml**	**600µg/ml**	**Calculations**		**400 µl/ml**	**500 µl/ml**	**600 µl/ml**	
Roots	0.589667	0.314333	0.199667		**Roots**	259.1941	138.1685	87.76557	
Stems	0.083333	0.09	0.091		**Stems**	36.63004	39.56044	40	
Leafs	0.094333	0.115	0.092333		**Leafs**	41.4652	50.54945	40.58608	
Peroxide	0.01785	0.01785	0.01785		**Peroxide**	7.846154	7.846154	7.846154	
DMSO	0.221	0.221	0.221		**DMSO**	97.14286	97.14286	97.14286	
Negative	0.2275	0.2275	0.2275						

8.5 APPENDIX E: FLOW CYTOMETRY VALUES (ANNEXIN V)

Events	***Stems 400 µg/ml***	***Stems 500 µg/ml***	***Leafs 400 µg/ml***	***Leafs 500 µg/ml***	***Untreated Cells***	***Peroxide***
Viable	40.2	43.7	40.9	37.8	99.8	6.4
Early apoptosis	12.1	11.2	13.8	14.3	0.1	0.6
Late apoptosis	18.4	18.4	22.2	27.1	0.1	13.5
Necrosis	29.3	26.7	23.1	20.8	0.1	79.4

8.6 APPENDIX F: THE MIRS SEQUENCES AND PRIMERS

1. MiR-7

Stem loop

hsa-mir-7-1 MI0000263

UUGGAUGUUGGCCUAGUUCUGUGUGGAAGACUAGUGAUUUUGUUGUUUU
UAGAUAACUAAAUCGACAACAAAUCACAGUCUGCCAUAUGGCACAGGCCA
UGCCUCUACAG

Mature miR-7

>hsa-miR-7-5p MIMAT0000252

UGGAAGACUAGUGAUUUUGUUGU

>hsa-miR-7-1-3p MIMAT0004553

CAACAAAUCACAGUCUGCCAUA

Primers

Forward Primer: 5'UUG GAU GUU GGC CUA GUU CU 3'

Reverse Primer: 5' CAC AGG CCA UGC CUC UAC AG 3'

2. MiR-20a

Stem loop

hsa-mir-20a MI0000076

GUAGCACUAAAGUGCUUAUAGUGCAGGUAGUGUUUAGUUAUCUACUGCA
UUAUGAGCACUUAAAGUACUGC

Mature miR-20a

hsa-miR-20a-5p MIMAT0000075

UAAAGUGCUUAUAGUGCAGGUAG

>hsa-miR-20a-3p MIMAT0004493

ACUGCAUUAUGAGCACUUAAAG

Primers

Forward primer: 5’ GUA GCA CUA AAG UGC UUA 3’

Reverse primer: 5’ UGA GCA CUU AAA GUA CUG 3’

3. MiR-21

Stem loop

hsa-mir-21 MI0000077

UGUCGGGUAGCUUAUCAGACUGAUGUUGACUGUUGAAUCUCAUGGCAAC
ACCAGUCGAUGGGCUGUCUGACA

Mature miR-21

hsa-miR-21-5p MIMAT0000076

UAGCUUAUCAGACUGAUGUUGA

>hsa-miR-21-3p MIMAT0004494

CAACACCAGUCGAUGGGCUGU

Primers

Forward: 5’ UGU CGG GUA GCU UAU CAG 3’

Reverse: 5’ UCG AUG GGC UGU CUG ACA 3’

4. MiR-27b

Stem loop

hsa-mir-27b MI0000440

ACCUCUCUAACAAGGUGCAGAGCUUAGCUGAUUGGUGAACAGUGAUUGG
UUUCCGCUUUGUUCACAGUGGCUAAGUUCUGCACCUGAAGAGAAGGUG

Mature miR-27b

hsa-miR-27b-5p MIMAT0004588

AGAGCUUAGCUGAUUGGUGAAC

hsa-miR-27b-3p MIMAT0000419

UUCACAGUGGCUAAGUUCUGC

Primers

Forward: 5'ACC UCU CUA ACA AGG UGC 3'

Reverse: 5' GCA CCU GAA GAG AAG GUG 3'

5. MiR-29a

Stem loop

hsa-mir-29a MI0000087

AUGACUGAUUUCUUUUGGUGUUCAGAGUCAAUAUAAUUUUCUAGCACCA
UCUGAAAUCGGUUAU

Mature miR-29a

hsa-miR-29a-5p MIMAT0004503

ACUGAUUUCUUUUGGUGUUCAG

hsa-miR-29a-3p MIMAT0000086

UAGCACCAUCUGAAAUCGGUUA

Primers

Forward: 5'AUG ACU GAU UUC UUU UGG 3'

Reverse: 5' CCA UCU GAA AUC GGU UAU 3'

6. MiR-34a

Stem loop

hsa-mir-34a MI0000268

GGCCAGCUGUGAGUGUUUCUUUGGCAGUGUCUUAGCUGGUUGUUGUGAG
CAAUAGUAAGGAAGCAAUCAGCAAGUAUACUGCCCUAGAAGUGCUGCACG
UUGUGGGGCCC

Mature miR-34a

>hsa-miR-34a-5p MIMAT0000255

UGGCAGUGUCUUAGCUGGUUGU

hsa-miR-34a-3p MIMAT0004557

CAAUCAGCAAGUAUACUGCCCU

Primers

Forward: 5’ GGC CAG CUG UGA GUG UUU 3’

Reverse: 5’ CUG CAC GUU GUG GGG CCC 3’

7. MiR-99a

Stem loop

hsa-mir-99a MI0000101

CCCAUUGGCAUAAACCCGUAGAUCCGAUCUUGUGGUGAAGUGGACCGCAC
AAGCUCGCUUCUAUGGGUCUGUGUCAGUGUG

Mature miR-99a

hsa-miR-99a-5p MIMAT0000097

AACCCGUAGAUCCGAUCUUGUG

hsa-miR-99a-3p MIMAT0004511

CAAGCUCGCUUCUAUGGGUCUG

Primers

Forward: 5’ CCC AUU GGC AUA AAC CCG 3’

Reverse: 5’ UGG GUC UGU GUC AGU GUG 3’

8. MiR-99b

Stem loop

hsa-mir-99b MI0000746

GGCACCCACCCGUAGAACCGACCUUGCGGGGCCUUCGCCGCACACAAGCU
CGUGUCUGUGGGUCCGUGUC

Mature miR-99b

>hsa-miR-99b-5p MIMAT0000689

CACCCGUAGAACCGACCUUGCG

hsa-miR-99b-3p MIMAT0004678

CAAGCUCGUGUCUGUGGGUCCG

Primers

Forward: 5’ GGC ACC CAC CCG UAG AAC 3’

Reverse: 5’ UGU CUG UGG GUC CGU GUC 3’

9. MiR-143

Stem loop

hsa-mir-143 MI0000459

GCGCAGCGCCCUGUCUCCCAGCCUGAGGUGCAGUGCUGCAUCUCUGGUCA
GUUGGGAGUCUGAGAUGAAGCACUGUAGCUCAGGAAGAGAGAAGUUGUU
CUGCAGC

Mature miR-143

hsa-miR-143-5p MIMAT0004599

GGUGCAGUGCUGCAUCUCUGGU

hsa-miR-143-3p MIMAT0000435

UGAGAUGAAGCACUGUAGCUC

Primers

Forward: 5’ GCG CAG CGC CCU GUC UCC 3’

Reverse: 5’ GAG AAG UUG UUC UGC AGC 3’

10. MiR-182

Stem loop

hsa-mir-182 MI0000272

GAGCUGCUUGCCUCCCCCCGUUUUUGGCAAUGGUAGAACUCACACUGGUG
AGGUAACAGGAUCCGGUGGUUCUAGACUUGCCAACUAUGGGGCGAGGAC
UCAGCCGGCAC

Mature miR-182

hsa-miR-182-5p MIMAT0000259

UUUGGCAAUGGUAGAACUCACACU

hsa-miR-182-3p MIMAT0000260

UGGUUCUAGACUUGCCAACUA

Primers

Forward: 5’ GAG CUG CUU GCC UCC CCC 3’

Reverse: 5’ CGA GGA CUC AGC CGG CAC 3’

11. MiR-200a

Stem loop

hsa-mir-200a MI0000737

CCGGGCCCCUGUGAGCAUCUUACCGGACAGUGCUGGAUUUCCCAGCUUGACUC
UAACACUGUCUGGUAACGAUGUUCAAAGGUGACCCGC

Mature miR-200a

hsa-miR-200a-5p MIMAT0001620

CAUCUUACCGGACAGUGCUGGA

>hsa-miR-200a-3p MIMAT0000682

UAACACUGUCUGGUAACGAUGU

Primers

Forward: 5’ CCG GGC CCC UGU GAG CAU 3’

Reverse: 5’ UGU UCA AAG GUG ACC CGC 3’

12. MiR-497

Stem loop

hsa-mir-497 MI0003138

CCACCCCGGUCCUGCUCCCGCCCCAGCAGCACACUGUGGUUUGUACGGCACU

GUGGCCACGUCCAAACCACACUGUGGUGUUAGAGCGAGGGUGGGGGAGGCA

CCGCCGAGG

Mature miR-497

hsa-miR-497-5p MIMAT0002820

CAGCAGCACACUGUGGUUUGU

hsa-miR-497-5p MIMAT0002820

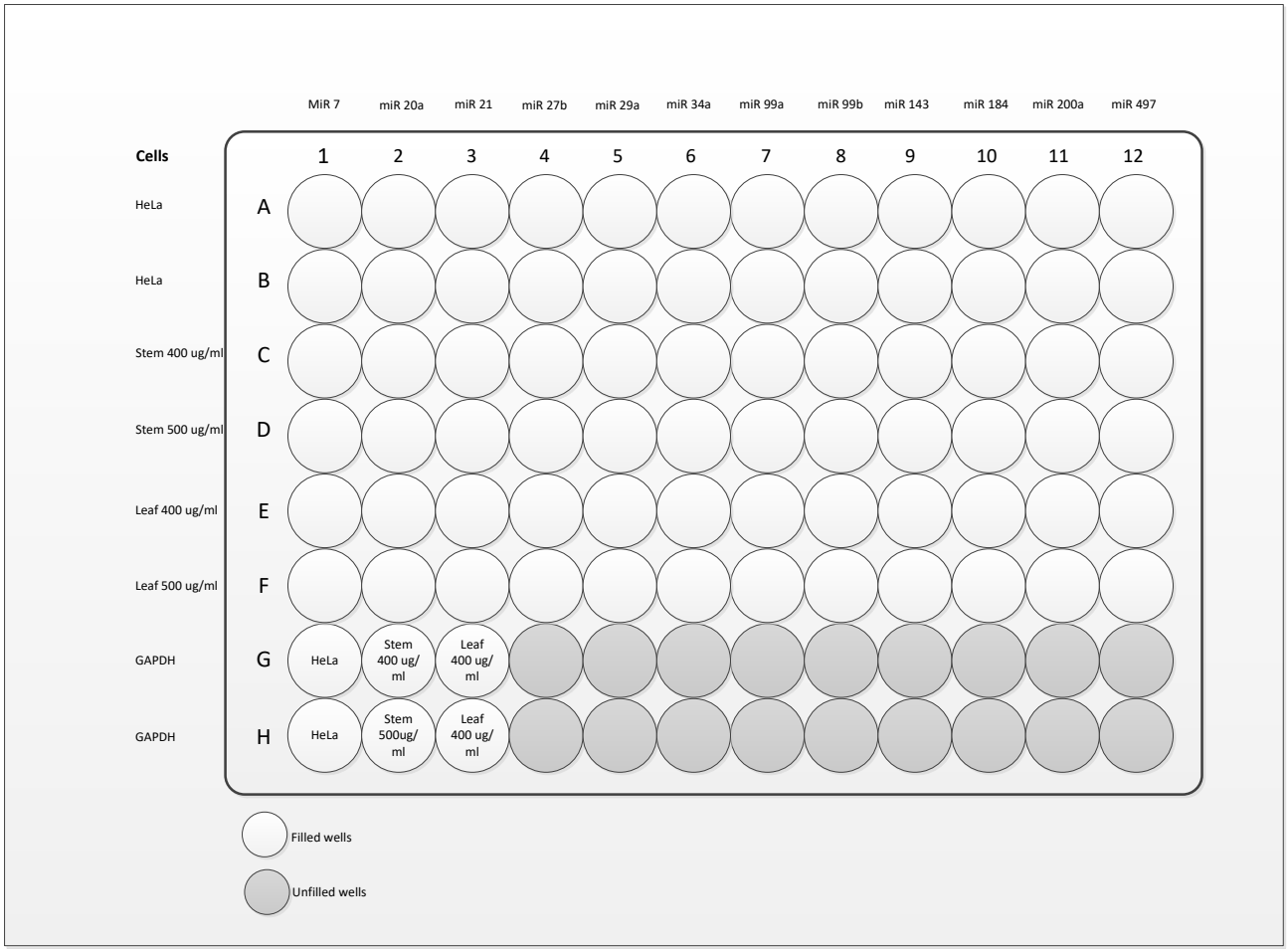
CAGCAGCACACUGUGGUUUGU

Primers

Forward: 5’ CCA CCC CGG UCC UGC UCC 3’

Reverse: 5’ GGG GAG GCA CCG CCG AGG 3’

8.7 APPENDIX G: SETUP OF THE 96 WELL PCR PLATE FOR MIRS AND CELLS



8.8 APPENDIX H: CALCULATIONS OF REAL TIME PCR RESULTS DONE IN EXCEL.

8.8.1 H1. STANDARD DEVIATION

	Stems 400 µg/ml							
MiRs	**Stem 400 µg/ml plate 1**	**Stems 400 µg/ml plate 2**	**Stems 400 µg/ml average**	**HeLa plate 1**	**HeLa plate 2**	**HeLa average**	**STD MiR treated**	**STD HeLa untreated**
MiR 7	36.2	36.55	36.375	26.56	35.515	31.0375	0.247487	6.332141
MiR 20a	38.71	26.58	32.645	35.425	43.2	39.3125	8.577205	5.497755
MiR 21	35.51	25.8	30.655	27.89	29.33	28.61	6.866007	1.018234
MiR 27b	41.45	26.59	34.02	29.37	31.61	30.49	10.50761	1.583919
MiR29a	33.23	35.28	34.255	26.36	32.89	29.625	1.449569	4.617407

MiR 34a	44.33	38.73	41.53	37.15	39.26	38.205	3.959798	1.491995
miR 99a	33.61	24.49	29.05	28.49	30.685	29.5875	6.448814	1.552099
MiR 99b	35	24.98	29.99	30.63	30.155	30.3925	7.08521	0.335876
MiR 143	35.6	31.73	33.665	41.11	30.01	35.56	2.736503	7.848885
MiR 182	49.06	35.9	42.48	42.55	35.845	39.1975	9.305525	4.741151
MiR 200a	38.99	31.18	35.085	27.97	31.85	29.91	5.522504	2.743574
MiR 497	46.95	28.7	37.825	29.78	33.205	31.4925	12.9047	2.421841
GAPDH (Reference Control)	40.34	33.305	36.8225	35.11	39.605	37.3575	4.974496	3.178445
	Stems 500 µg/ml							
MiRs	**Stems 500 µg/ml plate 1**	**Stems 500 µ/ml plate 2**	**Stems 500 µg/ml average**	**Hela plate 1**	**Hela plate 2**	**Hela average**	**STD miR treated**	**STD HeLa untreated**
MiR 7	34.09	37.05	35.57	26.56	35.515	31.0375	2.093036	6.332141
MiR 20a	37.34	42.41	39.875	35.425	43.2	39.3125	3.585031	5.497755
MiR 21	32.85	27.48	30.165	27.89	29.33	28.61	3.797163	1.018234
MiR 27b	34.48	34.19	34.335	29.37	31.61	30.49	0.205061	1.583919
MiR29a	31.82	35.4	33.61	26.36	32.89	29.625	2.531442	4.617407
MiR 34a	47.3	40.31	43.805	37.15	39.26	38.205	4.942676	1.491995
MiR 99a	30.42	26.72	28.57	28.49	30.685	29.5875	2.616295	1.552099
MiR 99b	32.88	23.95	28.415	30.63	30.155	30.3925	6.314464	0.335876
MiR 143	30.47	24.17	27.32	30.01	30.01	30.01	4.454773	7.848885
MiR 182	43.66	34.15	38.905	42.55	35.845	39.1975	6.724585	4.741151
MiR 200a	33.69	30.81	32.25	27.97	31.85	29.91	2.036468	2.743574
MiR 497	35.87	31.64	33.755	29.78	33.205	31.4925	2.991062	2.421841
GAPDH (Reference Control)	38.95	36.97	37.96	35.11	39.605	37.3575	1.400071	3.178445
	Leaf 400 µg/ml							
MiRs	**Leaf 400 µg/ml plate 1**	**Leaf 400 µg/ml plate 2**	**Leaf 400 µg/ml average**	**HeLa plate 1**	**HeLa plate 2**	**HeLa average**	**STD miR treated**	**STD HeLa untreated**
MiR 7	31.92	30.35	31.135	26.56	35.515	31.0375	0.785	6.332141
MiR 20a		42.01	42.01	35.425	43.2	39.3125	0	5.497755
MiR 21	34.02	26.76	30.39	27.89	29.33	28.61	3.63	1.018234
MiR 27b	32.2	26.09	29.145	29.37	31.61	30.49	3.055	1.583919
MiR29a	29.08	28.91	28.995	26.36	32.89	29.625	0.085	4.617407
MiR 34a	41.54	35.77	38.655	37.15	36.26	36.705	2.885	1.491995
miR 99a	29.13	26.4	27.765	28.49	30.685	29.5875	1.365	1.552099
MiR 99b	29.11	25.03	27.07	30.63	30.155	30.3925	2.04	0.335876

MiR 143	30.05	25.14	27.595	30.01	30.01	30.01	2.455	7.848885
MiR 182	42.98	31.98	37.48	42.55	35.845	39.1975	5.5	4.741151
MiR 200a	31.72	25.01	28.365	27.97	31.85	29.91	3.355	2.743574
MiR 497	39.52	35.65	37.585	29.78	33.205	31.4925	1.935	2.421841
GAPDH (Reference Control)	35.54	38.505	37.0225	35.11	39.605	37.3575	1.4825	3.178445
	Leaf 500 µg/ml							
MiRs	**Leaf 500 µg/ml plate 1**	**Leaf 500 µg/ml plate 2**	**Leaf 500 µg/ml average**	**HeLa plate 1**	**HeLa plate 2**	**HeLa average**	**STD miR treated**	**STD HeLa untreated**
MiR 7	27.56	28.88	28.22	26.56	35.515	31.0375	0.933381	6.332141
MiR 20a	35.38	39.98	37.68	35.425	43.2	39.3125	3.252691	5.497755
MiR 21	27.93	26.44	27.185	27.89	29.33	28.61	1.053589	1.018234
MiR 27b	36.23	24.69	30.46	29.37	31.61	30.49	8.160012	1.583919
MiR29a	29.46		29.46	26.36	32.89	29.625	0	4.617407
MiR 34a	36.51	37.3	36.905	37.15	39.26	38.205	0.558614	1.491995
MiR 99a	27.17	24.79	25.98	28.49	30.685	29.5875	1.682914	1.552099
MiR 99b	28.74	24.22	26.48	30.63	30.155	30.3925	3.196123	0.335876
MiR 143	30.06	24.54	27.3	41.11	30.01	35.56	3.903229	7.848885
MiR 182	36.77	29.6	33.185	42.55	35.845	39.1975	5.069956	4.741151
MiR 200a	32.8	23.87	28.335	27.97	31.85	29.91	6.314464	2.743574
MiR 497	35.54	31.64	33.59	29.78	33.205	31.4925	2.757716	2.421841
GAPDH (Reference Control)	35.225	38.505	36.865	35.11	39.605	37.3575	2.31931	3.178445

8.8.2 H2. DELTA CT CALCULATIONS

Stem 400 g/ml								
	Average	**GAPDH average**	**dCt miR7-GAPDH**	**ddCT dCt treated-dCT untreated)**	**Fold difference =2-(ddCT)**	**ST miRs treated**	**STD GAPDH**	**dCt STD(ct target-ct ref)**
MiR 7 Stems Average	36.375	36.8225	-0.4475	5.8725	0.017069	0.247487	4.974496	-4.72701
MirR 7 HeLa Average	31.0375	37.3575	-6.32	0	1	6.332141	3.178445	3.153696
MiR 20aStem 4%	32.645	36.8225	-4.1775	-2.245	4.740371	8.577205	4.974496	3.602709
MiR 20a	35.425	37.3575	-1.9325	0	1	5.497755	3.178445	2.31931

HeLa								
MiR 21 Stems 4%	30.655	36.8225	-6.1675	2.58	0.167241	6.866007	4.974496	1.891511
MiR 21 HeLa	28.61	37.3575	-8.7475	0	1	1.018234	3.178445	-2.16021
MiR 27b Stems 4%	34.02	36.8225	-2.8025	4.065	0.059747	10.50761	4.974496	5.533111
MiR 27b HeLa	30.49	37.3575	-6.8675	0	1	1.583919	3.178445	-1.59453
MiR29a Stems 4%	34.255	36.8225	-2.5675	5.165	0.027873	1.449569	4.974496	-3.52493
MiR29a HeLa	29.625	37.3575	-7.7325	0	1	4.617407	3.178445	1.438962
MiR 34a Stems 4%	41.53	36.8225	4.7075	-3.86	14.52031	3.959798	4.974496	-1.0147
MiR 34a HeLa	38.205	37.3575	0.8475	0	1	1.491995	3.178445	-1.68645
MiR 99a Stems 4%	29.05	36.8225	-7.7725	0.0025	0.998269	6.448814	4.974496	1.474318
MiR 99a HeLa	29.5875	37.3575	-7.77	0	1	1.552099	3.178445	-1.62635
MiR 99b Stems 4%	29.99	36.8225	-6.8325	-0.1325	1.096192	7.08521	4.974496	2.110714
MiR 99b HeLa	30.3925	37.3575	-6.965	0	1	0.335876	3.178445	-2.84257
MiR 143 Stems 4%	33.665	36.8225	-3.1575	-4.19	18.25222	2.736503	4.974496	-2.23799
MiR 143 HeLa	30.01	37.3575	-7.3475	0	1	7.848885	3.178445	4.67044
MiR 182 Stems 4%	42.48	36.8225	5.6575	-3.8175	14.0988	9.305525	4.974496	4.331029
MiR 182 HeLa	39.1975	37.3575	1.84	0	1	4.741151	3.178445	1.562706
MiR 200a Stems 4%	35.085	36.8225	-1.7375	5.71	0.019104	5.522504	4.974496	0.548008
MiR 200a HeLa	29.91	37.3575	-7.4475	0	1	2.743574	3.178445	-0.43487
MiR 497	37.825	36.8225	1.0025	6.8675	0.008564	12.9047	4.974496	7.930203

stems 4%								
MiR 497 HeLa	31.4925	37.3575	-5.865	0	1	2.421841	3.178445	-0.7566
Stems 500 µg/ml								
MiRs	**Average**	**GAPDH average**	**dCt miR7-GAPDH**	**ddCT dCt treated-dCT untreated)**	**Fold difference =2-(ddCT)**	**STD MiRs treated**	**STD GAPDH**	**dCt STD(ct target-ct ref)**
MiR 7 Stems 5%	35.57	37.96	-2.39	3.93	0.065607	2.093036	1.400071	0.692965
MiR 7 HeLa average	31.0375	37.3575	-6.32	0	1	6.332141	3.178445	3.153696
MiR 20a Stem 5%	39.875	37.96	1.915	-0.04	1.028114	3.585031	1.400071	2.18496
MiR 20a HeLa	39.3125	37.3575	1.955	0	1	5.497755	3.178445	2.31931
MiR 21 Stem 5%	30.165	37.96	-7.795	0.9525	0.516736	3.797163	1.400071	2.397092
MiR 21 HeLa	28.61	37.3575	-8.7475	0	1	1.018234	3.178445	-2.16021
MiR 27b Stems 5%	34.335	37.96	-3.625	3.2425	0.10566	0.205061	1.400071	-1.19501
MiR 27b HeLa	30.49	37.3575	-6.8675	0	1	1.583919	3.178445	-1.59453
MiR29a Stems 5%	33.61	37.96	-4.35	3.3825	0.095888	2.531442	1.400071	1.131371
MiR29a HeLa	29.625	37.3575	-7.7325	0	1	4.617407	3.178445	1.438962
MiR 34a Stems 5%	43.805	37.96	5.845	-4.9975	31.9446	4.942676	1.400071	3.542605
MiR 34a HeLa	38.205	37.3575	0.8475	0	1	1.491995	3.178445	-1.68645
MiR 99a Stems 5%	28.57	37.96	-9.39	1.62	0.325335	2.616295	1.400071	1.216224
MiR 99a HeLa	29.5875	37.3575	-7.77	0	1	1.552099	3.178445	-1.62635
MiR 99b Stems 5%	28.415	37.96	-9.545	2.58	0.167241	6.314464	1.400071	4.914393

MiR 99b HeLa	30.3925	37.3575	-6.965	0	1	0.335876	3.178445	-2.84257
MiR 143 Stems 5%	27.32	37.96	-10.64	3.2925	0.102061	4.454773	1.400071	3.054702
MiR 143 HeLa	30.01	37.3575	-7.3475	0	1	7.848885	3.178445	4.67044
MiR 182 stems 5%	38.905	37.96	0.945	0.8925	0.53868	6.724585	1.400071	5.324514
MiR 182 HeLa	39.195	37.3575	1.8375	0	1	4.741151	3.178445	1.562706
MiR 200a stems 5%	32.25	37.96	-5.71	1.7375	0.299889	2.036468	1.400071	0.636397
MiR 200a HeLa	29.91	37.3575	-7.4475	0	1	2.743574	3.178445	-0.43487
MiR 497 stems 5%	33.755	37.96	-4.205	1.66	0.316439	2.991062	1.400071	1.590991
MiR 497 HeLa	31.4925	37.3575	-5.865	0	1	2.421841	3.178445	-0.7566
Leaf 400 µg/ml								
MiRs	**Average**	**GAPDH average**	**dCt miR7-GAPDH**	**ddCT dCt treated-dCT untreated)**	**Fold difference =2-(ddCT)**	**STD miRs**	**STD GAPDH**	**dCt STD**
MiR 7 Leaf 4%	31.135	37.0225	-5.8875	0.4325	0.740977	0.785	1.4825	-0.6975
MirR 7 HeLa average	31.0375	37.3575	-6.32	0	1	6.332141	3.178445	3.153696
MiR 20a leaf 4%	42.01	37.0225	4.9875	3.0325	0.122216	0	1.4825	-1.4825
MiR 20a HeLa	39.3125	37.3575	1.955	0	1	5.497755	3.178445	2.31931
MiR 21 Leaf 4%	30.39	37.0225	-6.6325	2.115	0.230846	3.63	1.4825	2.1475
MiR 21 HeLa	28.61	37.3575	-8.7475	0	1	1.018234	3.178445	-2.16021
MiR 27b Leaf 4%	29.41	37.0225	-7.6125	-0.745	1.675974	3.055	1.4825	1.5725
MiR 27b HeLa	30.49	37.3575	-6.8675	0	1	1.583919	3.178445	-1.59453
MiR29a	28.995	37.0225	-8.0275	-0.295	1.226885	0.085	1.4825	-1.3975

leaf 4%								
MiR29a HeLa	29.625	37.3575	-7.7325	0	1	4.617407	3.178445	1.438962
MiR 34a leaf 4%	38.655	37.0225	1.6325	-2.285	4.873641	2.885	1.4825	1.4025
MiR 34a HeLa	36.705	37.3575	-0.6525	0	1	1.491995	3.178445	-1.68645
MiR 99a leaf 4%	27.765	37.0225	-9.2575	1.4875	0.35663	1.365	1.4825	-0.1175
MiR 99a HeLa	29.5875	37.3575	-7.77	0	1	1.552099	3.178445	-1.62635
MiR 99b leaf 4%	27.07	37.0225	-9.9525	2.9875	0.126088	2.04	1.4825	0.5575
MiR 99b HeLa	30.3925	37.3575	-6.965	0	1	0.335876	3.178445	-2.84257
MiR 143 leaf 4%	27.595	37.0225	-9.4275	2.08	0.236514	2.455	1.4825	0.9725
MiR 143 HeLa	30.01	37.3575	-7.3475	0	1	7.848885	3.178445	4.67044
MiR 182 leaf 4%	37.48	37.0225	0.4575	1.3825	0.383554	5.5	1.4825	4.0175
MiR 182 HeLa	39.1975	37.3575	1.84	0	1	4.741151	3.178445	1.562706
MiR 200a Leaf 4%	28.365	37.0225	-8.6575	-1.21	2.313376	3.355	1.4825	1.8725
MiR 200a HeLa	29.91	37.3575	-7.4475	0	1	2.743574	3.178445	-0.43487
MiR 497 leaf 4%	37.585	37.0225	0.5625	6.4275	0.011618	1.935	1.4825	0.4525
MiR 497 HeLa	31.4925	37.3575	-5.865	0	1	2.421841	3.178445	-0.7566
Leaf 500 µg/ml								
MiRs	**Average**	**GAPDH average**	**dCt miR7-GAPDH**	**ddCT dCt treated-dCT untreated)**	**Fold difference =2-(ddCT)**	**STD miRs**	**STD GaPDH**	**dCT STD**
MiR 7 leaf 5%	28.22	36.865	-8.645	-2.325	5.010658	0.933381	2.31931	-1.38593
MiR 7 HeLa Average	31.0375	37.3575	-6.32	0	1	6.332141	3.178445	3.153696
MiR 20a leaf 5%	37.68	36.865	0.815	-1.14	2.20381	3.252691	2.31931	0.933381
MiR 20a HeLa	39.3125	37.3575	1.955	0	1	5.497755	3.178445	2.31931

MiR 21 leaf 5%	27.185	36.865	-9.68	-0.9325	1.90858	1.053589	2.31931	-1.26572
MiR 21 HeLa	28.61	37.3575	-8.7475	0	1	1.018234	3.178445	-2.16021
MiR 27b leaf 5%	30.46	36.865	-6.405	0.4625	0.725728	8.160012	2.31931	5.840702
MiR 27b HeLa	30.49	37.3575	-6.8675	0	1	1.583919	3.178445	-1.59453
MiR29a leaf 5%	29.46	36.865	-7.405	0.3275	0.796916	0	2.31931	-2.31931
MiR29a HeLa	29.625	37.3575	-7.7325	0	1	4.617407	3.178445	1.438962
MiR 34a leaf 5%	36.905	36.865	0.04	0.8075	0.571371	0.558614	2.31931	-1.7607
MiR 34a HeLa	38.205	37.3575	0.8475	0	1	1.491995	3.178445	-1.68645
MiR 99a leaf 5%	25.98	36.865	-10.885	3.115	0.115423	1.682914	2.31931	-0.6364
MiR 99a HeLa	29.5875	37.3575	-7.77	0	1	1.552099	3.178445	-1.62635
MiR 99b leaf 5%	26.48	36.865	-10.385	3.42	0.093428	3.196123	2.31931	0.876812
MiR 99b HeLa	30.3925	37.3575	-6.965	0	1	0.335876	3.178445	-2.84257
MiR 143 leaf 5%	27.3	36.865	-9.565	7.7675	0.004589	3.903229	2.31931	1.583919
MiR 143 HeLa	35.56	37.3575	-1.7975	0	1	7.848885	3.178445	4.67044
MiR 182 leaf 5%	33.185	36.865	-3.68	5.52	0.021793	5.069956	2.31931	2.750645
MiR 182 HeLa	39.1975	37.3575	1.84	0	1	4.741151	3.178445	1.562706
MiR 200a leaf 5%	28.335	36.865	-8.53	-1.0825	2.117703	6.314464	2.31931	3.995153
MiR 200a HeLa	29.91	37.3575	-7.4475	0	1	2.743574	3.178445	-0.43487
MiR 497 leaf 5%	33.59	36.865	-3.275	2.59	0.166086	2.757716	2.31931	0.438406
MiR 497 HeLa	31.4925	37.3575	-5.865	0	1	2.421841	3.178445	-0.7566

8.8.3 H3. FOLD CHANGE CALCULATIONS

Stem 400 µg/ml fold change				
MiRs	**Stems**	**STD**	**HeLa**	**STD**

	400 µg/ml	Stems 400 µg/ml	untreated	HeLa
MiR 7	0.017069	-4.72701	1	3.153696
MiR 20a	4.740371	3.602709	1	2.31931
MiR 21	0.167241	1.891511	1	-2.16021
MiR 27b	0.059747	5.533111	1	-1.59453
MiR 29a	0.027873	-3.52493	1	1.438962
MiR 34a	14.52031	-1.0147	1	-1.68645
MiR 99a	0.998269	1.474318	1	-1.62635
MiR 99b	1.096192	2.110714	1	-2.84257
MiR 143	18.25222	-2.23799	1	4.67044
MiR 182	14.0988	4.331029	1	1.562706
MiR 200a	0.019104	0.548008	1	-0.43487
MiR 497	0.008564	7.930203	1	-0.7566
Stem 500 µg/ml fold change				
MiRs	**Stems 500 µg/ml**	**HeLa**	**STED stems 500 µg/ml**	**STD HeLa**
MiR 7	0.065607	1	0.692965	3.153696
MiR 20a	1.028114	1	2.18496	2.31931
MiR 21	0.516736	1	2.397092	-2.16021

MiR 27b	0.10566	1	-1.19501	-1.59453
MiR 29a	0.095888	1	1.131371	1.438962
MiR 34a	31.9446	1	3.542605	-1.68645
MiR 99a	0.325335	1	1.216224	-1.62635
MiR 99b	0.167241	1	4.914393	-2.84257
MiR 143	0.102061	1	3.054702	4.67044
MiR 182	0.53868	1	5.324514	1.562706
MiR 200a	0.299889	1	0.636397	-0.43487
MiR 497	0.316439	1	1.590991	-0.7566
Leaf 400 µg/ml fold change				
MiRs	**Leaf 400 µg/ml**	**HeLa**	**STD leaf 400 µg/ml**	**STD HeLa**
MiR 7	0.740977	1	-0.6975	3.153696
MiR 20a	0.122216	1	-1.4825	2.31931
MiR 21	0.230846	1	2.1475	-2.16021
MiR 27b	1.675974	1	1.5725	-1.59453
MiR29a	1.226885	1	-1.3975	1.438962
MiR 34a	4.873641	1	1.4025	-1.68645
MiR 99a	0.35663	1	-0.1175	-1.62635

MiR 99b	0.126088	1	0.5575	-2.84257
MiR 143	0.236514	1	0.9725	4.67044
MiR 182	0.383554	1	4.0175	1.562706
MiR 200a	2.313376	1	1.8725	-0.43487
MiR 497	0.011618	1	0.4525	-0.7566
Leaf 500 µg/ml fold change				
MiRs	**Leaf 500 µg/ml**	**HeLa**	**STD leaf 500 µg/ml**	**STD HeLa**
MiR 7	5.010658	1	-1.38593	3.153696
MiR 20a	2.20381	1	0.933381	2.31931
MiR 21	1.90858	1	-1.26572	-2.16021
MiR 27b	0.725728	1	5.840702	-1.59453
MiR 29a	0.796916	1	-2.31931	1.438962
MiR 34a	0.571371	1	-1.7607	-1.68645
MiR 99a	0.115423	1	-0.6364	-1.62635
MiR 99b	0.093428	1	0.876812	-2.84257
MiR 143	0.004589	1	1.583919	4.67044
MiR 182	0.021793	1	2.750645	1.562706
MiR 200a	2.117703	1	3.995153	-0.43487

MiR 497	0.166086	1	0.438406	-0.7566